

FINAL REPORT

Project Title

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**Effect of fatty acid oxidation on the mitochondrial respiratory chain through
the respiratory chain supercomplex formation in *Caenorhabditis elegans***

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รายงานโครงการวิจัยฉบับสมบูรณ์

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โครงการ การศึกษาผลของการออกซิเดชันของกรดไขมันต่อระบบการถ่ายทอดอิเล็กตรอนในไมโทคอนเดรีย
ผ่านการสังเคราะห์ซูเปอร์คอมเพล็กซ์ของระบบการถ่ายทอดอิเล็กตรอน ของหนอนตัวกลม *C. elegans*

Effect of fatty acid oxidation on the mitochondrial respiratory chain
through the respiratory chain supercomplex formation in *Caenorhabditis elegans*

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[หัวหน้าโครงการวิจัยผู้รับทุน]

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[สถาบันของหัวหน้าโครงการวิจัยผู้รับทุน]

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[สถาบันของนักวิจัยที่ปรึกษา]

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Physical interaction between enzymes in the fatty acid oxidation (FAO) and the electron transport chain (ETC) has been demonstrated in mammal. Evidently, this interaction enhanced the function of the ETC. However, whether such physical interaction is required for the formation and integrity of the mitochondrial supercomplexes remains unknown. To address this question, the nematode *C. elegans* is subjected to RNAi knockdown for ten acyl-CoA dehydrogenases which include the short-chain acyl-CoA dehydrogenases (*acdh-2*, *acdh-4*), the medium-chain acyl-CoA dehydrogenases (*acdh-7*, *acdh-8*, *acdh-10*), and the very long-chain acyl-CoA dehydrogenases (*acdh-11* and *acdh-12*). The knockdown nematodes exhibit the delayed post-embryonic development, decreased fecundity and significantly shortened lifespan. Interestingly, we demonstrate that a deficiency of ACDH-12 affects the formation and function of complex I. We suggest that in *Caenorhabditis elegans*, ACDH-12 is required for the assembly of the respiratory complex I. Nonetheless, the enzymatic activities of the ETC complexes of the other knockdown worms appear unchanged but the formation of the respiratory supercomplexes is not similar to that of wildtype. These results suggest that physical interaction of the ACADs and the ETC is required for maintaining respiratory supercomplex formation and integrity.

บทคัดย่อ

ปฏิสัมพันธ์ทางกายภาพระหว่างเอนไซม์ในกระบวนการออกซิเดชันของกรดไขมันกับกระบวนการขนส่งอิเล็กตรอนถูกค้นพบในสัตว์เลี้ยงลูกด้วยนม ปฏิสัมพันธ์ทางกายภาพดังกล่าวยังเพิ่มประสิทธิภาพของการทำงานของกระบวนการขนส่งอิเล็กตรอนอีกด้วย อย่างไรก็ตามยังไม่มีข้อพิสูจน์แน่ชัดว่าปฏิสัมพันธ์ทางกายภาพดังกล่าวจำเป็นสำหรับการคงสภาพของไมโทคอนเดรียซูเปอร์คอมเพล็กซ์หรือไม่ ผู้วิจัยได้ทำการทดสอบปัญหาดังกล่าวโดยยับยั้งการแสดงออกของยีนสำหรับเอนไซม์เอซิลโคเอ ดีไฮโดรจีเนสทั้งหมด 10 ชนิดในหนอนตัวกลมซีอีลีแกนส์ เอนไซม์ดังกล่าวได้แก่เอซิลโคเอ ดีไฮโดรจีเนสสำหรับกรดไขมันสายสั้น (เอซีดีเอช-2 เอซีดีเอช-4) เอซิลโคเอ ดีไฮโดรจีเนสสำหรับกรดไขมันสายกลาง (เอซีดีเอช-7 เอซีดีเอช-8 เอซีดีเอช-10) และเอซิลโคเอ ดีไฮโดรจีเนสสำหรับกรดไขมันสายยาวมาก (เอซีดีเอช-11 เอซีดีเอช-12) ผู้วิจัยพบว่าการยับยั้งการแสดงออกของเอนไซม์เอซิลโคเอ ดีไฮโดรจีเนสมีผลในการเจริญเติบโตของตัวอ่อนหลังระยะเอ็มบริโอยาวนานผิดปกติ การวางไข่ลดลง และอายุขัยสั้นลง เป็นที่น่าสนใจว่าการยับยั้งการแสดงออกของเอซิลโคเอ ดีไฮโดรจีเนสสำหรับกรดไขมันสายยาวมาก ชนิดเอซีดีเอช-12 ส่งผลให้คอมเพล็กซ์ที่ 1 ของกระบวนการขนส่งอิเล็กตรอนถูกสังเคราะห์ลดลง และทำงานลดลง ข้อมูลดังกล่าวทำให้สามารถสรุปได้ว่าเอซีดีเอช-12 ทำหน้าที่เป็นแฟลคเตอร์รวบรวมสำหรับการสังเคราะห์คอมเพล็กซ์ที่ 1 ของกระบวนการขนส่งอิเล็กตรอน ผลจากการวิจัยยังแสดงให้เห็นว่าการยับยั้งการแสดงออกของเอซิลโคเอ ดีไฮโดรจีเนสชนิดที่เหลือไม่ได้ทำให้การทำงานของเอนไซม์ในการขนส่งอิเล็กตรอนลดลง แต่ส่งผลให้การกระจายตัวของไมโทคอนเดรียซูเปอร์คอมเพล็กซ์แตกต่างไปจากปกติ งานวิจัยนี้จึงสรุปได้ว่าปฏิสัมพันธ์ทางกายภาพระหว่างเอนไซม์เอซิลโคเอ ดีไฮโดรจีเนสกับไมโทคอนเดรียซูเปอร์คอมเพล็กซ์มีความจำเป็นสำหรับการคงสภาพของไมโทคอนเดรียซูเปอร์คอมเพล็กซ์ในหนอนตัวกลมซีอีลีแกนส์

Executive Summary

- Physical interaction between the acyl-CoA dehydrogenases and respiratory supercomplexes exist in *C. elegans* similar to that of mammal.
- Deficiencies in acyl-CoA dehydrogenases lead shortened lifespan in *C. elegans*
- Complex I activity of the other acyl-CoA dehydrogenases knockdown nematode appear unchanged but the formation of the respiratory supercomplexes is not similar to that of wildtype. These results suggest that physical interaction of the acyl-CoA dehydrogenases and the electron transport chain is required for maintaining respiratory supercomplex formation and integrity.
- A deficiency of ACDH-12 results in decreased respiratory complex I formation and function. This result suggests that ACDH-12 is required for the assembly of the respiratory complex I in *C. elegans*

Introduction

The mitochondrial electron transport chain (ETC) is composed of five multimeric enzyme complexes, e.g. complexes I - V. These enzyme complexes assemble into large molecules called mitochondrial supercomplexes or respirasomes. Supercomplex is regarded as a functional entity of the ETC (1). It is conserved from prokaryotes to human (2-10). Supercomplex consists of two or more different respiratory complexes. Supercomplex architecture suggests a kinetic advantage which increases the respiratory rate by providing substrate channeling between components of the supercomplex as well as stabilizing the complexes (2, 9-11).

Fatty acid oxidation (FAO) is a catabolic pathway that take place in the mitochondrial matrix. The pathway involves fatty acid breakdown for energy production. The acyl-CoA dehydrogenase (ACAD) is the enzyme at the first step of FAO. This enzyme makes a double bond into a carboxylic acid between α and β carbons. ACAD requires FAD as a cofactor, which will the transfer electrons into the ETC.

The physiological relationship between FAO and the respiratory chain has long been established but poorly characterized. Identification of a complex consisting of MCAD, ETF, ETF:coenzyme Q oxidoreductase (ETFD), and complex III suggests that a physical connection between these two pathways may partially exist (12). Moreover, patients with deficiencies of either FAO or the respiratory chain often show clinical and biochemical findings indicative of a disorder of the other pathway. For instance, many patients with respiratory chain deficiencies showed accumulation of metabolizes from FAO suggesting dysfunction of enzymes in FAO (13-15). However, the mechanism for dysfunctional FAO related to the respiratory chain deficiency has not been well understood.

Recently, Wang *et al* (1) has demonstrated that several enzymes in FAO are physically

associated with supercomplexes isolated from rat liver. They showed that such as association facilitates metabolic channeling, resulting in an increased efficiency of the respiratory chain to produce ATP. Interestingly, in humans ACAD9, a protein initially identified as the a very long chain acyl-CoA dehydrogenase (VLCAD) (16), plays a role in complex I assembly. A mutation in the coding region of the *ACAD9* caused complex I deficiency (17, 18). Complementation with dehydrogenase-inactive ACAD9 was sufficient to rescue complex I formation and function in fibroblasts from patients with an *ADAC9* mutation (17, 19). RNAi knockdown of *ACAD9* in two different VLCAD-deficient fibroblasts led to significant increased levels of acyl-carnitine C18:1-carnitine and C16-carnitine, suggesting the role of ACAD9 in fatty acid oxidation (19). Analysis of 3-dimensional models of ACAD9 and VLCAD shows their high structural similarity, particularly at the binding site for fatty acid oxidation. An important difference between the ACAD9 and VLCAD exists at a region of 35 amino acids, forming helices in ACAD9 on the opposite sides of a cleft of the dimer at the membrane face. This evidence suggests that ACAD9 is closely related to VLCAD and it is might be evolved from VLCAD (17). Clearly, ACAD9 plays dual roles as a respiratory complex I assembly factor and an acyl-CoA dehydrogenase enzyme in human (19).

Association between FAO and respiratory chain supercomplexes leads to a hypothesis that dysfunction of enzymes in FAO that are found to be associated with supercomplex could potentially lead to dissociation of supercomplex and cause respiratory chain deficiency. We show here that, in *C. elegans*, the FAO machinery has physical interaction with the electron transport chain similar to mammal (1). We have also demonstrated that the ACAD-knockdown nematodes were short-lived. ADAC deficiency affected the electron transport chain function. Furthermore, it also altered supercomplex profile, especially complex I-containing supercomplexes. Our study suggested that physical interaction between the ACAD and the electron transport chain may be required for supercomplex complex function and integrity.

Material and methods

Nematodes and bacteria

Wild type *C. elegans* (N2) and *Escherichia coli* strains HT115 and OP50 were obtained from *Caenorhabditis* Genetic Center. RNAi bacteria strains C17C3.12, T10E9.9, T25G12.5, K05F1.3, T08G2.3, Y45F3A.3, and E04F6.5, which were originally from Open Biosystems ORFeome Library (GE Healthcare Dharmacon Inc), were obtained from Drs. Phil Morgan and Margaret Sedensky, University of Washington, Seattle, WA, USA.

Worm cultures and RNAi knockdown

Caenorhabditis elegans wild-type strain N2 Bristol and the *E. coli* strain HT115 were obtained from the *Caenorhabditis* Genetics Center (Minnesota, USA). The control N2 was fed with HT115 on the Nematode Growth Medium (NGM) as previously describes (20). RNAi knockdown of the target genes was achieved by growing N2 on C17C3.12, T10E9.9, T25G12.5, K05F1.3, T08G2.3, Y45F3A.3, and E04F6.5, the *E. coli* clones containing the plasmids that express dsRNA corresponding to the target genes *acdh-2*, *acdh-4*, *acdh-7*, *acdh-8*, *acdh-10*, *acdh-11*, and *acdh-12*, respectively. All RNAi strains were validated by the Sanger sequencing using the pL4440 primers (the forward primer: 5'CCTTTGAGTGAGCTGATACC3', and the reverse primer: 5'TTAAGTTGGGTAACGCCAGG3'). Analysis of the inserted sequence in plasmid DNA was also performed using the nucleotide BLAST to determine the specificity of their target genes. 5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was used to induce dsRNA expression for RNAi knockdown as previously described (21). Temperature was controlled at 20 °C throughout.

Lifespan study and analysis

Synchronized N2 adults were allowed to lay eggs for 2-4 hours on plates containing lawns of HT115 or RNAi strains. The adult nematodes were removed and eggs were allowed to hatch.

The day of egg laying was defined as day 0 of life. Animals were then moved to new plates every two days and a density of 20-25 animals per 35 mm plate was strictly controlled. Death was defined as failure to respond to a light touch. Bagging and flee animals were scored as censor and discarded from the study. Each experiment consisted of 100-150 animals and was duplicated. Log-rank survival statistical analysis and a survival graph plot were used for data analysis, which was done through the *OASIS*, an online-based analysis system (<https://sbi.postech.ac.kr/oasis>).

Mitochondrial isolation

Adults *C. elegans* were harvested from the liquid culture by centrifugation at 500g for 10 min, and then washed with 10 mL MSM-E (20 mM mannitol, 70 mM sucrose, 5 mM MOPS, and 2 mM EDTA). The worm pellet was homogenized with glass homogenizer at 300 RPM for 3 strokes on ice. The homogenate was then subjected to protease digestion by subtilisin for 10 min on ice. After enzyme digestion, the mixture was homogenized again at 300 RPM for 10 strokes, and then mixed with an equal volume of MSM-EB (MSM-E with 0.4 % bovine serum albumin). The homogenate was centrifuged at 300g for 10 min, and then supernatant was collected. The supernatant was centrifuged at 7000g for 10 min to pellet down the mitochondrial particles. The mitochondrial pellet was washed twice with MSM-E and MSM (MSM-E without EDTA) followed by centrifugation at 7000g 10 min for each wash. The mitochondrial pellet was suspended in a proper volume of MSM. This protocol was previously established (20, 22).

Measurement of enzymatic activity

Enzyme activities were measured at 37 °C using cholate-solubilized fresh mitochondria in KME solution (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA). Agilent 8453 UV-visible Spectroscopy system and Agilent ChemStation for UV-visible Spectroscopy (Agilent Technologies Inc, Santa Clara, CA, USA) were the hardware and the software used for the enzymatic assay, respectively.

NADH:ferricyanide reductase (NFR) and cytochrome c:oxygen oxidoreductase (COX) assays-
NFR and COX assays were performed as previously described (21).

NADH-decylubiquinone:2,6-dichloroindophenol oxidoreductase (NADH-DUQ: DCPIP) assay- NADH-DUQ:DCPIP assay determines enzymatic activity of the respiratory complex I by measuring the reduction rate of DCPIP in a presence of NADH as an electron donor and DUQ as an electron acceptor. A subsequent reaction acquires DCPIP as the electron acceptor from reduced DUQ. The assay was performed in 1 mL of solution containing 25 mM KH₂PO₄, 3.5 g/L BSA, 1 μ M antimycin A, 60 μ M DCPIP, 70 μ M decylubiquinone, 0.2 mM NADH, and 20 μ g mitochondrial protein. Another NADH-DUQ:DCPIP reaction with an addition of 1 mM rotenone was assayed in parallel to determine the rotenone-insensitive NADH oxidation. The absorbance of DCPIP was monitored at 600 nm. Coefficient of DCPIP, 21 mM⁻¹ cm⁻¹, was used to calculate the rate of DCPIP reduction. This method is modified from Janssen *et al.* (23).

Blue native gel (BNG) electrophoresis and complex I-in gel staining

A non-denaturing polyacrylamide gradient gel was casted at room temperature with a gradient mixer as described in Wittig *et al.* (24). To perform BNG, 150 - 250 μ g of mitochondrial protein was solubilized by 20% digitonin to obtain a ratio of detergent/protein as 6/1 (w/w) at room temperature for 10 min, followed by centrifugation at 15,000 x g for 20 min, 4 °C. After centrifuged, the mitochondrial debris was packed as a pellet whereas the respiratory complexes were dissolved in supernatant. Supernatant was collected, and then added 5% Coomassie blue to obtain detergent/dye ratio of 8/1 (w/w) before loading onto the 3-11% polyacrylamide gel. Cold cathode buffer was used to overlay the samples in each well. Electrophoresis was performed at 4 °C at constant 300 V. After the blue running front has moved about one-third of the desired total running distance, the first cathode buffer was replaced by the second cathode buffer that contained 10-fold dilution of Coomassie blue, and run until the dye front reached the end of the gel. Coomassie blue staining was performed by soaking a gel slice in the staining

solution (0.1% Coomassie blue in 50% methanol, and 10% glacial acetic acid) for 1 h at room temperature, and then destained. Complex I in-gel staining was performed by incubating a gel slice in 0.5 mM nitroblue tetrazolium, 5 mM NADH, 50 mM Tris, pH 7.4 at room temperature for 60 min. This method is modified from Suthammarak *et al.* (21).

Native immunoblotting analysis

Native proteins in BNGs were transferred to polyvinylidene difluoride (PVDF) membrane in transfer buffer (7.5 mM imidazole, and 50 mM tricine) at 4 °C with constant 20 V for 3 h. Membranes were blocked with 5% nonfat milk in phosphate-buffer saline/Tween 20 (0.05%). Antibodies used for subsequent immunodetection included anti-NDUFS3 antibody (Abcam, Cambridge, UK; AB14711) and anti-ATP5A1 antibody (Abcam, Cambridge, UK; AB14748). Visualization of the target proteins was made by chemiluminescence reaction.

Identification of amino acid homology

For multiple sequence alignment, amino acid sequences of *C. elegans* ACDH-11 and ACDH-12 (cACDH-11 and cACDH-12) and human VLCAD, and ACAD9 (hVLCAD and hACAD9) were aligned by “Multiple Sequence Comparison by Log-Expectation” (MUSCLE) algorithm. Percent Identity Matrix and phylogenetic tree of the 4 genes were analyzed from the alignment by MUSCLE algorithm.

For pairwise sequence alignment, each pair of amino acid sequences of the previously mentioned proteins was aligned by EMBOSS Needle algorithm. Percent of identical residues and of similar residues and score were reported.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze groups of data for significant differences. Student t-test was used to calculate statistical significance of specific pairs. Log-rank survival statistical analysis was used to analyze lifespan. Error bar in the figures represent standard deviation (SD) or standard error of mean (SEM) as indicated

Results

Physical interaction between components in the FAO and the mitochondrial supercomplexes exists in *C. elegans*.

We first investigated whether physical interaction between the FAO machinery and the respiratory chain supercomplexes is conserved in *C. elegans*. BNG and native electroblotting revealed that medium-chain acyl-CoA dehydrogenase and electron transfer flavoprotein, components in the FAO, physically interacted with the respiratory chain supercomplexes in *C. elegans* (**Fig. 1**).

Acyl-CoA dehydrogenase deficiency in *C. elegans* results in shortened lifespan.

ACAD-deficient nematodes were created by RNAi knockdown of the ACAD-encoding genes which include *acdh-2*, *acdh-4* (short-chain acyl-CoA dehydrogenase or SCAD); *acdh-7*, *acdh-8*, *acdh-10* (medium-chain acyl-CoA dehydrogenases or MCAD) and *acdh-11* and *acdh-12* (very long-chain acyl-CoA dehydrogenases or VLCAD). The result showed that median lifespan, mean lifespan and maximum lifespan of most of the ACAD-deficient worms were significantly shortened (2-3 days shorter) compared to wildtype. (**Fig. 2** and **Table 1**).

Biochemical Characterization of medium chain-acyl CoA dehydrogenase deficient nematode

Effect of MCAD deficiency on function of the electron transport chain

We proposed that MCAD might have a physical association with the ETC. MCAD deficiency may then alter the function of the ETC. *acdH-7* showed marginal decreased NFR activity whereas CI activity was spared. *acdH-8* showed decreased CI activity despite normal NFR activity. *acdH-10* showed severely compromised complex I function as CI, NFR activities were decreased. The triple knockdown of MCAD (*acdH-7;acdH-8;acdH-10* RNAi) exhibited robust decreased activities of respiratory complex I which included CI, NFR but normal CIV activity (Fig. 3).

Alteration of complex I-containing supercomplex profile in the MCAD-deficient nematodes.

BNG of the mitochondria revealed that the ratio of the I•III•IV to I•III supercomplex in the MCAD-deficient worms was different from that of wildtype (Fig. 4). The I•III supercomplex of the knockdown worms, especially *acdH-8* and triple knockdown (*acdH-7;acdH-8;acdH-10* RNAi) appeared more pronounced than that of the wildtype. However, the amount of supercomplex in all MCAD-deficient worms appeared unchanged compared to that of wildtype. Dimeric complex III, dimeric complex V and monomeric complex V in all MCAD-deficient strains appeared normal.

Biochemical Characterization of very long chain-acyl CoA dehydrogenase deficient nematode

Homology between ACDH-11, ACDH-12, ACAD9 and VLCAD

Two VLCADs of *C. elegans*, ACDH-11 and ACDH-12, were analyzed for their homology to human VLCAD and ACAD9 by multiple sequence alignment using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. MUSCLE calculates the percentage of identical amino acid residues (**Table 2, Overall identity**) and evolutionary relationship as shown in the phylogenetic tree (**Fig. 5**). These analyses suggest that overall amino acid sequence of ACDH-12 is more similar to those of VLCAD and ACAD9 (46.57% to VLCAD and 42.76% to ACAD9) than the overall amino acid sequence of ACDH-11 (21.70% to VLCAD and 20.21% to ACAD9), indicating a closer genetic relationship between the human VLCADs and ACDH-12 as also visualized in the phylogenetic tree.

In addition, we examined important domains of these proteins, i.e. the FAD (flavin adenine dinucleotide)-binding site, the substrate-binding pocket, and the VLCAD domain (25). Since the FAD-binding site and the substrate-binding pocket contain 7-10 residues that are not continuous, the percentage of identical residues was counted and calculated manually based on multiple alignment by MUSCLE; VLCAD domains were continuous and sufficiently long enough to be analyzed by pairwise alignment by EMBOSS Needle. Similar to the overall sequences, all 3 domains of ACDH-12 are more similar to the corresponding domains of VLCAD and ACAD9 than the same domains on ACDH-11 (**Table 2** and **Fig. 5**). These analyses suggest that ACDH-12 may have more similar functions to human VLCADs and ACAD9 than ACDH-11 does.

***acdH-12*-knockdown nematodes exhibits decreased complex I function**

ETC assay shown that *acdH-12* knockdown exhibited compromised complex I function as shown by decreased NFR and NADH-DUQ:DCPIP activities (**Fig. 6**). Decreased complex I function was also shown in *nuaf-1* knockdown. However, *acdH-11* knockdown did not affect complex I function. Complex IV activity was also assayed as an internal control. Complex IV activity in all RNAi knockdowns remained unchanged.

Complex I-containing supercomplexes are diminished in *acdH-12* knockdown

Blue native gel (BNG) electrophoresis was used to determine the amounts of respiratory complexes and supercomplexes. Compared to N2, both *acdH-12* and *nuaf-1* knockdowns decreased the amounts of the I•III and I•III•IV supercomplexes (**Fig. 7A**). The amounts of fully assembled complex I in *acdH-12* and *nuaf-1* knockdown as determined by complex I-in gel staining were remarkably decreased compared to N2 (**Fig. 7B**). Native immunoblotting of BNGs with the anti-NDUFS3 monoclonal antibody was also used to determine the amount of complex I. The result was consistent to those observed from BNGs and complex I-in gel staining in which complex I-containing supercomplexes were diminished in *acdH-12* and *nuaf-1* knockdowns compared to N2 (**Fig. 7C**). However, *acdH-11* knockdown did not affect the amount of complex I.

Discussion

We showed that in *C. elegans* the FAO machinery has physical interaction with the ETC similar to mammal (1). We have also demonstrated that the ACAD-knockdown nematodes were short-lived. ACAD deficiency also affected the ETC function and altered supercomplex formation, especially the profile of complex I-containing supercomplexes. Our study suggested that physical interaction between the ACAD and the ETC is necessary for supercomplex complex function and integrity. Intriguingly, we also demonstrated that *acdH-12*, one of ACADs that we studied, involved in complex I assembly process in *C. elegans*.

We originally hypothesized that the physical interaction between the FAO machinery and the ETC is necessary to maintain supercomplex integrity, i.e. function and formation since Wang *et al* (1) has demonstrated that such an interaction facilitates metabolic channeling, resulting in an increased efficiency of the respiratory chain to produce ATP. Here we showed for the first time that such interaction exists in *C. elegans*. The MCAD and ETF, components in the FAO, were found in the BNG as co-migrating entities with supercomplex I•III and I•III•IV. Unfortunately, we did not test other components in the FAO due to unavailability of antibodies. However, this conservation may be necessary for the integrity of the ETC supercomplex.

ACAD-deficient nematodes exhibited shortened lifespan. Although short lifespan is not specific to any molecular defect, this phenotype can often be found in the ETC deficiency such as in *gas-1* (26) and *mev-1* (27) which are complex I mutant and complex II mutant, respectively.

We examined the formation of the ETC complexes and supercomplexes in MCAD-deficient nematodes. We found that MCAD deficiency altered supercomplex profile. In wildtype, an appearance of supercomplex I•III in BNGs was less pronounced than that of supercomplex I•III•IV. However, supercomplex I•III of MCAD-deficient nematodes especially *acdH-8* and triple

knockdown (*acdh-7;acdh-8;acdh-10* RNAi) became more pronounced in both the BNG and CI-in gel staining than that of wildtype. This finding has been observed in the condition that supercomplex integrity is compromised such as in complex IV-deficient nematode (21), complex III mutation, i.e. *isp-1* and *isp-1;ctb-1* (28). In human, Schagger *et al.* demonstrated that a patient with decreased complex IV caused by a *SURF1* mutation had a decreased ratio of supercomplex I•III•IV to supercomplex I•III (10). It can also be observed in the worms that are not well fed (data not shown). Therefore, our data suggested that the MCADs is necessary for proper formation of the respiratory supercomplexes.

We measured enzymatic activities of the ETC enzymes to determine the effect of ACAD deficiency toward the ETC function. Although MCAD knockdown nematodes exhibited altered supercomplex profile, only *acdh-10* and triple knockdown of MCAD (*acdh-7;acdh-8;acdh-10* RNAi) showed severely compromised complex I function

For MCAD deficiency, our data supported our hypothesis in which the physical interaction between the FAO enzyme and the ETC is required for integrity of the ETC supercomplexes. This finding could potentially explain how patients with deficiencies of either FAO or the respiratory chain often show clinical and biochemical findings indicative of a disorder of the other pathway.

For VLCAD, we analyzed the amino acid sequence of the *C. elegans* VLCADs, i.e. ACDH-11 and ACDH-12, and found that both proteins possess the FAD-binding domain, the substrate-binding domain and the VLCAD domain, which are necessary for acyl-CoA dehydrogenase activity. We also analyzed their homology to human VLCAD and ACAD9 since ACAD9 has initially been categorized as a VLCAD but later found to also play a role in complex I assembly (19). ACDH-12 shares higher score of amino acid identity with ACAD9 and VLCAD than ACDH-11 does. The identity score is extremely high considering invertebrate to mammal comparison,

suggesting their functional homology. ACDH-11 was shown to have a tetrameric form or dimers of a dimer (29, 30) whereas ACAD9 is a homodimer as previously determined (17). Although a crystal structure of ACDH-12 has not been investigated, this information suggests that ACDH-12 might be functionally closer to ACAD9 than ACDH-11.

The knockdown of the target genes was considered effective to exert phenotypic changes since it demonstrated more than 50% knockdown efficiency in *acdH-11* and *acdH-12*. We analyzed the inserted sequence in plasmid DNA of all RNAi bacteria by the nucleotide BLAST to determine their specificity to their target genes. We found that the inserted sequence is specific to target genes and is less likely to target other genes of the ETC complexes. The shortened lifespans observed in *acdH-12* and *nuaf-1* knockdowns could reflect the alteration in mitochondrial function since this phenotype has been observed in some mitochondrial mutants such as *gas-1* (31) and *mev-1* (32). Nonetheless there are also other mitochondrial mutants that have long lifespan such as *nuo-1* (33) and *clk-1* (34). Therefore, shortened lifespan is not essentially correlated with a deficiency of the respiratory chain function. We then performed the fecundity test in the knockdown nematodes since this phenotype also indicates a deficiency in mitochondrial function (28). We found that not only *acdH-12* and *nuaf-1* knockdowns but also *acdH-11* knockdown exhibit significantly decreased fecundity. This could suggest that decreased fecundity may also indicate a deficiency in the fatty acid oxidation (35). Interestingly *acdH-12* knockdown exhibited the most severe fecundity defect. This observation could mean that *acdH-12* knockdown leads to a defect in both mitochondrial function and fatty acid oxidation. Besides those phenotypes, we also noticed a lag in egg laying in *acdH-12* and *nuaf-1* knockdowns. We found that these two RNAi strains spent a few more hours than N2 and *acdH-11* knockdown to start to lay eggs (data not shown). Although there are other phenotypes that can be associated with ETC knockdown in worms such as activation of the

mitochondrial unfolded protein response, and mitochondrial morphology, these phenotypes were not assessed.

RNAi knockdown of *acdH-12* exhibited a biochemical hallmark of complex I deficiency which includes the decreased function of complex I as determined by NFR and NADH-Q:DCPIP activity assays. *acdH-12* knockdown also exhibited decreased formation of complex I or complex I-containing supercomplexes as determined by BNG. Decrease in complex I function and formation in *acdH-12* knockdown was similar to *nuaf-1* knockdown as previously reported (36). However, *nuaf-1* knockdown had a more severe complex I formation defect compared to *acdH-12* knockdown.

Our study suggests that ACDH-12 is required for the formation of the respiratory complex I as an assembly factor. A deficiency in ACDH-12 leads to complex I deficiency, shortened lifespan and decreased fecundity. However, it is also possible that ACDH-12 involves in an assembly process of complex I as a stabilizer. An assembly factor facilitates the assembly process by a come-and-go mechanism in which it finally dissociates from the protein complex when a fully assembled complex is completely formed such as NDUFAF-1, NDUFAF-3 and C3orf1 (37, 38). In contrast, a stabilizer remains associated permanently with a fully assembled complex, e.g. cardiolipin (39), and COX7RP (40). Unfortunately, further investigation cannot be achieved since an antibody for ACDH-12 is not currently available. A prospective study may include the 2D BNG and immunoblotting to further characterize the role of ACDH-12 in complex I assembly.

Based on its amino acid sequence, ACDH-12 might also play a role in fatty acid oxidation, although our Nile Red staining failed to show any effects of RNAi knockdown on storage fat. We suspect that *C. elegans* might not mobilize very long-chain fatty acids as a major source of energy derived from fat. Noteworthy, a prospective study to test whether ACDH-12 crucially involves in

fatty acid oxidation may include a more specific approach such as the acyl-carnitine profile study. If ACDH-12 is crucial for fatty acid oxidation, accumulation of the very long-chain fatty acyl-CoA can be detected in *acdh-12* knockdown.

In summary, we proposed that ACDH-12 is required for the formation of the respiratory complex I in *C. elegans*. It may co-operate the assembly process of complex I as an assembly factor, or play a role as a complex I stabilizer. This observation is similar to ACAD9 which is a protein that has initially been identified as a VLCAD but later found that have dual functions in fatty acid oxidation and complex I assembly (19).

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Figures and Tables

Figure 1

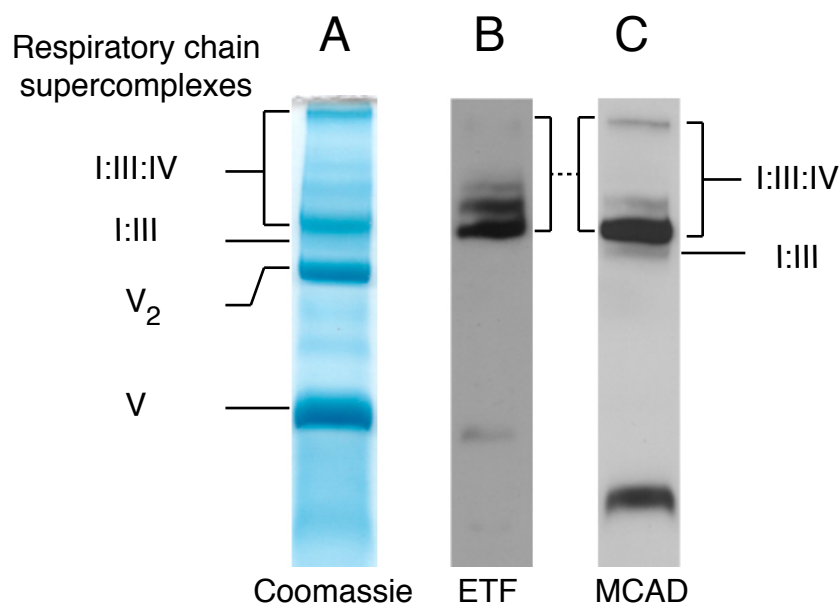


Figure 1. Physical interaction between the FAO machinery and the respiratory chain supercomplexes exists in *C. elegans*. BNG was performed on mitochondria of wildtype *C. elegans*. A gel was subsequently stained with Coomassie blue (A). Western blot analysis, using antibodies against electron transfer flavoprotein (ETF) and medium-chain acyl-CoA dehydrogenase (MCAD), was further analyzed as shown in (B) and (C), respectively.

Figure 2

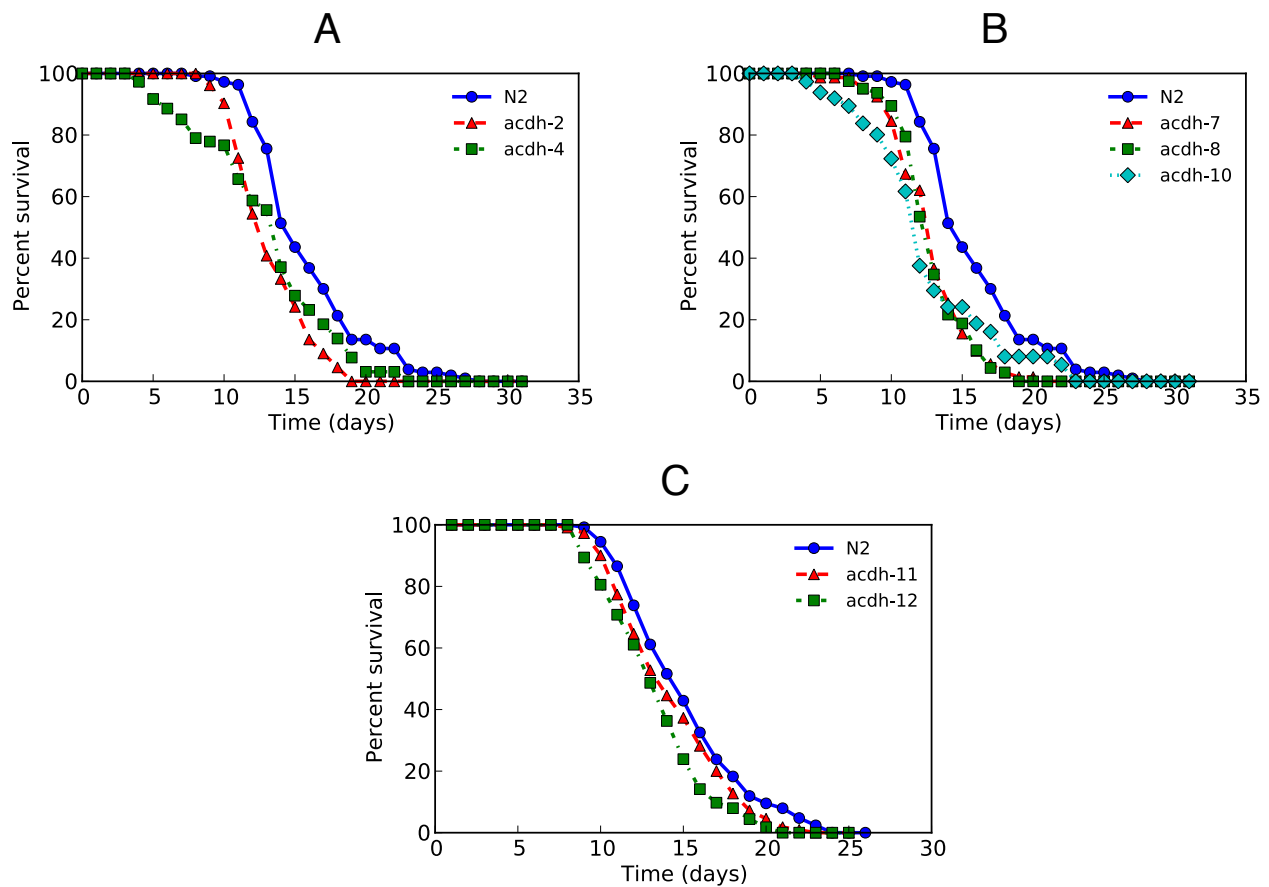


Figure 2. Lifespan of RNAi-knockdown worms. Wild-type *C. elegans* were fed on either *E. coli* strain OP50 as a control (N2), or RNAi bacteria to knock down the acyl-CoA dehydrogenases. Nematodes with acyl-CoA dehydrogenase deficiency exhibit short lifespan as shown. (A) RNAi inhibition of short-chain acyl-CoA dehydrogenases i.e. *acd-2*, *acd-4*. (B) RNAi inhibition of medium-chain acyl-CoA dehydrogenases i.e. *acd-7*, *acd-8*, *acd-10*. (C) RNAi inhibition of very long-chain acyl-CoA dehydrogenases *acd-11* and *acd-12*.

Figure 3

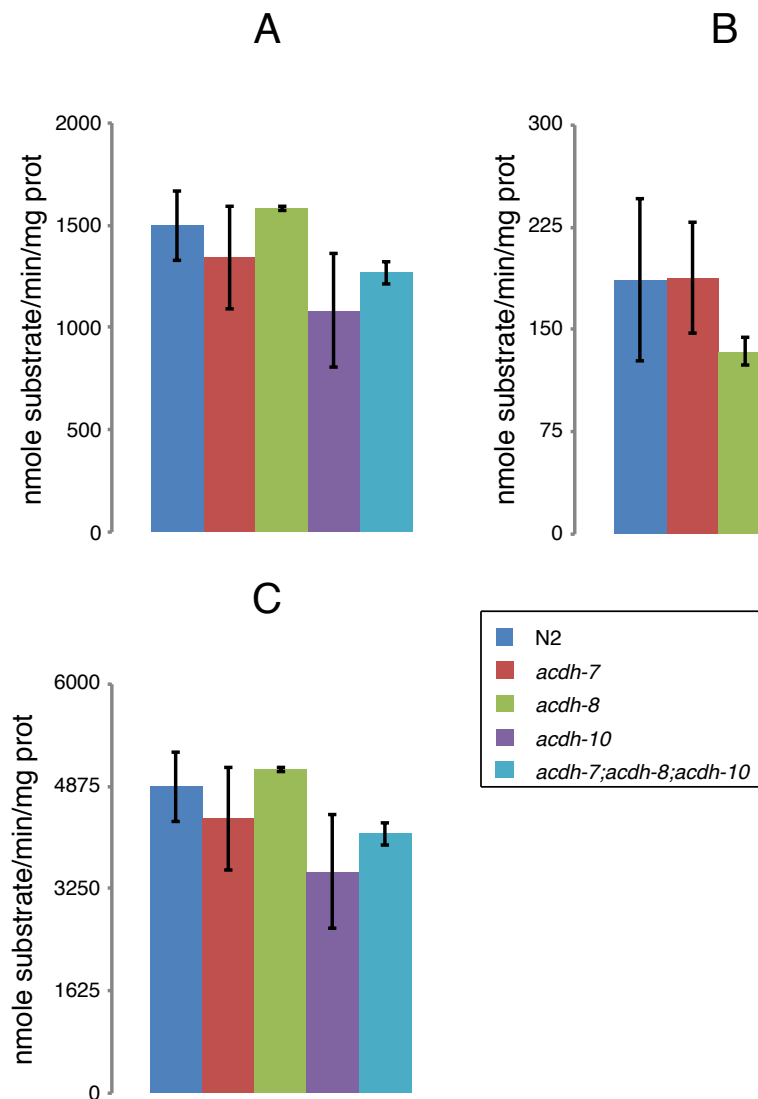


Figure 3. MCAD deficiency does not affect the ETC function. Respiratory enzyme activities of MCAD knockdowns (*acdh-7*, *acdh-8*, *acdh-10* and *acdh-7;acdh-8;acdh-10*) and wild-type worms were determined by spectrophotometric analysis. The assays included NFR; NADH-ferricyanide reductase, CI; rotenone sensitive NADH-decylubiquinone-DCPIP oxidoreductase, and CIV; KCN-sensitive cytochrome c reductase (A, B and C, respectively). The activities were reported as mean $\pm$ S.D. (mmol substrate/min/mg protein).

Figure 4

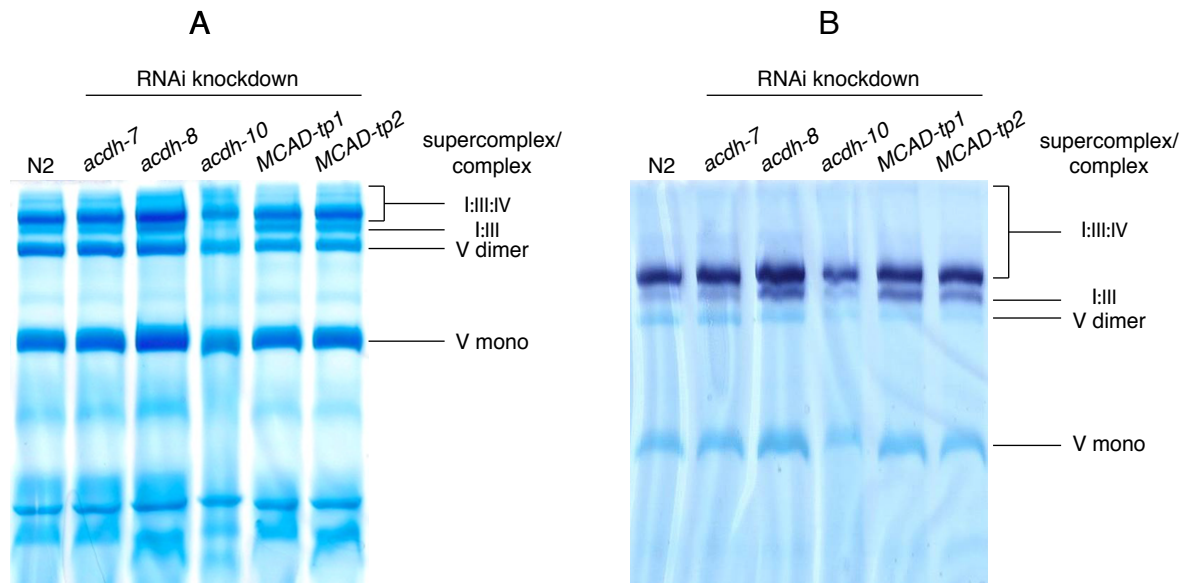


Figure 4. MCAD deficiency affects supercomplex profile. Mitochondria isolated from MCAD-knockdown nematodes (*acdh-7*, *acdh-8*, *acdh-10* and *acdh-7;acdh-8;acdh-10* labeled as MCAD-tp1 and MCAD-tp2) and wildtype were resolved in 3-12% gradient BN-PAGE. Coomassie blue (A) and complex I-in gel (B) staining were performed to visualize supercomplexes (blue bands) and complex I-containing supercomplexes (dark blue bands), respectively.

Figure 5

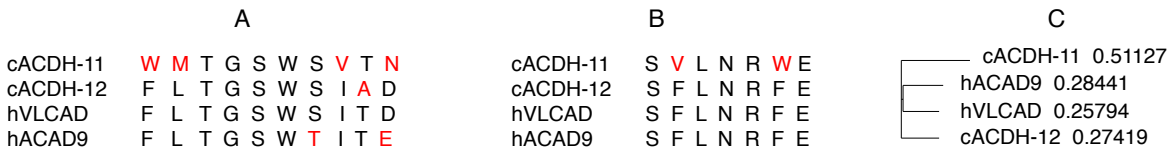


Figure 5. Homology between cACDH-11, cACDH12, hVLCAD, and hACAD9. Amino acid residues in the four proteins in the FAD-binding site (A) and the substrate-binding pocket (B) are shown with those in color signifying non-synonymous residues. Their evolutionary relationship was shown in the phylogenetic tree as extrapolated by MUSCLE. c and h indicates *C. elegans* and human, respectively.

Figure 6

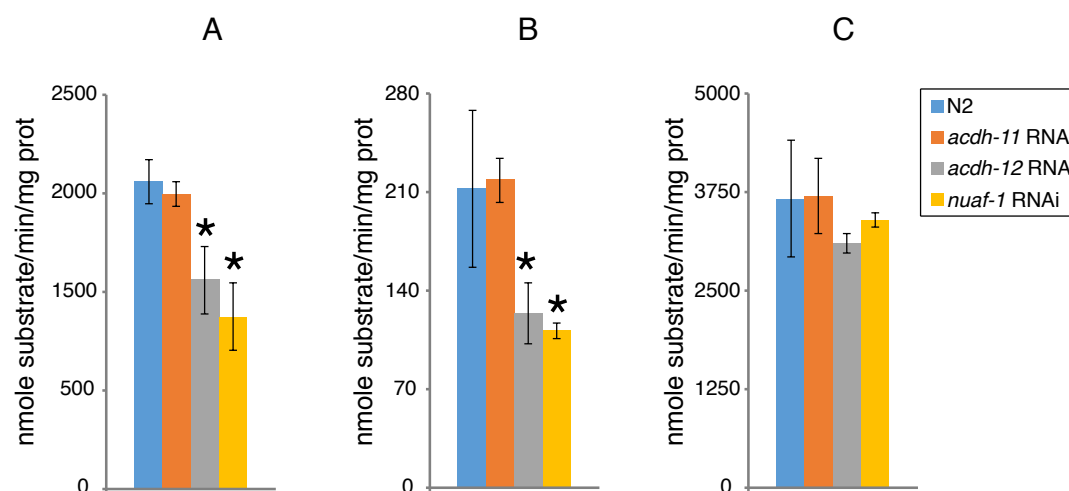


Figure 6. Effect of RNAi knockdown on the respiratory complex function. Enzymatic activities of the respiratory complexes of nematodes were determined by spectrophotometric analysis. Complex I activity was measured by NFR and NADH-DUQ:DCPIP assays (A and B, respectively), where as complex IV activity was measured by cytochrome c oxidoreductase assay (C). Data are presented as mean $\pm$ S.D. from three to five independent experiments. * indicates statistical significance as $p < 0.05$ (t -test) in comparison to N2.

Figure 7

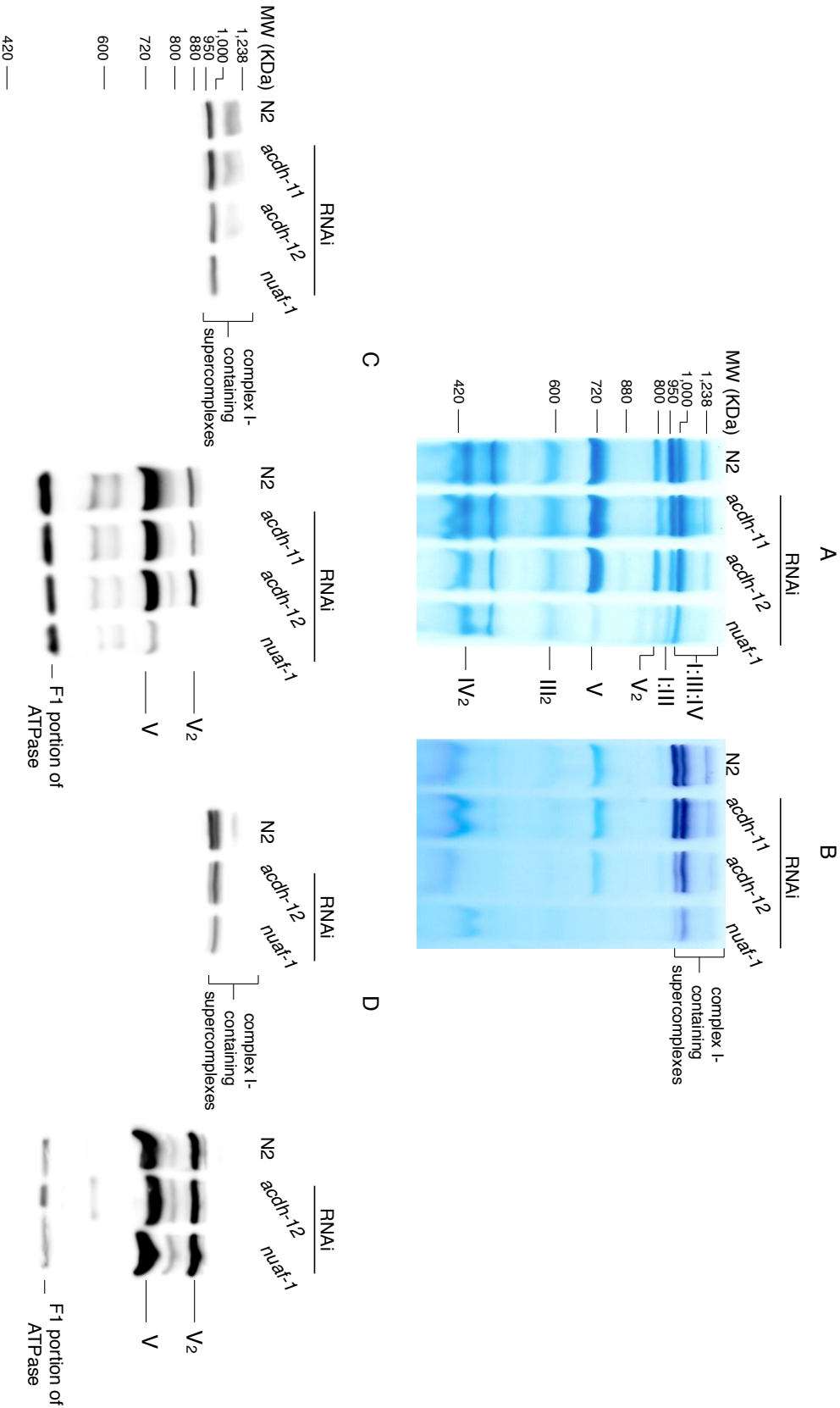


Figure 7. RNAi knockdown of *acdh-12* affected respiratory complex I formation. **(A)** Blue native gel electrophoresis (BNG). Coomassie blue staining of BNG showed bands of respiratory complexes and supercomplexes as indicated. The Roman numeral represents respiratory complexes and supercomplexes. I•III•IV supercomplex is composed of complexes I, III and IV. I•III supercomplex is composed of complexes I and III. V and V₂ represent monomeric and dimeric complex V, respectively. III₂ is a dimeric complex III. **(B)** In-gel activity staining of complex I. Dark blue bands indicate positive staining of complex I. **(C)** and **(D)** Native immunoblotting of BNGs. Monoclonal antibodies against the NDUFS3 (left panel) and ATP5A1(right panel) subunits were used to detect complex I in complex I-containing supercomplexes, and complex V (loading control for BNG) in two representative BNGs, respectively.

Table 1 Statistical analysis of lifespan of the ACAD-deficient worms. Lifespan assay was performed in the nematodes. Median lifespan, median lifespan and p-value were calculated by Log-Rank survival analysis.

Strain	No of worms	Mean lifespan		Median lifespan		Statistics (Log-Rank Test)	
		Days	S.D.	50%	95% median C.I.	p-value	Bonferroni p- value
N2	150	15.96	0.37	15	14~15		
<i>acdH-2</i>	100	13.39	0.32	13	12-13	8.4e-07	1.7e-06
<i>acdH-4</i>	148	13.14	0.47	14	12-13	0.0001	0.0001
<i>acdH-7</i>	150	12.96	0.31	13	12-13	5.9e-09	1.8e-08
<i>acdH-8</i>	150	13.01	0.28	13	12-12	2.1e-09	6.6e-09
<i>acdH-10</i>	150	12.58	0.61	12	11-12	5.0e-07	1.5e-06
<i>acdH-11</i>	150	14.39	0.31	14	13-14	0.0581	0.1162
<i>acdH-12</i>	150	13.49	0.28	13	13-13	0.0001	0.0002

Table 2

Proteins	Overall identity		FAD-binding site		Substrate-binding site		VLCAD domain	
	ACDH-11	ACDH-12	ACDH-11	ACDH-12	ACDH-11	ACDH-12	ACDH-11	ACDH-12
VLCAD	21.70%	46.57%	60%	90%	71.40%	100%	22.50%	58.30%
ACAD9	20.21%	42.76%	50%	70%	71.40%	100%	21.60%	51.70%

Table 2. Homology between cACDH-11, cACDH-12, hVLCAD, and hACAD9. Multiple sequence alignment by MUSCLE generates percentage of overall identity as tabulated. In terms of homology in important domains, amino acid sequences of the 4 proteins were examined at their FAD-binding site, substrate-binding pocket, and VLCAD domain. The FAD-binding site and the substrate-binding pocket contain 10 and 7 amino acid residues, respectively; the percentage tabulated are residues that are identical between each pair after alignment by MUSCLE. The VLCAD domain consists of 408-422 residues; each pair's percentage of identical residues was generated following EMBOSS Needle pairwise alignment.

Outputs

1. Publication in an international journal

- **Title:** Identification of a novel mitochondrial complex I assembly factor ACDH-12 in *Caenorhabditis elegans*.
- **Authors:** Chuaijit S, Boonyatistan W, Boonchuay P, Metheetrairut C, **Suthammarak W.**
- **Published:** Mitochondrion. 2018. doi: 10.1016/j.mito.2018.02.010 (Article In Press)
- **Impact factor (JCI 2017):** 3.704

2. Poster presentation and proceeding at national and international conferences

- Worawich Boonyatistan, Sirithip Chuaijit, Wanpen Katanyoo, **Wichit Suthammarak.** Functional Characterization of the *C. elegans* ACDH-12 as an assembly factor of the respiratory complex I. Siriraj Graduate Research forum, 13-15 June 2016, Bangkok, Thailand.
- Sirithip Chuaijit, Wanpen Katanyoo, **Wichit Suthammarak.** Effect of RNAi knockdown of the fatty acyl-CoA dehydrogenases on mitochondrial supercomplex integrity in *Caenorhabditis elegans*. The 3rd CAS National and International Conference 2015 (CASNIC 2015), 11 September 2015, Khon Kaen, Thailand

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Identification of a novel mitochondrial complex I assembly factor ACDH-12 in *Caenorhabditis elegans*

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ABSTRACT

Assembly of complex I of the mitochondrial respiratory chain (MRC) requires not only structural subunits for electron transport, but also assembly factors. In the nematode *Caenorhabditis elegans*, NUA1-1 and NUA1-3 are the only two assembly factors that have been characterized. In this study, we identify ACDH-12 as an assembly factor of the respiratory complex I. We demonstrate for the first time that a deficiency of ACDH-12 affects the formation and function of complex I. RNAi knockdown of *acdH-12* also shortens lifespan and decreases fecundity. Although ACDH-12 has long been recognized as a very long-chain acyl-CoA dehydrogenase (VLCAD), the knockdown nematodes did not exhibit any change in body fat content. We suggested that in *Caenorhabditis elegans*, ACDH-12 is required for the assembly of the respiratory complex I, but may not be crucial to fatty acid oxidation. Interestingly, sequence analysis shows high homology between ACDH-12 and the human ACAD9, a protein that has initially been identified as a VLCAD, but later found to also be involved in the assembly of complex I in human.

1. Introduction

Assembly of complex I of the MRC is an intricate process that occurs in a stepwise manner. The formation of assembly intermediates from several structural subunits precedes the assembly of the fully functional complex I. Complex I consists of 3 functional modules (Brandt, 2006): the N-module (NADH binding and oxidation), the Q-module (transfer of electrons to ubiquinone), and the P module (proton pumping). The central building block of complex I is submodule P which contains four out of the seven mitochondrially encoded subunits. Submodules N and Q are initially assembled separately. Formation of a Q/P submodule precedes the addition of additional subunits of the P-module to the developing complex. The final step in the assembly process is incorporation of the N-module to the Q/P submodule along with the remaining accessory subunits (Guerrero-Castillo et al., 2017). Protein chaperones called assembly factors which do not play any role in enzymatic function of complex I, assist in multiple steps of the assembly process at different steps (McKenzie and Ryan, 2010; Andrews et al., 2013; Heide et al., 2012). Assembly factors incorporate into complex I intermediates during the assembly process, but dissociate completely once it is fully formed. In addition, assembly factors can be reused in a new round of assembly. Defects in assembly factors, e.g. NDUFAF1 (Dunning et al., 2007), NDUFAF3 (Saada et al., 2009), NDUFAF4

(Saada et al., 2008), FOXRED (Calvo et al., 2010), can also compromise complex I function and cause isolated complex I deficiencies.

Interestingly, in humans ACAD9, a protein initially identified as the a very long chain acyl-CoA dehydrogenase (VLCAD) (He et al., 2007), plays a role in complex I assembly. A mutation in the coding region of the ACAD9 caused complex I deficiency (Nouws et al., 2010; Haack et al., 2010). Complementation with dehydrogenase-inactive ACAD9 was sufficient to rescue complex I formation and function in fibroblasts from patients with an ADAC9 mutation (Nouws et al., 2010, 2014). RNAi knockdown of ACAD9 in two different VLCAD-deficient fibroblasts led to significant increased levels of acyl-carnitine C18:1-carnitine and C16-carnitine, suggesting the role of ACAD9 in fatty acid oxidation (Nouws et al., 2014). Analysis of 3-dimensional models of ACAD9 and VLCAD shows their high structural similarity, particularly at the binding site for fatty acid oxidation. An important difference between the ACAD9 and VLCAD exists at a region of 35 amino acids, forming helices in ACAD9 on the opposite sides of a cleft of the dimer at the membrane face. This evidence suggests that ACAD9 is closely related to VLCAD and it might be evolved from VLCAD (Nouws et al., 2010). Clearly, ACAD9 plays dual roles as a respiratory complex I assembly factor and an acyl-CoA dehydrogenase enzyme in human (Nouws et al., 2014).

In *Caenorhabditis elegans* (*C. elegans*), little is known about complex I

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assembly. Only NUA-1 and NUA-3 have been identified as complex I assembly factors (van den Ecker et al., 2012). Deficiencies in NUA-1 and NUA-3 decrease biosynthesis of complex I and reduce body size and brood size (van den Ecker et al., 2012).

In this study, we hypothesize that *C. elegans* VLCADs homologues (ACDH-11 and ACDH-12) may also play a role in complex I assembly, similarly to dual roles of ACAD9 in human. We find that ACDH-11 and ACDH-12 share high amino acid homology with human VLCAD and ACAD9. Interestingly, ACDH-12 share higher percentage of amino acid identity with ACAD9 and human VLCAD than ACDH-11 does. A previous study on crystal structure of ACDH-11 shows that ACDH-11 exists as a tetramer in solution (Li et al., 2010; Ma et al., 2015), whereas ACAD9 forms dimers according to a modelling study (Nouws et al., 2010). We then hypothesize that ACDH-12 may be functionally homologous to ACAD9 as a complex I assembly factor in *C. elegans*. To address this hypothesis, we use RNAi technique to inactivate *acdH-11* and *acdH-12*. Formation and function of complex I as well as body fat content, lifespan and fecundity are examined in knockdown nematodes. Our result suggests that ACDH-12 acts primarily as a complex I assembly factor, with no measurable role as a VLCAD.

2. Materials and methods

2.1. Worm culture and RNAi knockdown

Caenorhabditis elegans wild-type strain N2 Bristol and the *E. coli* strain HT115 were obtained from the *Caenorhabditis* Genetics Center (Minnesota, USA). The control N2 was fed with HT115 on the Nematode Growth Medium (NGM) as previously describes (Kayser et al., 2001). RNAi knockdown of the target genes was achieved by growing N2 on E04F6.5, Y45F3A.3 or C50B8.3, the *E. coli* clones containing the plasmids that express dsRNA corresponding to the target genes, *acdH-11*, *acdH-12* and *nuaf-1*, respectively. All RNAi strains were validated by the Sanger sequencing using the pL4440 primers (the forward primer: 5'CCTTTGAGTGAGCTGATACC3', and the reverse primer: 5'TTAAGTTGGGTAACGCCAGG3'). Analysis of the inserted sequence in plasmid DNA was also performed using the nucleotide BLAST to determine the specificity of their target genes. 5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was used to induce dsRNA expression for RNAi knockdown as previously described (Suthamarak et al., 2009). Temperature was controlled at 20 °C throughout. Gene knockdown was checked by RT-qPCR. The *tba-1* was used as a housekeeping gene. RNA isolation and quantitative RT-qPCR (SYBR green) were done by standard techniques. Calculation of the target gene expression was done as previously described (Livak and Schmittgen, 2001). Primers for qPCR are tabulated below.

Gene	Forward primer	Reverse Primer
<i>acdH-11</i>	5'AGAGACACAGGCCAAAAT3'	5'AATTGGGTCATCTTTATAAGG3'
<i>acdH-12</i>	5'TTACTACGTTGATAAGGGGA3'	5'CTAAAAGAGCTTCTTACGATAAA3'
<i>tba-1</i>	5'AACTGTTGTTGATGAGATCC3'	5'ATGAAAGACGAAGAATCCTT3'

2.2. Lifespan and fecundity

Synchronized N2 adults were allowed to lay eggs for 2–4 h on plates containing lawns of HT115 or RNAi strains. The adult nematodes were removed and eggs were allowed to hatch. The day of egg laying was defined as day 0 of life. Animals were then moved to new plates every two days and a density of 20–25 animals per 35 mm plate was strictly controlled. Death was defined as failure to respond to a light touch. Bagging and flee animals were scored as censor and discarded from the study. Each experiment consisted of 100–150 animals and was duplicated.

For fecundity, 3–5 L4 N2 were placed on plates containing lawns of HT115 or RNAi strains. The nematodes were transferred to new plates every 24 h, and eggs were counted after adults were removed.

2.3. Nile red staining and microscopy

Synchronized L1 s were fed on HT115 or RNAi strains at 20 °C until adulthood. Young adults were then transferred onto new NGM plates containing lawns of HT115 or RNAi strains and 5 μ M Nile Red for overnight. After dye treatment, worms were mounted for microscopy on a 2% agar pad containing 10 mM sodium azide for immobilization. For microscopic observation and photography, images were obtained using a fluorescent microscope with a FITC filter set (emission 560–590 nm). All Nile Red images were acquired using identical settings and exposure times to allow direct comparisons.

2.4. Mitochondrial isolation

Worm preparation and mitochondrial isolation were performed using established protocols (Kayser et al., 2001; Kayser et al., 2004a).

2.5. Measurement of enzymatic activity of the respiratory complexes

Enzyme activities were measured at 37 °C using cholate-solubilized fresh mitochondria in KME solution (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA). Agilent 8453 UV–visible Spectroscopy system and Agilent ChemStation for UV–visible Spectroscopy (Agilent Technologies Inc, Santa Clara, CA, USA) were the hardware and the software used for the enzymatic assay, respectively.

NADH:ferricyanide reductase (NFR) and cytochrome c: oxygen oxidoreductase (COX) assays- NFR and COX assays were performed as previously described (Suthamarak et al., 2009).

NADH:decylubiquinone:2,6-dichloroindophenol oxidoreductase (NADH-DUQ: DCPIP) assay- NADH-DUQ:DCPIP assay determines enzymatic activity of the respiratory complex I by measuring the reduction rate of DCPIP in a presence of NADH as an electron donor and DUQ as an electron acceptor. A subsequent reaction acquires DCPIP as the electron acceptor from reduced DUQ. The assay was performed in 1 mL of solution containing 25 mM KH_2PO_4 , 3.5 g/L BSA, 1 μ M antimycin A, 60 μ M DCPIP, 70 μ M decylubiquinone, 0.2 mM NADH, and 20 μ g mitochondrial protein. Another NADH-DUQ:DCPIP reaction with an addition of 1 mM rotenone was assayed in parallel to determine the rotenone-insensitive NADH oxidation. The absorbance of DCPIP was monitored at 600 nm. Coefficient of DCPIP, 21 $\text{mM}^{-1} \text{cm}^{-1}$, was used to calculate the rate of DCPIP reduction. This method is modified from Janssen et al. (2007).

2.6. Blue native gel (BNG) electrophoresis and complex I-in gel staining

A non-denaturing polyacrylamide gradient gel was prepared at room temperature with a gradient mixer as described in Wittig et al. (2006). 150 μ g of mitochondrial protein was solubilized by 20% digitonin with a detergent/protein mass ratio of 6/1 at room temperature for 10 min, followed by centrifugation (15,000 $\times$ g, 20 min, 4 °C). After centrifugation, supernatants were collected and 5% Coomassie blue G-250 was added to the supernatants with a dye/detergent mass ratio of 8/1 before loading onto the 3–11% polyacrylamide gradient gel

0.15 × 14 × 16 cm (Hoefer Inc, Holliston, MA, USA). The gels were run for 100 V until all samples entered the separating gel and then at 300 V for the remainder. When the blue running from reached one-third of the gel, the first cathode buffer (7.5 mM imidazole, 50 mM tricine, and 0.02% coomassie blue) was replaced by the second cathode buffer containing a 10-fold dilution of Coomassie blue G-250. Complex I in-gel staining was performed by incubating a gel slice in 0.5 mM nitroblue tetrazolium, 2.5 mM NADH, 50 mM Tris, pH 7.4 at room temperature for 60 min. This method is modified from Suthammarak et al. (2009).

2.7. Native electroblotting

Native proteins in BNGs were transferred to polyvinylidene difluoride (PVDF) membrane in transfer buffer (7.5 mM imidazole, and 50 mM tricine) at 4 °C with constant 20 V for 3 h. Membranes were blocked with 5% nonfat milk in phosphate-buffer saline/Tween 20 (0.05%). Antibodies used for subsequent immunodetection included anti-NDUFS3 antibody (Abcam, Cambridge, UK; AB14711) and anti-ATP5A1 antibody (Abcam, Cambridge, UK; AB14748). Visualization of the target proteins was made by chemiluminescence reaction.

2.8. Identification of amino acid homology

For multiple sequence alignment, amino acid sequences of *C. elegans* ACDH-11 and ACDH-12 (cACDH-11 and cACDH-12) and human VLCAD, and ACAD9 (hVLCAD and hACAD9) were aligned by “Multiple Sequence Comparison by Log-Expectation” (MUSCLE) algorithm. Percent Identity Matrix and phylogenetic tree of the 4 genes were analyzed from the alignment by MUSCLE algorithm.

For pairwise sequence alignment, each pair of amino acid sequences of the previously mentioned proteins was aligned by EMBOSS Needle algorithm. Percent of identical residues and of similar residues and score were reported.

2.9. Statistical analysis

Analysis of variance (ANOVA) was used to analyze groups of data for significant differences. Student *t*-test was used to calculate statistical significance of specific pairs. Log-rank survival statistical analysis was used to analyze lifespan. Error bar in the figures represent standard deviation (SD) or standard error of mean (SEM) as indicated.

3. Results

3.1. Homology between ACDH-11, ACDH-12, ACAD9 and VLCAD

Two VLCADs of *C. elegans*, ACDH-11 and ACDH-12, were analyzed for their homology to human VLCAD and ACAD9 by multiple sequence alignment using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. MUSCLE calculates the percentage of identical amino acid residues (Table 1, Overall identity) and evolutionary relationship as shown in the phylogenetic tree (Fig. 1). These analyses suggest that overall amino acid sequence of ACDH-12 is more similar to those of VLCAD and ACAD9 (46.57% to VLCAD and 42.76% to ACAD9)

Table 1

Homology between cACDH-11, cACDH-12, hVLCAD, and hACAD9. Multiple sequence alignment by MUSCLE generates percentage of overall identity as tabulated. In terms of homology in important domains, amino acid sequences of the 4 proteins were examined at their FAD-binding site, substrate-binding pocket, and VLCAD domain. The FAD-binding site and the substrate-binding pocket contain 10 and 7 amino acid residues, respectively; the percentage tabulated are residues that are identical between each pair after alignment by MUSCLE. The VLCAD domain consists of 408–422 residues; each pair's percentage of identical residues was generated following EMBOSS Needle pairwise alignment.

Proteins	Overall identity		FAD-binding site		Substrate-binding site		VLCAD domain	
	ACDH-11	ACDH-12	ACDH-11	ACDH-12	ACDH-11	ACDH-12	ACDH-11	ACDH-12
VLCAD	21.70%	46.57%	60%	90%	71.40%	100%	22.50%	58.30%
ACAD9	20.21%	42.76%	50%	70%	71.40%	100%	21.60%	51.70%

than the overall amino acid sequence of ACDH-11 (21.70% to VLCAD and 20.21% to ACAD9), indicating a closer genetic relationship between the human VLCADs and ACDH-12 as also visualized in the phylogenetic tree.

In addition, we examined important domains of these proteins, i.e. the FAD (flavin adenine dinucleotide)-binding site, the substrate-binding pocket, and the VLCAD domain (Marchler-Bauer et al., 2017). Since the FAD-binding site and the substrate-binding pocket contain 7–10 residues that are not continuous, the percentage of identical residues was counted and calculated manually based on multiple alignment by MUSCLE; VLCAD domains were continuous and sufficiently long enough to be analyzed by pairwise alignment by EMBOSS Needle. Similar to the overall sequences, all 3 domains of ACDH-12 are more similar to the corresponding domains of VLCAD and ACAD9 than the same domains on ACDH-11 (Table 1 and Fig. 1). These analyses suggest that ACDH-12 may have more similar functions to human VLCADs and ACAD9 than ACDH-11 does.

3.2. Phenotypes- lifespan, fecundity and storage body fat of knockdown nematodes

Phenotypes that may be related to complex I deficiency were determined in the nematodes. We used *nuaf-1* knockdown as a positive control for a deficiency of a complex I assembly factor. The median lifespans of *acdH-12* and *nuaf-1* knockdown were shortened by 2–3 days compared to N2 whereas that of *acdH-11* knockdown was not significantly affected (Fig. 2A, and Supplemental Data 3). Fecundity as determined by a number of eggs laid was significantly affected in all knockdown nematodes. Interestingly, *acdH-12* knockdown appeared to affect fecundity more severely than *nuaf-1* knockdown (Fig. 2B). We also investigated body fat storage since a deficiency these putative homologues of VLCADs may affect fat metabolism. Nile red staining of whole animals showed that RNAi knockdown of *acdH-11* and *acdH-12* did not affect stored body fat (Fig. 2C). However, *nuaf-1* knockdown showed decreased fat content which is consistent with a previous report (van den Ecker et al., 2012).

3.3. *acdH-12*-knockdown nematodes exhibits decreased complex I function

Electron transport chain assays were used to determine complex I function in the nematodes. It was shown that *acdH-12* knockdown exhibited compromised complex I function as shown by decreased NFR and NADH-DUQ:DCPIP activities (Fig. 3A and B). Decreased complex I function was also shown in *nuaf-1* knockdown. However, *acdH-11* knockdown did not affect complex I function. Complex IV activity was also assayed as an internal control. Complex IV activity in all RNAi knockdowns remained unchanged (Fig. 3C).

3.4. Complex I-containing supercomplexes are diminished in *acdH-12* knockdown

Blue native gel (BNG) electrophoresis was used to determine the amounts of respiratory complexes and supercomplexes. Compared to N2, both *acdH-12* and *nuaf-1* knockdowns decreased the amounts of the

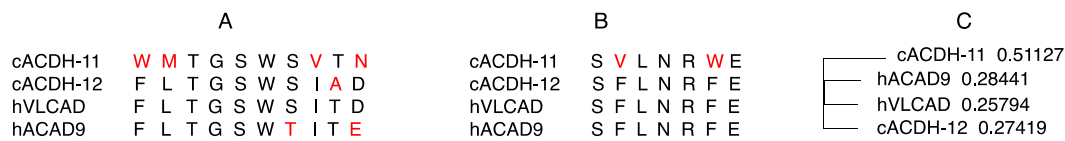


Fig. 1. Homology between cACDH-11, cACDH12, hVLCAD, and hACAD9. Amino acid residues in the four proteins in the FAD-binding site (A) and the substrate-binding pocket (B) are shown with those in colour signifying non-synonymous residues. Their evolutionary relationship was shown in the phylogenetic tree as extrapolated by MUSCLE. c and h indicates *C. elegans* and human, respectively.

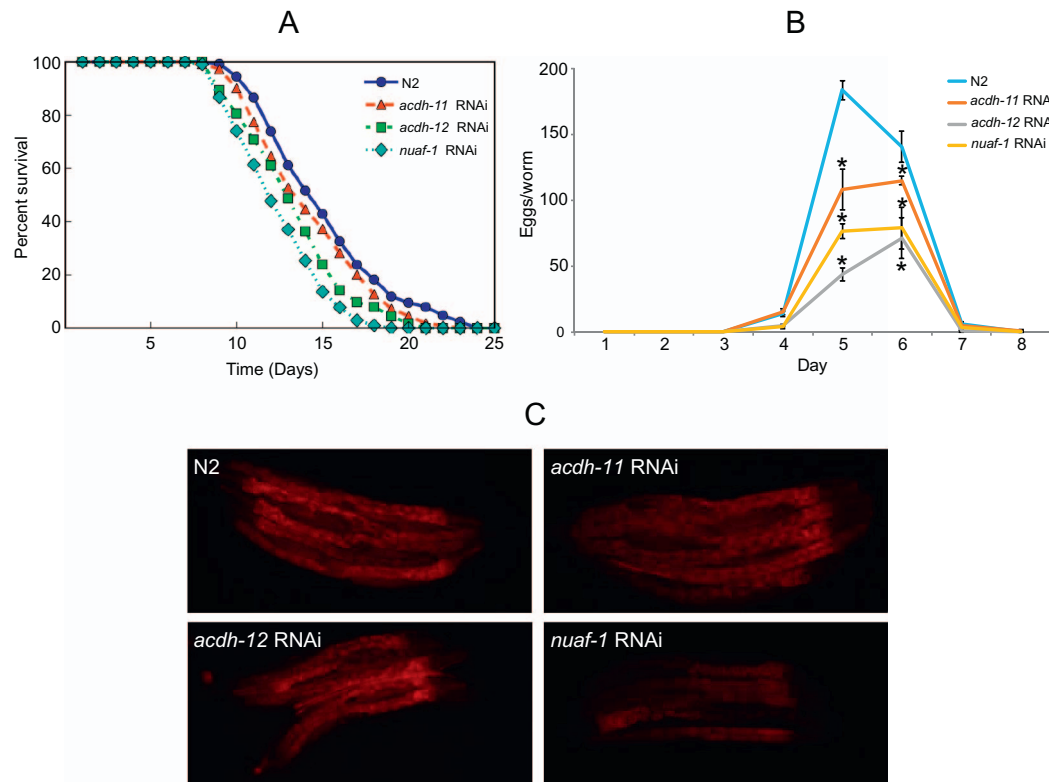


Fig. 2. Phenotypic changes in the knockdown nematodes. Phenotypes of N2 and RNAi-knockdown strains including *acdh-11*, *acdh-12*, and *nuaf-1*, were determined. Lifespan (A) of N2 ($n = 147$), *acdh-11* ($n = 150$), *acdh-12* ($n = 150$), and *nuaf-1* ($n = 150$) is presented in blue circle, red triangle, green box, and cyan diamond, respectively. Fecundity of the nematodes (B) was investigated from 3 independent experiments, and represented as mean $\pm$ S.D. as shown. *Indicates statistical significance as $p < 0.05$ (t -test), compared to N2. Storage fat of the whole animals was stained by Nile red (C) and visualized under the fluorescent microscope. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

I-III and I-III-IV supercomplexes (Fig. 4A). The amounts of fully assembled complex I in *acdh-12* and *nuaf-1* knockdown as determined by complex I-in gel staining were remarkably decreased compared to N2 (Fig. 4B). Native immunoblotting of BNGs with the anti-NDUFS3 monoclonal antibody was also used to determine the amount of

complex I. The result was consistent to those observed from BNGs and complex I-in gel staining in which complex I-containing supercomplexes were diminished in *acdh-12* and *nuaf-1* knockdowns compared to N2 (Fig. 4C). However, *acdh-11* knockdown did not affect the amount of complex I.

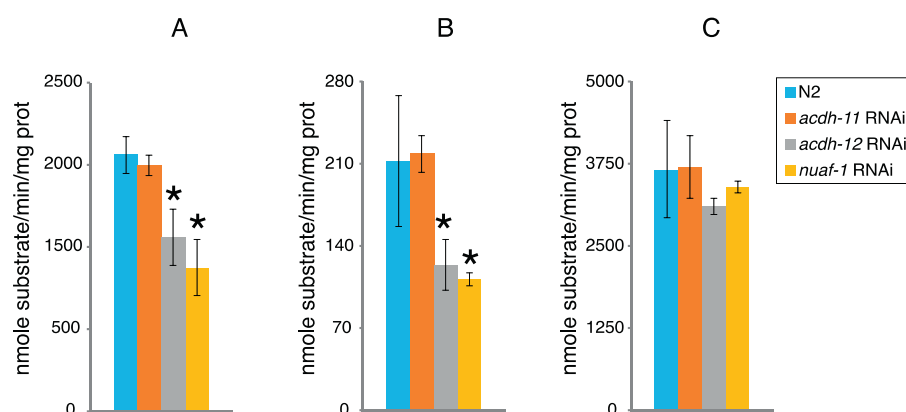


Fig. 3. Effect of RNAi knockdown on the respiratory complex function. Enzymatic activities of the respiratory complexes of nematodes were determined by spectrophotometric analysis. Complex I activity was measured by NFR and NADH-DUQ:DCPIP assays (A and B, respectively), where as complex IV activity was measured by cytochrome *c* oxidoreductase assay (C). Data are presented as mean $\pm$ S.D. from three to five independent experiments. *Indicates statistical significance as $p < 0.05$ (t -test) in comparison to N2.

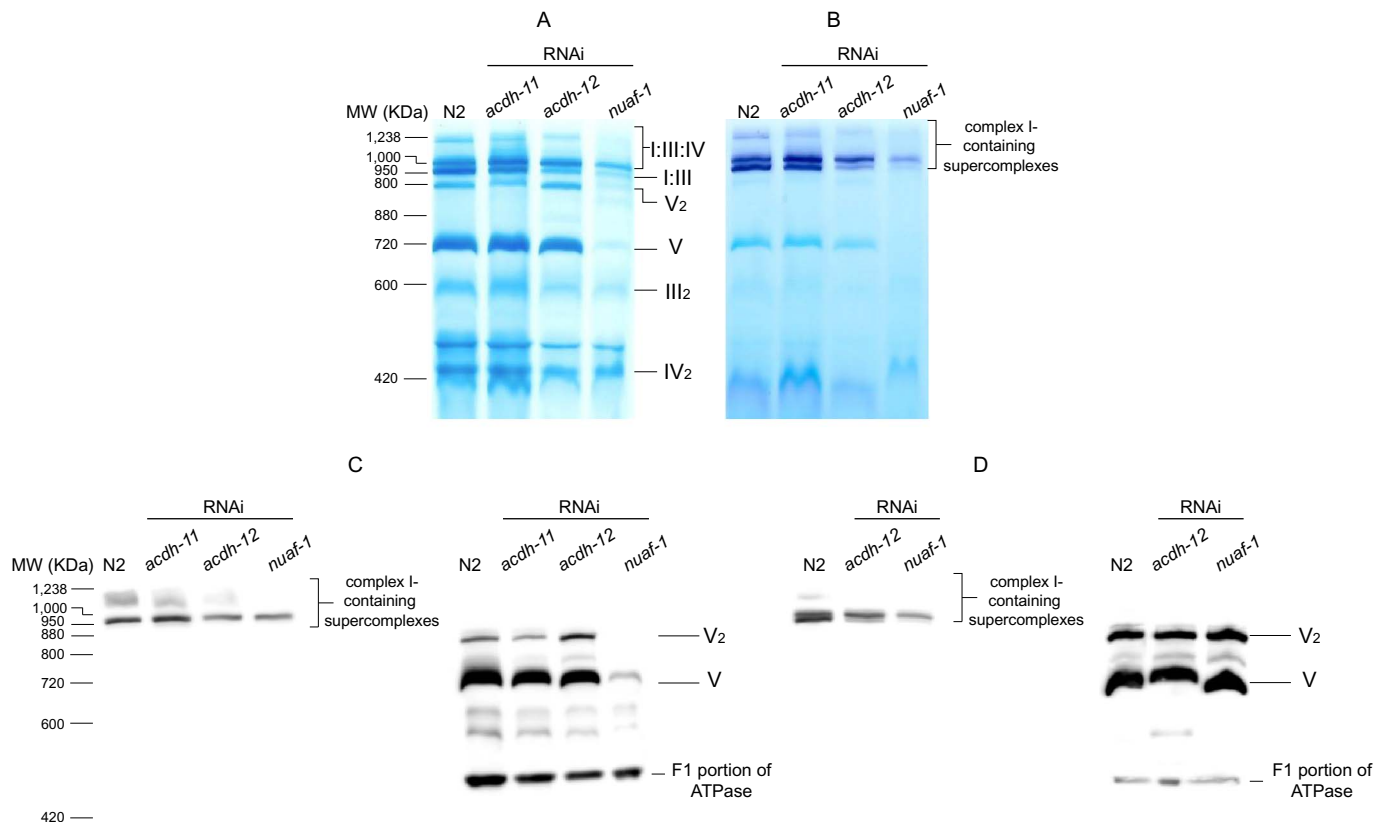


Fig. 4. RNAi knockdown of *acdh-12* affected respiratory complex I formation. (A) Blue native gel electrophoresis (BNG). Coomassie blue staining of BNG showed bands of respiratory complexes and supercomplexes as indicated. The Roman numeral represents respiratory complexes and supercomplexes. I:III:IV supercomplex is composed of complexes I, III and IV. I:III supercomplex is composed of complexes I and III. V and V₂ represent monomeric and dimeric complex V, respectively. III₂ is a dimeric complex III. (B) In-gel activity staining of complex I. Dark blue bands indicate positive staining of complex I. (C) and (D) Native immunoblotting of BNGs. Monoclonal antibodies against the NDUFS3 (left panel) and ATP5A1 (right panel) subunits were used to detect complex I in complex I-containing supercomplexes, and complex V (loading control for BNG) in two representative BNGs, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, we analyzed the amino acid sequence of the *C. elegans* VLCADs, i.e. ACDH-11 and ACDH-12, and found that both proteins possess the FAD-binding domain, the substrate-binding domain and the VLCAD domain, which are necessary for acyl-CoA dehydrogenase activity. We also analyzed their homology to human VLCAD and ACAD9 since ACAD9 has initially been categorized as a VLCAD but later found to also play a role in complex I assembly (Nouws et al., 2014). ACDH-12 shares higher score of amino acid identity with ACAD9 and VLCAD than ACDH-11 does. The identity score is extremely high considering invertebrate to mammal comparison, suggesting their functional homology. ACDH-11 was shown to have a tetrameric form or dimers of a dimer (Li et al., 2010; Ma et al., 2015) whereas ACAD9 is a homodimer as previously determined (Nouws et al., 2010). Although a crystal structure of ACDH-12 has not been investigated, this information suggests that ACDH-12 might be functionally closer to ACAD9 than ACDH-11.

The knockdown of the target genes was considered effective to exert phenotypic changes since it demonstrated > 50% knockdown efficiency in *acdh-11* and *acdh-12* (Supplemental data 2). We analyzed the inserted sequence in plasmid DNA of all RNAi bacteria by the nucleotide BLAST to determine their specificity to their target genes. We found that the inserted sequence is specific to target genes and is less likely to target other genes of the ETC complexes (inserted sequences shown in Supplemental Data 1; BLAST analysis data not shown). The shortened lifespans observed in *acdh-12* and *nuaf-1* knockdowns could reflect the alteration in mitochondrial function since this phenotype has been observed in some mitochondrial mutants such as *gas-1* (Suthammarak

et al., 2013) and *mev-1* (Yanase et al., 2002). Nonetheless there are also other mitochondrial mutants that have long lifespan such as *nuo-1* (Tsang et al., 2001) and *clk-1* (Kayser et al., 2004b). Therefore, shortened lifespan is not essentially correlated with a deficiency of the respiratory chain function. We then performed the fecundity test in the knockdown nematodes since this phenotype also indicates a deficiency in mitochondrial function (Suthammarak et al., 2010). We found that not only *acdh-12* and *nuaf-1* knockdowns but also *acdh-11* knockdown exhibit significantly decreased fecundity. This could suggest that decreased fecundity may also indicate a deficiency in the fatty acid oxidation (Hansen et al., 2013). Interestingly *acdh-12* knockdown exhibited the most severe fecundity defect. This observation could mean that *acdh-12* knockdown leads to a defect in both mitochondrial function and fatty acid oxidation. Besides those phenotypes, we also noticed a lag in egg laying in *acdh-12* and *nuaf-1* knockdowns. We found that these two RNAi strains spent a few more hours than N2 and *acdh-11* knockdown to start to lay eggs (data not shown). Although there are other phenotypes that can be associated with ETC knockdown in worms such as activation of the mitochondrial unfolded protein response, and mitochondrial morphology, these phenotypes were not assessed.

RNAi knockdown of *acdh-12* exhibited a biochemical hallmark of complex I deficiency which includes the decreased function of complex I as determined by NFR and NADH-Q:DCPIP activity assays. *acdh-12* knockdown also exhibited decreased formation of complex I or complex I-containing supercomplexes as determined by BNG. Decrease in complex I function and formation in *acdh-12* knockdown was similar to *nuaf-1* knockdown as previously reported (van den Ecker et al., 2012). However, *nuaf-1* knockdown had a more severe complex I formation defect compared to *acdh-12* knockdown.

Our study suggests that ACDH-12 is required for the formation of the respiratory complex I as an assembly factor. A deficiency in ACDH-12 leads to complex I deficiency, shortened lifespan and decreased fecundity. However, it is also possible that ACDH-12 involves in an assembly process of complex I as a stabilizer. An assembly factor facilitates the assembly process by a come-and-go mechanism in which it finally dissociates from the protein complex when a fully assembled complex is completely formed such as NDUFAF-1, NDUFAF-3 and C3orf1 (Andrews et al., 2013; Fassone et al., 2011). In contrast, a stabilizer remains associated permanently with a fully assembled complex, e.g. cardiolipin (Pfeiffer et al., 2003), and COX7RP (Ikeda et al., 2013). Unfortunately, further investigation cannot be achieved since an antibody for ACDH-12 is not currently available. A prospective study may include the 2D BNG and immunoblotting to further characterize the role of ACDH-12 in complex I assembly.

Based on its amino acid sequence, ACDH-12 might also play a role in fatty acid oxidation, although our Nile Red staining failed to show any effects of RNAi knockdown on storage fat. We suspect that *C. elegans* might not mobilize very long-chain fatty acids as a major source of energy derived from fat. Noteworthy, a prospective study to test whether ACDH-12 crucially involves in fatty acid oxidation may include a more specific approach such as the acyl-carnitine profile study. If ACDH-12 is crucial for fatty acid oxidation, accumulation of the very long-chain fatty acyl-CoA can be detected in *acdH-12* knockdown.

In summary, we proposed that ACDH-12 is required for the formation of the respiratory complex I in *C. elegans*. It may co-operate the assembly process of complex I as an assembly factor, or play a role as a complex I stabilizer. This observation is similar to ACAD9 which is a protein that has initially been identified as a VLCAD but later found that have dual functions in fatty acid oxidation and complex I assembly (Nouws et al., 2014).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mito.2018.02.010>.

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PROCEEDING OF THE

GRADUATE RESEARCH FORUM 2016

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IN SIRIRAJ INTERNATIONAL CONFERENCE IN MEDICINE
AND PUBLIC HEALTH 2016 (SICMPH 2016)
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GRADUATE RESEARCH FORUM 2016

An Academic Conference for Graduate Student in Faculty of Medicine Siriraj Hospital, Mahidol University. In Graduate Research Forum 2016 (GRF 2016)

Poster Presentation: May 18th, 2016

Oral Presentation: May 19th, 2016

Electronic Poster Presentation: June 13th, 2016

Venue:

May 18th, 2016:

1st Floor, SiMR Building, Faculty of Medicine Siriraj Hospital, Mahidol University

May 19th, 2016:

201 Conference Room, 2nd Floor, Srisavaringhira Building,
Faculty of Medicine Siriraj Hospital, Mahidol University

June 13th, 2016:

101 Conference Room, 1st Floor, SiMR Building
Faculty of Medicine Siriraj Hospital, Mahidol University

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Graduate Research Forum 2016 in Siriraj International Conference in Medicine and Public Health 2016 (SICMPH 2016)

Dateline for Abstract Submission: Before March 31st, 2016

Language: English

Type of Presentation:

All abstract must be presented by poster presentation on May 18th, 2016 (1st Round). After that, the committees will select 11 Posters for oral presentation on May 19th, 2016 (Final Round)

Time for Presentation:

1st Round (May 18th, 2016)

- Poster Presentation: 5 Minutes for Poster Presentation and 3 Minutes for Q&A

- Final Round (May 19th, 2016): 12 Minutes for Oral Presentation and 3 Minutes for Q&A

Poster Size: Vertical Poster size 62 x 95 cm

Rewards: separated into 3 Groups as below;

Group I: Ph.D. Student

- 1st Prize : 7,000 baht (1 Reward)
- 2nd Prize : 5,000 baht (1 Reward)
- 3rd Prize : 3,000 baht (1 Reward)
- Consolation prize (Poster Presentation): 1,000 Baht (2 Reward)

Group II : M.Sc. Student in Basic Biomedical Research

- 1st Prize : 7,000 baht (1 Reward)
- 2nd Prize : 5,000 baht (2 Rewards)
- 3rd Prize : 3,000 baht (2 Rewards)
- Consolation prize (Poster Presentation): 1,000 Baht (4 Rewards)

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- 1st Prize : 7,000 baht (1 Reward)
- 2nd Prize : 5,000 baht (1 Reward)
- 3rd Prize : 3,000 baht (1 Reward)
- Consolation prize (Poster Presentation): 1,000 Baht (2 Reward)

Conference Fees: Free

Electronic Poster Presentation:

Oral presentation for awardee on June 13th, 2016

Qualifications for Delegate and Abstract Preparation Guidelines;

1. Presenter must be a graduate student who studying a Ph.D. / M.Sc. program or a Graduate Scholarship / Thesis Scholarship Recipient who already graduated, but has never presented in any GRF as specified in the Scholarship contract.
2. Presenter who had presented in a previous graduate research forum also allowed submitting for the GRF 2016, but a previous abstract are not accepted.
3. The submitting abstract must never be published in any kind of abstract book or proceedings.
4. Major advisor and delegate bear responsibility for ensuring that your contents, academic English writing and formatting meet the criteria for scholarly writing. It is not a responsibility of the committee to verify.
5. All abstract must be presented by poster presentation on 18 May, 2016 for the 1st round. After that, the committees will select 11 poster to be oral presentation on 19 May, 2016 (* **If oral presenter can not present on that date, he/she will be disclaimed for any reward**)
6. All participant must submit for E-Poster presentation in Siriraj International Conference in Medicine and Public Health 2016 (SICMPH2016)
7. The award recipient will present by oral presentation in SICMPH2016 on June 13th, 2016 and join the award ceremony.

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Time	Event						
11.30-12.00	Evaluation Committee Meeting at 1 st Floor SiMR Building						
12.00-12.50	Lunch for Evaluation Committee						
12.50-13.00	Registration for presenter at 1 st Floor, SiMR Building						
Poster Presentation							
Time	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
13.00-13.10	G1-01 Phatthamon Laphanuwat	G2-01 Perapon Nitayanon	G2-14 Rapeepun Shompoopueng	G2-27 Sataporn Butnampet	G2-40 Nantapon Rawarak	G2-53 Peerapa Techaniyom	G3-01 Pongsakorn Rungwittayanuwat
13.10-13.20	G1-02 Sirithip Chuaitjit	G2-02 Nuttaporn Nakkerd	G2-15 Tarmphong Luangboribun	G2-28 Thatchaya Rodrad	G2-41 Lapatsanant Chaisiriwong	G2-54 Varittha Trangan	G3-02 Kanya Pengrungreungwong
13.20-13.30	G1-03 Ekapot Singsuksawat	G2-03 Nattawat Chaiyawong	G2-16 Kedsara Tayong	G2-29 Orawan Tulyaprawat	G2-42 Jirawan Panachan	G2-55 Imran Sama-Ae	G3-03 Annop Mingkwan
13.30-13.40	G1-04 Prem Lamichhane	G2-04 Wiraya Eamsaard	G2-17 Nonthporn Piskanok	G2-30 Benjamaporn Dulnee	G2-43 Worawit Boonyatistan	G2-56 Thapanee Poompoung	G3-04 Krittidech Mingmai
13.40-13.50	G1-05 Penkhae Utaijaratrasmi	G2-05 Siriporn O-Thong	G2-18 Kittima Sakorn	G2-31 Kanokwan Samon	G2-44 Kessirin Sukarom	G2-57 Suthatip Kainu	G3-05 Wunphen Punchanung
13.50-14.00	G1-06 Kanchana Suksri	G2-06 Sarinrat Jitjum	G2-19 Soraya Sangjan	G2-32 Nitinae Srising	G2-45 Phyoe Kyawe Myint	G2-58 Patpannee Khanthagan	G3-06 Nardnaree Karnchanachettanee
14.00-14.10	G1-07 Anyapat Atipimonpat	G2-07 Wuttisan Saengkeaw	G2-20 Worawan Dachavichitlead	G2-33 Sandeepa Ghimire	G2-46 Janya Grainok	G2-59 Samaporn Yongyod	G3-07 Somruetai Satmun
14.10-14.20	G1-08 Nutchanawan Promnakhon	G2-08 Chayanee Kaewthong	G2-21 Kanchariya Kuljiraseth	G2-34 Natthaphorn Kaewchot	G2-47 Arnat Pasena	G2-60 Wannasiri Chiraphappaiboon	G3-08 Nithanthaya Klungploy
14.20-14.30	MD-01 Pornlada Likasitwatanakul	G2-09 Wanida Boonmark	G2-22 Chompunuch Klinmalai	G2-35 Tanwarat Thanwisate	G2-48 Jariphorn Sukjaijearoenphorn	G2-61 Wasinee Rattanasirirat	G3-09 Nattawut Chaychum
14.30-14.40	MB-01 Wipawan Thangnipon	G2-10 Kanlayanee Sawangsalee	G2-23 Sujintana Janesomboon	G2-36 Chawin Korsirikoon	G2-49 Petlada Yongpitakwattana	G2-62 Thanaporn Mahutchariyakul	G3-10 Buntita Tuntichusak
14.40-14.50	MB-02 Wipawan Thangnipon	G2-11 Teerawin Bouma	G2-24 Chanin Hatthakaroon	G2-37 Farhana Padungraksart	G2-50 Sutthicha Matamnan	G2-63 Paveena Somboon	G3-11 Mareeyah Nirunrat
14.50-15.00	-	G2-12 Porntep Tanyut	G2-25 Sasiwimon Utama	G2-38 Thitipong Naowasiri	G2-51 Apinya Sayinta	G2-64 Kamonporn Sengking	G3-12 Duangkamon Eakkunnathum
13.50-15.10	-	G2-13 Pornsuk Romputtan	G2-26 Sujiraphong Pharkjaksu	G2-39 Sukanya Chumchuen	G2-52 Pimporn Reoksrungruang	G2-65 Jittiporn Wongpun	G3-13 Thanaporn Poophalee
15.10-15.20	-	-	-	-	-	G2-66 Paninee Mongkolsuk	G3-14 Teeraphan Chalermrungroj
15.20-15.30	-	-	-	-	-	-	G3-15 Weeraphong Chalermrungroj
15.30-16.15	Coffee Break						
16.15	Awards Announcement						

Schedule of Graduate Research Forum 2016

Oral Presentation May 19th 2016, 201 Conference Room, 2nd Floor, Srisavarindhira Building

Time	Event
8.00 – 8.20	Registration
8.20 – 8.30	Opening Ceremony by Prof. Suwannee Suraseranivongse
Session I; M.Sc. Research in Basic Biomedical Research Chair: Assoc. Prof. Chatchawan Srisawat, MD, PhD Co-Chair: Asst.Prof.Iyarit Thaipisuttikul, MD, PhD	
8.30 – 9.00	Invited lecture I: Lect. Phatchariya Pannasilp, PhD Topic : Roles of Pyruvate Carboxylase in breast cancer
9.00 – 9.15	G2-46 Pooled mRNA-pulsed Dendritic Cells Enhance Efficacy of Cellular Immune Responses against Cholangiocarcinoma – Janya Grainok
9.15 – 9.30	G2-56 The Role of MiR-125b in Drug Resistance in Acute Myeloid Leukemia (AML) - Thapanee Poompoung
9.30 – 9.45	G2-12 Simultaneous Detections of Thalassemia Mutations, HLA haplotyping and Chromosomal Aneuploidy for Preimplantation Genetic Diagnosis (PGD) in a Single Workflow combining Comprehensive Molecular Analyses and Next Generation Sequencing (NGS) - Porntep Tanyut
9.45 – 9.55	Coffee Break
Session II; M.Sc. Research in Basic Biomedical Research Chair: Asst. Prof. Wattana Watanapa, MD, PhD Co-Chair: Assoc. Prof. Suwattanee Kooptiwut, MD, PhD	
10.00 – 10.30	Invited lecture II : Lect. Chinnavuth Vatanashevanopakorn, MD, PhD Topic : DKK1 and SHH suppress the enkephalinergic phenotype in mouse embryonic stem cell-derived medium spiny neurons
10.30 – 10.45	G2-20 Identification of 25 kDa protein from Leptospira interrogans and application for immunodiagnosis of leptospirosis – Worawan Dachavichitlead
10.45 – 11.00	G2-38 Retinal and White Matter Damage Related to Memory Deficits in Voice-Cued Fear Test attenuated by Sea Cucumber Extracts (Holothuria Scabra) – Thitipong Naowasiri
Session III; Ph.D. Research in Basic Biomedical Research Chair: Assoc. Prof. Adisak Wongkajornsilp, MD, PhD Co-Chair: Assoc. Prof. Uraiwan Panich, MD, PhD	
11.00 – 11.30	Invited lecture III : Lect. Prapaporn Juengtrakool, PhD Topic : Exome sequencing for identification of sequence variants causing autosomal dominant diabetes
11.30 – 11.47	G1-05 MiR-15a-modulated PAI-2 and VEGF-A Expressions by Cancer-associated Fibroblasts Promote Aggressiveness of Cholangiocarcinoma – Penkhae Utaijaratrasmi
11.47 – 12.04	G1-01 A novel anti-ROS role of cyclin D1 in breast cancers – Phatthamon Laphanuwat
12.04 – 12.15	G1-04 Infectivity and Cytokine Production by Influenza Infected Monocytic Cells and their Association with NS Genomic Segment – Prem Lamichhane
12.15 – 13.30	Lunch Break
Session IV; M.Sc. Research in Clinical and Social Science Research Chair: Asst. Prof. Chalangkwan Tangbanluekal, PhD. Co-Chair: Asst. Prof. Peti Thuwajit, MD, PhD.	
13.30 – 14.00	Invited lecture IV: Lect. Wanlop Atsariyasing, MD Topic : Enhanced stress reactivity to emotional stimuli in a discrete subset (polydipsic) of schizophrenia patients
14.00 – 14.15	G3-06 Emission of volatile organic compounds from the immature stages of blow fly Chrysomya megacephala - Nardnaree Karnchanachettanee
14.15 – 14.30	G3-12 Genetic Analysis of Sodium Voltage-gated Channel Alpha Subunit 5 in Thai Cadavers Died with Sudden Unexplained Nocturnal Death Syndrome - Duangkamon Eakkunnathum
14.30 – 14.45	G3-08 The Association of Firing Distances and Terminal Ballistics as Evaluated by a 5.56 mm Bullet - Nithanthaya Klungploy
14.45 – 15.00	Coffee Break
15.00	AWARDS ANNOUNCEMENT

List of Presenters

Graduate Research Forum 2016

No	CODE	Research Title	Firstname	Lastname	Department	Level
01	G1-01	A Novel Anti-ROS Role of Cyclin D1 in Breast Cancers	Phatthamon	Laphanuwat	Pharmacology	Doctoral Degree
02	G1-02	Effect of RNAi knockdown of the Fatty Acyl-CoA Dehydrogenases on Mitochondrial Supercomplex Integrity in <i>Caenorhabditis elegans</i>	Sirithip	Chuaijit	Biochemistry	Doctoral Degree
03	G1-03	Effect of Estrogen on Experimental Cholangiocarcinoma Progression	Ekapot	Singsuksawat	Immunology	Doctoral Degree
04	G1-04	Infectivity and Cytokine Production by Influenza Infected Monocytic Cells and their Association with NS Genomic Segment	Prem Prasad	Lamichhane	Medical Microbiology	Doctoral Degree
05	G1-05	MiR-15a-modulated PAI-2 and VEGF-A Expressions by Cancer-associated Fibroblasts Promote Aggressiveness of Cholangiocarcinoma	Penkhae	Utaijaratrasmi	Immunology	Doctoral Degree
06	G1-06	Effect of Genistein in Prevention of Dexamethasone-induced Pancreatic β -cell Apoptosis	Kanchana	Suksri	Medical Physiology	Doctoral Degree
07	G1-07	Role of Interleukin-33 in Cholangiocarcinoma Progression	Anyapat	Atipimonpat	Immunology	Doctoral Degree
08	G1-08	Ovarian Cancer-Associated Fibroblasts Attenuate Autophagy and Promote Ovarian Cancer Cell Migration	Nutchanawan	Promnakhon	Immunology	Doctoral Degree
09	G2-01	Serotype Distribution and Antimicrobial Susceptibility of <i>Streptococcus pneumoniae</i> Causing Invasive Pneumococcal Disease (IPD)	Perapon	Nitayanon	Medical Microbiology	Doctoral Degree
10	G2-02	Correlation of <i>gyrA/B</i> Mutations with Level of Susceptibility to Fluoroquinolone of MDR-TB Isolates	Nuttaporn	Nakkerd	Medical Microbiology	Doctoral Degree
11	G2-03	Changes in Asporin Expression of Newborn Mouse Lungs after Hyperoxia	Nattawat	Chaiyawong	Medical Physiology	Master Degree
12	G2-04	The Expression of Major Zinc Transporters in The Duodenum of Thalassemia Mice: A Preliminary Study	Wiraya	Eamsaard	Biochemistry	Master Degree

No	CODE	Research Title	Firstname	Lastname	Department	Level
13	G2-05	Levels of Ethambutol Resistance and <i>embB</i> Mutations in Clinical Isolates of Multidrug Resistant <i>Mycobacterium tuberculosis</i> (MDR-TB)	Siriporn	O-Thong	Medical Microbiology	Master Degree
14	G2-06	Teratogenic Effects of Pesticides Dicofol in Chick Embryos	Sarinrat	Jitjum	Anatomy	Master Degree
15	G2-07	The Teratogenic Effect of High-dose Caffeine Exposure on The Development of Chick Embryos	Wuttisan	Saengkeaw	Anatomy	Master Degree
16	G2-08	The Teratogenic Effect of a Single Dose of Carbaryl Insecticide on <i>in ovo</i> Chick Embryo	Chayanee	Kaewthong	Anatomy	Master Degree
17	G2-09	Teratogenic Effect of Monosodium Glutamate on Chick Embryo Development	Wanida	Boonmark	Anatomy	Master Degree
18	G2-10	The Teratogenic Effect of <i>Curcuma comosa</i> to the Development <i>in ovo</i> Chick Embryos	Kanlayanee	Sawangsalee	Anatomy	Master Degree
19	G2-11	Transforming Growth Factor- β Receptor Kinase Inhibitors Enhance The Anti-Cholangiocarcinoma Cytotoxicity of Cytokine-Induced Killer Cells	Teerawin	Bouma	Pharmacology	Master Degree
20	G2-12	Simultaneous Detections of Thalassemia Mutations, HLA haplotyping and Chromosomal Aneuploidy for Preimplantation Genetic Diagnosis (PGD) in a Single Workflow combining Comprehensive Molecular Analyses and Next Generation Sequencing (NGS)	Porntep	Tanyut	Immunology	Master Degree
21	G2-13	Genetic diversity of human polyomavirus (BK) in organ transplant patients at Siriraj hospital during 2012-2015	Pornsuk	Romputtan	Medical Microbiology	Master Degree
22	G2-14	Copy Number Variations (CNVs) of Alpha Globin Genes Influence Hematological Phenotypes in Beta-Hemoglobinopathies	Rapeepun	Shompoopong	Immunology	Master Degree
23	G2-15	Screening of Anti-amoebic Activity on <i>Neogleria fowleri</i> in Extracts of Thai Medicinal Plants	Tarmphong	Luangboribun	Microbiology	Master Degree

No	CODE	Research Title	Firstname	Lastname	Department	Level
24	G2-16	The Effects of Additional Potassium Periodate Treatment in Combination with The Standard Heat-RDE Inactivation of Post Influenza Vaccination Sera Using ELISA, HI and Microneutralization Assay	Kedsara	Tayong	Microbiology	Master Degree
25	G2-17	Determination of The Respiratory Complex I Activity in Fibroblasts of Patients with Leber Hereditary Optic Neuropathy	Nonthporn	Piskanok	Biochemistry	Master Degree
26	G2-18	Exposure to Cold Impairs Interferon-induced Antiviral Defense in A Human Respiratory Epithelial Cell Line	Kittima	Sakorn	Medical Microbiology	Master Degree
27	G2-19	Identification of Antibody Dependent Cell Mediated Cytotoxicity (ADCC) Antibody Epitopes of HIV-1 CRF01_AE in Early HIV-1 Infected Individuals	Soraya	Sangjan	Medical Microbiology	Master Degree
28	G2-20	Identification of 25 kDa protein from <i>Leptospira interrogans</i> and application for immunodiagnosis of leptospirosis	Worawan	Dachavichitlea d	Medical Microbiology	Master Degree
29	G2-21	Study of <i>ZHX2</i> Expression in β^0 -Thalassemia/HbE Erythroid Cells	Kanchariya	Kuljiraseth	Biochemistry	Master Degree
30	G2-22	Comparison Between Avicel based- and Low Melting Agarose Based-overlay Media in Plaque assays of Enteroviruses	Chompunuch	Klinmalai	Microbiology	Master Degree
31	G2-23	Development of a PCR-based Method for Detection of <i>Burkholderia thailandensis</i> and <i>Burkholderia pseudomallei</i> DNA from Soil Samples	Sujintana	Janesomboon	Immunology	Master Degree
32	G2-24	Molecular epidemiology of cryptococcosis in Siriraj Hospital	Chanin	Hatthakaroon	Medical Microbiology	Master Degree
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PROCEEDINGS

Serotype distribution and antimicrobial susceptibility of *Streptococcus pneumoniae* causing invasive pneumococcal disease (IPD)

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Abstract

Objective: To describe the trends in serotype distribution and antimicrobial susceptibility testing of *S. pneumoniae* causing invasive pneumococcal disease (IPD).

Materials and Methods: The serotype of 113 *S. pneumoniae* isolates obtained from normally sterile sites (109 from blood, 4 from CSF) during September 2012 to March 2015 was evaluated. The catchment area in this study included 27 hospitals from 8 provinces in Thailand. Multiple isolates from different sites from the same patients were counted only once. Serotypes were determined by quellung test, using various specific group and factor antisera. Antimicrobial susceptibility was performed by microbroth dilution test and standard disk diffusion methods.

Results: Ninety-six percent of clinical specimens were blood, while CSF was the minority. The most common vaccine serotypes were 6B (26.47%), 14 (21.57%) and 19A (18.63%). The overall serotype coverage by PCV10, PCV13 and PCV15 were 60.18%, 81.42% and 81.42%, respectively. The corresponding coverage of these pneumococcal conjugate vaccines for children ≤ 5 years were 62.65%, 80.72% and 80.72% respectively. Pneumococci were sensitive to penicillin [if nonmeningitis (83.15%), if meningitis (28.33%)] and cefotaxime likewise [(95.28%), (87.72%)], chloramphenicol (82.46%), erythromycin (49.22%), tetracycline (24.49%) and trimethoprim-sulfamethoxazole (30.77%). All were susceptible to ofloxacin, linezolid and vancomycin.

Conclusion: We demonstrated changes in serotype distribution and antimicrobial susceptibility of pneumococcal isolates causing IPD in Thailand. The results of this study may guide to prevention and treatment strategies of invasive pneumococcal disease.

Keywords: *Streptococcus pneumoniae*, pneumococcal conjugate vaccine, invasive pneumococcal disease, antimicrobial susceptibility

1. Introduction

Streptococcus pneumoniae is an important cause of bacteremia, sepsis, meningitis, pneumonia in children and the elderly with high morbidity and mortality worldwide. The recent estimates suggested the annual burden in the US included 30,000 cases of invasive pneumococcal disease (IPD), 500,000 cases of community-acquired pneumonia, and 25,000 deaths⁽¹⁾. It is important because of the high incidence of pneumococcal infections and the widespread increase in drug-resistant isolates. To date, 94 immunologically distinct serotypes of *S. pneumoniae* have been described⁽²⁾. Protection depends on an antibody which is serotype-specific. Despite this diversity, a relatively small number of serotypes are associated with the majority of diseases. The selection of antigens to include in pneumococcal vaccine is based on the predominant serotypes that cause the disease. There are 10-valent, 13-valent and 15-valent conjugate vaccines (PCV). PCV10 contained serotypes that were the most common causes of IPD in children and often confer drug-resistance (serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). PCV13 contains serotypes in PCV10 plus serotypes 3, 6A and 19A. PCV15 contains serotypes in PCV13 plus serotypes 22F and 33F.

We present here the prevalence of serotypes causing disease to assess the percentage coverage of the pneumococcal conjugate vaccines and the in vitro susceptibility of commonly used antibacterial agents in addition to new drugs against *S. pneumoniae* Thai isolates.

2. Materials and Methods

S. pneumoniae isolates from normally sterile sites were obtained from patients age 2 months -92 years from the collaborative network including 3 tertiary care public hospitals (Siriraj Hospital, King Chulalongkorn Memorial Hospital, Bhumipol Aduljadej Hospital), and 27 other alliance hospitals (18 private and 9 public hospitals), from September 2012 - March 31, 2015. The catchment area in this study included hospitals from 8 provinces in Thailand (Bangkok, Khonkaen, Nakhonratchasima, Phuket, Rayong, Samutprakarn, Samutsakorn and Songkla). The isolates were cultured on sheep blood agar and were sent to the Department of Microbiology, Siriraj Hospital, for serotyping and drug susceptibility testing. The isolates were confirmed to be *S. pneumoniae* by optochin test and bile solubility test. Only one isolate from each patient was studied. Each isolate was kept at -70°C in 5% trypticase soy broth plus 20% (v/v) glycerol until use⁽³⁾.

Capsular serotyping of isolates was performed by Quellung reaction⁽⁴⁾, using various specific group and factor antisera obtained from the State Serum Institute, Copenhagen, Denmark, according to the manufacturer's guideline. Serotyping for those including in the PCV13 vaccine was conducted using a phase contrast microscope [Olympus BX 50 Model U-MD08 with phase contrast lens (magnification, 10x100), Olympus Corporation, Tokyo, Japan]. When the isolates were not one of the PCV13 serotypes, they were labeled as nonvaccine types.

The isolates were tested for susceptibility to 9 drugs following the CLSI methods and criteria⁽⁵⁾. *S. pneumoniae* ATCC 49619 was used as the reference control. For penicillin and cefotaxime, the susceptibility was carried out by using standard microbroth dilution method with cation-adjusted Mueller-Hinton broth supplemented with 3% lysed horse blood. The susceptibilities to other antibiotics including chloramphenicol, clindamycin, erythromycin, levofloxacin, ofloxacin, tetracycline, trimethoprim/sulfamethoxazole, linezolid, and vancomycin, were tested by using the disk diffusion method. The vaccine coverage rates were presented statistically with 95% confidential interval.

3. Results

Over the study period, a total of 113 pneumococcal isolates were collected from clinical specimens (Table 1). From serotyping system, 102 (90.27%) of the isolates were typeable, whereas 11 (9.73%) were nontypeable (Table 2). These isolates were from the patients with mean age 13 (range 2 months - 92 years), and male:female ratio of 1.5:1. The serotype distribution of pneumococcal isolates causing IPD in various age groups was shown in Figure 1. The overall serotype coverage by 10-, 13- and 15-valent pneumococcal conjugate vaccines in all age group were: 60.18%, 81.42% and 81.42%. The corresponding coverage for children ≤ 5 years were 62.65%, 80.72% and 80.72% for patients aged 6-49, 50-64 and >65 years, respectively (Table 3).

The five most common serotypes were 6B (26.47%), followed by serotypes 14 (21.57%), 19A (18.63%), 19F (7.84%) and 9V (3.92%), respectively (Table 4 and Figure 1). Nonvaccine types were found in 18.58% of the isolates.

The penicillin susceptibility rates of the isolates were 93.15% using nonmeningitis and 28.33% using meningitis criteria, respectively. Likewise, 95.28% and 87.72% of the isolates were susceptible to ceftriaxone using nonmeningitis and meningitis criteria, respectively (Table 5) (Table 6). Overall, pneumococcal isolates were sensitive to chloramphenicol (82.46%), erythromycin (49.33%), tetracycline (24.49%), and trimethoprim-sulfamethoxazole (30.77%). All were susceptible to linezolid, ofloxacin and vancomycin.

4. Conclusion and discussion

Our results confirm the high incidence of pneumococci isolated from blood but rarely from cerebrospinal fluid or sterile sites, observed by other studies⁽⁶⁾. This implied pathogenesis of IPD that affected specific organs were from hematogenous spread. We found male predominate, similar to that found in other studies, although the reason for this is unclear⁽⁷⁾.

Pneumococcal serotypes may vary according to geographic location in that serotypes which are prevalent in some countries may not cause disease in others. In general, geographical differences in serotype distribution interfere with the development of one unique vaccine for worldwide use. In Palestinian territories, serotypes 6A/B, 14, 1 and 9V were common⁽⁸⁾. It is obvious that from this study, the five most common serotypes i.e., 6B, 14, 19A, 19F, 9V were different from those isolates.

We previously reported that patients in all age groups, the overall serotype coverage were 41.5% for PCV10, 59.3% for PCV13 and 59.7% for PCV 15. The corresponding coverage for children ≤ 5 years were 48.8%, 73.2%, and 73.2% respectively⁽⁹⁾.

Further studies regarding serotype coverage over *S. pneumoniae* by pneumococcal conjugate vaccines and antimicrobial susceptibility may help provide the better analysis of potential benefit of vaccines over time and cross-country comparison.

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Table 1. Distribution of pneumococcal isolates among specimen sources

Specimens	No. of isolates	Percentage
Blood	109	96.46
CSF	4	3.54
Total	113	100

Table 2. Percentage of pneumococcal serotyping

	No. of isolates	Percentage
Typeable	102	90.27
Nontypeable	11	9.73
Total	113	100

Figure 1 The serotype distribution of pneumococcal isolates causing IPD in various age groups

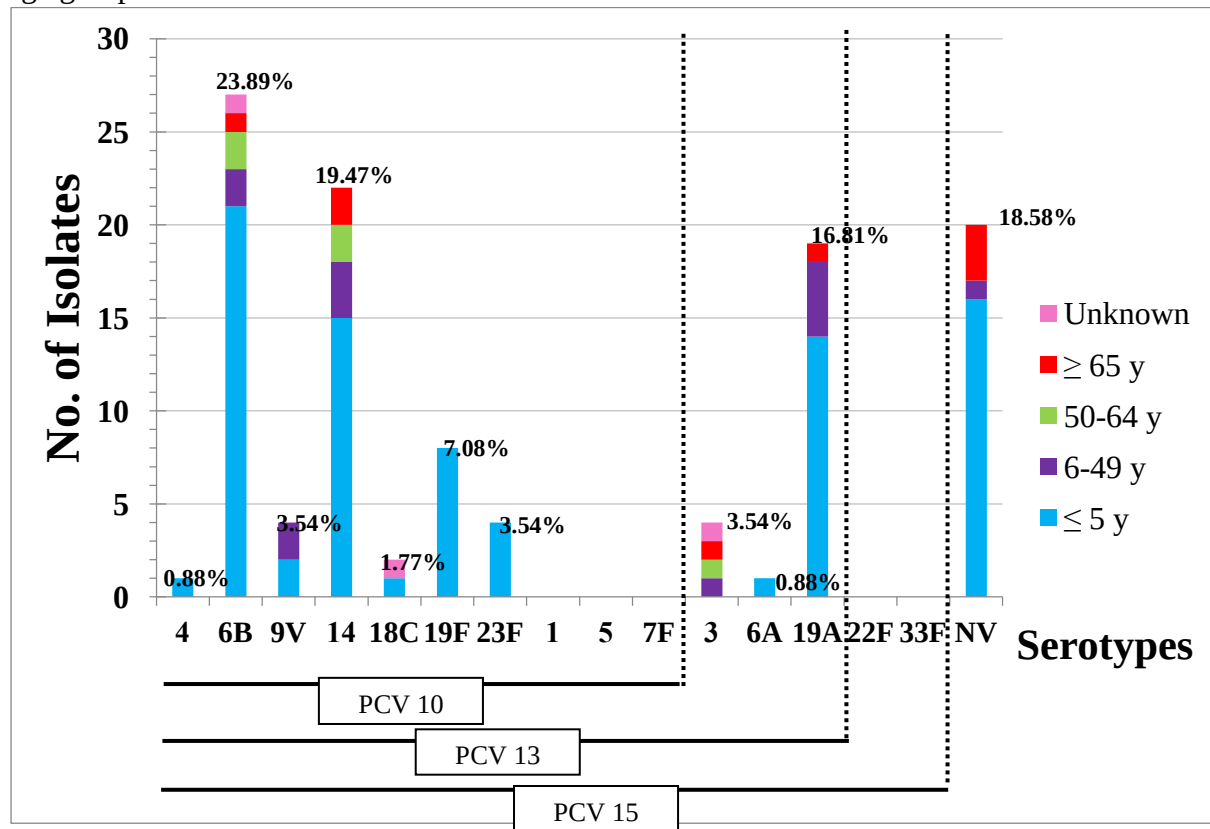


Table 3. Serotype coverage by 10-, 13- and 15-valent pneumococcal conjugate vaccine in various age groups

Serotype coverage by PCV	Serotype coverage rate (%)					
	Age ≤ 5 y (n = 83)	Age 6-49 y (n = 13)	Age 50-65 y (n = 6)	Age > 65 y (n = 8)	Unknown (n = 3)	All ages (n = 113)
PCV10	52 (62.65)	7 (53.85)	4 (66.67)	3 (37.50)	2 (66.67)	68 (60.18)
95% CI of percentage	(51.90,72.28)	(29.14,76.79)	(30.00,90.32)	(13.68,69.43)	(20.77,93.85)	(50.96,68.73)
PCV13	67 (80.72)	12 (92.31)	5 (83.33)	5 (62.50)	3 (100)	92 (81.42)
95% CI of percentage	(70.96,87.77)	(66.69,98.63)	(43.65,96.99)	(30.57,86.32)	(43.85,100)	(73.25,87.51)
PCV15	67 (80.72)	12 (92.31)	5 (83.33)	5 (62.50)	3 (100)	92 (81.42)
95% CI of percentage	(70.96,87.77)	(66.69,98.63)	(43.65,96.99)	(30.57,86.32)	(43.85,100)	(73.25,87.51)

Table 4. Serotypes of pneumococcal isolates

Order of Frequency	Serotype	No. of isolates	Percentage	Order of frequency	Serotype	No. of isolates	Percentage
1	6B	27	26.47	11	6A	1	0.98
2	14	22	21.57	12	8	1	0.98
3	19A	19	18.63	13	10	1	0.98
4	19F	8	7.84	14	1	-	-
5	15	8	7.84	15	5	-	-
6	9V	4	3.92	16	7F	-	-
7	23F	4	3.92	17	22F	-	-
8	3	4	3.92	18	33F	-	-
9	18C	2	1.96				
10	4	1	0.98				
					Total	102	100

Table 5. Susceptibility of penicillin and cefotaxime by broth microdilution method against *S. pneumoniae*

Antimicrobial agents	Susceptibility (%)			MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)
	Susceptible	Intermediate	Resistant		
Penicillin					
-nonmeningitis	93.15	0.0	6.85	0.38	1.00
-meningitis	28.33	6.67	65.00		
Cefotaxime					
-nonmeningitis	95.28	3.08	1.54	0.25	0.75
-meningitis	87.72	7.02	5.26		

Table 6. Susceptibility of other drugs by disk diffusion method against *S. pneumoniae*

Antimicrobial agents	Susceptibility (%)		
	Susceptible	Intermediate	Resistant
Chloramphenicol	82.46	0.0	17.54
Erythromycin	49.33	1.33	49.33
Linezolid	100	0	0
Tetracycline	24.49	2.04	73.47
TMP-SMX	30.77	13.85	55.38
Ofloxacin	100	0.0	0.0
Vancomycin	100	0.0	0.0

Changes in Asporin Expression of Newborn Mouse Lungs after Hyperoxia

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Abstract

Objectives: To determine the effect of high oxygen exposure on expression of asporin, an extracellular matrix molecule, in newborn mouse lungs with an arrest of postnatal alveolar formation (alveolarization).

Materials and Methods: Newborn C57BL/6 mice on postnatal day 3 were randomized for 7-day exposure of fraction of inspired oxygen (FI_{O_2}) of 0.21 (room air) or $\text{FI}_{\text{O}_2} \geq 0.90$ (hyperoxia group). Mean alveolar linear intercept (MLI) was measured to assess the airspace size. Lung asporin gene expression was determined by quantitative real time PCR.

Results: MLI of mouse lungs was significantly increased in hyperoxia compared with normoxia (42.33 ± 1.88 vs. $25.11 \pm 1.2 \mu\text{m}$; $n = 5-11$; $P\text{-value} < 0.001$). Asporin gene expression was significantly decreased in newborn mouse lungs after hyperoxia exposure compared with normoxia (15.91 ± 5.05 vs. $222.92 \pm 14.94 [\times 10^{-3} \text{ arbitrary unit}]$; $n = 4-8$; $P\text{-value} < 0.001$)

Conclusion: This study showed that alteration of asporin gene expression was detected in newborn mouse lungs after hyperoxia exposure implying the role of asporin on alveolarization.

Keywords: Alveolarization, Asporin, Hyperoxia, Bronchopulmonary dysplasia

1. Introduction

Lung is the organ of respiration, the process of gas exchange between the air and the blood. Development of mammalian lung is divided into 5 stages consisting of embryonic, pseudoglandular, canalicular, saccular and alveolar stage (1-3). Airspace development involves saccular and alveolar stage. During saccular stage, saccules or terminal sacs are created by the formation of typical clusters of widened airspaces from the peripheral airways. Saccular walls or primary septa, which are thick and consist mainly of capillary bilayer, serve as the basis for the formation of the secondary septa. A new tissue ridges from the existing primary septa and gradually protrudes small crest-like secondary septum, which divides alveolar sacs into alveoli. This process is alveolarization or alveogenesis, which is continuously repeated to maximize gas-exchange surface area until the double capillaries in alveolar walls merge as a thinner single capillary layer. In human, alveolarization starts at 36th week of gestation and continues to develop until about 7 years of age whereas alveolarization in mouse occurs after birth during postnatal day (P) 4- 28 (3-5).

Globally, approximately 12.9 million infants were preterm babies with underdeveloped lungs, providing a prevalence of preterm birth of 9.6 percent in 2009 (6). Preterm birth with oxygen supplement is the common risk factor of bronchopulmonary dysplasia (BPD) (7-9). BPD is a chronic lung disorder in infants associated with premature birth (< 32 weeks gestation), very low birth weight (< 1,000 g) and oxygen therapy (9-12). BPD is characterized by an arrest of alveolarization leading to abnormal lung growth, impaired pulmonary vascular development and lung inflammation (8, 13, 14). Mechanical ventilation, prenatal and postnatal infections and genetic susceptibility are also factors that can lead to BPD (15). One of the BPD genetic mouse models using fibroblast growth factor receptor (FGFR) 3/4 compound knockout mice showed a failure of alveolarization with abnormal airspace enlargement (16). The mouse lung abnormalities caused by FGFR 3/4 compound knockout also displayed primitive lung saccules and impairment of alveolar septation (16, 17). Gene discovery by microarray in FGFR 3/4 mutant lungs was performed to identify molecular defects associated with abnormal lung development. These microarray findings suggested that FGFR signalings are essential for the regulation of ECM formation involving proper alveolarization in mouse lungs.

Several extracellular matrix (ECM) proteins have been reported to regulate alveolarization (18). ECM transmits essential information to pulmonary cells and regulates pulmonary cells differentiation, organization, and function. The alterations in gene expression levels of several ECM components, including the small leucine-rich proteoglycan (SLRP) family, can affect alveolarization during lung development (19, 20). Previously, upregulation of asporin, one of the ECM molecules in SLRP family, and growth factor-binding extracellular matrix proteoglycan involved in cellular differentiation using microarray analysis was detected in infant lung tissue with BPD (21). Significant upregulation of asporin gene was also detected in BPD genetic mouse model using compound FGFR3/4 mutation (16). All these findings suggested that the lung overexpression of asporin involves in abnormal cellular differentiation leading to impaired alveolarization. Moreover, the human RNA blot hybridization study by Lorenzo and colleagues reported higher asporin expression levels in human fetal tissues than adult lungs, suggesting its involvement in fetal lung development (19). Recently, Wongkhat *et al.* reported that asporin gene expression rose during mouse alveolarization and reached the peak during P7 - P14, suggesting that asporin expression in the lung was highly correlated with mouse alveolar formation or alveolarization (31).

Asporin has been reported to be associated in alveolarization and airspace enlargement-related lung injury in human and mouse studies. In this present study, we hypothesized that asporin gene expression would be changed during postnatal mouse lung injury associated with BPD. We exposed newborn mice to high oxygen concentration for one week to disrupt alveolarization and induce airspace enlargement mimicking BPD pathology (32, 33). We used quantitative real-time PCR to determine asporin gene expression in the lungs with airspace enlargement from hyperoxia-exposed mice in comparison with the lungs during alveolarization in age-matched control group. This study provided further evidence for the potential involvement of asporin gene in airspace enlargement of a mouse model of BPD using hyperoxia exposure.

2. Materials and methods

Animal

Animals used in this study were newborn C57BL/6 mice. Newborn mice are defined as mouse pups up to 10 days of age, which were bred from adult C57BL/6 mice (National Laboratory Animal Center, Mahidol University) (34). They were housed in an airtight Plexiglas chamber, at constant temperature. All mice were supplied with food and water ad libitum and kept on a 12 hour (h) light-dark cycle. All experimental procedures were approved by Institutional Animal Care and Use Committee, Faculty of Medicine Siriraj Hospital, Mahidol University (SI-ACUP 019/2557).

Hyperoxia induction

Mouse pups at P3 were randomized into two groups; exposure to room air ($\text{FIo}_2 = 0.21$, normoxia) and high oxygen concentration ($\text{FIo}_2 \geq 0.90$) or hyperoxia group. On P10, lungs were harvested from all groups for further analysis. For oxygen exposure, mouse pups along with their mothers were placed in an airtight Plexiglas chamber. To minimize excessive oxygen toxicity for adult mice, nursing dams were rotated between hyperoxia and room air-exposed litters every 24 h. Throughout the experiment, oxygen level was monitored by oxygen analyzer (Servoflex Mini MP [5200 Multipurpose], Servomex, East Sussex, UK).

Bronchoalveolar lavage (BAL)

BAL was performed by instilling 0.2 ml normal saline (General Hospital Products Co., Ltd., Pathum Thani, Thailand) into the airway through a 24-gauge tracheal catheter then gently withdrawing BAL fluid. The fluid was collected and spun down. Cells were then precipitated on a glass slide by Centrifuge 5810R (Eppendorf, Hamburg, Germany). Cell counts were determined using hemocytometer. Differential cell counts were performed on slides stained with eosin (C.V. Laboratories, Bangkok, Thailand) and 0.5% methylene blue (Ajax Finchem Pty., Ltd., Auckland, New Zealand)

Lung histology

Mouse lung was inflated with 10% buffered formalin (The United Drug [1996] Co., Ltd., Bangkok, Thailand) via tracheal catheter at transpulmonary pressure of 25 cmH₂O for at least 15 minutes. The lung was fixed and further processed for paraffin section. After fixation, lung was cut into 5- μm thickness in the midsagittal plane with a microtome and stained with hematoxylin and eosin (C.V. Laboratories, Bangkok, Thailand). Finally, lung slice was imaged using the Zeiss AxioCam MRc digital camera for microscopy (Carl Zeiss Inc., Göttingen, Germany).

Lung morphometry

Mean linear intercept (MLI) value was used to determine airspace size on lung histological section slides (2). Previous studies found an increase in MLI of BPD mouse lung, corresponding to airspace enlargement (14, 35, 36). MLI determination was performed on H&E-stained lung section. The whole lung slice was imaged. The lung images were magnified and divided in fields for further analysis. The fields of selection were the center of image to avoid the areas containing border of lung or areas with no lung tissue. The same set of field numbers was consistently used for random selection on all lung images for further analysis. The selected fields were viewed under gridlines of equally spaced area. Each

gridline passes the wall of the lung structures. If the line passes a wall of an alveolus, an alveolar intercept was counted. The numbers of all intercepts were collected by two independent researchers. Finally, the MLI was calculated as length of grid lines divided by the average of alveolar intercepts from each lung.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from whole lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with ribonuclease-free deoxyribonuclease (DNase) I digestion (New England Biolabs, Ipswich, MA) to eliminate contaminating genomic DNA. Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). VeriQuest™ SYBR® Green qPCR Master Mix with ROX (Affymetrix, Santa Clara, CA) was used as qPCR reagent in this experiment. qPCR was performed in triplicate for each sample using oligonucleotide primers (Table 1) for mouse asporin and peptidylprolyl isomerase A (PPIA) (Integrated DNA Technologies, Singapore) by the Agilent Mx3005P QPCR system (Agilent Technologies, Santa Clara, CA). PPIA was used as a housekeeping gene for normalization.

Table 1 Oligonucleotide primers used for real-time PCR (2).

Primers	Primer Sequence (5'→3')	Tm (°C)
Forward Asporin	CTGATCTAGGTCTGACATCGGTTCC	57
Reverse Asporin	GAATCAGAGCATAAAGTGAAGTGAGTCC	57
Forward PPIA	AGGATTCATGTGCCAGGGTGG	58.2
Reverse PPIA	CATCCAGCCATTCAGTCTTGGC	58.2

Statistical analysis

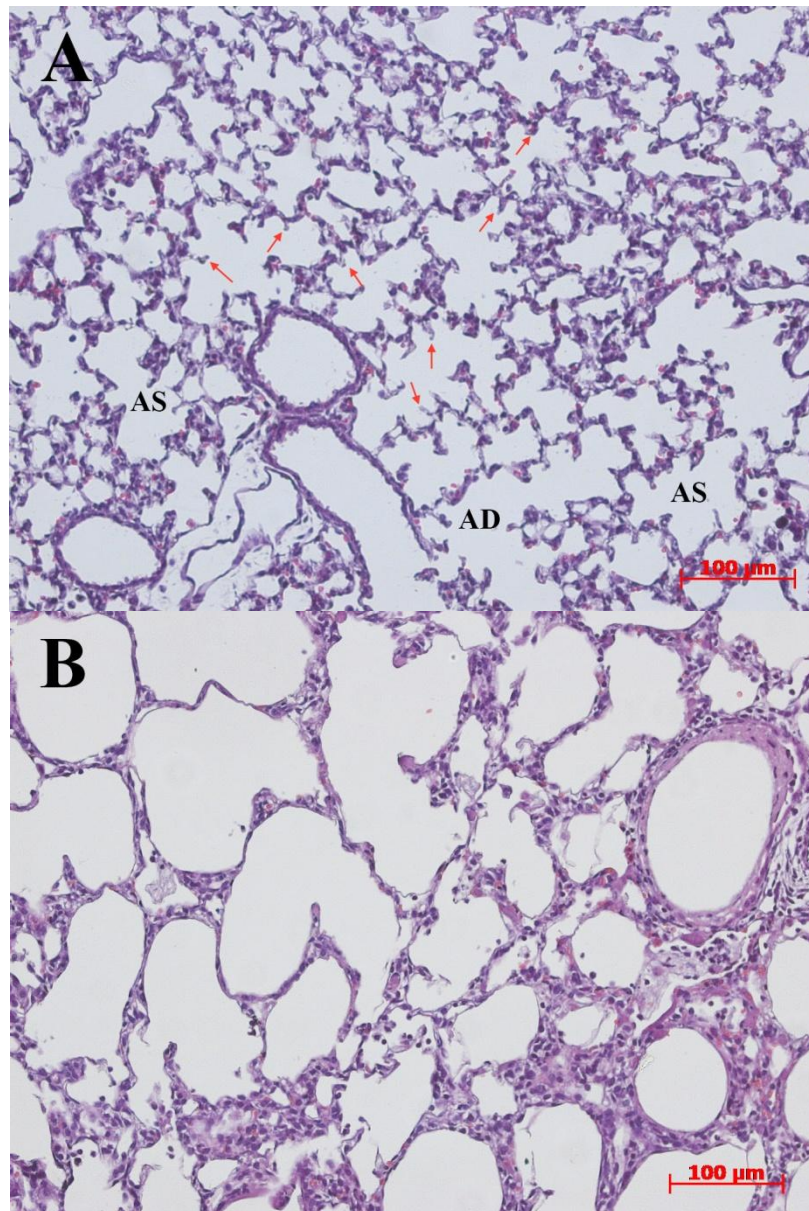
All data were presented as mean ± standard error of mean. Comparisons between normoxia and hyperoxia group were performed by unpaired t-test from SPSS Statistics version 17.0 (SPSS Inc., Armonk, NY). *P*-values less than 0.05 were considered statistically significant.

3. Results

Hyperoxia exposure in newborn mouse induces an arrest of alveolar septation and airspace enlargement

To induce BPD, newborn mice were exposed to hyperoxia ($FI_{O_2} \geq 0.90$) for 7 days. Hyperoxia-exposed mice developed a lung injury that mimicked human BPD. The mouse BPD model was characterized by lung inflammation and airspace enlargement. We detected the histology of normoxia and hyperoxia mouse lungs at P10 by H&E staining. Lung histology showed different size and structure of airspace between normoxia and hyperoxia group (Figure 1). The protrusion or the crest of the new tissue from the airspace wall was observed in normal mouse lung at P10 corresponding with a plentiful secondary septum partitioning saccules into alveolar ducts and alveoli, thus generating small airspace size, numerous alveolar numbers and eventually maximizing the alveolar surface area for gas exchange (Figure 1A). On the other hand, hyperoxia-exposed mouse lungs displayed an

incomplete development of lung structure similar to primitive lung saccules, no projection of alveolar crests due to lack of alveolar septation, alveolar walls breakdown resulting in airspace enlargement, and decrease in alveolar number leading to, significant decrease in alveolar surface area (Figure 1B). MLI assessment was performed to determine airspace enlargement. MLI of mouse lungs was significantly increased in hyperoxia compared with normoxia (42.33 ± 1.88 vs. 25.11 ± 1.2 μm ; P -value < 0.001). The MLI was a 68.58% increase in hyperoxia group, indicating an increase in airspace size or airspace enlargement (Figure 1C).



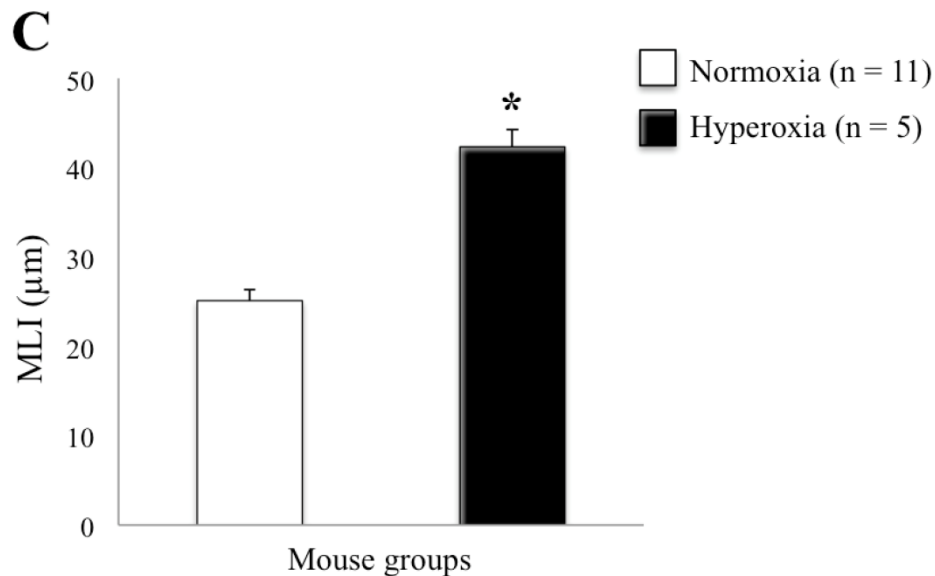


Figure 1 Hyperoxia exposure in newborn mouse induced airspace enlargement. Hematoxylin and eosin staining of C57BL/6 mouse lungs at P10 in normoxia (A) and hyperoxia (B). Mouse pups at P3 were exposed to room air and hyperoxia ($FI_{O_2} > 0.90$) for 7 days. Arrows indicate new tissue ridging from the existing primary septum, which divides saccules into alveolar ducts and alveoli. AS: alveolar sacs; AD: alveolar ducts. Mean linear intercept of normoxia and hyperoxia mouse lungs (C). Data are expressed as mean $\pm$ SEM. * indicates P -value < 0.05 compared with normoxia

Hyperoxia exposure in newborn mouse causes neutrophilic lung inflammation

To evaluate lung inflammation in newborn mouse after hyperoxia exposure, the percentage of white blood cells in BAL fluid was calculated, Macrophage was observed as highest percentage of differential cell count in normoxia (Figure 3). In contrast, neutrophil was observed as highest percentage of differential cell count in hyperoxia group. The percentage of neutrophil and lymphocyte was significantly increased in hyperoxia compared with normoxia (69.40 ± 4.7 vs. 3.55 ± 0.73 %; P -value < 0.001 for neutrophil; and 8.6 ± 1.86 vs. 1.55 ± 0.62 %; P -value < 0.016 for lymphocyte), whereas the percentage of macrophage was significantly decreased in hyperoxia compared with normoxia (21.00 ± 4.07 vs. 93.82 ± 1.64 %; P -value < 0.001). Total cell number was counted to estimate the number of each cell type. There was no significant difference in total cell number between hyperoxia and normoxia (6.25 ± 1.33 vs. $9.36 \pm 1.56 \times 10^3$; P -value = 0.17) (Figure 2A). The number of neutrophil was significantly increased in hyperoxia compared with normoxia indicating lung inflammation (3.96 ± 0.95 vs. $0.33 \pm 0.09 \times 10^3$; P -value < 0.01), whereas the number of macrophage was significantly decreased in hyperoxia compared with normoxia (1.94 ± 0.53 vs. $8.71 \pm 1.46 \times 10^3$; P -value < 0.01) (Figure 2B).

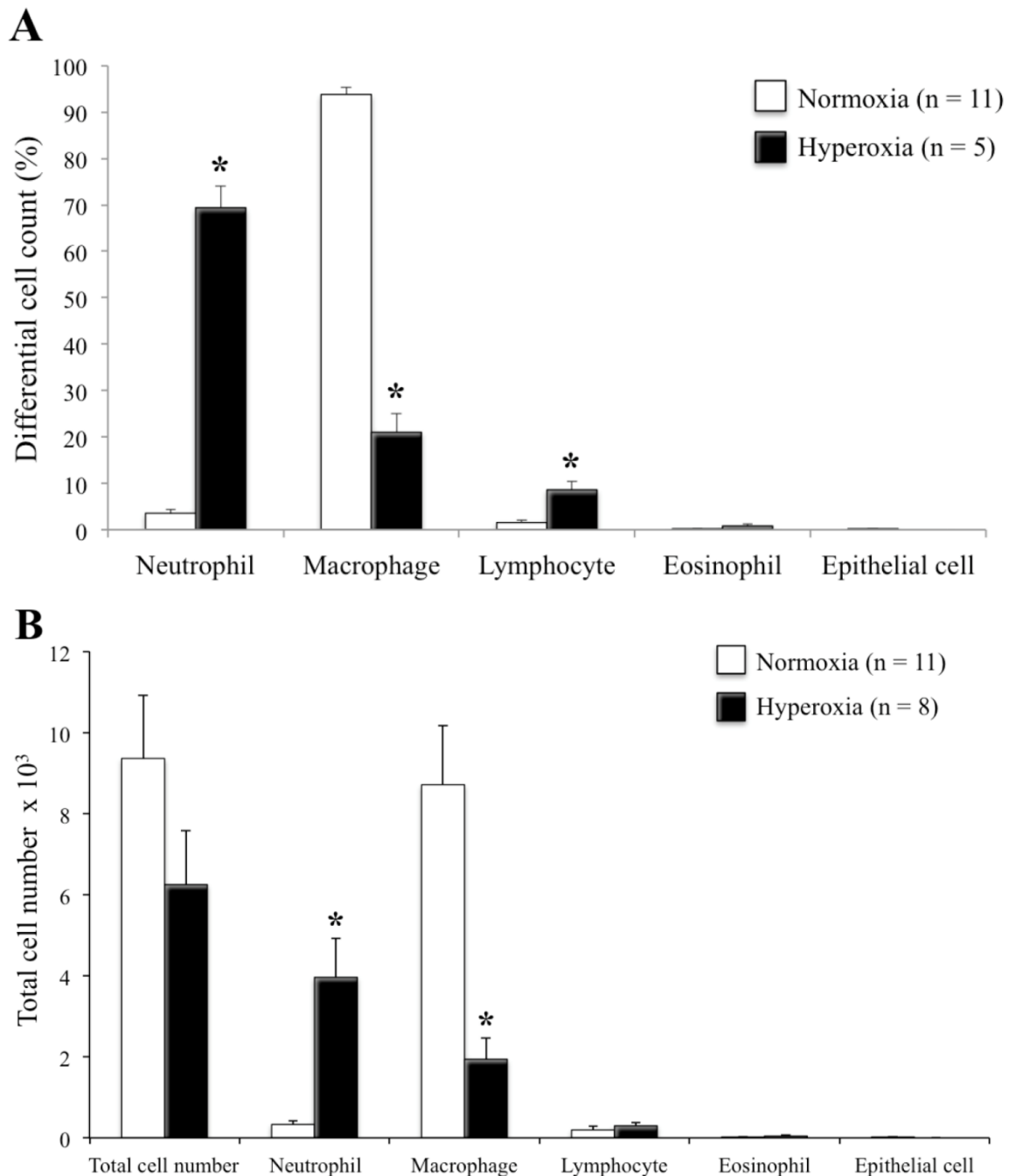


Figure 2 Hyperoxia exacerbates postnatal lung inflammation. The percentage of each cell type (A) and differential cell number (B) in BAL fluid was calculated after 7 day-exposure to room air or $\text{FIo}_2 \geq 0.90$. Data are expressed as mean $\pm$ SEM. * indicates P -value < 0.05 compared with normoxia

Hyperoxia exposure leads to asporin gene expression decrement in mouse lung

To investigate the alteration of asporin gene expression in newborn mouse after hyperoxia exposure, we detected the asporin gene expression in normoxia and hyperoxia mouse lungs at P10 by real time PCR. We found that lung asporin gene expression was significantly decreased in hyperoxia compared with normoxia (15.91 ± 5.05 vs. 222.92 ± 14.94 [$\times 10^{-3}$ arbitrary unit]; P -value < 0.001) (Figure 3). Asporin gene expression was a 92.86% decrease in hyperoxia.

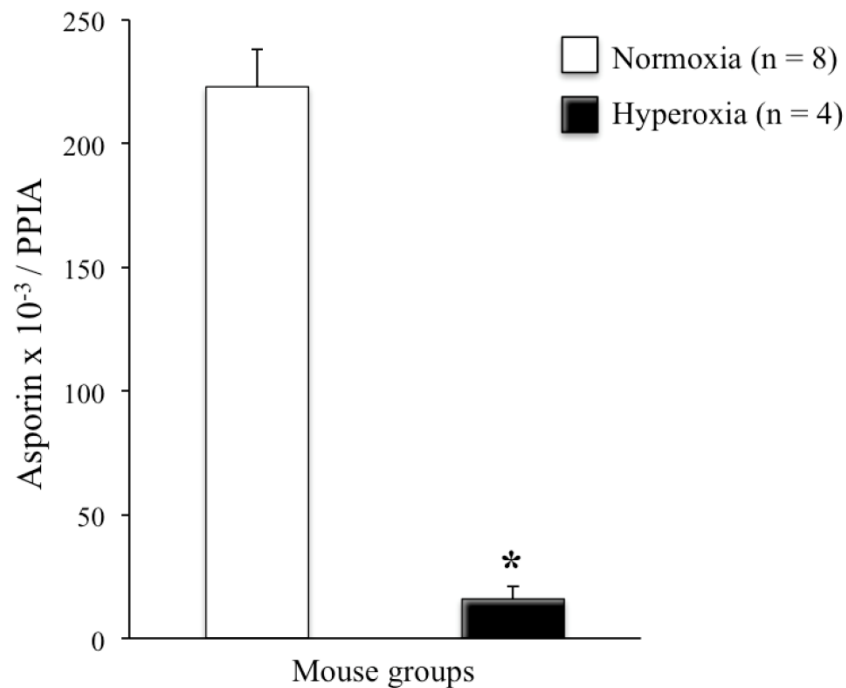


Figure 3 Asporin gene expressions in normoxia and hyperoxia mouse lungs. Data are expressed as mean ± SEM. * indicates *P*-value < 0.05 compared with normoxia

Discussion

Asporin gene expression has been reported to be associated with mouse lung alveolarization and its alteration may be involved in lung injury (16, 21, 31). An elevation of asporin gene expression was detected in lung tissues from human BPD subjects and BPD mice with genetic FGFR3/4 compound mutation. However, no data about the changes in asporin expression were available in a mouse model of injury-induced BPD. In this study, we tested the hypothesis that lung asporin gene expression was altered in newborn mouse after hyperoxia exposure. Our findings demonstrated that 7-day exposure of mice on postnatal day 3 to $\text{FIo}_2 \geq 0.90$ induced airspace enlargement, lung inflammation and significant decrease in asporin gene expression.

Alveolarization is the final stage of mammalian lung development. Perturbation of lung alveolarization results in abnormal lung structure, gas exchange impairment, and probably neonatal respiratory failure. Premature infants born with insufficient lung development often received oxygen supplementation for life support in the neonatal intensive care unit (7, 12, 37). However, supplementation of high levels of oxygen or oxygen therapy is toxic and damages the lung (7, 10). Lung injury in newborn infants can progress to pulmonary function impairment, decreased alveolarization and decreased vascular development, which are the pathological hallmarks of BPD (15).

Exposure to high oxygen tension is one of the critical risk factors in the development of infant chronic lung disease. High oxygen exposure in mouse model is widely used to mimic the pathophysiology of BPD (14, 32, 38-40). Hyperoxia-induced mouse lung injury showed lung inflammation consistent with histologic abnormalities similarly to infants with BPD. However, BPD infants mostly occur in preterm babies who received positive pressure ventilation and oxygen supplementation extending beyond 28 days of age (12). A hundred percent of oxygen exposure in newborn mice for 7 days showed high macrophage number indicating lung

inflammation, and an increase in alveolar chord length indicating airspace enlargement (32). Mouse lungs with high O₂ concentration exposure (FIO₂ = 0.75-0.85) for 10-14 days were found to develop macrophage and neutrophil recruitment into airspace, an increase in MLI, and a decrease in alveolar number associated with lung injury (14, 39). An increase in mortality rates up to 40% after a week of continuous exposure to 100% oxygen was reported (38). To minimize the mortality rates, injury to the lung was induced by using high O₂ concentration exposure for 7 days in this study. Mouse lungs at birth generally correspond with the saccular stage of lung development and further develop into alveolar stage during P4-28 (3). In our study, we selected to expose high O₂ tension to P3 mice whose lung development corresponds with saccular stage and histologically is comparable to preterm human infant lungs. At P10, their lung tissues were later harvested for analysis. Since mouse alveolarization or alveolar stage entirely takes place postnatally, we therefore selected to observe the effects of high oxygen exposure on alveolarization. We found an increase in neutrophil numbers indicating lung inflammation and an increase in MLI indicating alveolar enlargement, which are consistent with previous reports (14, 32, 38-40).

Normally, alveolarization in mouse starts at P4. The rise of asporin gene expression was observed in mouse lungs during P7-14, corresponding with alveolarization (2, 31). We identified an alteration of asporin gene expression in newborn mouse lungs after hyperoxia. Upregulation of asporin and growth factor-binding extracellular matrix proteoglycans involved in cellular differentiation using microarray analysis in BPD genetic mouse lung at P28 with double FGFR 3/4 mutation (16). These knockout mice developed postnatal abnormal airspace enlargement, similarly observed as in patients' lungs with BPD and pulmonary emphysema whereas pulmonary histological findings at birth looked comparably normal as age-match wild type control. Moreover, Bhattacharya et al further explored genome-wide expression data of human infant lung tissue with BPD from autopsy and detected upregulation of asporin gene in BPD lung tissue especially at early gestational age at birth (<27 week) in comparison with age-matched non-BPD lung samples (21). In contrast, we found that mouse lung asporin expression was significantly decreased in hyperoxia exposure, corresponding with disruption of alveolarization. We can conclude that high oxygen exposure can alter the regulation of asporin gene in association with affecting mouse lung development.

Asporin is an extracellular matrix protein member of class I small leucine-rich proteoglycan (SLRP) family. It was first identified in human cartilage (19). Asporin is commonly found in several tissues including articular cartilage, periodontal ligaments, uterus, aorta and lung (19, 27, 28). The role of asporin has been increasingly studied in articular cartilage and periodontal ligament because of its abundant expression (41, 42). Asporin expression has been reported with involvement in the pathogenesis of several diseases. Overexpression of asporin has been found in articular cartilage of patients with osteoarthritis and rheumatoid arthritis (41). It was shown that asporin suppresses chondrogenesis by blocking transforming growth factor beta 1 (TGF-β1). Maris and colleagues suggested that asporin directly bound TGF-β1 on the cell surface leading to TGF-β signaling inhibition (27). Generally, TGF-β plays a critical role in the regulation of cartilage homeostasis, including other ECM molecules of cartilage such as collagens (29, 43, 44). Inhibition of TGF-β can then contribute to impaired chondrogenesis, resulting in osteoarthritis. In addition, knockdown of asporin in human articular chondrocytes using small interfering RNA (siRNA) showed an increase in the expression of cartilage marker genes. They concluded that asporin has a physiological role as a negative regulator of chondrogenesis (29, 30). Asporin protein was

also produced and overexpressed in human breast cancer fibroblast (27).

Previous studies also found that asporin expression was induced by TGF- β 1 and it was suppressed by interleukin (IL)-1 β . Moreover, TGF- β 1 has been found to show a crucial role on the development, maintenance and repair of alveolar structure (45, 46). On the contrary, treatment with IL-1 β and tumor necrosis factor (TNF)- α , proinflammatory cytokines showed downregulation of asporin mRNA level in human articular chondrocytes during osteoarthritis process (41). The report suggested that asporin expression was regulated by proinflammatory cytokines. In similar fashion, we found the decrement of asporin gene expression after hyperoxia-induced lung inflammation. Proinflammatory cytokines and their role in the development of BPD infants and BPD mouse model were identified in recent years (47, 48). However, the molecular interactions between asporin and proinflammatory cytokines on lung injury are still unclear.

Currently, a few data have been reported about asporin regulation on lung development. In normal mouse lung, asporin gene expression showed the significant increase in wild type mouse lungs at P7 and 14 (31). These data suggested the possible involvement of asporin in alveolarization. Our data showed that asporin gene expression was significantly decreased in hyperoxia compared with normoxia. It is possible that disruption of alveolarization by hyperoxia, resulting in lung inflammation and airspace enlargement, may affect asporin gene expression. Moreover, hyperoxia may disrupt the association among ECM and cell signaling pathways during lung development (49-52). Excessive oxygen induced a series of cellular and molecular events including oxidative stress and inflammation. Reactive oxygen species and proinflammatory cytokines were produced leading to lung injury. On the contrary, asporin expression in BPD infants lungs was markedly increased in compared with age-matched control (21). This finding is not consistent with the result of our study. For animal model of BPD, only high oxygen concentration was used to induce BPD pathology. While BPD infants may be exposed to several factors other than oxygen supplement, such as mechanical ventilation, infection (8, 12, 33). Time point of oxygen exposure and abnormalities of lung development for investigation of these two studies might be different leading to distinction in severity of illness. Further investigations are needed to identify the relation between asporin expression and duration of hyperoxia exposure. Therefore, asporin protein expression and their mechanism on growth factors and cytokines including TGF- β signaling after hyperoxia induced mouse lung injury should be investigated. We proposed that asporin, might be a candidate for one of the mediators of aberrant lung alveolarization, causing abnormal airspace development in an animal model of BPD.

4. Conclusion

We noted that hyperoxia induced mouse lung injury in the developing lung leads to airspace enlargement and lung inflammation. These effects coincided with decrease in asporin gene expression after 1-week exposure of high oxygen tension. The data from this study suggested that hyperoxia affects the alteration of asporin gene expression in association with impaired alveolarization during mouse lung development.

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Teratogenic Effects of Pesticides Dicofol in Chick Embryos

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Abstract

Objective: This study was conducted to evaluate the teratogenic effect of pesticides, dicofol, in chick embryos which were exposed to different concentrations of dicofol. This study would reveal the dose specific of dicofol which caused teragenic effect. The morphological anomalies in total mount of day 3 of incubation were illustrated and finally, the results were compared with normal chick embryo and human.

Material and Method: Fertilized eggs of *Gallus demesticus* were randomly divided into 2 groups included control group which was injected with normal saline and the experimental groups which were injected with different concentrations of dicofol, 7, 8, 9, 11 g/L. Fertilized eggs were incubated at 37 °C in sterile incubator until day 3. The eggs were opened for observing and recording the survival and mortality rates, then examined by total mount technique.

Results: In the control group, survival rate of day 3 chick embryos was 100% and experimental group, survival and mortality rate of day 3 chick embryos showed non-consistent with the administrated doses. The highest survival rate was in 8 g/L gr, 54.16% and the highest mortality rate was in 9 g/L gr, 65.78%. All experimental groups showed morphological anomalies in several structures such as growth retardation, neural tube showed non-parallel dense lines, somites were not extend to caudal end, heart loop was dilated and was irregular U-shaped.

Conclusion: Dicofol can cause mortality and abnormal development in chick embryo although contamination at very low dose. Therefore people should avoid contaminating such pesticides especially the pregnant women.

Keywords: Teratogen, Dicofol, Chick embryos

1. Introduction

Dicofol, 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol is an organochlorine pesticide. It structurally compound is similar to dichlorodiphenyltrichloroethane (DDT). It is stored in fatty tissues. WHO estimated dicofol to be one of the class II (moderately hazardous) pesticide ⁽¹⁾. Trade names of dicofol including Benzenemethanol, Carbax, Cekudifol, Kelthane, Hilfol, Colonel and Acarin ⁽²⁾. It is widely used in agriculture for controlling spider mite. In Thailand, the annual import of dicofol was about 37,680 kilograms in 2007 ⁽³⁾. When

dicofol was used regularly for a long period of time, it retain in environment such as soil ⁽⁴⁾, river ⁽⁵⁾, vegetables and fruit too ⁽⁶⁾.

Reports regarding the dicofol toxicity in reproductive system of male albino rats, by decreasing testes and epididymis weight. It declined epididymus sperm count, percentage of sperm motility, viability and maturity and increased abnormal sperm morphology ⁽⁷⁾. In addition, report of female *Bufo Gargarizans*, it caused increase of ovary coefficient and the number of follicles and disordered the progress of follicle growth as well as induce the abnormality of the follicle structure ⁽⁸⁾. Dicofol therefore produced toxicity to reproductive system in both male and female animals. However the possible teratogenic effect were scanty in literature. This study was to study teratogenic effects of dicofol in chick embryos which was used as an animal model and compare results to human.

2. Materials and Methods

2.1 Pesticide

Dicofol (18.5 % EC) with commercial name ROTRAMITE was purchased from agrochemical markets selling in Thailand. Prepare working solution 20 ml every doses by dissolving dicofol in 0.9% normal saline.

2.2 Test animal

The fertilized eggs of leghorn hens (*Gallus demesticus*) were purchased from Suwanvajok-kasikit Research Station, Department of Animal Science, Faculty of Agriculture, Kasetsart University, Thailand.

2.3 Experimental design

Fertilized eggs were randomly divided into 2 groups, included the control group which was injected with normal saline and experimental groups which were subdivided into 4 subgroups by different concentrations of dicofol, 7, 8, 9, 11 g/L. The eggs were cleaned with 70% alcohol and incubated for 24 hours at 37 °C, 68-70% humidity in sterile incubator. Then they were injected with various concentrations of 7, 8, 9, 11 g/L at the volume of 0.15 mL into the yolk sac by sterile needle. In the control group, normal saline at the volume of 0.15 mL. was injected, then all were incubated until day 3. Then they were opened for observing and recording the survival and mortality rates. After that, the embryos were removed from the yolk sac, washed with normal saline, and fixed in Dietrich's FAA solution, transferred to 70% alcohol solution and processed for total mount preparation.

3. Results

The effects of dicofol on day 3 chick embryos were revealed by survival and mortality rates compared with control group. Survived chick embryo was determined by heart beating and blood circulation that according to stage 18 of Hamburger and Hamilton ⁽⁹⁾ in normal development chick embryo and compared to 4th week ⁽¹⁰⁾ of human embryo after fertilization.

Table 1. Percentages of survival and mortality rate in chick embryos day 3

Treatment group	Number of eggs for treatment	Survival		Mortality	
		(n)	(%)	(n)	(%)
Control	28	28	100	0	0
7 g/L	38	14	36.84	24	63.15
8 g/L	24	13	54.16	11	45.83
9 g/L	38	13	34.21	25	65.78
11 g/L	32	11	34.37	21	65.62

Table 1. showed the survival rate of the control group was 100% and the experimental groups showed the highest survival rate was in group of 8 g/L 54.16% and the highest mortality rate was in group of 9 g/L 65.78%.

The day 3 chick embryo showed that the control group was described as Figure A and experimental groups showed morphological anomalies of several structures such as neural tube, somites did not extend to caudal end, Unformed anterior and posterior limb buds and tail fold (Figure B), anterior and posterior neuropores were opened, severe retardation of the brain, unformed optic cup, lens vesicle, otocyst and branchial arch, heart loop was dilated irregular U-shaped (Figure D), and other growth retardations when compared with control group (Figure C, E).

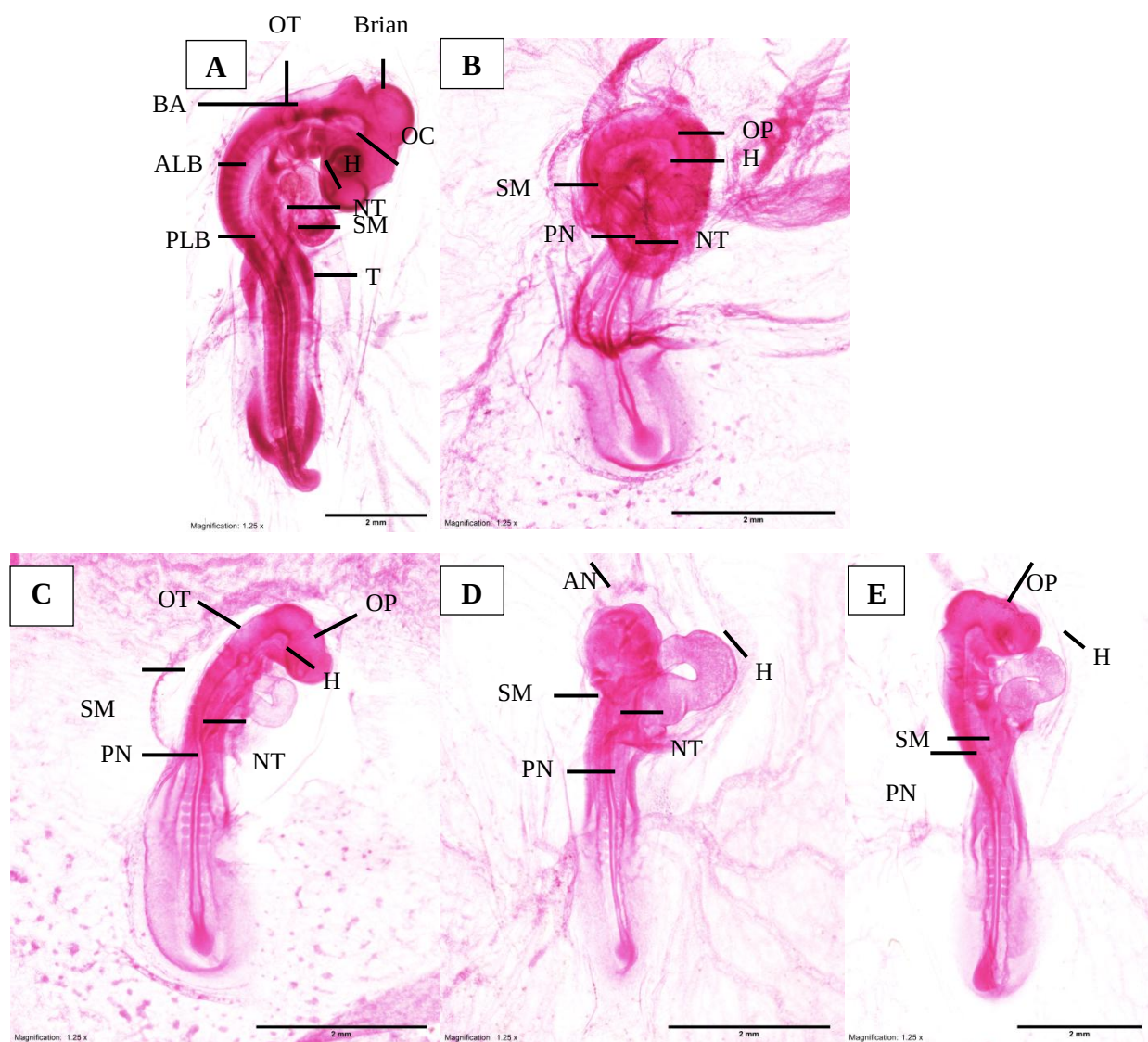


Figure 1 The micrograph of the day 3 chick embryos by total mount preparation. A, control group. B, a 7 g/L of dicofol treated embryo. C, a 8 g/L of dicofol treated embryo. D, a 9 g/L of dicofol treated embryo. E, a 11 g/L of dicofol treated embryo. (H: heart, OC: Optic cup, OT: Otocyst, BA: Branchial arch, ALB: Anterior limb buds, PLB: Posterior limb buds, NT: Neural tube, SM: Somite, T: Tail fold, OP: Optic primordia, OV: Optic vesicle, AN: Anterior neuropore, PN: Posterior neuropore)

4. Conclusion and discussion

This study was evaluated teratogenic effects of pesticides dicofol in chick embryos. Results revealed survival and mortality rate of the day 3 chick embryos was non-consistent with the dosages this was consistent with the study of Petrovova et al. (2009)⁽¹¹⁾ who described the exposure of bendiocarbamate on embryonic day 2, 3, 4, 5 cause sporadic mortality percent in chick embryo. In addition, morphological anomalies in several structures were similar to Nitu et al. (2012)⁽¹²⁾ who described the exposure of colonel-s on embryonic day 4 caused abnormal of various structures in chick embryo. Dose specific of dicofol which

caused teratogenic effect revealed at 7 g/L and 9 g/L so they could produce the most seriously morphological anomalies. As dicofol was teratogenic of the chick embryo it might also be harmful to human's if not necessary we should avoid contaminating such pesticides especially during pregnancy.

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The teratogenic effect of high-dose caffeine exposure on the development of chick embryos

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Abstract

Objective: To investigate the teratogenic effects of caffeine on the 3rd day chick embryos.

Material and Method: Fourty fertilized white leghorn hen eggs were randomly divided into 4 groups. They were injected at the 24th hours of incubation with NSS for the control group and 30 mg/ml, 40 mg/ml, 50 mg/ml of caffeine to the yolk sac of chick embryo at the equal volume of 0.15 ml for the experimental groups. After 72 hours of incubation of the embryos, the mortality and survival rates were recorded. The surviving embryos were harvested for the assessment of total mount preparation.

Results: The total mount showed several abnormalities such as looser U-shape of heart looping, dilated heart tube and microphthalmia of the eyes. Caffeine induced the growth retardation such as absence of cephalic flexure and cervical flexure. The five brain vesicle formation were incomplete. Moreover, the 2 dense lines of neural tube were unparallel, and absence of anterior limb buds and posterior limb buds were found. The experimental group of 50 mg/ml showed incomplete closure of the anterior neuropore. The percentage of mortality and surviving data showed the survival of chick embryos in the control group, at 30 mg/ml, at 40 mg/ml and 50 mg/ml were 100%, 60%, 63.63% and 40% respectively. The survival embryos were confirmed by the heart beating and blood circulation.

Keyword: caffeine, teratogenicity, chick embryo

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is a white crystal xanthine alkaloid which is worldwide consumed in various forms. Caffeine is a bitter substance found in many kinds of diet, such as coffee beans, tea, soft drinks, chocolate, kola nuts and also in medicines. It has many effects on the body's metabolism including the stimulation of the central nervous system (an increase in the activity of the sympathetic nervous system and a decrease in parasympathetic nervous system activity), diuresis, increasing heart rate and/or blood pressure after consumption (1). Caffeine consumption can boost energy level of the consumers, increase wakefulness, alleviate fatigue, and improve concentration and focus. On the other hand tachycardia, bradycardia, arrhythmia, hypertension, hypotension, vomiting, convulsions, coma and even death can occur when a caffeine overdose is consumed (2). Caffeine can also disturb the normal fetal development following overdose maternal intake.

Caffeine crosses the placenta to embryos. Although we are able to handle the amounts of caffeine we feed our body, but embryos cannot. The metabolism of embryos cannot fully

metabolize the caffeine. This experiment in the chick embryo will demonstrate whether caffeine is a teratogen or not. If so the pregnant woman should avoid drinking caffeine during pregnancy.

2. Materials and Methods

Fertilized white leghorn (*Gallus domesticus*) eggs were obtained from Suwanvajokasikit Research Station, Department of Animal Sciences, Faculty of Agriculture, Kasetsart University. The eggs were cleaned with 70% ethanol. The experimental group was subdivided into 3 groups by different dosages of caffeine, which were 30 mg/ml, 40 mg/ml and 50 mg/ml. The eggs were incubated at 37-38 °C and 68-70% humidity, for 24 hours before injection. All eggs were treated with the volume of 0.15 ml. solution to the yolk sac by sterile needle for all groups. After day 3 of incubation, the embryos were removed from the shells. The mortality rate were recorded, and surviving embryos washed with normal saline and underwent the process of total mount preparation.

3. Results

Table 1. The mortality and survival rate

Group (mg/ml)	n	Survival rate (%)	Mortality rate (%)
Control	10	10 (100)	0 (0)
30	10	6 (60)	4 (40)
40	10	6 (60)	4 (40)
50	10	4 (40)	6 (60)
Total	40	26 (65)	14 (35)

From The table 1 the percentage of mortality and surviving data showed the survival of chick embryos in the control group, at 30 mg/ml, at 40 mg/ml and 50 mg/ml were 100%, 60%, 60% and 40% respectively. The survival embryos were confirmed by the heart beating and blood circulation.

3.2 The total mount of the 3rd day chick embryos

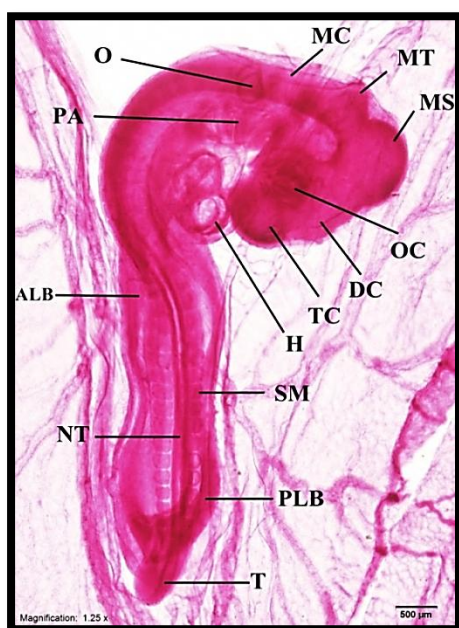


Figure 1.

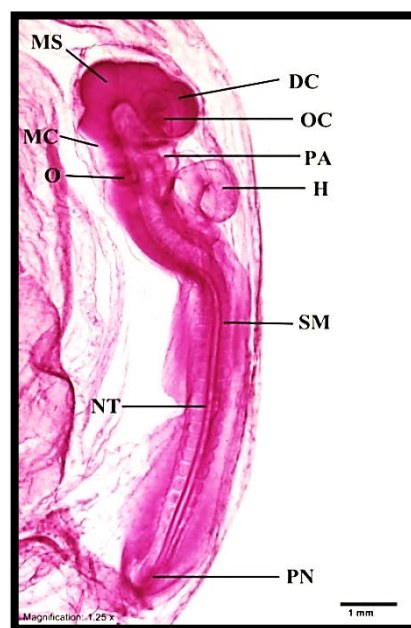


Figure . 2.

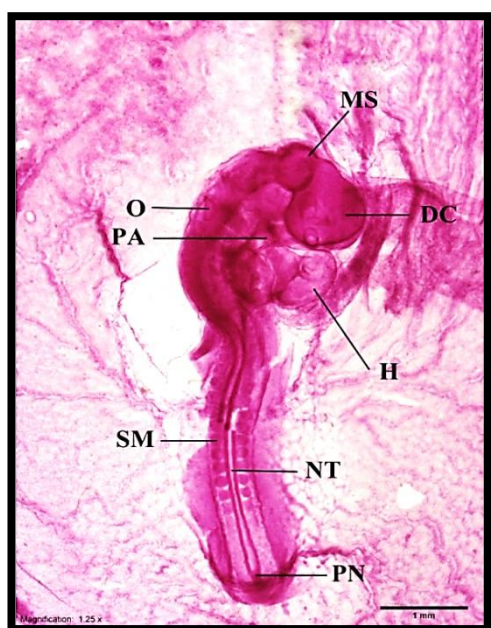


Figure . 3.

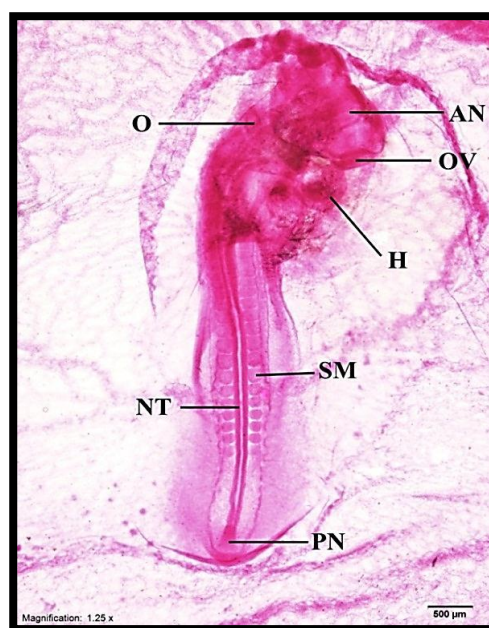


Figure . 4.

Figure 1. The micrograph showed normal development of the 3rd day chick embryo.

Figure 2. The micrograph showed growth retardation and abnormalities of the 3rd day chick embryo exposed to 30 mg/ml of caffeine concentration in experimental group

Figure 3. The micrograph showed growth retardation and abnormalities of the 3rd day chick embryo exposed to 40 mg/ml of caffeine concentration in experimental group.

Figure 4. The micrograph showed growth retardation and abnormalities of the 3rd day chick embryo exposed to 50 mg/ml of caffeine concentration in experimental group.

(TC = Telenchephalon, DC = Diencephalon, OC = Optic cup, MS = Mesencephalon, MT = Metencephalon, MC = Myelencephalon, O = Otocyst, PA = Pharyngal arch, H = Heart, ALB = Anterior limb bud, SM = Somite, NT= Neural tube, PLB = Posterior limb bud, T = tail fold, PN = Posterior neuropore, AN = Anterior neuropore).

In Figure 1., The micrograph showed normal development of the 3rd day chick embryo.

The 2 flexures of chick embryo were cervical flexure and cephalic flexure. The chick embryos had 5 secondary brain vesicles : telencephalon, diencephalon (forebrain), mesencephalon (midbrain), metencephalon and myelencephalon (hindbrain). There were optic cups with large size of horseshoe - shaped and complete lens at the middle of optic cup in the level of diencephalon. The heart was a S-shaped loop and the tail fold was visible at the caudal end (3).

In Figure 2., The micrograph showed growth retardation and abnormalities of the 3rd day chick embryo exposed to 30 mg/ml of caffeine concentration in experimental group. The body flexes slightly to ventral side at the point caudal to the heart. The heart was a dilated S-shaped heart looping. Only one pair of the pharyngeal arch existed. In addition, the brain was composed of primary brain vesicles (prosencephalon, mesencephalon, rhombencephalon) which continued to spinal cord and showed no cervical flexure as the cephalic flexure was visible at the level of mesencephalon. Moreover, the optic cup and lens at level of prosencephalon showed irregular shape and were smaller when compared with the control group, this was called microphthalmia.

In Figure 3., The micrograph showed growth retardation and abnormalities of the 3rd day chick embryo exposed to 40 mg/ml of caffeine concentration in experimental group. The body had 2 irregular twists either twisting to the ventral or the dorsal side. The heart was loosely dilation with irregular shaped heart looping. Only one pair of the pharyngeal arch existed. Moreover, the brain of the embryo showed primary brain vesicles (prosencephalon, mesencephalon, rhombencephalon) which continued to spinal cord at caudal end. The brain parts were incompletely separated from each other. Both the cephalic and cervical flexures were did not exist at the level of mesencephalon and myelencephalon. The optic cup and lens at the level of prosencephalon showed irregular shape, smaller in size and unclear visibility because this optic cup and lens were still in the primordium stage.

In Figure 4., The micrograph showed growth retardation and abnormalities of the 3rd day chick embryo exposed to 50 mg/ml of caffeine concentration in experimental group. The anterior neuropore was still open. The primary brain vesicles (prosencephalon, mesencephalon, rhombencephalo) continued to the spinal cord at caudal end and disappeared at the cephalic end. The pharyngeal arch caudal was absent. Moreover, the heart was very small, with loosely irregular shape looping when compared with the control group. The optic cup and lens at the level of prosencephalon showed irregularly shaped and smaller in size when compared with the control group. The lower body showed developmental retardation of the limb buds and tail fold.

4. Conclusion and discussion

The total mount showed several major abnormalities. The eyes showed microphthalmia which was in accordance with Kujawa-Hadryoe Monika in 2010 who studied caffeine associated ultrastructural changes in developing chicken cornea and found that

caffeine caused a decrease in the total thickness of corneal layer in the 10th and 19th day of chick embryos (4). The heart showed loosely dilation and irregular shaped heart looping corresponding to Christopher C. Wendler in 2009 who suggested that caffeine caused reduce ventricular myocardial tissue of mice embryos (5). Moreover, the five brain vesicles were absent in each doses of caffeine. This result was corresponding to Zheng-lai Ma in 2012. They found that caffeine caused defect of the neural tube, unclosure and scattering of somites (6). This study indicated that caffeine can cause abnormalities and growth retardation in many body structures. Caffeine may be a toxic agent and cause embryonic death. The severity of teratogenic effects were increased with higher dose of caffeine.

Further studies should be carried out in the elderly embryos before hatching such as the 6th day, and visceral organs of 10th day, 14th day.

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The Teratogenic Effect of A Single Dose of Carbaryl Insecticide on *in ovo* Chick Embryo

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Abstract

Objective : Carbaryl insecticide, a widely used of carbamate group of insecticide act as acetylcholinesterase inhibitor. This study was conducted to observe the effect of several doses of Carbaryl insecticide on the development of chick embryo.

Materials and Methods : The fertilized white leghorn hen eggs (*Gallus domesticus*) were divided into 2 groups. The control was injected with 0.9% NSS, the experimental groups were injected with 4 different doses of carbaryl insecticide including 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L, respectively into the yolk sac at the 24th hour of incubation. At day 3 of incubation, the living embryos were studied by the total mount and serial section techniques.

Results : The total mount of the day 3 of carbaryl insecticide treated groups of 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L showed retardation of the brain, eye, branchial arch development, cephalic and cervical flexures, neural tube, U-shaped looping, loosen and dilatation of heart. The treated group with 250 mg/L showed an absence of anterior, posterior limb buds and the tail fold, as well as opening of anterior neuropore. The serial section of the eye showed smaller of eye primordia and no elongation of the posterior lens fiber.

Conclusion : The results suggested that carbaryl insecticide could cause stillbirth, growth retardation and congenital malformations of the chick embryos. Therefore, pregnant women should avoid the exposure of carbaryl insecticide.

Keywords : Carbaryl insecticide, Teratogenic effect, Chick embryo

1. Introduction

Carbaryl (1-naphthyl-N-methylcarbamate) or SevinTM is a carbamate insecticide which registered in the United States in 1959 (1) . It is classified as class II or moderately hazardous according to World Health Organization,2009 (2). It is used to control many types of pest and affected on the nervous system by disrupting an enzyme that regulates acetylcholine, a neurotransmitter. It is a famous insecticide because of its low toxicity and short half-life in the environment. However, it is commonly found persisting in aquatic ecosystem(3). Previous report showed a detectable level of carbaryl insecticide in Chao Phraya river, Thailand (4) and residues of carbaryl insecticide in vegetables and fruits of agricultural products in upper northeast, Thailand (5).

Previous researches showed teratogenic effect of insecticide which caused reproductive problem or fetal death due to congenital anomalies. Carbaryl insecticide caused malformation development of zebrafish embryos (6), affected on gonadal development of chick embryos (7) and affected on axial-skeletal defect of xenopus laevis embryo (8). Carbaryl insecticide caused toxicity on different species except for mammalian(9). However, the report of California, United State showed that carbaryl exposure was during the 3rd-8th week of pregnancy on human had a risk for fetal affect and fetal death (10). Many studies showed that carbaryl insecticide caused toxicity to several organs of embryo but some studies stated that carbaryl insecticide was non-toxic. Chick embryo is a good model for animal experiment because it is inexpensive, easy to observe and manipulate. Moreover, it has short gestation time and data comparable to human embryo. Therefore, this study was conducted to show the teratogenic effect of single dose of carbaryl insecticide on the development of chick embryo.

2. Materials and Methods

2.1 Animal collection and egg grouping

The fertilized white leghorn hen eggs (*Gallus domesticus*) were purchased from Department of Animal Science, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen, Nakornpatom, Thailand. All eggs were cleaned with 70% alcohol and maintained in incubator at 37°C. The fertilized eggs were randomly divided into 2 groups : control and experimental group.

2.2 Carbaryl exposure

The control group (Group C) was injected with 0.9% normal saline while the experimental group (Group CB) was injected with varies doses of carbaryl insecticide. Group CB was subdivided into 4 subgroups with different concentration of carbaryl insecticide at 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L, respectively of which 0.15 ml of each concentration was injected into the yolk sac at 24 hour of incubation and placed back to incubate until day 3 of the experimental period.

2.3 Data collection and histological analysis

At day 3 of incubation, the eggs from each concentration were collected to observe and record for the mortality rate. Then, embryos were prepared to make permanent slide, stained with Mayer's carmalum for 10 minutes, dehydrated with ascending alcohol series, cleared with xylene, mouted on slide and covered with roundshaped cover slip, then observed by total mount technique.

The serial section were dehydrated with ascending alcohol series, infiltrated, embedded, sectioned at 6 µm, stained with H&E, mouted and covered with cover slip. Histological analysis was performed under light microscope.

3. Result

3.1 Survival and mortality rate

Table 1 showed percentage of survival and mortality of the day 3 chick embryo, indicated that the mortality rates were 0%, 16.67%, 9.10%, 16.67%, 16.67% in control group, 100 mg/L CB, 150 mg/L CB, 200 mg/L CB and 250 mg/L CB, respectively.

Table 1. Percentage of survival and mortality of the day 3 chick embryos.

Groups	% Survival	%Mortality
Control (0.9 %NSS)	100	0
100 mg/L CB	83.33	16.67
150 mg/L CB	90.91	9.10
200 mg/L CB	83.33	16.67
250 mg/L CB	83.33	16.67

3.2 Total mount of chick embryo

Figure 1. A showed normal development of chick embryo for 36 somite in control group. There were a cephalic flexure at mesencephalon and a cervical flexure between myelencephalon and spinal cord. Brain study was divided into 3 parts : forebrain, midbrain and hindbrain. Forebrain was divided into telencephalon and diencephalon. Midbrain was derived for mesencephalon. Hindbrain was divided into metencephalon and myelencephalon. Eye located at the level of diencephalon showed large optic cup with a lens located at the center. At the level of myelencephalon, the closed vesicle of otocyst was identified. At cervical flexure, level of stomodeum showed 4 branchial arches. Right side of the body showed heart tube. Normal heart of this period showed S shaped loop. Heart was divided into 4 parts: sinus venosus, atrium, ventricle and bulbus cordis, respectively. Middle of the body showed two parallel lines of neural tube with somites adjacent to it and extended to the caudal end. In addition the body showed anterior limb bud and posterior limb bud. The caudal end also showed a tail fold.

Figure 1B, 1C, 1E and 1F showed the development of chick embryo in total mount of day 3 which treated with carbaryl insecticide at the concentration of 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L, respectively. All of them showed growth retardation of several areas. Carbaryl treated at the concentration of 100 mg/L, 150 mg/L and 200 mg/L showed retardation with poorly development of cephalic and cervical flexures brain was not clearly developed, small eyes, small otocyst and one brachial arch. Carbaryl treated at the highest concentration of 250 mg/L showed the most severity of retardation. Figure 1E showed open of anterior neuropore, abnormal brain development. Eye, otocyst, branchial arch, cephalic and cervical flexures were absent. Heart showed U-shape looping, looser and dilatation. All showed unparallel dense line of neural tube. Somites extended to 75 % of body with the absent of anterior limb buds, posterior limb buds and tail fold.

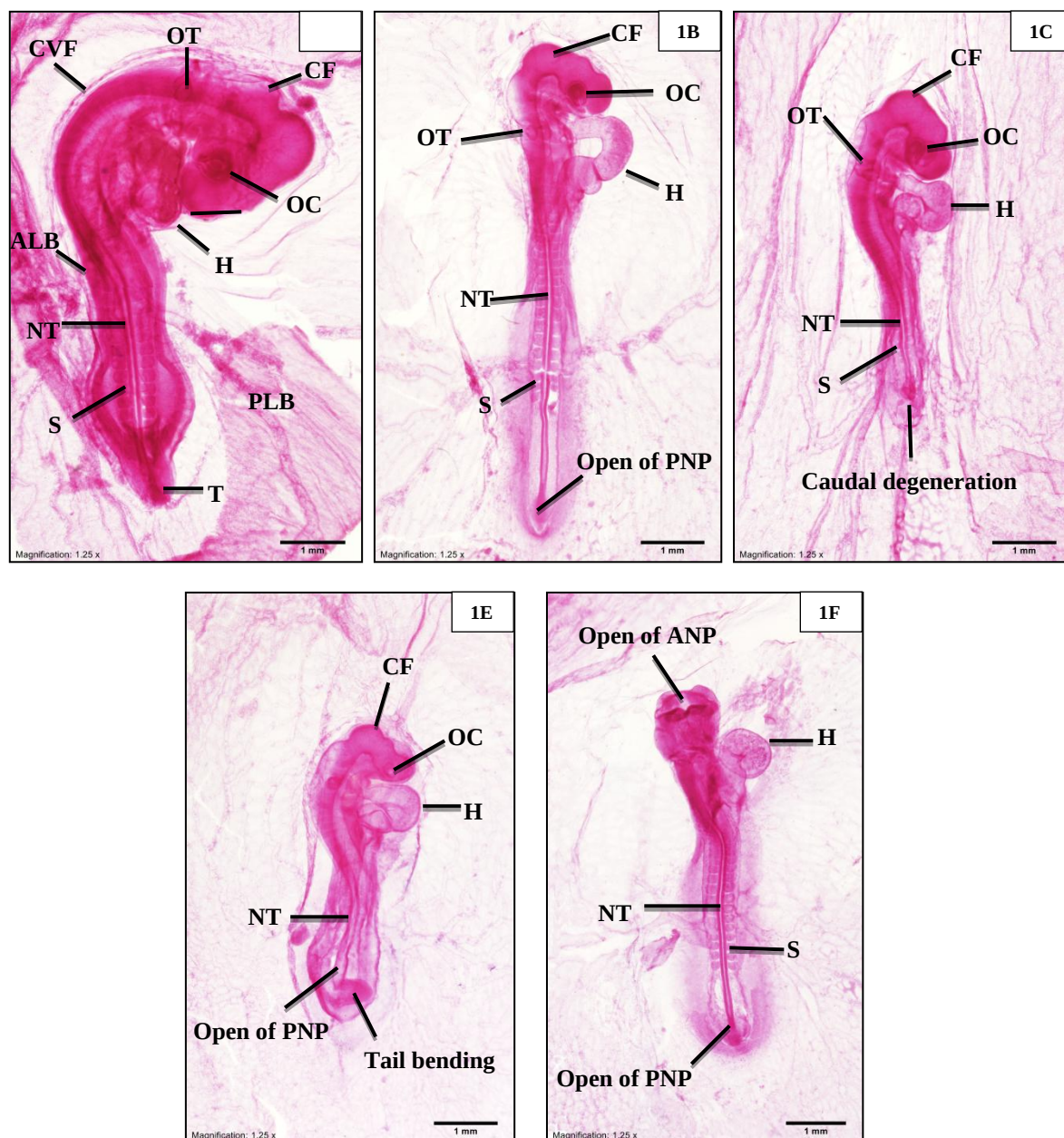


Figure 1 Development of chick embryo in total mount of day 3 in the control group (1A), 100 mg/L CB (1B), 150 mg/L CB (1C), 200 mg/L CB (1E), 250 mg/L CB (1F), respectively. (OC : optic cup, OT : otosis, CF : cervical flexure, CVF : cervical flexure, H : heart loop, ALB : anterior limb bud, NT : neural tube, S : somite, PLB : posterior limb bud, T : tail fold, ANP : anterior neuropore, PNP : posterior neuropore

3.3 Serial section of chick embryo

Figure 2A showed normal eye development of chick embryo 36 somite in the control group. Eye developed from diencephalon. Beginning with optic vesicle contacted to the ectoderm, induction of ectoderm thickened to form the “lens placode”. Consequently the distal end of the optic vesicle enlarged while proximal part became narrow. Invagination of the distal part to form a cup-shaped structure, so called the optic cup. Optic cup was attached with the diencephalon by the optic stalk. There was 2 layers of optic cup : outer pigment layer

and inner nervous layer which were separated by the intraretinal space. In the cavity of optic cup consisted of the invagination and double fusion of lens placode to form the lens vesicle which was divided into 2 walls, anterior lens epithelium and elongated posterior lens fiber.

Figure 2B, 2C, 2E and 2F showed retardation of eye development in the experimental group which was treated with carbaryl insecticide at the concentration of 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L, respectively. All of optic cups were smaller and wider of the intraretinal space than the control group. For the lens vesicles, all of experimental groups showed equal in thickness of lens epithelium without elongation of the posterior lens fiber while in the dosages of 150 mg/L, 200 mg/L and 250 mg/L showed opening of the lens pit.

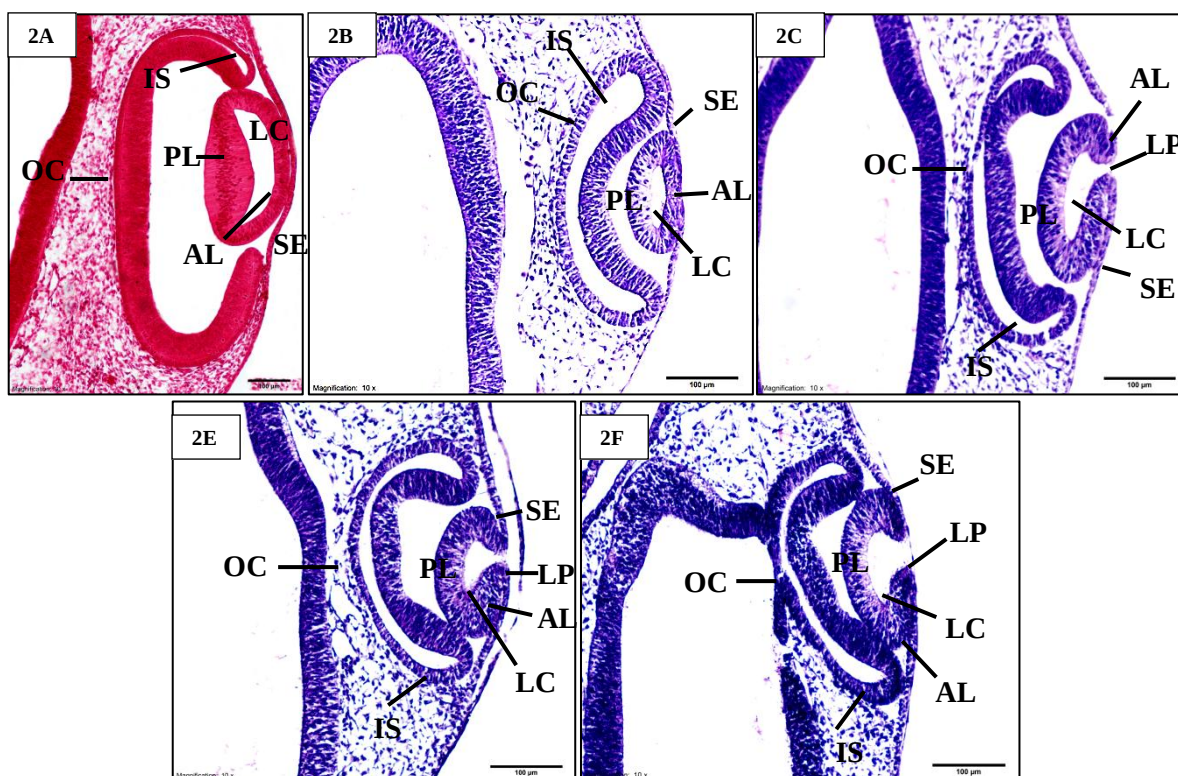


Figure 2 Eye development of day 3 chick embryo in the control group (2A), 100 mg/L CB (2B), 150 mg/L CB (2C), 200 mg/L CB (2E), 250 mg/L CB (2F), respectively. (OC : optic cup, LP : lens pit, SE : surface ectoderm, AL : anterior lens epithelium, PL : posterior lens fiber, LC : lens cavity, IS : intraretinal space)

4. Conclusion and discussion

Carbaryl was a carbamate insecticide which was widely used because of its low toxicity. However, it might be dangerous to pregnant mother who exposed to Carbaryl and to cause teratogenic effect in chick embryo development.

Development on day 3 of this study showed growth retardation and malformation from carbaryl exposure including the retardation of brain development, opening of anterior neuropore, microphthalmia, anophthalmia, abnormal heart looping, neural tube defect and absence of limb bud and tail fold. The finding of this study was paralleled to the study of Sahu and Ghatak in 2002 (11), Bacchetta *et al.* in 2008 (8), Uggini *et al.* in 2010 (12).

The present study indicated that exposure to carbaryl insecticide during pregnancy could cause teratogenic effects to several organs. The severity was corresponded with the

increased concentration of carbaryl, this suggested that pregnant women should avoid the exposure to carbaryl insecticide during pregnancy.

Acknowledgement

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Teratogenic Effect of Monosodium Glutamate on Chick Embryo Development

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Abstract

Objective(s): The present study was conducted to evaluate the toxicity and teratogenic effect of monosodium glutamate (MSG) on chick embryo development by injections the MSG solution into the fertilized hen's egg, then chick embryos at day 3 of development were studied and recorded contributed to the database of the congenital abnormalities which can be applied to the future experiment.

Material(s) and Method(s): Fertilized eggs of white leg horn hens (*Gallus domesticus*) were divided into 5 groups. MSG solutions of various concentrations of 1 mg, 1.5 mg, 2.5 mg and 3 mg of MSG/g egg weight and normal saline for the control group. After 24 hours of incubation the solutions were injected into the yolk sac and sacrificed on the 3rd embryonic day to observe the teratogenic effects.

Results: The effect of MSG on the day 3 chick embryo development in the experimental groups showed abnormalities in several structures and also showed growth retardation compared with the control group. In addition, the numbers of mortality rate showed lower in number in the higher dose, while the abnormalities were identified in all dosages such as a small size, anencephaly, microphthalmia, fewer somites and unclosed of posterior neuropore.

Conclusion: We concluded that various concentrations of MSG resulted growth retardation and congenital malformations. The excessive dose and long term used of MSG might affect to tissue organs and embryonic development. This might indicate the important of studying effect of MSG in pregnant woman.

Keyword(s): Teratogenic effect, Monosodium glutamate, Chick embryo

1. Introduction

Monosodium glutamate (MSG) has been used as a food additive and flavoring agent. In the early 1900s scientists discovered the ingredient which was extracted from seaweed of Japan (Kombu). An essential taste name "Umami" which was responsible for greatly enhancing flavor. At present, MSG is the most commonly produced used by housewives for cooking process in many countries around the world. United Kingdom was shown to use MSG about 4 grams (less than one teaspoon) per week while U.S. was about 0.55 grams per week and in Taiwan were much higher of averaging 3 grams per day^(1,2). Medical Doctors called a specific MSG-induced symptoms "Chinese restaurant syndrome" which showed after ingestion of Chinese meal characterized by headache, chest discomfort and facial

flushing⁽⁴⁾. Research reported that MSG caused abnormal function of glutamate receptors linked with neurological diseases, such as retinal degeneration, endocrine disorder, addiction, stroke, epilepsy, brain trauma, neuropathic pain, schizophrenia, anxiety, depression, Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis^(3,4).

The excessive dose and long term use of MSG in cooking might effect to tissue organ and embryonic development if taken by pregnant women. Therefore it was important to study to what extent the teratogenic effect of MSG was. Chick embryo had been an important animal model in the field of embryology and developmental biology, as its developing features were very well documented, which made it easier for the researcher to compare the control finding in his study to previous studies. The data of stages comparing to human were also well established by Hamburger and Hamilton since 1952⁽⁵⁾.

The present study was conducted to demonstrate the toxicity and teratogenic effect of MSG on the 3rd day on chick embryos development by injection MSG solution into the yolk sac of fertilized egg and study gross structure anomalies and histological changes of chick embryo exposing to different concentrations of MSG by the total mount preparation compared to control group.

2. Materials and Methods

2.1 Chemicals

MSG was sold in most open market in Bangkok, Thailand under the license of Ajinomoto co. INC. Tokyo, Japan.

2.2 Experimental design

Fertilized white leg hen eggs (*Gallus domesticus*) was collected fresh from Department of Animal Science, faculty of Agriculture, Kasetsart University. They were randomly divided into 5 groups. MSG in normal saline solution of various concentrations, 1 mg, 1.5 mg, 2.5 mg and 3 mg of MSG/g egg weight were used for experimental group and normal saline for the control group. All eggs were injected after 24 hours of incubation, at the equal volume of 0.15 ml/egg into yolk sac. After 3 days of incubation, eggs were removed from the incubator and sacrificed. The chick embryos were removed from egg and recorded the mortality rate. The embryos were kept the Dietrich's FAA fixative for 4 hours and transfer to 70% alcohol. The embryos were processed for total mount for study gross structure anomalies and histological study.

3. Results

The present study was conducted to evaluate the toxicity and teratogenic effect of MSG in experimental groups compared with the control. The living embryo was indicated by observing the heartbeat and blood circulation. The percentage of survival and mortality were presented in Table 1.

Table 1: Percentages of the survival and mortality of chick embryos day 3 of the experimental groups compared with control

Group (mg MSG/g. egg weight)	Survival (%)	Mortality (%)
Control (n=4)	100 (4)	0 (0)
1 (n=7)	57.14 (4)	42.86 (3)
1.5 (n=7)	71.43 (5)	28.57 (2)
2.5 (n=7)	100 (7)	0 (0)
3 (n=6)	83.33 (5)	16.67 (1)

Total mount

The total mount of the day 3 chick embryo showed that the control group (Figure 1A) had normal development as classified by Hamburger⁽⁵⁾ as stage 18 which composed of the 5 brain vesicles (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon), 2 flexures (cervical flexure and cephalic flexure), normal optic cup at diencephalon, normal S-shaped loop of heart, 3½ brachial arches, somite extended to the caudal end, anterior and posterior limbs and tail fold were established.

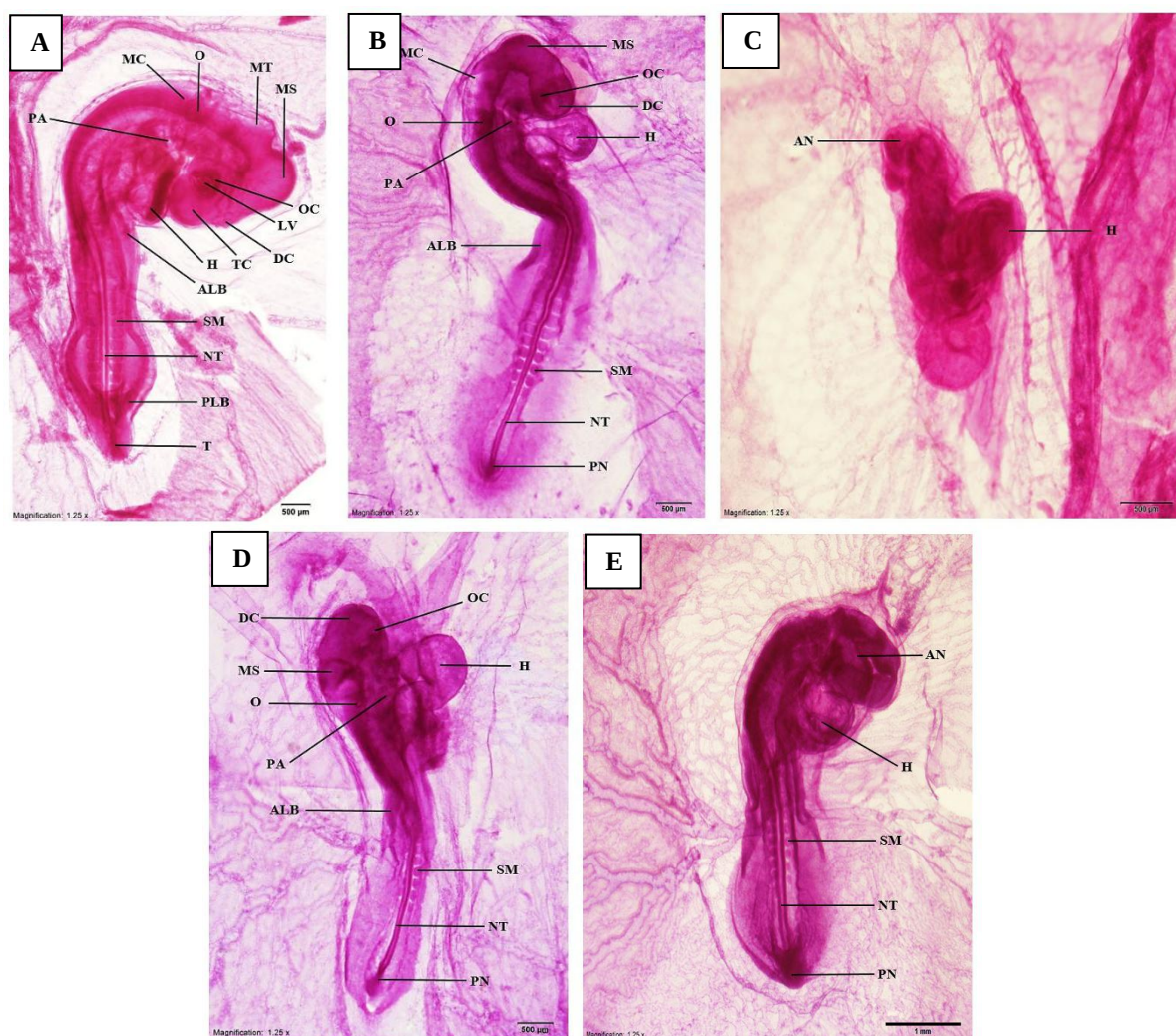


Figure 1: Micrographs of the total mount of day 3 chick embryos, control group (A), 1 mg of MSG group (B), 1.5 mg of MSG group (C), 2.5 mg of MSG group (D) and 3 mg of MSG group (E). (TC = Telencephalon, DC = Diencephalon, MS = Mesencephalon, MT = Metencephalon, MC = Myelencephalon, OC = Optic cup, LV = Lens vesicle, O = Otocyst, H = Heart, PA = Pharyngeal arch, ALB = Anterior limb buds, PLB = Posterior limb buds, NT = Neural tube, SM = Somite, T = Tail fold, AN = Anterior neuropore, PN = Posterior neuropore)

The total mount of the day 3 chick embryo in the experimental groups showed growth retardation and congenital malformations such as on illustrated (Figure 1B). The general appearance of the chick embryos was smaller in size, with 3 parts of primary brain vesicle and very irregular (diencephalon, mesencephalon and myelencephalon), with small size of optic cup or microphthalmia, small size of otocyst, pharyngeal arch was 1½ arches, heart was developed as U-shaped heart looping, neural tube showed unparalleled dense lines, somites were not exceeding to the caudal end, absent of anterior and posterior limb buds and tail fold and unclosed of posterior neuropore. The micrographs of the total mount of day 3 chick embryos in 1.5 mg of MSG group (Figure 1C) showed severe abnormality and growth retardation. The embryo was a small size, unclosed anterior neuropore with no brain subdivision called anencephaly, caudal degeneration, heart was developed as U-shaped heart looping, underdeveloped of otocyst, optic cup, pharyngeal arch, somites, neural tube, anterior limb buds and posterior limb buds. The micrographs of the total mount of day 3 chick

embryos in 2.5 mg of MSG group (Figure 1D) showed growth retardation and abnormality. The brain vesicle was not completed (diencephalon, mesencephalon), unclosed of anterior neuropore, small size of optic cup (microphthalmia), brachial arches was fewer than the control, heart loops was U-shaped, somites were not exceeding to caudal end, unparallel of neural tube, absent of otosis, posterior limb bud, tail fold and opened of posterior neuropore. The micrographs of the total mount of day 3 chick embryos in 3 mg of MSG group (Figure 1E) showed growth retardation and caudal degeneration. Anterior neuropore was not closed, brain vesicles was unformed which was called anencephaly, uncleared pharyngeal arches, irregular of heart looping like U-shaped, the neural tube was irregular shape and the somites were not exceeding to caudal end, absent of otocyst, limb bud, tail fold and opened of posterior neuropore.

4. Conclusion and discussion

Several concentration dosages of MSG might affect chick embryonic development causing growth retardation and congenital malformations. The lower concentration of MSG showed the higher mortality rate, but of the same time the abnormality of structures could be found in all dosage such as a small size, anencephaly, microphthalmia, slowly somites development and unclosed of posterior neuropore. This result was consistent to the study of Qudsi FA *et al* ⁽¹⁾. The excessive dose and long term used of MSG in cooking might affect to tissue organ and embryonic development and if it was consumed by pregnant women during pregnancy could produce clearly harmful effects. Women should avoid consumption this food additive during pregnancy.

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Determination of the respiratory complex I activity in fibroblasts of patients with Leber hereditary optic neuropathy

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Abstract:

Objective: Leber hereditary optic neuropathy (LHON) is a maternally inherited disease caused by point mutations on the mitochondrial DNA. The most prevalent mutation in Thailand is G11778A, affecting the ND4 subunit of the respiratory complex I. The effect of the G11778A mutation on respiratory complex I function has not been thoroughly investigated. Therefore, we proposed to study effect of the G11778A mutation in Thai LHON patients on the enzymatic activity of the respiratory complex I in comparison to normal controls.

Materials and Methods: Skin biopsy samples were taken 3 LHON patients carried the G11778A mutation, and from 7 normal controls. Fibroblasts were cultured from the biopsy samples according to the standard culture technique. Mitochondria were isolated from fibroblasts. Respiratory complex I activity was determined by spectrophotometric analysis, which included NADH:ferricyanide reductase (NFR) assay and NADH-decylubiquinone:2,6-dichloroindophenol oxidoreductase (NADH-DUQ:DCPIP) assay.

Results: NFR activity and NADH-DUQ:DCPIP activity measured from mitochondria from LHON patients with G11778A mutation was decreased by 35% ($P=0.1$) and 17% ($P=0.08$) compared to normal controls, respectively.

Conclusion: This study demonstrated that the G11778A mutation did not affect NADH oxidation activity of the respiratory complex I.

Keywords: Leber hereditary opticneuropathy, G11778A, respiratory complex I

1. Introduction

Leber hereditary optic neuropathy (LHON) is a maternally inherited disease, which is caused by point mutations on the mitochondrial DNA (mtDNA). The primary mutations include G3460A (1), G11778A (2) and T14484C (3) which affects the ND1, ND4, and ND6 subunits of the respiratory complex I, respectively. LHON patients present with acute or subacute bilateral loss of central vision. The pathology of LHON is optic nerve atrophy which results from the degeneration of retinal ganglion cells (4). The disease phenotype usually presents during young adult, and is predominantly found in male (5, 6). Incomplete

penetrance is another unique characteristic of LHON (7). In Thailand, the G11778A mutation is the most prevalent LHON mutation (8).

The respiratory complex I (NADH: ubiquinone oxidoreductase) is the largest enzyme complex in the respiratory chain (9). Electron microscopy studies of complex I revealed the L-shaped structure with a hydrophobic membrane arm and peripheral arm (10). It consists of 45 subunits with 980 kDa in size. Complex I plays a role in energy production by coupling the transfer of two electrons, from NADH to ubiquinone, to translocation of four protons across the membrane (11). Complex I is also believed to be a major source of ROS in mitochondria, which can damage mtDNA and cause many disease (12).

Although LHON mutations may result in defective respiratory complex I, the effect of the G11778A mutation on respiratory complex I function has not been thoroughly investigated. Therefore, we proposed to study the enzymatic activity of the respiratory complex I in Thai LHON patients with the G11778A mutation.

2. Materials and Methods

2.1 Subject recruitment

A total numbers of 3 LHON patients who have G11778A mutation, and 7 normal controls participated in this study upon informed consents. The G11778A mutation on the mtDNA was validated by Sanger DNA sequencing of all samples. This study has been granted an approval of the Ethics Committee of Faculty of Medicine Siriraj Hospital, Mahidol University.

2.2 Skin fibroblast culture

The skin biopsy samples were taken from all subjects by punch biopsy technique. Fibroblasts were cultured from the biopsy samples according to the standard culture technique (13). In brief, skin fibroblasts were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (BSA), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml amphotericin, 2 mM L-glutamine, 50 µg/ml uridine at 37°C in 5% CO₂ atmosphere. To initiate culture, the frozen skin fibroblasts from liquid nitrogen stock were thawed at 37°C in water bath. Cell suspension was transferred to a 15 ml centrifuge tube containing 5 ml stock DMEM, and then centrifuged at 1000g for 5 min. The pellet were resuspended with working DMEM. Cell suspension was transferred to a T25 flask, and kept in an incubator at 37°C. Culture medium was changed every 2-3 day.

Skin fibroblasts were subcultured when they reached 100% confluence. The medium was removed, and kept in a 15 ml centrifuge tube. Then, skin fibroblasts were washed with phosphate buffer solution (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) twice. Cells were detached by 0.25% trypsin. After cell detachment, the removed medium was added to the cell suspension in order to block trypsin activity. Cell suspension was transferred to a 15 ml centrifuge tube, and then centrifuged at 1000g for 5 min. Supernatant was discarded, and the pellet was resuspended with working DMEM. Cell suspension was transferred to a new T25 flask.

2.3 Mitochondrial isolation from the skin fibroblast

This method is modified from Frezza *et al.* (14). Mitochondrial isolation was performed using approximately 8-10 x 10⁶ fibroblast cells. Fibroblasts were washed with

PBS once, and then detached from the culture flask by a cell scraper. Cell suspension was centrifuged at 1,000g for 10 min. The pellet were resuspended in 25 μ l of ice-cold isolation buffer (1 M sucrose, 0.1 M Tris/MOPS, 0.1 M EGTA/Tris, pH 7.4) per mg of pellet. Cell suspension was subjected to homogenization by a glass homogenizer for 10 strokes at 1,600 rpm. The homogenized cells were centrifuged at 600g for 10 min at 4 °C. Supernatant was collected, and then centrifuged at 7,000g for 10 min at 4°C. After centrifugation, the pellet was resuspended with 30 μ l of ice-cold isolation buffer and centrifuged at 7,000g for 10 min at 4°C twice. Mitochondrial pellet was obtained. The concentration of mitochondrial protein was determined by the Lowry's method (15).

2.4 Measurement of enzymatic activity of the respiratory complex I

Spectrophotometric analysis was used to determine enzymatic activity of the respiratory complex I. Mitochondrial isolated from the skin fibroblasts were solubilized by 5% sodium cholate to obtain a ratio of protein/detergent as 5/1 (w/w). Solubilized mitochondria were mixed with KME solution (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA) to obtain the final mitochondrial protein concentration of 1 μ g/ μ l.

2.4.1 NADH:ferricyanide reductase (NFR) assay

NFR assay measures the respiratory complex I activity by monitoring a rate of NADH oxidation in a presence of NADH as an electron donor, and ferricyanide as an electron acceptor. The assay was performed in a mixture containing 0.1 mM KH_2PO_4 , 50 mg/l BSA, 15 mg/l asolectin, 0.01 mM EDTA, 0.01 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, 0.1 mM NaN_3 , 0.01 mM NADH, and 20 μ g mitochondrial protein. NADH oxidation was determined by measuring an absorbance at 340 nm for 120 sec at 37°C. Coefficient of NADH, 6.22, was used to calculate the rate of NADH oxidation This method is modified from Hatefi *et al.* (16).

2.4.2 NADH-decylubiquinone:2,6-dichloroindophenol oxidoreductase (NADH-DUQ:DCPIP) assay

NADH-DUQ:DCPIP assay determines enzymatic activity of the respiratory complex I by measuring the DCPIP reduction in the presence of NADH as an electron donor, and decylubiquinone as an electron acceptor. A subsequent reaction acquires DCPIP as the electron acceptor from reduced decylubiquinone (17). The reduction of DCPIP was measured in a 1 ml of mixed solution containing 25 mM KH_2PO_4 , 3.5 g/l BSA, 15 mg/l asolectin, 0.01 mM EDTA, 0.2 mM NADH, 1 μ M antimycin A, 60 μ M DCPIP, 70 μ M decylubiquinone and 20 μ g mitochondrial protein. The absorbance of DCPIP was read at 600 nm for 120 sec at 37°C. In addition, another NADH-DUQ:DCPIP reaction with an addition of 1 mM rotenone was assayed in parallel to obtain the rotenone-insensitive activities. Coefficient of DCPIP, 21, was used to calculate the rate of DCPIP reduction. This method is modified from Janssen *et al.* (18).

2.5 Measurement of citrate synthase activity

Citrate synthase activity is determined by a rate of thionitrobenzoic acid (TNB) production in the presence of acetyl-CoA, oxaloacetate (OAA), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The assay was performed in 1 ml mixed solution containing 1 mM DTNB, 10 mM OAA, 5 mM acetyl-CoA, and 20 μ g mitochondrial protein. TNB was read at the absorbance at 412 nm for 120 sec at 37°C. Coefficient of TNB, 13.6, was used to

calculate the production of TNB which reflected the activity of citrate synthase. This method is modified from Sere *et al.* (19).

2.6 Statistical analysis

Student t-test was used to analyze statistical significance of the enzymatic activities between LHON patients and normal controls. $P\text{-value} < 0.05$ was considered statistical significant.

3. Results

Enzymatic activities of the respiratory complexe I were normalized by citrate synthase activity for reducing the noise from the different of mitochondrial yeild in each sample. NFR activity in LHON patient's fibroblast was 35% decrease ($P=0.1$; Figure 1), compared to that of normal controls whereas the NADH-DUQ:DCPIP activity in LHON was found 17% decrease ($P=0.08$; Figure 2) from normal controls.

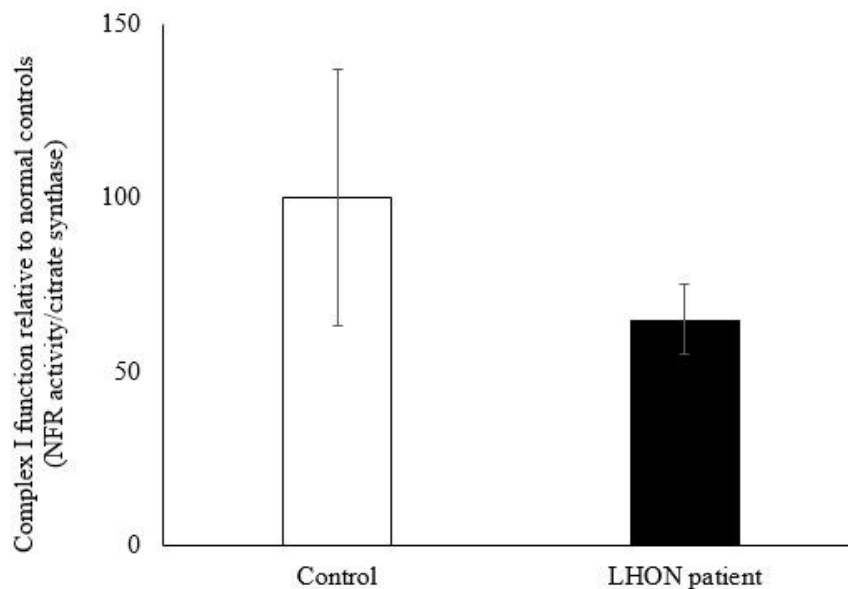


Figure 1 NADH:ferricyanide reductase activity- NFR activity in LHON patients with G11778A mutation remained unchanged compared to normal controls. Data are presented as mean $\pm$ SD.

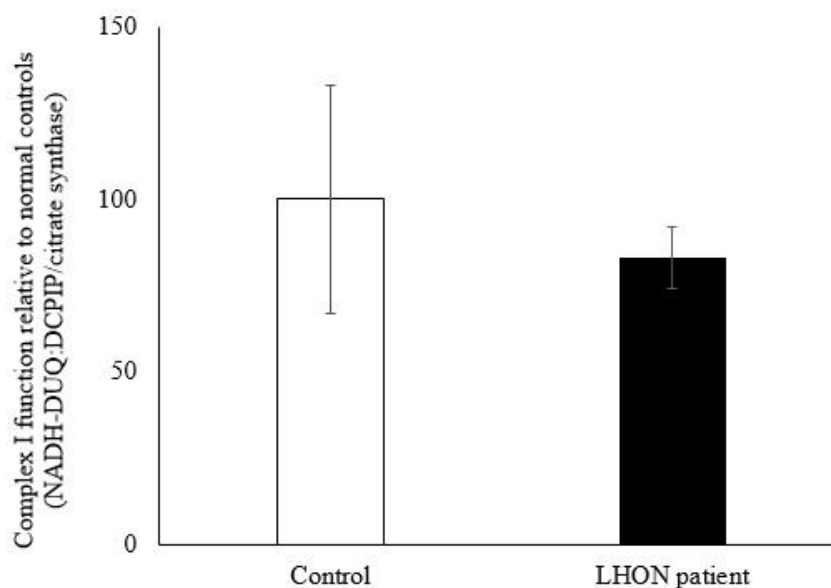


Figure 2 NADH-decylubiquinone:DCPIP oxidoreductase activity- NADH-DUQ:DCPIP activity in LHON patients with G11778A mutation remained unchanged compared to normal controls. Data are presented as mean $\pm$ SD.

4. Conclusion and discussion

Our study demonstrated that the G11778A mutation does not affect NADH oxidation activity of the respiratory complex I, which is consistent with previous studies (20) despite different models being investigated. Larsson *et al.* used muscle tissue from LHON patients who carry G11778A mutation to measure NFR activity, no significant differences were detected in LHON patient with G11778A mutation compared with normal controls (21). Majander *et al.* showed that NADH-DUQ:DCPIP activity in lymphoblastoid cell line of LHON patients was comparable to that of normal controls (22).

It is interesting to find that the G11778A mutation which causes an arginine to histidine amino acid substitution in the ND4 subunit of the respiratory complex I displayed no effect on the NADH oxidation of the respiratory complex I. One possible explanation could be that the ND4 subunit mainly involves proton pumping in the membranous arm of the respiratory complex I (23). The mutation may then affect only proton pumping function without disrupting NADH oxidation which takes place in the matrix arm of the respiratory complex I. This result suggests that molecular mechanism that leads to the phenotype of LHON may not be related to the NADH oxidation of the respiratory complex I.

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Molecular epidemiology of cryptococcosis in Siriraj Hospital

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Abstract

Objective: To examine molecular type and allele type distributions of the *Cryptococcus neoformans* in Siriraj Hospital.

Materials and Methods: All of cryptococcal isolates during 2012-2014 in Siriraj Hospital were collected. The major molecular types were determined by *URA5*-RFLP analysis PCR products were digested by restriction enzymes *HhaI* and *Sau96I*. Analysis of *URA5* gene sequences was performed with the neighbor-joining method to construct phylogenetic trees by MEGA 6 program. Allele types (AT) were assigned to each isolate according to ISHAM MLST database for *C. neoformans* (<http://mlst.mycologylab.org>).

Results: 51 cryptococcal isolates were obtained from clinical samples, 56.86% were from male and 43.14% were from female patients. 56.86% of the isolates were recovered from cerebrospinal fluid as the most common sites while 41.18% were from blood and 1.96% were from bone marrow. *URA5*-RFLP analysis revealed that all isolates of *C. neoformans* belonged to molecular type VNI. Allele types (AT) of *URA5* gene consisted of AT5 (80.4%, n = 41) and AT1 (19.6%, n = 10). No significant correlation between *URA5* AT and HIV status was found.

Conclusion: *C. neoformans* VNI is the most common molecular type as found globally. Although no significant association between allele type and HIV status was suggested, our current data is limited. Thus, further studies by MLST (multilocus sequence typing) are needed

Keywords: *Cryptococcus neoformans*, Molecular epidemiology

1. Introduction

Cryptococcosis occurs in humans and a broad range of animals. The disease causes by members of the *Cryptococcus neoformans*/*C. gattii* species complex ^(1, 2). This species complex is composed of two species, *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. neoformans* comprises of two varieties and three serotypes: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and a hybrid (serotype AD). The other two serotypes, serotype B and C, have been designated to *C. gattii* but no variety level is recognized. *C. neoformans* has been known to infect mainly immunocompromised hosts such as HIV patients while immunocompetent hosts are mostly infected by *C. gattii* ⁽¹⁾.

Identification of cryptococcal species typically can be achieved by phenotypic methods but genotyping methods are found to be more accurate^(2,3). Established genotyping methods, including: M13 fingerprinting, amplified fragment length polymorphism (AFLP) analysis, *URA5* restriction fragment length polymorphism (RFLP) analysis and multi-locus sequence typing (MLST) separate the organism into eight major molecular types: VNI, VNII, VNIII, VNIV, designated for *C. neoformans* and VGI, VGII, VGIII, VGIV, designated for *C. gattii*⁽⁴⁾.

A previous study in Thailand, based on MLST analysis, showed that almost all of the isolates belonged VNI molecular type and only few isolates were VNII⁽⁴⁾. As evident worldwide, VNI is also a major molecular type in Thailand. However, a correlation between allele types with demographic data was under studied. Thus, aim of the present study is to investigate a correlation between genotype and HIV status at the Siriraj Hospital, Bangkok, Thailand.

2. Material and Method

2.1 Culture and isolation

Fifty-one cryptococcal isolates from 2012-2014 were obtained from clinical samples in the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The isolates were cultured on standard yeast media, Sabouraud dextrose agar (dextrose 20 grams per liter, peptone 10 grams per liter, agar 15 grams per liter and final pH 5.6±0.2). The isolates were maintained at 30 °C for 48-72 h and, subsequently, kept in 10-15% glycerol stock for a long-term storage. Demography data were collected by approval from Siriraj Institutional Ethics Committee (No.Si405/2014).

2.2 Reference strains

A set of standard reference strains from Siriraj Hospital, representing each of the eight major molecular type of the *C. neoformans/C. gattii* species complex, were used for molecular type identification: WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype AD, VNIII), WM629 (serotype D, VNIV), WM179 (serotype B, VGI), WM178 (serotype B, VGII), WM175 (serotype B, VGIII), and WM779 (serotype C, VGIV)⁽⁵⁾.

2.3 DNA extraction

Isolates were cultured on Sabouraud dextrose agar and grew at 37 °C for 48–72 h. Genomic DNA were extracted from the cryptococcal colonies. Colonies of yeasts were transferred into a tube. Lysis of the cells was done by incubating at 65 °C for 1 h in water. The lysate were mixed and cleaned with phenol–chloroform method. The aqueous phase was transfer to a new tube. DNA precipitation step was done by alcohol in cool condition. The DNA pellet were washed with 70% ethanol, centrifuged and air-dried. Then, DNA was resuspended with sterile water and ready for subsequent amplification steps.

2.4 *URA5* gene RFLP

The major molecular types of the studied strains were determined by *URA5*-RFLP⁽⁵⁾. PCR amplification of the *URA5* genes were performed using 10 µM of forward and reverse *URA5* primers with 50 ng of DNA template concentration. The PCR condition were be performed as followed: 94°C for 2 min of initial denaturation and followed by 35 cycles of 45 s denaturation at 94°C, 1 min annealing at 61°C, and 2 min extension at 72°C. The

reaction will be finished by a final extension for 10 min at 72°C ⁽⁶⁾. PCR products were double digested by *Sau96I* and *HhaI* and incubated at 37 °C overnight. RFLP pattern were determined from digested products by running on 3 % of agarose gel electrophoresis and visualized under UV light.

2.5 Sequencing and phylogram construction

The *URA5* PCR products were purified and sequenced at Macrogen company (Seoul, Korea). *URA5* gene sequence data were adjusted manually. The AT was defined according to ISHAM MLST database for *C. neoformans* (<http://mlst.mycologylab.org>) ⁽⁷⁾. *URA5* gene phylograms were constructed using the neighbor-joining method by program MEGA 6.0 and bootstraps of 1000 replications.

2.6 Statistics analysis

Statistical analysis was performed using the Fischer exact test. Unknown data were regarded as lost data and not included in the calculations. Fisher Exact test was done using an online program SISA (<http://www.quantitativeskills.com/sisa/statistics/fisher.htm>). Statistical significance was defined when p value ≤ 0.05 .

3. Results

3.1 Demography and molecular type distribution

Cryptococcosis occurred more frequently in HIV positive (88.57%; 31 out of 35 cases) than HIV negative (11.43%; 4 out of 35 cases) patients. Source of isolate distribution were shown in the table 1. All isolates belonged to VNI molecular type. Patterns of *URA5* RFLP method were shown in Figure 1.

Table 1. Molecular types and sources of the cryptococcal isolates

Source			Total (%)
Number (%)			
CSF	Blood	Bone marrow	
29)56.86%(	21)41.18%(	1)1.96%(	51 (100%)

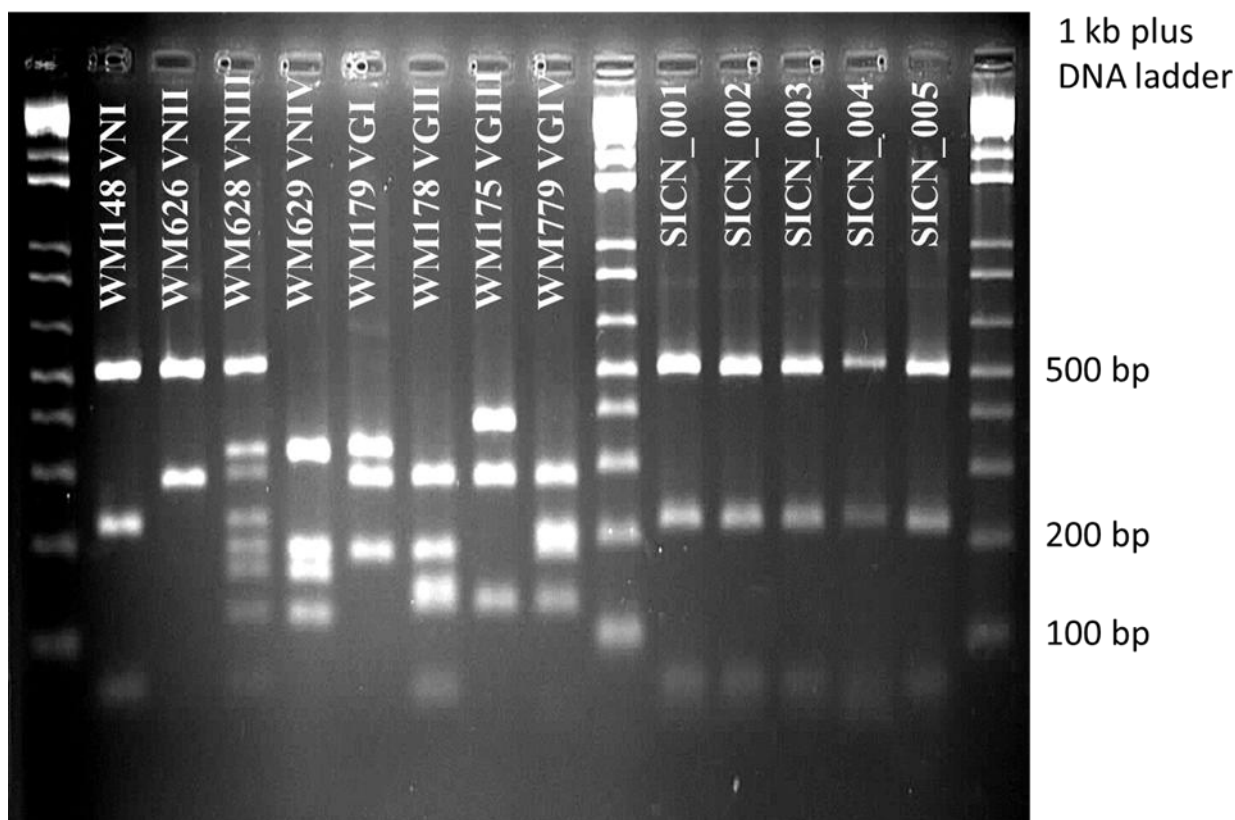


Figure 1. An example of major molecular type determination by *URA5*-RFLP. Strain names and their corresponding molecular types were labeled. 1 Kb plus DNA maker (Invitrogen®) was used.

3.2 Allele type determination

A phylogram show that all of isolates were in the same clade as the molecular type VNI standard (WM148). Two allele types, AT1 (19.6%; 10 out of 51 cases) and AT5 (80.4%; 41 out of 51 cases) were found among isolated in this study (Figure 2.)

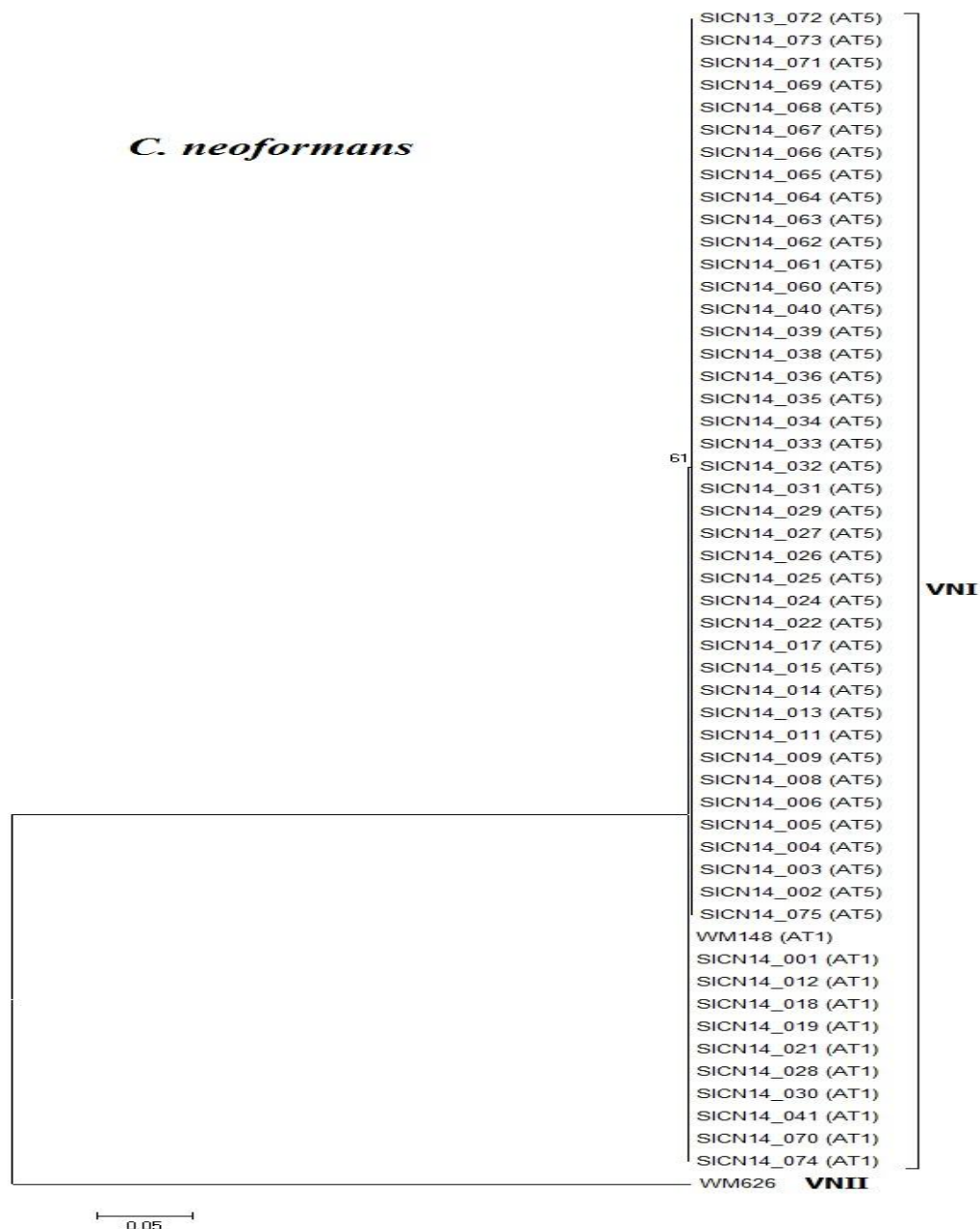


Figure 2 A phylogram of *URA5* loci. A phylogram depicts genetic relationships between the cryptococcal isolates in Siriraj Hospital based on neighbor joining analysis of *URA5* loci using the program MEGA 6.0. AT = allele type.

3.3 A correlation of *URA5* allele type and HIV status

Allele types and HIV status were not correlated significantly ($p = 0.913$), the data was presented in the table 3.

Table 3. Comparison of allele type and HIV status

URA5 Allele type (AT)	HIV status		P-value
	Positive	Negative	
Allele type 1	7 (20.0%)	1 (2.8%)	0.913
Allele type 5	24 (68.6%)	3 (8.6%)	

*Only patients with available HIV status (35 cases) were included.

4. Conclusion and discussion

Cryptococcosis mostly occurs in immunocompromised host, particularly, HIV infected persons. Likewise, most of the cryptococcal isolates in this study were recovered from HIV patients. Using genotyping techniques, all of the isolates was designated as VNI molecular type as reported globally as the most common molecular type ⁽⁸⁾.

Most infections with *C. neoformans* occur in the central nervous system as cryptococcal meningitis. It is believed that human gets primary infection at lungs by inhalation of the fungal spores then the fungus disseminated to the central nervous system through blood stream. Aligned with these facts, we also found CSF (56.86%) and blood (41.18%) were the most common sources of *Cryptococcus*. This emphasize the cryptococcal pathogenesis in Thailand is most likely similar to what described elsewhere ⁽⁴⁾.

In nature, *Cryptococcus* reproduces asexually enabling its population to be largely clonal ⁽⁹⁾. Analysis of URA5 allele type in our study showed that cryptococcal isolates were highly homogenous, consisting of only two allele type, AT5 and AT1. Moreover, the association analysis showed no signification correlation between AT and HIV status. This could be explained by the difference in AT is very minimal thus phenotypic. However, a clear association between genotype and virulence of *Cryptococcus* was previously shown in mice model that VGIIa subtype was more virulent than VGIIb isolates ⁽¹⁰⁾. Genotyping techniques have powerful for epidemiological studies of medically important fungi ^(11, 12). In order to understand the epidemiological studies more clearly, the more discriminatory genotyping methods, such as MLST analysis, is needed.

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Mitochondrial ATP production in fibroblasts from Thai individuals with homoplasmic m11778G>A mutation

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Abstract

Leber Hereditary Optic Neuropathy (LHON) is a mitochondrial inherited disease that is caused by optic nerve degeneration. The m11778G>A mutation was regarded as one of the most common mutations in LHON. It results in complex I deficiency, which could lead to inefficient oxidative phosphorylation. However, the effect of this mutation on ATP synthesis was still controversial. In this study, we investigated the effect of m11778G>A mutation on complex I-dependent ATP synthesis in digitonin-permeabilized fibroblasts. We found that the rate of complex I-dependent ATP synthesis in fibroblasts of normal controls was not significantly different from that in LHON patients.

Objective: To study the effect the m11778G>A mutation on rate ATP production in fibroblasts.

Material and Method: Skin fibroblasts obtained from normal controls and LHON patients were permeabilized by digitonin. Glutamate and malate were used to stimulate complex I-dependent ATP synthesis. Luciferin/luciferase assay was used to levels of ATP.

Results: ATP synthesis rates from normal controls and LHON fibroblasts were found to be not significantly different.

Conclusion: It was shown that the m11778G>A mutation does not affect rate of complex I-dependent ATP synthesis. It suggested that ATP production may not be the major cause of optic nerve degeneration in LHON patients.

Keyword(s): Digitonin permeabilization, ATP measurement, m11778G>A, LHON

1. Introduction

Mitochondrion (mitochondria; plural) is the cell organelle that governs energy production. Mitochondria harvest energy from food and store it in a form of ATP (adenosine 5'-triphosphate). Electrons from NADH and FADH₂, which are generated from oxidation of the nutrients, will be transferred in the electron transport chain. Electrons from NADH and FADH₂ are transferred to complex I and complex II, respectively. Then, coenzyme Q will carry those electrons to complex III. Complex III transferred electrons to complex IV via cytochrome c. Electrons finally reach O₂, the last electron acceptor which is finally reduced to H₂O. Electrons transfer is coupled with protons pumping from the mitochondrial matrix

across the inner mitochondrial membrane into the inter membrane space. Proton gradient across the inner mitochondrial membrane drives the protons back to the matrix via ATP synthase or complex V where ATP synthesis takes place. This whole process is called oxidative phosphorylation (1).

LHON is regarded as the most common mitochondrial inherited disease (2). It is caused by death of retinal ganglion cells, which leads to optic nerve degeneration (3). There are three common mutations causing LHON which are m3460G>A at MTND1 gene, m11778G>A at MTND4 gene and m14484T>C at MTND6 gene (4). These mutations have been shown to alter complex I function (5). The mean age of onset is in mid second decade of life for both sexes (6). The symptom starts with blurring vision without pain. Then, the visions will loss bilaterally within 6 to 8 weeks (7).

There are several LHON models that have been used to study the disease pathogenesis. However, it is still inconclusive whether LHON mutations result in decreased ATP production or not. For instance, it was shown that ATP production in the m3460G>A mutated fibroblasts was not significantly different from normal (8). ATP production was decreased in peripheral blood mononuclear cells of LHON patients (9) but not in LHON mouse models (10). These findings lead to a question whether rate of ATP production in normal fibroblasts is different from LHON fibroblasts or not.

2. Materials and methods

2.1 Skin fibroblasts culture and preparation

Skin fibroblasts were obtained from normal controls and LHON patients and grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified incubator at 37°C under 5% CO₂. The frozen skin fibroblast was thawed in 37°C and resuspended in the working DMEM and kept in humidified incubator at 37°C and 5% CO₂. When the cells had reached 80-90% confluent, the cells were detached by 0.25% trypsin-EDTA for another passage culture. The fibroblasts cells used in the present study were between the 1st to 6th passages.

2.2 Digitonin permeabilization

The cells were counted in a counting chamber. A total of 5×10^5 cells were used for each reaction in a subsequent ATP measurement assay. Cells were washed 2 times in 1 ml phosphate buffer saline (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM K₂HPO₄ pH 7.4) at 800g, for 5 min at room temperature. The pellets were resuspended with 160 µl respiratory buffer (100mM KCl, 5mM K₃PO₄, 1mM EGTA, 3mM EDTA, 10 mM Tris-HCl, pH 7.4). Digitonin was added at the final concentration of 25 µg /ml (11), then incubated for 1 min and wash once in 1 ml respiratory buffer. P₁, P₅-di(adenosine-5') pentaphosphate (Ap₅A) at the final concentration of 0.15 mM in 160 µl respiratory buffer was used to resuspend the pellet. Cells were kept on ice for an immediate used in ATP measurement assay.

2.3 ATP measurement assay

ADP at the final concentration of 4 mM was used as substrate of ATP synthesis for each reaction. The substrates, 1 mM malate and 1 mM glutamate were used to stimulate complex I-dependent ATP production. Each assay was replicated with the substrates and 10 µg/ml oligomycin. Rotenone was used as complex I inhibitor at the final concentration of 2

µg/ml. ATP production was measured by luciferin-luciferase system of Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich). Luminescent signal was detected at 560 nm. The experiment was performed for 20 min.

2.4 Statistical analysis

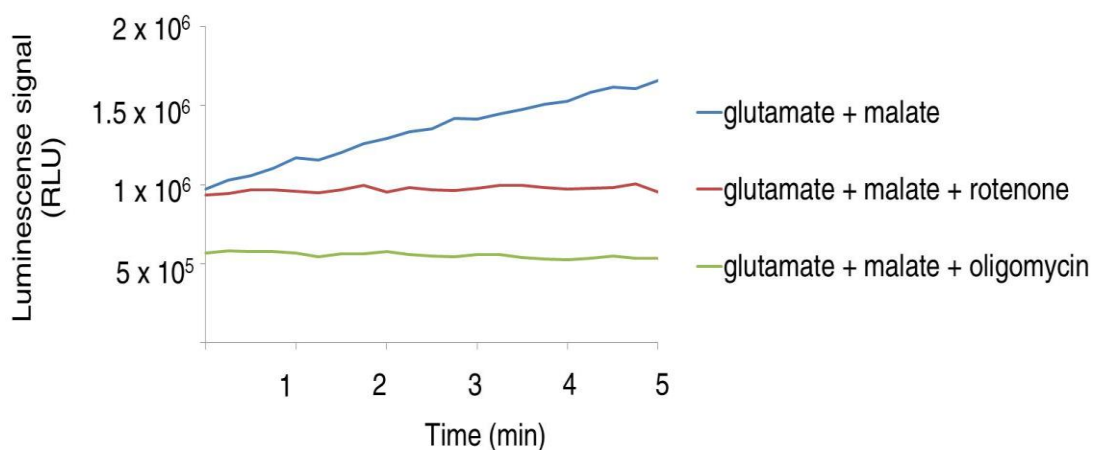
Student t-test was used to analyze statistical significance. Experiment data were calculated as mean $\pm$ SEM. *P-value* < 0.05 was considered statistical significant.

3. Results

3.1 Luciferin luciferase based measurement of ATP synthesis in alive fibroblasts

Complex I dependent ATP synthesis in digitonin-permeabilized fibroblasts was driven by glutamate and malate. Luminescent signal linearly correlated with the amount of ATP (Figure 1). Rate of ATP production was calculated during the first 5 min of the assay using the extrapolation of the luminescence signal from the ATP standard curve that was done in parallel. (Figure 2)

Figure 1. Measurement of ATP production. Glutamate and malate were used to stimulate



complex I-dependent ATP synthesis in digitonin-permeabilized fibroblasts. Luciferin-luciferase assay was used to determine level of ATP. Luminescent signal (RLU) linearly correlated with the amounts of ATP produced over time. Rotenone and oligomycin were used as complex I and V inhibitors, respectively.

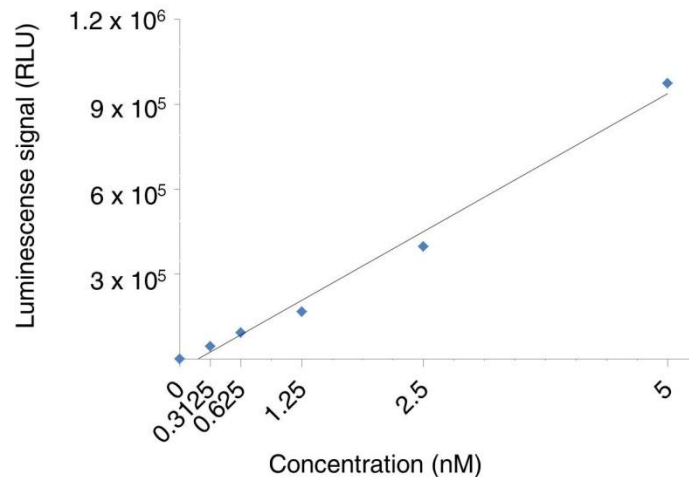


Figure 2. The ATP assay standard curve. ATP levels were measured based on luciferin-luciferase assay. Luminescence signals (RLU) was measured at each ATP standard concentrations of 0, 0.3125, 0.625, 1.25, 2.5, 5 nM as shown.

3.2 Complex I-dependent ATP synthesis in LHON patients

The rate of ATP synthesis driven by complex I substrates (glutamate and malate) in fibroblasts from LHON patients remained unchanged compared to that of normal controls ($p=0.23$) (Figure 3).

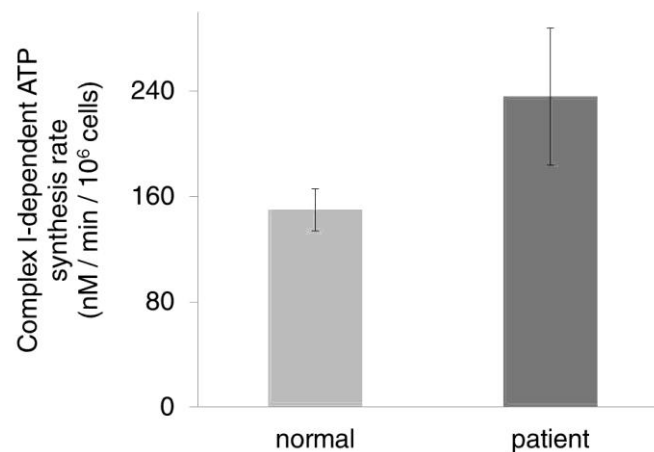


Figure 3. Quantitative analysis of ATP production from digitonized fibroblasts. Rates of ATP production (nmol/min/10⁶cells) from complex I-dependent ATP synthesis were determined in 9 normal controls and 3 LHON patients. Data are shown as mean. Error bars represent SEM.

4. Discussion and conclusion

We demonstrated that complex I-dependent ATP synthesis in fibroblasts of LHON patients was not impaired compared to that of normal controls. It might be explained that the m11778G>A mutation is the ND4 mutated part of complex I, this might not affected the oxidative phosphorylation. Our results was also consistent with a study from Yen *et al.* (12) which found that levels of ATP, ADP and AMP were not reduced in the m11778G>A

lymphocytes. However, Baracca *et al.* (13) reported that complex I dependent ATP synthesis was decreased in the cybrid cells containing LHON mutations (m3460G>A, m11778G>A and m14484T>C). Interestingly, the m11778G>A mutation remained the least affected in ATP synthesis. However, the total cellular ATP contents in those cybrid cells were not significantly different compared to that of normal controls. Therefore, it can be postulated that although the mitochondrial ATP synthesis was defective, the cells were effectively compensate cellular ATP to normal level by other energy-producing pathways such as glycolysis. It also suggested that ATP production may not the major cause of optic nerve degeneration in LHON patients.

Nevertheless, it has been documented that the variables on substrates that are used to stimulate ATP production can affect the results (14). For instance, respiratory rate in human skeletal muscle using glutamate-malate was higher than that when using pyruvate-malate (15). Additionally, each cell type should be optimized for digitonin concentration (11). This might be the limitation of mitochondrial ATP synthesis measurement.

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Molecular Epidemiology of *Candida parapsilosis* complex in Siriraj Hospital

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Abstract

Objectives: *Candida parapsilosis* complex is an important opportunistic pathogen in human. Over the past decade, incidence of infection by this microorganism has increased. This research aimed to study species distribution of *C. parapsilosis* complex isolated from clinical specimens and its association with patient demography.

Materials and Methods: *Candida parapsilosis* complex isolates between 2011 and 2015 from sterile body sites were collected from Department of Microbiology, Siriraj hospital and used in this study. Genomic DNA was extracted from yeast isolates. Species identification was done by ITS sequence analysis. A correlation of patient's data with the species was also correlated.

Results: Majority (68.87%) of the isolates belonged to Clade A (*C. parapsilosis*) while 29.25% and 1.88% of the isolates belonged to Clade IB (*C. orthopsilosis*) and Clade IIB (*C. metapsilosis*), respectively. Most strains were isolated from blood (73.88%). Majority (66.67%) of patients were male and had mean age of 54.33. No significant correlation was related between clade and patient's demography.

Conclusion: *Candida parapsilosis* was the most common species in the yeast complex. A correlation between molecular epidemiology and patient's data of Thai isolates need further investigations on a great variety of *C. parapsilosis* complex isolates.

Keywords: *Candida parapsilosis* complex, ITS region, patient's data

1. Introduction

An increase in prevalence of invasive fungal infection caused by *Candida* was noted in the past decade. *Candida* species is a fungal opportunistic pathogen. It causes a variety of diseases ranging from superficial mucocutaneous diseases to systemic infections and can be isolated from bloodstream or other sterile sites in the body. Approximately, 8 to 15% of patients in United States had *Candida* isolated from bloodstream⁽¹⁾.

Furthermore, a recent shift from *C. albicans* to non-albicans *Candida* (NAC) as the predominant causative pathogens has been described^(2, 3). Among NAC, *C. parapsilosis* complex has emerged as a major cause of human diseases especially in immunocompromised patients and hospitalized patients with underlying diseases associated with parenteral nutrition and intravascular catheters. *C. parapsilosis* complex affects mainly critically ill patients from surgical intensive care units, neonates, and cancer patients⁽⁴⁻⁶⁾. Previous studies

showed that *C. parapsilosis* complex could be separated into three groups. Group I strains are predominate in clinical sample, whereas group II and group III account for approximately 10% of isolates ⁽⁷⁾. In 2005, *C. parapsilosis* complex was divided into three closely related species, *C. orthopsilosis*, *C. metapsilosis*, and *C. parapsilosis*, based on ITS region sequences analysis ⁽⁷⁻⁹⁾. The three species were found to be differences in geographical distribution, antifungal susceptibility, and expressions of their virulence factors ⁽¹⁰⁾.

In Thailand, Boonyasiri *et. al* studied *Candida* prevalence in a tertiary hospital during 2007-2012 showed approximately 6 % of the cases were caused by *C. parapsilosis* complex ⁽¹¹⁾. On the other hand, a study of Petlum *et. al* showed 10% increase in prevalence of *C. parapsilosis* complex isolated in 2012 comparing to in 2007 ⁽¹²⁾. However, none of the studies reported distribution of species in the complex. Thus, we reported here the species distribution and its correlation with patient demography in Thailand.

2. Materials and Methods

2.1 Strains and demography

All *C. parapsilosis* complex isolated from sterile body sites were collected from the Department of Microbiology Laboratory, Siriraj Hospital during January 2011 to December 2015. Demographic data of the isolates, including: isolation sites, ages, and genders were collected. Ethics approval was granted by the Siriraj institutional review board No. SI 091/2016

2.2 Species distribution

Identification of isolates were done by ITS sequence analysis. All *C. parapsilosis* complex isolates were grown on Sabouraud Dextrose Agar (2% peptone, 2% dextrose and 2% agar). DNA isolation was performed by the boiling method as described previously ⁽¹³⁾. ITS regions were amplified using primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') (BioDesign Co., Ltd., Thailand). PCR amplification was performed as followed: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 1 minute and extension at 72°C for 75 seconds; and a final extension at 72 °C for 10 minutes. The amplicons were checked by 1.5% agarose gel electrophoresis ⁽¹⁴⁾. The amplified the DNA product has a size of approximately 520 bp ⁽¹⁵⁾. Then, PCR product was purified by GenepHlow™ Gel/PCR kit as described by manufacturer (Geneaid Biotech Ltd., Taiwan). Approximately 50 ng of DNA sample will be sequenced using ITS1 primer and ITS4 primer with 3730xl DNA analyzer (By MacroGen Inc., Korea). The nucleotide sequences were manually adjusted before used in multiple alignment and construction of phylogenetic tree.

The ITS nucleotide sequences of *C. parapsilosis* complex were analyzed by BlastN program in NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide sequences were aligned using the MEGA 6 program. Phylogenetic tree constructed using Neighbor joining analysis by MEGA 6 program. Sequences of *C. parapsilosis* MITS 1000, *C. orthopsilosis* MITS 907, *C. metapsilosis* MITS 868 were used as control group and sequence of *C. albican* MITS 440 were used as an out group (data available in <http://its.mycologylab.org/>). Phylogenetic tree was statistically tested by using bootstrap of 1,000 replications.

2.3 Data analysis

Statistical analysis was defined using Microsoft Excel 2013 software (Microsoft Corporation, Redmond, WA, USA). Any unknown data were regarded as missing data and not included into the calculations. Fisher Exact test was done using an online program QuickCalcs (<http://graphpad.com/quickcalcs/contingency1/>). Statistical significance were defined when p value ≤ 0.05 .

3. Results

3.1 Demographic data

One hundred and six *C. parapsilosis* complex isolates were isolated from blood and non-blood such as cerebrospinal fluid, peritoneal dialysis fluid, bronchoalveolar lavage, eye, and tissues. Blood (73.88%) was the most common site. The age of the patients ranged from 10 days to 85 years with a mean of 54.33 years. The majority of the patients (66.67%) were male (Table 1).

3.2 Distribution of molecular clade from clinical specimens

The phylogenetic tree of one hundred and six ITS nucleotide sequences of *C. parapsilosis* complex isolates were constructed. The tree revealed two clades of isolates (Clade A and B) that branching patterns were supported by high bootstrap values.

The major isolates were classified to clade A (68.87%) representing *C. parapsilosis*. Blood (71.23%) was the most common site of isolation in clade A. Clade B was composed of 31.13% of *C. parapsilosis* complex and could be divided into two subgroups (I_B, II_B). The most common specimen type in this clade was blood (78.79%). Clade I_B was related to *C. orthopsilosis* and clade II_B was related to *C. metapsilosis* (Figure 1).

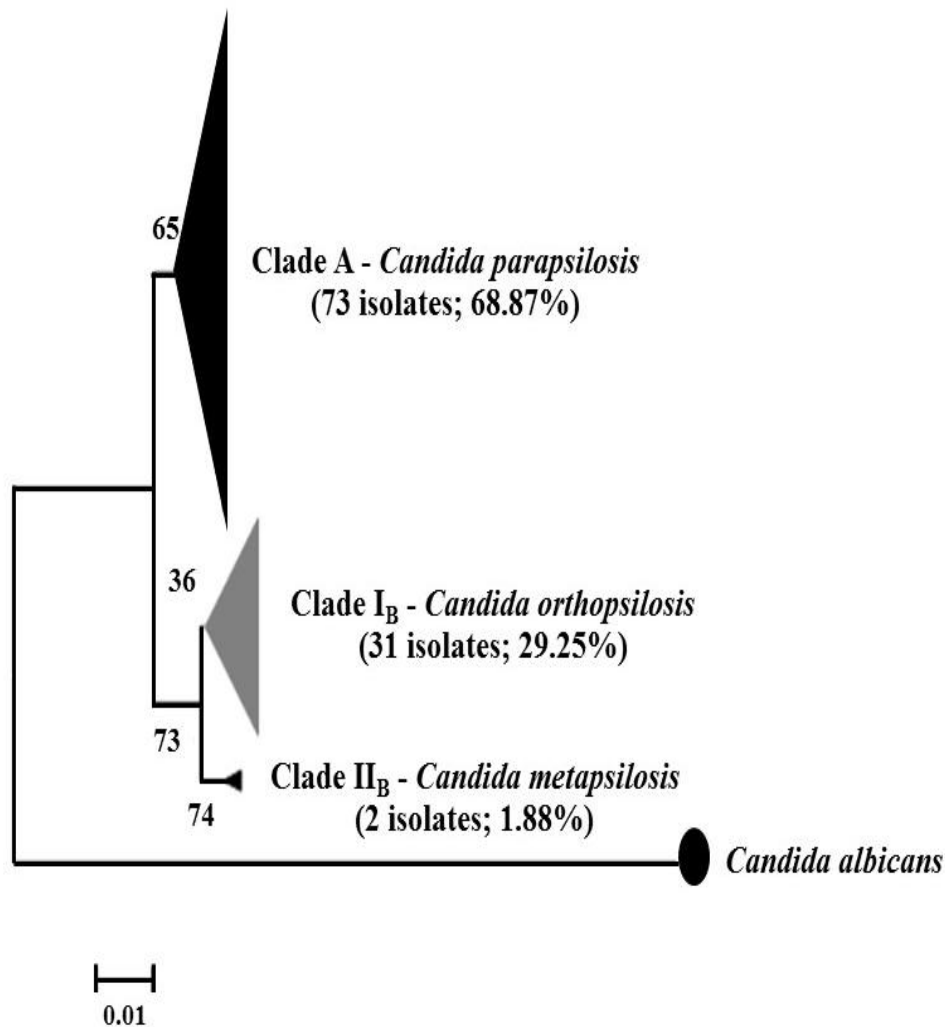


Figure 1. The phylogenetic tree of *C. parapsilosis* complex constructed based on ITS nucleotide sequencing.

3.3 Correlation between patient's demography and species distribution

No significant correlation was shown between clade and patient's demography, namely, sources, age range, and gender (Table 1).

Table 1. Demographic features of *C. parapsilosis* complex patients of clinical isolates classified in clade A and Clade B by ITS nucleotide sequences.

Characteristic	No. (%) of patients				p-value**
	Clade A	Clade B			
		I _B	II _B	Total	
Source of isolation (n = 106)					
Blood	52 (71.23%)	25 (80.65%)	1 (50.00%)	26 (78.79%)	0.482
Non-blood*	21 (28.77%)	6 (19.35%)	1 (50.00%)	7 (21.21%)	
Total	73 (100.00%)	31 (100.00%)	2 (100.00%)	33 (100.00%)	
Age (n = 70)					
< 15 years	14 (28.57%)	8 (42.11%)	0	8 (38.10%)	0.575
≥15 – > 65 years	35 (71.43%)	11 (57.89%)	2 (100.00%)	13 (61.90%)	
Total	49 (100.00%)	19 (100.00%)	2 (100.00%)	21 (100.00%)	
Gender (n = 85)					
Male	40 (66.67%)	15 (65.22%)	1 (50.00%)	16 (64.00%)	0.807
Female	20 (33.33%)	8 (34.78%)	1 (50.00%)	9 (36.00%)	
Total	60 (100.00%)	23 (100.00%)	2 (100.00%)	25 (100.00%)	

*Non-blood: cerebrospinal fluid, peritoneal dialysis fluid, bronchoalveolar lavage, eye tissues, and other source

**Calculated correlation of each characteristic between clade A and clade B (total) by Fisher Exact test

4. Discussion and Conclusion

Prevalence of infections by the *C. parapsilosis* complex has been increased in Thailand⁽¹²⁾. Three groups in the yeast complex are recognized by ribosomal DNA analysis and represent three separate species: *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*⁽⁹⁾.

Two clades were identified by ITS sequence analysis in our study. The most common clade A (68.87%), representing *C. parapsilosis*, is also the most common species in the yeast complex isolated in Malaysia (69%) and Taiwan (59%)^(16, 17). The less common clade B (31.13%), representing *C. orthopsilosis* and *C. metapsilosis*, is also the less common species elsewhere^(18, 19). Thus, species distribution in the *C. parapsilosis* complex in Thailand aligns with global prevalence.

To the best of our knowledge, this study provides the first data on the species distribution in the *C. parapsilosis* complex in Thailand. Although no significant correlation between the species and patient's demography were observed, analysis with more isolates is needed before drawing any further conclusion.

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Annotating Genetic Materials in Human Blood Plasma Based on Vesicular Type with Bioinformatics Analyses

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Abstract

Objective: A major problem in pathogen identification in plasma specimen is the host genetic materials persistently remaining in the sample after pathogen enrichment process. This study aimed to identify patterns of host genetic materials left in human blood plasma sample after enrichment methods with bioinformatics analyses.

Materials and Methods: Next-Generation Sequencing (NGS) data of genetic materials in plasma after pathogen enrichment was cleaned by PRINSEQ program. Then, the data were aligned with human genome using 'BWA-MEM'. Alignment results were annotated to mitochondrial genome, genes and non-gene regions using an annotation data from European Molecular Biology Laboratory (EMBL) database. The genes found from annotation were clustered based on their associated vesicle types using Vesiclepedia database.

Results: Cleaned NGS data were classified into genomic DNA (92.90%), mitochondrial DNA (3.95%) and sequences that cannot map to human genome (3.15%). The Genomic DNA was composed of gene (57.70%) and non-gene regions (43.30%). 43.50% of reads mapped to gene regions were also mapped with genes associated vesicle. 42.79% of reads mapped to genes associated with vesicle were associated with microvesicles.

Conclusion: From this study, around 23% of human genetic materials contaminating plasma sample originate from genes previously detected in vesicles. Therefore, the proper vesicle elimination method may be crucial in pathogen enrichment. Finally, contaminating human genetic materials not associated with vesicles and non-gene region must be studied to improve an elimination strategy for pathogen enrichment.

Keywords: Pathogen identification, Genome annotation, Pathogen enrichment, Next generation sequencing: NGS, Blood plasma

1. Introduction

In clinical research, pathogen identification is a process for detecting known pathogens in a specimen to help disease investigation (1, 2). Pathogen identification is applied to various sample, such as serum (3), cerebrospinal fluid (CSF), nasopharyngeal aspirate (NPA) (4) and plasma (5). The molecular methods for pathogen identification include conventional PCR, multiplex PCR, microarray and Next Generation Sequencing (NGS) (6). A major problem in pathogen identification by NGS technology is that the genetic materials of pathogen are overwhelmed by host genetic materials. Since NGS sequences DNA from any organisms without using specific primers very few sequencing reads originating from pathogens will be sequenced compared to the host DNA (7, 8). A study of viral pathogen identification by NGS technology without viral enrichment process found a contamination of host DNA more than 90% of all sequencing reads (9). Therefore, the enrichment of pathogens or their genetic materials is the crucial step. The goal of this process is to remove host DNA and RNA that contaminate the samples. Centrifugation and DNase treatment are commonly used for isolating pathogen from contaminant (7, 8).

Plasma specimen is often used for pathogen identification (5). Plasma components are comprised of water, protein, metabolites, some microorganisms and circulating DNA and RNA (10). The host DNA in plasma is released from nucleus and mitochondria. The host DNA are in cell-free forms or vesicular bound forms in exosomes, microparticles and apoptotic bodies (11-14). To enrich pathogens in a plasma sample, low-speed centrifugation is used to eliminate cell debris, while high-speed centrifugation eliminates microvesicles. DNase treatment is used to deplete host DNA in cell-free forms. However, after centrifugation and DNase treatment, some host genetic materials persistently remain in the sample (7). Possibly, host genetic materials are under conditions prohibiting an access of DNase, such as vesicle or protein bound DNA (7). Therefore, better understanding of the nature of host genetic materials in plasma may help design an effective method for host DNA elimination (15, 16). In this research project, we aim to use NGS data from a human plasma for alignment and annotation analyses to identify patterns of host genetic materials. In this research project, we aim to use NGS data from a human plasma for alignment and annotation analyses to identify patterns of host genetic materials.

2. Materials and Methods

2.1 Source of data

NGS data of this study were from the sequencing of plasma sample used in a previous study. Briefly, the sample was from an anonymous patient with infection. The plasma sample was passed through pathogen enrichment processes including centrifugation and DNase treatment before paired-end sequencing for 100 cycles by Illumina (Miseq) platform.

2.2 Bioinformatics analysis

NGS data were analyzed by bioinformatics methods including data cleaning by PRINSEQ program, sequence alignment with human genome (Hg38), genome annotation with EMBL database. (Figure 1).

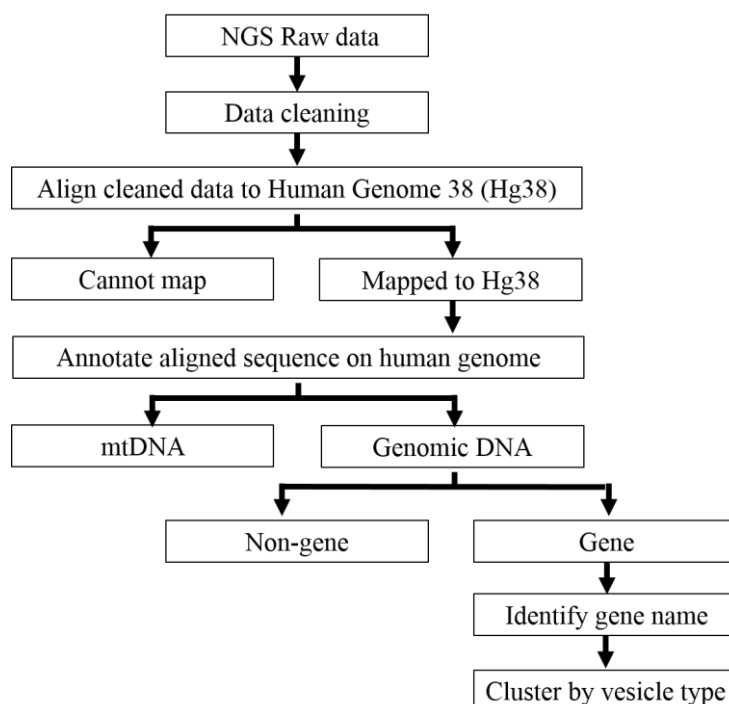


Figure 1: schematic of analytical process by bioinformatics methods

Part 1: Data cleaning

NGS data in FASTQ format were cleaned by PRINSEQ program (standalone lite version, available at: <http://prinseq.sourceforge.net/>). Data cleaning was composed of three steps. First, the reads with ambiguous N bases were filtered out while bases with quality score <30 were trimmed from the reads. Then, reads with sequence duplications, both exact duplication and reverse complement exact duplication, were deduplicated. Finally, reads with sequence complexity below 70 by entropy method were removed (17).

Part 2: Alignment

Cleaned data were passed through an analysis pipeline on DNAnexus (available at <https://www.dnanexus.com/>). First, data were aligned with human genome (Hg38, available at <https://genome.ucsc.edu/>) by BWA-MEM. The alignment results were then converted from SAM format to BAM format by Samtools view (18). Alignment file in BAM format was converted to BED format by BEDTOOLS bamtobed function (19). BED file was merged to generate larger contigs by BEDTOOLS merge (19) at the end of the DNAnexus pipeline.

Part 3: Annotation

Contigs from the previous step were compared to the annotation data of Hg38 (available at <https://genome.ucsc.edu/cgi-bin/hgTables>) from EMBL database with in-house python scripts in order to locate gene positions. Python scripts kept genes or parts of gene in query sequences overlapping with the reference genome (Figure 2). “A intersect B” region indicated the regions on query sequence mapped on gene regions of the reference genome. When the annotation for “A intersect B” regions was completed, human gene names were collected from the regions. In addition, in-house python scripts were used for categorizing merged contigs into two groups including genes (i.e. protein coding and non-protein coding genes) and non-gene regions.

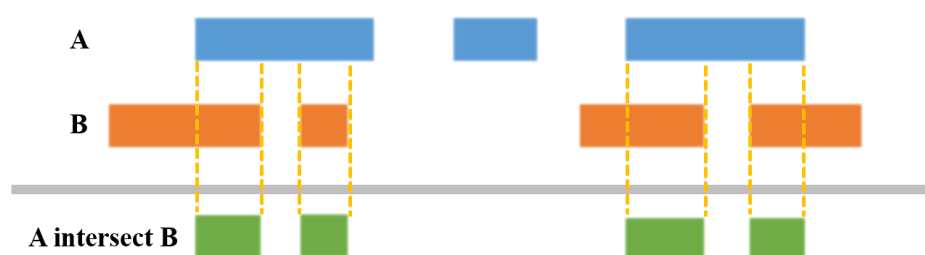


Figure 2: Diagram of a process for mapping query sequences to reference genome. (A) Blue rectangles represents a reference genome. (B) Orange rectangles represents query sequences. “A intersect B” (green rectangles) indicates parts of query sequences overlapping with the reference.

Part 4: Gene clustering

Gene names were clustered according to their associated vesicle type based on data from Vesiclepedia database Version 3 (<http://microvesicles.org/>, Release date: 9 Jan 2015). Types of vesicles in the database were exosome, microparticle, microvesicle and apoptotic blebs. The clustering process was performed by using in-house python scripts. A file containing gene names found in vesicles and a file containing details of experiment were downloaded from Vesiclepedia database.

The annotated gene names from the previous analysis were matched with the names in the file containing gene names to extract experimental IDs. The experimental ID in Vesiclepedia database is the unique number referring to a scientific report showing genes and their associated vesicles. Next, the extracted experimental IDs were matched with the experimental IDs in the details of experiment file to extract types of the vesicles and details of experiment that identified association between genes and vesicles types.

3. Results

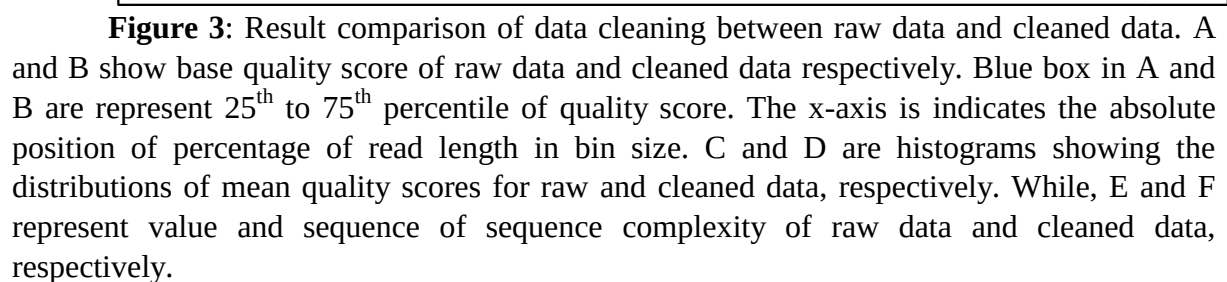
3.1 Cleaned NGS data

The sequencing data were composed of two files, forward and reverse reads. NGS data were cleaned by PRINSEQ program as described in materials and methods (17). The total reads and bases of raw data and cleaned data were showed in table 1.

Table 1: The total reads and bases of raw data and cleaned data

Number of sequence and total base	Raw data	Cleaned data
Sequence file 1 (reads number)	10,627,083	7,255,135
Total bases file 1 (base pair)	787,925,687	537,690,154
Sequence file 2 (reads number)	10,627,083	7,255,135
Total bases file 2 (base pair)	789,050,787	536,707,465

After the cleaning step, 7,255,135 reads (68.27%) remained. Base quality score of raw data and cleaned data were shown in Figure 3 (A and B) showing that the mean scores were around 39. Sequence complexity of cleaned data was cut at 70 as a minimum entropy value. (Figure 3, E and F)



Out of 14,510,270 reads, 14,053,810 reads could be mapped to human genome. After sequences merged and annotated with EMBL database, 573,819 reads (4.08%) were from mitochondrial DNA, and 13,479,991 reads (95.92%) were from genomic DNA. 7,643,446 reads (56.70%) of genomic DNA were mapped to gene consisting of 48,643 genes (protein-coding and non-protein coding gene) while 5,836,545 reads (43.30%) were from a non-gene regions. (Figure 4)

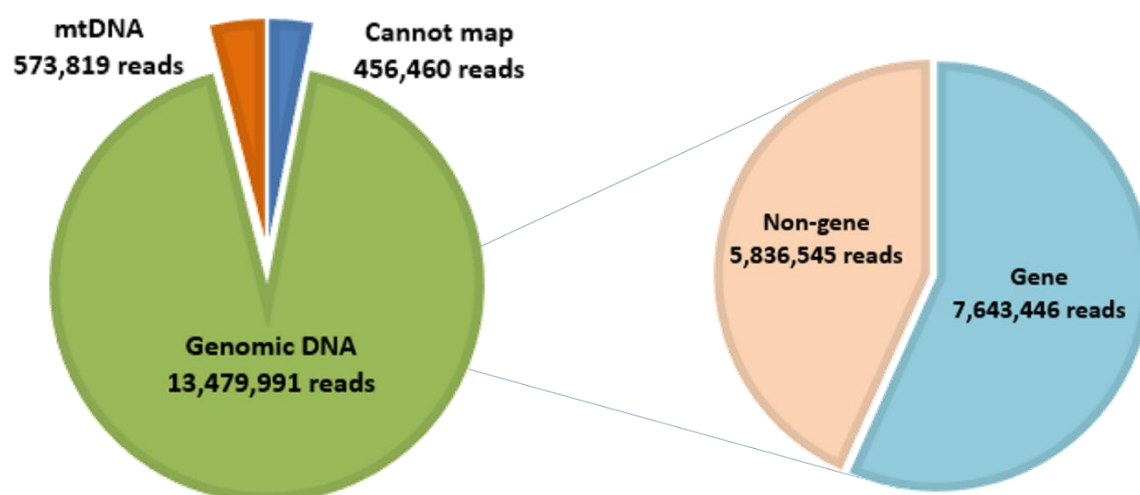


Figure 4: Proportion of cleaned sequences classified by alignment and annotation analyses.

Out of 48,643 genes obtained from annotation analysis, 35,906 genes (4,318,647 reads) were not associated with any vesicle type. While, the number of genes associated with any vesicle were 12,737 genes (3,324,799 reads). The details of number of genes, number of reads and associated vesicle types were showed in table 2 and Figure 5.

Table 2: Number of genes and reads classified by vesicle type from Vesiclepedia database

Vesicle type	Number of genes	Number of reads
Exosomes/ extracellular vesicles	7	1,350
Exosomes	11	3,700
Exosomes/ microvesicles	18	8,373
Exosomes/ membrane vesicles	183	40,502
Microvesicles	12,518	3,270,874
Total	12,737	3,324,799

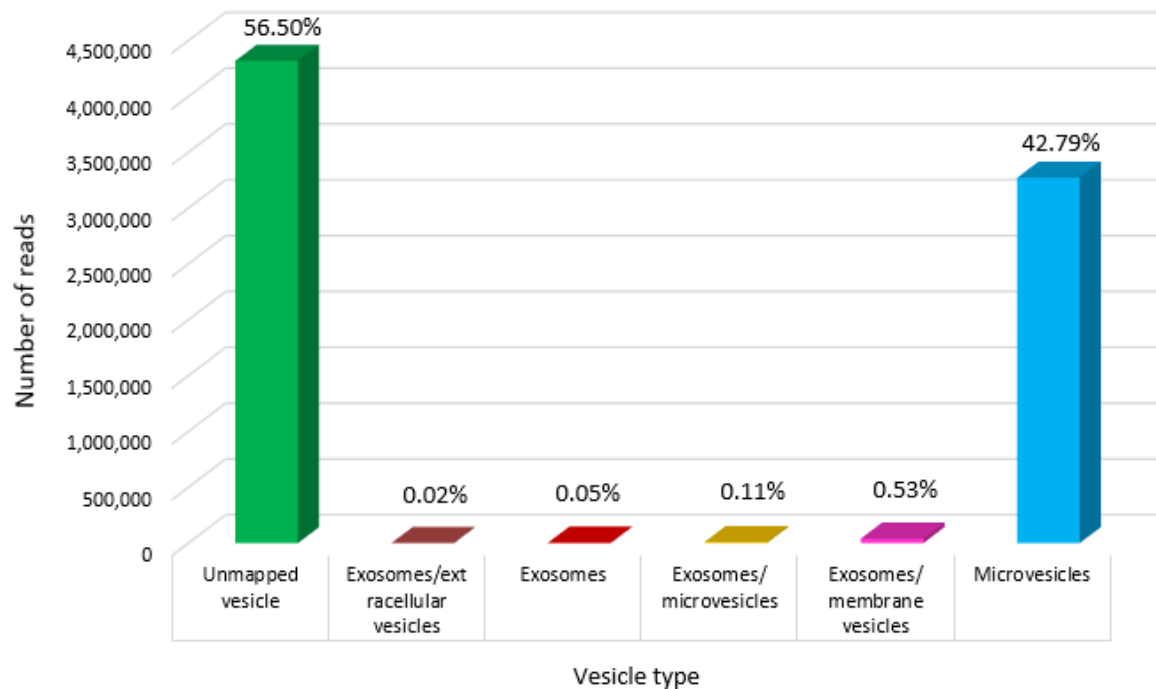


Figure 5: Number and percentage of reads classified by vesicle type

4. Discussion and conclusion

The data cleaning is an important step before perform further sequence analyses because data from NGS technology may contain low quality sequence reads. The low quality reads can disturb analytical process, such as sequence alignment and de novo assembly, so elimination of low quality data is necessary step. The important considerations for data cleaning are presence of ambiguous bases, distribution of read lengths, quality score of bases, complexity of sequences and duplication of sequences (17, 20). The observed raw error rate of Illumina (Miseq) is 0.80% (21). The probability of an error rate of an individual base is predicted by quality score (21). The highest possible quality score is Q = 40 meaning the sequenced base is almost absolutely correct. The mean quality scores for raw data of this study was high. So, our data were cleaned with a cut-off at Q = 30 which meant error probability was 1 in 1,000 bases (22). A low-complexity sequence is usually consisted of repetitive bases. Low-complexity sequences are nonsensical and likely to not provide information for further analysis (23). Sequence duplication is an occurrence of a set of exactly identical copies of sequences which must be removed at the beginning of analytical pipeline. Sequence duplication is usually produced by basic steps of library preparation and sequencing especially PCR amplify of template fragments with adapters. Without deduplication, duplicated sequence may skew the proportion of reads mapped in alignment step (24).

Since our sample was from a plasma of a patient with infection, we expected to see sequence reads not belonging to either human or mitochondrial genome. However, the alignment data showed the amount of sequence reads originating from human genome and mitochondrial overwhelmed the sequence reads possibly originating from pathogens. This is because human DNA in various forms, such as cell free DNA (cfDNA) and vesicular bound form, normally circulates in blood stream (11-14).

Genetic materials in vesicle are protected against DNase treatment (7). Sequence reads in our data were found to associate with exosome, microvesicle, extracellular vesicle and membrane vesicle. The majority of genes found in the study were associated with microvesicle. Only a small proportion of the genes were associated with exosome/extracellular vesicle, exosome only, exosome/microvesicle and exosome/membrane vesicle. It is worth noting that gene clustering in our study were performed only with Vesiclepedia database and may not be comprehensive. Therefore, our data may be further expanded with an inclusion of other vesicle databases such as Exocarta database (25), which collect data of genetic contents that found in exosome, and EVpedia database (26), which contain another vesicle such as apoptotic body. Knowing types of vesicle may help select an effective method for host depletion. For example, apoptotic bodies may be depleted from plasma by using an immunoaffinity column such as annexin V affinity assay (27).

cfDNA released into blood stream are poorly degraded by DNA-degrading enzyme during pathogen enrichment because they are often bound by DNA-binding proteins such as transcription factors (28). Transcription factors bind to regulatory regions and shield the DNA from enzymes. Therefore, information about regulatory regions found in cfDNA could help design an effective method for pathogen enrichment, such as applying chromatin immunoprecipitation to deplete cfDNA. Additionally, a previous study showed that EDTA, an anticoagulant regularly used for blood collection and preparation, could inhibit DNase possibly resulting in the persistence of cfDNA (29). Thus, a new method is still required to help better eliminate host genetic materials.

Acknowledgement

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Effect of *Curcuma longa* rhizome on expression of *HMGCR* gene involving in the cholesterol biosynthesis

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Abstract

Objective: Hypercholesterolemia is a major risk factor for the development of atherosclerosis which leads to premature CVD. Statins are widely used for treatment of hypercholesterolemia. However, natural dietary compounds might be an alternative choice for lowering cholesterol levels. Therefore, we studied the effect of *Curcuma longa* rhizome on the expression of the *HMGCR* gene involving in the cholesterol biosynthesis.

Materials and methods: Human hepatocellular carcinoma (HepG2) cells were treated with 0.5%DMSO, *C. longa* rhizome, or simvastatin for 24 hr. After the treatment, total RNA was extracted from the cells before expression of the *HMGCR* gene was studied using quantitative real-time PCR.

Results: The expression of the *HMGCR* gene was significantly down-regulated in *C. longa* rhizome-treated HepG2 cells at the concentration of 0.03 mg/mL compared with the negative control ($52 \pm 3\%$, $P < 0.05$). It was also found that 0.03 mg/mL of *C. longa* rhizome could more down-regulate the expression *HMGCR* gene than the concentrations of 0.06 and 0.13 mg/mL, respectively.

Conclusion: The study indicated that *C. longa* rhizome might have a cholesterol-lowering potential by downregulation of the *HMGCR* gene expression in HepG2 cells. This herb might be an alternative choice for treatment of hypercholesterolemia and early onset CVD.

Keywords: Atherosclerosis, *Curcuma longa*, CVD, *HMGCR*, hypercholesterolemia

1. Introduction

Cardiovascular disease (CVD) is currently the most common cause of death worldwide. An over 17.5 million people died from CVD in 2012, representing 31% of all global deaths and it has been expected to grow to more than 23.6 million deaths by 2030 ⁽¹⁾. In Thailand, CVD has become a leading cause of death of both men and women ⁽²⁾.

Hypercholesterolemia is a risk factor for the development of atherosclerosis which leads to premature CVD ⁽³⁾. Elevated levels of plasma low-density lipoprotein cholesterol (LDL-C) are well documented risk factor for CVD ⁽⁴⁾.

3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (HMGCR), membrane-bound enzyme, is the rate-limiting enzyme for cholesterol biosynthesis, primarily in the liver where it is responsible for synthesizing 2/3rd of the total cholesterol in the body (5).

HMGCR synthesis is increased by 200 fold when cellular cholesterol concentrations decrease ⁽⁶⁾. HMGCR activity declines by 90% when cholesterol is adequate ⁽⁷⁾. HMGCR is tightly regulated by a supply of cholesterol. Thus, the enzyme has been a therapeutic target of the statin drugs to lower blood LDL-C concentration and reduces the risk of CVD.

Curcuma longa L. (known as turmeric) is widely cultivated in tropical and subtropical parts of the world. Curcumin, the main yellow bioactive constituents in the rhizome of *C. longa* has been shown to exert various biological actions including hypocholesterolemic effects via the hepatic regulation of lipoprotein cholesterol metabolism ^(8, 9). In Thailand, the powder of *C. longa* rhizome is mainly used as a spice in foods and herbal medicines in forms of capsules/tablets. Therefore, it also might exert any hypocholesterolemic effects and could be alternative choice for treatment of hypercholesterolemia, which can further develop to CVD.

In this study, we examined whether the *C. longa* rhizome powder alters the expression of the *HMGCR* gene in the cholesterol biosynthesis.

2. Materials and methods

2.1 Study design

Human hepatocellular carcinoma (HepG2) cells used in this study were divided into 3 groups: negative control group (0.5%DMSO), *C. longa* rhizome-treated group, and simvastatin-treated group. After the treatment, total RNA was extracted from the cells before expression of the *HMGCR* gene was studied using quantitative real-time PCR (qRT-PCR).

2.2 Materials

Dried *C. longa* rhizome powder was obtained from Dr. Pathompong Prupong, Phana Hospital Thailand Medicine Center, Amnat Charoen province. Simvastatin was purchased from Berlin Pharmaceutical Co., Ltd., Bangkok, Thailand.

A stock solution was prepared by dissolving the *C. longa* rhizome powder or simvastatin in DMSO and stored at -20°C for subsequent uses. All dilutions were freshly prepared in a cell culture medium.

2.3 Cell culture

The culture condition was modified from Lu *et al.* ⁽¹⁰⁾. HepG2 cells were cultured in Dulbecco's modified of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin and 100 µg/mL of streptomycin) at 37°C in a humidified atmosphere of 5% CO₂ in air as a monolayer culture in steriled plastic culture plates or flasks. The medium was changed every 2 days, and the cells were subcultured when they reach 80-90% confluence.

2.4 Cytotoxicity assay

Cytotoxicity effect of *C. longa* rhizome was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay modified from Sun *et al.* ⁽¹¹⁾. HepG2 cells were plated in a 96-well cell culture plate (5×10^4 cells/well) in DMEM supplemented with 10% FBS and antibiotics. After an overnight culture, the cells were incubated with 0.5%DMSO, different concentrations of *C. longa* rhizome (0.03, 0.06, 0.13, and 0.25 mg/mL) or simvastatin (0.01, 0.03, 0.05, and 0.10 mg/mL) in triplicate wells for 24 h. After incubation, the medium was removed. A volume of 20 µL/well of MTT (5 mg/mL)

was added and incubated for 2 h. After incubation, the medium was replaced with 100 μ L of DMSO and the amount of formazan formed from the reaction was determined by measuring the absorbance at 570 nm using an ELISA plate reader. The cell density of treatment groups was expressed as a percentage of the control.

2.5 RNA isolation and quality control

HepG2 cells (2×10^6 cells) were treated with 0.5%DMSO, different concentrations of *C. longa* rhizome or simvastatin. After 24 h, total RNA was extracted using TRIzol[®] reagent according to the manufacturer's instructions. For quality control, RNA purity and integrity were evaluated by measuring the ratio of absorbance at 260 and 280 nm (~ 1.9) using a nanophotometer (Implen, Germany) and quality of the extracted total RNA will be observed from the 28S/18S rRNA ratio (2 : 1) using agarose gel electrophoresis.

2.6 cDNA synthesis

Total RNA was reverse transcribed into cDNA using two-steps RT-PCR (Reverse transcription-polymerase chain reaction). In reverse transcription step, RNA template was transcribed into a single-stranded cDNA catalyzed by oligo(dT)₁₈ primer (Thermo Scientific, Lithuania). In PCR step, single-stranded cDNA was amplified to yield a double-stranded cDNA by RevertAid reverse transcriptase (Thermo Scientific, Lithuania).

2.7 qRT-PCR analysis

Gene expression was quantified by qRT-PCR, using Stratagene Mx3005P (Agilent Technologies, USA) and SYBR green detection system according to the manufacturer's instructions. The PCR parameters were 95°C for 1 min, 1 cycle; 95°C for 1 s, specific annealing temperature 60°C for 1 min (depending on product length), 72°C for 1 min for 40 cycles and a further melting curve step for 1 cycle. Each sample was run in triplicates and C_T data was normalized using *GAPDH*, which is stably expressed in experimental groups. Relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method⁽¹²⁾.

2.8 Statistical analysis

Differences between the negative control and simvastatin-treated groups were determined by Student's *t*-test. Differences between the negative control and *C. longa* rhizome-treated groups were examined by one-way ANOVA. Any significant differences were further analyzed using Dunnett's test. Statistical significance was considerably accepted at *P*-value < 0.05. All data are presented as mean $\pm$ SEM (n = 3).

3. Results

3.1 Cytotoxicity effect of *C. longa* rhizome on HepG2 cells

To evaluate the effect of *C. longa* rhizome on cell viability, HepG2 cells were treated with 0.5%DMSO, different concentrations of *C. longa* rhizome or simvastatin for 24 h. The results from MTT assay showed a dose-response decrease in cell viability in both *C. longa* rhizome and simvastatin-treated groups. After 24 h of the treatment, the results were found that *C. longa* rhizome treatment at concentrations of 0.03-0.13 mg/mL had no effect on HepG2 cell viability but *C. longa* rhizome treatment at concentration of 0.25 mg/mL was significantly toxic to the cells (IC₅₀ = 0.24 mg/mL; Figure 1A). In addition, simvastatin

treatment was more toxic to HepG2 cells than *C. longa* rhizome (IC₅₀ = 0.07 mg/mL; Figure 1B).

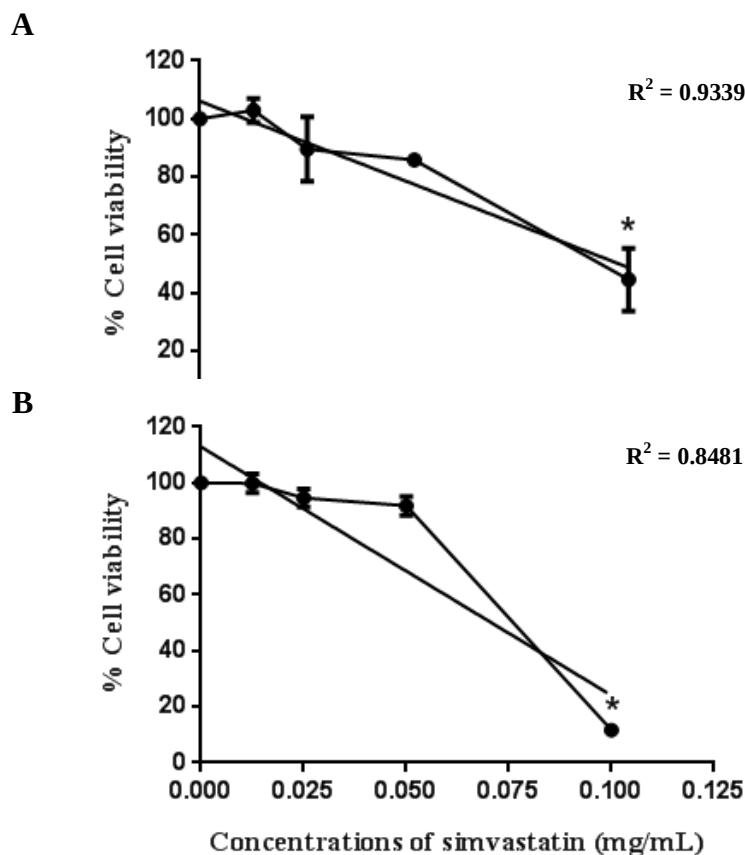


Figure 1. Effect of *C. longa* rhizome and simvastatin on HepG2 cell viability. The data are shown as mean $\pm$ SEM (n = 3).

3.2 Effect of *C. longa* rhizome on *HMGCR* gene expression

To determine whether *C. longa* rhizome alters the expression of the *HMGCR* gene in the cholesterol biosynthesis, we treated HepG2 cells with 0.5%DMSO, different concentrations of *C. longa* rhizome, or simvastatin for 24 h. The *C. longa* rhizome treatment significantly down-regulated the *HMGCR* gene expression at the concentrations of 0.03 and 0.25 mg/mL compared with the negative control ($52 \pm 3\%$ and $64 \pm 7\%$, respectively). Whereas, simvastatin treatment did not change the *HMGCR* gene expression compared with the negative control (Figure 2).

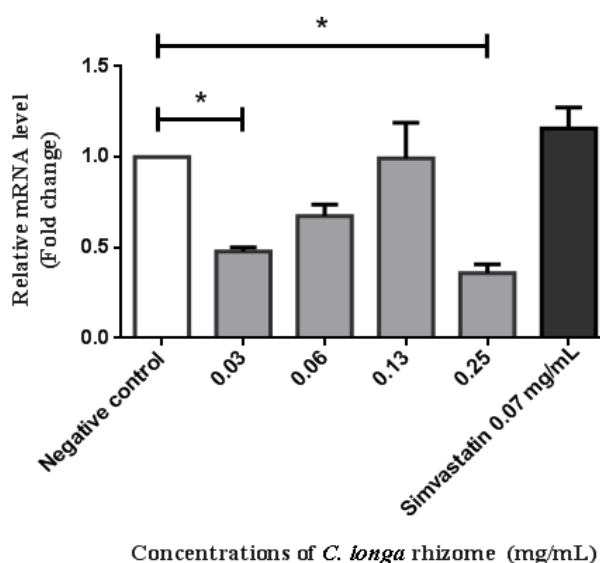


Figure 2. qRT-PCR analysis of the *HMGCR* gene expression in the *C. longa* rhizome or simvastatin-treated HepG2 cells for 24 h. The data are shown as mean $\pm$ SEM (n = 3).

4. Discussion and conclusion

In this study, we demonstrated that *C. longa* rhizome treatment significantly down-regulated the *HMGCR* gene expression in HepG2 cells at the lowest concentration of 0.03 mg/mL. It was also found that 0.03 mg/mL of *C. longa* rhizome could more down-regulated than the concentrations of 0.06 and 0.13 mg/mL, respectively. Notably, The *HMGCR* gene expression was also dramatically down-regulated in *C. longa* rhizome-treated HepG2 cells at concentration of 0.25 mg/mL. However, it might be due to the cytotoxicity effect of the herb at that concentration. Furthermore, simvastatin treatment did not change the *HMGCR* gene expression in HepG2 cells.

The effect of *C. longa* on the *HMGCR* gene expression have had controversial results. *In vivo* studies, long-term curcumin treatment in LDLR^{-/-} mice fed a high-cholesterol diet was reported to lower plasma and hepatic cholesterol and decrease the activity and mRNA levels of the *HMGCR* enzyme⁽¹³⁾. Oral administration of the extract of *C. longa* in rats fed a high-cholesterol diet also exhibited a significant decrease in the *HMGCR* gene expression⁽¹⁴⁾. Our findings supported the studies in the animals fed with a high-cholesterol diet. However, Peschel *et al.* found that curcumin-treated HepG2 slightly increased the level of the *HMGCR* mRNA⁽⁹⁾. Different responsiveness might be caused by the different in constituents of *C. longa* and the model study.

In conclusion, the powder of *C. longa* rhizome seems to be more effective than simvastatin at inhibiting the *HMGCR* gene expression. However, simvastatin has a primary action to inhibit the enzyme activity. Therefore, further studies on effect of *C. longa* rhizome and its active compounds on protein levels and other genes involving in the cholesterol homeostasis may help provide a better understanding of cholesterol-lowering potential of *C. longa* rhizome.

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Distribution of Spoligotyping defined genotypic lineages of ethambutol-resistant *Mycobacterium tuberculosis* complex clinical isolates in Thailand

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Abstract

Objective: Tuberculosis (TB), caused by *M. tuberculosis* complex, is a leading cause of morbidity and mortality worldwide. Along with attempts to treat patients, multidrug resistant-TB (MDR-TB) emerged. Ethambutol (EMB) is a first-line anti-TB drug to reduce emergence of other anti-TB drug resistance, including isoniazid and rifampicin. However, the resistance rate of EMB has gradually increased in some regions. In Thailand, EMB resistance was reported to be the most common among the first line drugs. The aim of this study is to explore the genetic diversity and identify predominant spoligotypes of ethambutol-resistant *M. tuberculosis* clinical isolate in Thailand using spacer oligonucleotide typing (spoligotyping).

Materials and Methods: A total of 153 *M. tuberculosis* clinical isolates were collected from Siriraj Hospital in 2012-2014. After performed spoligotyping, the result was compared with the SITVITWEB international database to designate Shared International Type (SIT) and phylogenetic clade. The relationship between identified clade and ethambutol resistance was examined by statistical analysis.

Results: Spoligotyping resulted in 28 distinct patterns, of which 5 patterns had not been reported in the global database. Among these spoligotypes, SIT1 was the most common (71.2%, n = 109) followed by SIT89 (3.9%, n = 6). Phylogenetic lineage designation found that Beijing genotypes were predominant in both ethambutol susceptible group (69.6%) and resistant group (83.3%). Moreover, ethambutol resistant strains of Beijing genotype was significantly higher than those of non-Beijing genotype (83.3% and 16.7% respectively, $p = 0.044$).

Conclusion: Beijing family genotypes were the most predominant genotype, which exhibited significant correlation with ethambutol-resistance.

Keyword: *Mycobacterium tuberculosis*, ethambutol resistance, genotyping, spoligotyping

1. Introduction

Tuberculosis (TB) is a leading cause of mortality worldwide. Every minute, three people in developing countries die of tuberculosis. With more than 6 million new cases of active diseases and almost 1.5 million deaths each year, tuberculosis is a global health

emergency of overwhelming magnitudes ⁽¹⁾. To treat patients with tuberculosis, a combination of first-line anti-TB drugs, isoniazid, rifampicin, ethambutol and streptomycin, is the most effective treatment. After 6 months of a treatment with the first-line drugs, more than 90% cure rate is typically achieved. However, an estimated 480,000 people per year developed multidrug-resistant TB (MDR-TB), which is resistant to at least isoniazid and rifampicin. Moreover, there were approximately 190,000 deaths due to MDR-TB worldwide ⁽²⁾.

Ethambutol (EMB) is an important first-line anti-TB drug, particularly when treating MDR-TB and XDR-TB (extensively drug-resistant TB). EMB has also been found to reduce emergence of resistance strains to other companion drugs, particularly isoniazid and rifampicin ⁽³⁾. Initially, EMB was effective for preventing treatment failures caused by *M. tuberculosis* isolates resistant to other anti-TB drugs. However, resistance rate of EMB has gradually increased in some regions and is close to 50% in retreatment patients ⁽²⁻⁵⁾. In the northeastern part of Thailand, EMB resistance was reported to be the most common among the first line drug (3.8%), more than the isoniazid (2.3%) and rifampicin resistance (2.8%). However, the high proportion of EMB resistance may result from region-specific population or a cause of outbreak during the study period ⁽⁶⁾.

To better understand epidemiology of EMB resistance, we explored the genetic diversity of EMB-resistant *M. tuberculosis* clinical isolate in Thailand. Furthermore, spoligotyping-defined phylogenetic lineages were investigated relationship to the drug resistance.

2. Materials and Methods

2.1 Strains and anti-mycobacterial susceptibility testing

M. tuberculosis clinical isolates were obtained from Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University for a span of three years (2012-2014). The first-line anti-tuberculosis drug susceptibility (isoniazid, rifampicin, ethambutol, and streptomycin) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described by Abate *et al.* ⁽⁷⁾. This study was approved by Siriraj Institutional Ethic Committee (No. Si504/2014).

2.2 Host demography or spoligotyping-defined *M. tuberculosis* lineages and their associations to ethambutol resistance

Association of ethambutol resistance and predisposing factors such as age, gender, and phylogenetic lineage of spoligotypes were considered. Statistical analysis of the results were carried out by using the SPSS V-19.0 software (IBM, New York, USA). Chi square test was conducted to determine the statistical difference along with crosstab analysis on *p* values. The *p* value of less than 0.05 was considered as statistically significant.

2.3 Spoligotyping

Spoligotyping, a PCR based reverse hybridization technique, was performed according to manufacturer's specifications ⁽⁸⁾. Briefly, highly polymorphic Direct Repeat (DR) locus was investigated by detecting presence or absence of 43 spacers between DR. The first step was to isolate *M. tuberculosis* genomic DNA boiling method ⁽⁹⁾. The isolated DNA was used to amplify spacers by using biotinylated DRa and DRb primer. The amplified spacers were hybridized to membrane immobilized the set of 43 spacers oligonucleotide.

Detection of hybridization signals was optimized by the enhanced chemiluminescence (ECL) detection system.

Positive signal was interpreted as 1 while negative signal was represented by 0. Then, each binary triplet except the last spacer was converted into octal code designation⁽⁸⁾. Using SITVITWEB, database of the Pasteur Institute of Guadeloupe ([http://www.pasteur-guadeloupe.fr:8081/SITVIT ONLINE/](http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/))⁽¹⁰⁾, octal code format was entered to assign Shared International Type (SIT) and phylogenetic lineage. The new SITs was designate lineage based on SpotClust database (http://tbinsight.cs.rpi.edu/about_spotclust.html), as previously described⁽¹¹⁾.

3. Results

A total of 153 *M. tuberculosis* strains were collected. There were consisted of 84 ethambutol-resistant, 35 ethambutol-susceptible with multi-drug resistance (resistance to isoniazid and rifampicin) and 34 pan-susceptible (susceptible to isoniazid, rifampicin, ethambutol, and streptomycin) isolates. The isolates were mostly from females (60.1%, n =92). The median age of the studied patients was 50 years (range 5-84 years). Most patients aged between 25 and 44 years (43.2%, n = 51), followed by those aged more than 45 years (41.5 %, n =49). According to statistical analysis, no correlation between ethambutol resistance and sex or age of individuals was found ($p = 0.200$ and $p = 0.147$, respectively), as indicated in table1.

Table 1: Isolate characteristics and their associations with ethambutol resistance

Isolate characteristics	Number of subjects	Ethambutol susceptibility testing		
		Susceptible Number (%)	Resistant Number (%)	<i>p</i> -value
Gender				
Female	92	45 (66.2)	47 (55.3)	0.200
Male	60	23 (33.8)	37 (44.0)	
Unknown	1			
Age (years)				
< 25	18	6 (10.2)	12 (20.3)	0.147
25-44	51	24 (40.7)	27 (45.8)	
>45	49	29 (49.2)	20 (33.9)	
Unknown	35			
Lineage		48 (69.6)	70 (83.3)	0.044
Beijing	118	21 (30.4)	14 (16.7)	
Non-Beijing	35			
Total	153	69	84	

The 153 isolates were classified into 28 distinct spoligotypes as shown in table 2. 23 SITs (n = 144) and 5 unclassified SITs (n = 9) were identified. The most common spoligotype was SIT1 (71.2%, 109 out of 153 strains). Upon database comparison, strains were grouped into 15 major lineages. The largest lineage was Beijing family (SIT 1, SIT 941, SIT 190, and SIT 255) accounted for 75.2% (n=115), followed by the EAI2-nonthaburi family (4.6%, n=7) and the EAI5 family (4.6%, n=7), as illustrated in the table 2.

Table 2: Spoligotype patterns of *Mycobacterium tuberculosis* complex clinical isolates in this study

[illegible]

Orphan: An extremely rare SIT, New: The newly identified spoligotype pattern in this study

Distribution of spoligotype-defined lineages against drug resistant profiles showed that Beijing family was more predominant in the MDR groups (91.4% for ethambutol sensitive and 76.2% for ethambutol resistant) than the pan-susceptible group (38.2%), as shown in table 3. The ethambutol resistance comparison between Beijing family and non-Beijing family showed that the percentage of Beijing genotype was significantly higher than the non-Beijing genotype (83.3% and 16.7%, respectively; $p = 0.044$), as indicated in table 1.

Table 3: Association between spoligotypes and drug resistance patterns of *Mycobacterium tuberculosis* complex in Thai clinical isolates from 2012 to 2014

SIT	Phylogenetic lineage	Drug resistance profile		
		Pan-susceptible Number (%)	MDR-EMB ^S Number (%)	MDR-EMB ^R Number (%)
1	Beijing	13 (38.2)	32 (91.4)	64 (76.2)
941	Beijing			2 (2.4)
190	Beijing	1 (2.9)		
255	Beijing		1 (2.9)	
Orphan	Beijing			2 (2.4)
89	EAI2-nonthaburi	4 (11.8)	1 (2.9)	1 (1.2)
Orphan	EAI2-nonthaburi	1 (2.9)		
256	EAI5	3 (8.8)		
236	EAI5	2 (5.9)		
618	EAI5	1 (2.9)		
591	EAI6-BGD1	1 (2.9)		1 (2.9)
53	T1	1 (2.9)		2 (2.4)
123	T1			1 (1.2)
2673	T2	1 (2.9)		
149	T3-ETH			1 (1.2)
943	Ambiguous:T2T			1 (1.2)
742	H3	1 (2.9)		
Orphan	H3	1 (2.9)		
54	MANU2			1 (1.2)
523	Manu_ancestor		1 (2.9)	
246	Orphan			1 (1.2)
1149	Orphan			1 (1.2)
1462	Orphan	1 (2.9)		
New	New	3 (8.8)		6 (7.1)
Total		34	35	84

Orphan: An extremely rare SIT, New: The newly identified spoligotype pattern in this study, Pan-susceptible: Strains that were susceptible to isoniazid, rifampicin, streptomycin, and ethambutol, MDR-EMB^S: Strains that were classified to MDR (resistant to at least isoniazid and rifampicin) and susceptible to ethambutol, MDR-EMB^R: MDR strains that were resistant to ethambutol.

4. Discussion and Conclusion

In Thailand, the resistance rate of ethambutol, one of the first-line anti-TB drug, is considered high comparing to the other major first-line drugs, isoniazid and rifampicin (6). Thus, patient demography, including age and gender were investigated for their correlation with ethambutol resistance. The chi-square test with crosstab analysis found that there were no significant difference between ethambutol resistance and age or gender of individuals. To better understand the increasing rate of ethambutol resistance, spoligotyping was used to investigate predominant genotype among *M. tuberculosis* in Thai clinical isolates.

Spoligotyping demonstrated that the Beijing family was the most prevalent lineage with more than 75%. This result indicated an important role for the transmission cause of Beijing strains in Thai community. Similar to several findings, incidence of Beijing clinical strains were also considered high in many countries, including 54% in Vietnam ⁽¹²⁾, 70% in Hong Kong ⁽¹³⁾, and 41% in Taiwan ⁽¹⁴⁾. Not only in Asia, there was also report global

dissemination of *M. tuberculosis* Beijing family strains⁽¹⁶⁾. Several studies proposed that Beijing genotype had high transmission rate,⁽¹⁷⁾ and high virulence, including resistance to BCG vaccine⁽¹⁸⁾ and several antibiotics with low fitness cost⁽¹⁹⁾. These may lead to the high incidence of Beijing genotype in many countries including in Thailand. However, the present study could not conclude that the high prevalence of ethambutol resistance in Thailand caused by the Beijing genotype outbreaks as the Beijing strains has emerged in China and spread throughout the world since 1995⁽¹⁶⁾. In addition, epidemiological investigation with spoligotyping alone may overestimate the proportion of Beijing lineage⁽²²⁻²⁴⁾. Therefore, alternative genotyping technique with a higher discriminatory power such as IS6110-RFLP or MIRU-VNTR is suggested to be used in combination with the spoligotyping to further evaluate the clustering.

Nevertheless, analysis of relationship between ethambutol resistance and Beijing genotype found that the drug resistance frequency among Beijing strains (83.3%) was significantly higher than that of the non-Beijing strains (16.7%). The result implied that Beijing strains might gain resistance by a selective advantages comparing to non-Beijing strains. Besides, ethambutol resistance might be a driving force of spread and high prevalence of Beijing family. These results in agreement with previous studies proved that the Beijing lineage was highly correlated to resistances of isoniazid, rifampicin⁽²⁰⁾, and fluoroquinolones⁽²¹⁾. Thus, it implied that the higher resistance rates of the Beijing isolates facilitates spread of *M. tuberculosis* Beijing genotype.

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Expressions of stratifin and cytokeratin7 as biomarkers in gallbladder cancer

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Abstract

Objective: Gallbladder cancer (GBC) is the most frequent biliary tract. GBC in early stage is difficult to be detected due to lack of obvious specific symptoms. More than 50% of GBC cases develop metastasis at the time of diagnosis and most of the resectable cases develop recurrences or metastases within two years after surgery. The detection of specific markers for GBC might help physicians to predict the prognosis, recurrence rate, overall survival rate and disease-free survival of their patients. This study aims to search for biomarkers for GBC from Formalin-Fixed, Paraffin-Embedded (FFPE) samples by real-time RT-PCR. Moreover, the biomarkers we found might be further developed for targeted treatment options in GBC patients.

Materials and Methods: All FFPE tissue samples of GBC and corresponding non-tumourous gallbladder from 11 patients were subjected to RNA extraction and the expression of biomarkers Stratifin (*SFN*) and Cytokeratin7 (*CK7*) were examined by real-time PCR. The expressions were then analyzed by one sample *t*-test. The data in each experiment was calculated as mean $\pm$ SEM. *P*-value < 0.05 was considered significant.

Results: The expression of biomarkers *SFN* and *CK7* were studied in 11 pairs of GBC FFPE tissues by real-time PCR. The significant overexpression of *SFN* (73%) and *CK7* (82%) were found in GBC tissue compared with corresponding non-tumourous tissue from the same patient.

Conclusion: The overexpression of biomarkers *SFN* and *CK7* were found significantly in GBC. Therefore, their roles as molecular biomarkers in GBC will be further investigated whether they could be useful to predict the prognosis, recurrence rate, overall survival rate and disease-free survival of GBC patients

Keyword: Gallbladder cancer, Biomarkers, Expression

1. Introduction

Gallbladder cancer (GBC) is the most frequent biliary tract malignancy (1). The most common risk factors for the development of GBC is cholesterol gallstone (2). GBC in early stage is difficult to be detected due to lack of obvious specific symptoms. More than 50% of GBC cases develop metastasis at the time of diagnosis and most of the resectable cases develop recurrences or metastases within two years after surgery (3).

A specific marker for GBC might help physicians to predict the prognosis, recurrence rate, overall survival rate and disease-free survival of the patients. From previous studies, there are some markers used for detection of biliary tract cancer, for instance, Stratifin (*SFN*) and Cytokeratin7 (*CK7*) (4, 5). The *SFN* plays a crucial role in anoikis resistance of cholangiocarcinoma cells in the recent data of Thai and Asian people (5-7). This mechanism is very important for metastatic process of cancer and may explain why biliary tract cancer is usually followed by metastasis early after surgery (8). For *CK7*, it is the fundamental intermediate filament protein mostly found in epithelial cells. It is reported as a marker for biliary epithelial cells, bile duct damage, and cholestasis (9).

This study aims to search for the expressions of *SFN* and *CK7* as the biomarkers for the GBC from FFPE samples by real-time RT-PCR. Moreover, the biomarkers we found might be further developed for targeted treatment options in GBC patients.

2. Materials and methods

2.1 FFPE tissue

All FFPE tissue samples are from GBC and corresponding non-tumourous gallbladder of 11 GBC patients who underwent the surgical resection at Siriraj Hospital during 2007 to 2015. The FFPE tissues were prepared at Department of Pathology. All human studies were approved by The Institution review Board (IRB), Faculty of Medicine, Siriraj Hospital, Mahidol University (EC no. SI244/2015).

2.2 RNA extraction and cDNA synthesis

FFPE tissue samples were cut into serial section with a thickness of 10 μ m. Using an RNase-free microtome on RNase-free slides. The total RNA was extracted from FFPE sections by Absolutely RNA FFPE Kit (Agilent, USA) and eluted in a final volume of 30 μ l. After RNA isolation, amount of RNA was estimated for the concentration and the purification by NanophotometerTM (Implen GmbH, Germany) and reverse-transcribed to cDNA by SuperscriptTM III First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA).

2.3 Real-Time PCR

The expressions of 2 genes of interest, *SFN* and *CK7*, and a reference gene, beta actin (*ACTB*) were detected by real-time PCR (CFX 96TM Real-Time PCR detection system, Bio-Rad, USA).

2.4 Data analysis for relative gene expression

To compare the relative levels of gene expression in GBC and corresponding non-tumourous gallbladder FFPE tissue, the comparative cycle (Ct) method, also known as $2^{-\Delta\Delta C_t}$ was employed. *ACTB* was used as a reference gene (where Ct is the threshold cycle for the target gene amplification)

$$\Delta C_t = C_{t \text{ target gene}} - C_{t \text{ reference gene}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{GBC FFPE tissue}} - \Delta Ct_{\text{non-tumour gallbladder FFPE tissue}}$$

The expressions in GBC FFPE tissues was shown by fold change in expression of the same patient, then, calculated to log fold change by apply base-10 logarithm (Log10).

2.5 Statistical analysis

All result data was analyzed by the software package GraphPad Prism version 5.0. The Kolmogorov Smirnov's test represents the normality of base-10 logarithm of the fold change at the significant level of $p < 0.05$ with 95% confidence interval. The expressions were analyzed by one sample *t*-test. The data in each experiment was calculated as mean $\pm$ SEM. *P*-value < 0.05 was considered significant.

3. Results

3.1 Analysis of pair GBC FFPE tissue and corresponding non-tumorous gallbladder FFPE tissue by real-time RT-PCR showed a significant up-regulation of *SFN* and *CK7*.

We observed a consistently up-regulated of *SFN* expression in GBC when compared with the corresponding non tumour gallbladder tissue. Eight of eleven (73%) of the tumour samples showed significant up-regulation of *SFN* (Figure 1). Similarly, nine of eleven (82%) of the tumour samples showed significant up-regulation of *CK7* (Figure 2)

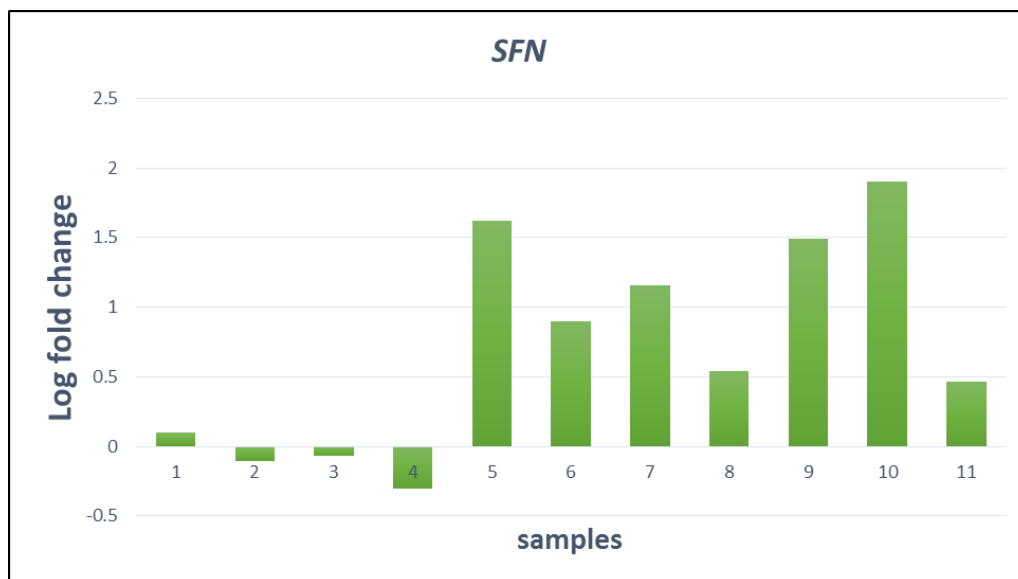


Figure 1 Log fold change of *SFN* expression in GBC relative to corresponding non-tumorous gallbladder FFPE tissue. The data showed 8/11 (73%) up-regulation of *SFN* in GBC FFPE tissue

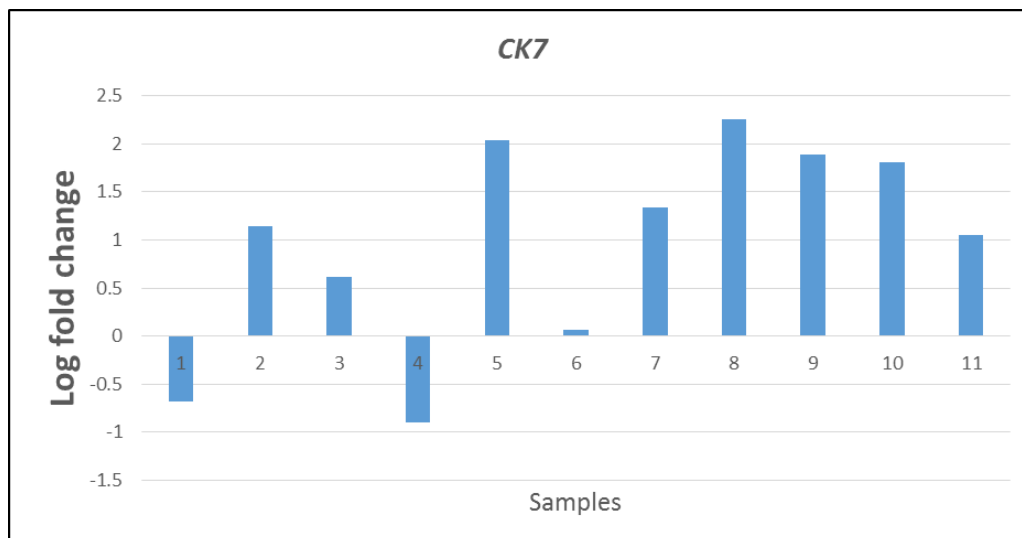


Figure 2 Log fold change of *CK7* expression in GBC relative to corresponding non-tumourous gallbladder FFPE tissue. The data showed 9/11 (82%) up-regulation of *CK7* in GBC FFPE tissue.

The summary of the expression data of *SFN* and *CK7* was shown in Figure 3. The mean of fold change of *SFN* expression is 16.7 times of the corresponding non-tumourous FFPE tissue (log fold change= 0.7) (P -value < 0.05; one-sample t -test), similarly, the mean of fold change of *CK7* expression is 43.9 times of the corresponding non-tumourous FFPE tissue (log fold change= 1.0) (P -value < 0.05; one-sample t -test).

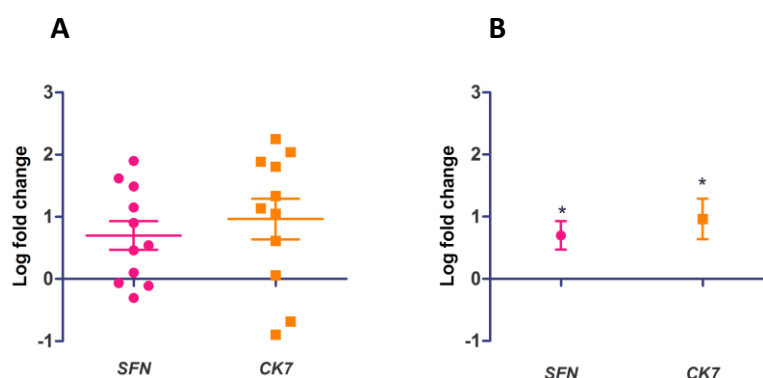


FIGURE 3 SUMMARY OF THE EXPRESSIONS OF *SFN* AND *CK7* IN GBC.

A) Scatter plot showed log fold change of expression of *SFN* and *CK7*

B) Cell plot showed mean $\pm$ SEM of log fold change of expression

*Significant P -value < 0.05 one sample t -test, *SFN* and *CK7*: n=11

4. Conclusion and discussion

Our study showed that *SFN* is significantly up-regulated in GBC FFPE tissue compared with corresponding non-tumorous gallbladder FFPE tissue. Seventy-three percent of GBC FFPE tissues showed overexpression of *SFN*. The *SFN* plays a crucial role in anoikis resistance of cholangiocarcinoma cells and this mechanism is very important for metastatic process of cancer and may explain why biliary tract cancer is usually followed by metastasis early after surgery (8).

For *CK7*, our study showed that *CK7* is significantly up-regulated in GBC FFPE tissue compared with corresponding non-tumorous gallbladder FFPE tissue. Eighty-two percent of GBC FFPE tissues showed overexpression of *CK7*. *CK7* is the fundamental intermediate filament protein mostly found in epithelial cells. It is reported as a marker for biliary epithelial cells, bile duct damage, and cholestasis (9).

Both *SFN* and *CK7* have previously been demonstrated as biomarkers for other biliary tract cancers except GBC. Therefore, their roles as molecular biomarkers in GBC will be further investigated whether they could be useful to predict the prognosis, recurrence rate, overall survival rate and disease-free survival of GBC patients.

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Prunetin Protects Against Dexamethasone-Induced Pancreatic β -cells Apoptosis

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Abstract

Objective(s): Dexamethasone is an anti-inflammatory and immunomodulation drugs. Dexamethasone causes steroid-induced diabetes through increased insulin resistance and decreased β -cell mass. Prunetin, a phytoestrogen, was shown to prevent diabetes. This study aims to identify the dosage of prunetin that protects dexamethasone-induced pancreatic β -cell apoptosis.

Material(s) and Method(s): INS-1 cells were cultured in RPMI-1640 medium containing 11.1mM glucose. To investigate the effective dose of dexamethasone that induced cell death, INS-1 cells were incubated in dexamethasone concentration from 0.01, 0.1, 1 and 10 μ M for 72 hours. Then, propidium iodide (PI) staining was performed to evaluate cell death. To identify the protective dosage of prunetin, INS-1 cells were treated at 10, 20, and 50 μ M of prunetin in the presence or absence of dexamethasone for 72 hours. After that, cellular apoptosis was assessed by Annexin V/PI staining.

Results: The percentage of cell death was significantly increased in INS-1 cultured in 0.1, 1 and 10 μ M of dexamethasone, respectively. While, 0.01 μ M of dexamethasone did not have any effect on cell death when compared to control. All concentrations of prunetin significantly decreased dexamethasone-induced cell apoptosis while prunetin alone showed no effect on cell apoptosis.

Conclusion: Our study demonstrated that prunetin concentration at 10 μ M is the most effective dosage to protect dexamethasone-induced pancreatic β -cell apoptosis. However, the molecular mechanism that prunetin protects dexamethasone-induced pancreatic β -cell apoptosis needs further investigation.

Keyword(s): Prunetin, Dexamethasone, Pancreatic β -cells, Apoptosis, Diabetes

1. Introduction

Diabetes is a common chronic disease occurred world-wide including Thailand (1, 2). Type 2 diabetes is a result of chronic insulin resistance and loss of functional pancreatic β -cell mass. Pancreatic β -cell apoptosis is caused by excessive nutrients including glucotoxicity, lipotoxicity and glucolipotoxicity (3). Also, pancreatic β -cell apoptosis can be induced by drugs such as dexamethasone (4). Dexamethasone, synthetic glucocorticoids (GCs), has been used widely for several diseases including asthma, autoimmune diseases and

organ transplanted (5-8). Chronic usage of dexamethasone has been shown to associate with development of diabetes (5). It is well known that dexamethasone produced hyperglycemia by increase insulin resistance (9). Other studies showed that dexamethasone induced pancreatic β -cell death by several mechanisms (5, 9) including dephosphorylation of BAD leading to influx of cytochrome C and activate caspase activity (10). Also, dexamethasone was shown to suppress insulin secretion and inhibit β -cell proliferation by reduced PDX-1 and induced Foxo1 expression (11). Dexamethasone also induced cellular stress lead to increase ROS production (12). In addition, dexamethasone directly suppresses Glutathione Peroxidase-4 (13) and thioredoxin through MAPK and p38 signaling (14). Dexamethasone has also been shown to induce endoplasmic reticulum homeostasis which led to pancreatic β -cell death (15). Several studies used different dosages of dexamethasone to induce cell death in cell lines. For example, primary cell culture of hepatocyte and islet cells apoptosis occurred at 0.1 μ M dexamethasone (16, 17). Hamster β -cells (HIT-15) can induce apoptosis at 1 μ M dexamethasone (4). A few studies have been reported that dexamethasone induced INS-1 cell death at concentration from 10-1000 nM (6, 18, 19). Therefore, the first aim of this study is to identify the dose of dexamethasone that induced pancreatic β -cell death.

Prunetin is one of phytoestrogen (20), that belongs to *Prunus* family. It was extracted from *Prunus yedoensis* (21). Prunetin is also found in soy products, bitter cherry, red clover, legume, and forage plant (22, 23). Prunetin has several effects including weak estrogenic effect (24), tyrosine kinase inhibitor effect (20), and prevention of metabolic disorders related to obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, aging, and cancer (25). Moreover, previous study demonstrated that prunetin may have anti-inflammatory effect, which suppressed NF- κ B pathway both *in vitro* and *in vivo* (21). Also, prunetin has been shown to protect against obesity by decreasing adipogenesis in high fat diet fed mice (26). Role of prunetin on pancreatic β -cell has not been studied. Whether prunetin protected pancreatic β -cell against dexamethasone-induced apoptosis is still unknown. Thus, the second aim of this study is to investigate the protective effect of prunetin on pancreatic β -apoptosis that induced by dexamethasone.

2. Materials and Methods

2.1 Cell culture

INS-1, rat insulinoma β -cells, were cultured in RPMI 1640 medium with L-glutamine without phenol red containing 11.1 mM glucose supplemented with 10% heat inactivated fetal calf serum (FCS), 10 mM HEPES, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin, pH 7.4 at 37 °C in humidified air containing 5% CO₂. The culture medium was changed every 2 days.

2.2. Propidium iodide (PI) staining assay

Cell death was performed by PI staining (BD Pharmingen, USA). INS-1 cells were seeded in RPMI-1640 medium containing 11.1 mM glucose. After 24 hours, INS-1 cells were treated with or without dexamethasone different doses at 0.01, 0.1, 1, and 10 μ M. After 72 hours, cells were collected and fixed with 70% (v/v) ethanol in PBS at -20°C for 2 hours. After centrifugation, the pellet was suspended in 100 μ l of PBS with 1 μ l of RNase A (1 ng/ml) and incubated at 37°C for 1 hour. After that, PI (1 mg/ml) in 400 μ l of PBS was added into the samples and incubated for 3 minutes. Cell cycle was analyzed by flow cytometry using FACSCalibur Flow Cytometer (Becton Dickinson, USA).

2.3. Annexin V-FITC/ Propidium Iodine (PI) assay

Cellular apoptosis were determined by FITC Annexin V Apoptosis detection Kit I (BD Pharmingen™, USA). The experiments were performed as described by the company. Briefly, INS-1 cells were plated and pre-treated with or without 10 μ M, 20 μ M, and 50 μ M of prunetin for 1 hour after that 0.1 μ M dexamethasone was added. After 72 hours, cell pellets were obtained after centrifugation. After that, cells from each condition was added with Annexin V-FITC and further incubated on ice in dark room for 15 minutes. After that, PI was added into each condition. Cellular apoptosis were analyzed by flow cytometry using FACSCalibur Flow Cytometer (Becton Dickinson, USA).

2.4 Statistical analysis

All studies were performed at least 3 independent experiments. Data were expressed as mean $\pm$ standard error of the mean (SEM). The data obtained in experiments with multiple treatments were analyzed by one-way ANOVA followed by *post Hoc* Tukey's multiple comparison test of significant using GraphPad Prism version 5.0 software. A *p*-value less than 0.05 were considered to be statistically significant.

3. Results

3.1 The effective dose of dexamethasone that induced cell death in INS-1 cells.

To identify dose of dexamethasone induced pancreatic β -cell death, INS-1 cells were treated with or without dexamethasone at 0.01, 0.1, 1, and 10 μ M for 72 hours. Then, the numbers of cell death were measured by PI staining. The percentage of cell death was increased in correspondent with dose of dexamethasone when compared to control. Dexamethasone at dose 0.1, 1 and 10 μ M significantly increased when compared to those cultured without dexamethasone. Only dexamethasone at dose 0.01 μ M, the number of cell death was increased but not significant when compared to control (Figure 1).

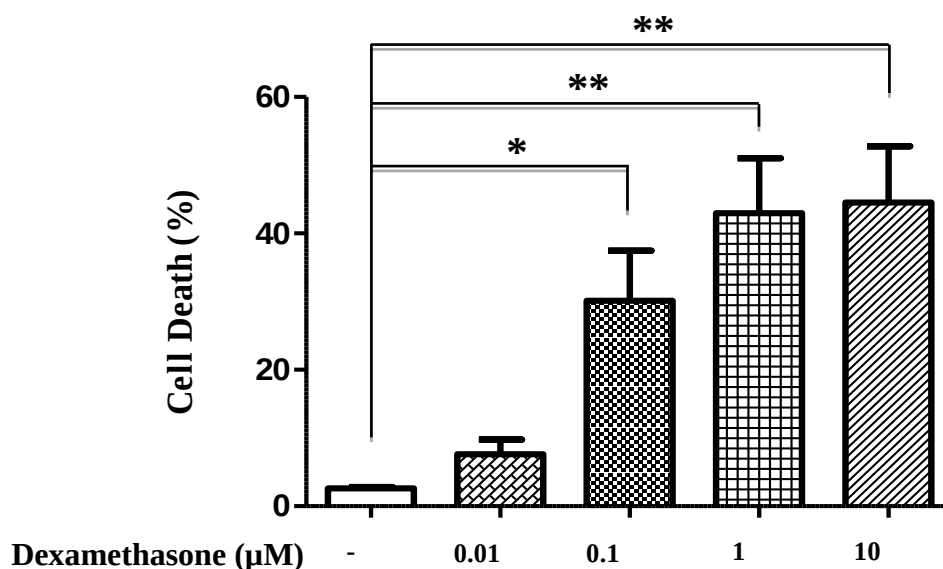


Figure 1. Cell death was measured by PI staining

INS-1 cells were treated with or without 0.01, 0.1, 1, and 10 μ M of dexamethasone for 72 hours in complete growth medium. Then, the numbers of cell death were measured by PI

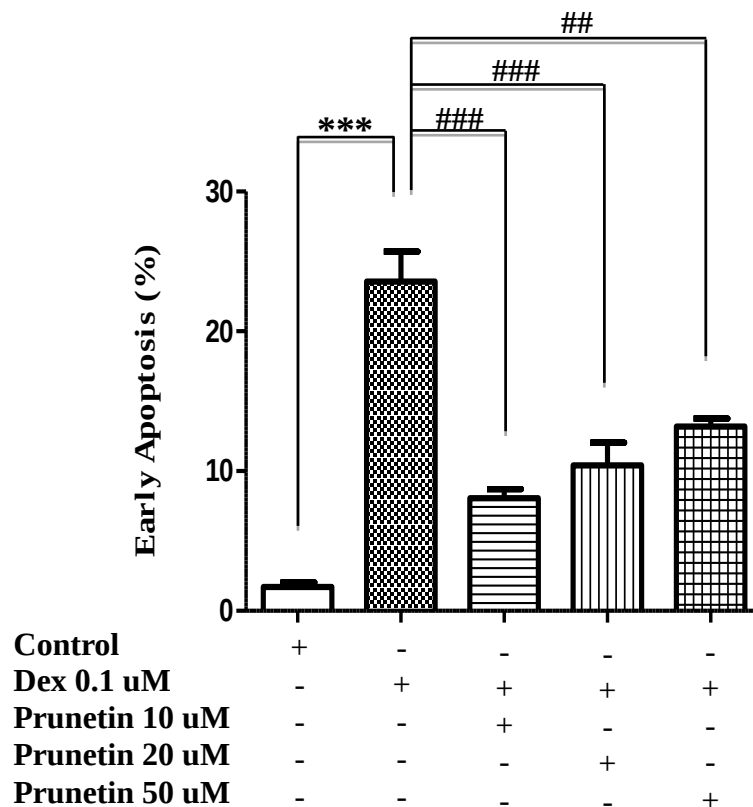
staining. The mean $\pm$ SEM of four individual experiments are shown. $*P < 0.05$, $**P < 0.01$ significant different from control.

3.2 Protective dosage of prunetin in INS-1 cells after treated with dexamethasone.

To examine whether prunetin can protect against dexamethasone induced pancreatic β -cell apoptosis, INS-1 cells were cultured with prunetin at 10, 20, and 50 μ M in the presence of 0.1 μ M dexamethasone. Dexamethasone significantly induced pancreatic β -cell apoptosis, whereas, prunetin significantly reduced pancreatic β -cell apoptosis when co-cultured with dexamethasone (Figure 2A). Our results suggested that prunetin at 10 μ M has the most effective in reduction of pancreatic β -cell apoptosis.

To examine whether prunetin alone affects β -cell death, INS-1 cells were cultured with prunetin at 10, 20, and 50 μ M. Then, cell apoptosis was assessed by AnnexinV/PI staining. Prunetin alone at the concentration 10, 20, and 50 μ M did not show any significant change on the cellular apoptosis (Figure 2B).

A



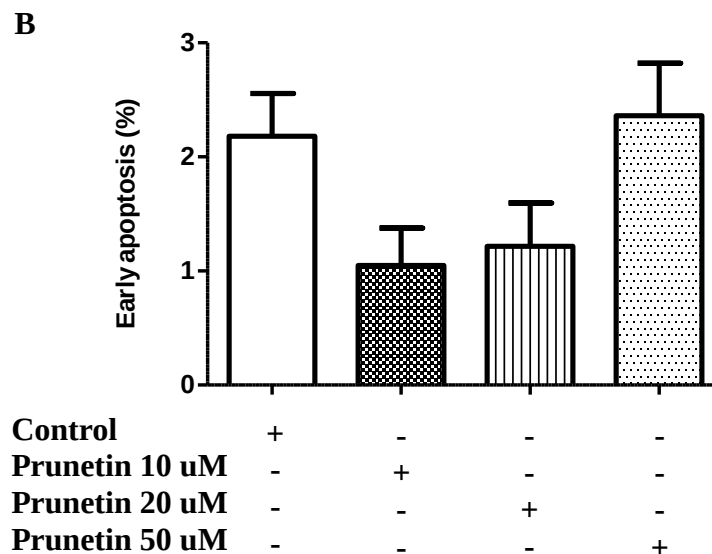


Figure 2. Cell apoptosis was detected by AnnexinV-FITC/PI staining.

INS-1 cells were pre-treated with various concentration of prunetin for 1 hour followed by treated with (A) or without 0.1 μ M dexamethasone (B). After 72 hours, cellular apoptosis was performed. This bar graph shows the average percentage undergoing early apoptosis of INS-1 cells. Data were represented mean $\pm$ SEM of three independent experiments. *** $P < 0.001$ significant different from control. ### $P < 0.001$ significant different from dexamethasone treatment.

4. Conclusion and Discussion

Glucocorticoids treatment has been demonstrated to induce pancreatic β -cell dysfunction and ensue in apoptosis (6). Previous study has been shown that prednisolone directly inhibited insulin secretion, decreased β -cell proliferation, impaired glucose metabolism and induced endoplasmic reticulum (ER) stress (8, 15). Prednisolone has less potency than dexamethasone. Thus, this study firstly aimed to identify dose of dexamethasone induced pancreatic β -cell death in 72 hours. Our result showed that dexamethasone increased pancreatic β -cell death in dose dependent manner at concentration range from 0.1, 1 and 10 μ M. However, dexamethasone at concentration 0.01 μ M did not significantly increased pancreatic β -cell death when compared to control. Our finding is comparable to other study showed that dexamethasone at concentration range from 0.1 and 1 μ M induced mitogen-inducible gene 6 (Mig6), which led to pancreatic β -cell death in a dose dependent manner (18). Similar to our result, recent study reported that IC_{50} value of 0.1 μ M dexamethasone is 0.092 μ M in INS-1 cells (27). In addition, dexamethasone at concentration 1 μ M can also significantly increase cell death in pancreatic β -cell line from hamster (HIT-15) (4). Thus, in this study used dexamethasone at concentration 0.1 μ M to induce pancreatic β -cell death. This concentration can be found in plasma in person who took dexamethasone with mean diurnal levels ranging from 100-510 nmol/L (28).

Prunetin is a phytoestrogen. The effect of prunetin on pancreatic β -cells apoptosis has not been studied. This study examined dose of prunetin in reduction of pancreatic β -cells apoptosis that induced by dexamethasone. Our results suggested that INS-1 cells cultured with prunetin at concentration 10, 20 and 50 μ M and dexamethasone significantly decreased

pancreatic β -cells apoptosis. The most effective dose of prunetin in reduction of dexamethasone-induced pancreatic β -cells apoptosis is 10 μ M. INS-1 cells cultured with prunetin at concentration 10, 20 and 50 μ M alone did not show significantly change in number of cell apoptosis. It is possible that high concentration of prunetin is high toxicity in cell culture. In agreement with this study, it has been reported that prunetin has IC_{50} value approximately at 15 μ M (20). Similar to previous study reported that 100 μ M of prunetin significantly decreased viability of macrophage cells than 25 and 50 μ M but not significant (21). In accordance with another study showed that prunetin stimulated osteoblast function at concentration range from 0.1 nM to 10 μ M (29). However, in human epithelium, prunetin inhibited effect of TNF $-\alpha$ -induced MUC5AC gene expression at concentration 10, 50, and 100 μ M (30). Thus, it is worth to mention that effective dose of prunetin are different in each cell types.

Several previous data have been showed beneficial effects of prunetin both *in vitro* and *in vivo* (21, 26, 29, 31). Prunetin prevented high fat induced-obesity in mice (26). Classically, type 2 diabetes mellitus is the result of insulin resistance associated with relative insulin deficiency that caused by dysfunction of β -cells and reduced β -cells mass. This report suggested that prunetin inhibits adipogenesis in visceral adipose tissue. Reduction in visceral adipose tissue might reduce insulin resistance. Moreover, in this study showed that prunetin induced adiponectin receptor 1, adiponectin receptor 2 and AMP-activated protein kinase (AMPK). Adiponectin action has been shown to protect pancreatic β -cell apoptosis and increase insulin production and secretion (32). It can be noted that prunetin protected pancreatic β -cells apoptosis against dexamethasone might be due to these effects. However, this study did not examine the mechanisms that prunetin protects pancreatic β -cells apoptosis from dexamethasone.

In summary, our results suggested that 10 μ M of prunetin is the most effective dosage to protect dexamethasone-induced pancreatic β -cell apoptosis in INS-1 cells. However, the molecular mechanism that prunetin protects dexamethasone-induced pancreatic β -cell apoptosis needs further investigation.

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The Aneurysm of Coronary Artery in Thais: The Cadaveric Study

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Abstract

Objectives: To study the location, type, size, abnormal change of the arterial wall of coronary aneurysm and the comparison between sex and age group in Thai cadaveric heart. To study the pathological changes of the arterial wall of aneurysms and the histological structure of the normal arterial wall in light microscope.

Materials and Methods: The arterial coronary aneurysms were studied and classified by age, sex and cause of death. The type, size and location of aneurysms were recorded. Later, coronary artery aneurysms were cut and fixed in 10% formalin. The tissue was processed of hematoxylin and eosin staining. Some sections were chosen for light microscopic study.

Results: In 83 cases of heart cadavers, the coronary aneurysms were found in 62 cases (74.70%), 41 males (66.13%) and 21 females (33.87%). The coronary aneurysms were found in age between 39-97 years old. Arterial aneurysm was classified into 3 types, the saccular type (46.61%), the fusiform type (36.44%) and the ectasia type (16.95%). The location of the coronary aneurysm was the right coronary artery (RCA), the left anterior descending artery (LAD), the left circumflex artery (LCX), the right transverse branch (RTB), the right posterior descending branch (RPD), the right marginal branch (RMB) and the left coronary artery was 46.61%, 27.12%, 16.10%, 4.24%, 2.54%, 1.69%, and 1.69% respectively. The maximum size of the arterial diameter was 7.77 mm. and the minimum size was 1.04 mm. The pathological structure of saccular aneurysm was the degeneration of tunica media and the thickness of the tunica intima.

Conclusion: The incidence of the arterial wall of coronary aneurysm in Thais was 74.70% (male 66.13% and female 33.87%) and the age between 39-97 years old. The most common type was saccular type 46.61% and the most location was the right coronary artery 46.61%. The maximum diameter of the arterial dilatation was 7.77 mm. The histological structure of saccular aneurysm was the hypertrophy of the tunica intima in light microscopic study.

Keywords: coronary artery aneurysm, aneurysm, coronary artery

1. Introduction

Heart disease is a main problem of the world, leading to the cause of death. The epidemiology in Poland found that the mortality rate of cardiovascular disease increased to the maximum in ten countries [1,2]. Cardiovascular disease, including stroke and coronary heart disease, was the major problem in Asian countries. Coronary heart disease is an important problem of public health in Thailand which increases continuously and is found in late middle-aged patients. A major cause of ischemic heart disease is the disease of coronary artery such as aneurysm, atherosclerosis. These arterial diseases are generally found in both men and women [3,4,5]. The risk factors of heart disease are environmental, in particular smoking, high cholesterol, diabetes mellitus, obesity, family history or genetic factor. The coronary artery disease is chronic disease that the process of disease progresses slowly depending on lifestyle.

Coronary artery aneurysm is an important heart disease because the coronary artery contains the oxygen rich blood that supplies the heart muscle. The arterial wall of coronary artery consists of three layers and it has the structural proteins of inside the arterial wall; collagen, elastin and smooth muscle cell [6]. The inflammation of the wall of coronary artery causes the formation of plaque, blood clots, fatty deposition or thrombus formation inside the vessel which effects on the lumen narrowing. Otherwise, inflammation of the arterial wall can cause the abnormal microscopic structure; internal elastic lamina, tunica intima or tunica media were destroyed and the artery becomes enlarge. The lack of blood supply of heart due to coronary artery aneurysm or stenosis can induce the ischemic heart disease. While thrombosis, embolization or rupture incur myocardial infarction and sudden death. The main cause of coronary artery aneurysm is atherosclerosis and the other causes are Kawasaki disease, polyarteritis nodosa, systemic lupus erythematosus, infection, trauma, dissection, angioplasty, congenital malformation, behcet disease, abnormal connective tissue syndrome (Marfan syndrome), cocaine addict [5,7]. Coronary artery aneurysms are asymptomatic but symptom was shown often the rupture. The symptoms of coronary heart disease are chest pain, tiredness and weakness. In severe case, heart attack, heart failure and sudden cardiac muscle death will be occur [8].

Arterial aneurysm can be classified into 2 types, true aneurysm and false aneurysm (pseudoaneurysm). True aneurysm has pathological changes in all three layers of the vessel wall (tunica intima, tunica media and tunica adventitia) but pseudoaneurysm has only the outer layer of the vessel wall (tunica adventitia) is changable. True aneurysm can be classified as saccular shape and fusiform shape. Saccular aneurysm is a bubble-shaped or burry-like. It's always form at the bifurcation of vessel or the side wall. It wall involves the partial circumference of the vessel wall. Fusiform aneurysm is cigar-shaped or spindle-shaped. It locates at the bifurcation or stem of branch. Its wall involves the full circumference of vessel. The most common locations were abdominal aorta, intracranial artery, iliac artery, femoral artery and popliteal artery [9]. In general arterial aneurysm is 50% increase in diameter of normal vessel. In case which the diameter increase more than 4 cm. is called the giant coronary artery aneurysm. The right coronary artery, the left anterior descending artery, the left circumflex artery and the left main artery in frequent coronary artery aneurysm was discovered respectively [6]. Computed tomographic angiography (CTA), magnetic resonance image (MRI), echocardiography are used for the diagnosis confirmation about the size, shape or location of coronary artery aneurysm. These disease are treated by some drugs (statin, warfarin, aspirin, clopidogrel, simvastatin, lovastatin, and cerivastatin) for small aneurysm or

no symptom and nonsurgical (stent and coil embolization) or surgical repair for aneurysm diameter exceeding 3 cm. [5,10].

In this thesis, the location, type, size, abnormal change of the arterial wall of coronary aneurysm, the comparison between sex and age group will be studied in Thai cadavers from the Department of Anatomy, Faculty of Medicine Siriraj Hospital in Mahidol University.

2. Materials and Methods

2.1 Cadaveric specimen

Thai cadaveric specimens from the Department of Anatomy, Faculty of Medicine Siriraj Hospital in Mahidol University between 2015 to 2016 were studied. In 83 cases of cadavers (50 males and 33 females) were classified by age, sex and cause of death. The type, size and location of aneurysms were studied and recorded.

2.2 Human cadaver collection

At the beginning, the heart's cadavers were removed from pericardial cavity and the study of the major vascular supplies the heart were made. These are called "coronary artery". Both right and left coronary arteries originate from ascending aorta. After that the coronary artery and their branches were observed for aneurysm. The type, size and location of aneurysms were recorded. While the Vernier digital caliper were using to measure the size of aneurysms. Then, coronary artery aneurysms were cut and fixed in 10% formalin. The tissue was washed in water, dehydration, clearing and infiltration respectively. Later on, the tissue was embedding in paraffin. The tissue was cut at 6 micron thickness and then the process of hematoxylin and eosin staining were done. Some sections were chosen for light microscopic study. The histological structure of the arterial wall aneurysms were compared to those of the normal arterial wall. Pathological changed of the arterial wall aneurysms were studied.

3. Result

In 83 cases of heart cadavers, the coronary aneurysms were found the normal vessel in 21 case and the abnormal dilation of the coronary artery in 62 cases (74.70%), 41 males (66.13%) and 21 females (33.87%). The age between 39-97 years old were found in the coronary aneurysm. In 83 cases were found the normal vessel 21 cases, the single aneurysm 25 cases and the multiple aneurysm 37 cases. Arterial aneurysm was classified into 3 types, 55 the saccular type (46.61%), 43 the fusiform type (36.44%) and 20 the ectasia type (16.95%) [Figure 1]. The location of the coronary aneurysm was 55 the right coronary artery (RCA), 32 the left anterior descending artery (LAD), 19 the left circumflex artery (LCX), 5 the right transverse branch (RTB), 3 the right posterior descending branch (RPD), 2 the right marginal branch (RMB) and 2 the left coronary artery respectively [Table 1]. The maximum size of the arterial diameter was 7.77 mm. and the minimum size was 1.04 mm. The histological structure of the saccular aneurysm was the thin vessel wall of the tunica media that smooth muscle cell were migrated to the tunica intima and the thickness of the tunica intima [Figure 2].

Table 1: The type and the location of coronary artery aneurysm

Location	Saccular	Fusiform	Ectasia
RCA	22	21	12
RMB	2	0	0
RTB	1	4	0
RPD	2	1	0
LCA	1	1	0
LAD	16	11	5
LCX	11	5	3
Cases	55	43	20
Total (%)	46.61%	36.44%	16.95%

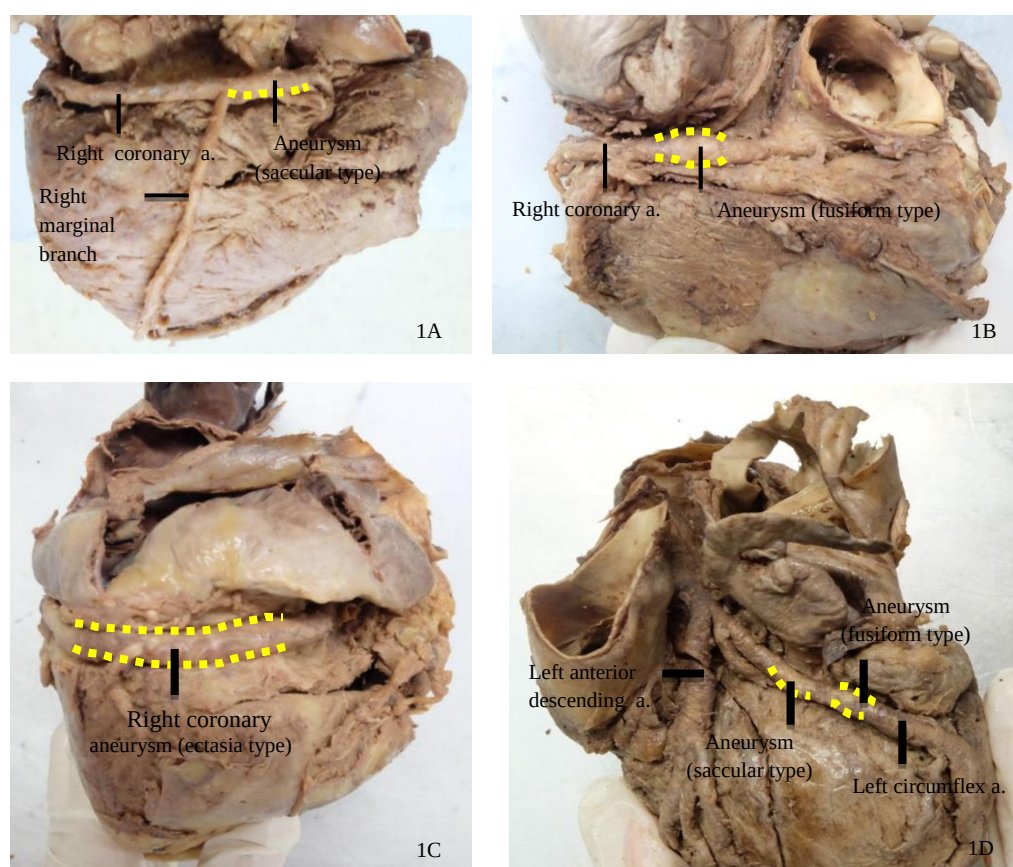


Figure 1: The saccular type aneurysm at 1A, the fusiform type aneurysm at 1B, and the ectasia type aneurysm at 1C of the right coronary artery (RCA) were the single aneurysm. The multiple aneurysms at 1D were shown the saccular type and the fusiform type of the aneurysmal the left circumflex artery (LCX).

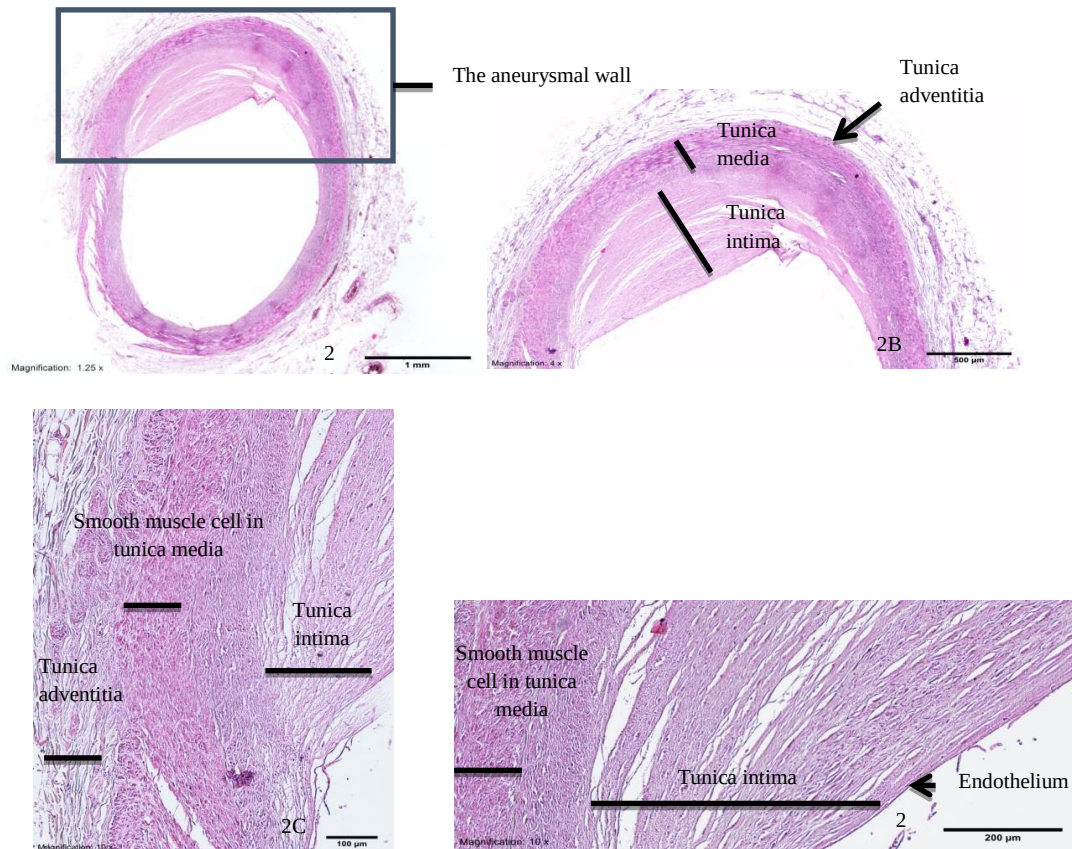


Figure 2: The histological structure of the cross section of the left anterior descending artery (LAD) was abnormal arterial wall in Figure 2A. The thickening of the tunica intima was shown in 4X, Figure 2B and 10X, Figure 2C. The degenerative area in tunica intima showed 10X, Figure 2D.

3. Discussion

Previously report the most location of coronary artery aneurysms were 68% of the left anterior descending in 1990 [10]. This finding differs from Paul et al. found 40%-87% of the right coronary artery [11]. The incidence of coronary artery aneurysm was 46.61% the right coronary artery (RCA) and the right marginal branch was found rarely in this study.

The histological structure of coronary artery aneurysms were the destruction of the tunica media, which thinned of the arterial wall and connective tissue replaced the smooth muscle cell and elastic fiber in tunica media report by Nichols et al. in 2008 [4]. The present study found the degeneration of the tunica media, which was thinned and the dilatation of tunica intima in the saccular aneurysm of the left anterior descending artery.

4. Conclusion

This study showed the incidental finding of the coronary artery aneurysm in Thais that was more male (66.13%) than female (33.87%) and age between 39-97 years old. The coronary artery aneurysm found more saccular type than fusiform type and ectasia. The most location was 46.61% the right coronary artery. The histological structure of saccular aneurysm was the hypertrophy of the tunica intima in light microscopic study.

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***HBS1L* Expression in β^0 -Thalassemia/Hemoglobin E Derived Erythroid Cells**

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Abstract

Objectives: β^0 -Thalassemia/HbE disease has a variety of symptoms from mild to severe form, of which HbF is one of the key factors that can relieve the severity. Despite the fact that *HBS1L* is shown associated with HbF and disease severity in β^0 -thalassemia/HbE, its function in red cells is entirely unknown. Therefore, this study aims to explore the expression of *HBS1L* during erythropoiesis of normal compared with β^0 -thalassemia/HbE subjects.

Materials and Methods: CD34+ progenitors were collected from peripheral blood of β^0 -thalassemia/HbE and healthy normal subjects for *ex vivo* erythroblast culture. The transcript levels of *HBS1L* during erythroid development were measured by quantitative RT-PCR and the protein levels were measured by Western blot.

Results: The mRNA expression of *HBS1L* showed similar pattern but different levels between normal and β^0 -Thalassemia/HbE derived erythroblasts. Western blot analysis revealed the present of HBS1L protein in both nucleus and cytoplasm, but the levels were displayed in opposite trend between the two fractions.

Conclusion: This study suggested the potential roles of *HBS1L* in erythropoiesis and hemoglobin production. However, further experiments are needed to confirm the hypotheses and explore the mechanisms.

Keyword: *HBS1L*, β^0 -thalassemia, hemoglobin E, erythroid cells

1. Introduction

β^0 -Thalassemia/HbE disease causes a highly variable symptoms ranging from nearly asymptomatic state (mild form) to transfusion-dependent anemia (severe form). The basis of this variation is not fully understood (1, 2). Fetal hemoglobin (HbF) is one of the major modifiers of disease severity in β^0 -thalassemia/HbE i.e. the higher levels of HbF could ameliorate the disease severity by reducing the degree of imbalance globin chain (3-5).

HBS1L (or HBS1-like translational GTPase), a homolog of yeast *hbs1*, is located on chromosome 6q23.3 encoding a member of the GTP-binding elongation factor family (6, 7). The intergenic region of *HBS1L* and *MYB* genes is known as one of HbF quantitative trait loci (or QTLs) (1) and genome wide association study (GWAS) in mild versus severe β^0 -thalassemia/HbE patients has revealed a handful of SNPs (single nucleotide polymorphism) associated with the disease severity and HbF levels (8). Moreover, expression of *HBS1L* negatively correlates with HbF levels (9). The SNP in exon 1 of *HBS1L* is shown to be

associated with HbF level and severity in β^0 -thalassaemia/HbE patients (4). Although, substantial evidences implicate the potential roles of *HBS1L* in red blood cells development, the functions of *HBS1L* in that process are still unknown.

To gain more insight about the functions of *HBS1L* during erythropoiesis and HbF production, its expression during erythropoiesis in β^0 -thalassemia/HbE was investigated and compared with normal erythropoiesis.

2. Materials and Methods

2.1 Subjects

Healthy normal and β^0 -thalassemia/HbE subjects were recruited from Nakhon Pathom Hospital and Siriraj Hospital, Thailand. Peripheral blood samples were collected from 3 healthy normal and 3 β^0 -thalassemia/HbE patient volunteers who are at least 18 years old. The healthy normal subjects must not be carriers of α -thalassemia, β -thalassemia, or any Hb variants. The β^0 -thalassemia/HbE patients must not receive blood transfusion for at least 1 month before participation. This study was approved by the Ethical Committee, Siriraj Institutional Review Board (Si 290/2015).

2.2 Primary erythroblast culture

Primary CD34⁺ cells were positively selected from peripheral blood mononuclear cells (PBMCs) using CD34 MicroBead kit (Miltenyi Biotec) according to manufacturer's protocol. The selected CD34⁺ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) with addition of 20% fetal bovine serum (FBS; Sigma-Aldrich), 300 ng/mL human holotransferrin (holo-TF; Sigma-Aldrich), 100 U/mL penicillin-streptomycin (P/S; Sigma-Aldrich), including 3 cytokines; 50 ng/mL human stem cell factor (hSCF; R&D system), 25 ng/mL interleukin-3 (IL-3; Promokine), and 2 U/mL erythropoietin (EPO; Cilag-Janssen) (11) and incubated at 37°C in 5% CO₂ humidified atmosphere. After 4 days, only 5 U/mL EPO was added as cytokine and culture was continued to day 14.

2.3 RNA isolation and reverse transcription

Total RNA were extracted from cultured erythroblast at day 4, 6, 8, 10, 12 and 14 using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) were synthesized by reverse transcription using SuperScript III reverse transcriptase with oligo (dT) primers (Invitrogen).

2.4 Quantitative polymerase chain reaction (qPCR)

The synthesized cDNAs were amplified with specific primers for *HBS1L* transcripts (Forward: 5'-GCATCGGAATGTTTCGAGGC-3'; Reverse: 5'-CTTCCACAGGCTCAACGGA-3') using SYBR master mix (Applied Biosystems) according to the manufacturer's recommended conditions, on CFX96™ Real-Time system (Bio-Rad). The relative expression levels were represented by 2^{-ΔCt} value, normalizing with β -actin (*ACTB*).

2.5 Western blot analysis

The cultured erythroblasts were collected for protein extraction by NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Concentrations of proteins were determined by Quick Start™ Bradford Protein Assay (Bio-Rad). Then, proteins were separated on 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF)

membrane, and blocked with 5% skim milk. The membrane was hybridized with HBS1L specific primary antibody (Novus Biologicals) which then bind to goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam). Enhanced chemiluminescence (ECL; GE Healthcare Life Sciences) was used as a substrate. The proteins were visualized by chemiluminescence exposed on x-ray films.

2.6 Statistical analysis

Differences in expression between normal and patients were tested by unpaired t-test in program GraphPad Prism 5. P-values equal to or less than 0.05 are considered statistical significance.

3. Results

3.1 *HBS1L* mRNA expression during normal and β^0 -thalassemia/HbE erythroblasts differentiation

The expressions of *HBS1L* in normal and β^0 -thalassemia/HbE derived erythroblasts were analyzed during differentiation at day 4, 6, 8, 10, 12, and 14. The results showed that *HBS1L* expression exhibited indistinguishable pattern in both normal and β^0 -thalassemia/HbE erythroblasts. The levels were highly expressed in early to intermediate stages (day 4-10) and declined in late stages (day 12-14) (Figure 1).

Being compared to normal, β^0 -thalassemia/HbE derived erythroblasts displayed slightly higher levels of *HBS1L* from early to intermediate stages (day 4-10), which showed significantly different at day 8, and afterward both appeared in lower levels in the late stages (day 12-14).

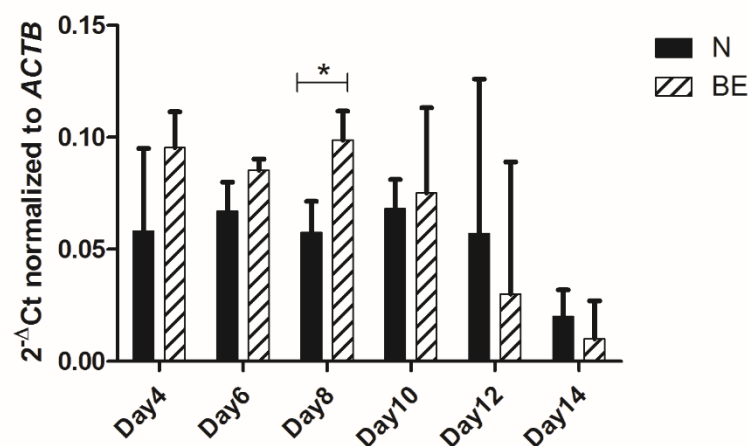


Figure 1. *HBS1L* mRNA expression in normal (N) and β^0 -thalassemia/HbE (BE) derived erythroid cells. The relative expressions of *HBS1L* were shown in mean $\pm$ SD values of $2^{-\Delta C_t}$ by using *ACTB* as a normalizer (n=3 each group). *p-value = 0.0210 compared to normal.

3.2 *HBS1L* protein expression in human normal erythroblast

HBS1L protein was measured at day 4, 6, 8, 10, 12, and 14 of normal cultured erythroid cells. The crude proteins were separated into cytoplasmic and nuclear fractions and analyzed by Western blot technique. The result revealed the localization of *HBS1L* protein in both nucleus and cytoplasm (Figure 2). The level of *HBS1L* increased during differentiation in cytoplasmic fraction. The opposite trend was observed in nuclear fraction.

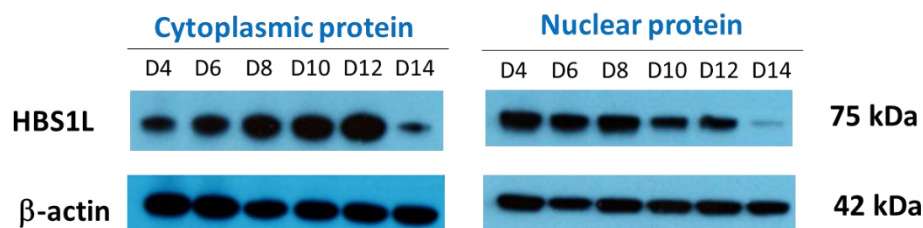


Figure 2. HBS1L protein expression during normal erythropoiesis. Western blot analysis of nuclear and cytoplasmic protein fractions from various days of normal erythroblasts (n=1) using β -actin as loading control.

4. Discussion

To our knowledge, this study has reported for the first time the expression of *HBS1L* during erythropoiesis. The differences in expression levels of *HBS1L* transcripts between normal and β^0 -thalassemia/HbE derived erythroblasts suggested its role in hemoglobin production, especially fetal hemoglobin. The presence of HBS1L protein in both cytoplasmic and nuclear fractions and their distinct expression levels during erythropoiesis further potentiate its proposed roles.

Taken together, this study suggested that *HBS1L* might be really important in erythropoiesis and hemoglobin production. However, further experiments are needed to confirm the hypotheses and explore the mechanisms.

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Proteome Profiling of Low-abundance Plasma Proteins in β -thalassemia/Hb E Patients Treated with Curcuminoids Antioxidant Cocktail

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Abstract

Objective: β -thalassemia/Hb E is characterized by a partial or total lack of β -globin chain synthesis resulting in accumulation of unpaired α -globin chains, the primary cause of oxidative stress and iron overload in patients with thalassemia. The administration of curcuminoids antioxidant cocktail (curcuminoids, N-acetyl cysteine, and deferoxamine) has been demonstrated as an alternative treatment to relieve oxidative damage and iron overload in previous study. To gain insights into the therapeutic effect of curcuminoids antioxidant cocktail on thalassemic patients, we compared plasma proteome profiling of β -thalassemia/Hb E patients after receiving curcuminoids antioxidant cocktail at three different time periods (baseline, month 6, and month 12).

Materials and Methods: High-abundance proteins in the plasma of thalassemic patients were removed using ProteoMiner enrichment technology. Proteomic analysis was then performed to determine the changes in plasma protein content during curcuminoids cocktail treatment, the results were further confirmed by western blot.

Results: Plasma proteomic profiles were compared between β -thalassemia/Hb E patients and normal subjects. In total, 37 differentially expressed spots representing 24 proteins were identified. Among these, treatment with the cocktail altered the plasma level of 8 proteins including apolipoprotein A-I, apolipoprotein E, complement C3, complement C4-A, prothrombin, plasma glutathione peroxidase, tropomyosin alpha-3 chain, and transthyretin towards improving the conditions of thalassemic patients. Western blotting results of prothrombin and apolipoprotein E were consistent with proteomic data.

Conclusion: This study showed the existence of oxidative stress, hypercoagulation, and abnormal lipid homeostasis in β -thalassemia/Hb E patients. Supplementation with curcuminoids antioxidant cocktail may ameliorate these pathological conditions.

Keyword: β -thalassemia/Hb E, curcuminoids, plasma proteome, low-abundance protein

1. Introduction

β -thalassemia/Hb E is a heterogeneous group of genetic disorders arising from deficiency in the synthesis of β -globin chains⁽¹⁾. The absence or decrease of β -globin chain synthesis subsequently lead to surplus α -globin chains which are rapidly dissociated, precipitated, and released toxic iron species. These iron generate free radicals which oxidize intracellular macromolecules including nucleic acids, lipids, proteins, and carbohydrates^(2, 3). The structural red cell membrane is the principal target for oxidation leading to less flexibility, more lysis susceptibility, and phospholipid rearrangement, especially phosphatidylserine (PS) externalization followed by activations of platelets and various coagulation factors. This may explain the chronic anemia and hypercoagulable state in patients with thalassemia. Eventually, increased iron absorption and blood transfusions to compensate anemia cause iron overload and the oxidative injury of organs in which excess iron is accumulated^(4, 5).

Our previous study of 1-year treatment with curcuminoids antioxidant cocktail improved anemia, decreased iron overload, reduced oxidative stress, and reversed hypercoagulable state in β -thalassemia/Hb E patients⁽⁶⁾. Since no proteomic study has been performed in thalassemic patients treated with antioxidant cocktail, we therefore established a follow-up study using ProteoMiner enrichment technology and two-dimensional gel electrophoresis to determine plasma proteins which their levels were altered relative to the treatment. Proteomic data may provide an insight into treating thalassemic patients with antioxidant cocktail.

2. Materials and Methods

2.1 Subjects

This study was approved by Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, number Si 687/2556 (EC2). Plasma of 6 normal subjects were obtained from the project "Proteomic analysis of plasma proteins in responded and non-responded β -thalassemia/Hb E patients treated with antioxidant cocktail" (Si 309/2011). Plasma of 6 patients at baseline, month 6, and month 12 after receiving curcuminoids antioxidant cocktail treatment were obtained from the project "Effect of antioxidant cocktail in β -thalassemia/Hb E patients" (Si 063/2009). All plasma samples were stored at -80°C until used.

2.2 Sample preparation

High-abundant proteins were depleted and precipitated from plasma samples using ProteoMiner™ Protein Enrichment Kit (Bio-Rad Laboratories, Inc.) and 2-D Clean-Up Kit (GE Healthcare), respectively, according to manufacturers' instructions. The protein pellets were suspended with lysis buffer and their concentrations were determined using the Bradford method (Bio-Rad Laboratories, Inc.).

2.3 Two dimensional gel electrophoresis

One-hundred fifty microliters of the depleted plasma were incubated overnight with immobilized pH gradient (IPG) strips (7 cm, pH 3-10 non-linear; GE Healthcare). Isoelectric focusing (IEF) was performed using Ettan IPGphor 3 (GE Healthcare) at 7,000 Vhr, 50 μA per gel strip and the strips were then separated in 12.5% SDS-PAGE using SE260 Mini-

Vertical Electrophoresis Unit (GE Healthcare). The gels were stained with SYPRO® Ruby Protein Gel Stain (Invitrogen™) and visualized using Typhoon FLA 7000 (GE Healthcare). The ImageMaster 2D Platinum 7.0 software (GE Healthcare) was used for image analysis.

2.4 In-gel digestion and protein identification by LC-MS/MS

In-gel tryptic digestion and protein identification of differentially expressed protein spots using Nanoflow liquid chromatography coupled with the amaZon speed ion trap mass spectrometry (Bruker) were performed as previously described^(7, 8). MASCOT search engine with NCBI nr version 20130630 sequence databases was used in order to identify proteins from MS/MS data.

2.5 Western blotting

Ten microgram of plasma proteins from normals and patients were separated in 12.5% SDS-PAGE at constant 10 mA per for 3 h. The separated proteins were transferred onto PVDF membrane (Millipore) at constant 100 V for 45 min. The membranes were then probed with primary mouse monoclonal anti-albumin antibody (1:2,000 dilution; Abcam, USA), mouse monoclonal anti-apolipoprotein E antibody (1:2,000 dilution; Abcam) and rabbit polyclonal anti-prothrombin antibody (1:2000 dilution; Abcam) at 4°C overnight. After that, the membranes were incubated with respective secondary antibody conjugated with horseradish peroxidase (1:6,000 dilution, Dako, Denmark) at room temperature for 1 h. Bands on immunoblots were detected and captured images using WesternBright ECL (Advansta) and ImageQuant™ LAS 4000 digital imaging system (GE Healthcare).

2.6 Data analysis

The data analysis was performed with SPSS 18.0. Unpaired t-test were used to test the statistical difference between data of normal subjects and thalassemic patients, whereas paired t-test was used to compare data of the patients at different time points of the treatment. Values were considered statistically significant at $p < 0.05$.

3. Results

Proteome profiles of depleted plasma from normal subjects and patients treated with curcuminoids cocktail were resolved using two-dimensional gel electrophoresis as shown in Figure 1 and 2. The software detected approximate 1200 protein spots on each gel. Thirty-seven spots were differentially expressed in plasma of normal subjects compared to those of thalassemic patients (Figure 1). A total of 24 proteins were identified and they could be classified into 7 functional groups including antioxidant enzyme, blood coagulation, cell structure, immunity, lipid metabolism, protease inhibitor and protein transporter (data not shown). Among these, 8 protein spots were significantly increased in the plasma of patients compared to normal subjects; including apolipoprotein A-I, apolipoprotein E, complement C3, complement C4-A, glutathione peroxidase 3 and transthyretin (Table 1). In contrast, 2 protein spots identified as prothrombin and tropomyosin alpha 3 chain were significantly decreased in patients' plasma. Interestingly, curcuminoids cocktail treatment could alter the levels of those spots towards improving oxidative stress, hypercoagulation, and abnormal lipid homeostasis as observed in Table 1. Western blot was performed to validate prothrombin, prothrombin fragment 1 and apolipoprotein E. The results were consistent with proteomic data (Figure 3).

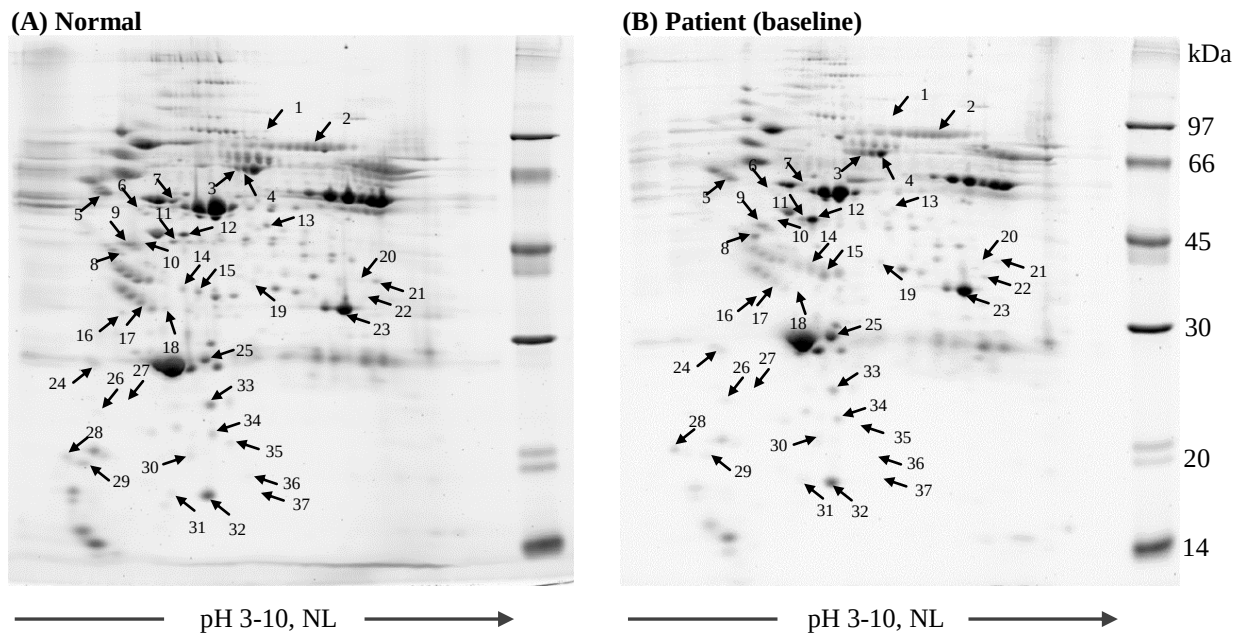


Figure 1 Representative 2-DE gel images of depleted plasma proteins from normal subjects (A) and patients with β -thalassemia/Hb E (B). Arrows indicate protein spots with significantly differential expression compared between normal subjects and thalassemic patients.

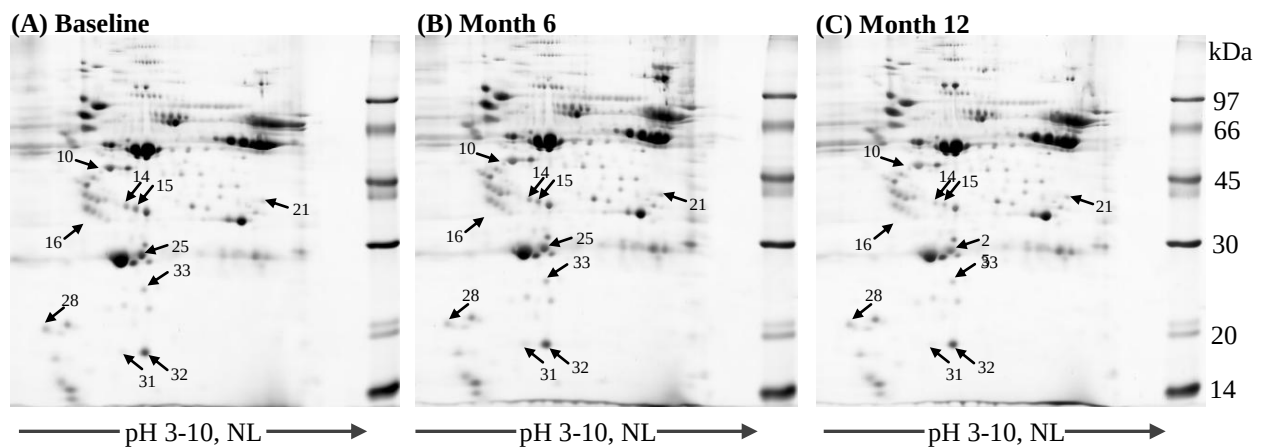
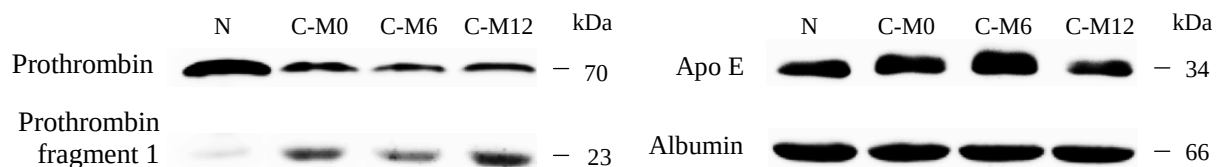


Figure 2 Representative 2-DE gel images of depleted plasma proteins from β -thalassemia/Hb E patients treated with curcuminoids cocktail at baseline (A), month 6 (B), and month 12 (C). Arrows indicate spots with significantly differential expression compared between time points.

Table 1 Differentially expressed plasma proteins of normal subjects and thalassemic patients treated with curcuminoids antioxidant cocktail.

Spot no.	Proteins	pI/MW	Average fold change		
			Normal vs baseline	Month 6 vs baseline	Month 12 vs baseline
10	Complement C3	40.20/4.79	+IINF***	+1.81±0.40*	+1.54±0.34
14	Apolipoprotein E	36.25/5.65	+2.01±0.39**	-1.24±0.15*	-1.77±0.22**
15	Apolipoprotein E	36.25/5.65	+2.42±0.35***	-1.15±0.12	-1.51±0.28*
16	Tropomyosin alpha-3 chain	28.91/4.72	-6.56±1.96**	+3.21±0.70*	+4.80±1.06*
21	Prothrombin	71.48/5.64	-2.81±0.65*	+1.11±0.26	+1.26±0.27*
25	Apolipoprotein A-I	30.76/5.56	+2.74±0.49**	-1.34±0.33**	-1.23±0.25
28	Complement C4-A	194.26/6.65	+2.1±0.26***	-1.41±0.24**	-1.54±0.32**
31	Transthyretin	15.99/5.52	+2.82±0.85**	+1.21±0.26*	-1.45±0.28*
32	Transthyretin	15.99/5.52	+2.02±0.20***	-1.11±0.07	-1.33±0.10*
33	Glutathione peroxidase 3	25.76/8.26	+1.22±0.12*	-1.24±0.11*	-1.32±0.10*

p*-value < 0.05, ** *p*-value < 0.01, * *p*-value < 0.001.



INF represents the differential protein expression more than 50-fold.

+ :Refer to increase of average fold change in patients as compared to normal; in month 6/12 as compared to baseline.

- :Refer to decrease of average fold change in patients as compared to normal; in month 6/12 as compared to baseline.

Figure 3 Western Blot analysis of prothrombin, prothrombin fragment 1, and apolipoprotein E of normal subjects (N), thalassemic patients at baseline (C-M0), month 6 (C-M6), and month 12 (C-M12) after treatment with curcuminoids cocktail. Albumin was served as loading control.

4. Conclusion and discussion

Treatment with curcuminoids cocktail in β -thalassemia/Hb E patients has demonstrated the improvement of anemia, oxidative stress, iron overload, and also hypercoagulable state in our previous study⁽⁶⁾. Proteomic approaches were therefore applied to gain insight into the therapeutic effect of curcuminoids cocktail on these thalassemic patients. In agreement with that study, proteome profiling of low-abundance plasma proteins revealed the improvement of proteins involved in oxidative stress (plasma glutathione peroxidase) and hypercoagulation (prothrombin and prothrombin fragment 1+2) in response to the treatment. Prothrombin fragment 1+2 is considered as a sensitive marker for diagnosis of thrombosis^(12, 13). In the present study, protein levels of prothrombin fragment 1 was remarkably increased in β -thalassemia/Hb E plasma at baseline compared to normal subjects. This finding might support the existence of chronic hypercoagulable state in thalassemic

patients as mentioned earlier. Interestingly, similar to Weeraphan *et al.* ⁽⁷⁾, hypercoagulation in β -thalassemia/Hb E patients are improved during the first 6 months of treatment with curcuminoids alone.

In addition, the levels of apolipoprotein E were significantly increased in β -thalassemia/Hb E patients as compared to those of normal subjects. However, the levels of apolipoprotein E were significantly decreased at month 12 of curcuminoids cocktail treatment. Apolipoprotein E is a well-known lipid transport protein, acts as a scavenger of free radicals and iron chelator. Apolipoprotein E is also play importance role for lipid homeostasis in brain, artery wall and adipose tissue. Recently, Mooijaart *et al.* ⁽³⁾ has reported that high levels of apolipoprotein E may indicate a detrimental lipoprotein profile and/or a proinflammatory response contributing to cardiovascular mortality in elderly people. Apolipoprotein E-4 alleles are associated with increasing risk of central nervous system and cardiovascular disorders in thalassemia major patients ⁽¹⁴⁾. Therefore, these findings revealed the first proteomic pattern of low-abundance plasma proteins under the thalassemic state and also shows the ameliorating role of curcuminoids cocktail towards oxidative stress, hypercoagulable state and abnormal lipid homeostasis ⁽⁹⁾.

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Functional Characterization of the *C. elegans* ACDH-12 as an assembly factor of the respiratory complex I

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Abstract *C. elegans* ACDH-12 (acyl-CoA dehydrogenase 12) is an enzyme in fatty acid oxidation that shares high homology to the human ACAD9. ACAD9 was initially categorized as the very long-chain fatty acyl-CoA dehydrogenase (VLCAD), a mitochondrial enzyme that catalyzes the first reaction of fatty acid oxidation. However, several studies have shown that ACAD9 also functions as an assembly factor of the respiratory complex I. In this study, we attempted to study the function of the *C. elegans* ACDH-12 whether it is homologous to the human ACAD9 as a complex I assembly factor or not. We used RNAi feeding technique to knockdown *acdH-12* in *C. elegans*. We found that knockdown worms showed decrease in formation and activity of the respiratory complex I.

Objective: To investigate a role of the *C. elegans* ACDH-12 in an assembly process of the respiratory complex I.

Materials and Methods: The *C. elegans* wild type strain N2 Bristol was obtained from the *Caenorhabditis elegans* Genetic Center. RNAi knockdown was done by feeding wild type animals the RNAi bacteria feeder, E04F6.5, for specific *acdH-12* knockdown. Lifespan, mitochondrial function and formation of the respiratory complexes were investigated in the RNAi-knockdown animals in comparison to wild type animals.

Results: RNAi knockdown of *acdH-12* in *C. elegans* resulted in significantly shortened lifespan, decreased function and formation of the respiratory complex I.

Conclusion: The result suggested that ACDH-12 is required for formation and function of the respiratory complex I in *C. elegans*.

Keywords: *Caenorhabditis elegans*, mitochondria, acyl-CoA dehydrogenase, respiratory complex I assembly factor, ACAD9, *acdH-12*

1. Introduction

Mitochondria are organelles in most eukaryotic cells. One of the major functions of mitochondria is to generate ATP via the oxidative phosphorylation (OXPHOS) system. The OXPHOS system is composed of five enzyme complexes, e.g. complexes I-V. Complex I is the largest complex of the respiratory chain (1). It is composed of 45 subunits (38 nuclear subunits and 7 mitochondrial subunits). McKenzie *et.al* (2) have demonstrated that the formation of complex I is a sequential process. Several subunits will be assembled into the assembly intermediates which will then fuse together to become a fully functional complex I. There are proteins called assembly factors that facilitate the assembly process. In mammals,

the assembly factors for complex I include NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4 and ECSIT (3).

Nouws *et.al* (4) have recently found that ACAD9, one of the acyl-CoA dehydrogenases in human functions as an assembly factor of the respiratory complex I. Interestingly, the human ACAD9 was initially characterized as a very-long chain acyl-CoA dehydrogenase (VLCAD) (5). However, another separate study from Nouws *et.al* (6) revealed that ACAD9 plays dual roles as a respiratory complex I assembly factor and an acyl-CoA dehydrogenase enzyme.

In *C. elegans*, ACDH-11 and ACDH-12 are characterized as VLCAD according to the homology study (7). We found that ACDH-12 shares higher percentage of amino acid identity with human ACAD9 than ACDH-11 does. Therefore, we asked the question whether ACDH-12 is functionally homologous to ACAD9 as a respiratory complex I assembly factor or not.

2. Materials and Methods

2.1 Worm strain

The wild type *C. elegans* strain N2 Bristol was obtained from the *Caenorhabditis elegans* Genetic Center (Minnesota, USA).

2.2 Worm culture

Normal control- Wild type *C. elegans* was fed *E. coli* strains OP50 and K12 while growing on the Nematode Growth Medium (NGM) (50 mM NaCl, 1.7% agarose, 2.5 g/L peptone, 1% cholesterol, 1 M KPO₄, 1 M MgSO₄, and 1 M CaCl₂) and in S-basal (100 mM NaCl, 25 mM K₂HPO₄, and 25 mM KH₂PO₄), respectively. The cultures were strictly maintained at 20 °C.

RNAi-knockdown- Wild type *C. elegans* was fed RNAi bacteria strain E04F6.5 for *acdh-12* knockdown in the presence of 5 mM isopropyl β-D-1-thiogalactopyranoside and 100 µg/mL ampicillin.

2.3 Lifespan study

Adult nematodes laid eggs for 2– 4 h on lawns of *E. coli* OP50 or RNAi bacteria, and were then removed. The day of hatching was defined as day 0. The first day of adulthood was recorded by the appearance of eggs on the plate. All studies were done at 20 °C (10).

2.4 Lifespan data collection and analysis

The data was collected from a numbers of alive or dead worms in each strain everyday, which was then used calculate the proportion of worms alive on each day. Worms that flee were excluded from an analysis. Data analysis using log-rank survival statistical analysis, and a survival graph plot, were done through an OASIS, online-based analysis system (11).

2.5 Mitochondrial isolation

Mitochondria were isolated form the adult worms that were grown in the liquid culture (1 L S-basal, 1% cholesterol, 5 mg/mL nystatin in Dulbacco's PBS). The worm pellet was collected from the liquid culture by centrifugation at 500g, and then washed with 10 mL MSM-E (20 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EDTA) twice. The

homogenate was then subjected to protease digestion by subtilisin for 10 min on ice. After protease digestion, the mixture was homogenized again at 300 rpm for 10 strokes, and then mixed with an equal volume of MSM-EB (MSM-E with 0.4 % bovine serum albumin (BSA)). The homogenate was centrifuged at 350 rpm for 10 min, and then supernatant was collected. The supernatant from the previous step was centrifuged at 7000g for 10 min to pellet down the mitochondrial particles. The mitochondrial pellet was washed twice with MSM-E and MSM (MSM-E without EDTA) followed by centrifugation at 7000g 10 min for each wash. The mitochondrial pellet was suspended in a proper volume of MSM.

2.6 Measurement of enzymatic activity of the respiratory complex I

Spectrophotometric analysis was used to determine the respiratory complex I activity (12). Freshly isolated mitochondria were solubilized by 5% sodium cholate to obtain a ratio of protein/detergent as 5/1 (w/w). Solubilized mitochondria were mixed with KME solution (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA) to obtain the final mitochondrial protein concentration of 1 µg/µl.

2.6.1 NADH:ferricyanide reductase (NFR) assay

NFR assay measures the respiratory complex I activity by monitoring a rate of NADH oxidation in a presence of NADH as an electron donor and ferricyanide ($K_3Fe(CN)_6$) as an electron acceptor. The assay was performed in a mixture containing 0.1 mM KH_2PO_4 , 50 mg/L BSA, 15 mg/L asolectin, 0.01 mM EDTA, 0.01 mM $K_3Fe(CN)_6$, 0.1 mM NaN_3 , 0.01 mM NADH, and 20 µg mitochondrial protein. NADH oxidation was determined by measuring an absorbance at 340 nm for 120 sec at 37°C. Coefficient of NADH, 6.22, was used to calculate the rate of NADH oxidation. This method is modified from Hatefi *et al.* (13)

2.6.2 NADH-decylubiquinone:2,6-dichloroindophenol oxidoreductase (NADH-DUQ:DCPIP) assay

NADH-DUQ:DCPIP assay determines enzymatic activity of the respiratory complex I by measuring the 2,6-dichloroindophenol (DCPIP) reduction in a presence of NADH as an electron donor, and decylubiquinone (DUQ) as an electron acceptor. A subsequent reaction acquires DCPIP as the electron acceptor from reduced decylubiquinone (14). DCPIP reduction was measured in a 1 mL of mixed solution containing 25 mM KH_2PO_4 , 3.5 g/L antimycin A, 60 µM DCPIP, 70 µM decylubiquinone and 20 µg mitochondrial protein. The absorbance of DCPIP was read at 600 nm for 120 sec at 37°C. In addition, another NADH-DUQ:DCPIP reaction with an addition of 1 mM rotenone was assayed in parallel to obtain the rotenone-insensitive activities. Coefficient of DCPIP, 21, was used to calculate the rate of DCPIP reduction. This method is modified from Janssen *et al.* (15).

2.7 Blue native gel (BNG) electrophoresis and complex I-in gel staining

A non-denaturing polyacrylamide gradient gel was poured at room temperature with a gradient mixer as described in Wittig *et al.* (16). To perform BNG, 150 - 250 µg of mitochondrial protein was solubilized by 20% digitonin to obtain a ratio of detergent/protein as 6/1 (w/w) at room temperature for 10 min, followed by centrifugation at 15,000 g for 20 min, 4°C. After centrifuged, the mitochondrial debris was packed as a pellet and respiratory complexes were dissolved in supernatant. Supernatant was then collected. 5% coomassie blue was added to supernatant to obtain detergent/dye ratio of 8/1 (w/w) before loading onto the 3-

11% polyacrylamide gel. Overlay the samples in each well with cold cathode buffer B (7.5 mM imidazole, 50 mM tricine and 0.02% coomassie blue). Electrophoresis was performed at 4 °C at constant 300 V. After the blue running front has moved about one-third of the desired total running distance, cathode buffer B was replaced by cathode buffer B/10, which contains 10 fold dilution of coomassie blue and run until the dye front reached the end of the gel. The gel was cut into two, one for coomassie blue staining (CMB) and the other for in-gel activity staining (In-gel). CMB was performed by soaking a gel slice with staining solution (0.1% coomassie blue in 50% methanol 10% glacial acetic acid) for 1 h at room temperature, and then destained a gel slice in destaining solution (40% methanol and 10% glacial acetic acid) overnight at room temperature. Complex I in-gel staining was performed by incubating a gel slice in 0.5 mM nitroblue tetrazolium, 5 mM NADH, 50 mM Tris, pH 7.4 at room temperature for 60 min.

2.8 RNA isolation

Total RNA was isolated from the adult worms using Trizol extraction (17). The worms were washed with chilled S-basal 3 times. A total 2 mL of Trizol was mixed with the pellet before subjected to manual homogenization in a glass homogenizer for 35 strokes. Addition of 400 μ L chloroform to the homogenate was done before shake vigorously for 15 sec. The mixture was incubated at room temperature for 10 min before centrifugation at 12,000g for 15 min. An upper aqueous phase was collected followed by an addition of 1 mL isopropanol and then incubated at room temperature for 10 min. The solution was centrifuged at 12,000g for 10 min. The white pellet of RNA was kept while supernatant was discarded. Addition of 2 mL 75% ethanol to the pellet was done before centrifugation at 7,500g for 5 min. The pellet of RNA was then dissolved in RNase-free H₂O.

2.9 RT-qPCR

RNA from the whole worm was subjected to reverse transcription using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit following the manufacturer protocol. The cDNA from the previous step was subjected to qPCR using Thermo Scientific Quantitative PCR Kit. The specific primers for the target gene, *acdh-12*, and the housekeeping gene, *tba-1*, were used in qPCR step. The primer sequences were tabulated below.

Gene	Forward primer	Reverse primer
<i>acdh-12</i>	5'TTACTACGTTGATAAGGCGA3'	5'CTAAAAGAGCTTCTTACGATAAA3'
<i>tba-1</i>	5'AACTGTTGTTGATGAGATCC3'	5'ATGAAAGACGAAGAATCCTT3'

3. Results

3.1 RNAi knockdown of *acdh-12* resulted in shortened lifespan in *C. elegans*

Lifespans of *acdh-12* knockdown worms and wild type control were determined. Interestingly, *acdh-12* knockdown worms exhibited shortened lifespan compared to wild type (Figure 1). Median lifespan of *acdh-12* knockdown worms and wild type animals was 13 and 15 days, respectively. Log rank analysis revealed that *acdh-12* knockdown resulted in significantly shortened lifespan compared to wild type.

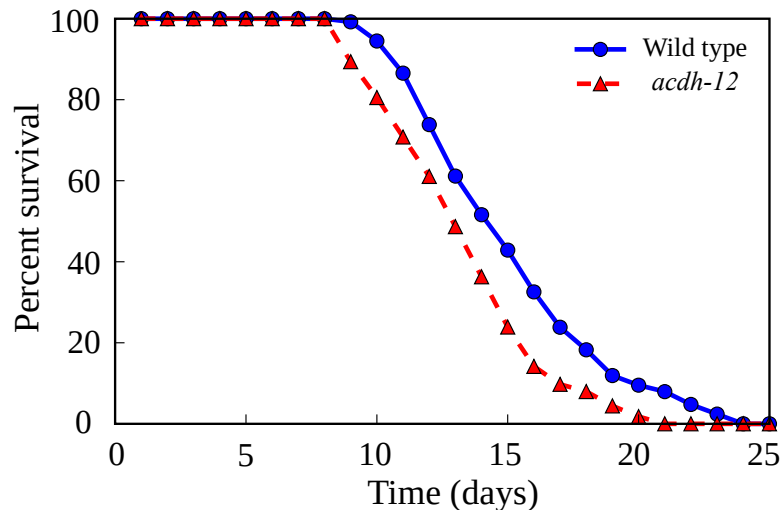


Figure 1. Effect of *acd-12* knockdown on lifespan.

Lifespan study was done on *acd-12* knockdown worms in comparison to wild type. Red triangle represents *acd-12* (n = 125), and blue dot represents wild type control (n = 125).

3.2 Effect of *acd-12* knockdown on enzymatic activity of the respiratory complex I

Enzymatic activity assay was performed to determine the effect of *acd-12* knockdown on respiratory complex I function. It was shown that respiratory complex I activities as determined by NFR (Figure 2A) and NADH-DUQ:DCPIP (Figure 2B) assays in *acd-12* knockdown worms were significantly decreased compared to wild type.

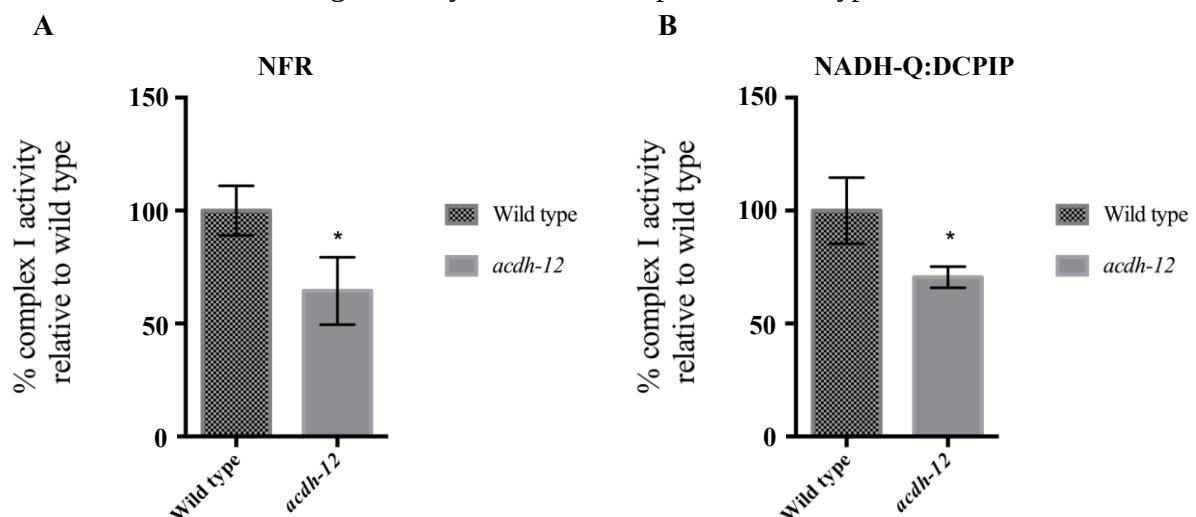


Figure 2. Decreased respiratory complex I function in *acd-12* knockdown worms.

Respiratory complex I activities as determined by NFR (A), and NADH-DUQ:DCPIP (B) assay were measured from *acd-12* knockdown and wild type animals. Enzymatic activity was expressed in relative to wild type control. Data are presented as mean $\pm$ S.D. from three independent experiments. * indicates statistical significant as $p < 0.05$ (t -test) in comparison with wild type.

3.3 Effect of *acdH-12* knockdown on formation of the respiratory complex I

BNG was used to determine the formation of respiratory complexes and supercomplexes. Identification of respiratory complexes and supercomplexes has been done by Suthammarak *et al.* (19). It was shown that *acdH-12* knockdown resulted in a decreased amount of the complex I-containing supercomplexes, e.g. supercomplexes I:III and I:III:IV, as seen in both coomassie staining and in-gel activity staining (Figure 3A and 3B).

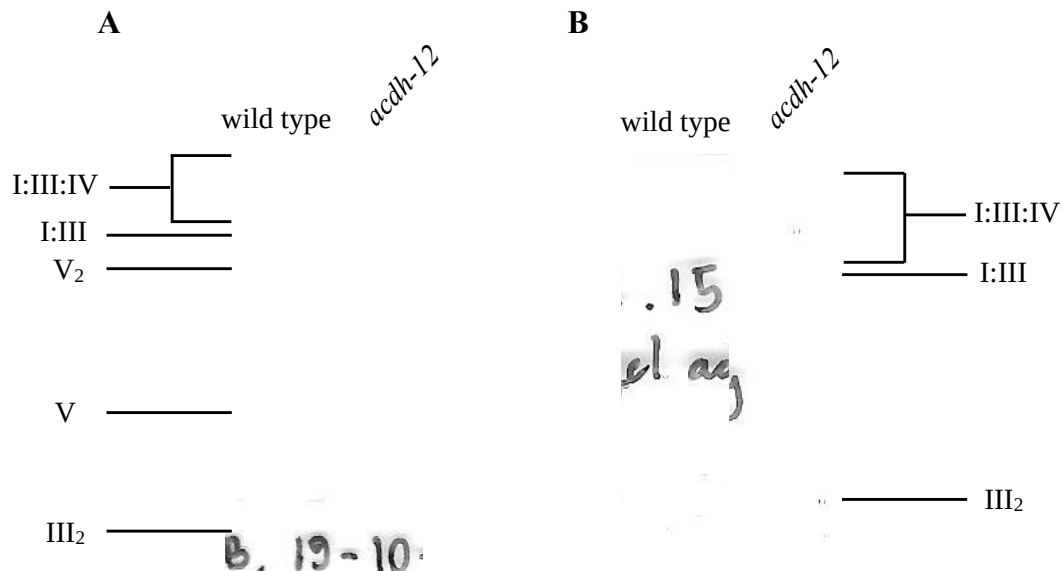


Figure 3. *acdH-12* knockdown resulted in decreased formation of the respiratory complex I. (A), Coomassie blue staining shows bands of respiratory complexes and supercomplexes. The Roman numeral represents respiratory complexes and supercomplexes. I:III:IV supercomplex is composed of complexes I, III and IV. I:III supercomplex is composed of complexes I and III. V and V₂ represent monomeric and dimeric complex V, respectively. III₂ is a dimeric complex III. (B), In-gel activity staining of complex I. Dark blue bands indicate positive staining of complex I.

4. Discussion and conclusion

In this study, we hypothesized that *C. elegans* ACDH-12 may be a homologous counterpart of human ACAD9 due to its high amino acid identity. ACAD9 has been characterized as VLCAD. Interestingly, Nouws *et.al* (4, 6) showed that loss of function mutation in ACAD9 resulted in decrease in respiratory complex I, together with compromised fatty acid oxidation. In addition, Rhein *et.al* (20) described that ACAD9 worked together with other assembly factors which included NDUFAF1 (21) and ECSIT (22) in an assembly process of the respiratory complex I. We addressed our hypothesis by knocking down *acdH-12* to see the effect towards complex I formation and function. In comparison to wild type, *acdH-12* knockdown not only resulted in shortened worms lifespan but also decreased complex I activity and formation.

Our results indicated that loss of *acdH-12* function led to respiratory complex I dysfunction and increased worms mortality. We concluded that ACDH-12 is required for respiratory complex I function and formation, similar to human ACAD9. However, the role of ACDH-12 in fatty acid oxidation was not investigated in our study.

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Determination of NS1 expression pattern between wild-type and mutant (10RQ) dengue viruses

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Abstract

Objectives: Dengue fever is a major public health problem caused by the dengue virus (DENV). Free circulating levels of nonstructural protein 1 (NS1) correlated with the viremia levels in dengue patients, and were higher in dengue hemorrhagic fever (DHF) than dengue fever (DF) patients. This suggested that the severity of the disease related on levels of NS1 secretion. The changes in West Nile Virus (WNV) NS1 at amino acid positions 10 and 11 from Arginine (R) and Glutamine (Q) to Asparagine (N) and Lysine (K), decreased surface NS1, and increased NS1 secretion in WNV-infected cells. If NS1 expression pattern could be changed by modifying dengue virus, so it is possible that these viruses could be reduced NS1 secretion. This study aims to construct wild-type and mutant (10RQ) dengue viruses and determine NS1 expression pattern between wild-type and mutant DENV-infected cells.

Materials and Methods: Wild-type and mutant (10RQ) dengue viruses were constructed using Gibson assembly. Intracellular and surface NS1 were determined using indirect immunofluorescence method

Results: Wild-type and mutant DENVs all serotypes except serotype 1 could be generated using Gibson assembly method. Surface NS1 on mutant DENV-infected cells is different to surface NS1 from wild type infected cells.

Conclusion: These results suggested that NS1 mutation in dengue NS1 at amino acid positions 10 and 11 change NS1 expression pattern. If these viruses decrease NS1 secretion, so it is possible that these viruses could be applied for studying how NS1 involved in severe dengue.

Keywords: Dengue virus (DENV), DENV NS1, NS1 expression

1. Introduction

Dengue virus infection is a major public health problem in tropical and sub-tropical areas around the world. Up to 390 million people are infected globally each year ⁽¹⁾. The therapy of dengue infection in patients is a supportive treatment. Dengue virus (DENV) is a member of *Flaviviridae* family, which consists of 4 serotypes, DENV-1, 2, 3, and 4. DENV is an enveloped virus. The genome of DENV is approximately 11,000 nucleotides, and its genome is composed of a positive sense, single stranded RNA, which codes for three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) ⁽²⁾. DENV infection produces a vary symptoms of clinical illness which are mild to severe disease. The degree of severity can range from dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Previous reports showed that free circulating levels of NS1 in DHF were higher than those of DF patients. This suggested that the severity of disease related on levels of NS1 secretion ^(3, 4). The precise mechanisms of how NS1 contributes to pathogenesis have remained unclear.

NS1 is a ~48-kDa glycoprotein. NS1 is absent from the infectious viral particle ⁽⁵⁾. The plasma membrane expression patterns of NS1 from two different flaviviruses were compared in the study from Youn *et al* in 2010. WNV-infected cells and DENV-infected cells showed a distinct NS1 expression pattern on the plasma membrane. Surface NS1 on WNV-infected cells were higher expressed than surface NS1 on DENV-infected cells. The researchers looked for the basis of the difference in the NS1 surface expression patterns of WNV and DENV NS1. NS1 chimeras were generated and characterized to map which residues's responsible for the different phenotype. The changes in WNV NS1 at amino acid positions 10 and 11 from Arginine (R) and Glutamine (Q) to Asparagine (N) and Lysine (K) (RQ10NK) switched the WNV NS1 expression pattern to DENV NS1 expression pattern. This substitution decreased surface expression and increased secretion of NS1 in WNV-infected cells ⁽⁶⁾. We hypothesized that NS1 mutations at amino acid positions 10 and 11 (RQ) in DENVs, surface NS1 expression pattern will be changed. In this study, we generated wild type and mutant (10RQ) dengue viruses using Gibson assembly method. The NS1 expression pattern between mutant viruses and wild-type viruses was determined. The expression pattern of intracellular NS1 and surface NS1 between wild type and mutant DENVs was determined by indirect immunofluorescence assay (IFA).

2. Materials and methods

2.1 Preparation of DENV NS1

DENV NS1s were amplified from cDNA of DENVs by PCR. The PCR was performed by Phusion High-fidelity DNA polymerase (NEB). PCR product of DENV NS1s was purified using Purelink® Quick PCR purification kit (Invitrogen).

2.2 Ligation PCR of DENV NS1s with TOPO vector and Cloning of plasmid TOPO-DENV NS1s

To insert DENV NS1s into plasmid vector, the plasmid PCRtmII-blunt-TOPO (Invitrogen) was ligated with DENV NS1s. After ligation of DENV1 NS1s with TOPO plasmid, these plasmids were transformed into competent *E coli*. Then, plasmid was extracted using PureLink® Quick Plasmid Miniprep Kit (Invitrogen).

2.3 Generation of DENV NS1 (10RQ) mutation by site-directed mutagenesis

DENV NS1s were aligned and applied for DENV NS1 (10RQ) mutation construction. DENV NS1 mutation was performed by site-directed mutagenesis method. Mismatch primers were designed for generating mutated PCR. PCR product was digested with *DpnI* restriction enzyme for elimination of template PCR. After cloning, recombinant plasmid DNA was digested for analysis of DENV NS1 insertion. All nucleotide sequences of DENV NS1s and DENV NS1s (10RQ) were aligned using Multiple Sequence Alignment program.

2.4 Wild type and mutant DENV-1, 2, 3, and 4 construction

Wild-type and mutant DENV all serotypes were constructed for determination of NS1 expression pattern. First, overlapping genes were designed for virus construction. PCR products were designed with an overlapping nucleotide sequences with its flank genes. DENV NS1s, DENV NS1s (10RQ), and other fragment of viral genes were amplified from plasmids. Next process, PCR of NS1, expression gene, and other fragments was assembled using Gibson assembly master mix (NEB). The reaction protocol was followed manufacturer's protocol. The assembled gene was transfected separately into 293T cells for virus construction. Transfection of wild-type and mutant (10RQ) DENVs into 293T cells was performed by lipofectamine transfection method. Next day, supernatant was collected and centrifuged at 8,000 rpm 4 °C for 10 minutes.

The supernatant was titrated for checking virus production. Viruses were then propagated for large-scale upscale. Briefly, C6/36 cell lines were infected with virus. After that, Vero cells were infected with virus supernatant for raising virus production. Supernatant was collected and centrifuged at 8,000 rpm for 10 minutes. 20 % FBS was added into supernatant. Virus supernatants were stocked at -70 °C. Nucleotide sequences from wild-type and mutant (10RQ) DENVs were aligned in Serial cloner 2.6.1.

2.5 Determination of NS1 expression pattern on wild-type and mutant DENVs by indirect immunofluorescence assay (IFA)

Vero cells grown on coverslips were individually infected with wild-type and mutant (10RQ) DENV-2, 3, and 4. After 24 hours post infection, wild-type or mutant DENV-infected cells coverslips was pried from plate. Infected cells were stained for determination of surface NS1 and intracellular NS1, 2 ml of 3.7% formaldehyde was added for fixing cells. For staining of intracellular NS1, 1 ml of 2% Triton X was added after fixing cells. 1F11 (anti-NS1 antibody) was used as NS1 captured-antibody. Mixture of Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody with Hoechst was used as secondary antibody. Images of NS1 expression pattern on intracellular and surface of wild type DENV-infected cells and mutant DENV-infected cells were acquired from fluorescence microscope.

3. Results

3.1 Generation of wild type DENVs and mutant DENVs (10RQ)

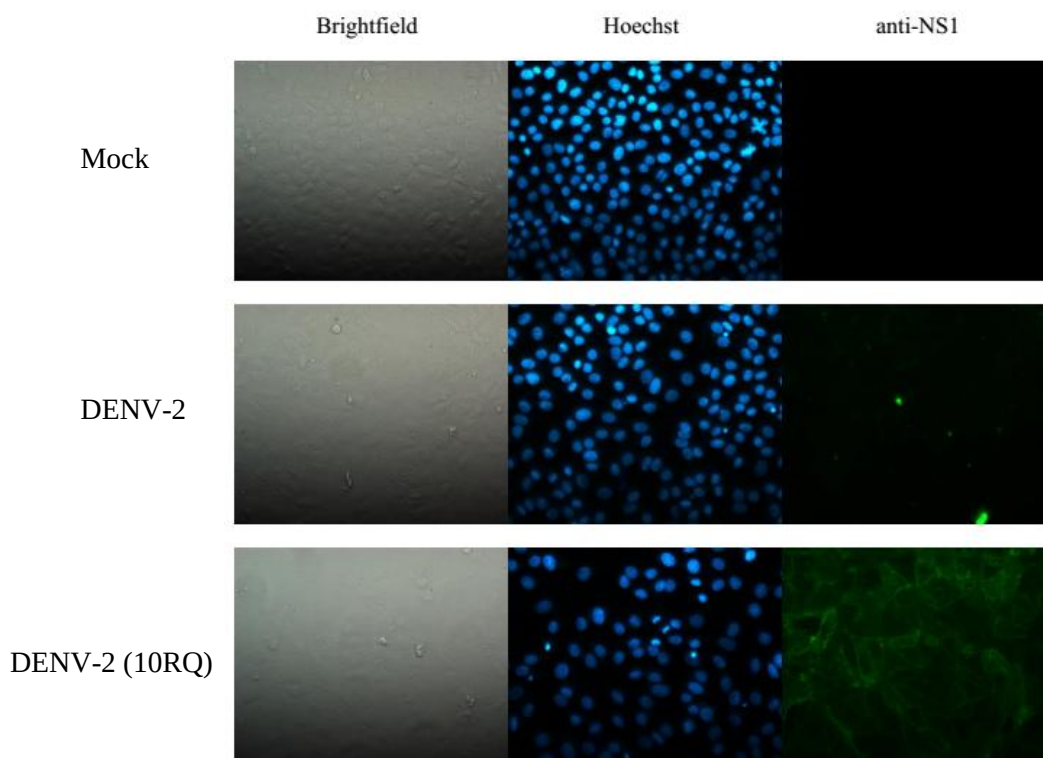
DENV NS1s were obtained from cDNA of DENVs by PCR method. DENV NS1s were used as template for DENV NS1s (10RQ) construction. Then, this DENV NS1s were assembled with other DENV genes and expression plasmid gene. Assembled gene of each DENVs was transfected into 293T cells. Wild-type and mutant (10RQ) DENV-2, 3, and 4 were successfully generated from this method. The different amino acids at positions 10 and 11 between wild-type and mutant DENVs were shown in table 1.

Table 1 The difference between NS1 of wild-type and mutant DENVs at positions 10 and 11

	Wild-type DENV	Mutant DENV
DENV-2	Asparagine (N), Lysine (K)	Arginine (R), Glutamine (Q)
DENV-3	Glycine (G), Lysine (K)	Arginine (R), Glutamine (Q)
DENV-4	Glycine (G), Lysine (K)	Arginine (R), Glutamine (Q)

3.2 NS1 protein expression pattern on surface and intracellular NS1 in wild-type and mutant (10RQ) DENV-2, 3, and 4

Vero cells were infected with wild-type and mutant (10RQ) DENV-2, 3, and 4. After 24 hours post infection, infected cells were stained for determination of surface and intracellular. Images of infected cells were captured using fluorescence microscope. Surface NS1 expression pattern differs between wild-type and mutant (10RQ) DENV-2, 3, and 4. In surface staining without permeabilization, surface NS1 on mutant DENV-infected cells is clearly different to surface NS1 from wild-type DENV-infected cells. Mutant DENV-infected cells show surface NS1 expression in contrast to wild-type DENV-infected cells (Figure 1). For permeabilization intracellular NS1 staining, producing a result of correspondence with surface staining, NS1 on cell surface in mutant DENV-infected cells was found but not in wild type DENV- infected cells (Figure 2). This result shows that NS1 mutations as amino acid positions 10 and 11 to RQ change NS1 expression pattern. This mutations show surface NS1 expression.



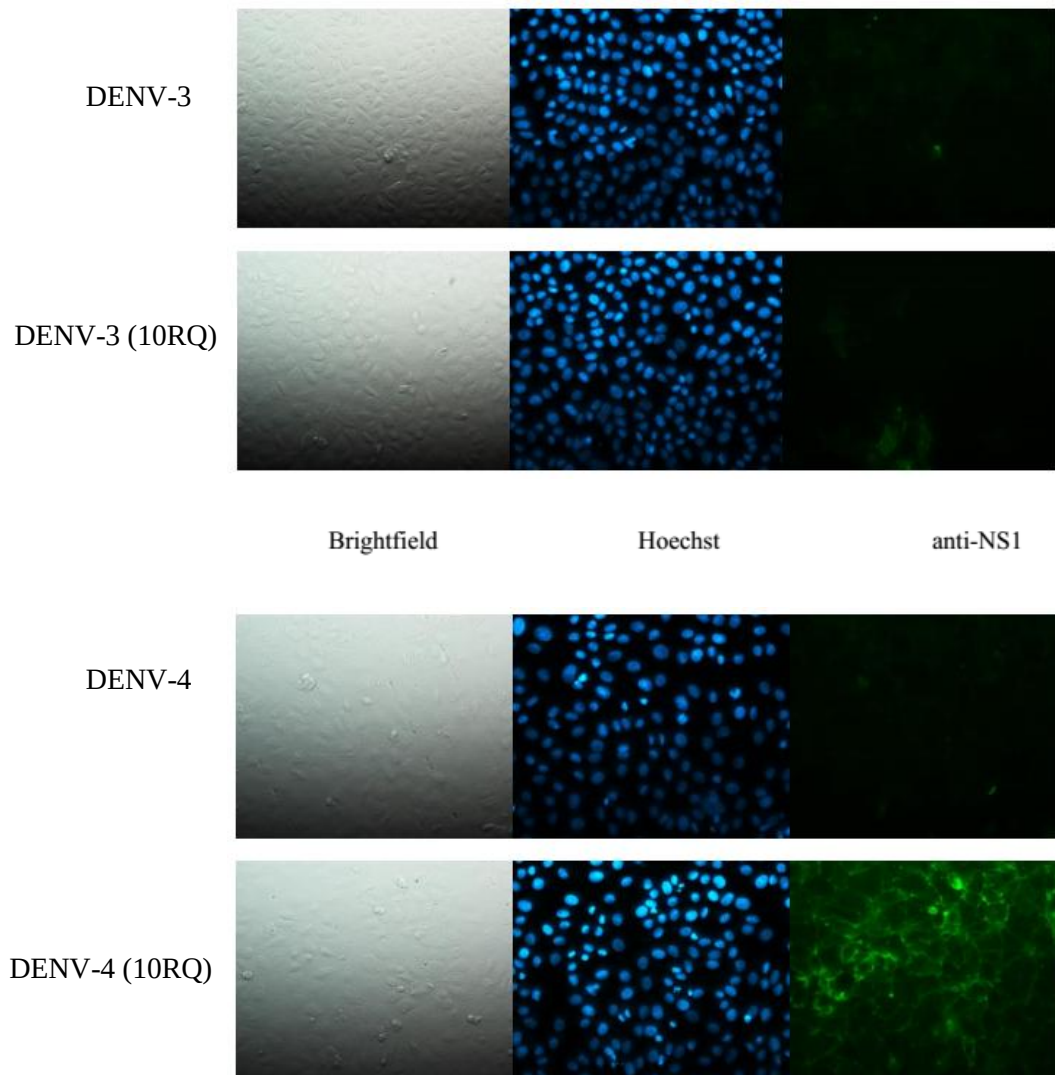


Figure 1 Surface staining of the wild-type and mutant (10RQ) DENV-2, 3, and 4 infected cells

Vero cells were infected with wild-type or mutant DENV-2, 3, and 4. After 24 hours post infection, infected cells were stained for determination of surface NS1. Mock and infected cells were stained with anti-NS1 MAb (1F11), followed by mixture of Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody with Hoechst. Images were captured by fluorescent microscopy. Infected cells were observed under visible light or fluorescence microscope at 488 nm.

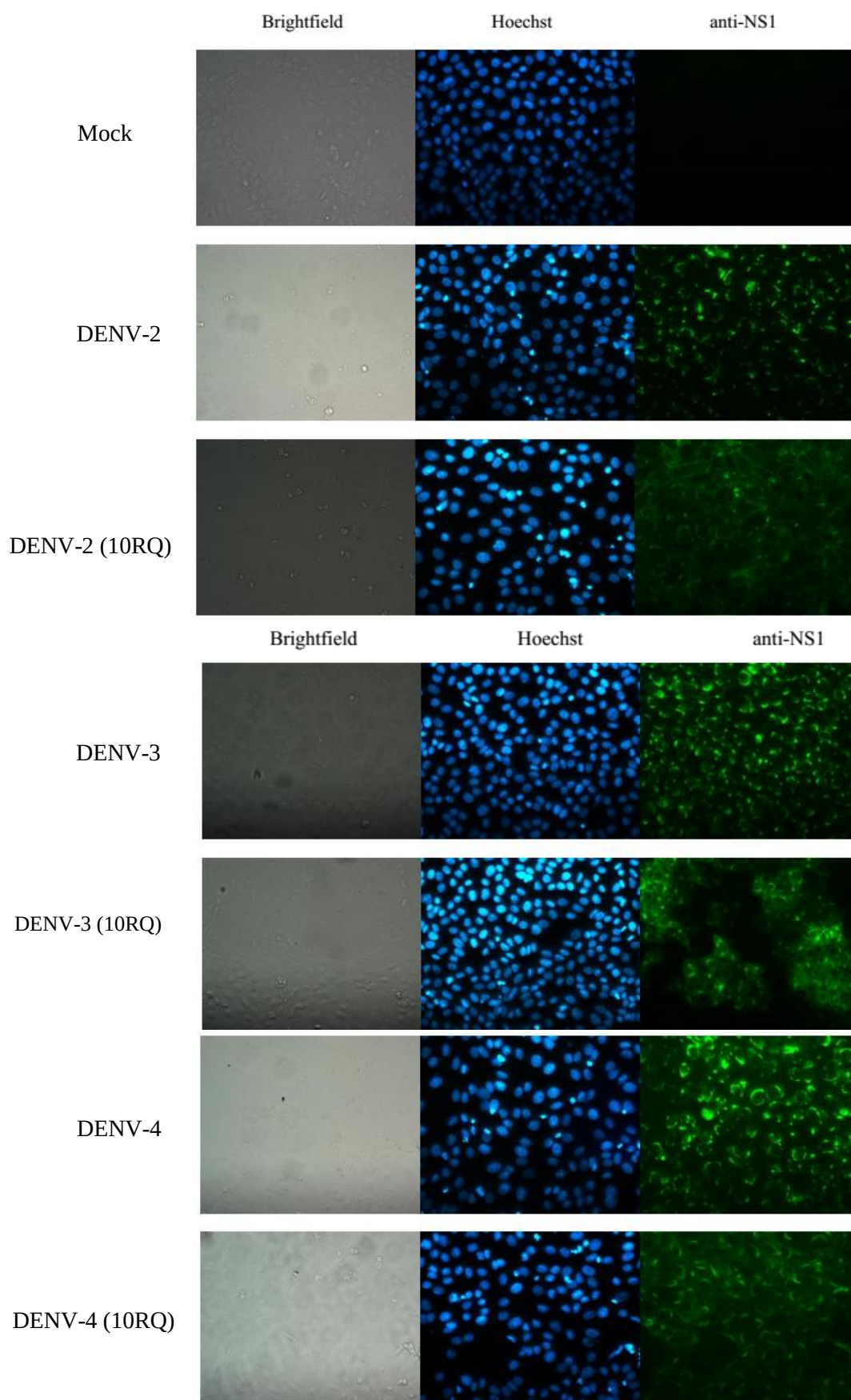


Figure 2 Intracellular staining of the wild-type and mutant (10RQ) DENV-2, 3, and 4 infected cells

Vero cells were infected with wild-type or mutant DENV-2, 3, and 4. After 24 hours post infection, infected cells were stained for determination of surface NS1. Mock and infected cells were stained with anti-NS1 MAb (1F11), followed by mixture of Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody with Hoechst. Images were captured by fluorescent microscopy. Infected cells were observed under visible light or fluorescence microscope at 488 nm.

4. Conclusion and discussion

In flavivirus, NS1 is secreted into extracellular space through secretory pathway, expressed in intracellular, and directly transported into surface of infected cells. Precise mechanisms that NS1 is expressed on different locations remain unclear. NS1 on the cell surface in Flavivirus infection comes from two sources. First from directly transported to plasma membrane ⁽⁷⁾. Second, NS1 is from bind back mechanism. The interaction between plasma membrane and NS1 is through Glycosaminoglycan ⁽⁸⁾. Previous study reported that amino acids of NS1 at positions 10 and 11 regulate NS1 distribution on WNV-infected cells. Switching of amino acids at positions 10 and 11 of WNV NS1 into amino acids of DENV NS1 at the same positions changed the pattern of NS1 secretion, NS1 on plasma membrane, and replication of WNV ⁽⁶⁾. We hypothesized that mutations of NS1 at amino acid positions 10 and 11 into RQ in DENVs, NS1 expression pattern will be changed. To find out, wild type and mutant DENV were generated. Mutant viruses contained NS1 that comprised of RQ at amino acid positions 10 and 11. In this study, virus construction was successfully performed by Gibson assembly method. This method allows for assembly of multiple DNA fragments with an overlapping ⁽⁹⁾.

In this study, wild type and mutant DENV-2, 3, and 4 were successfully generated. Then, these viruses were used for determination of NS1 expression pattern. Expression pattern of intracellular and surface NS1 from wild type and mutant DENV-2, 3, and 4 were determined. In mutant DENV-2, 3, and 4, pattern of NS1 on plasma membrane of infected cells differs from their wild type. NS1 expression on cell surface is distinctly different between wild-type and mutant infected-cells. This pattern is compatible with previous study that a change of WNV NS1 (RQ) to DENV NS1 (NK) at amino acid position 10 and 11 (RQ10NK) switched the WNV expression pattern by reducing surface NS1 ⁽⁶⁾. In summary, mutant DENV-infected cells that contained NS1 (10RQ) have different surface NS1 expression pattern to wild-type DENV-infected cells. In further study, NS1 secretion and replication will be examined in wild-type and mutant DENVs. If mutant viruses can reduce NS1 secretion, it could be applied to studying how NS1 drives disease.

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Genistein Reduces Fasting Plasma Glucose in Prolonged Dexamethasone Treated Mice

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Abstract

Objective: Prolonged administration of dexamethasone is known to induce diabetes. Genistein, a phytoestrogen, was reported to reduce hyperglycemia in high fat-induced diabetic mice. This study was designed to investigate the effect of genistein on prolonged dexamethasone treated mice.

Material(s) and Method(s): Eight-week old wild type ICR male mice were randomly divided into 3 groups (9 mice per group) and treated with DMSO (Control), dexamethasone (Dexa), and genistein with dexamethasone (Dexa+Gen) by daily injection for four weeks. Food intake was measured daily, and body weight and fasting blood glucose (FBG) were measured weekly. After four weeks, mice were sacrificed and pancreas, epididymal fat pad and spleen were collected for weight measurement.

Results: Dexamethasone treated mice had significantly elevated FBG at the fourth week (p value < 0.05) when compared to control group. Co-treatment of genistein with dexamethasone brought the FBG back similar to control level (p value < 0.001). Dexamethasone treated mice had pancreas and spleen weight significantly lower than control group (p value < 0.001). Dexamethasone treated mice significantly increased epididymal fat pad weight than control and Dexa+Gen groups. However, food intake of Dexa and Dexa+Gen groups significantly decreased when compared with control group (p value < 0.01).

Conclusion: Our findings demonstrated that genistein protects against dexamethasone-induced impaired FBG in mice. The mechanisms of protective effects require further study.

Keyword(s): Genistein, Dexamethasone, Fasting blood glucose, Mice, Pancreas

1. Introduction

Glucocorticoids (GCs) are steroid hormones that are produced from the zona fasciculata of the adrenal gland (ref). GCs are released in response stress (1, 2). GCs exert their action in different target tissues by binding to intracellular receptors, either GC receptor (GR) or the mineralocorticoid receptor (MR) (3). Glucocorticoids have been used to treat inflammatory diseases such as rheumatoid arthritis (4), inflammatory bowel disease, multiple sclerosis, psoriasis and eczema, as well as in treatment of autoimmune diseases (5) and in immunosuppressive regimes (6, 7). Among them, dexamethasone is the most commonly used synthetic glucocorticoid due to high potency with longer half-life (8-10). However, long-term treatment of glucocorticoids (GC) can induce insulin resistance and frank diabetes, clinically known as "steroid diabetes" (11).

GCs are member of catabolic hormones whose primary responsibility is to modulate carbohydrate metabolism (12, 13). GCs principally mobilize glucose to the systemic circulation by not only stimulating gluconeogenesis but also glycogenolysis in liver. Moreover, GCs promote muscle wasting and breakdown of triglycerides in the adipose tissue to supply substrate for gluconeogenesis, enhancing the hyperglycemic effect (14). In vitro studies have shown that GCs exert many detrimental effects on pancreatic β -cell function, including reduced glucose-stimulated insulin secretion (GSIS) by interfering with the translocation of GLUT2 transporter (15), increased activity of Kv1.5 channel (repolarizing potassium channel) (16), endoplasmic reticulum dysfunction (17), and increased β -cell apoptosis (18, 19). In addition, GCs induce whole body insulin resistance, especially at skeletal muscle and adipose tissues (11, 20, 21). Altogether, GCs can cause diabetes that is found in Cushing's syndrome or patients with prolonged use of GCs (22-24).

Phytoestrogens, structurally similar to estrogen, are plant-derived compounds with weak estrogenic activity (25) and have been extensively studied recently as potential curative candidates for many diseases (26, 27). Estrogen receptors are expressed in many tissues such as brain, bone, heart, liver and reproductive tissues (28, 29). The effects of phytoestrogens in body are controversy depending on type of tissues, it might has estrogenic effect in some while anti-estrogenic effect in others (30). Genistein is a class member of isoflavone, commonly found in soybeans, soy products, legumes and other plants (31, 32). Isoflavones have raised considerable interest because of their anticarcinogenic, antiarthrogenic, antidiabetic, immunomodulatory and antioxidant potentials (33-36).

Previous studies showed that genistein prevented diabetes via many mechanisms both in vitro and in vivo. Genistein stimulated glucose-induced insulin secretion in INS-1 cells via cAMP dependent protein kinase pathway (37). In human islets, genistein prevented high glucose induced cell damage by counterbalancing reductions in cell growth and DNA fragmentation via estrogen receptor beta (ER- β) and Bcl2 pathway (38). In consistent with that, another study proved that genistein reduced pancreatic β -cell apoptosis induced by fluoride and pertussis in isolated rat and human islets (39). Furthermore, it has been shown that genistein stimulated pancreatic β -cell proliferation by enhancing cell replication and increasing protein abundance of cyclin D1 in INS-1 cells (40). Similar results are observed in animals and human subjects. Genistein improved hyperglycemia, glucose intolerance, and serum insulin level in streptozotocin (STZ)-induced diabetic mice (40), high fat-induced diabetic mice (HFD) (41), and non-obese diabetic mice (NOD) (42) together with significant proliferation of pancreatic β -cell mass. In rats with alloxan-induced pancreatic damage, daily supplementation of genistein reduced hyperglycemia and minimized islet cell loss in dose

dependent manner (43). Daily soy intake was associated with lower body mass index and fasting circulation insulin levels, according to a cross-sectional study involving post-menopausal women (44). A randomized placebo controlled study in 2009 had demonstrated that genistein administration decreased fasting glucose and improved glucose tolerance and insulin sensitivity in post-menopausal women (45). These data suggested that genistein has not only insulinotropic action but also effect on β -cell survival by inhibiting apoptosis and stimulating proliferation. Although there were intensive researches regarding protective roles of genistein in different diabetic mouse models, whether genistein can prevent prolonged dexamethasone-induced hyperglycemia has not been studied.

Thus, this study aims to examine effect of genistein in prevention of dexamethasone induced high blood glucose in mice. This study might reveal a possible substance that prevents dexamethasone induced abnormal glucose metabolism.

2. Materials and Methods

2.1 Animals

Experiments were performed on 8-week old wild type male ICR mice purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. Prior to the experiments, animals were group-housed (n=3-5) in ventilated cages with solid floors and wooden dust bedding. Animals had free access to food and water *ad libitum* and were housed in 12 hours of light/12 hours of dark cycles at $22 \pm 2^\circ\text{C}$ with 60% humidity. The study was performed according to the guidelines of Mahidol University, and the animal experimental protocol was approved by Siriraj Animal Care and Use Committee.

2.2 Treatment

After acclimatization for a week, animals were randomly assigned into three groups (9 mice per group), control group (DMSO), 10mg/kg body weight dexamethasone treated group (Dexa) (T.P Drug Laboratories, Thailand) or dexamethasone with 10mg/kg body weight genistein (**Sigma-Aldrich**, USA) treated group (Dexa+Gen). All groups received injections daily for 4 weeks. The dose of genistein approximately reflects the twice of daily genistein intake in a soy-enriched diet in human (46-48). DMSO and dexamethasone were given subcutaneously, whereas genistein was given via intra-peritoneal injection.

2.3 Measurement

Food intake was measured daily, and body weight and fasting blood glucose level were measured once a week. Food pellets (CP Mice Feed, Thailand) were stored in hoppers within each cage to prevent spillage and cages were visually inspected daily to check for loose pellets. First, weight of food was measured using Jadever digital weighting scale (JadeverScale, Taiwan) on Day 0. Then the left-over pellets were measured every day before adding new food pellets. The difference between the initial recording and the final gave the food intake of total mice in a cage. Finally, food intake per day per mouse was averaged. Body weight of individual mouse was measured in a similar manner by Jadever digital scale weekly. Fasting blood glucose were measured by using a hand-held glucometer (OneTouch[®]Ultra[™], LifeScan, USA). After four weeks of treatment, mice were sacrificed using i.p Nembutal injection. Then epididymal fat pad, pancreatic tissue, and spleen were gently removed and weighted.

2.4 Statistical analysis

GraphPad Prism 5.0 (GraphPad software) and Microsoft excel were used for statistical analysis. The data in each experiment were calculated as mean $\pm$ SD using Student's t-test to compare between two groups. A *p*-value < 0.05 was considered significant.

3. Results

3.1 Prolonged treatment of dexamethasone lowers food intake and body weight.

We examined the effect of prolonged treatment of dexamethasone and genistein in term of food intake and body weight in compared with control. Food intake was not significantly affected by 10mg/Kg body weight treatment of either dexamethasone alone or genistein with dexamethasone in first three weeks. However, both Dexa and Dexa+Gen group showed significantly decreased food intake in 4th week when compared with control (*p* value = 0.006 and 0.007 respectively) (Figure 1a). Animals in all groups had similar body weight at the start of the experiment. Throughout the experiment, control group had significantly higher body weight than other groups (*p* value < 0.01) (Figure 1b). There was no significant body weight difference between Dexa and Dexa+Gen group.

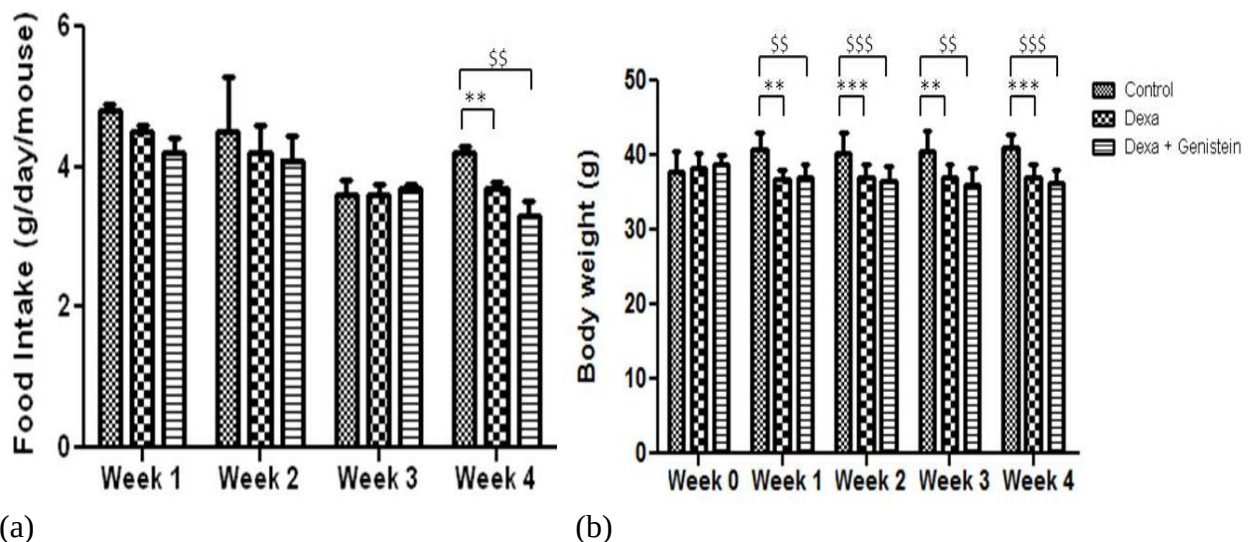


Figure 1 The effect of prolonged treatment of dexamethasone in (a) food intake (b) body weight. Mice were treated with either DMSO, dexamethasone (Dexa) or Dexa with genistein. Data are expressed as mean $\pm$ SD. Student's t-test. ** *p* < 0.01 , *** *p* < 0.001 for control vs Dexa group (N = 6-9). \$\$ *p* < 0.01 , \$\$\$ *p* < 0.001 for control vs Dexa+Gen group (N = 6-9)

3.2 Prolonged dexamethasone treatment elevates fasting blood glucose

Fasting blood glucose were similar in all 3 groups at first week (Figure 2). During the first three weeks, FBG of both Dexa and Dexa+Gen groups showed a decreasing trend in compare with control group. However, after 4 weeks, mice treated with dexamethasone showed significantly increased fasting blood glucose in compared with control (*p* value = 0.025). Co-treatment of genistein with dexamethasone attenuated the elevated blood sugar level (*p* value = 0.0002 in compared with desamethasone alone, *p* value = 0.028 in compared with control).

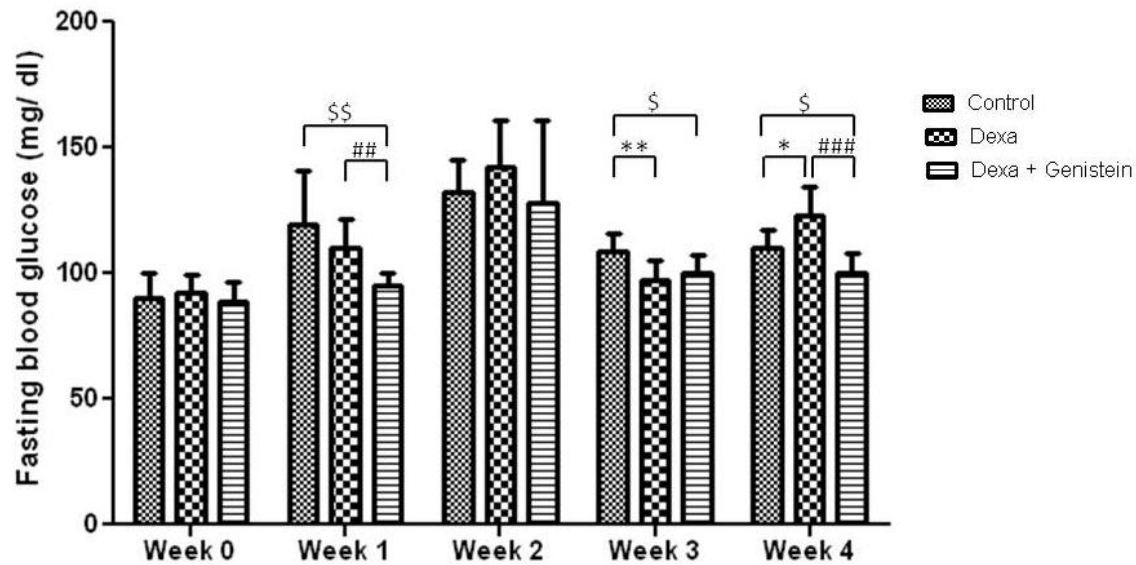
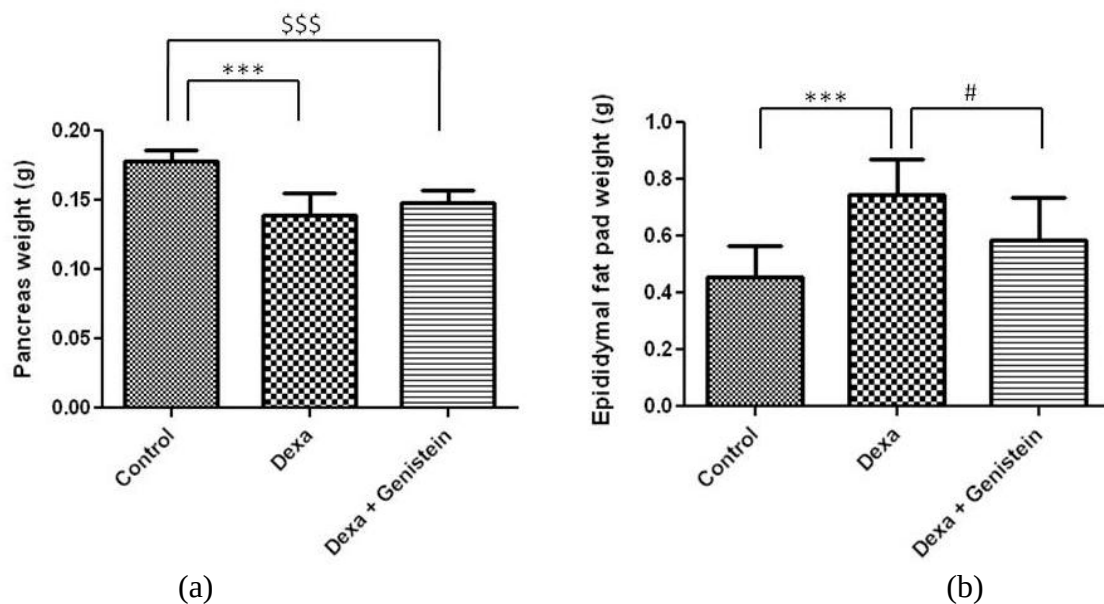


Figure 2 The effect of prolonged treatment of dexamethasone in fasting blood glucose level. Data are expressed as mean $\pm$ SD. Student's t-test. * $p < 0.05$, ** $p < 0.01$ for control vs Dexa. \$ $p < 0.05$, \$\$ $p < 0.01$ for control vs Dexa+Gen. ## $p < 0.01$, ### $p < 0.001$ for Dexa vs Dexa+Gen (N=6-9)

3.3 Prolonged treatment of dexamethasone affects organ weights

We examined the effect of prolonged treatment of dexamethasone and genistein in pancreas, epididymal fat pad, and spleen. Chronic administration of dexamethasone significantly reduced weight of pancreas when compared with control (p value = 0.0004). Co-treatment of genistein could not ameliorate the reduction in pancreatic weight (Figure 3a). Regarding epididymal fat pad, dexamethasone prolonged treatment caused significantly increased fat pad weight when compared to control. Co-administration of genistein with dexamethasone could reverse the change in weight of epididymal fat pad (Figure 3b). Mice treated with dexamethasone had significantly lower spleen weight than control group (p value = 0.0001). Dexa+Genistein group increased spleen weight to near normal value (Figure 3c).



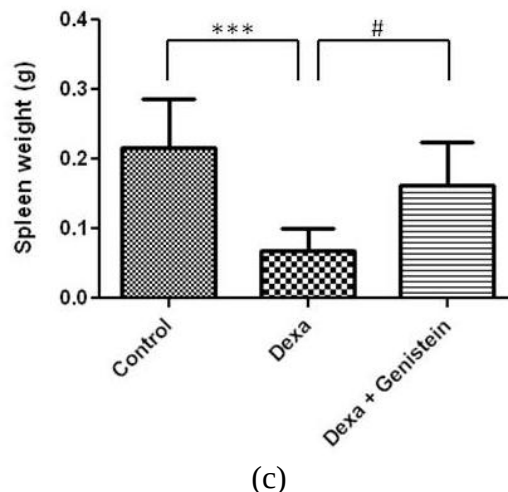


Figure 3 The effect of prolonged treatment of dexamethasone in organ weights. (a) Pancreas (b) Epididymal fat pad (c) Spleen. Data are expressed as mean $\pm$ SD. Student's t-test. *** $p < 0.01$ for control vs Dexa. \$\$\$ $p < 0.001$ for control vs Dexa+Gen. # $p < 0.05$ for Dexa vs Dexa+Gen (N = 5 for control, 9 for dexamethasone, 8 for Dexa+Genistein)

4. Discussion and Conclusion

Many recent studies have shown that genistein is a potential candidate for diabetic treatment (40, 41, 49). To our best knowledge, the effect of genistein on "steroid diabetes" has not been investigated. Our result demonstrated that, after four-week, administration of 10 mg/kg dexamethasone significantly decreased food intake when compared with control. Our result is in parallel with other study that intraperitoneal dexamethasone treated rats reduced their body weights (50). The report suggested that the reduction of body weights might be due to central effect of compensatory hyperinsulinemia (51). There are studies indicating hyperphagia in GC treated mice (52, 53). The discrepancy between the effects of dexamethasone on body weight might be due to several factors such as different potency of GC used, duration, dosage and mode of administration of GCs. It is possible that short term treatment of dexamethasone lowered hypothalamus serotonin level (54), thereby stimulating appetite (55). However, in prolonged dexamethasone treatment, brain serotonin level was unchanged (54) and compensatory hyperinsulinemia might suppress the feeding center in hypothalamus (51). Body weight of dexamethasone treated mice was significantly lower than control mice since 1st week (p value < 0.01) and it was maintained towards the end of experiment as indicated in a recent study (52). Even though the mechanism of reduction in body weight is not certain, dexamethasone is well known for its proteolytic effect and suppression of growth hormone axis in growing animals (56). Also, GC therapy was reported to cause the muscle wasting and a reduction in bone density (11). Increased lipolysis by dexamethasone may also lower body weight (56). Therefore, it is possible that the weight reduction of dexamethasone treated mice in this study might be due to its metabolic effects and reduction of food intake. Co-treatment of genistein with dexamethasone did not restore the body weight. Although, the dose of genistein used in this study approximately reflects the twice of daily genistein intake in a soy-enriched diet in human (46-48), this dosage of genistein reduced hyperglycemia and protected islet cell loss in alloxan- (43) or STZ-treated rats (57). Genistein was reported to decrease food intake and body weight in ovariectomized female mice (58). This study might explain our result that genistein did not increase body weight in dexamethasone treated mice.

Dexamethasone treated mice had significantly elevated fasting blood glucose level on 4th week when compared with control mice. This is in parallel with other studies stating that dexamethasone treatment can stimulate hepatic gluconeogenesis and glycogenolysis (59, 60), hyperinsulinemia (61), and whole body insulin resistance (50, 62), resulting in increased fasting blood glucose. In line with elevated FBG, dexamethasone treated mice showed significant reduction of pancreatic weight when compared to control. This data suggests that dexamethasone might increase pancreatic β -cell apoptosis, leading to impair insulin secretion and glucose homeostasis. Dexamethasone is a known inducer of cell apoptosis, including insulin secreting pancreatic β -cell (63, 64). Moreover, it can suppress pancreatic β -cell proliferation in long term (50). This would contribute to reduction in pancreatic weight. Moreover, mice treated with dexamethasone had a markedly significant epididymal fat pad weight in compared with control. It has been reported that glucocorticoid can induce lipolysis at peripheral tissue and increase central fat deposition. Prolonged treatment of glucocorticoid can stimulate expression of fatty acid synthase, and hyperplasia of adipose tissue, leading to visceral fat cell hypertrophy (56, 65). Increased circulating free fatty acid level and ectopic lipid deposition can also induce whole body resistance themselves (66-68). Insulin resistance also contributes to elevate fasting blood glucose.

Co-treatment of genistein with dexamethasone ameliorated the elevated fasting blood glucose. Our results suggested that genistein has protective effect on carbohydrate metabolism against dexamethasone in mice. Other study reported that genistein reduced blood glucose by decreasing hepatic glucose production by modulating enzymes involved in glucose metabolism (42, 69). Genistein administration was shown to associate with higher hepatic glucokinase activity but significantly lower activities of glucose-6-phosphatase and phosphoenolpyruvate carboxylase in liver in obese diabetic (db/db) mice (69). Other possible protective effect of genistein is antioxidant effect. Genistein was shown to reduce ROS production (70) and promote insulin secretion (49, 70, 71). However, our results showed that co-treatment of genistein with dexamethasone did not increase pancreatic weight when compared to dexamethasone treated group. Although genistein protected STZ induced pancreatic cell death (57), the high dose and long-term effect of dexamethasone might off-set the protective effect of genistein. It is possible that genistein might protect pancreatic β -cell function but might not protect pancreatic weight. Co-treatment of genistein with dexamethasone restored epididymal fat pad weight. This finding may be explained by genistein effects that decreased hepatic lipogenic activity (42) and/or increased apoptosis of adipose tissue (58). Genistein might alleviate insulin resistance by inhibiting adipogenesis, promoting hepatic glucose uptake and insulin secretion. All of these restored fasting glucose blood level. Prolonged dexamethasone treatment dramatically decreased spleen weight in comparison to control. This finding is similar to previous study that dexamethasone reduced splenic weight (72) and induced splenic cell apoptosis (73). Co-treatment of genistein with dexamethasone ameliorated the reduction in spleen weight. This result suggested that genistein reduced dexamethasone action. These data are supported by studies stating that genistein treatment can increase splenic cell production and proliferation. This might lead to increment in splenic weight in rat (74) and in mice (75).

Our results demonstrated that genistein attenuates the elevated fasting blood glucose induced by prolonged treatment of dexamethasone with lowered food intake and body weight. This is the first time that genistein has been used to prevent dexamethasone induced hyperglycemia. The mechanism how genistein prevents dexamethasone-induced impaired fasting blood glucose level requires further investigations.

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Tryptophan-Aspartate Repeat Domain 72 (WDR72) mRNA and Protein Expressions in Kidney Cells and Tissue

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Abstract

Objectives: Compound heterozygous mutations in *Tryptophan-Aspartate Repeat Domain 72 (WDR72)* gene, encoding the WD repeat domain 72 possibly regulating intracellular protein trafficking, have previously been identified as a candidate gene causing distal renal tubular acidosis (dRTA) in a Thai family by exome sequencing. This study thus aims to investigate *WDR72* mRNA and protein expressions in kidney cells and tissue and to further evaluate the effect of its mutations in pathogenesis of dRTA.

Materials and Methods: *WDR72* mRNA and protein expressions were examined in kidney cells and tissue by using reverse transcription-polymerase chain reaction (RT-PCR), western blot analysis, immunohistochemistry (IHC), and immunofluorescence assays (IFA). Subsequently, plasmids containing *WDR72* wild-type and identified mutants were generated and transfected into HEK293T cells following by evaluation of the protein expression and sub-cellular localization by western blot analysis and IFA.

Results: *WDR72* mRNA and protein were found to be expressed in kidney cells and tissue. By IHC and IFA, *WDR72* is localized in cytoplasm of both glomeruli and tubules of human kidney. Furthermore, all generated plasmids containing wild-type, R593G, or L841Q of *WDR72* were found to be equally expressed in transfected HEK293T cells. Likewise, the wild-type *WDR72* and mutants had no difference in their cellular localization.

Conclusion: The presence of *WDR72* mRNA and protein in the kidney suggests that it may have a function in this organ, and may be involved in dRTA disease. Moreover, wild-type and mutant *WDR72* plasmids could successfully be generated and expressed for further investigation into their effects on the pathogenesis of dRTA.

Keywords: Distal Renal Tubular Acidosis (dRTA), *Tryptophan-Aspartate Repeat Domain 72 (WDR72)*, Compound Heterozygous Mutation

1. Introduction

Hereditary distal renal tubular acidosis (dRTA) is a rare genetic disease characterized by a defect in acid secretion of α -intercalated cells located at the distal nephron of the kidney, resulting in persistent metabolic acidosis (1). Autosomal dominant dRTA is caused by mutations in *SLC4A1* (*AE1*) gene encoding anion exchanger 1 (*AE1*) while autosomal recessive dRTA is caused by mutations of *AE1* and two other genes, *ATP6V1B1* and *ATP6V0A4*, encoding B1 and a4 subunits of H^+ -ATPase (1, 2). In Thailand, mutations in the *AE1* gene are a main cause of dRTA, especially the recessive *AE1* G701D mutation is frequently observed in both homozygous and compound heterozygous conditions (3-6). However, there are many patients that mutations in these reported causative genes are not detected and the cause of the disease remains unknown. From a previous study by our group that aimed to identify the disease causing gene in this group of patients by exome sequencing, it was revealed that compound heterozygous mutations of *WDR72* gene, encoding the WD repeat domain 72, are a possible cause of dRTA in an affected family. Novel mutations, c.1777A>G: pR593G and c.2522T>A: pL841Q, of this gene have an impact on the structure and function of WD repeat domain 72 protein analyzing by using 6 mutation prediction web-based programs. These mutations segregated with dRTA disease in the affected family and were not found in 216 normal control subjects (unpublished data). Here, we present the protein study of this gene in the kidney and the successfulness in generated plasmids containing *WDR72* wild-type and identified mutants for further study into their effects on the pathogenesis of dRTA.

2. Materials and Methods

2.1 Kidney cell culture and maintenance

HEK293, HEK293T and HK2 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin G (Sigma, USA) and 100 μ g/ml of streptomycin (Sigma, USA), at 37°C in humidified atmosphere with 5% CO₂. For transfection, HEK293T were grown on 6-well plate and each recombinant plasmid were transfected with lipofectamine™ 2000 (Invitrogen, USA) in serum free DMEM and incubated at 37°C in 5% CO₂ incubator for 4-6 h. After incubation, serum free DMEM were removed and DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin G and 100 μ g/ml of streptomycin were added following by further incubation for 48 h at the same conditions.

2.2 *WDR72* mRNA expression study by RT-PCR

The protocol of this study was approved by Siriraj International Review Board (SIRB). Normal human kidney tissue that was discarded after tumor removal from kidney cancer patients were used in this study. Human kidney cell lines (HEK293 and HK2) and human kidney tissue were subjected to total RNA isolation by following the manufacturer's instruction of TRIzol® Reagent RNA Isolation Kit (Invitrogen, USA). Then, all purified total RNA were reversed transcribed to cDNA by using SuperScript™ III First-Strand Synthesis Kit (Invitrogen, USA). After that, conventional PCR was performed to investigate RNA expression by using primers specific to *WDR72* cDNA (forward, 5'-GGTGCTGGGAATGACATTTT-3', and reverse, 5'-CCGAGTTTGAGTTGCTGTCA-3'). The PCR products were detected by using agarose gel electrophoresis.

2.3 WDR72 protein expression study by western blot analysis

Human kidney cell lines (HEK293 and HK2) and human kidney tissue were homogenized in 8 M urea lysis buffer, followed by centrifugation to collect the total protein lysate. Protein concentration was estimated by using a Bradford Protein Assay (Bio-Rad, USA) and subjected to SDS-PAGE and western blotting. The membrane was blocked and primary antibodies specific to WDR72 at 1:1000 (ab190268, Abcam, UK) and β -actin at 1:1000 (Santa Cruz, USA) were added and incubated overnight at 4°C. Secondary antibody conjugated-HRP at 1:1000 (Dako, Japan) was applied and detected by using chemiluminescence assay (Thermo Scientific, USA).

2.4 Immunohistochemistry (IHC) and immunofluorescence (IFA)

To prepare kidney tissue sections, fresh human kidney tissues were fixed with 4% formalin buffer, paraffin-embedded, and cut at 4 μ m thickness. After rehydration in ethanol series, kidney tissue sections were treated in sodium citrate buffer (10 mM sodium citrate, 2 mM EDTA, 0.05% Tween-20, pH 6.0) for retrieval of antigenic sites followed by blocking endogenous HRP by 3% hydrogen peroxide. After blocking with 1% BSA, primary antibody specific to WDR72 at 1:100 (ab188518, Abcam, USA) was applied and incubated overnight at 4 °C. Secondary antibody conjugated-HRP at 1:250 (Dako, Japan) was applied. Kidney tissue sections were stained with 3% 3,3'-diaminobenzidine followed by counter-staining with hematoxylin. Pictures were captured by Axio Star Plus Light microscope (Carl Zeiss). For IFA, secondary antibody conjugated-Alexa 488 (Invitrogen, USA) was applied, followed by Hoechst staining (Molecular Probes, USA). Normal rabbit IgG (Cell Signaling Technology, USA) in the equal concentration to primary antibody was used as isotype control. Kidney tissue sections were examined by using LSM 510 Meta confocal microscope (Carl Zeiss).

2.5 Generation of wild-type and mutant WDR72 recombinant plasmids

Full length WDR72 coding sequence tagged with FLAG or HA were amplified from pEGFP plasmid containing wild-type WDR72 tagged GFP sequence (a gift from Dr. Jung-Wook Kim, Seoul National University, Korea) by using specific primers, and inserted into a pcDNA3.1/Hygro plasmid followed by transformation into *E. coli* DH5 α . The positive clones containing wild-type WDR72 recombinant plasmids were screened by colonies PCR and confirmed by DNA sequencing (First BASE Laboratory, Malaysia). Plasmids containing mutants c.1777A>G (R593G) or c.2522T>A (L841Q) of WDR72 gene tagged with GFP, FLAG, or HA were generated by site-directed mutagenesis using Pfx enzyme (NEB, USA). Mutant plasmids were transformed and screened for positive clones. The mutations were confirmed by DNA sequencing.

2.6 Expression of WDR72 recombinant protein in HEK293T cells

One μ g of each wild-type or mutant WDR72 recombinant plasmids were transfected into HEK293T cells by using lipofectamine™ 2000 (Invitrogen, USA). Transfected cells were harvested by using RIPA lysis buffer, subjected into SDS-PAGE and western blotting. Primary antibodies specific to each tag protein; ANTI-FLAG®M2 (Sigma, USA), anti-HA, anti-GFP (Invitrogen, USA), and β -actin (Santa Cruz, USA) were used to detect the WDR72 protein expression.

2.7 WDR72 sub-cellular localization in HEK293T cells by IFA

HEK293T cells were seeded onto the coverslip and transfected with 1 µg of *WDR72*-GFP plasmids. Forty eight hours after transfection, cells were fixed with 4% paraformaldehyde before staining of their nuclei with Hoechst dye (Molecular Probes). The fluorescence signal of WDR72 protein was captured by using LSM 510 Meta confocal microscope (Carl Zeiss).

2.8 Statistical analysis

The data of protein expression in Figure 4 was analyzed by one-way ANOVA for comparison between wild-type and mutants. A p-value <0.05 was considered as statistical significance.

3. Results

3.1 Investigation of *WDR72* mRNA and protein expressions in human kidney cell and tissue

To study whether WDR72 is expressed in the kidney, its mRNA and protein were examined by RT-PCR and western blot analysis. Figure 1 shows the expression of *WDR72* mRNA and protein in human kidney cells and tissue. In addition, by IHC and IFA, the WDR72 protein was observed in the cytoplasm of both kidney glomerular and tubular cells (Figure 2).

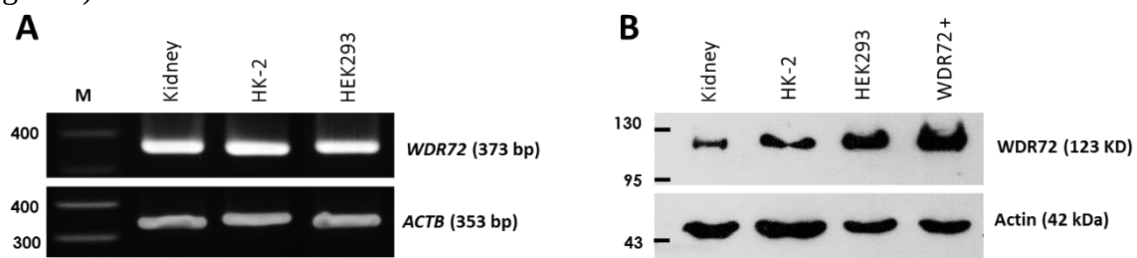


Figure 1 *WDR72* mRNA and *WDR72* protein expression in human kidney cells and tissue. **(A)** Detection of *WDR72* mRNA expression by RT-PCR method in human kidney cell lines, HK-2 and HEK293 cells, and tissue. The specific *WDR72* mRNA fragment is 373 bp and *ACTB* mRNA of β -actin (353 bp) was used as an internal control. **(B)** Full-length WDR72 protein (123 kDa) was detected by western blot analysis, β -actin (42 kDa) was used as loading control. WDR72+ is WT-WDR72 obtained from transfected HEK293T cells.

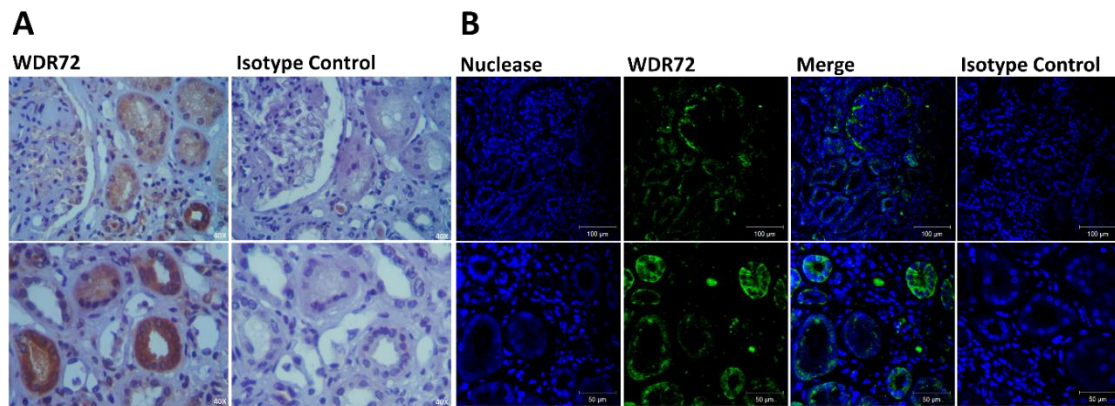


Figure 2 Immunohistochemistry (IHC) and immunofluorescence (IFA) staining of WDR72 in human kidney tissues. (A) IHC using specific antibody indicated the expression of WDR72 in glomerular and tubular cells (upper and lower left) comparing to isotype antibody (right). The Figure shows at 40X objective lens captured from light microscope. (B) IFA shows WDR72 localization in the cytoplasm of kidney glomerular and tubular cells (green). Blue indicates DAPI staining of nuclei. Signals were visualized by confocal microscopy, scale bar; top 100 µm, bottom 50 µm.

3.2 Construction of mammalian expression plasmid containing wild-type WDR72 and mutant R593G or L841Q tagged with GFP, FLAG, or HA.

Full length wild-type *WDR72* tagged with FLAG and HA were amplified and inserted into pcDNA3.1 to generate 3 recombinant plasmids containing wild-type *WDR72*. All plasmids were confirmed by DNA sequencing (data not shown). Moreover, 6 recombinant plasmids containing mutant R593G or L841Q of *WDR72* tagged with GFP, FLAG, or HA sequences were also generated. The sequencing chromatograms of mutant plasmids are shown in Figure 3.

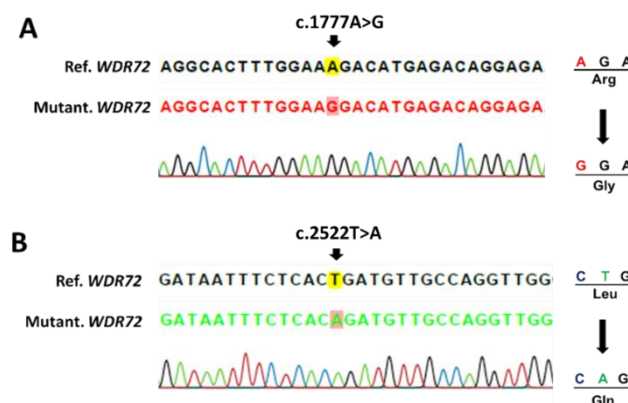


Figure 3 Sequencing profiles of mutant WDR72 recombinant plasmids. (A) Sequencing chromatogram of mutant R593G plasmid that altered nucleotide position 1777 from A to G; amino acid position 593 changed from arginine to glycine, and (B) mutant L841Q plasmid that altered nucleotide position 2522 from T to A; amino acid position 841 changed from leucine to glutamine. Reference *WDR72* is human *WDR72* sequence (NM_182758).

3.3 Expression of wild-type WDR72 and mutants tagged with GFP, FLAG, or HA recombinant proteins

The expressions of wild-type and mutants WDR72 recombinant proteins were evaluated by western blot analysis by using specific antibody to each tag protein. All plasmids were equally expressed. The protein bands of WDR72 tagged with GFP, FLAG, or HA are 150, 123, and 123 kDa, respectively (Figure 4).

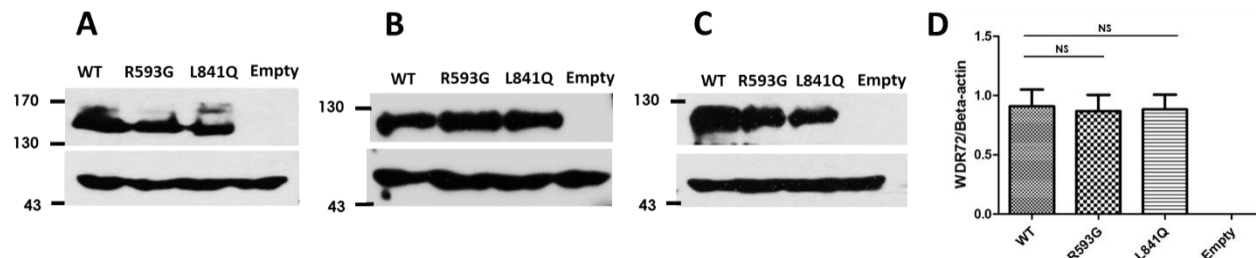


Figure 4 Western blot analysis of wild-type and mutant WDR72 protein expression. Plasmids expressing WDR72 protein tagged with GFP, FLAG or HA were analyzed by western blotting using specific antibodies. The size of recombinant WDR72-GFP is 150 kDa (A), WDR72-FLAG (B) and WDR72-HA (C) are 123 kDa. Empty is pcDNA3.1 transfected cells. (D) The results of 3 independent experiments of WDR72-FLAG expression, normalized with β -actin. The data was analyzed by one-way ANOVA. NS is not significance.

3.4 Sub-cellular localization of wild-type and mutant WDR72-GFP proteins

Protein localization of wild-type WDR72-GFP and two mutants in transfected HEK293T cells were examined; no differences between wild-type, R593G, and L841Q were found. Mutant WDR72 was observed in the cytoplasm, similar to the wild-type (Figure 5).

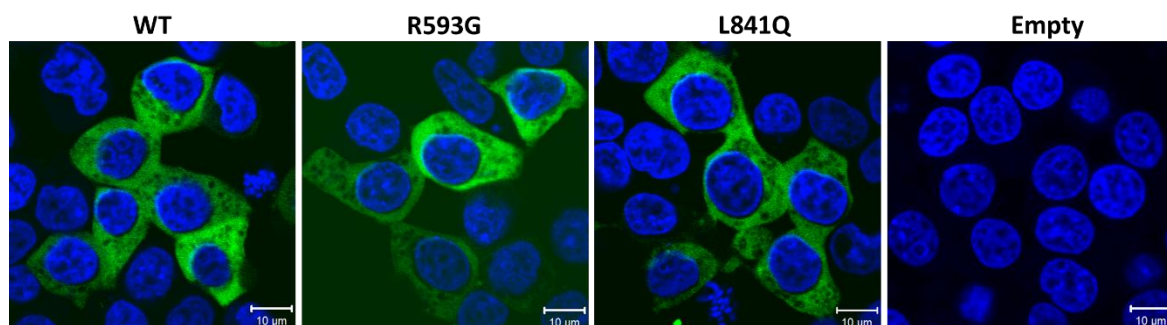


Figure 5 Sub-cellular localization of wild-type and mutant WDR72-GFP proteins. The expression of wild-type WDR72-GFP and two mutants is shown in green. Blue indicates DAPI staining of nuclei. Empty is pcDNA3.1 transfected cells. Signals were visualized by confocal microscopy, scale bar, 10 μ m.

4. Conclusion and discussion

In our previous study, we identified compound heterozygous mutations of *WDR72* in a Thai family with autosomal recessive dRTA. However, the effect of mutations leading to dRTA have not been elucidated. Mutations of *WDR72* have been reported to be a cause of dental defect, amelogenesis imperfecta (AI) (7-12), and a recent study has described AI phenotype in a dRTA patient (13). In addition, AI is may also associate with biochemical change elsewhere in the body (14).

Several previous genetic studies showed that *WDR72* is a gene that has a role in kidney function associated with kidney disease (15-17). The results of our present study showing that *WDR72* is expressed in human kidney cells and tissue by several methods, suggest that *WDR72* may have a function in the kidney. This is consistent with transcriptomes database that indicate the expression of this protein in kidney tissue. Normally, expression of a gene in a specific tissue or organ indicates its protein function in that organ (18, 19). Therefore, this study provides supplementary evidence supporting that *WDR72* is a functional protein in kidney as demonstrated by mRNA and protein expressions. Previous studies in ameloblast cells, *WDR72* has been predicted to be a vesicle-coated protein that involves in membrane protein trafficking. Ameloblasts of *Wdr72* knockout mice were found to have decreased levels of sodium/potassium/calcium exchanger 4 (NCKX4) at the membrane (8, 12). *WDR72* modeling study shows highest homology to *Saccharomyces cerevisiae* β' -COP protein. N-terminal part is formed into WD40 β -sheet folds of two β -propellers with an α -solenoid fold protein at its C-terminus. Having the structure-like β' -COP, *WDR72* that expresses in cytoplasm is suspected to function as a vesicle coat protein that involves in protein trafficking. Considering the cytoplasmic expression of *WDR72* in the kidney tubular cells and also in transfected HEK293T cells, it is possible that *WDR72* is necessary for intracellular protein trafficking function, similar to what that found in ameloblasts.

The expression of mutant proteins is equally to wild-type protein. Likewise, sub-cellular localization is the same for both mutants comparing to wild-type, suggesting that the mutant proteins do not affect the expression and their sub-cellular localization. However, their effects on the protein function are unknown. R593G mutation located in β -propeller domains may affect the binding activity to partner proteins and L841Q mutation in the α -solenoid domain may affect the stability of this protein during trafficking function. Taken together, both mutations may affect to membrane protein trafficking of α -intercalated cells such as AE1 protein. However, the precise mechanism of these mutants leading to dRTA should be further elucidated.

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***Orientia tsutsugamushi* induces cathepsin and inflammatory caspase-dependent cell death in human endothelial cells**

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Abstract

Objectives: *Orientia tsutsugamushi* (OT), the causative agent of scrub typhus, is an intracellular bacterium which escapes from phagosome and replicates in the cytosol of host cells. Inflammatory caspases-1,-4, and -5 are involved in inflammasome activation pathways and cell death. OT can induce caspase-1 –dependent IL-1 β secretion from macrophages, the suggestive evidence of inflammasome activation. Therefore, this study aims to determine the involvement of inflammatory caspases in cell death in human endothelial cells.

Materials and Methods: The human dermal microvascular endothelial cells (HMECs) were infected with OT at an multiplicity of infection (MOI) of 20 : 1. Cell death was measured by LDH release assay. The inflammatory caspase-1,-4, -5 and cathepsin (lysosomal enzyme) inhibitors were used for blocking OT-induced cell death. The gene expression of IL-1 β was determined by qRT-PCR, whereas the secretion of IL-1 β was measured by ELISA.

Results: Cell death determined by LDH release was observed in OT-infected HMECs since 24 h post-infection. The inflammatory caspase and cathepsin inhibitors significantly reduced the percentage of cytotoxicity at 48 and 72 h post infection. IL-1 β mRNA was upregulated in LPS-stimulated and OT-infected HMECs. However, in contrast to the macrophage response, IL-1 β secretion by HMECs was undetectable.

Conclusion: This study demonstrates that OT can induce cell death in HMECs. Inflammatory caspases and lysosomal enzymes (cathepsins), which may be released during bacterial escape, play a role in cell death following OT infection. However, OT cannot IL-1 β secretion from HMECs. Further studies are required to determine the exact caspase(s) activated after the infection.

Keywords: Cathepsins, Cell death, Endothelial cells, Inflammatory caspases, *Orientia tsutsugamushi*,

1. Introduction

Orientia tsutsugamushi (OT) is an obligate intracellular bacterium and causative agent of scrub typhus. This disease is transmitted to humans by the bite of the larval trombiculid mites (*Leptotrombidium* spp.) or chigger (1). The invasion of OT into endothelial cells (ECs) results in inflammatory process characterized by perivascular cell infiltration. Endothelial cell damage in visceral organs is probably the cause of complications in scrub typhus (2).

ECs are targets of OT infection. OT-induced cell death has been reported in human endothelial cell line (ECV304) (3). These cells exhibited the features of cell death including DNA fragmentation and TUNEL positive nuclei. Moreover, OT induces cell death and release of IL-1 β from monocytes/macrophages (4, 5), suggesting that OT may induce caspase-1-dependent cell death or pyroptosis. However, the mechanisms and biochemical pathways that are involved in cell death during OT infection are still unknown.

There are many cell death pathways involved during microbial infection. The well known cell death pathways are apoptosis and pyroptosis (inflammatory cell death). Apoptosis is mediated by effector caspases (caspase -3, -6, and -7) (6), whereas pyroptosis is mediated by inflammatory caspases (caspase-1, -4, and -5). Regarding pyroptosis, caspase-1 is induced by inflammasome activation and leads to IL-1 β secretion. Human caspase-4 and -5, the newly recognized caspases that are homologs to murine caspase-11, recognize cytoplasmic LPS, induce non-canonical inflammasome activation and eventually lead to pyroptotic cell death (7).

This study focuses on the mechanism of OT-induced cell death in endothelial cells. Knowledge from this study may improve the current understanding in the pathogenesis of scrub typhus.

2. Materials and methods

2.1 Cultivation of human dermal microvascular endothelial cells (HMECs)

The human dermal microvascular endothelial cells were cultured in complete MCDB131 (Sigma) supplemented with 10% fetal bovine serum, EGF, IGF-1, FGF-basic and VEGF-165 (Immunotools) at 37°C in a 5% CO₂ atmosphere in 75-cm² tissue culture flasks. The old culture medium was replaced by new medium every 2 days. The cells were sub-cultured every week by using 0.05% trypsin/EDTA.

2.2 Stimulation of host cell by *O. tsutsugamushi* (OT)

OT (karp strain) stock stored at -80°C was thawed and resuspended with RPMI 1640. Then, the bacterial suspension was centrifuged at 2200 g for 10 min. The OT pellet was resuspended with RPMI. Endothelial cells were infected with OT at an multiplicity of infection (MOI) of 20 : 1 and incubated for 1 h at 37°C. Infection medium was removed and replaced by new complete MCDB131 medium.

2.3 The percentage of cytotoxicity in OT-infected HMECs by lactate dehydrogenase (LDH) release assay

After infection, supernatants were collected at 8, 24, 48, 72 and 96 h post-infection. Lactate dehydrogenase was determined by LDH release assay (Biovision). Absorbent was measured with an ELISA reader set at 450 nanometers. The percentage of cytotoxicity was calculated, using this formula: % cytotoxicity = (OD at test sample – low control) $\times$ 100 / (high control – low control).

2.4 Inhibition of inflammatory caspases and cathepsins

Inflammatory caspase-1, -4, -5 inhibitors, namely Z-LEVD-FMK, Z-WEHD-FMK (A.G. scientific), cathepsin inhibitors (Z-FA-FMK and FF-FMK) and pan-caspase (Z-VAD-FMK) inhibitors (Biovision) were used in this study. The cells were incubated with these inhibitors for 2 h before the infection process. The supernatants were collected for determining cell death by LDH release assay.

2.5 Gene expression of IL-1 β by qRT-PCR technique

Total RNA was extracted from the infected cells by GenUP total RNA kit (Biotechrabbit). RNA was converted to cDNA by superscriptTM III first strand synthesis system (Invitrogen). *IL-1 β* real-time PCR was performed using iTaq SYBR Green master mix (Bio-Rad). Quantification cycle (Cq) of IL-1 β and GAPDH was used to calculate the fold change by the following formula : $FC = 2^{\Delta\Delta Cq}$, $\Delta\Delta Cq = (Cq \text{ of target} - Cq \text{ of GAPDH}) \text{ sample} - (Cq \text{ of target} - Cq \text{ of GAPDH}) \text{ uninfected}$.

2.6 The secretion of IL-1 β by ELISA

The supernatants were collected and stored at -80°C. IL-1 β secretion was assessed by IL-1 β ELISA kit (Komabiotech), according to the manufacturer's protocol.

2.7 Statistical analysis

GraphPad Prism 3.0 software was used for statistical analysis. The data of LDH assay and cytokine release were compared by a t test or non-parametric method. The data in each experiment were expressed as mean $\pm$ SEM. p-value lowers than 0.05 was considered significant.

3. Results

3.1 OT induced cell death in HMECs

HMECs were infected with OT at an MOI of 20 : 1. The percentages of cytotoxicity shown at 8, 24, 48, 72 and 96 h post infection were approximately 2.5, 15.3, 21.0, 29.5 and 43.6, respectively. This result suggested that OT-infected cells exhibited a feature of cell death, characterized by loss of cell membrane integrity and subsequent release of intracellular LDH.

Since the loss of cell membrane integrity was observed as early as 24 h after infection, we hypothesized that inflammatory caspases and lysosomal membrane permeabilization may be involved in OT-induced cell death. Accordingly, the next experiment therefore determined the caspase(s) involved in OT-induced cell death in primary endothelial cell HMECs.

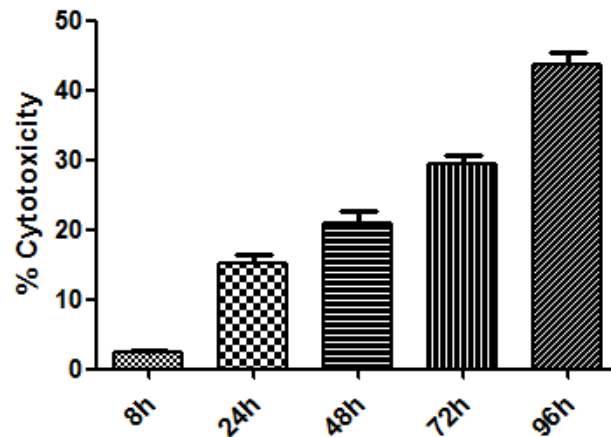


Figure 1. The percentages of cytotoxicity of OT-infected HMECs were shown for different time periods. The results represent, each performed in triplicate.

3.2 Inflammatory caspase and cathepsin inhibitors reduced the cell death in OT-infected HMECs

We determined the effect of inflammatory caspase and cathepsin inhibitors in OT-infected HMECs. HMECs were treated with caspase inhibitors and then infected with OT at MOI of 20. The inflammatory caspase inhibitors are not specific to one caspase and can probably block all of inflammatory caspases. The supernatants were collected and the cell death was determined by LDH assay.

LEVD-FMK, WEHD-FMK (caspase-1, -4, -5 inhibitors), FA-FMK, FF-FMK (cathepsin inhibitors) and VAD-FMK (pan-caspase inhibitor) significantly decreased the cytotoxicity, compared to culture with 0.1% DMSO without inhibitor. This result suggested that inflammatory caspases and cathepsins (lysosomal enzymes) are important to OT-induced cell death. Bacterial escape from phagosomes into the cytosol likely results in cathepsin release that subsequently induces cell death of host cells.

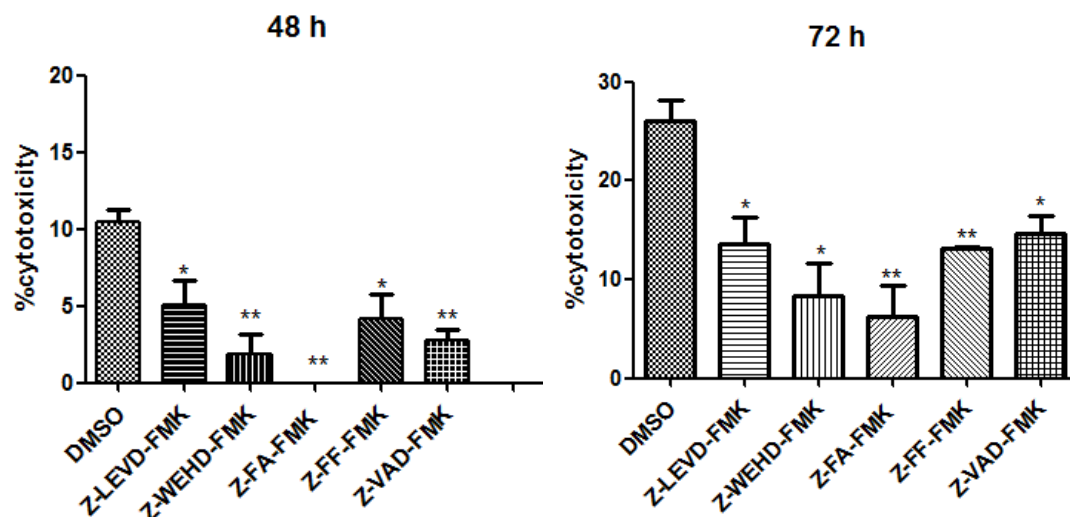


Figure 2. Effect of inflammatory caspase inhibitors on OT-induced cell death was assessed by the percentages of cytotoxicity. Data were expressed as means $\pm$ s.d. of triplicate wells and the statistical significance was calculated by the student's t-test. * $p < 0.05$, ** $p < 0.01$. The results represent, each performed in triplicate.

3.3 OT upregulated IL-1 β expression in HMECs but failed to induce IL-1 β secretion

The proinflammatory cytokine IL-1 β plays a role in the inflammatory cell death. Hence, the next objective was to study proinflammatory cytokine expression in OT-infected HMECs. *IL1B* gene was measured in HMECs stimulated with 200 ng/ml LPS or infected with OT at MOI of 20. The mRNA expression at 6 h after stimulation was determined by qRT-PCR. Lipopolysaccharide (LPS)-induced HMECs was used as the positive control for gene expression.

The results indicated both LPS-stimulated and OT-infected HMEC could upregulate IL-1 β mRNA expression.

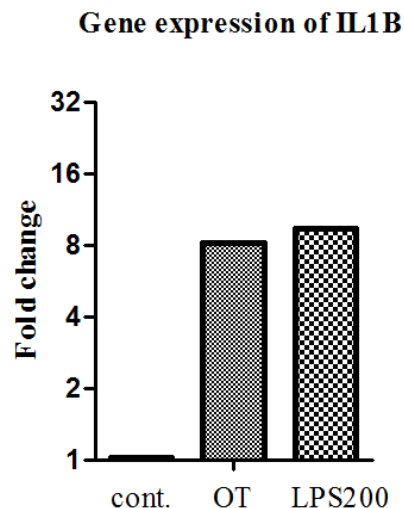


Figure 3. Fold change of gene expression was performed at 6 h after 200 ng/ml LPS stimulation or OT infection. The results represent, each performed in triplicate.

Next, the IL-1 β secretion was determined in supernatants by ELISA. HMECs were primed with LPS 1 μ g/ml for 6 h or unprimed, and infected with OT for 6 and 24 h. The lower limit of IL-1 β detection is 4 pg/ml. Unexpectedly, Neither LPS-primed nor unprimed OT-infected HMECs secreted detectable levels of IL-1 β (Figure not shown). This result is different from the previous studies using monocytes or macrophages that showed high levels of IL-1 β secretion from those phagocytic cells.

4. Conclusion and discussion

This study emphasizes the mechanism of OT-induced cell death in primary endothelial cell line HMECs. It can be concluded that OT can induce cell death in HMECs characterized by LDH release. Inflammatory caspases and cathepsins play a role in the cell death of HMECs during OT infection since inflammatory caspase and cathepsin inhibitors can block that response. Moreover, OT induces the upregulation of IL-1 β gene expression. Although previous studies have shown that OT induces release of IL-1 β from monocytes and macrophages (4, 5), this study shows that HMECs cannot secrete IL-1 β after OT infection. Therefore, the difference in cell types may affect the ability to secrete cytokine.

In conclusion, this study suggests that OT-infected endothelial cells exhibit the feature of cell death but induce poor cytokine secretion. Further studies by Western blot that detects cleaved caspase(s) are required to determine the exact caspase(s) involved during OT

infection. OT-induced vascular damage with poor inflammatory response may be involved in the pathogenesis of scrub typhus.

Acknowledgement

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Molecular identification and susceptibility of *Trichosporon* species isolated from bloodstream infections

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Abstract

Objective: Although disseminated trichosporonosis is an emerging mycosis as the second- or third-most-common agents of yeast fungemia with high mortality, information on its causative agents is still limited. Then, DNA sequence to support species and genotyping information, including antifungal drug susceptibility patterns were investigated.

Materials and Methods: All *Trichosporon* strains isolated from blood culture of individual patients during December 2011- August 2014 at Siriraj Hospital were investigated into species by sequence analysis of the IGS1 region, ITS region and D1/D2 domain. They were also investigated for genotyping by IGS1 sequence analysis and antifungal drug susceptibility by broth microdilution method.

Results: The total of ten strains of *Trichosporon* species were isolated. Two species were attained, 6 *T. mycotoxinivorans*, 3 *T. asahii* (genotype 3 for 2 isolates and genotype 7 for 1 isolate) and including 1 *Trichosporon* novel species. Almost 50% of all *T. asahii* isolates exhibited amphotericin B MICs of 2 µg/ml. Nine isolates were susceptible to voriconazole, posaconazole and itraconazole at MICs ≤ 0.25 µg/ml. The echinocandins, caspofungin and anidulafungin demonstrated no activity against all isolates with high MIC >8 µg/ml.

Conclusion: *T. mycotoxinivorans* was the most common recovered isolates from blood cultures. Different genotypes from other country of *T. asahii* were revealed. Therefore, molecular analysis was required for species identification. According to high MIC to antifungal drugs, *in vitro* drug susceptibility testing was necessary.

Keyword: *Trichosporon*, blood, susceptibility, genotyping

1. Introduction

Trichosporon species are yeast-like fungi that produces septate hyphae, arthroconidia, yeasts, and pseudohyphae. They are widely distributed in nature and constitute normal flora of the human skin, vagina, respiratory and gastrointestinal tracts (1, 2). Although most *Trichosporon* spp. isolated in clinical laboratories are related to colonization or superficial infections, this fungus has been recognized as an emergent opportunistic agent causing invasive infections in tertiary care hospitals worldwide (1, 3-6). In this decade, *Trichosporon* spp. have been an emerging fungal pathogen in immunocompromised hosts that represent the second or third most common non-*Candida* yeast infections causing invasive disease in patients with hematological cancer (1, 3).

Presently, the nomenclature within the genus *Trichosporon* has been revised base on molecular analysis. Nowadays, at least 50 *Trichosporon* species have been described from different regions, including 16 species with clinical relevance such as *T. asahii*, *T. asteroides*, *T. coremiiforme*, *T. cutaneum*, *T. dermatis*, *T. domesticum*, *T. faecale*, *T. inkin*, *T. japonicum*, *T. jirovecii*, *T. loubieri*, *T. montevidense*, and *T. mucoides* (1, 6, 7).

In clinical laboratories, genus level of *Trichosporon* isolates are routinely identified through their gross morphology of colonies and microscopic morphology. Colonies were white-cream and round with wrinkle and radiating margin. Microscopic characteristics are budding yeast cells with arthroconidia including positive urease test as a supplement result to distinguish from the genus *Geotrichum* (8). Species level identification by physiological and biochemical characteristics or commercial kits usually are unreliable (1, 6, 8-10). Therefore, sequencing becomes necessary for *Trichosporon* diagnosis.

Currently, identification of *Trichosporon* isolates from culture to species level based on the sequencing of ribosomal DNA (rDNA) genes such as conserved genes (D1/D2 domain of the 26S rDNA) (11, 12) or variable non-coding regions such as Internal Transcribed Spacers 1 and 2 (ITS) or Intergenic Spacer 1 region (IGS1, located between the 26S and 5S rDNA) are recommended (1, 13, 14). The D1/D2 and ITS regions are each highly homologous within the genus *Trichosporon*, they achieve to distinguish at least the 37 generally recognized *Trichosporon* species (13), with the exception of *T. montevidense* and *T. domesticum*, which have indistinguishable ITS sequences. However, a few strains had D1/D2 domain and ITS regions sequences that showed high similarity to any of the groups of closely related species, making it difficult to gain an accurate identification (15). Whereas, IGS1 showed more discriminatory power between closely related species and so it is helpful for distinguishing phylogenetically closely related species (1, 13). Consequently, it has been successfully adopted for the identification of clinical *Trichosporon* isolates in a number of recent studies (9, 10, 15-19).

Currently, IGS1 show intraspecies variability for genotyping within *Trichosporon* species as a tool in epidemiological studies (13). The geographical distribution of the genotypes of the IGS1 is variable for *T. asahii* which is the most common found species (1). Then, at least 9 *T. asahii* IGS1 genotypes have been described from many countries (6, 9, 10, 18, 20, 21). The first seven genotypes constitute the major ones while genotypes 8 and 9 have only been reported from Turkey (6).

This study elucidated the *Trichosporon* strains isolated from blood of patients. Characterization was based on the molecular identification of the IGS1, ITS and D1/D2 regions using DNA sequence analysis, genotyping of *T. asahii* and drug susceptibility of the isolates.

2. Materials and methods

2.1 Strains and DNA extraction.

The total of 10 *Trichosporon* spp. isolates from individual patients' blood cultures submitted to the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, during December 2011 – August 2014 were evaluated. They were genus identified base on macroscopic and microscopic observation of elongated budding yeast with hyphae and/or arthroconidia on Saboraud dextrose agar (SDA) including urease positive. The isolates were cultured on SDA at room temperature for 3 days before molecular identification and antifungal drugs susceptibility test. DNA was released from pure cultures of each of the isolates by boiling for 20 min. After centrifugation, clear supernatant was used as template in the polymerase chain reaction.

2.2 PCR and DNA sequencing.

Three primer pairs were used to amplify 3 regions of the ribosomal DNA from the genomic DNA template. The IGS1 region was amplified by primers 26SF (5'-ATCCTTTGCAGACGACTTGA-3') and 5SR (5'-AGCTTGACTTCGCAGATCGG-3') (4). The internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) was amplified by primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), (14, 22). D1/D2 domain was performed with primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') (22). Each 50 µl of PCR mixture contained 25 µl of KAPA Taq readymix (Applied Biosystems, US), 21 µl of nuclease free water, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer and 2 µl of DNA template. Nuclease free water was used as a negative control. All PCR were performed in a CFX96™ Real-Time PCR Detection Systems (Bio-Rad, Hercules, US). The PCR conditions consist of an initial denaturation at 95°C for 5 min follow by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for primers 26Sf/5SR, 57°C for ITS1/ITS4 and 59°C for NL1/NL4, for 30 sec and extension at 72°C for 30 sec, follow by one cycle of final extension at 72°C for 10 min. After amplification, the PCR products were electrophoresed through a 1% agarose gel to confirm amplification and purified to remove unincorporated nucleotides, primers and the remaining template DNA with QIAquick PCR Purification Kit (Qiagen, Germany). They were used as templates for DNA sequencing with a forward primer at the 1st BASE Sequencing INT, Malaysia, using the BigDye® Terminator v3.1 cycle sequencing kit chemistry on the ABI PRISM 3730XL Analyzer.

2.3 Alignment and Phylogenetic Analysis

All DNA sequence chromatograms were checked manually to ensure high-quality sequences before submitted for BLAST analysis at the NCBI site (<http://www.ncbi.nlm.nih.gov>) for species identification. Phylogenetic analysis were compared with those corresponding *Trichosporon* IGS1 region sequences database downloaded from GenBank. Multiple-sequence alignments with the corresponding sequences, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (23). The evolutionary distances were computed using the Kimura two-parameter method. One thousand bootstrap replicates were conducted for the reliabilities of the nodes on the phylogenetic trees. The IGS1 region was further targeted to determine the genotypes of the *T. asahii* isolates as described previously (4, 13, 21).

2.4 Antifungal susceptibility testing.

Antifungal susceptibility testing was performed by the broth microdilution method of Sensititre® YeastOne® susceptibility system (Trek Diagnostic Systems, UK) to determine minimal inhibitory concentration (MIC) of amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole (PSC), caspofungin (CAF), micafungin (MIF), anidulafungin (AND) and 5-flucytosine (5FC) as described by the manufacture instruction. The microdilution plates were incubated at 35°C for 48 before visual reading. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as the quality control strains.

3. Results

3.1 Molecular identification of *Trichosporon* isolates.

All ten isolates of *Trichosporon* from blood were identified by querying their unknown IGS, ITS and D1/D2 sequences against sequences of *Trichosporon* species in the GenBank database and results are summarized in Table 1. The three loci simultaneously identified the strains to species level of 3 isolates of *T. asahii* and 6 isolates of *T. mycotoxinovorans* with 99-100% maximal identity. Although isolates TC140 and TC153 had matches with *T. loubieri* 99% by ITS and D1/D2 regions, it's IGS1 was only 97% identity. Therefore, these 2 isolates was identify as *T. mycotoxinovorans*.

One isolates (TC075) could not be species level identified. This was form discrepancy of species determination by all 3 region. Although, it had 99% ITS and D1/D2 identity with *T. asahii*, it was also 99% matching to *T. insectorum*, *T. facale* *T. japonicum*, and *Trichosporon* sp. EU258671. Likewise, IGS sequence of TC075 had low level matched (83-88%) to *T. asahii*, *T. facale* *T. insectorum*, *T. japonicum*, and *T. asteriodes*. However, TC075 IGS sequence was identical to unspecified species of *Trichosporon* sp. GenBank accession number JX476304.

Table 1. Pairwise alignment results for species identification by Intergenic Spacer (IGS, located between the 26S and 5S rDNA), Internal Transcribed Spacers 1 (ITS), and D1/D2 domain of the 26S rDNA).

Strain no.	Species identification	Sequencing analysis					
		IGS		ITS		D1D2	
		region ^a	Identity ^b , %	region ^a	Identity ^b , %	region ^a	Identity ^b , %
TC003	<i>T. asahii</i>	KT438846	602/602, 100%	KT900123	503/503, 100%	KT900123	507/507, 100%
TC089	<i>T. asahii</i>	JX111959	609/609, 100%	KT900123	503/503, 100%	KT900123	507/507, 100%
TC111	<i>T. asahii</i>	KR233248	605/605, 100%	KU363042	491/491, 100%	EU258673	605/605, 100%
TC032	<i>T. mycotoxinivorans</i>	AJ601390	511/513, 99%	JQ266092	488/489, 99%	EU118984	592/592, 100%
	<i>T. loubieri</i>	KT356593	111/114, 97%	KC254110	485/489, 99%	JN939448	585/594, 98%
TC171	<i>T. mycotoxinivorans</i>	AJ601390	514/516, 99%	JQ266092	499/499, 100%	EU118980	603/603, 100%
	<i>T. loubieri</i>	KT356593	111/114, 97%	KC254110	496/499, 99%	JN939448	596/605, 99%
TC173	<i>T. mycotoxinivorans</i>	AJ601390	523/524, 99%	JQ266092	502/502, 100%	EU118980	601/601, 100%
	<i>T. loubieri</i>	KT356593	111/114, 97%	JN712655	506/506, 100%	JN939448	593/602, 99%
TC096	<i>T. mycotoxinivorans</i>	AJ601390	504/505, 99%	JQ266092	502/502, 100%	EU118979	596/596, 100%
	<i>T. loubieri</i>	KT356593	111/114, 97%	KC254110	498/502, 99%	JN939118	589/589, 98%
TC140	<i>T. mycotoxinivorans</i>	AJ601390	499/500, 99%	JQ266092	500/500, 100%	EU118979	603/603, 100%
	<i>T. loubieri</i>	KT356593	111/114, 97%	KC254110	497/500, 99%	JN939118	596/605, 99%
TC153	<i>T. mycotoxinivorans</i>	KR132600	527/528, 99%	JQ266092	502/502, 100%	EU118979	594/595, 99%
	<i>T. loubieri</i>	KT356593	111/114, 97%	KC254110	499/502, 99%	JN939118	587/598, 98%
TC075	<i>Trichosporon. sp</i>	JX476304*	493/493, 100%	LN808895	502/505, 99%	EU258671	600/602, 99%
	<i>T. faecale</i>	KM488281	520/589, 88%	EF153624	503/504, 99%	EF121872	596/602, 99%
	<i>T. asahii</i>	KR872659	522/619, 84%	KT900116	504/505, 99%	FJ455110	601/602, 99%
	<i>T. asteroides</i>	EU934800	506/602, 84%	KC881079	501/504, 99%	AF075513	588/591, 99%
TC075	<i>T. japonicum</i>	JF302988	481/577, 83%	JQ425388	501/505, 99%	AF308657	589/591, 99%
	<i>T. insectorum</i>	Not found	Not found	HM802133	503/504, 99%	HM802133	597/602, 99%
	<i>T. coremiiform</i>	JF153606	311/372, 84%	NR073249	501/503, 99%	JX111949	593/602, 99%

^a GenBank accession number

^b maximum query/matching species bp

*clinically relevant *Trichosporon* species from Argentina (data from GenBank)

3.2 Phylogenetic analysis of IGS1 region and genotyping.

Phylogenetic analysis was performed on the basis of the results of sequence analysis of the IGS region (Figure 1). The 3 isolates of *T. asahii* belong to genotype 3 (2 isolates) and genotype 7 (1 isolate) with high genetic similarity at bootstrap 1000. The number assigned to genotypes corresponding to Sugita's data (13, 21). All 6 isolates of *T. mycotoxinivorans* had 2 IGS1 sequence types. They clustered together and showed high genetic relatedness within the group (bootstrap 1000) and show in different branch from others species. Interestingly, the TC075 isolate was in separated branch from *T. asahii*, *T. faecale* and *T. japonicum* with a bootstrap support higher than 90.

3.3 Antifungal susceptibility testing.

The susceptibilities of ten isolates to nine antifungal agents are shown in Table 2. MIC for AND, MIF and CAS were consistently higher than 8 µg/ml for all *Trichosporon* isolates. Of note, ITC, PC, and VRC were the most potent agents *in vitro* against all *Trichosporon* isolates with MIC ranging 0.03-1 µg/ml. Four of 6 *T. mycotoxinivorans* isolates show 1-3 two-fold dilution of MIC of FLC (4-16 µg/ml) higher than all 3 *T. asahii* isolates (2 µg/ml). One isolate (TC140) of *T. mycotoxinivorans* show high MIC to all drugs.

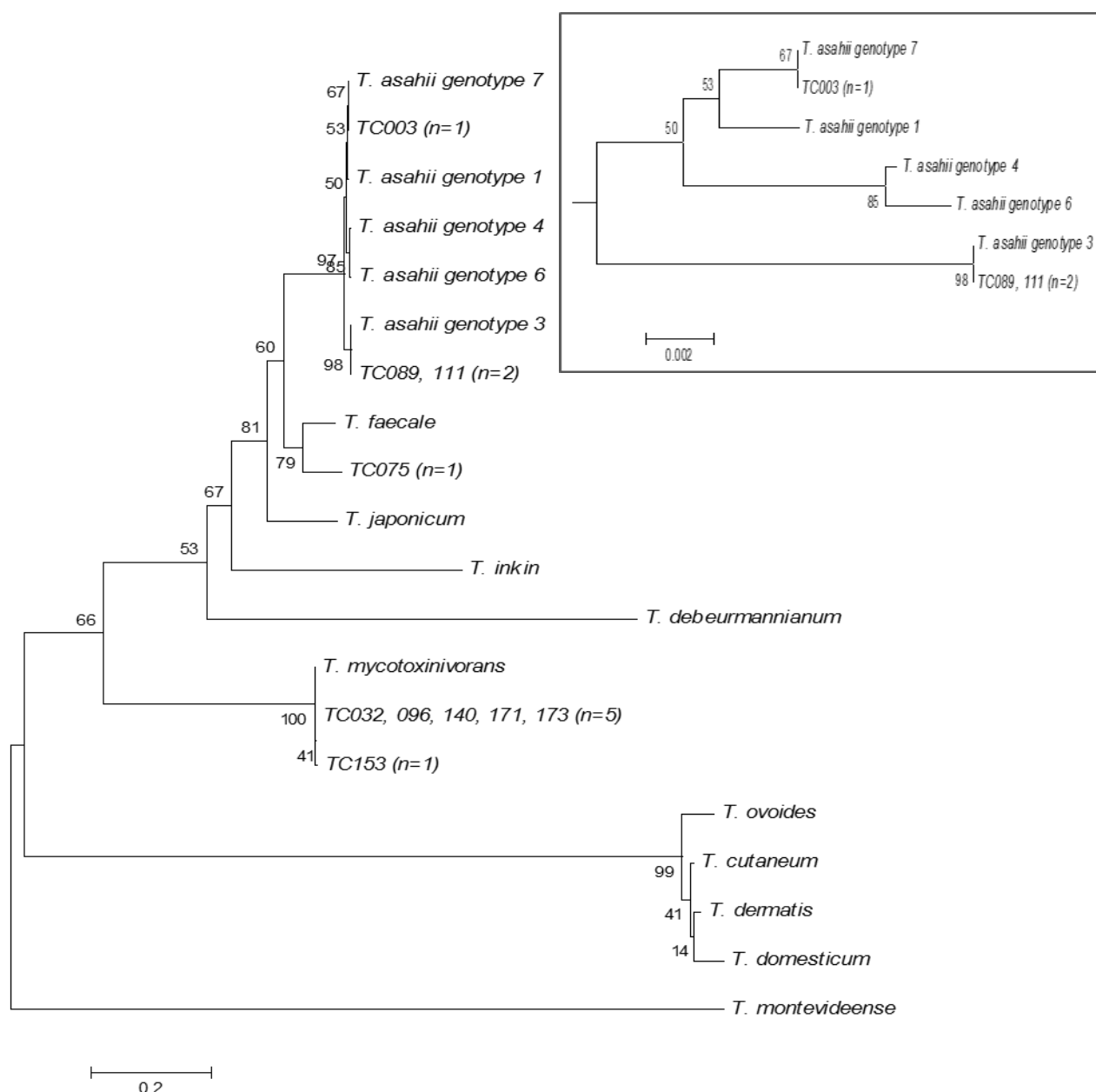


FIGURE 1 Phylogenetic relationships of 610 base positions in the final dataset of IGS region of 10 *Trichosporon* spp. from blood cultures and the 15 corresponding *Trichosporon* from GenBank under the indicated accession numbers: *T. asahii* FJ754250 (genotype 1), *T. asahii* JX111959 (genotype 3), *T. asahii* FJ896484 (genotype 4), *T. asahii* FJ754248 (genotype 6), *T. asahii* JX111955 (genotype 7), *T. faecale* KM488281, *T. japonicum* JF32988, *T. inkin* FJ153608, *T. asteroid* EU938057, *T. mycotoxinivorans* AJ601390, *T.*

debeurmannianum FJ754245, *T. montevidense* JX476302, *T. cutaneum* AF075483, *T. dermatitis* AY143555, *T. ovoides* AF075483 and *T. domesticum* AF075512. Phylogenetic analyses were constructed by neighbor-joining method using MEGA 6 (23). The evolutionary distances were computed using the Kimura two-parameter method. Bootstrap values of 1000 resamplings are indicated at the branch nodes. Bar scale represent evolutionary distance.

Table 2. Species, genotyping and antifungal susceptibilities of 10 *Trichosporon* blood isolates

Strain no.	Molecular ID ^a (IGS1 genotype) ^b	MIC (µg/ml) for indicated antifungal agent								
		AMB	FLC	ITC	PC	VRC	5FC	AND	MIF	CAS
TC003	<i>T. asahii</i> (genotype 7)	0.5	2	0.12	0.25	0.12	16	>8	>8	>8
TC089	<i>T. asahii</i> (genotype 3)	1	2	0.12	0.12	0.06	2	>8	>8	>8
TC111	<i>T. asahii</i> (genotype 3)	1	2	0.12	0.12	0.03	2	>8	>8	>8
TC032	<i>T. mycotoxinivorans</i>	0.5	4	0.25	0.25	0.25	4	>8	>8	>8
TC171	<i>T. mycotoxinivorans</i>	1	2	0.12	0.12	0.06	16	>8	>8	>8
TC173	<i>T. mycotoxinivorans</i>	2	2	0.12	0.12	0.12	8	>8	>8	>8
TC096	<i>T. mycotoxinivorans</i>	2	4	0.25	0.25	0.25	16	>8	>8	>8
TC153	<i>T. mycotoxinivorans</i>	2	4	0.25	0.25	0.12	8	>8	>8	>8
TC140	<i>T. mycotoxinivorans</i>	2	16	0.5	1	1	64	>8	>8	>8
TC075	<i>T. sp</i> unidentified	2	2	0.25	0.25	0.06	32	>8	>8	>8

^a ID, identification by sequence analysis of the ITS region, D1/D2 domain, and IGS1 region

^b IGS genotype number of *T. asahii* isolates followed those in Sugita's research (13)

4. Conclusion and discussion

Trichosporon has been reported to be the most common cause of non-*Candida* yeast infections in patients with hematological malignancies with poor prognosis, despite antifungal therapy (3). Accurate identification of *Trichosporon* species is important, since different species may have different antifungal susceptibilities (4-6). *T. asahii*, *T. faecale* and *T. coremiiforme* exhibited high MICs for amphotericin B, while other species showed lower MICs (21). Moreover, trichosporonosis has been recognized as a breakthrough infection following treatment with candidin derivatives, such as micafungin or caspofungin (24) and also some resistant strains to multiple azole agents have been recovered from patients.

This present study establishes the first analysis of blood culture *Trichosporon* isolates in Thailand, with *T. mycotoxinivorans* involving the dominant isolate. *T. mycotoxinivorans* had been described since 2004 (25). However, *T. mycotoxinivorans* was only identified as a novel respiratory pathogen in patients with cystic fibrosis (26), with one case of a disseminated fatal infection after lung and liver transplantation in a cystic fibrosis patient (27). So this is the most isolates of the species reported in disseminated infections.

Although *T. asahii* has been published as the most common species associated with clinical specimens, it is not true for disseminated infection as in this study. Until now, IGS 1

sequencing has shown nine genotypes identification of *T. asahii*, including genotypes 1–5 from the United States and Japan, genotype 6 from Spain, Argentina, and Brazil, and genotype 7–9 from Turkey (13, 20, 21). From Thai patients, there was only one report of 101 *T. asahii* isolates during 1997-2007. The predominated IGS1 genotype was genotype 1 (44.5%) followed by genotype 3 (34.7%), genotype 7 (17.8%), genotype 2 (2%) and genotype 5 (1%) (20). From our data, 2 of 3 *T. asahii* isolates were genotype 3 and one of genotype 1.

Considering *Trichosporon* fungemia reports, *Trichosporon* species from 76 blood isolates has been recently reviewed from publications during 2005-2014. *T. asahii* was the most commonly isolated *Trichosporon* species (75%) and the other were *T. mucoides* (6.58%), *T. inkin* (5.26%), *T. asteroides* (5.26%), *T. loubieri* (2.63%), and 1 case each (1.31%) of *T. cutaneum*, *T. dermatis*, *T. mycotoxinivorans*, and *T. coremiiforme* (28). The report from Brazil has presented that the most common species of 22 bloodstream *Trichosporon* isolates recovered from patients between 1995 and 2007 was *T. asahii* (15 in 22, 68.18 %) which the majority belonged to genotype 1 (86.7%) follow by genotype 3 and 4 (4). The others isolates were 5 *T. asteroides*, 1 *T. coremiiforme*, and 1 *T. dermatis* (4). In 2009, the report from Taiwan, present 13 of all 14 blood *Trichosporon* isolates were *T. asahii* without genotyping and the other was *T. asteroides* (29).

This study, caspofungin, anidulafungin and migafungin confirmed no *in vitro* activity against all *Trichosporon* species. The general echinocandins should not be used to treat *Trichosporon* infections. The four azoles tested in our study were all active *in vitro*, confirming previous reports particularly voriconazole, posaconazole and itraconazole with low MIC (10, 16, 20). Only one isolate of *T. mycotoxinivorans* exhibited a high fluconazole MIC at 16 µg/ml. However, our results presented low MIC of amphotericin B to *T. asahii* whereas most *T. mycotoxinivorans* isolates had MIC at 2 µg/ml.

In conclusion, *Trichosporon* should be species identified by molecular method for precise identification and drug susceptibility testing. For more information, genotyping for epidemiology had better be investigated in prevention and pathogenesis.

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Study of mitochondrial protein modifications by 4-hydroxynonenal and phenotypic expression in Thai's Leber hereditary optic neuropathy patients

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Abstract

Objective: Leber hereditary optic neuropathy (LHON) is an inherited disease caused by point mutations in the mitochondrial DNA, affecting the ND1, ND4 and ND6 subunits of the respiratory complex I. The disease is uniquely manifested by partial blindness of both eyes. It has been known that underlying pathology of LHON is retinal ganglion cell death which could be resulted from excessive reactive oxygen species (ROS) damage. ROS initiates lipid peroxidation resulting in production of 4-hydroxynonenal (HNE) which can covalently bond with other molecules causing HNE modification. HNE modification has been widely used as the biomarker for oxidative damage. In this study, we quantitated HNE modification in mitochondrial proteins of fibroblasts from LHON patients with the G11778A mutation compared to normal controls. We found that HNE modification in mitochondrial proteins of the patients' fibroblast remained unchanged compared to that in normal controls.

Materials and Methods: Mitochondrial proteins were isolated from cultured skin fibroblasts of LHON patients and normal controls. Western blot analysis was performed using specific antibody to HNE. Level of protein modification by HNE was analyzed.

Results: Level of HNE modification in mitochondrial proteins of LHON patients' fibroblasts remained unchanged compared to that of normal controls.

Conclusion: Our result demonstrated that the G11778A mutation does not affect level of HNE modification in mitochondrial proteins of LHON patients' fibroblasts.

Keywords: Leber hereditary optic neuropathy (LHON), oxidative damage, 4-hydroxynonenal (HNE), mitochondria.

1. Introduction

Leber hereditary optic neuropathy (LHON) is one of the most common mitochondrial genetic disorders. The disease prevalence is approximately 1 in 30,000 – 1 in 50,000 in Europe (1). Clinical manifestation of LHON is painless acute or subacute visual loss in one eye at the central part of the visual field. The onset of visual loss is approximately 15 - 35 years (2). LHON is caused by one of three missense mutations at G3460A (3), G11778A (4), and T14484C (5) in the mitochondrial DNA (mtDNA). These mutations affect the ND1, ND4, and ND6 subunits of the respiratory complex I, which leads to defective function of complex I (6). Defective complex I can cause overproduction of ROS (7, 8), which can ultimately cause oxidative damage to proteins, lipids and nucleic acids. It has been proposed that ROS damage may be one of the events that contribute to the death of retinal ganglion cell (RGC), which is an underlying pathology of LHON (9).

Lipid peroxidation is one of the detrimental events from ROS damage. It is a chain reaction of oxidative damage to unsaturated fatty acid, which is a component of the biological membranes. By-products of lipid peroxidation include malonaldehyde and 4-hydroxynonenal (HNE) (10, 11). HNE covalently binds to any proteins at the side chains of histidine, lysine and cysteine residues to form HNE adducts (12). HNE adducts will cripple protein functions (13). Interestingly, HNE adduct is found to be involved in many diseases such as liver disease (14), Alzheimer's disease, (15) and atherosclerosis (16). HNE is easy to detect and very stable when compared to very short half-life of ROS. Hence, HNE is widely used as a biomarker for oxidative damage (17).

There are several studies indicating that ROS production is increased in LHON patients (7, 8). However, extent of ROS damage in LHON patients has not been thoroughly investigated. In this study, HNE protein modification was used as the biomarker for oxidative damage in mitochondrial proteins from skin fibroblast of LHON patients and normal controls.

2. Materials and methods

2.1 Sample preparation

2.1.1 Sample collection and subject recruitment

The samples used in this study were skin fibroblast taken from skin biopsy of the subjects. The subjects include 3 Thai's LHON patients and 5 normal controls. LHON patients carrying the G11778A mutation were recruited from 3 different families whereas 5 normal controls were recruited from 5 other families. This study was conducted upon an approval of the Ethics Committee of Faculty of Medicine Siriraj Hospital, Mahidol University.

2.1.2 Skin fibroblast culture

Skin fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 ug/mL amphotericin B, 100 U/mL penicillin, 100 ug/mL streptomycin, 2 mM L-glutamine and 50 ug/mL uridine. Frozen skin fibroblasts taken from liquid nitrogen were thawed in 37°C, and suspended into working DMEM, then the cells were centrifuged at 1,000 g for 5 min. Pellet was resuspended with working DMEM, and then transferred to a T25 flask and kept at 37°C, 5% CO₂ atmosphere. When the cultured cells reached nearly 100% confluent, the cells were detached from the flask by 0.25% trypsin for another passage culture. Medium was refreshed every 3-4 days. The cells were used until passage 6 in order to avoid the effect of cell senescence.

2.1.3 Mitochondrial isolation

This method is modified from Frezza *et al.* (18). Approximately $8-10 \times 10^6$ skin fibroblasts were harvested using a cell scraper, then the ice-cold isolation buffer (1 M sucrose, 0.1 M Tris/MOPS, 0.1 EGTA/Tris, pH 7.4) was mixed by using a ratio of 1 mL per 40 mg of the fibroblast cells in order to break the cell membrane and purify mitochondria. Cell suspension was then homogenized with 15 mL glass homogenizer at 1,600 rpm for 10 times. The homogenate was centrifuged at 600g for 10 min at 4°C. Supernatant was collected and centrifuged at 7,000g for 10 min at 4°C. After centrifugation, the pellet was kept and resuspended with 50 μ L isolation buffer, then the suspension was centrifuged again at 7,000g for 10 min at 4°C. Supernatant was discarded. The pellet containing mitochondria was resuspended in the remaining isolation buffer. The suspension was transferred to a 0.6 mL microtube and kept in -80°C until used.

2.2 Western blot analysis

2.2.1 Protein concentration measurement

Concentration of mitochondrial protein was determined by Bradford assay. The assay was performed by mixing 10 μ L of protein sample with 200 μ L of Bradford reagent in microplate, then the solution was incubated and shaken at room temperature for 5 min. Coomassie Brilliant Blue G-250 in the reagent bound to proteins in acidic condition, resulting in the color changed from brown to blue. The color was detected at 595 nm by microplate reader. Protein concentration of the sample was calculated from extrapolation of the standard curve which was done in parallel. This method is modified from Bradford *et al.* (19).

2.2.2 SDS-PAGE electrophoresis

The samples were mixed with Laemmli buffer (20), containing 10% (v/v) β -mercaptoethanol before denatured at 95°C for 5 min. A total amount of 25 μ g protein was loaded onto an SDS-polyacrylamide gel. The gel was run at 80 V at 4°C until finished.

2.2.3 Western transfer and immunoblotting

A polyvinylidene difluoride (PVDF) membrane was used for Western blot transfer using Towbin's buffer (25 mM Tris, 192 mM glycine, and 12% methanol (v/v)). The transfer was under a steady current of 300 mA for 1 hour at 4°C. After the transferring completed, the PVDF membrane was blocked with 5% skimmed milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 and 0.05% polyxyethelene-20) for 1 hour at room temperature. The membranes were incubated with anti-HNE antibody and anti-voltage dependent anion channel (VDAC) antibody as a loading control in 5% skimmed milk PBS-T at 4°C overnight. The membrane was washed 3 times with PBS-T before incubated with horseradish peroxidase (HRP)-conjugated secondary antibody in 5% skimmed milk in PBS-T for 2 hours at room temperature. The Enhanced chemiluminescence plus western blotting detection kit was used to develop the HRP reaction to visualize HNE modification. The antibody complex was detected by using ImageQuant LAS4010 imaging system then the images were analyzed by using Image J software. The experiment was performed from 2-3 independent cultures for statistical analysis.

2.3 Statistical analysis

Statistical t-test was used to analyze the quantification of HNE modifications in mitochondrial proteins of LHON patients and normal controls. P-value < 0.05 was considered statistical significant.

3. Results

HNE modification in mitochondrial proteins from skin fibroblast of LHON patients and normal controls was investigated by Western blot analysis (Figure 1). Quantitative analysis of HNE modification from Western blot was also performed (Figure 2). It was shown that level of HNE modification in mitochondrial proteins from skin fibroblast of LHON patients was not significantly different from that of normal controls.

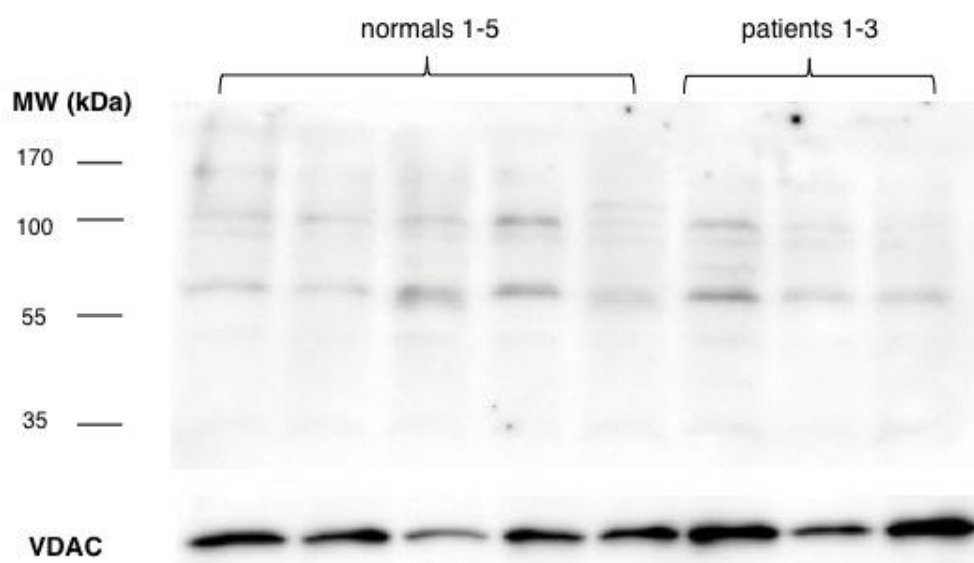


Figure 1. HNE modification of mitochondrial proteins. Mitochondrial protein from fibroblasts of 5 normal controls and 3 LHON patients were dissolved in SDS-PAGE before subjected to Western blots analysis using specific antibody to HNE. VDAC was used as a loading control.

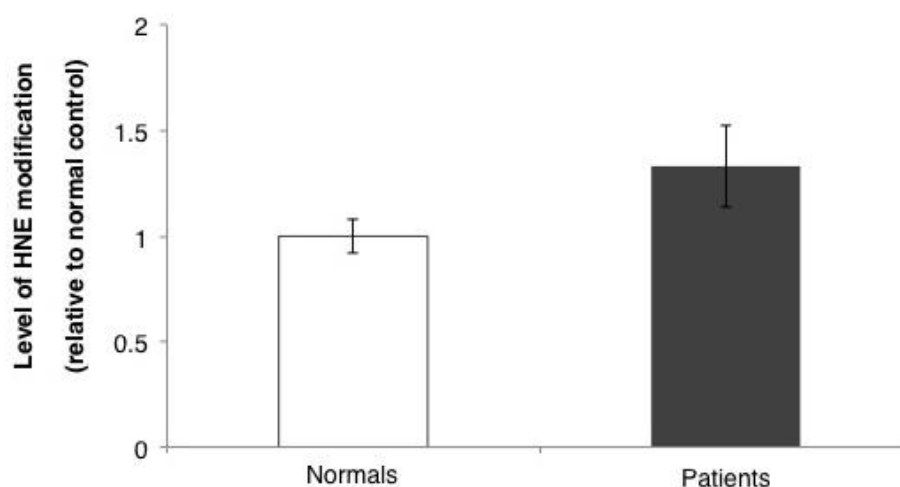


Figure 2. Quantitative analysis of HNE Western blot- Level of HNE modification of mitochondrial protein was quantitated from band intensity in Western blot analysis. The value was expressed in relative to normal controls. The data shown are represented as mean $\pm$ SD.

4. Discussion

Our study aimed to investigate oxidative damage in mitochondrial proteins from skin fibroblasts using HNE as a marker. Surprisingly, we found that level of HNE modification in mitochondrial proteins of LHON patients remained unchanged compared to normal controls. Our result was not consistent with other studies which showed that ROS was increased in LHON patients. For instance, Lin *et al.* showed that the rate of hydrogen peroxide production was significantly higher in mouse's mitochondria harboring T14484C mtDNA mutation (9). A study from Wong *et al.* also showed that the amount of mitochondrial superoxide was increased in cybrid cells containing the G11778A mutation, and even higher in the cell harboring the G3460A mutation (21). Interestingly, the study from Gonzalo *et al.* showed that not only the production of hydrogen peroxide was found increased in cybrid cells containing the T14484C mutation but extent of oxidative damage to both lipid and mtDNA were also significantly higher in comparison to normal cells (22).

However, HNE is a biomarker for oxidative damage. Extent of HNE protein modification is a net result of ROS production versus antioxidant system, which were not investigated in our study. In addition, fibroblast is relatively a quiescent cell in comparison to the RGCs. The G11778A mutation may not exert its effect toward the oxidative damage in mitochondrial proteins in this circumstance.

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Species Identification of *Nocardia* Clinical Isolates by using 16S rRNA, *secA1*, and *gyrB* gene sequencing

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Abstract

Species identification of *Nocardia* is very useful for epidemiological purpose and also studying the correlation between species and disease manifestation or species and antimicrobial resistance. This study was aimed at the evaluation of species identification of *Nocardia* clinical isolates by using DNA sequencing of 16S rRNA, *secA1*, and *gyrB* genes and investigation of some phenotypic tests for common species differentiation. Two sets of DNA sequencing including those of 16S rDNA and *secA* genes, were carried out to identify species of 80 *Nocardia* clinical isolates collected at Siriraj Hospital. *Nocardia* species obtained from those two target genes analyses were compared. The isolates yielding discordant results were subjected to *gyrB* gene sequencing. The same species generated from sequences of two genes were accepted. Top seven common species obtained were used for the investigation of phenotypic tests for species differentiation. Among 80 *Nocardia* clinical isolates studied, 58 (72%) and 74 (92.5%) isolates could be identified into species level by partial 16S rDNA and *secA1* gene sequence analyses, respectively. However, the combination analyses of these two genes yielded concordant results for 73 (91%) isolates. For the remaining seven isolates, sequence analyses of all three genes (16S rDNA, *secA* and *gyrB*) could identify five isolates into species level. Top seven common species obtained were *N. cyriacigeorgica* (24%), *N. farcinica* (21%), *N. otitidiscaviarum* (12%), *N. asiatica* (11%), *N. brasiliensis* (5%), *N. beijingensis* (4%), and *N. nova* (4%). Some phenotypic tests could be used for supplementary identification of those 7 common species such as the ability to grow at 50°C for differentiating *N. farcinica* from other 6 species. Of all three genes, *secA1* was shown to be the most appropriate target for species identification of *Nocardia*. Additional *gyrB* gene sequencing maybe useful for some isolates that cannot be identified by using *secA1* gene alone.

Keyword(s): *Nocardia*, species identification, *secA1*, 16S rDNA, *gyrB*

1. Introduction

The genus *Nocardia* is one of the members of aerobic actinomycetes. It can be found in the environment such as soil, dust, and water. This organism is the most commonly isolated and clinically significant aerobic actinomycetes (1, 2). Infection caused by *Nocardia* is called nocardiosis, ranging from cutaneous infection in normal host (3) to pulmonary and disseminated infection in immunocompromised host (4). The appropriate methods for diagnosis of nocardiosis are isolation and identification of the organisms from clinical samples. Microscopic examinations by Gram stain and modified acid-fast stain are important for presumptive diagnosis. Lysozyme resistance and aerial hyphae production are the

important characteristics to confirm and differentiate *Nocardia* from other aerobic actinomycetes (5-8).

Species identification of the genus *Nocardia* can be performed by either phenotypic or genetic method. For phenotypic identification, the battery of biochemical tests is required. Although, the basic biochemical tests can provide preliminary identifications of frequently isolated of *Nocardia* species from clinical specimens, the disadvantages of phenotypic species identification are time-consuming (3-4 weeks), difficult to perform the tests and interpret the results. Moreover, the number of *Nocardia* species has been increasing, the use of a limited number of phenotypic tests to determine exact species seems to be impossible. Therefore, the molecular identification such as gene sequencing is recommended (2, 5, 8, 10-12).

Gene sequencing provides rapid and usually reliable identifications for most *Nocardia* isolates. Sequence analysis of 16S rRNA gene has been used for the species identification of these organisms since this gene contains highly genus conserved region and also variable regions for different species (10, 13-16). However, there is a limited amount of interspecies heterogeneity of 16S rRNA gene among some closely related species, for examples, *N. kruczakiae*/ *N. veterana*, and *N. brevicatena*/ *N. paucivorans* (12, 17).

In 2006, Conville (18) reported the sequence of *secA1* gene coding for an essential secretory protein SecA. The *secA1* gene showed more interspecies heterogeneity than 16S rDNA sequence. In 2010, the phylogenetic analysis of 56 type species of *Nocardia* was studied by using the sequence of 1,200 nucleotides of the beta subunit of DNA topoisomerase (*gyrB*) gene. The results showed that the diversity of this gene was greater than those of 16S rDNA and *secA1* gene (19, 20). The aims of this study were to differentiate species of *Nocardia* clinical isolates using DNA sequencing of 16S rRNA, *secA1*, and *gyrB* genes and investigate specific phenotypic characteristics of common species.

2. Materials and Methods

2.1 Bacterial isolates

The study was conducted on a total of 80 isolates of *Nocardia* species isolated from clinical specimens sent to Microbiology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital. All isolates were obtained from different patients from 2008 to 2012 and identified to the genus *Nocardia* by phenotypic methods (modified acid-fast stain, the presence of aerial hyphae, and lysozyme resistance). From 80 isolates, 48 isolated from male and 32 from female patients. Specimen types from which *Nocardia* spp. were isolated were shown in table 1.

Table 1. Specimen types from which *Nocardia* spp. were isolated

Specimen	No. of isolates
Respiratory (sputum, BAL)	49
Soft tissue	16
Blood	5
Brian abscess	4
Eye (cornea)	1
Lymph node	2
Pericardium	1
Synovial fluid	1
Pleural fluid	1
Total	80

2.2 Species identification of *Nocardia* by DNA sequencing of 16S rRNA, *secA*, and *gyrB* genes

The organism was cultured on blood agar incubated at 35±2°C in ambient air for 3 to 7 days. Few colonies of the organisms were added to 100 µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The cell suspension was heated at 95°C for 35 minutes and centrifuged at 13,500 rpm for 5 minutes. The supernatant was collected and kept at -20°C until used. This crude DNA was used as template in the PCR. Five microliters of DNA sample were added to 50 µl of PCR mixture consisting of 1x PCR buffer, 2 mM of MgCl₂ (Invitrogen, Brazil), and 200 µM of dNTPs (Fermentas, USA). Sixty nanograms each of forward and reverse primers specific to 16S rDNA (25), 100 ng each of *secA* primers (18), and , 30 ng each of *gyrB* primers (19) were used for each PCR mixture of 16S rDNA, *secA*, and *gyrB* genes, respectively. DMSO (7.5%) was added into the PCR mixture for *gyrB* gene. *Taq* DNA polymerase (Invitrogen, Brazil) was added into the PCR mixture at concentrations of 1U, 0.5U, and 0.3U for 16S rDNA, *secA*, and *gyrB* genes, respectively. After an initial denaturation step (5 min at 94°C), 30 cycles of amplification were performed. For 16S rDNA and *secA* genes the amplification reactions were performed under the following conditions: DNA denaturation at 94°C for 30 s, primer annealing at 54°C for 30 s and extension at 72 °C for 45 s with an increment 3 s per cycle, and final extension at 72 °C for 7 min. For *gyrB* gene, denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min with an increment 3 s per cycle, and final extension at 72 °C for 10 min. Purification of PCR products were performed by using GenepHlow™ Gel/PCR Kit (Geneaid, Taiwan) according to the manufacturer's instructions. The partial sequences of each gene (16S rDNA, *secA*, and *gyrB* genes) were compared to type or reference sequences in the GenBank database and identified by using BLAST. The sequence lengths were adjusted to match the length of the shortest sequence and align using ClustalX algorithm and analyzed by using GeneDoc software. Criteria for species identification by 16S rDNA sequencing were as follows: the sequence shared more than or equal to 99.0% similarity to type/ reference strain and differed more than 0.5% to next closest species (24). For species identification by *secA1* and *gyrB* gene sequencing, the best-match to type/ reference strain sequences were used to assign the species (36).

2.3 Biochemical tests

The organism was cultured on blood agar and incubated at $35\pm 2^{\circ}\text{C}$ in ambient air for 3 to 7 days. Heavy suspension of the organism was, then, prepared in a small volume of broth culture. The organism clumps were broken up by using vortex mixer, and the suspension was allowed to sit for 15 minutes for the large clumps to settle to the bottom of the tube. Several drops of the supernatant were added to 3 ml of sterile distilled water for adjusting the turbidity of the suspension to approximately 0.5 McFarland standard for being used in biochemical tests. For all tests, the results were observed every day until 4 weeks.

2.3.1 Citrate utilization test

A few drops of the organism suspension were added to citrate medium and incubated at $35\pm 2^{\circ}\text{C}$. A positive reaction was observed by the blue color on the slant.

2.3.2 Urease test

A few drops of the organism suspension were added to urea medium and incubated at $35\pm 2^{\circ}\text{C}$. A positive reaction was observed by the red color on the slant.

2.3.3 Esculin hydrolysis test

A few drops of the organism suspension were added to esculin medium and incubated at $35\pm 2^{\circ}\text{C}$. A positive reaction was observed by the brown-black color in the medium.

2.3.4 Test for ability to growth at 45°C and 50°C

A few colonies of the organism were subcultured on BA and incubated at 35°C , 45°C , and 50°C for 3 days.

3. Results

3.1 Species identification of *Nocardia*

3.1.1 Species identification of *Nocardia* by using partial 16S rDNA sequencing

The 16S rDNA sequences of all *Nocardia* isolates studied were determined. These sequences were, then, adjusted to match the length of the shortest one, 613 base pairs (bp) before analyses. By using partial 16S rDNA gene sequencing, 58 of 80 isolates (72.5%) were identified into species including *N. cyriacigeorgica*, *N. farcinica*, *N. otitidiscaviarum*, *N. brasiliensis*, *N. nova*, *N. pseudobrasiliensis*, *N. asteroides*, *N. takedensis*, *N. blacklockiae*, and *N. thailandica*, respectively.

3.1.2 Species identification of *Nocardia* by using partial *secA1* gene sequencing

The *secA1* gene sequences of all *Nocardia* isolates studied were determined. These sequences were, then, adjusted to match the length of the shortest one, 429 base pairs (bp) before analyses. By using partial *secA1* gene sequencing, 74 of 80 isolates (92.5%) were identified into species including *N. cyriacigeorgica*, *N. farcinica*, *N. otitidiscaviarum*, *N. asiatica*, *N. brasiliensis*, *N. beijingensis*, *N. nova*, *N. pseudobrasiliensis*, *N. asteroides*, *N. takedensis*, *N. blacklockiae*, *N. terpenica*, *N. neocaledoniensis*, *N. thailandica*, *N. testacea*, and *N. mexicana*, respectively.

3.1.3 Species identification of *Nocardia* by using partial 16S rDNA, and *secA1* gene sequencing

Based on the highest similarity scores of 16S rDNA and *secA1* gene sequences to type/reference strains, the species identification results for 73 out of 80 (91.3%) *Nocardia* clinical isolates were concordant and identified into 15 species (Table 2). Those species included *N. cyriacigeorgica* (19 isolates), *N. farcinica* (17 isolates), *N. otitidiscaviarum* (10 isolates), *N. asiatica* (9 isolates), *N. brasiliensis* (4 isolates), *N. beijingensis* (3 isolates), *N. nova* (3 isolates) and one isolate each of *N. pseudobrasiliensis*, *N. asteroides*, *N. takedensis*, *N. blacklockiae*, *N. terpenica*, *N. neocaledoniensis*, *N. thailandica* and *N. testacea*.

Table 2. Concordant species identification by 16S rDNA and *secA1* gene sequencing

Group	No. of isolates	Highest similarity species obtained by the analysis of	
		16S rDNA	<i>secA1</i> gene
1	19	<i>N. cyriacigeorgica</i>	<i>N. cyriacigeorgica</i>
2	17	<i>N. farcinica</i>	<i>N. farcinica</i>
3	10	<i>N. otitidiscaviarum</i>	<i>N. otitidiscaviarum</i>
4	9	<i>N. abscessus</i> / <i>N. asiatica</i>	<i>N. asiatica</i>
5	4	<i>N. brasiliensis</i>	<i>N. brasiliensis</i>
6	3	<i>N. beijingensis</i>	<i>N. beijingensis</i>
7	3	<i>N. nova</i>	<i>N. nova</i>
8	1	<i>N. pseudobrasiliensis</i>	<i>N. pseudobrasiliensis</i>
9	1	<i>N. asteroides</i>	<i>N. asteroides</i>
10	1	<i>N. takedensis</i>	<i>N. takedensis</i>
11	1	<i>N. blacklockiae</i>	<i>N. blacklockiae</i>
12	1	<i>N. terpenica</i>	<i>N. terpenica</i>
13	1	<i>N. neocaledoniensis</i>	<i>N. neocaledoniensis</i>
14	1	<i>N. thailandica</i>	<i>N. thailandica</i>
15	1	<i>N. testacea</i> / <i>N. sienata</i>	<i>N. testacea</i>
Total	73		

Discordant species identification by 16S rDNA and *secA1* gene sequencing were observed for 7 isolates (Table 3). All these seven isolates were, therefore, further identified by *gyrB* gene sequencing.

Table 3. Discordant species identification by 16S rDNA and *secA1* gene sequencing

Group	No. of isolates	Highest similarity species obtained by the analysis of	
		16S rDNA	<i>secA1</i> gene
16	5	<i>N. niwae</i>	<i>N. arthritis</i>
17	1	<i>N. miyunensis</i>	<i>N. mexicana</i>
18	1	<i>N. pseudobrasiliensis</i>	<i>N. otitidiscaviarum</i>

3.1.4 Species identification of *Nocardia* by using partial *gyrB* gene sequencing

The partial *gyrB* gene sequences of 7 *Nocardia* isolates exhibiting discordant results of species identification determined by 16S rDNA and *secA1* gene sequencing were obtained and then, adjusted to match the length of the shortest one, 609 base pairs (bp) before analyses. Based on the highest similarities of *gyrB* gene sequences, 7 *Nocardia* clinical isolates were identified as followed.

For 5 isolates (group 16), the *gyrB* gene sequences ranged from 97.8-100% similarities to one another and the highest similarity was from 96.0-96.5% to that of *N. araoensis* DSM 44729. The next similar scores were 95.1-95.7% to that of *N. beijingensis* JCM 10666, and 94.0-94.7% to *N. niwae* DSM 45340.

One isolate (group 17), the highest similarity of its *gyrB* gene sequence was 95.1% to *N. transvalensis* ATCC 6865. The next similar score was 94.1% to that of *N. mexicana* DSM 44952.

One isolate (group 18), the highest similarity score was 92.9% to that of *N. lijiangensis* DSM 44933, the next similar score was 92.6% to that of *N. concava* JCM 12351, *N. xishanensis* JCM 12160, and *N. uniformis* JCM 3224.

Nine isolates of group 4 and 1 isolate of group 15 were also identified by partial *gyrB* gene sequencing to confirm the results obtained by 16S rDNA and *secA1* gene sequencing. Concordant results were obtained for all 10 isolates. For isolates of group 4 the highest similarity score was 97% to that of *N. asiatica* DSM 44668. For one isolate of group 15 the highest similarity score was 94% to that of *N. testacea* JCM 12235.

3.1.5 Species identification of *Nocardia* by using partial 16S rDNA, *secA1*, and *gyrB* gene sequencing

Based on the analyses of 16S rDNA, *secA1*, and *gyrB* gene sequences, the species identification results of 17 *Nocardia* clinical isolates (groups 4, 15, 16, 17, and 18) were as follows: 9 isolates (group 4) were identified as *N. asiatica* and 1 isolate (group 15) was identified as *N. testacea* whereas the remaining 7 isolates could not be identified (Table 4).

Table 4. Result of species identification of *Nocardia* by using partial 16S rDNA, *secA1*, and *gyrB* gene sequencing

Group (no. of isolates)	Top three similarity species obtained by the analysis of each gene (% of similarity score)		
	16S rDNA	<i>secA1</i> gene	<i>gyrB</i> gene
16 (5)	<i>N. niwae</i> (99.3-99.6%) <i>N. beijingensis/ N. pneumoniae</i> (99.0-99.3%) <i>N. araoensis</i> (98.6-99.1%) <i>N. arthritidis</i> (98.2-98.6%)	<i>N. arthritidis</i> (96.0-97.4%)	<i>N. araoensis</i> (96.0-96.5%)
17 (1)	<i>N. miyunensis</i> (98.0%)	<i>N. mexicana</i> (98.8%)	<i>N. transvalensis</i> (95.1%)
18 (1)	<i>N. pseudobrasiliensis</i> (97.8%)	<i>N. otitidiscaviarum</i> (91.6%)	<i>N. lijaingensis</i> (92.9%)

3.2 Phenotypic characteristics used for differentiation of seven most common species of *Nocardia* in this study

The selected phenotypic characteristics for differentiation of seven most common species of *Nocardia* in this study were evaluated and the results were demonstrated in Table 5. For the ability to grow at 45°C and 50°C observed at day 3 of incubation, only *N. farcinica* (15 of 17 isolates) was able to grow at both temperatures. The remaining two isolates that did not grow at 50°C but grew at 45°C were repeated for this test and the results were still the same. The reactions of urease and esculin could be read in 3 days of incubations. For *N. cyriacigeorgica*, 18 of 19 isolates could grow at 45°C but were unable to grow at 50°C and all 19 isolates showed negative urease test. The reaction of citrate for all tested isolates was negative until 28 days of incubation.

Table 5. Phenotypic characteristics of 7 most common species of *Nocardia* in this study

Species (no. of isolates)	Biochemical characteristics (no. of isolates)				
	Growth at 45°C	Growth at 50°C	Urease production	Citrate utilization	Esculin hydrolysis
<i>N. cyriacigeorgica</i> (19)	G (18)	NG (19)	- (19)	- (19)	- (12)
<i>N. farcinica</i> (17)	G (17)	G (15)	+ (16)	- (17)	+ (15)
<i>N. otitidiscaviarum</i> (10)	G (7)	NG (10)	+ (10)	- (10)	+ (6)
<i>N. asiatica</i> (9)	NG (9)	NG (9)	+ (5)	- (9)	- (6)
<i>N. brasiliensis</i> (4)	NG (4)	NG (4)	+ (4)	- (4)	+ (4)
<i>N. beijingensis</i> (3)	G (2)	NG (3)	+ (3)	- (3)	- (3)
<i>N. nova</i> (3)	NG (3)	NG (3)	+ (2)	- (3)	- (2)

4. Discussion

Gene sequencing provides rapid and usually reliable identification for most *Nocardia* isolates when compared with conventional phenotypic identification method. It has become a powerful tool for the identification of *Nocardia* species. Partial 16S rDNA and *secA1* gene sequencing are mostly used for *Nocardia* species identification (5). In this study, only 58 out of 80 isolates (72.5%) of *Nocardia* clinical isolates could be identified into species level by using only partial 16S rDNA sequencing compared with 74 of 80 isolates (92.5%) that could be identified by *secA1* sequencing alone.

By using both 16S rDNA and *secA1* gene sequence analyses, the results revealed identifiable species (73/80, 91.3% of isolates) as followed: *N. cyriacigeorgica* (24%), *N. farcinica* (21%), *N. otitidiscaviarum* (12%), *N. asiatica* (11%), *N. brasiliensis* (5%), *N. beijingensis* (4%), and *N. nova* (4%). The remaining isolates were *N. pseudobrasiliensis*, *N. asteroides*, *N. takedensis*, *N. blacklockiae*, *N. neocaledoniensis*, *N. terpenica*, *N. thailandica*, and *N. testacea* (one isolate each).

Seven isolates remained unidentifiable by using both 16S rDNA and *secA1* gene sequence analyses, analysis of *gyrB* gene sequence of these isolates still could not resolve the problem. This result may be due to the analysis of too short sequences (609 bp). However, five isolates in group 16 that were probably *N. araoensis* as determined by *gyrB* gene sequence analysis might be correct identification since this species was also present among those assigned by 16S rDNA and *secA1* gene analyses. In conclusion, the results obtained from this study indicate that *secA1* gene sequencing is appropriate for species identification of *Nocardia* clinical isolates and >90% of isolates should be identified. If some isolates cannot be identified into species by *secA1* sequence analysis, the *gyrB* gene sequencing maybe helpful.

The most prevalent species in this study (*N. cyriacigeorgica*, *N. farcinica* and *N. otitidiscaviarum*) were also shown to be prevalent in the studies from Ontario, Canada, 2010 (21), and the Quebec, Canada, 2011 (22). For the studies from Thailand, 2005 (26) and Japan, 2004 (27), they reported similar results of the most prevalent species but only *N. cyriacigeorgica* and *N. farcinica* were the most prevalent species in Japan. In contrast, *N. brasiliensis*, *N. otitidiscaviarum*, and *N. abscessus* were found to be prevalent in Greece, 2009 (23), whereas *N. nova*, *N. brasiliensis*, *N. farcinica*, and *N. cyriacigeorgica* were found to be prevalent in the United States, 2010 (24). The difference of prevalence of *Nocardia* species in each region support the hypothesis that the prevalence may be related to geographical area or climate (1). The time of study and sample collection method may also affect the prevalence of *Nocardia*.

Attempt to use some phenotypic characteristics such as colony morphology, urease test, esculin hydrolysis and ability to grow at 45°C and 50°C for species differentiation among seven most common *Nocardia* species in this study was carried out. The results obtained strengthen the difficulty of phenotypic methods for species identification of *Nocardia*. By using the ability to grow at 50°C, *N. farcinica* could be able to be differentiated from other species.

Since the most common species of *Nocardia* were different in each region, the study on species prevalence should be done for the selection of appropriate methods for species identification. As the results obtained from this study, phenotypic characteristics could be used for presumptive species identification and molecular method, *secA1* gene sequencing, was shown to be useful for definitive identification of *Nocardia* species.

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Germline Mutation Study of *STK11* Gene in Thai patients with Peutz-Jeghers syndrome

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Abstract

Objectives: To identify *STK11* mutations in Thai patients with Peutz-Jeghers syndrome (PJS) and apply semi-quantitative multiplex polymerase chain reaction (PCR) coupled with denaturing high-performance liquid chromatography (DHPLC) for detection of large deletions/duplications in *STK11*.

Materials and Methods: Mutational analysis of *STK11* gene was performed in three unrelated PJS probands. Genomic DNA was PCR-amplified, screened for point mutations by DHPLC, followed by direct sequencing. Mutation-negative samples were further examined using semi-quantitative multiplex PCR/DHPLC to evaluate *STK11* copy-number variations in all exons.

Results: Mutation screening revealed no pathogenic *STK11* mutations in all PJS patients. Interestingly, however, there was a heterozygously single-nucleotide variant (SNV) with conflicting interpretations of pathogenicity, c.1062C>G, p.Phe354Leu (rs59912467) in two patients. No copy-number changes were found in all exons except exon 1 of *STK11*. The peak-height fluctuation of exon 1 may be resulted from copy-number variations which presented in both PJS patients and normal controls. These inconclusive results may require a more appropriate method to analyze large deletions/duplications in *STK11* gene.

Conclusion: This study demonstrated that no well-defined pathogenic *STK11* mutations were identified in all PJS patients, but one heterozygous missense variant, p.Phe354Leu with uncertain pathogenicity was found in two patients. The results may reflect the limitation of our test to detect *STK11* mutations in deep-intronic regions and promoters elsewhere. Other possibilities may include the rare genetic-mosaicism event as well as the misdiagnosis according to an overlapping signs and symptoms of PJS with other similar diseases. Further studies are required for the better understanding in molecular basis of PJS.

Keywords: Peutz-Jeghers syndrome, *STK11*, Germline mutations, DHPLC, multiplex PCR

1. Introduction

Peutz-Jeghers syndrome (PJS) is an autosomal dominant inherited disorder with high penetrance and variable expression. This syndrome is generally characterized by hamartomatous gastrointestinal polyps, mucocutaneous melanin pigmentation, and a higher risk of developing various cancers including gastrointestinal and extra-gastrointestinal origin. The common complications including intussusception, intestinal obstruction, abdominal pain, and bleeding increase the frequency of recurrent laparotomy and bowel resection leading to life-threatening conditions and mortality in PJS. The estimated incidence of PJS ranges from 1 in 50,000⁽¹⁾ to 1 in 200,000 liveborns⁽²⁾.

Germline mutations in the serine-threonine kinase 11/liver kinase B1 (*LKB1/STK11*) tumor suppressor gene have been identified as a major cause of PJS⁽³⁾. This gene is located at 19p13.3 and consists of nine coding exons (Figure 1). It plays an important role in regulation of cell metabolism, cell polarity, cell cycle arrest, and cell apoptosis. Defective epithelial polarity and abnormal apoptosis are proposed to be a cause of polyp formation⁽⁴⁾. In addition, the haploinsufficiency of *STK11* with second hit by allelic loss and additional mutations is suggested for malignant transformation.

To date, more than 370 mutations were identified in *STK11*⁽⁵⁾ and most of them affect the catalytic kinase domain. Two-thirds of *STK11* mutations are point mutations including single base substitution, small insertions or deletions, and splicing mutations^(6, 7). One-third are large genomic deletions including exonic and whole gene deletions⁽⁸⁻¹¹⁾. The combination of direct sequencing and multiplex ligation-dependent probe amplification (MLPA) is mostly recommended for *STK11* with the detection rate between 80% and 94%^(9, 11). However, MLPA is expensive, laborious, and time-consuming. We therefore developed a simpler and faster alternative technique for analysis of copy number alterations in *STK11* by semi-quantitative multiplex polymerase chain reaction (multiplex PCR)/denaturing high-performance liquid chromatography (DHPLC). This method has a low cost, but provide a high accuracy⁽¹²⁾. Additionally, the designed primers were worthy used for both single and multiplex PCR reactions.

This study aims to investigate *STK11* mutations in Thai patients with PJS. The mutation analysis was initially performed with mutation screening in the entire coding sequence of *STK11* using partially denaturing mode of DHPLC. Then, the direct sequencing was carried out in the positive screening PCR amplicons. The semi-quantitative multiplex PCR/DHPLC under non-denaturing condition was applied to evaluate *STK11* copy number variations (CNVs).

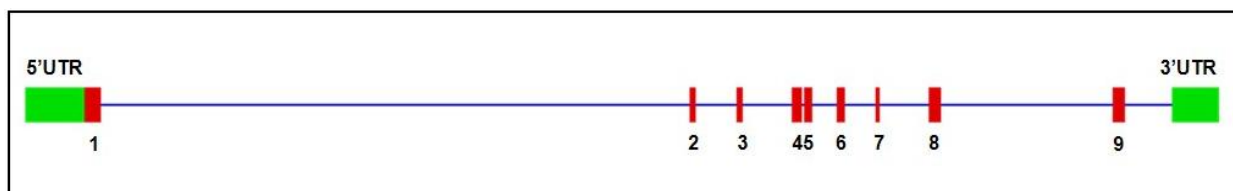


Figure 1 The structure of *STK11* gene

The entire nine coding exons are displayed with the red boxes and the introns are indicated by the blue line. The 5' and 3' untranslated region (UTR) are shown by the green boxes. This Figure is modified from RefGene (<http://refgene.com/gene/6794>).

2. Materials and Methods

2.1 Subjects and Genomic DNA extraction

The study was approved by Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University (COA no. Si711/2015). Peripheral blood samples were collected from three unrelated PJS patients at Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. Genomic DNA was extracted from lymphocytes by the standard salting out method.

2.2 DNA analysis for *STK11* mutations

Point mutations in *STK11* were initially screened using partially denaturing mode of denaturing high-performance liquid chromatography (DHPLC). This method was performed using Transgenomic WAVE[®] DNA Fragment Analysis System equipped with DNASep[®] HT cartridge (Transgenomic, USA). All the nine coding exons of *STK11* were amplified by polymerase chain reaction (PCR) using *PhusionTM DNA polymerase* (Finnzymes, Finland) and Veriti[®] Thermal Cycler (Thermo Fisher Scientific Inc., USA). The PCR products were denatured and reannealed by gradual decreasing temperature resulting in the heteroduplex formation before being injected into DHPLC column. The samples showing abnormal peak pattern were further analyzed by re-amplification and subsequent direct sequencing. PCR products were purified using ExoSAP-IT[®] and sequenced using BigDye[®] Terminator 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and Applied Biosystems[®] 3130xl Genetic Analyzer (Thermo Fisher Scientific, USA).

Semi-quantitative multiplex PCR/DHPLC was performed to determine *STK11* copy number alterations in the samples showing negative DHPLC-screening results. Two sets of multiplex PCR were established. The first set included exon 7-9 and exon 1 of *STK11*. The second set contained exon 2-6 of *STK11*. *IGF1* gene was used as an internal control for autosomal chromosome, while *DMD* or *ACOT9* represented an internal control for X-chromosome. The multiplex PCRs were carried out using quantitated DNA concentrations and limited PCR cycle number at the exponential phase (25 cycles in this study). Non-denaturing mode of DHPLC using a temperature of 50°C was applied for the separation of multiple DNA fragments from multiplex PCR reactions depending on their sizes. A normal control sample of the same gender with patients was always injected in the same run to compare the results. The DHPLC peak heights reflect the quantity of PCR products in each amplicon.

3. Results

3.1 Clinical findings

A total of three patients presented with gastrointestinal polyposis and mucocutaneous pigmentation. Two patients had definitely diagnosis of PJS and one had suspected PJS. Only one case is familial and had undergone enterotomy due to intussusception. None of the patients has developed cancer. The clinical characteristics of three PJS patients are shown in Table 1.

Table 1. Clinical characteristics of PJS patients

Patient	Sex	Age of diagnosis (Current age)	Family history	Clinical presentation				Cancer
				Pigmentation (Location)	Polyps (Histology)	Location of polyps	Intussusception	
PJS01	F	22 (34)	No	Yes (Lp-Fg)	Yes (Adenoma)	St-Sb-Lb	No	No
PJS02	M	13 (32)	Yes	Yes (Lp-Bm-Fg)	Yes (Hamartoma)	St-Sb-Lb	Yes	No
PJS03	M	S (44)	NA	Yes (Lp)	Yes (Normal histology)	St-Lb	No	No

PJS: Peutz-Jeghers syndrome, **F:** female, **M:** male, **NA:** not available, **S:** suspected PJS, **Lp:** lips, **Bm:** buccal mucosa, **Fg:** finger, **St:** stomach, **Sb:** small bowel, **Lb:** large bowel

3.2 *STK11* mutations and variations

No *STK11* mutations were detected in all the three PJS patients. The DHPLC screening revealed aberrant DHPLC elution profiles in PJS01 and PJS02. The fragments showing abnormal peak were identified by direct sequencing. There was no detectable aberrant DHPLC peak in PJS03. In order to confirm the DHPLC results, some exons in all patients were randomly selected to be sequenced. Accordingly, only homozygous variants were found in PJS03 and selected fragments in other two patients. The results of DHPLC in conjunction with DNA sequencing are shown in Table 2. Most of the identified *STK11* variants were common amongst all ethnic population studied in the 1000 Genomes Project. Data from KHV (Kinh in Ho Chi Minh City, Vietnam) and CDX (Chinese Dai in Xishuangbanna, China) which represent Southeast Asian populations were chosen to be references for allele frequencies for Thai population⁽¹³⁾ as shown in Table 3.

Table 2. DHPLC elution profiles and sequence electropherograms showing sequence variations

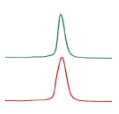
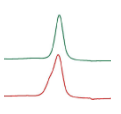
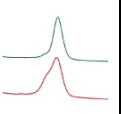
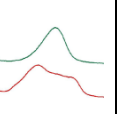
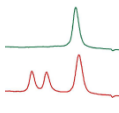
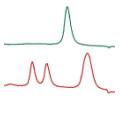
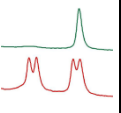
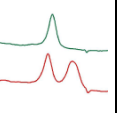
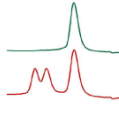
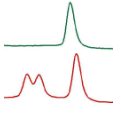
<i>STK11</i> PCR fragments	Aberrant DHPLC elution profile As compared to the normal control (upper)				DNA sequence tracing	Genotype (Location)	PJS Patients identified
	Temp. 1	Temp. 2	Temp. 3	Temp. 4			
Exon 2					TGG GACC G CGGGGC C c.374+24G>T	c.374+24G>T (Intron 2)	PJS02
	59.9°C	61°C	64°C	66°C			
Exon 3					GCAAGGT G GGTGCA G c.375-99 G>T	c.375-99 G>T (Intron 2)	PJS01
	64.7°C	65°C	66°C	68°C			
Exon 8			-	-	ACCTCTT C GACATCG c.1062C>G	c.1062C>G, p.Phe354Leu or p.F354L (Exon 8)	PJS01, PJS02
	64.3°C	64.7°C					

Table 3. *STK11* variations identified in PJS patients

Patient	Variation	Location	dbSNP ID ^a	KHV		CDX	
				Major ^b	Minor ^b	Major ^b	Minor ^b
PJS01	Homo c.290+36G>T	Intron 1	rs3764640	G=0.3030	T=0.6970	G=0.3441	T=0.6559
	Homo c.290+78C>T	Intron 1	rs3764641	C=0.5909	T=0.4091	C=0.6022	T=0.3978
	Homo c.375-49G>A	Intron 2	rs34928889	A=0.8182	G=0.1818	A=0.8172	G=0.1828
	Het c.375-99G>T	Intron 2	rs78995829	G=0.8535	T=0.1465	G=0.8548	T=0.1452
	Homo c.464+46_464+47dupGGGGGCC ^c	Intron 3	rs58579265	-	-	-	-
	Het c.1062C>G, p.F354L^d	Exon 8	rs59912467	C=0.9192	G=0.0808	C=0.9677	G=0.0323
PJS02	Homo c.290+36G>T	Intron 1	rs3764640	G=0.3030	T=0.6970	G=0.3441	T=0.6559
	Homo c.290+78C>T	Intron 1	rs3764641	C=0.5909	T=0.4091	C=0.6022	T=0.3978
	Het c.374+24G>T	Intron 2	rs2075604	G=0.5404	T=0.4596	G=0.5591	T=0.4409
	Homo c.375-49G>A	Intron 2	rs34928889	A=0.8182	G=0.1818	A=0.8172	G=0.1828
	Homo c.464+46_464+47dupGGGGGCC ^c	Intron 3	rs58579265	-	-	-	-
	Het c.1062C>G, p.F354L^d	Exon 8	rs59912467	C=0.9192	G=0.0808	C=0.9677	G=0.0323
PJS03	Homo c.290+36G>T	Intron 1	rs3764640	G=0.3030	T=0.6970	G=0.3441	T=0.6559
	Homo c.375-49G>A	Intron 2	rs34928889	A=0.8182	G=0.1818	A=0.8172	G=0.1828
	Homo c.920+7G>C	Intron 7	rs2075607	G=0.7475	C=0.2525	G=0.7097	C=0.2903

KHV: Kinh in Ho Chi Minh City (Vietnam), **CDX:** Chinese Dai in Xishuangbanna (China),

Major: major allele frequency, **Minor:** minor allele frequency, **Homo:** homozygous, **Het:** heterozygous

^aData from db SNP database ⁽¹⁴⁾, ^bData from 1000 Genomes Browser ⁽¹⁵⁾

^cThis variant was found in 7 alleles out of 10 normal chromosomes (70%).

^dLikely benign variant and conflicting interpretations of pathogenicity (Data from ClinVar database ⁽¹⁶⁾)

It was noticed that one rare missense variant (c.1062C>G, p.F354L) was found heterozygously in two out of the three PJS patients (PJS01 and PJS02). Pathogenicity of this variant remains elusive according to ClinVar database and global minor allele frequency (MAF) as shown in Table 4. However, p.F354L was found more often in Asian population (around 1-8%), while being absent in several ethnic groups. Computational prediction tools for pathogenicity have predicted this rare variant (p.F354L) to be either a disease causing (by MutationTaster ⁽¹⁹⁾) or a neutral variant (by PolyPhen-2 ⁽¹⁷⁾ and Meta-SNP ⁽¹⁸⁾).

Table 4. Allele frequencies of rs59912467 (c.1062C>G, p.F354L) from 1000 Genomes⁽¹⁵⁾

Global MAF/MinorAlleleCount: G = 0.0112/56 (1000 Genomes), 0.0058/583 (ExAC), 0.0038/49 (GO-ESP)						
Populations		Major allele	Minor allele	Populations		Major allele
ACB	African Caribbeans in Barbados	C = 0.9948	G = 0.0052	IBS	Iberian Population in Spain	C = 1.0000
ASW	Americans of African Ancestry in SW USA	C = 1.0000	G = 0.0000	ITU	Indian Telugu from the UK	C = 0.9902
BEB	Bengali from Bangladesh	C = 0.9884	G = 0.0116	JPT	Japanese in Tokyo, Japan	C = 0.9663
CDX	Chinese Dai in Xishuangbanna, China	C = 0.9677	G = 0.0323	KHV	Kinh in Ho Chi Minh City, Vietnam	C = 0.9192
CEU	Utah Residents (CEPH) with North and Western European Ancestry	C = 1.0000	G = 0.0000	LWK	Luhya in Webuye, Kenya	C = 1.0000
CHB	Han Chinese in Beijing, China	C = 0.9515	G = 0.0485	MSL	Mende in Sierra Leone	C = 1.0000
CHS	Southern Han Chinese	C = 0.9476	G = 0.0524	MXL	Mexican Ancestry from Los Angeles USA	C = 1.0000
CLM	Colombians from Medellin, Colombia	C = 1.0000	G = 0.0000	PEL	Peruvians from Lima, Peru	C = 1.0000
ESN	Esan in Nigeria	C = 1.0000	G = 0.0000	PJL	Punjabi from Lahore, Pakistan	C = 1.0000
FIN	Finnish in Finland	C = 1.0000	G = 0.0000	PUR	Puerto Ricans from Puerto Rico	C = 1.0000
GBR	British in England and Scotland	C = 0.9945	G = 0.0055	STU	Sri Lankan Tamil from the UK	C = 1.0000
GIH	Gujarati Indian from Houston, Texas	C = 1.0000	G = 0.0000	TSI	Toscan in Italia	C = 1.0000
GWD	Gambian in Western Divisions in the Gambia	C = 1.0000	G = 0.0000	YRI	Yoruba in Ibadan, Nigeria	C = 1.0000
						G = 0.0000

In semi-quantitative multiplex PCR/DHPLC analysis, comparison of DHPLC peak heights of the amplicons from PJS patients with those of the relevant normal controls demonstrated all concordance except the peak height of exon 1 in *STK11* which revealed the fluctuation in not only the three patients but also all normal controls analyzed. Their peak heights could be classified into three levels of 1, 2, and 3 copies of exon 1 of *STK11*. This inconstancy was confirmed by several repeated tests, indicating the true variations of copy number in this exon of *STK11* gene of Thai population which impeded the test for deletion/duplication analysis in exon 1. DHPLC profiles from the two multiplex PCR sets of all patients are shown in Figure 2.

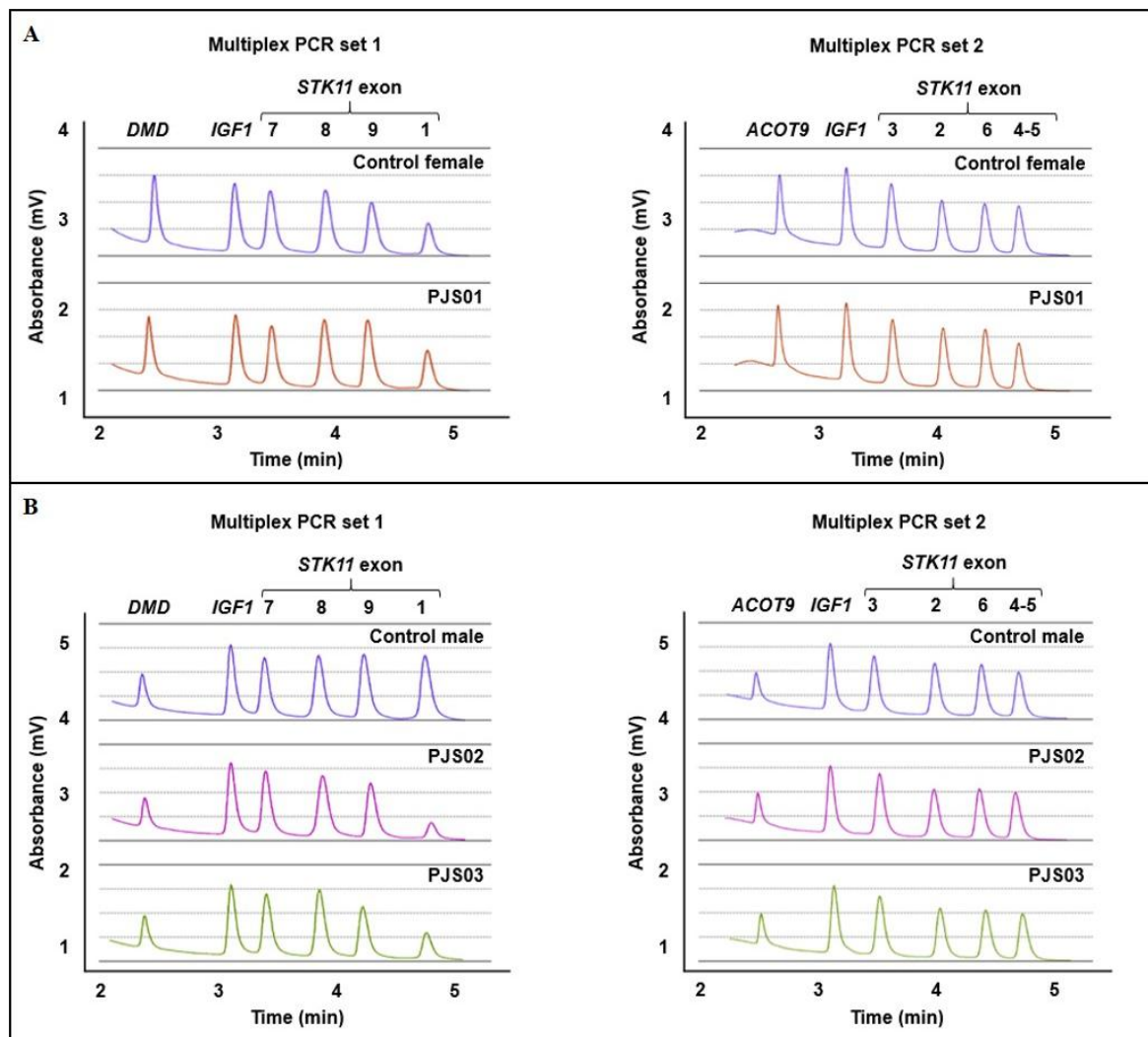


Figure 2 DHPLC profiles of two multiplex PCR sets used for *STK11* deletion analysis. Genes and exons under study are indicated on the top. The chromatograms in blue represent the normal control. A) The chromatograms of PJS01 are shown compared to a control female. B) From top to bottom, the chromatograms of a control male, PJS02, and PJS03 are shown respectively.

4. Conclusion and discussion

This study demonstrated that *STK11* mutations were not found in all patients with PJS. Some harbored mutations may be undetectable because of the limitations of our methods. For example, the designed primers in this study do not cover deep intronic regions, promoters, and untranslated regions. It is possible that another chromosomal locus or genetic heterogeneity involved in PJS, but until now no second PJS locus has been affirmed and this hypothesis remains controversial. However, Alhopuro et al. (2008) identified a heterozygous pathogenic variant in *MYH11* gene in one out of 25 patients who had PJS but negative *STK11* mutations⁽²⁰⁾. In addition, genetic mosaicism may be found in 1-2% of PJS cases⁽²¹⁾. PJS02 has obvious clinical manifestations and family history of PJS suggested hidden mutations or locus heterogeneity as mentioned above. While the phenocopy may be considered in PJS01 and PJS03 because they have no significant family history and their pathology of polyps are not hamartoma. There are many syndromes which show overlapping signs and symptoms

with PJS such as juvenile polyposis syndrome (JPS), familial adenomatous polyposis (FAP), Cowden's syndrome, and Bannayan-Ruvalcaba-Riley syndrome⁽²²⁾.

DHPLC is a major versatile method applied in this study. One certain limitation of partially denaturing mode of DHPLC is an incapability to detect homozygous changes. Nevertheless, PJS is inherited in an autosomal dominant trait, therefore, this weakness can be ignored. Using DHPLC for mutation screening provided the rapid and cost-effective method for PJS.

In semi-quantitative multiplex PCR/DHPLC analysis, the peak-height fluctuation of exon 1 in *STK11* presenting in both PJS patients and normal controls may be resulted from the copy number variations. The Database of Genomic Variants (DGV) shown that CNV cover some parts or the whole region of *STK11*⁽²³⁾. Moreover, a highly Alu repetitive sequence in *STK11* gene accounted for more than 1 element every 1 kb may be associated with Alu-mediated homologous recombination resulting in genomic rearrangements and copy number alterations⁽²⁴⁾. Further investigations, such as multiplex ligation-dependent probe amplification (MLPA), long-range PCR, quantitative PCR, DNA microarray, and next-generation sequencing (NGS) are required to gain a better understanding regarding the copy number variations in *STK11*.

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Virulence Genes in *Enterococcus faecalis* and *Enterococcus faecium* Clinical Isolates from Siriraj Hospital

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Abstract

Objective: The aim of this study was to determine the detection rates of virulence genes (*asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl*) in vancomycin-susceptible *Enterococcus faecalis* (VSEI) and *E. faecium* (VSEf) and vancomycin-resistant *E. faecium* (VREf) isolated from Siriraj Hospital.

Material and Method: Two multiplex PCR assays were developed for detecting 4 (*cylA*, *efaA*, *esp*, *gelE*) and 2 virulence genes (*asa1*, *hyl*), respectively, in enterococci. A total of 264 isolates of VSEI (n = 100, 50 each from blood and urine), VSEf (n = 100, 50 each from blood and urine) and VREf (n = 64) were included. Chi-square test was used for the statistical analysis.

Results: The detection rates of *asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl* in VSEI and VSEf were 73% and 0%, 53% and 0%, 100% and 100%, 50% and 81%, 91% and 0%, 0% and 9%, respectively. Significant difference was found between the detection rates of all genes in EI and Ef, except *efaA*. Detection rates of *asa1* and *cylA* genes in blood and urine isolates were significantly different. Virulence genes in VSEf and VREf were similar except for *hyl* that was detected in VSEf only. Up to 58% of EI had ≥ 4 genes whereas 92% of VSEf and 100% of VREf were found to carry ≤ 2 genes.

Conclusion: EI was generally found to contain more virulence genes than Ef. However, *esp* gene was more prevalent in the latter. Detection rates of virulence genes in VSEf and VREf were similar except for *hyl* gene.

Keyword(s): *Enterococcus faecalis*/ *Enterococcus faecium*/ multiplex PCR/ virulence genes

1. Introduction

Enterococci consist of at least 54 species and a few species have been usually associated with human infection ⁽¹⁾. Two common species of human pathogens are *E. faecalis* (El) and *E. faecium* (Ef). These organisms are important causative agents caused nosocomial infections. Another problem is the resistance to antimicrobial agents of enterococci especially those that are resistant to vancomycin (vancomycin-resistant enterococci, VRE). Moreover, Ef is usually more resistant than El. From VRE epidemiological study worldwide, it has been revealed that VREf is more prevalent than VREl ⁽²⁻⁵⁾.

Several enterococcal virulence factors have been used to enhance the ability to cause disease, for examples, aggregation substances, cytolysin, enterococcal surface protein, endocarditis antigen A, gelatinase and hyaluronidase ⁽⁶⁾. Determinants and genes encoding these virulence factors have been characterized and reported ⁽⁷⁻⁹⁾. The studies of virulence genes and factors of enterococci especially El and Ef could provide the information for understanding the enterococcal pathogenic mechanism. Prevalence of each enterococcal virulence gene has been reported from many countries in Europe ⁽¹⁰⁾, America ⁽¹¹⁾ and also in Asia ⁽¹²⁻¹⁴⁾. However, there is no/little information on isolates in Thailand. The aim of this study was to determine the detection rates of virulence genes (*asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl*) in El and Ef (VSEf and VREf) isolated from blood and urine specimens in Siriraj Hospital.

2. Materials and Methods

2.1 Clinical isolates

This study was conducted on a total of 264 enterococcal isolates from patients at Microbiology laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital. They were composed of 100 isolates of El (50 each from blood and urine), 100 isolates of VSEf (50 each from blood and urine) and 64 isolates of VREf (37 from urine). All of blood isolates were collected during January 2012 to May 2013, and urine isolates, collected during May 2012 to June 2013. All of VRE isolates were collected during 2004 to 2013. All isolates were non-repetitive and derived from individual patients.

2.2 Control strains

E. faecalis ATCC 29212 carrying *asa1*, *cylA*, *efaA*_{El} and *gelE* gene, strain U30 (clinical isolates in this study) carrying *efaA*_{Ef}, *esp* and *hyl*, strain H14 (clinical isolates in this study) carrying *asa1*, *cylA*, *efaA*_{El}, *esp* and *gelE*, were used as positive controls in multiplex PCR assays.

2.3 Virulence genes detection

2.3.1 DNA extraction

Crude DNA extraction from enterococcal isolates was carried out as described by Leelaporn *et al.* ⁽¹⁵⁾. Few colonies of the organisms were added to 100 µl of Tris-EDTA (TE) buffer. The cell suspension was incubated at 95°C for 40 minutes. After incubation, the cell suspension was centrifuged at 13,500 rpm for 5 minutes. The supernatant, then, was carefully transferred as much as possible with a pipette into a new sterile microcentrifuge tube. The DNA was used immediately or kept no longer than 3 days.

2.3.2 Oligonucleotide primers

A total of 16 nucleotide primers were used in this study (Table 1). Amplification product sizes generated by each pair of primers were also presented. Primers PS3 and PS4 targeting 16S rDNA were used for internal control of multiplex polymerase chain reaction (mPCR).

2.3.3 DNA amplification

Two mPCR assays (assay 1 and assay 2) were used for the detection of virulence genes (*asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hly*). PCR mix without template was used as a negative control.

2.3.3.1 Multiplex PCR assay 1 for detection of *cylA*, *efaA*, *esp* and *gelE* genes

Two tests were set up for mPCR assay 1, viz., assay 1a and assay 1b, because of the difference in *efaA* sequences of EI and Ef. The mPCR assay 1a was used for detection of *cylA*, *efaA_{EI}*, *esp* and *gelE* genes in EI whereas the other was used for the detection of those genes (*cylA*, *efaA_{Ef}*, *esp* and *gelE*) in Ef. Internal control primers were also included in each assay. In mPCR assay 1a, two microlitres of DNA templates were added into 18 µl of mPCR mixture consisting of 1X PCR buffer (Invitrogen), 4 mM MgCl₂ (Invitrogen), 0.2 mM of deoxynucleoside triphosphates (dNTPs: dATP, dCTP, dGTP and dTTP) (Invitrogen), 40 ng each of primers specific for *esp*, 25 ng each of primers specific for *efaA_{EI}*, 15 ng each of primers specific for *cylA* and *gelE*, 15 ng each of primers specific for 16S rDNA, 0.4 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Invitrogen) and 0.4% bovine serum albumin (BSA). PCR condition was used one cycle of initial denaturation at 94°C for 5 minutes and 30 cycles of PCR amplifications as follows: denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 45 seconds with increment of 3 seconds per cycle. The reaction was achieved with a final extension at 72°C for 7 minutes.

For mPCR assay 1b, two microlitres of DNA templates were added into 18 µl of mPCR mixture consisting of 1X PCR buffer, 2 mM MgCl₂, 0.2 mM of dNTPs, 30 ng each of primers specific for *esp*, 20 ng each of primers specific for *efaA_{Ef}*, *cylA* and *gelE*, 15 ng each of primers specific for 16S rDNA, 0.4 units of *Taq* DNA polymerase and 0.4% BSA. PCR condition used was one cycle of initial denaturation at 94°C for 5 minutes and 30 cycles of PCR amplifications as follows: denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, extension at 72°C for 45 seconds with increment of 3 seconds per cycle. The reaction was achieved with a final extension at 72°C for 7 minutes.

2.3.3.2 Multiplex PCR assay 2 for detection of *asa1* and *hly* genes

For both of *E. faecalis* and *E. faecium* isolates, *asa1* and *hly* were detected by the same set of primers specific to those genes (see section 2.3.2). Internal control primers were also included. In assay 2, two microlitres of DNA templates were added into 18 µl of mPCR mixture consisting of 1X PCR buffer, 2 mM MgCl₂, 0.2 mM of dNTPs, 15 ng each of primers specific for *hly*, 10 ng each of primers specific for *asa1* and 16S rDNA, 0.2 units of *Taq* DNA polymerase and 0.4% BSA. PCR condition used was the same as mPCR assay 1a.

2.3.4 Detection of PCR products by gel electrophoresis

Amplification products of assay 1a and assay 2 were determined on 1.5% (wt/v) agarose gel (SeaKem® LE agarose) performed in 1X TAE buffer, whereas the products of assay 1b were examined on 2% agarose gel. The gels, then, were stained with GelRed™ (Biotium) for 15 minutes. After destaining with distilled water for 15 minutes, DNA fragments were visualized under ultraviolet transilluminator (Bio-Rad).

2.4 Statistical analysis

The numbers of each virulence genes were compared between EI/Ef, VSEf/VREf and blood/urine isolates by Chi-square test. A p -value < 0.05 was considered statistically significant.

3. Results

To determine the detection rates of virulence genes in clinical enterococcal isolates, multiplex PCR assay 1 detecting *cylA*, *efaA*, *esp* and *gelE*, and multiplex PCR assay 2 detecting *asa1* and *hyl*, were developed and used. Gel electrophoreses of virulence genes products from mPCR assays 1 and 2 performed on representative isolates were shown in Figure 1 and 2. The detection rates of *asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl* in VSEI and VSEf were 73% and 0%, 53% and 0%, 100% and 100%, 50% and 81%, 91% and 0%, 0% and 9%, respectively (Table 2). The *asa1*, *cylA* and *gelE* genes were detected only in VSEI whereas the *hyl* gene was detected only in VSEf. Both species were found to carry *esp* gene but the detection rates in VSEf was higher than that in VSEI. Interestingly, *efaA* gene was detected in all isolates tested. Significant difference was found between the detection rates in VSEI and VSEf, of all genes except *efaA* (p -value < 0.0001 , only *hyl* showing p -value = 0.002). The detection rates of *asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl* of all VREf isolates are shown in Table 2. For VREf, 100% and 79.7% of isolates contained *efaA* and *esp*, respectively. No significant difference was found between the detection rates of *esp* gene in VSEf and VREf (p -value = 0.966). Interestingly, the *hyl* gene was detected in VSEf only.

Beside the comparison between two species, the detection rates of virulence genes between different sources were also compared. The detection rates of *asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl* in blood and urine were 29% and 44%, 18% and 35%, 100% and 100%, 64% and 67%, 43% and 48%, 6% and 3%, respectively (Table 3). Detection rates of *asa1*, *cylA*, *esp* and *gelE* genes in urine isolates were higher than those in blood isolates whereas *hyl* gene was more prevalent in blood isolates. Between blood and urine isolates, significant difference was found only in the detection rates of *asa1* and *cylA* genes (p -values = 0.027 and 0.006, respectively).

Of all six virulence genes studied, the maximum number of genes detected in each isolate in this work was five as shown in Table 4. Patterns of virulence genes are demonstrated in Tables 4 and 5. All isolates that contained five virulence genes were VSEI. Isolate without any virulence gene was not found. Up to 58% of VSEI isolates had ≥ 4 genes whereas 92% of VSEf and 100% of VREf were found to carry ≤ 2 genes.

4. Discussion

In this study, virulence genes including *asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl* were detected by using two multiplex PCR assays. The results obtained were similar to those of several previous studies^(10, 16, 17). Those investigators reported that EI was generally found to contain more virulence genes than Ef. However, detection rates of some genes were different in each location. Detection rates of *efaA* and *esp* genes in VSEf and VREf in this study were similar. However, the *asa1*, *cylA* and *gelE* were not found in Ef whereas *hyl* gene was detected in VSEf only. In contrast, a study from Brazil⁽¹⁸⁾ reported that the high detection rates of *asa1* and *cylA* genes were found in VREf. Moreover, Vankerckhoven et al.⁽¹⁹⁾ reported that the detection rates of *esp* gene in VSEf was less than VREf whereas the detection rates of *hyl* gene was similar. Furthermore, one study⁽²⁰⁾ found that EI carried *hyl* gene in the rate similar to that of Ef. However, those data received from a study on enterococcal isolates from food stuffs.

The comparison of virulence gene detection rates between blood and urine isolates was carried out. It was found that enterococcal urine isolates contained more virulence genes than blood isolates except *hyl* gene that was more prevalent in blood isolates. These results were concordant with that from a study of Creti et al.⁽²¹⁾. However, that study did not detect the *hyl* gene. Interestingly, all isolates tested carried *efaA* gene. The impact of this gene in enterococcal infection should be further studied.

Data obtained from this study may contribute to better understanding in enterococcal pathogenesis. However, further study in this field is needed.

Acknowledgement

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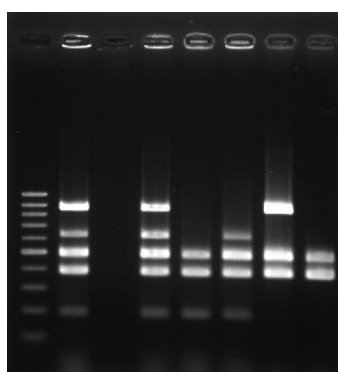


Figure 1 Agarose gel (2%) electrophoresis of amplification products of virulence genes (*cylA*, *efaA*_{EI/Ef}, *esp* and *gelE*) and 16S rDNA sequence detected in vancomycin-susceptible *Enterococcus faecalis* (VSEI) and VS *E. faecium* (VSEf) obtained from multiplex PCR assay 1

Lane M	100 bp DNA ladder
Lane 1	Positive control for detection of <i>cylA</i> , <i>efaA</i> _{EI/Ef} , <i>esp</i> and <i>gelE</i>
Lane 2	Negative control (without DNA template)
Lane 3-5	Representative VSEI isolates
Lane 6-7	Representative VSEf isolates

The lengths of molecular size markers and amplified products in basepairs (bp) are indicated on the left and right, respectively.

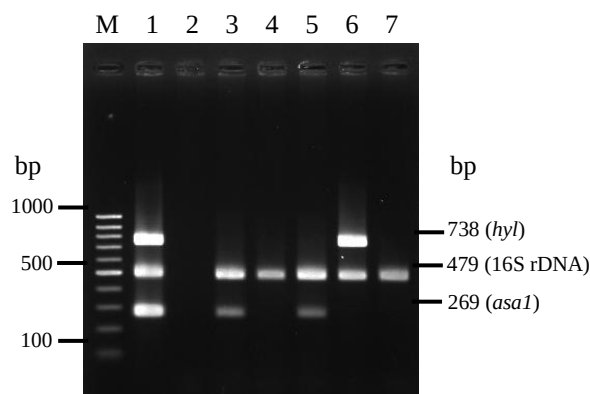


Figure 2 Agarose gel (2%) electrophoresis of amplification products of virulence genes (*asa1* and *hyl*) and 16S rDNA sequence detected in vancomycin-susceptible *Enterococcus faecalis* (VSEI) and VS *E. faecium* (VSEf) obtained from multiplex PCR assay 2

Lane M	100 bp DNA ladder
Lane 1	Positive control for detection of <i>asa1</i> and <i>hyl</i>
Lane 2	Negative control (without DNA template)
Lane 3-5	Representative VSEI isolates
Lane 6-7	Representative VSEf isolates

The lengths of molecular size markers and amplified products in basepairs (bp) are indicated on the left and right, respectively.

Table 1 Oligonucleotide primers used in this study

Target	Primer name	Product size (bp)	Reference
<i>gelE</i>	<i>gelE_F</i> <i>gelE_R</i>	188	This study
<i>asa1</i>	<i>asa1_F</i> <i>asa1_R</i>	269	This study
<i>efaA_{EI}</i>	<i>efaA_{EI}_F</i> <i>efaA_{EI}_R</i>	375	This study
<i>efaA_{Ef}</i>	<i>efaA_{Ef}_F</i> <i>efaA_{Ef}_R</i>	379	This study
16S rDNA	PS3 PS4	479	(22)
<i>cylA</i>	<i>cylA_F</i> <i>cylA_R</i>	615	This study
<i>hyl</i>	<i>hyl_F</i> <i>hyl_R</i>	738	This study
<i>esp</i>	<i>esp_{EI/Ef}_F</i> <i>esp_{EI/Ef}_R</i>	859	This study

Table 2 Virulence genes in vancomycin-susceptible *Enterococcus faecalis* (VSEI) and VS *E. faecium* (VSEf) isolated from blood (B) and urine (U) and vancomycin-resistant Ef (VREf) isolated from non-urine (N) and U

Organism	Source	Number (%) of isolates containing following genes					
		<i>asa1</i>	<i>cylA</i>	<i>efaA</i>	<i>esp</i>	<i>gelE</i>	<i>hyl</i>
VSEI	B+U	73 (73)*	53 (53)*	100 (100)	50 (50)*	91 (91)*	0*
	B	29 (58)	18 (36)	50 (100)	23 (46)	43 (86)	0
	U	44 (88)	35 (70)	50 (100)	27 (54)	48 (96)	0
VSEf	B+U	0*	0*	100 (100)	81 (81)*	0*	9 (9)*
	B	0	0	50 (100)	41 (82)	0	6 (12)
	U	0	0	50 (100)	40 (80)	0	3 (6)
VREf	N+U	0	0	64 (100)	51 (79.7)	0	0
	N	0	0	27 (100)	21 (77.8)	0	0
	U	0	0	37 (100)	30 (81.1)	0	0

*Significant difference between the detection rates in *E. faecalis* and *E. faecium*, of all genes except *efaA*_{El/Ef} at p -value < 0.0001, only *hyl* showing p -value = 0.002

Table 3 Virulence genes in blood and urine isolates of vancomycin-susceptible enterococci

Source	Organism	Number (%) of isolates containing each of following genes					
		<i>asa1</i>	<i>cylA</i>	<i>efaA</i>	<i>esp</i>	<i>gelE</i>	<i>hyl</i>
Blood	El+Ef	29 (29)*	18 (18)*	100 (100)	64 (64)	43 (43)	6 (6)
	El	29 (58)	18 (36)	50 (100)	23 (46)	43 (86)	0
	Ef	0	0	50 (100)	41 (82)	0	6 (12)
Urine	El+Ef	44 (44)*	35 (35)*	100 (100)	67 (67)	48 (48)	3 (3)
	El	44 (88)	35 (70)	50 (100)	27 (54)	48 (96)	0
	Ef	0	0	50 (100)	40 (80)	0	3 (6)

El, *E. faecalis*; Ef, *E. faecium*

*Significant difference the detection rates of *asa1* and *cylA* genes between blood and urine isolates at p -value < 0.05

Table 4 Virulence gene patterns of vancomycin-susceptible isolates of *Enterococcus faecalis* (Ef) and *E. faecium* (Ef) isolated from blood (B) and urine (U)

No. of genes detected	Virulence gene patterns						Number (%) of Ef isolates			Number (%) of Ef isolates		
	<i>asa1</i>	<i>cylA</i>	<i>efaA</i>	<i>esp</i>	<i>gelE</i>	<i>hyl</i>	B+U	B	U	B+U	B	U
5	+	+	+	+	+	-	35 (35)	12 (24)	23 (46)	0	0	0
4	+	+	+	-	+	-	11 (11)	1 (2)	10 (20)	0	0	0
	+	+	+	+	-	-	7 (7)	5 (10)	2 (4)	0	0	0
	+	-	+	+	+	-	5 (5)	3 (6)	2 (4)	0	0	0
	Total						23 (23)	9 (18)	14 (28)	0	0	0
3	+	-	+	-	+	-	15 (15)	8 (16)	7 (14)	0	0	0
	-	-	+	+	+	-	2 (2)	2 (4)	0	0	0	0
	-	-	+	+	-	+	0	0	0	8 (8)	5 (10)	3 (6)
	Total						17 (17)	10 (20)	7 (14)	8 (8)	5 (10)	3 (6)
2	-	-	+	-	+	-	23 (23)	17 (34)	6 (12)	0	0	0
	-	-	+	+	-	-	1 (1)	1 (2)	0	73 (73)	36 (72)	37 (74)
	-	-	+	-	-	+	0	0	0	1 (1)	1 (2)	0
	Total						24 (24)	18 (36)	6 (12)	74 (74)	37 (74)	37 (74)
1	-	-	+	-	-	-	1 (1)	1 (2)	0	18 (18)	8 (16)	10 (20)
Total							100 (100)	50 (100)	50 (100)	100 (100)	50 (100)	50 (100)

Table 5 Virulence gene patterns of vancomycin-susceptible *Enterococcus faecium* (VSEf) and vancomycin-resistant Ef (VREf) isolates

No. of genes detected	Virulence gene patterns						Number (%) of VSEf isolates	Number (%) of VREf isolates
	<i>asa1</i>	<i>cylA</i>	<i>efaA</i>	<i>esp</i>	<i>gelE</i>	<i>hyl</i>		
3	-	-	+	+	-	+	8 (8)	0
2	-	-	+	+	-	-	73 (73)	51 (79.7)
	-	-	+	-	-	+	1 (1)	0
	Total						74 (74)	51 (79.7)
1	-	-	+	-	-	-	18 (18)	13 (20.3)
Total							100 (100)	64 (100)

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FACTORS AFFECTING HEART RATE VARIABILITY IN HEALTHY THAIS

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Abstract

Objective(s): To identify factors influencing Heart Rate Variability (HRV) in healthy Thais.

Material(s) and Method(s): Healthy Thai participants, 44 men and 73 women, 21-72 years old, were screened by thorough history, physical examination and blood profile. Using a finometer, 5-min short-term HRV parameters (time domain: SDNN, SDSD, RMSD, pNN50; frequency domain: Total power, LF, LFnu, HF, HFnu, LF/HF) were obtained noninvasively during spontaneous breathing.

Results: Age was significantly inversely correlated with HRV, except for LFnu, HFnu, and LF/HF. Significant differences in HRV among age groups, were demonstrated for all HRV parameters. Men and women were significantly different only in SDNN and LF (higher in men). Stepwise multiple regressions showed that age and resting heart rate were negatively correlated with all parameters, except LFnu, and LF/HF, whereas gender was not associated with any HRV parameters. In addition, BMI associated positively with LFnu and negatively with HFnu, while systolic blood pressure associated positively with LFnu. Finally, LF/HF only weakly correlated with white blood cell count and triglyceride. No effects of regular caffeine, alcohol, exercise, menstrual cycle and assessment time could be demonstrated.

Conclusion: In health, age and resting heart rate are independent factors which negatively influencing most HRV parameters, except LFnu and LF/HF. BMI associates with LFnu and HFnu. However, gender was not an independent factor of any HRV parameter. These results will be the basis for determining age-dependent HRV reference values.

Keyword(s): Autonomic function, age, gender, resting heart rate, finometer

1. Introduction

Heart rate variability (HRV) is the oscillation of the interval between consecutive heart beats (RR interval) produced by the autonomic influence at the sinoatrial node (SA node) of the heart.^(1, 2) This variability can be analyzed by time domain, frequency domain or non-linear methods.⁽³⁻⁶⁾ Time domain analysis makes use of statistical methods, such as standard deviation, or geometric indices to quantify the variation. Frequency domain analysis extracts at least three frequency components from the oscillation in RR intervals: very low, low and high frequency components (VLF, LF and HF). HF component has been shown to

represent vagal activity.^(7, 8) However, the origin of LF component is less clear, with at least three postulates: a) it may represent both sympathetic and parasympathetic outflows,^(9, 10) b) it may reflect only the sympathetic activity^(3, 4, 11) and c) LF may be related to the ability to modulate cardiac autonomic outflows by the baroreflex.^(7, 8) Due to its simplicity, accuracy, and noninvasive application, HRV analysis has become quantitative marker of autonomic activity.⁽¹²⁻¹⁴⁾ Furthermore, decreased HRV correlate well with disease diagnosis and prognosis, such as diabetic neuropathy,^(15, 16) multiple sclerosis,^(17, 18) beta-thalassemia,^(19, 20) Brugada syndrome,^(21, 22) and several cardiovascular diseases.^(23, 24)

HRV values can depend on external and internal environments, as well as the subjects' conditions. External environment affecting HRV values includes temperature, the laboratory protocol and equipment used.⁽³⁾ Internal environment refers to age, gender and race. HRV in healthy population is strongly affected by age,^(12, 14, 25-31) and may be influenced by race.^(2, 25, 31, 32) On the other hand, it is still unclear whether gender disparity exist for HRV. Although most reports found gender differences,^(12-14, 25, 26, 29, 33) a few studies did not^(28, 30, 34). In addition, HRV of healthy human is affected by conditions, such as stress or exercise.^(3, 14)

A few studies in Thailand reported HRV values, comparing between controls and patients.^(20, 21) We aimed to identify factors influencing resting HRV values in healthy Thais, including age, gender, resting hemodynamic parameters, BMI, and blood profile. This information is needed as a basis for further compilation of reference values to be used clinically.

2. Materials and Methods

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University (574/2555).

2.1 Participants

Participants were recruited by advertising on posters and through social networks. Elderly subjects were recruited from several elders' associations in Bangkok. All participants signed written informed consent.

Inclusion criteria Participants must be healthy 20-80 year-old Thais, with normal body mass index (BMI; 18.5-22.9 kg/m²)^(35, 36) and a normal blood profile obtained from a standardized laboratory (mostly Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital) within six months of assessment time, consisting of complete blood count, fasting blood glucose (FBG), blood urea nitrogen (BUN), creatinine, albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (BILI), total cholesterol (CHOL), triglyceride (TG), high-density lipoprotein cholesterol (HDL), and calculated high-density lipoprotein (cal-LDL). All blood values, except lipid profile, must fall within the reference values. Normal lipid profile was defined as normal triglyceride (< 200 mg/dl) and total cholesterol (< 200 mg/dl); but if total cholesterol was $\geq$ 200 mg/dl, the blood profile would still be accepted as normal if cal-LDL < 160 mg/dl and HDL > 50 mg/dl (women) or > 40 mg/dl (men).⁽³⁷⁾

Exclusion criteria Participants with the following conditions were excluded: diabetes mellitus; cardiovascular diseases; abnormal ECG; hypertension (systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg, as taken by sphygmomanometry); pulmonary diseases, including a diagnosis of obstructive sleep apnea;

psychiatric diseases; taking drugs affecting the autonomic nervous system, e.g., β -adrenergic blockers, antihypertensive medication and contraceptives⁽³⁸⁾ (but statins and baby aspirins were allowed); smokers, or ex-smokers who had stopped smoking for at least one year before the assessment;⁽³⁹⁾ heavy drinkers, i.e. drinking more than an equivalent of 28 grams of pure alcohol (two standard drinks) per day;⁽⁴⁰⁾ having occupations affecting the autonomic nervous system, such as security guards and athletes; acute fever; and pregnancy. Furthermore, female participants were not assessed during menstruation to avoid low blood volume and other effects on cardiovascular autonomic activity.^(41, 42)

Sample size calculation Sample size were calculated according to the equation: $n = [Z_{\alpha/2} \text{ sd} / d]^2$; where n = sample size, α = probability of type I error (2-sided) = 0.05; $Z_{0.025} = 1.96$; sd = estimated standard deviation of the measured parameter = 15.6 ms (standard deviations of RMSSD in Park et al., 2007,⁽²⁾ a study in 20 to 87-year old Koreans); and d = margin of error = ± 3 ms. Therefore, $n = [(1.96 \times 15.6) / 3]^2 = 103.88$, i.e. at least 104 volunteers were needed for the study.

Participants were asked to avoid all alcoholic beverages at least 48 hours before the assessment. In addition, caffeinated beverages, vitamins, herb and heavy exercise were not allowed for at least 12 hours before testing.

2.2 Data Collection

The assessment was performed between 8 am to noon or 1 to 3 pm. Participants' characteristics and medical history were recorded upon arrival at the Laboratory for Cardiovascular Autonomic Assessment, Department of Physiology. These included gender, age, body mass index (BMI), health-related habits, such as exercise (type and frequency), smoking, alcohol intake, and menstrual history in women. Then all participants underwent physical examination by a physician. Vital signs, general appearance, and physical examination by body systems, such as cardiovascular, pulmonary, abdominal and nervous systems, were assessed and recorded. Blood pressures and heart rate were initially measured with an automated sphygmomanometer, which had been regularly calibrated with a standard mercury sphygmomanometer every three months.

ECG recording was obtained using Finometer[®] Pro device with ECG module (lead II) and BeatScope[®] Easy program (Finapres Medical System BV, Amsterdam, Netherlands) at 200 Hz sampling rate. Standardized conditions during the test included a room temperature of 23-24°C and a humidity of 50-60% in a quiet room. The actual recording started at least two hours after the participant's last meal. After resting for at least ten minutes in supine position, 12-minute ECG was recorded. During that time, participants were asked to keep relaxed, awake, quiet and still, without meditating, and they did not see the monitor during recording. To prevent sleeping, they were gently reminded every 4 minutes.

2.3 Data Analysis

The ECG signal was exported for HRV analysis in LabChart[®] 7 Pro program (ADInstruments, Castle Hill, Australia). From 12-minute ECG, data of the 1st and 12th minutes were excluded. Then a 5-minute ECG data with stable baseline was chosen for HRV analysis. The presence of ectopic beats was rare in these subjects, and if found, the ectopic beats were excluded from analysis. Analysis and interpretation followed the guidelines set by the Task Force of the European Society of Cardiology and the North American Society, 1996.⁽³⁾

Five time domain parameters of HRV were obtained: SDNN, the standard deviation of all NN intervals (normal-to-normal RR intervals) in ms, is an estimate of overall heart rate variability; SDSD is the standard deviation of the differences between adjacent NN intervals in ms; RMSSD is the square root of the mean squared differences of successive NN intervals (ms); NN50 is the number of NN interval pairs differing more than 50 ms; and pNN50 is the percentage of NN50 / total NN number.

Seven frequency domain components were extracted: Total power is the variance of all NN intervals (ms^2); VLF, or the very low frequency component (ms^2), is the power in the very low frequency range (≤ 0.04 Hz); LF, or the low frequency component (ms^2), is the power in the low frequency range, 0.04-0.15 Hz; LFnu, the normalized unit of LF (in n.u.) = $\text{LF} \times 100 / (\text{total power} - \text{VLF})$; HF, the high frequency component (ms^2), is the power in the high frequency range, 0.15-0.4 Hz (around the breathing frequency of each individual); HFnu, the normalized unit of HF (in n.u.) = $\text{HF} \times 100 / (\text{total power} - \text{VLF})$; and LF:HF ratio or LF/HF.

Simple statistical analysis were calculated in Microsoft Excel[®]. Data were expressed as mean $\pm$ standard deviation (SD), for normally distributed data, and both mean $\pm$ SD and median (interquartile range, i.e. P_{25} - P_{75}) for non-normally distributed data. Statistical tests for differences and correlations were performed using the program GraphPad Prism[®] 5 (GraphPad Software, La Jolla, CA, USA). Statistical significance was taken at $P < 0.05$.

Kolmogorov-Smirnov test was employed to demonstrate data normality. Differences between two independent groups were tested with Student's unpaired t test (normally-distributed data) or Mann-Whitney U test (non-normally distributed data). For three-group comparisons, one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test or Kruskal-Wallis test followed by *post hoc* Dunn's test was applied, for normally or non-normally distributed data, respectively. Linear correlations among factors and parameters were tested with Pearson correlation. For moderate or strong correlation, the absolute value of the correlation coefficient, $|r|$, must be ≥ 0.36 ,⁽⁴³⁾ and the correlation must be statistically significant ($P < 0.05$). Stepwise multiple regression was performed using Statview[®] statistical package (SAS Institute, Inc., Cary, North Carolina, USA), with a general multiple linear equation: $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_n X_n$; where $X_1, X_2 \dots X_n$ are the independent factors; Y is the dependent variable (HRV parameter); $\beta_1, \beta_2 \dots \beta_n$ are partial regression coefficients associated with each X ; and α is the intercept.

Statistical analysis was not performed for 1) NN50, due to its dependent on total NN intervals, and pNN50 already reflected this fact; and 2) VLF due to our short-term (5 minutes) analysis which may not give any meaningful information regarding the VLF.⁽³⁾

3. Results

Of the 144 Thai subjects who passed the initial interview as healthy, 27 were excluded due to abnormalities in blood profile, physical examination, blood pressure, electrocardiogram, medical history or taking contraceptives. Thus, 117 participants remained in this study.

3.1 Participant General Characteristics

The 117 healthy Thai participants consisted of 44 men and 73 women, aged 21-72 years. There were six ex-smokers who quit the habit for at least seven years, 11 non-heavy alcohol drinkers, 85 with regular caffeine intake (e.g. coffee, tea, or chocolate drink), and 81

with regular exercise. Table 1 compares general characteristics between men and women in the study. The mean age of both genders were comparable. Men were significantly higher in BMI, resting systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and respiratory rate (RespR), while significantly lower in resting heart rate (HR), consistent with previous studies.⁽⁴⁴⁻⁴⁶⁾ Blood values which were higher in men were hemoglobin (Hb), hematocrit (Hct), BUN, creatinine, ALT, AST, ALP, and BILI. These are known gender differences mostly attributed to the higher testosterone in men, leading to higher red blood cell,⁽⁴⁷⁾ bone and muscle mass,⁽⁴⁸⁾ and consequently liver profile. Those which were not statistically different between genders were FBG, CHOL, TG, cal-LDL, and albumin. Only HDL was higher in women, a finding attributable to estrogen.^(12, 49, 50)

When participants were divided into three age groups (20-39, 40-59 and ≥ 60 years old), the age difference between men and women in each group was not statistically significant, except for the age group ≥ 60 years, where women were significantly older (Table 2). This may be due to the very small sample size of men in this age group.

Table 1 *Participant general characteristics*

Characteristics	Men (n=44)	Women (n=73)	P value
Age (years)	39.00 ± 12.82	44.11 ± 14.94	ns ^a
BMI (kg/m ²)	21.29 ± 1.21, 21.65 (20.22 - 22.32)	20.75 ± 1.09	0.0128 ^b
SBP (mmHg)	118.16 ± 10.50	109.89 ± 11.77, 108.00 (101.00 - 115.50)	0.0002 ^b
DBP (mmHg)	73.39 ± 8.10	69.01 ± 8.69	0.0079 ^a
MAP (mmHg)	88.31 ± 8.193	82.64 ± 8.975	<0.0001 ^a
HR (/min)	67.25 ± 8.20	72.10 ± 9.75, 72.00 (66.00 - 76.50)	0.0054 ^b
RespR (/min)	15.98 ± 9.69, 13.00 (12.00 - 16.00)	15.38 ± 2.13, 16.00 (14.00 - 16.00)	0.0040 ^b
Hb (g/dl)	14.55 ± 0.87	12.84 ± 0.73	< 0.0001 ^a
Hct (%)	44.35 ± 2.34	39.25 ± 2.12	< 0.0001 ^a
FBG (mg/dl)	90.86 ± 5.27	89.67 ± 6.03	ns ^a
BUN (mg/dl)	12.56 ± 2.24	11.12 ± 2.85	0.0017 ^a
Creatinine (mg/dl)	1.01 ± 0.13	0.75 ± 0.11	< 0.0001 ^a
CHOL (mg/dl)	193.45 ± 31.68	199.66 ± 29.18	ns ^a
TG (mg/dl)	77.57 ± 32.88, 68.50 (52.00 - 93.25)	74.97 ± 32.39, 68.00 (46.50 - 93.50)	ns ^b
HDL (mg/dl)	60.58 ± 13.01	73.45 ± 15.01	< 0.0001 ^a
cal-LDL (mg/dl)	116.48 ± 24.57	111.45 ± 24.18	ns ^a
AST (U/l)	21.29 ± 6.25, 20.00 (17.00 - 23.00)	18.78 ± 4.11, 18.00 (16.00 - 20.50)	0.0455 ^b
ALT (U/l)	21.62 ± 9.68, 18.00 (14.00 - 27.00)	14.14 ± 4.84	< 0.0001 ^b
ALP (U/l)	61.36 ± 13.21	55.63 ± 15.98	0.0208 ^a
Albumin (g/dl)	4.50 ± 0.22, 4.50 (4.40 - 4.70)	4.99 ± 4.65, 4.50 (4.30 - 4.60)	ns ^b
BILI (mg/dl)	0.69 ± 0.285, 0.70 (0.50 - 0.80)	0.53 ± 0.25, 0.50 (0.40 - 0.60)	0.0013 ^b

Data are mean ± SD. Median (P₂₅ – P₇₅) are additionally shown for non-normally distributed data. ^aUnpaired t-test (normally distributed data); ^bMann-Whitney U test (non-normally distributed data); ns, not statistically significant or $P \geq 0.05$. Abbreviations are given in 2.1 and 3.1.

Table 2 Age comparison between genders, by age group.

Age (years)	Men (n)	Women (n)	P value
20-39	29.40 ± 5.85 (25)	29.42 ± 5.37 (31)	ns ^a
40-59	49.27 ± 6.05 (15)	48.52 ± 5.70 (25)	ns ^a
≥ 60	60.50 ± 1.00 (4), 60.00 (60.00 - 61.50)	64.41 ± 3.81 (17)	0.0297 ^b

Data are mean ± SD. Median (P₂₅ – P₇₅) are additionally shown for non-normally distributed data. ^aUnpaired t-test (normally distributed data); ^bMann-Whitney U test (non-normally distributed data); ns, not statistically significant or $P \geq 0.05$.

3.2 Independent Group Comparisons

Table 3 compares HRV parameters among three age groups, 20-39, 40-59, and ≥ 60 years old. Age difference was demonstrated for all parameters. Most parameters were highest in the 20-39 age group, except for LFnu and LF/HF, where the values were lowest in this youngest group. The 40-59 group was generally not significantly different from the ≥ 60 group, exceptions being SDNN, Total power and LF, where the values were higher in the 40-59 group.

Table 4 compares HRV parameters between men and women. Men were significantly higher only in the HRV parameters SDNN (time domain) and LF (frequency domain), while LFnu, HFnu, and LF/HF were not different between genders.

No statistically significant difference was found in any of the HRV parameters when the following groups were compared (n): Regular caffeine drinkers (85) vs non-caffeine drinkers (32); (non-heavy) alcohol drinkers (11) vs non-alcohol drinkers (106); participants with regular exercise (81) vs sedentary ones (36); women in follicular phase (n=28) vs those in luteal phase (n=17) of their menstrual cycle; and HRV results obtained in the morning (8 am-12 pm; n=77) vs in the afternoon (1-3 pm; n=40). Apparently, no effect of regular caffeine intake, alcohol, exercise, menstrual cycle and assessment time could be demonstrated in this population.

Table 3 Comparison of HRV parameters among age groups.

Parameters	20-39 years (n = 56)	40-59 years (n = 40)	≥ 60 years (n = 21)	P value
SDNN (ms)	56.98 ± 28.43, 46.51 (38.04-70.12)	46.42 ± 18.22, 42.31 (34.29-56.85)	32.34 ± 13.69 ^{*,#}	<0.0001 ^b
SDSD (ms)	50.88 ± 30.92, 41.07 (33.40-64.85)	31.76 ± 16.69 [*]	22.78 ± 11.59 [*] , 19.15 (15.98-26.16)	<0.0001 ^b
RMSSD (ms)	50.80 ± 30.86, 41.01 (33.36-64.74)	31.71 ± 16.66 [*]	22.74 ± 11.56 [*] , 19.12 (15.95-26.12)	<0.0001 ^b
pNN50 (%)	26.43±20.55, 21.54(10.60±41.89)	10.76±13.96 [*] , 4.77(1.58-17.98)	5.82±9.20 [*] , 2.11(0.80-6.02)	<0.0001 ^b
Total power (ms)	4083.4 ± 4348.0, 2414.0(1235.0-4845.0)	2366.2±2036.6, 1671.0(1303.0-2742.0)	1215.9 ± 1336.9 ^{*,#} , 900.1(487.1-1326.0)	<0.0001 ^b
LF (ms ²)	1173.8 ±1509.9, 681.2 (307.1-1398.0)	495.1 ± 385.0 [*] , 373.6 (255.0-729.6)	270.8 ± 309.1 ^{*,#} , 184.4(104.2-304.0)	<0.0001 ^b
LFnu	42.93 ± 15.51	56.73 ± 15.84	47.21 ± 18.23	<0.0436 ^a
HF (ms ²)	1491.1 ±2040.5, 763.3 (418.1-1552.0)	444.8 ± 571.8 [*] , 286.4 (167.5-535.9)	226.9 ± 225.3 [*] , 151.4 (98.0-212.0)	<0.0001 ^b
HFnu	51.43 ± 16.11	40.00 ± 15.71 [*]	44.95 ± 18.67	<0.0058 ^a
LF/HF	1.09 ± 0.78, 0.86 (0.45-1.52)	1.89 ± 1.77 [*] , 1.22 (0.79-2.21)	1.62 ± 1.45, 1.42 (0.67-2.00)	<0.0289 ^b

Data are mean ± SD. Median (P₂₅ – P₇₅) are additionally shown for non-normally distributed data. ^aOne-way ANOVA followed by *post hoc* Tukey's test (normally distributed data); ^bKruskal-Wallis test followed by *post hoc* Dunn's test (non-normally distributed data). *Significant difference compared to age group 20-39 years or [#]age group 40-59 years; ns, not statistically significant or *P* ≥ 0.05. Abbreviations are given in 2.3.

Table 4 Comparisons of HRV parameters between men and women participants.

Parameters	Men (n=44)	Women (n=73)	P value
SDNN (ms)	54.25 ± 26.33	45.75 ± 23.28, 41.46 (28.36 - 57.93)	0.0293 ^b
SDSD (ms)	44.05 ± 29.98, 35.33 (21.33 - 58.08)	36.44 ± 23.98, 31.83 (21.67 - 45.87)	ns ^b
RMSSD (ms)	43.97 ± 29.92, 35.28 (21.30 - 57.98)	36.38 ± 23.90, 31.78 (21.64 - 45.80)	ns ^b
pNN50 (%)	21.80 ± 22.25, 15.10 (1.44 - 35.99)	14.70 ± 16.24, 8.31 (2.23 - 23.34)	ns ^b
Total power (ms ²)	3655.09 ± 3794.16, 2108.00 (1276.00 - 4959.00)	2575.74 ± 3194.82, 1633.00 (814.60 - 2832.00)	ns ^b
LF (ms ²)	924.10 ± 1030.66, 489.20 (285.60 - 1143.00)	692.64 ± 1197.83, 347.90 (160.90 - 791.10)	0.0286 ^b
LFnu	50.51 ± 16.50	44.10 ± 16.60	ns ^a
HF (ms ²)	1063.16 ± 1444.74, 549.90 (171.30 - 1211.00)	812.03 ± 1618.67, 384.50 (185.00 - 788.20)	ns ^b
HFnu	44.80 ± 15.97	47.24 ± 17.83, 49.12 (34.23 - 54.54)	ns ^b
LF/HF	1.57 ± 1.43, 1.21 (0.69 - 1.93)	1.39 ± 1.32, 0.90 (0.68 - 1.70)	ns ^b

Data are mean ± SD. Median (P₂₅ – P₇₅) are additionally shown for non-normally distributed data. ^aUnpaired t-test (normally distributed data); ^bMann-Whitney U test (non-normally distributed data); ns, not statistically significant or $P \geq 0.05$. Abbreviations are given in 2.3.

3.3 Correlation analysis

Correlation analysis was performed between each HRV parameter and all factors (general characteristics and blood profile). Table 5 shows significant correlations ($P < 0.05$) between HRV parameters vs age, BMI, SBP, DBP, MAP, and HR, with moderate-to-strong correlations ($|r| \geq 0.36$) shown in bold. Results indicate that most HRV parameters, i.e., SDNN, SDSD, RMSSD, pNN50, Total power, LF, and HF, were significantly and negatively correlated with age, while LFnu, HFnu, and LF/HF vs age yielded only weak correlation. Additionally, SDSD and RMSSD were moderately correlated with resting HR. Other factors, including BMI, resting SBP, DBP, and MAP, and those not shown were either not significantly correlated with HRV parameters ($P \geq 0.05$), or the correlations were significant, but weak ($P < 0.05$, $|r| < 0.36$).

Table 5 Correlations between HRV parameters vs age, BMI, resting BP and HR.

Parameters	Age	BMI	SBP	DBP	MAP	HR
SDNN	$r = -0.45$ ****	ns	$r = -0.19$ *	ns	ns	$r = -0.34$ **
SDSD	$r = -0.50$ ****	ns	$r = -0.29$ **	ns	$r = -0.20$ *	$r = -0.37$ ****
RMSSD	$r = -0.50$ ****	ns	$r = -0.29$ **	ns	$r = -0.20$ *	$r = -0.37$ ****
pNN50	$r = -0.53$ ****	ns	ns	ns	ns	ns
Total power	$r = -0.38$ ****	ns	ns	ns	ns	$r = -0.32$ **
LF	$r = -0.37$ ****	ns	ns	ns	ns	$r = -0.22$ *
LFnu	$r = 0.21$ *	$r = 0.34$ ***	$r = 0.22$ *	ns	$r = 0.18$ *	ns
HF	$r = -0.40$ ****	ns	$r = -0.24$ **	ns	ns	$r = -0.26$ **
HFnu	$r = -0.26$ **	$r = -0.26$ **	$r = -0.26$ **	$r = -0.19$ *	$r = -0.24$ *	$r = -0.19$ *
LF/HF	$r = 0.23$ *	$r = 0.22$ *	$r = 0.24$ **	$r = 0.21$ *	$r = 0.23$ *	ns

r , Pearson's correlation coefficient; ns, not statistically significant or $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Significant correlations with $|r| \geq 0.36$ are shown in bold type.

3.4 Stepwise multiple regression

To demonstrate the contribution extent of factors with significant correlation with each HRV parameter, and to correct for correlations among interdependent factors, stepwise multiple regression was performed. All factors with any significant correlation were included in this analysis. In all, 22 parameters were entered: gender (men = 0, women = 1), age, BMI, weight, height, resting SBP, DBP, MAP, HR, RespR, ambient temperature, humidity, FBG, TG, cal-LDL, ALT, Hb, Hct, red blood cell count (Rbc), white blood cell count (Wbc), neutrophil count (N), and percent eosinophile (%E). The resulting multiple linear equations are shown in Table 6. Most parameters were negatively correlated with age, except for LFnu, and LF/HF. Surprisingly, gender did not enter in any of the equations. This implied that differences between genders described above (Table 5) may result from the fact that fewer men could be recruited in the older age group. Notice also that none of the R^2 (coefficients of determination) exceeded 0.50, meaning that the factors in each equation could explain no more than 50% of the parameters' variance. Therefore, it is likely that factors other than those included in our study may be involved.

Table 6 Stepwise multiple regression results for HRV parameters

Regression equation					Adj. R ²
SDNN	= 159.929	- 0.836 Age	- 1.170 HR		0.335
	(90.992)	(40.820)	(24.619)		
SDSD	= 170.202	- 0.985 Age	- 1.381 HR		0.407
	(100.290)	(55.108)	(33.375)		
RMSSD	= 169.864	- 0.984 Age	- 1.377 HR		0.407
	(100.261)	(55.129)	(33.344)		
pNN50	= 117.848	- 0.748 Age	- 1.065 HR		0.468
	(104.949)	(69.339)	(43.356)		
TotalP	= 17092.348	- 99.590 Age	- 153.131HR		0.258
	(47.557)	(26.480)	(19.307)		
LF	= 4487.946	- 31.256 Age	- 36.928 HR		0.190
	(27.694)	(22.031)	(9.484)		
LFnu	= -97.336	+ 5.091 BMI	+ 0.337 SBP		0.143
	(9.417)	(14.765)	(5.916)		
HF	= 6645.337	- 46.026 Age	-58.678 HR		0.236
	(34.556)	(27.188)	(13.627)		
HFnu	= 190.121	-4.392 BMI	-0.604 HR	-0.303 Age	0.177
	(35.468)	(11.759)	(10.511)	(8.997)	
LF/HF	= 2.063	+ 0.013 TG	- 0.248 Wbc		0.139
	(9.345)	(12.495)	(7.015)		

R², coefficient of determination; italicized numbers in parentheses are F-to-remove of the parameter immediately above.

4. Discussion

In the present study, short-term (5 minutes) heart rate variability (HRV) were determined in 117 healthy Thais (44 men and 73 women), 21-72 years of age. It was found, after correlation analysis and stepwise multiple regression study, that most HRV parameters, namely, SDNN, SDDSD, RMSSD, pNN50, Total power, LF, HF and HFnu, were negatively correlated with age and resting HR. In addition, BMI was associated with LFnu positively and HFnu negatively; SBP was positively associated with LFnu; and LF/HF was positively correlated with TG, but negatively correlated with Wbc. However, gender, most blood profile components, caffeine intake, alcohol drinking, exercise, phases of the menstrual cycle and assessment time did not significantly associate with HRV parameters.

4.1 HRV major determinants are age and resting heart rate

From our data, age appears to be the major independent factor influencing most HRV parameters in healthy subjects. The negative correlation of age with all parameters but LFnu and LF/HF could be demonstrated in both correlation study and stepwise multiple analysis (Table 5 and 6). Similar results were obtained when HRV parameters were compared between age groups (Table 3), consistent with several studies reporting decreased HRV with age in healthy participants.^(12, 14, 29, 51-53) In addition, age difference yielded low significance in LFnu, HFnu, and LF/HF (Table 3). Stepwise multiple regression indicated that age was not associated with LFnu and LF/HF, while age contributed little to and was only weakly correlated with HFnu. These findings are in agreement with Remaekers et al., 1998, who reported that LFnu, HFnu and LF/HF were not changed with age in 276 healthy subjects (18-71 years), using studied 24-hr Holter records.⁽¹³⁾ Moreover, it can be noticed in Table 3 that the difference existed mostly between the youngest group (20-39 years) and the two older groups, while the age group 40-59 and ≥ 60 were more similar and differed only in the SDNN, Total power and LF. Although, this could result from the small sample size in the oldest age group (21 participants), there have been studies demonstrating that older subjects (≥ 60) did not differ from the oldest middle age group (40- or 50-59), in line with our results.^(13, 28) Reduced HRV is believed to reflect decreased parasympathetic activity,^(12-14, 26, 29) found in aging and disease.^(3, 6, 54, 55) However, the mechanism responsible for this decrease in aging has not been fully elucidated. Explanations include reduction of arterial compliance with aging^(28, 56) which leads to decreased baroreceptor sensitivity;⁽⁵⁷⁾ a sympathetic decrease from, e.g. cardiac sympathetic input decline⁽⁵⁸⁾ and/or beta-adrenergic sympathetic receptor desensitization,^(59, 60) or decreased estrogen in aging women and testosterone in aging men.⁽⁶¹⁾

Another independent major factor of HRV derived from our data is resting HR, which is not frequently reported. We found that resting HR negatively correlated with most HRV parameters, similar to the age effect but smaller. Our results generally agree with Ramaekers, 1998⁽¹³⁾, who described negative correlations between HR and SDNN, SDSD, pNN50, RMSSD, Total power, LF, and HF, except LFnu and LF/HF ratio. Tsuji, 1996⁽³⁰⁾ had slightly different results: HR was negatively correlated with all HRV parameters, i.e., SDNN, RMSSD, pNN50, LF, HF, Total power and LF/HF ratio. Interestingly, the latter study suggested that β -blocker medication that reduced HR by more than 10 /min could increase HRV.

BMI appeared to have only a small influence on HRV. Consistent with previous studies,^(62, 63) BMI was not related to most HRV parameters, and only weakly to LFnu and HFnu in the present study. In contrast, Vallejo et al., 2005, found that age and BMI importantly affect HRV in 30 women with BMI ranging from <19 to >30 kg/m².⁽⁵³⁾ We ascribe this discrepancy to the narrow range of our healthy participants' narrow-ranged BMI, 18.5-22.9 kg/m². Likewise, the fact that all recruited participants had blood profile values in the normal range may explain the lack of correlations between HRV parameters and any of the blood profile components ($P \geq 0.05$, $|r| \leq 0.35$). Although some studies reported correlations between autonomic parameters and blood profile, the correlation coefficients were very low ($r = 0.08-0.27$, $P < 0.05$).^(64, 65) Finally, our data did not show the effects of caffeine, alcohol, exercise, menstrual cycle and assessment time on HRV parameters. In the literature, the effects of caffeine,^(63, 66) alcohol,^(30, 63) and menstrual cycle^(53, 67) on HRV are still controversial, while physical activity only had small effects.⁽⁶³⁾ Some studies reported

that circadian rhythm could affect HRV parameters^(29, 68) but our assessment time probably fell within sufficiently narrow time window that we could not demonstrate any circadian influence.

4.2 HRV parameters are probably not gender-dependent

In this study, no gender differences in HRV parameters were detected, except that men had significantly higher SDNN and LF (Table 5). However, gender did not turn up in any of the stepwise multiple regression equations (Table 6), indicating that gender was not an independent factors determining HRV parameters in healthy subjects of our study. These results agree well to Tsuji *et al.*, 1996, analyzing 2-hour ambulatory ECG recording of 2,722 subjects in Framingham heart studies (21-93 years old, 45% men) and, using stepwise multiple regressions, showing that gender was not a major determinant of HRV.⁽³⁰⁾ Laitinen *et al.*, 1998, studying in 117 healthy Finns (23-77 years old) by detailed history taking, physical examination, routine laboratory tests, and clinical exercise test. It was found, also after stepwise multiple regressions, that SDNN, RMSSD, Total power, LF, HF, and LF/HF (all HRV parameters studied except VLF) were independent of gender.⁽²⁸⁾ These two studies differed in the sample size and participants selection process. However, both reports and ours employed stepwise multiple regressions to find independent HRV determinants. Nevertheless, gender differences in HRV have been described in many reports,^(12-14, 29, 51, 62, 63, 69-71) of which some were very careful in selecting participants.^(13, 62, 69) However, multiple regression was not used in any of the study and this may be a possible source of the discrepancy. Interestingly, Corrales *et al.*, 2012, also found no difference between genders in 200 young healthy Mexicans (18-25 years old), including athletes, even though multiple regression was not employed.⁽³⁴⁾ The study was carried out in a young and healthy population, a probable piece of evidence supporting that there was no fundamental autonomic difference between genders.

4.3 Limitations

It was difficult to find participants in the older population, especially men. This precluded gender comparison in the oldest age group and may have caused apparent gender difference in some parameters. The latter problem was alleviated with stepwise multiple regression.

5. Conclusion

In healthy subjects, age is the most important independent determinant for SDNN, SDSD, RMSSD, pNN50, Total power, LF, HF and HFnu. These parameters correlate negatively with age, and also resting HR, which is another independent factor, but with a weaker contribution. LFnu and LF/HF are independent of age. LFnu associates positively with BMI and SBP, while LF/HF associates weakly with TG and Wbc. Interestingly, none of the HRV parameters is gender-dependent. These results are essential for further developing HRV reference values; e.g., at least age-dependent range is needed for clinical application of HRV measurement.

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Impairment of Meissner Corpuscle in Skin of Diabetic Rat

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Abstract

Objectives: Meissner corpuscle (MC) or tactile corpuscle is the sensitive structure for touch recognition on hairless skin. The reduction of tactile sensation (numbness) is a common symptom of patients with peripheral neuropathy, which is a devastating complication in diabetes mellitus. If the tactile deficiency is detected, it will greatly increases the chance for successful treatment in diabetic neuropathic complication. Therefore, primary aim of this study is to investigate the alterations of MCs in diabetic rats, compared to control.

Materials and Methods: Seven male adult Sprague-Dawley rats (5-8 weeks, 200-270 g) were divided into two groups, three control and four diabetes. Diabetes was induced by a single dose intraperitoneal injection of streptozotocin (60 mg/kg body weight). After 24 weeks, the histological changes of MCs in skin of forepaws were examined by hematoxylin and eosin staining technique.

Results: In diabetic rats, the number of MCs in dermal papillae were significantly reduced. The MCs appeared conspicuous disorganization of lamellar structure, degenerations of Schwann cells, and capsular fibroblasts.

Conclusion: The effects of hyperglycemia induced the impairment of MCs, such as decreased density and pyknosis of MCs with losses of capsular fibroblasts and capsules, which lead to impaired touch sensation in diabetic neuropathy.

Keywords: Peripheral neuropathy, Diabetes mellitus, Streptozotozin, Hyperglycemia

1. Introduction

Diabetic peripheral neuropathy is the most common and devastating complication in half of diabetic patients⁽¹⁾. Longstanding hyperglycemia is a main cause of nerve fiber damage in the peripheral nervous system. The general symptoms are numbness, burning or tingling of extremities, and decreases in tactile and proprioception⁽²⁻³⁾. Because of losses of mentioned sensations, the foot ulcers, amputation, and high tendency to fall have been developed. One of sensory apparatus, involving in pain, tactile, and fine touch is Meissner corpuscle (MC). In previous reports, reduced MCs density, distorted their structure and elevated thresholds of sensation have been reported in diabetic neuropathy⁽⁴⁻⁶⁾. Furthermore, earlier study showed a reduction of number MCs and their afferent myelinated nerve fibers in skin biopsy of diabetic polyneuropathy⁽⁷⁾. These study focused on changes of mechanoreceptive function in the diabetes. The structural alterations of MCs, correlated with their impairment, were not elucidated in diabetes mellitus (DM). As a consequence, the primary focus of this study is to investigate MCs morphological lesions in streptozotocin

(STZ)-induced diabetic rats compared with control using light microscopy. This study provide an insight into structural impairments of MCs on glabrous skin by DM. It may be benefit for development of innovative treatment to improve diabetic patients with or without neuropathy.

2. Materials and Methods

2.1. Animal Preparation and Diabetic Induction

Seven adult male Sprague–Dawley rats 5-8 weeks old and weighed 200-270 g, were used. The Guide for the Care and Use of Laboratory Animals by the National Research Council was performed. Each rats were fasted for at least 6 hours. Next, body weight, urine glucose levels and blood glucose levels were determined. The rats were divided into 2 experimental groups, three control and four long-termed diabetic groups.

Diabetes was induced by intraperitoneal injection with a single dose of 60 mg/kg body weight of the STZ in the citrate buffer at pH 7.4. Only citrate buffer was injected into control group. The mean body weights and urine glucose values for each group were measured every morning. Blood glucose levels were assessed at 48, 72 hours after induction and before sacrifice. Rats were anesthetized by halothane inhalation and sacrificed at 24 weeks after the induction as long-termed period. Finally, the rib cage were cut to expose the thoracic cavity, before the ascending aorta was perfused with 500 ml of 0.9% NSS solution.

2.2. Tissue Preparation for Histological Study

After 0.9% NSS solution, rats were perfused with 300 ml of the Bouin's solution perfusion to preserve the tissues. The skin of plantar side of forepaws was removed and cut into small cubes. Then, the cubes were immersed in the same fixative solution overnight and rinsed several times in 70% ethanol. Thereafter, the cubes were dehydrated in a graded series of ethanol, cleared in xylene, infiltrated, embedded in paraffin, serially sectioned at 7 μm thickness by microtome and stained with hematoxylin and eosin (H&E).

2.3. Quantitative Analysis of Meissner Corpuscles

Under the light microscope with scanscope software, the density of MCs in each groups were counted at plantar pad of the forepaw, as the ratio of number per surface area (SA); MCs/SA (area = 3 mm²) by using Axiostar plus version Rel. 4.8 program (Jena, Germany).

2.4. Statistical Analysis

MC densities were presented as mean $\pm$ standard error (SE). Statistical differences were tested using Mann-Whitney U test (SPSS 20.0 software) with $p < 0.001$ considered significant.

3. Results

Weights of diabetic rats were significantly reduced, at 239.50 ± 13.86 g, compared with control rats, at 405 ± 38.56 g ($p < 0.05$). In the STZ-induced diabetic rats, increased glucouria (more than 500 mg/dL) was detected by glucostrips. Plasma glucose values were higher in diabetes (451.62 ± 22.72 mg/dL), compared with control (122.00 ± 11.19 mg/dL). The MCs density were significantly lower in long-termed diabetic group (0.63 ± 0.098 MCs/SA), compared with control group (4.14 ± 0.189 MCs/SA) with $p < 0.001$ (Figure 1).

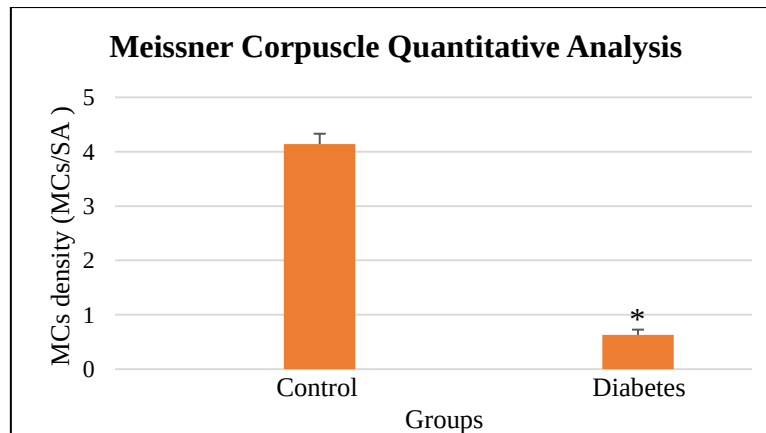


Figure 1 Comparisons in MCs density of long-termed diabetes, compared with control group. (*p-value<0.001)

In the diabetes, dermal papillae disappeared with few number of MCs (Figure 2B), compared with control group (Figure 2A).

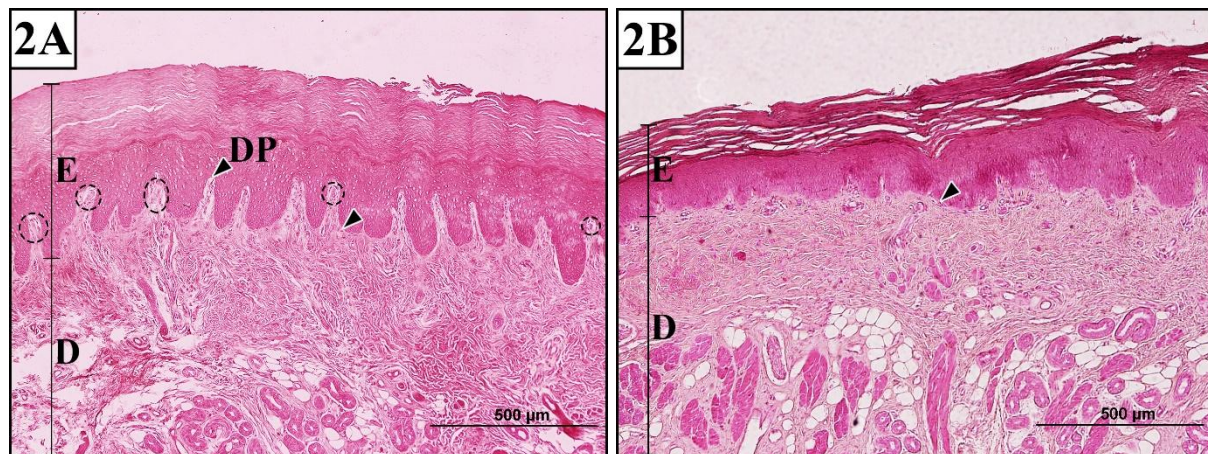


Figure . 2 Light micrographs of longitudinal sections at plantar pad of forepaw skin in control group (Figure 2A) and long-termed diabetic group (Figure 2B). At low magnification, MCs (dotted-line circles) were observed at dermal-papillae (DP) between epidermis (E) and dermis (D), whereas dermal-papillae and MCs in diabetes were absent (Figure 2B). H&E staining with 10x magnification. Arrowhead = dermo-epidermal junction. At high magnification, histological features of MCs in control rats showed an oval shape arranged vertically in the dermal papillae. They were surrounded by connective tissue (CNT) capsule and capsular fibroblasts. Within the corpuscle, there was a lamellar of Schwann cells in the horizontal plane (Figure 3A).

The major differences of MCs between control and diabetic rats that the stack of modified Schwann cells in the corpuscle became disorganization. Moreover, the abnormal MCs exhibited dark staining area and pyknotic nuclei of Schwann cells instead of lamellar and loss of capsule cells occurred (Figure 3B).

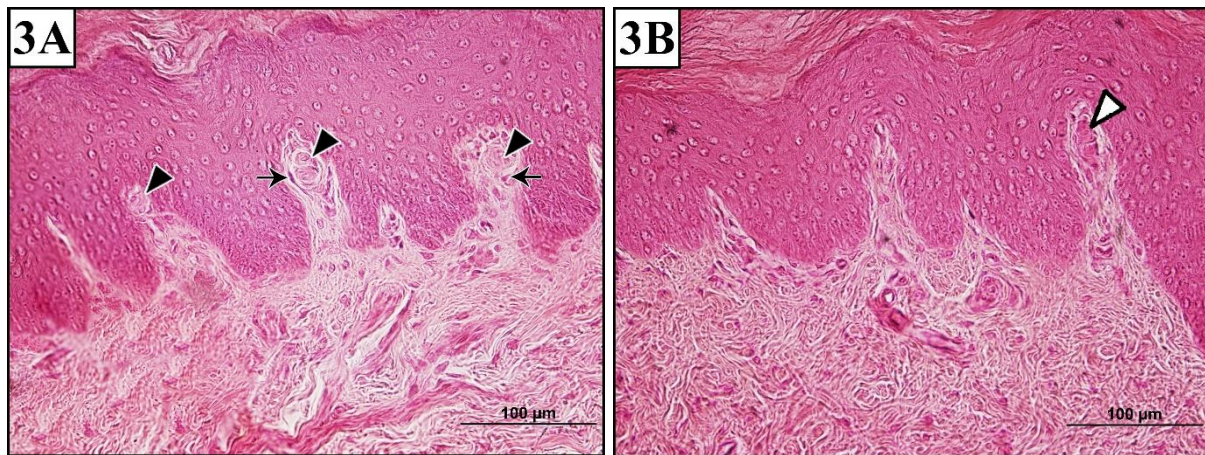


Figure . 3 Light micrographs of longitudinal sections at plantar pad of forepaw skin in control group (Figure 3A) and long-termed diabetic group (Figure 3B). MCs were located at the tip of dermal papillae (black arrowheads). In long-termed diabetic group (Figure 3B), MCs were represented by pyknosis and losses of CNT capsular and fibroblasts (white arrowhead). H&E staining with 40x magnification. Black arrow = fibroblasts.

4. Conclusion and Discussion

In the present study, the MCs density of diabetic group were significantly decreased, compared with the control group. The appearances of MCs were dark staining containing pyknotic Schwann cells with loss of fibroblasts and CNT capsule in the diabetes. During hyperglycemia, increased reactive oxygen species (ROS) and advanced glycation end products (AGEs) in both fibroblasts and Schwann cells can activate pro-apoptosis protein expression that stimulates caspase 3. Then, the caspase 3 promotes expression of endonuclease, proteinase and lipase, which damage chromatin, cell organelles, cytoskeleton, and unit membrane, respectively⁽⁸⁻¹²⁾. Therefore, lost fibroblasts, pyknotic nuclei of Schwann cells, decreased number of MCs, were observed. Moreover, it has been known that fibroblasts produce collagen fibers as CNT capsule in MCs. There was no fibroblast in the MCs, so that no CNT capsule encircled around the MCs. In the normal MCs, the Schwann cells extend lamellae inside the capsule. Additionally, there are dermal collagen fibers across the CNT capsule, which attach between Schwann lamellae in the MCs and dermal papillae of epidermis. It has been proposed that if skin is pressed, the dermal collagen fibers is pulled to stretch the nerve fibers inside the MCs. Consequently, the action potential progresses⁽¹³⁻¹⁴⁾. If CNT capsule in the MCs loses, the response to mechanical stimuli will reduce⁽¹⁵⁾. In addition, it has been reported that nerve plexuses in the epidermis and dermis became fragmented during hyperglycemia. Thus, destructive MCs and nerve plexuses lead to sensory impairment in the diabetic neuropathy patients⁽¹⁶⁻¹⁷⁾.

In conclusion, this study revealed the effects of hyperglycemia on the alteration of cells and CNT capsule of the MCs in the long-termed diabetes. In spite of, ultrastructures of cells, including nerve fibers in the MCs, should be investigated in the next experiments, which might be useful to relate to symptoms of diabetic neuropathy.

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Alterations of Hair Structure in Streptozotocin-diabetic Rats

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Abstract

Objectives: Diabetes Mellitus, a chronic metabolic disease, causes many serious health complications. The disease can lead to hair loss and infection, that affects on the quality of life in body image and community. Therefore, the purpose of this study was to examine the histopathological changes of hair and hair follicles in chronic diabetes.

Materials and Methods: Seven male Sprague-Dawley rats were arranged into two groups: control and streptozotocin (60 mg/kg body weight) -induced diabetic groups. Rats were sacrificed at 24 weeks. Skin at mid dorsal back were removed and allowed for light microscopic investigation.

Result: Chronic diabetes showed abnormal hair follicles including undifferentiated inner root sheath, separation between inner root sheath and cuticle, swollen cells in dermal papilla with cell death, and thin layer of outer root sheath.

Conclusion: These findings indicated that chronic diabetes causes the abnormal structures in hair follicles that relate to hair function and might involve in hair loss later.

Keyword: Hair loss, Hair follicle, Streptozotocin, Diabetes mellitus

1. Introduction

One of the epidermal derivatives of skin is hairs, which are characteristic in mammals. The hair apparatus plays a role in the regulation of body temperature, facilitation in evaporation of perspiration, sensation, and self-expression in human^(1, 2). When the hair cycle is disrupted or the hair follicle is destroyed, hair loss occurs⁽³⁾. Moreover, the causes of hair loss include heredity, hormonal change, medication, and diabetes mellitus (DM). The potential effects of DM on hair loss are impaired processes of hair growth via induction of hormonal imbalance, reduced blood circulation to hair follicle, and development of an infection to destroy the hair follicles. Alopecia areata, round patches of hair loss on the scalp, has been reported in the diabetic patients⁽⁴⁾. Diabetic animal researches have been shown the deformation of hair follicles including a decrease in their density and a retarded growth during utero life⁽⁵⁾ and postnatal period⁽⁶⁾. Consequently, the aim of this study was to illustrate the histopathological properties of hair in experimentally induced long-termed diabetic rats, compared to the normal condition. The results of this study might provide the alterations of hair structure in the diabetic state, that may be supported information to reduce hair loss in the diabetic patients.

2. Materials and methods

2.1. Animals and Diabetic Induction

Seven male adult Sprague-Dawley rats, 5-8 weeks of age, weighing 200-270 g from National Laboratory Animal Center, Mahidol University, Salaya, were used in this study. Animal housing, care and application of experimental procedures were performed in accordance with National Research Council's Guide for the Care and Use of Laboratory Animal.

Prior to experiments, rats were fasted for 10-12 hours followed by assessment of body weights, urine and blood glucose levels to confirm non-diabetic signs and symptoms. Healthy rats were chosen randomly and separated into two groups. The first group (n=4) was diabetic-induced using 60 mg/kg body weight of streptozotocin (STZ) in citrate buffer (7.4 pH) via intraperitoneal injection. The second group was injected with only citrate buffer served as the control. After the induction, the body weights and urine glucose levels were detected daily. Additionally, at 48 and 72 hours after STZ injection, the blood glucose levels were measured. The glucose levels above 300 mg/dl were accepted as diabetes. At the end of experimental period (24 weeks), blood glucose levels were also taken, and the rats were sacrificed. The thoracic cage was opened to perfuse with 500 ml of 0.9% NaCl through ascending aorta.

2.2. Histological Observation

The skin at mid dorsal back were removed. Then, tissues were fixed in Bouin's solution and washed many times in 70% ethanol before dehydration in ascending grades of ethanal. Thereafter, tissues were cleared in xylene and embedded in paraffin. Paraffin-embedded skin samples were cut into 7-8 μ m thick slices, stained by hematoxylin and eosin (H&E), and observed under a light microscope.

3. Result

The average body weight of diabetic rats (239.50 ± 13.86 g) was less than that of control (405 ± 38.56 g). The blood glucose levels increased as 451.62 ± 22.72 mg/dL in the diabetic group, compared to 122.00 ± 11.19 mg/dL in control group. The difference between two groups reached statistical significance ($p < 0.05$) by using Mann-Whitney U test.

The normal hair apparatus consisted of hair shaft, the visible part of hair protruded through the skin surface, and hair follicles, which enclosed the hair. Hair follicle were aligned obliquely to the skin. The base of the hair follicle in the dermis was called a hair bulb (Figure 1A). In the STZ-induced diabetic rats (Figure 1B), less complete and discontinuous structural hair follicles were observed.

At the high magnification (Figure 2A), the normal hair bulb of hair follicle consisted of a dermal hair papilla at its center with a cluster of fusiform shaped cells surrounding by hair matrix, which were differentiated into the hair shaft, inner root sheath (IRS) and outer root sheath (ORS). The flat cells in the IRS act as a mold of hair shaft, and they tightly attached to cells in the cuticles (Figure 3A). The ORS surrounded the IRS and consisted of multiple layers of epithelial cuboidal cells (Figure s 2A, 3A). In the diabetic stage, cells in the dermal papilla underwent hypertrophy (Figure 2B), compared to control (Figure 2A). Moreover, deeply eosinophilic staining line, suggested cell death, was observed in the dermal papilla near the matrix. In the IRS, cells remained oval shaped nuclei and tended to increase cell size (Figure 2B).

Above the bulbar region, hair follicle made up of three layers: the medulla, cortex, and cuticle covered by IRS and ORS (Figure 3A). The medulla was the most central layer of the hair follicle. In the suprabulbar region, the cells of medulla were cuboidal shape with vesicles in their cytoplasm. Lateral to the medulla was the cortex which contained a pack of fusiform shaped cells and arranged longitudinally parallel to the shaft. Cuticle was a single flattened cell layer that interlocked with the surrounding IRS (Figure 3A). Moreover, this was separation between IRS and cuticle in the diabetic group (Figure 3B).

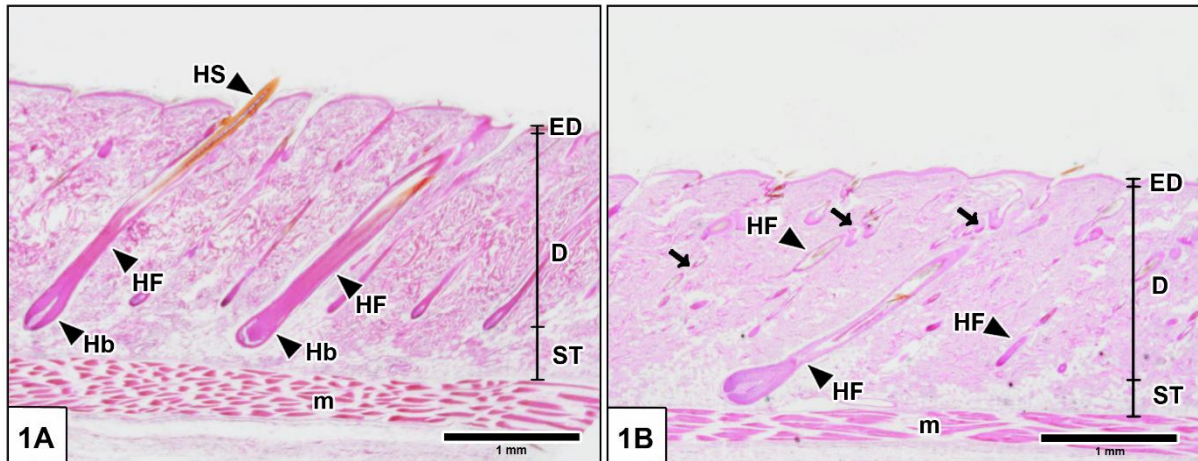


Figure 1. Light micrographs of hair follicles in skin at mid dorsal back of control (1A) and diabetic groups (1B). Normal hair follicles (HF) were sunken in either dermis (D) or subcutaneous tissue (ST) and elongated to epidermis (ED). In the diabetic rats, elongation of hair follicle was discontinuous (black arrows). Muscle (m), hair shaft (HS), hair bulb (Hb)

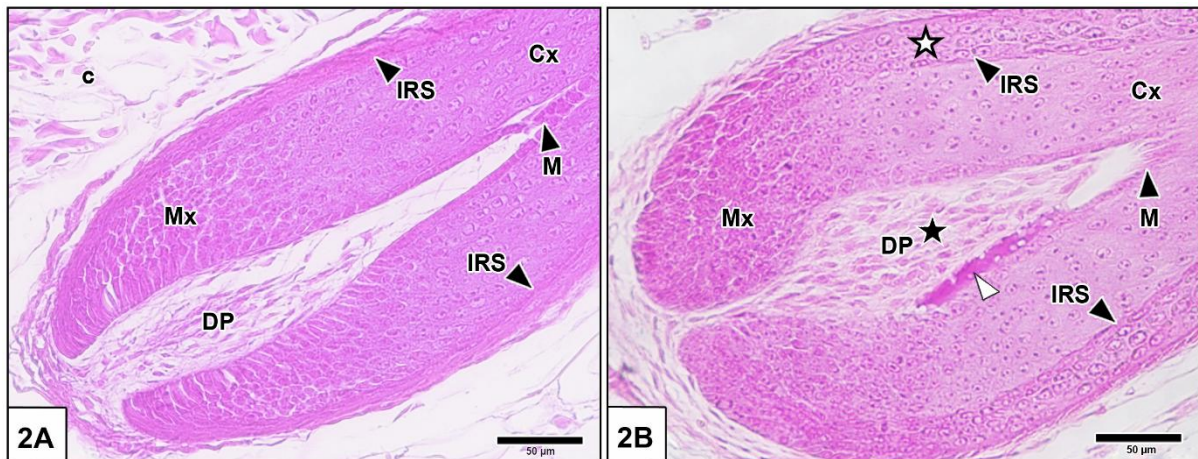


Figure 2. Light micrographs of hair follicles at the bulbar region of control (2A) and diabetic groups (2B). Hair bulb consisted of the dermal papilla (DP) at the center and surrounding matrix (Mx). In the diabetic stage, the cluster of cells in dermal papilla swelled (a black star). Deeply eosinophilic stained cytoplasm of dermal papilla cell was found as a dark pink line near the matrix (white arrowhead). Furthermore, the IRS cells increased its size (white star). Cortex (Cx), medulla (M), collagen fibers (c)

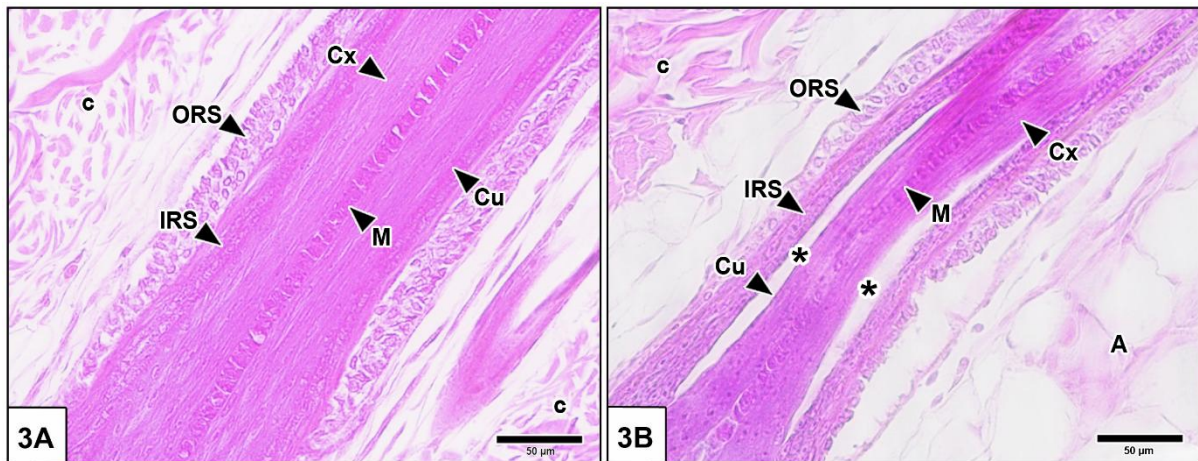


Figure 3. Light micrographs of hair follicles at the area above the bulbar region from control (3A) and diabetic groups (3B). Normal hair follicle were divided into medulla (M), cortex (Cx), cuticle (Cu), inner root sheath (IRS), and outer root sheath layer (ORS). The separation of cuticle from the inner root sheath (asterisks) and the thinner outer root sheath layer were observed in diabetic hair follicle, compared to control. Collagen fibers (c), adipose tissue (A)

4. Conclusion and discussion

In the long-termed diabetic hair follicles, balanced keratinization process is disturbed. Therefore, there were undifferentiated IRS with swollen cuboidal cells and discontinuous hair follicles⁽⁷⁾. In addition, abnormal differentiation and keratinization also affected on interlocking between IRS and cuticles, which occurred in late stage of keratinization⁽⁸⁾. As a result, there were clefts between IRS and cuticle. Finally, loss of hair may occur. The numbers of hair in control and DM will be counted to compare in the further study. Additionally, it has been known that intracellular reactive oxygen species (ROS) increases in the diabetic fibroblasts and keratinocytes, that cause water influx into the cells⁽⁹⁾. Consequently, the IRS and cells in dermal papilla became swollen. Furthermore, elevated ROS in fibroblasts and keratinocytes during DM mediates endonuclease, protease, and lipase for cell destruction⁽¹⁰⁾. Moreover, thin layer of keratinocytes in ORS, that increase discontinuation of hair and deeply eosinophilic staining line as cell death were obviously observed.

In conclusion, the malformation of hair follicles in the diabetic condition promotes hair loss and diabetic dermopathy. However, numbers of hair follicles, ultrastructures and junctional complexes of hair follicles should be further studied to understand the characteristics of diabetic hair follicles.

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Effects of the alternative sigma factor B on growth behavior and antiseptic exposure in bovine mastitis causing *Staphylococcus aureus*

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Abstract

Objective(s): The alternative sigma factor B (σ^B) has been reported to play a role in stress response and expression of virulence factors resulting in an increase in survivability and pathogenesis of *Staphylococcus aureus*. Therefore, this study aimed to observe the effects of σ^B in growth behavior, survival ability in the presence of chlorhexidine gluconate (CG), and identify σ^B -dependent genes based on presence of putative σ^B binding sites.

Material(s) and Method(s): $\Delta sigB$ mutant strain was constructed from a wild type RF122 bovine mastitis strain by an in-frame deletion. To characterize the role of σ^B in this RF122 strain, growth curves, survival assays in CG exposure and determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in wild type and mutant were compared. Furthermore σ^B -promoters were searched and predicted by MOODS algorithm to determine genes directly regulated by σ^B .

Results: Growth behaviors of RF122 wild type and $\Delta sigB$ mutant were not significantly different. The average doubling times of WT and $\Delta sigB$ were 28.1 and 31.6 min, respectively. For CG exposure, MIC and MBC between the two strains were not significantly different. In order to identify the putative genes dependent on σ^B in this strain, *in silico* search for σ^B promoters was performed using training sequences from known σ^B promoters of other strains. This study has identified 43 genes with putative σ^B -dependent promoters upstream, five of which were confirmed to be regulated by σ^B i.e., catalase, SAB2028c (unknown function), SAB0772 (unknown function), tyrosine phosphatase (SAB1813), and alkaline shock protein (*asp23*).

Conclusion: The role of σ^B in bovine mastitis causing *Staphylococcus aureus* was minimal in terms of contribution to growth in complex medium and resistance to antiseptic chlorhexidine gluconate.

Keyword(s): Alternative sigma factor B, *Staphylococcus aureus*, Bovine mastitis, Chlorhexidine gluconate

1. Introduction

Staphylococcus aureus is a bacterial pathogen causing diseases in both of human and livestock. In zoonotic infections such as bovine mastitis, *S. aureus* is one of the major problems leading to lose of dairy industry economically (1). Moreover, *S. aureus* from livestock can transfer to human by direct or indirect contact; therefore, people who work with livestock have higher risk to zoonotic *S. aureus* infection. In addition, antibiotic resistance genes can be transmitted from bacteria in animals to humans by direct contact or along the food chain (2). Understanding the factors causing *S. aureus* persistence in the environment and leading to zoonotic infections can help identify disease preventive measures and potentially alternative treatment target.

Alternative sigma factor B (σ^B) is a bacterial-specific subunit of RNA polymerase involving in transcription initiation process (3). In *S. aureus*, σ^B promotes expression of stress response genes, virulence factors, and antibiotic resistance genes in human *S. aureus* isolates (4-6). Previous studies have reported that deletion of *sigB* dramatically reduced methicillin and glycopeptide resistance (4,6). Besides, the over-expression of σ^B in *S. aureus* resulted in increased cell wall thickness and resistances cell wall-active antibiotics (5). As mentioned earlier, σ^B in *S. aureus* is; therefore, a potential pathogen-specific target of prophylactics and therapeutics development. This study aimed to construct a $\Delta sigB$ null strain and determined the effect of σ^B in a common sequence type bovine *S. aureus* strain which can be used to prevent bovine mastitis and human infections.

2. Material and methods

2.1 Bacterial strains

In-frame deletion mutant in *sigB* gene was constructed in *S. aureus* RF122 wild type (WT) using splicing by overlapping extension and allelic exchange mutagenesis (7). $\Delta sigB$ null mutant strains was checked by PCR and confirmed by DNA sequencing (Table 1). *S. aureus* RF122 WT and $\Delta sigB$ null mutant strains were stored in 20% glycerol stock at -80 °C until use.

Table 1 Primers used in this study.

Primers	DNA sequences (5' -> 3')
SigB-Ex1	ACACCTATGAGACAAGATGGAACTC
SigB-Ex2	CCTCAGTAAGTAATCCTTGCTGTTC

2.2 Growth curve

S. aureus RF122 WT and $\Delta sigB$ null mutant strains were inoculated into 5 ml of tryptic soy broth (TSB) and incubated at 200 rpm 37 °C overnight. Fifty μ l of overnight culture was inoculated into 5 ml TSB and cultured until OD₆₀₀ = 0.4. Five hundred μ l of OD₆₀₀ = 0.4 culture was added into 50 ml of TSB. The two strains were cultured at 200 rpm with agitation 37°C for 12 h. The OD₆₀₀ values were measured and recorded every hour. To determine colony forming unit (CFU), bacterial cultures were serially diluted (ten folds) with phosphate buffer saline (PBS) pH7.4 before spreading onto TSA every 2 h. Plates were incubated at 37°C overnight. Growth characteristics of WT and $\Delta sigB$ null mutant strains were tested in triplicates.

2.3 Chlorhexidine gluconate exposure

Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of *S. aureus* WT and $\Delta sigB$ null mutant strains were determined by the following steps. A single colony was inoculated into TSB and cultured overnight. Fifty μ l of overnight culture was inoculated into 5 ml of TSB and culture until $OD_{600} = 0.4$. Five hundred μ l of $OD_{600} = 0.4$ culture was added into 50 ml of TSB flask and culture was continued for 6 h. The culture was inoculated into Mueller Hinton (MH) broth (inoculum of 4×10^5 CFU/ml) containing varying concentrations of chlorhexidine gluconate (CG). The final concentrations of CG were 0, 0.00001, 0.00002, 0.00005, 0.0001, 0.0002, and 0.0004%. Bacterial cultures were grown at 37 °C for 18 h. The growth of bacteria in MH containing CG was measured by monitoring OD_{600} using spectrophotometer. After observation, the concentrations that did not show visible turbidity were sub-cultured onto MH agar by streaking to determine MBC.

For survival assay, after the stationary phase cultures of WT and $\Delta sigB$ null mutant strains were reached, 0.00005% of CG (final concentrations) was added. The treated cultures were incubated at 37°C, 200 rpm, and plated out at 1 and 2 h after exposure for enumeration of survived cells.

2.4 σ^B -promoters binding sites

σ^B -promoter sequences were searched in Pubmed literature. Table 2 shows the training sequences used for DNA motif search in *S. aureus* RF122 genome by MOODS algorithm (<http://pepper.molgenrug.nl/index.php/dna-motifs>).

Table 2 Training sequences of σ^B –dependent promoters from other bacteria

σ^B binding site of <i>Staphylococcus aureus</i>				
Strain	Gene	Promoter sequence	Distance from +1	References
		(-35)(No.of space)(-10)		
COL	<i>asp23</i>	GTTTAA(N14)GGGTAT	7	(8)
COL	<i>opuD</i>	GATTAA(N14)GGGTAT	9	(8)
COL	<i>clpL</i>	GTTTA(N14)TGGA	9	(8)
COL	<i>clfA</i>	GATTAA(N13)GGGTAT	7	(8)
COL	<i>csb8</i>	GTTTAG(N13)TGGTAT	8	(8)
COL	<i>csb9</i>	GTTTTA(N14)TGGTAT	10	(8)
COL	sa0455	GTTTAA(N14)GGGTAT	8	(8)
COL	sa0572	GTATAT(N12)GGGTAT	7	(8)
COL	sa1143	GTTTAT(N13)TGGTAA	8	(8)
COL	sa0772	GTTTAG(N13)GGGTAA	9	(8)
COL	sa2298	GTTTCA(N14)TGGTAT	9	(8)
COL	sa0752	GTTTAA(N14)GGGTAA	8	(8)
COL	sa2451	GATTCA(N13)GGGTAA	9	(8)
COL	sa0633	GTTTTA(N13)GGGTAT	9	(8)
COL	sa2309	GTTTAA(N14)GGGAAA	7	(8)
COL	sa2219	GACTAA(N13)GGGTAT	10	(8)
COL	sa0359	GAATAA(N13)GGGTAA	10	(8)
COL	Mw0922	GTTTAA(N15)GGGTAT	9	(8)
COL	<i>yabJ-spoVG</i>	GTTTAA(N14)GGGTAT	8	(6)
COL	sa0678	GTTTAA(N13)GGCAAT	7	(9)
COL	sa0742	GTTTAT(N14)GGGTAT	8	(9)
COL	sa2563(<i>clpL</i>)	GTTTTA(N14)TGGA	9	(9)
COL	sa2056 (<i>rsbV</i>)	GATTAG(N13)GGGTAT	9	(9)
COL	sa2293	GTTTAA(N14)GGTTAT	7	(9)
RN6390	<i>hla</i>	TTGATT(N16)TATTTT	6	(10)
N315	<i>bglG/sacY</i>	GTTATT(N14)GGGTAT	-	(11)
N315	<i>yabJ</i>	GTTTAA(N14)GGGTAT	8	(11)
N315	<i>sbrA</i>	GTTT(N17)GGGTAT	-	(12)
N315	<i>sbrB</i>	GTTT(N16)GGGTAA	-	(12)
N315	<i>sbrC</i>	GTAT(N14)GGGTAA	-	(12)
N315	<i>sbrD</i>	GTAT(N14)GGGTAT	-	(12)
N315	<i>sbrE</i>	GAAT(N17)GGGTTT	-	(12)
SH1000	<i>katA</i>	GTTTAA(N14)GGGTAA	9	(13)
MA11	egc operon	GTTATA(N10)TGGTTT	6	(14)

σ^B binding site of <i>Staphylococcus aureus</i>				
Strain	Gene	Promoter sequence	Distance from +1	References
		(-35)(No.of space)(-10)		
RN6390	<i>sarA</i> P3	AGTGAT(N14)GGGTAT	-	(15)
8325-4	<i>aur</i>	TTTACT(N17)TAATAT	6	(16)
8325-4	<i>sspA</i>	TTGACT(N16)TATATT	6	(16)
8325-4	<i>scpA</i>	TTTATA(N22)TATAAT	6	(16)
SH1000	<i>agr</i> P3 (RNAIII)	TTGGAA(N19)TTTAAT	6	(17)
MA13	<i>asp23</i>	GTTTAA(N14)GGGTAT	10	(18)
ATCC 29213	<i>sar</i>	GTGA(N16)GGGTAT	-	(19)
ATCC 29213	<i>asp23</i>	GTTT(N16)GGGTAT	-	(19)
ATCC 29213	<i>coa</i>	GTTT(N14)GGGCAA	-	(19)
8325-4	<i>sarH1</i>	GTTT(N17)GGGTAT	13	(20)
RN6390	<i>arl</i>	TTGAGT(N15)GATTGT	5	(21)

2.5 Statistical analysis

Mann-Whitney U test was used to analyze all experiment data. Statistical difference was indicated when *P*-value was less than or equal to 0.05.

3. Results

3.1 Mutant construction

Figure 1 shows the putative mutant clones with confirmed deletion using sigB Ex1-Ex2 primers. The expected product size for wild type (WT) is 2,137 bp while that of the mutant is 1,420 bp, corresponding to 717 bp deletion (or 239 amino acid deletion).

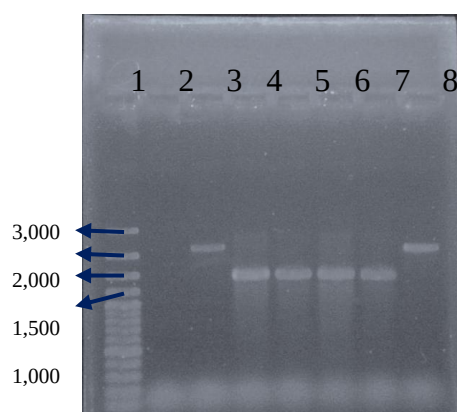


Figure 1 PCR check for $\Delta sigB$ clones. Expected size of $\Delta sigB$ clone is 1,420 bp. Lane 1: 100 bp Plus DNA marker, Lane 2: Negative control, Lane 3: Wild type control, Lane 4-Lane 7: $\Delta sigB$ clones, Lane 8: WT clone.

3.2 Growth rate

WT RF122 and $\Delta sigB$ were grown in TSB at 37°C (200 rpm) and growth characteristics were monitored via OD₆₀₀ measurements and colony forming unit (CFU) enumeration (Figure 2). The calculated doubling times of WT and $\Delta sigB$ were 28.1 and 31.6 min, respectively. However, the difference was not statistically significant (Mann-Whitney U test, $P = 0.513$).

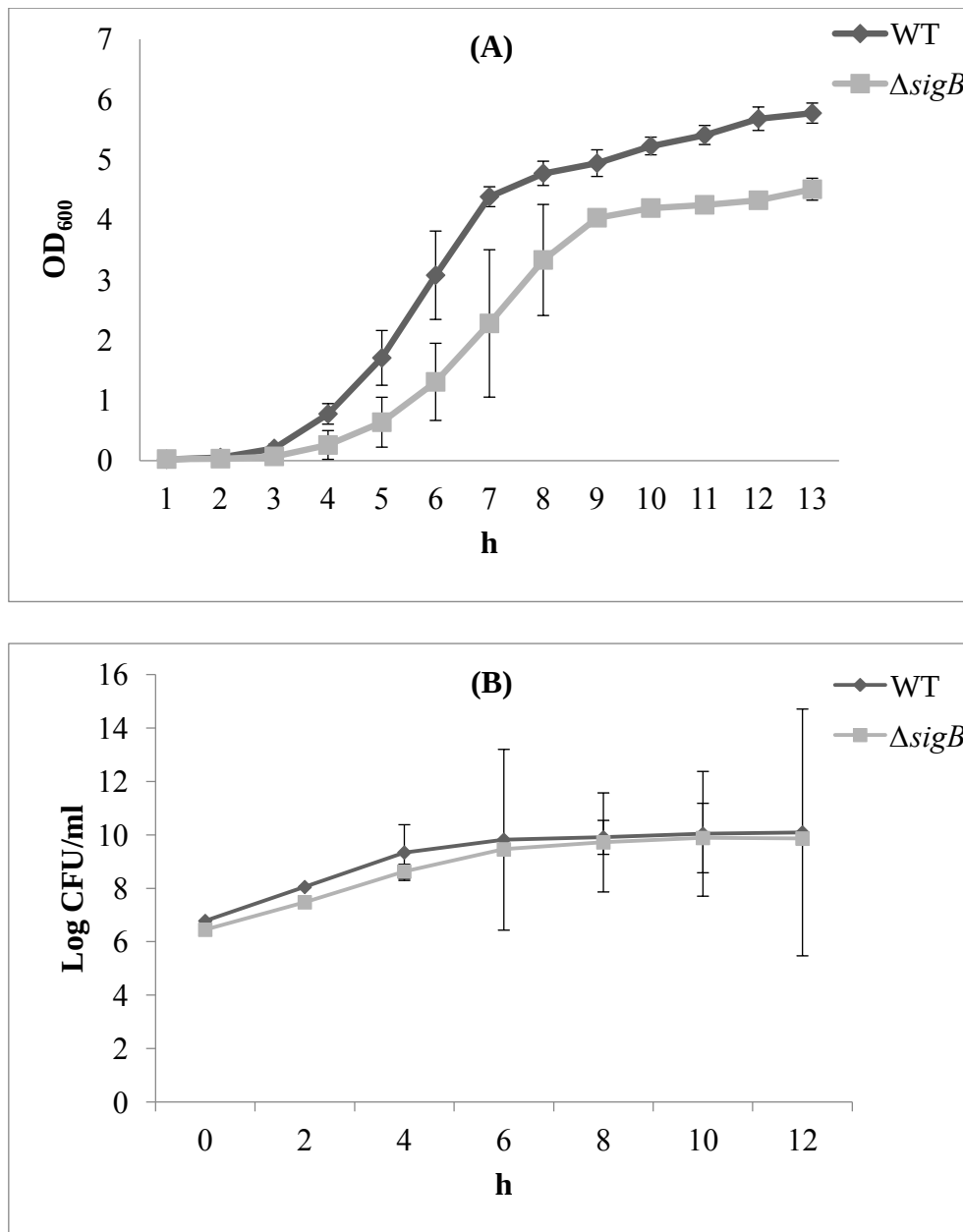


Figure 2 Growth curves of WT RF122 and $\Delta sigB$ in TSB at 37°C for 12 h.
(A) OD₆₀₀ measurement (B) Log CFU/ml

3.3 Chlorhexidine gluconate (CG) exposure

3.3.1 MIC and MBC of CG

Figure 3 showed that MIC values of CG for WT and $\Delta sigB$ were 0.0001% and 0.00005%, respectively. MBC of CG varies greatly from 0.00005-0.0004% in WT and 0.00002-0.0002% in $\Delta sigB$. Since MIC and MBC between strains were less than 4 folds, the values were considered not significantly different (22).

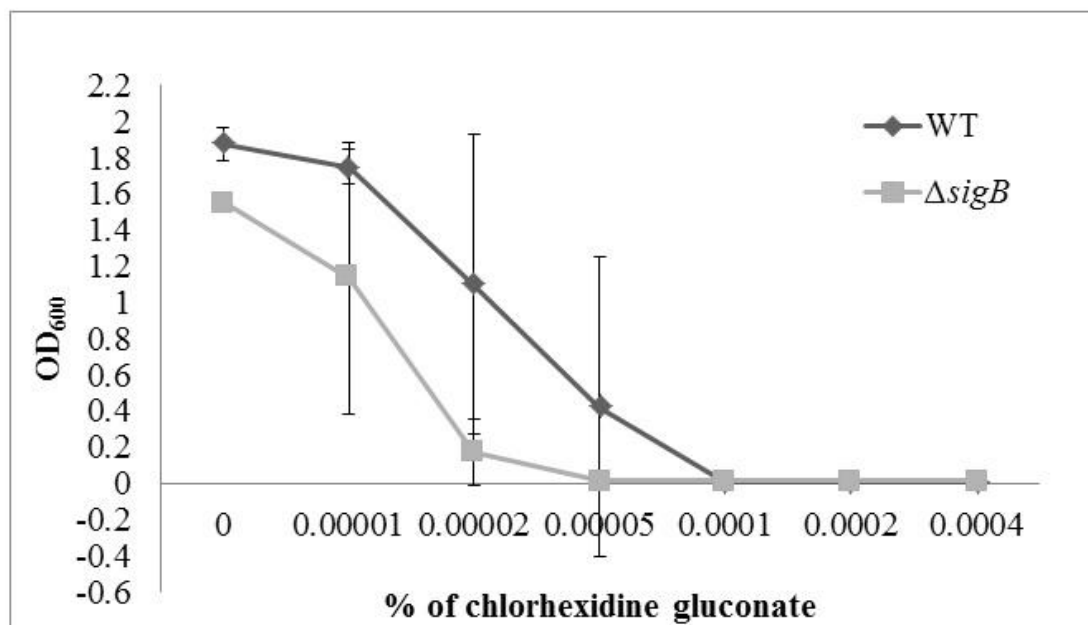


Figure 3 OD₆₀₀ of *S. aureus* RF122 wild type and $\Delta sigB$ cultures exposed to varying concentrations of chlorhexidine gluconate.

3.3.2 Chlorhexidine gluconate survival assay

After determination of MIC, WT and $\Delta sigB$ strains were exposed to chlorhexidine gluconate (0.00005%). Each strain was assessed for survival after 1 and 2 h. Log CFU/ml values of WT and $\Delta sigB$ strains were not statistically significant at both 1 and 2 h (Figure 4). Log reductions (0-2 h) in WT and $\Delta sigB$ strains were also compared and the difference was not statistically significant.

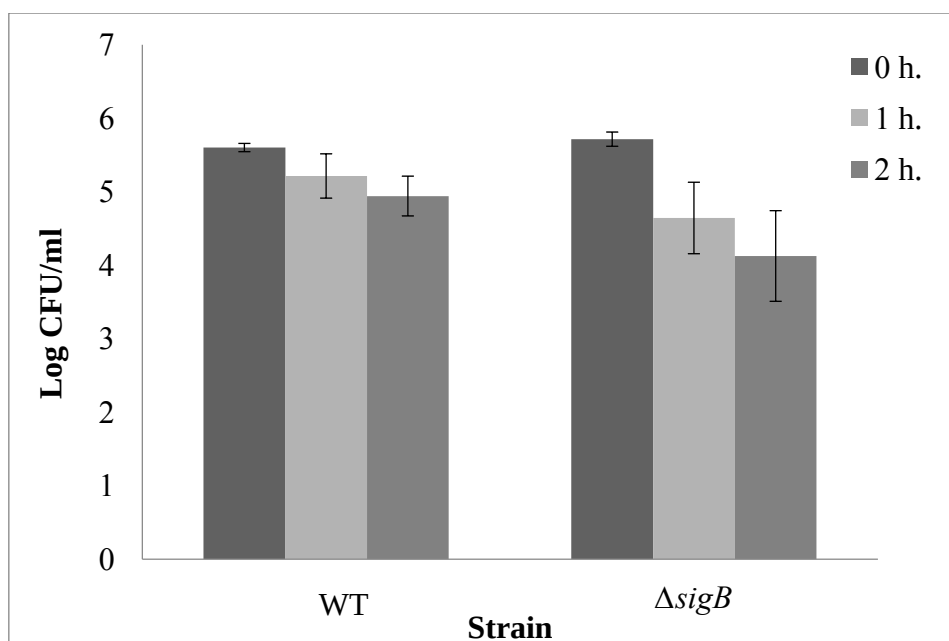


Figure 4 Survival assay of WT and $\Delta sigB$ after 0.00005% chlorhexidine gluconate exposure at 0, 1, and 2 h.

3.4 σ^B binding sites

To determine the putative genes dependent on σ^B in *S. aureus* RF122 strain, *in silico* search for σ^B promoters was performed using training sequences from confirmed σ^B binding sites of other *S. aureus* strains. Position weight matrix σ^B promoters was generated and used in MOODS algorithm to search RF122 genome. This study had identified 43 genes with putative σ^B -dependent promoters (Table 3).

Table 3 Genes with predicted σ^B binding sites upstream of the open reading frame (ORF)

Gene ID	Position of predicted promoter upstream of ORF	Function
SAB0257	319	Glycerolipid metabolism
SAB0256c	249	Unknown
SAB0523	331	Major Facilitator Superfamily (MFS) transporter
SAB0534	68	Amino acid transporters
SAB0588	40	Membrane transport
SAB0625	26	Unknown
SAB0627	36	Unknown
SAB0680	123	Unknown
SAB0744	197	<i>clfA</i> ; clumping factor A
SAB0755c	158	Unknown
SAB0758c	319	Unknown
SAB0759c	88	Unknown
SAB0772*	66	Unknown
SAB0786	290	Unknown
SAB0824	199	Amino acid metabolism
SAB0838	245	Unknown
SAB1040c	149	Unknown
SAB1177	382	Unknown
SAB1192*	126	Catalase
SAB1211	57	DNA topoisomerase IV subunit B
SAB1229	234	Glycan biosynthesis and metabolism, peptidoglycan pentaglycine glycine transferase
SAB1274c	229	Unknown
SAB1317c	282	DNA replication initiation protein
SAB1437	293	Glycyl-tRNA synthetase
SAB1589c	75	Transposase
SAB1596c	95	LacI family transcriptional regulator
SAB1630	163	Arsenical resistance operon repressor, ArsR family transcriptional regulator
SAB1813*	93	Protein-tyrosine phosphatase
SAB1815	199	Unknown
SAB1854c	56	Unknown
SAB1917	56	Unknown

Table 3 Genes with predicted σ^B binding sites upstream of the ORF (cont.)

Gene ID	Position of predicted promoter upstream of ORF	Function
SAB2028c*	92	Unknown
SAB2065c*	12	Unknown
SAB2134c	20	Methicillin resistance factor peptidoglycan pentaglycine interpeptide biosynthetic protein
SAB2151c	225	Membrane transport
SAB2174c	116	Unknown
SAB2255	49	Unknown
SAB2281c	80	Fumarate and nitrate reduction regulatory protein
SAB2313c	138	Unknown
SAB2333	74	Carboxyl esterase
SAB2380c	82	MerR family transcriptional regulator
SAB2423	251	ATP-dependent Clp protease ATP-binding subunit ClpL
SAB2533	72	Unknown

* σ^B -dependent genes as confirmed by other methods e.g., qRT-PCR and RNA sequencing.

4. Conclusion and discussion

Our study demonstrates that lacking the *sigB* gene did not affect the growth in complex media as the $\Delta sigB$ mutant can grow as well as the WT strain. Similar findings were found in human *S. aureus* clinical strain such as WCUH29 (23). σ^B also showed a minor or no role in chlorhexidine gluconate resistance as both WT and $\Delta sigB$ strains showed the same survival abilities and had comparable MIC and MBC values. However, the dose of chlorhexidine gluconate typically used as antiseptic (0.55%) could still to inhibit growth of both strains (24). Future study will be done to (i) further characterize the phenotypes of $\Delta sigB$ in comparison to WT, (ii) confirm the identified σ^B promoters, and (iii) compare the σ^B -dependent genes in bovine mastitis strain and human clinical methicillin-resistant strain.

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The Association of Firing Distances and Terminal Ballistics as Evaluated by a 5.56 mm Bullet

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Abstract

Objectives: An M-16 assault rifle with a 5.56 mm ammunition tends to be more intense causing life destruction, asset and also national security; however, firing distances and bullet impact characteristics are not clearly elucidated. This study aimed to investigate the relationship of firing distances and terminal ballistics from a 5.56 mm bullet including firing distance, bullet kinetic energy, wound characteristics related to bullet fragmentation pattern.

Materials and Methods: The firing model was performed by M-16A1 assault rifle along with 5.56 mm ammunition (M193) which was fired directly to impact human tissue simulant (soap block). The firing distance was performed at 5, 15, 25, 50, and 100 m, respectively. Bullet fragmentation was observed and recorded. Bullet velocity and soap block characteristics were also determined and analyzed. The data were statistically analyzed by using one-way ANOVA at $p < 0.05$ significant difference.

Results: Factors affecting firing distance such as bullet velocity, bullet kinetic energy, initial upset depth, wound cavity, and penetration depth showed statistically significant difference at different firing distances. The bullet fragmentation pattern also showed a significant difference at each firing distance.

Conclusion: These data suggest that the association of firing distances from M-16 assault rifle affected terminal ballistics as well as bullet fragmentation, wound characteristics of cavity and penetration and could be applied for criminal investigation.

Keywords: M-16, M193, Kinetic energy, Bullet fragmentation, Terminal ballistics

1. Introduction

At present, the trend of using M-16 assault rifle on sedition has been found in the southern border provinces of Thailand consisting of Yala, Pattani and Narathiwat, and Bangkok, as well. M-16 assault rifle power was designed for shooting up to 400 m in expected distance leading to the immense power that propels 5.56 mm bullets. This can attack to the target body and object with a maximum velocity of 3,250 f/s (990 m/s) and cause a grave danger on country subsistence and social security (1).

The firing study in human tissue simulants found that specific bullet fragmentation characteristic was only performed with conflicted target via high velocity and less mass bullets that almost found from 5.56 mm bullet firing cases as defined as lead snowstorm, the white delicate fragments in X-ray films dispersed within target area body that resemble with dust or snowstorm characteristic, in X-ray films (2-4).

Bullet kinetic energy was another factor related to bullet velocity (5). The study of 5.56 mm bullet kinetic energy changing whether affect to the target wound characteristic along with how the bullet fragmentation be did not find out.

The lead snowstorm, a special characteristic, refers to the study of 5.56 mm bullet (M193) fragmentation pattern from M-16A1 assault rifle impact to human tissue simulant in various 5 firing distances of 5, 15, 25, 50, and 100 m, respectively. The changed of bullet kinetic energy in various firing distances and effect of specific bullet fragmentation characteristics within impacted human tissue simulant were studied for the advantage of evaluating possible firing distance and criminal circumstance from the disturbance created by M-16A1 assault rifle.

2. Materials and Methods

2.1 Bullet velocity determination

Twenty-five 5.56x45 mm ammunitions (M193) 6th product lot of Fábrica Nacional de Munições de Armas Ligeiras, Chelas, Portugal (FNM) in 1981 were shot using M-16A1 assault rifle of COLT manufacturer in each 5 firing distances of 5, 15, 25, 50, and 100 m, respectively. Chronograph Model 8TM (Shooting Chrony Incorporate, Canada) was used to detect and calculate bullet velocity, then bullet kinetic energy was determined.

2.2 Terminal ballistics determination

Twenty-five 5.56x45 mm ammunitions (M193) 6th product lot of Fábrica Nacional de Munições de Armas Ligeiras, Chelas, Portugal (FNM) in 1981 were shot using M-16A1 assault rifle of COLT manufacturer into 25 human tissue simulants. Soap blocks, human tissue simulants were made from translucent glycerine with base size of 18x18x30 cm for each 5 firing distances of 5, 15, 25, 50, and 100 m, respectively. Initial upset depth, wound cavity height and penetration depth were measured and taking photo with scale. Dispersed bullet fragmentations in soap blocks were collected. The data achieved from various firing distances were compared.

2.3 Statistical analysis

GraphPad Prism version 6 was used for statistical analysis. The values of wound ballistics were calculated using mean $\pm$ SD and analyzed by one-way ANOVA at $p < 0.05$.

3. Results

3.1 Characteristic of fired human tissue simulants

To mimic a wound channel found in a shot body, a 5.56 mm ammunition (M193) were fired into human tissue simulant using a soap block. The wound channel showed similar characteristics in 5 firing distances of which presented the initial upset depth in the first period of bullet impact to the soap block. Then the bullet transfers the large amount of energy through soap block leading to wound cavity. The penetration depth is defined as a penetration of bullet and its fragmentation through a soap block until the bullet stopped (Figure 1). Although the 5 firing distances directed toward the soap block found similar, the initial upset depth, wound cavity, and penetration depth were significantly difference according to the firing distances.

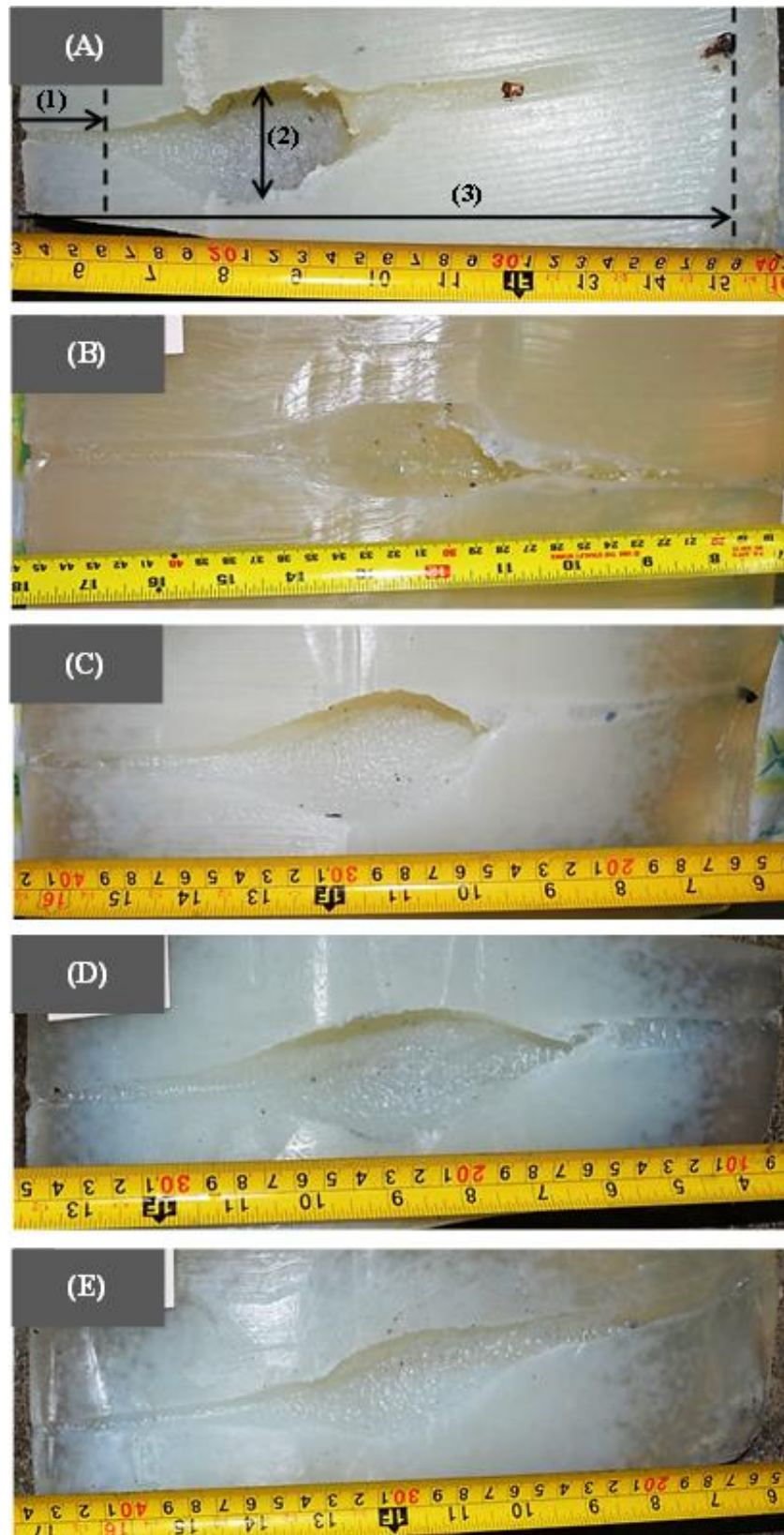


Figure 1 Wound channel in different firing distances: (A) 5 m, (B) 15 m, (C) 25 m, (D) 50 m, (E) 100 m. Note: (1) initial upset depth, (2) wound cavity, and (3) penetration depth.

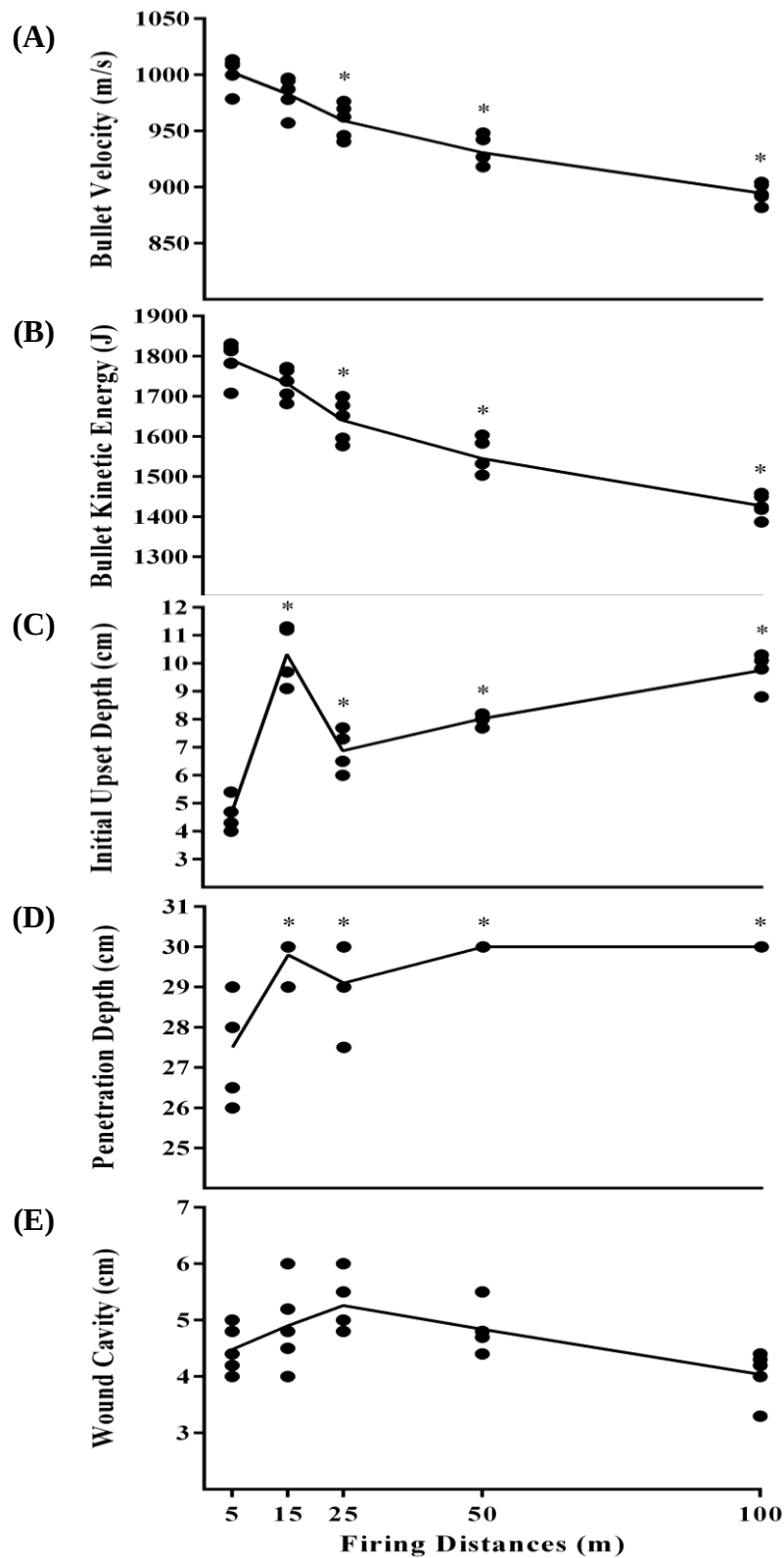


Figure 2 Factors affected firing distances: (A) bullet velocity (n=5); (B) bullet kinetic energy (n=5); (C) initial upset depth (n=4); (D) penetration depth (n=5); and (E) wound cavity (n=5). Note: * indicate $p < 0.05$ compared with 5 m firing distance.

Table 1 Factors affected firing distances

	5 m	15 m	25 m	50 m	100 m
Bullet velocity (m/s)	1,002.22 ± 14.10	982.83 ± 16.16 c, d	959.07 ± 15.36 *, c, d	930.72 ± 13.99 *, a, b, d	894.72 ± 8.87 *, a, b, c
Bullet kinetic energy (J)	1,791.21 ± 50.03	1,732.49 ± 38.26 c, d	1,640.35 ± 52.49 *, c, d	1,544.79 ± 46.52 *, a, b, d	1,427.44 ± 28.27 *, a, b, c
Initial upset depth (cm)	4.60 ± 0.61	10.33 ± 1.10 *, b, c	6.87 ± 0.77 *, a, d	8.02 ± 0.24 *, a, d	9.75 ± 0.67 *, b, c
Penetration depth (cm)	27.50 ± 1.22	29.80 ± 0.45 *	29.10 ± 1.02 *	30.00 ± 0.00 *	30.00 ± 0.00 *
Wound cavity (cm)	4.48 ± 0.41	4.90 ± 0.75	5.26 ± 0.49 d	4.84 ± 0.40	4.04 ± 0.44 b

Note: * indicate $p < 0.05$ compared with 5 m firing distance
a indicate $p < 0.05$ compared with 15 m firing distance
b indicate $p < 0.05$ compared with 25 m firing distance
c indicate $p < 0.05$ compared with 50 m firing distance
d indicate $p < 0.05$ compared with 100 m firing distance

Table.1 showed factors including bullet velocity, bullet kinetic energy, initial upset depth, wound cavity, and penetration depth that affect firing distances. In this study, the 5 m firing distance was used as the control group due to the safety and minimal distance that does not receive a return impact power from the firing.

The bullet velocity at 5 m firing distance showed a significant difference at $p < 0.05$ when compared to 25 m, 50 m, and 100 m, respectively (Figure 2A). However, 15 m firing distance showed a significant difference at $p < 0.05$ when compared to 50 m and 100 m firing distance. The study found that the bullet velocity showed a significant reverse relationship with the firing distance at $p < 0.05$.

The bullet kinetic energy showed similarly significant difference as bullet velocity due to the related value from calculation (Figure 2B).

The initial upset depth and penetration depth from soap blocks measurement showed similar tendency. At 5 m firing distance showed the minimal values as significant difference at p -value < 0.05 when compared to other firing distances. After the firing distance adding more at 15 m, both of their values rapidly increased whereas at 25 m firing distance showed a dropped tendency. At 50 m and 100 m firing distance, the initial upset depth and penetration depth tended to be increase from previous firing distances. The initial upset depth showed the gradual increased values from 50 m to 100 m firing distances. During the non-different values were observed from 50 m to 100 m firing distances of penetration depth (Figure 2C and 2D).

The wound cavity as measured from human tissue simulants at 5 m firing distances as showed no significant difference when compared to other firing distances (Figure 2E).

3.2 Bullet fragmentation pattern

The variation of bullet fragmentation pattern were found corresponding to different firing distance and bullet velocity (Figure 3).

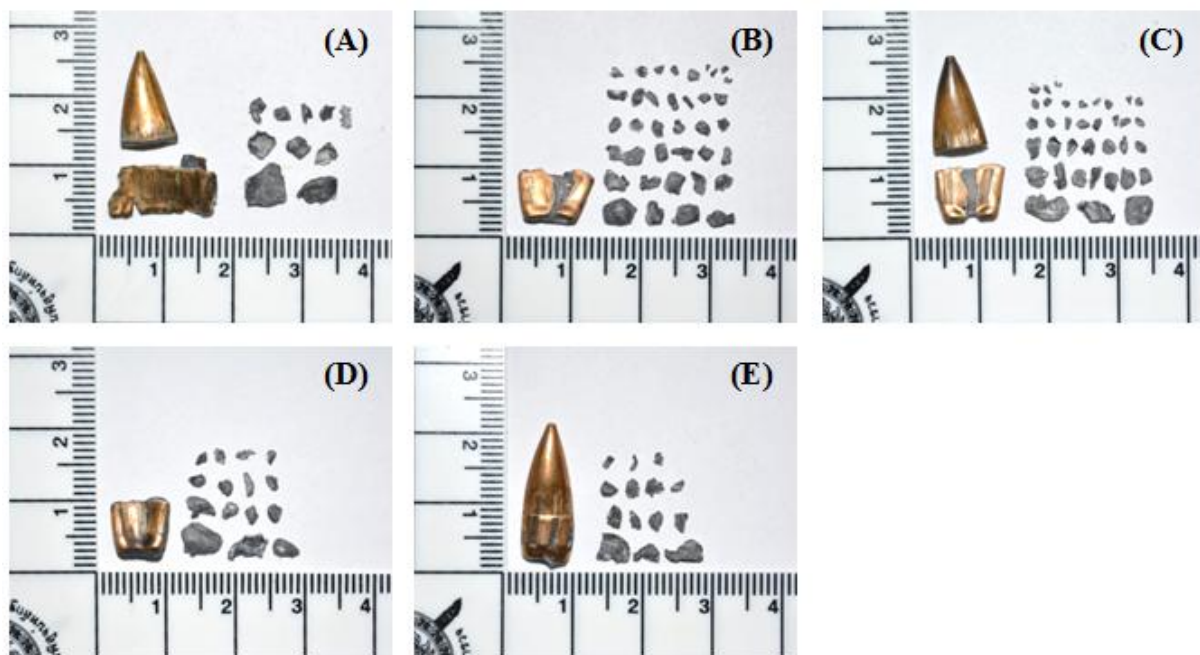


Figure 3 The bullet fragments in different firing distances: (A) 5 m, (B) 15 m, (C) 25 m, (D) 50 m, (E) 100 m.

4. Discussion

At 5 m firing distance, it was found that at first, abundant bullet fragmentation occurred from high velocity of bullet as it left the muzzle. After the bullet left the muzzle for 5 m, high bullet velocity still remained and introduced a large energy impact to the soap block whereas bullet gyrostatic moment is unstable oscillation; therefore, the precession on impacted soap block was span vigorously upon bullet contact and subsided along the distance. As a result of high velocity of yaw bullet and high deformation of bullet shape leading to jacket base tearing from large impact power and energy causing lead component inside bullet broke down into small pieces as well as dust particle which dispersed within bullet passage cavity. In opposite, the bullet tip was not major impact receiving part so bullet still existed and moved on by base-forward with kinetic energy in more distance until it stopped. The study found that bullet tip from 5 m firing distance seemed to more distortion and deformation than other firing distances. Upon bullet contact to the soap block with high impact power, it slightly penetrated to the soap block while the bullet yaw occurred (6, 7). In addition, there was a large energy transfer from bullet to the soap block. Then, bullet was deformed and fragmented. Meanwhile, the fragmented bullet still remains its velocity and kinetic energy; hence, leading to the dispersion to various directions. The wound cavity formed at 5 m firing distance occurred rapidly due to the massive energy transfer to the soap block from the beginning and subsequently declined. Therefore, this cause a less penetration to the soap block with the least distance when compared to other firing distances.

At 15 m firing distance, high bullet velocity still remained which showed no significant difference when compared to 5 m of firing distance. However, the result showed

more stability on gyrostatic moment causing the better existence of bullet upon contact to the soap block resulting in longer distance of bullet penetration in soap block prior to bullet deformation when compared to 5 m firing distance leading to decrement of bullet deformation which found only small tearing of jacket base. Lead particles of bullet fragmentation which everted from the bullet base contained a large number of small fragments caused by the high energy. Bullet tip, non-major impact power receiving part, still existed and moved forward to penetrate the soap block by its kinetic energy; therefore, higher penetration power was achieved when compared to 5 m of firing distances. The wound cavity at 5 m, 15 m, 25 m, and 50 m firing distances from 4.45 ± 0.40 to 5.26 ± 0.49 cm showed no significant difference; however, the wound cavity at 15 m firing distance found to have more penetration power from the beginning prior to bullet deformation when compared to 5 m, 25 m and 50 m firing distance. According to the at most of bullet deformation, the greatest penetration at 15 m firing distance appeared to have the most severity to human tissue when compared to other firing distances.

At 25 m firing distance, bullet velocity reduced significantly when compared to 5 m firing distance. Although the bullet velocity at 25 m and 15 m firing distance did not differ, the penetration power of bullet at the first period before deformed bullet occurring was less than the penetration power at 15 m firing distance. The reduction of penetration power at first period affected the faster yawing of bullet leading to faster break-off of bullet at the cannellure and more tearing of jacket base. The bullet tip could not move far enough and was remained within the soap block at the tip-forward position.

At 50 m firing distance, bullet velocity and bullet kinetic energy significantly reduced from 5 m, 15 m, 25 m and 50 m firing distance, respectively. When compared to 25 m of firing distance, although the bullet penetration power in first period prior to bullet deformation and also throughout bullet travelling were not different; however, the difference was found in deformed bullet collection from soap block due to bullet yawing which led to the bullet break-off at the cannellure and showed the kidney-shaped instead of jacket base tearing (8). The bullet tip, non-major part of impact power receiving, still existed and moved forward by kinetic energy until penetration through soap block (9) due to the better relationship between proper bullet velocity and gyrostatic moment in 50 m when compared to 25 m of firing distances.

At 100 m of firing distance, bullet velocity showed the smallest value when compared to other firing distances. The result showed the difference in all factors affecting firing distances except wound cavity size when compared to 5 m firing distance similar to 50 m firing distance. Four of five bullets were totally penetration through the soap block; however, one bullet was found remaining in the distal part of soap block with a perfect bullet shape at the base-forward position. None of bullet break-off at the cannellure was observed except for small lead fragment everted out from bullet base. The bullet direction in the soap block also found only one way exit. The finding in this study was paralleled to Fackler in 1984 and 1989 (10, 11). This indicated that 100 m firing distance also found the smallest of bullet fragmentation due to the less precession from the stability of bullet trajectory. The decreased bullet velocity at 100 m firing distance was related to the decreased kinetic energy of the bullet resulted in the decreased impact power. The more stability of the bullet, the more penetration of bullet and less impact as well as less of energy transferring to soap block. The remained energy could propel the bullet penetration through the soap block with quite non-deformed bullet (7).

5. Conclusion

The study found different findings in various firing distances with 5.56 mm bullet (M193) such as wound channel, factors affected firing distance and bullet fragmentation pattern. The data from the present study suggest that firing distances affect terminal ballistics in association with the evaluation of possible firing distance and criminal circumstance from the disturbance created by M-16A1 assault rifle. Further studies should be conducted in depth for the characteristic of fired human tissue simulant in different firing distances on X-ray film evaluation which may help to provide the better understanding in lead snowstorm, fragmentation pattern. In addition, the effect of intermediate targets and bullet brand would be interested for further investigation.

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Evaluation of Urinary Iodine in Patients with Well-Differentiated Thyroid Cancer before Radioiodine Therapy

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Abstract

Objective: To increase efficacy of I-131 ablation in differentiated thyroid cancer patients after total thyroidectomy, patients are usually instructed to deplete the whole-body iodine pool by restriction of daily low iodine diet (LID) intake. The aim of the study was to compare the urinary iodine excretion (UIE) median values after LID preparation to indicate patient compliance with LID.

Patients and Method: A total of 70 well-differentiated thyroid cancer patients with total thyroidectomy preparing for first ablation were enrolled. They were 54 (77%) females and 15 (23%) males age ranged between 18 to 79 years and the average was 45 years. All had I-131 uptake lower than 15% and serum thyroid stimulating hormone (TSH) concentrations above 30 mIU/L. All patients had undergone two week of LID before I-131 administration. From each patient, morning spot urine specimens were obtained twice, before and two weeks after methods based on the Sandell-Kolthoff reaction. According to WHO criteria, UIE concentrations below 50 µg/L were considered moderately iodine deficient, and the levels ≥ 50 µg/L but < 100 µg/L correspond to mild iodine deficiency. These criteria are regarded as adequate LID preparation for I-131 ablation therapy. The cut off value of UIE, which indicates poor LID preparation, was ≥ 100 µg/L.

Results: After LID preparation for 2 weeks, UIE levels lower than 50 µg/L were noted in 15 out of 70 patients (21.4%). The levels ≥ 50 µg/L but < 100 µg/L was noted in 22 of 70 patients (31.4%). There were 33 patients (47.1%) inadequately prepared with UIE levels over 100 µg/L after 2 weeks. The highest UIE value in these patients was 601.12 µg/L. The median values of UIE before LID and 2 weeks after LID were 133.54 µg/L and 99.99 µg/L. These values were not significantly different ($p=0.09$ by Wilcoxon signed-rank test).

Conclusion: Our results show that successful iodine depletion was accomplished only in 52.8% (37/70) of patients after LID for 2 weeks. To obtain a higher LID success rates, a more strict LID preparation program to all patients by specially trained personnel may be required to increase patient compliance with LID to achieve an adequate decreased of the body iodine pool before I-131 therapy.

Keyword: Well-differentiated thyroid cancer, Low iodine diet, Urinary iodine excretion.

1. Introduction

Thyroid carcinoma is one of the most common endocrine malignancies. Well-differentiated thyroid carcinomas, primarily papillary or follicular, accounts for over 90% of the malignant lesions ⁽¹⁾. The first treatment of choice is surgical excision followed by postoperative radioiodine treatment (RAIT) to destroy any remaining thyroid tissue in the thyroid bed area and any microscopic traces of thyroid cancer that remain in the body. For successive RAIT, appropriate radiation dose to the thyroid remnant or tumor must be delivered. In preparation for receiving RAIT, patients are usually asked to go on a low-iodine diet (LID) for a period of 1–2 weeks. Radioiodine is more binding to the cancer cells with a lower level condition of iodine and increasingly transfers to the thyroid gland via the sodium-iodine symporter ⁽²⁾. The purpose of a low-iodine diet is to deplete the whole-body iodine pool to help increase the effectiveness of the RAIT from increasing the binding ability of radioiodine to the cancer cell ⁽³⁾. The ATA (American Thyroid Association) recommended limited dietary iodine to less than 50 micrograms per day for 1–2 weeks ⁽⁴⁾. To assess iodine nutritional status, evaluation of body iodine pool by urine analysis is recommended before RAIT. The European Association of Nuclear Medicine (EANM) ⁽⁵⁾ remarked that a condition with a content of iodine higher than 150-200 µg/l in a patient blood is not suggested to be treated by radioiodine. This indicates that the restriction of consuming food containing high levels of iodine is strongly recommended for thyroid cancer therapy.

Urinary iodine excretion reflects very recent iodine intake. Thereby the analysis of urinary iodine concentrations can be an alternative method to indicate nutritional status of iodine. It is a more popular method than other ways. In addition, urine is more easily collected than blood or serum. WHO (World Health Organization) recommends the median urinary iodine excretion (UIE) of 100–199 µg/L as optimal iodine nutrition.

This study aims to measure and compare a concentration of urinary iodine excretion (UIE) in thyroid cancer patients before and after the patients had limited themselves from consuming food containing high amounts of iodine.

2. Materials and Methods

2.1 Patients

A total of 70 patients with differentiated thyroid cancer (54 females and 16 males) were enrolled. Their mean age was 45±15 years (range 18-79 years). The TSH levels were greater than 30 mU/L in all patients. They were preparing for first ablation after thyroidectomy due to papillary and follicular thyroid cancer. Sixty four patients (91%) had papillary and 9 patients (9%) had follicular cancer. Twenty seven patients have median peak TSH of 80.3 mU/L and more than 100 mU/L in 43 patients. All patients had undergone two weeks of LID before RAIT administration. The patient characteristics are summarized in Table 1. This study had been approved by the Institutional Review Board, Siriraj Hospital Medical School.

Table 1. Patient characteristics

	<i>All (n=70)</i>
Age (yr)	
Mean	45
Range	18-79
Sex	
Female	54 (77%)
Male	16 (22%)
Histological type	
Papillary	64 (91%)
Follicular	6 (9%)
Peak TSH (mU/L) (<i>n= 27</i>)	
Median	80.3
Range	30.52 – 99.11
TSH> 100 mU/L (<i>n=43</i>)	
Administrated activity of I-131 (mCi)	
Range	30-150

2.2 Low iodine diet

In preparation for receiving RAIT, patients are given instructions to deplete their body iodine stores by restriction of daily iodine intake through a low-iodine diet (LID). The usual time period is two weeks. Patients were also informed to strictly adhere to instructions, for their own benefits, to help increase the effectiveness of the RAIT.

Urine iodine depletion was categorized based on UIE concentrations. According to World Health Organization (WHO) criteria for an assessment of iodine nutritional status: severe deficiency, 0–19 µg/L; moderate deficiency, 20–49 µg/L; mild deficiency, 50–99 µg/L; optimal iodine nutrition, 100–199 µg/L; more than adequate iodine intake, 200–299 µg/L; and excessive iodine intake, >300µg/L) ⁽⁶⁾. Median urinary iodine concentrations of >100 µg/L define a population that has no iodine deficiency. UIE <50 µg/L were considered moderately iodine deficient, whereas levels ≥50 µg/L but <100 µg/L correspond to mild iodine deficiency. These two criteria are regarded as optimum LID for RAIT. The cutoff value of UIE indicating inadequate LID preparation was >100 µg/L.

2.3 Urine iodine analysis

The morning spot urine specimens were obtained twice from each patient because it is less subjective and also most convenient for the patient. The first urine specimen was collected a day before starting a low iodine diet and second sample after keeping low iodine diet for two weeks. The most common method recommended by WHO ⁽⁶⁾ was used. This method based on the spectrophotometric measurement of the Sandell–Kolthoff reaction ⁽⁷⁾ in which iodide catalyzes the reduction of ceric ammonium sulfate (yellow) to the colorless cerous form in the presence of arsenious acid. The ammonia persulfate was also used to dissolve organic substance that might influence the result ⁽⁸⁾. It has an advantage of taking short analysis time. The sensitivity of the method is 8 µg/L.

2.4 Statistical Analysis

The median UIE before and after instruction has been given were compared by using Wilcoxon Signed-Rank test. The statistical data was considered significant at $p < 0.05$.

3. Results

After LID preparation for 2 weeks, UIE levels lower than 50 $\mu\text{g/L}$ were noted in 15 out of 70 patients (21.4%). The levels 50–100 $\mu\text{g/L}$ was noted in 22 out of 70 patients (31.4%). There were 33 patients (47.1%) inadequately prepared with UIE levels over 100 $\mu\text{g/L}$ after 2 weeks (Table 2).

Table 2 Portion of patient in each criteria of UIE

Criteria of UIE	Patients (N=70)
<50 $\mu\text{g/L}$	15 (21.4%)
50-100 $\mu\text{g/L}$	22 (31.4%)
>100 $\mu\text{g/L}$	33 (47.1%)

The median values of UIE before LID and 2 weeks after LID were 133.54 $\mu\text{g/L}$ (range: 22.19 – 549.55) and 99.99 $\mu\text{g/L}$ (range: 6.52 – 601.12). These values were not significantly different ($p=0.09$ by Wilcoxon Signed-Rank test). The highest UIE value in the group of inadequately prepared was 601.12 $\mu\text{g/L}$.

4. Conclusion and discussion

According to the World Health Organization's (WHO) report, profile of UIE in the morning urine specimens provides an adequate assessment of the recent dietary iodine intake⁽⁶⁾. UIE concentrations $\leq 50 \mu\text{g/L}$ and 50–100 $\mu\text{g/L}$ indicates a moderately and mild iodine deficient state respectively. These criteria are regarded as adequate LID preparation for I-131 ablation therapy. The cut off value of UIE, which indicated poor LID preparation, was $> 100 \mu\text{g/L}$.

The results showed that a successful iodine depletion was accomplished only in 52.8% (37/70) of patients, and 47.1% had the iodine sufficient status (>100) after LID for 2 weeks. According to Tomoda, *et al*⁽⁸⁾, 70% of patients reduced their UIE to $< 100 \mu\text{g/L}$ after 2 weeks LID, and 35% patients had moderate iodine deficient. Margareta *et al*⁽⁹⁾ reported 88% of patients were able to achieve iodine deficient status (82% to mild iodine deficient state, and 6% patients had gained moderate iodine status deficient state). The possible reason behind iodine reduction is that differences of iodine level in each country's food and it can be the eating habit of the patients themselves. Some of the patients have a very high iodine level and it is difficult to get back to normal level. To obtain a higher LID success rates, a more precise LID preparation program to all patients by specially trained personnel may be required to increase patient compliance with LID for achievement an adequate reduction of the body iodine pool before RAIT. Refer to Margareta *et al*⁽⁹⁾, LID was explained to the patients and sent home with a list of dietary recommendations. Morsch *et al*⁽¹⁰⁾ and Morris *et al*⁽¹¹⁾, participants received the instructions about the diet except for the duration, and they were also counseled on avoiding non-dietary iodine sources.

In Thailand, ATA guidelines were adopted for management of thyroid cancer, which recommended a temporary LID prior to RAIT. The measurement of UIE is iodine sufficiency and direct reflection of dietary iodine intake. However, this method is not widespread

throughout Thailand. Therefore, this research aims to initiate evaluation of UIE with thyroid cancer patients following the ATA guidelines. These results provide an essential data for future studies regarding preparation for radioiodine therapy.

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Genetic Analysis of Sodium Voltage-gated Channel Alpha Subunit 5 (SCN5A) in Thai Cadavers Died with Sudden Unexplained Nocturnal Death Syndrome

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Abstract

Objective: Sodium voltage-gated channel alpha subunit 5 (SCN5A) polymorphisms have previously been identified and revealed association with sudden unexplained nocturnal death syndrome (SUNDS) patients in various populations. This study therefore aimed to characterize variations in this gene in Thai cadavers died with SUNDS or “Lai-Tai” in Thai.

Materials and Methods: Five genomic DNA samples collected from Thai SUNDS cadavers were processed for variation screening by polymerase chain reaction followed by Sanger sequencing. Sequence alignments with SCN5A reference sequence were then analyzed by computational programs. All variations detected were evaluated with the dbSNP database and unclassified variations were assessed for the deleterious effects using online prediction programs (MutationTaster, PROVEAN and Human Splicing Finder 3.0).

Results: A total of eleven previously reported polymorphisms in SCN5A were identified in Thai SUNDS cadavers; including two unclassified variations and nine variations related with cardiac conduction defects. The two unclassified variants (rs11414422 and rs4592631) were predicted to be polymorphisms. Of the nine variations, five (rs6599230, rs1805124, rs1805126, rs7429945 and rs41315485) were formerly reported to be associated with cardiac arrhythmias and another two variants (rs4073797 and rs4073796) were described to increase the risk of Brugada syndrome.

Conclusion: This study was able to identify a total of eleven variants in SCN5A, most of which would be possibly associated with SUNDS. Further study is needed to confirm the results and elucidate the association of these variations and SUNDS in Thai population.

Keywords: Genetic variation, Lai-Tai, SCN5A, SUNDS, Thais

1. Introduction

Sudden unexplained nocturnal death syndrome (SUNDS) or Lai-tai in Thai is a disorder that can cause a sudden death in young and healthy adults during sleep. The autopsy reports showed no identifiable abnormal structural heart and the history of any heart diseases or related disorders. The age of the deceased is between 21 and 40 years (mean age 40 ± 15 years).^(1, 2) Previous studies have shown that this disorder can affect males more than females with the ratio 10:1. Its incidence is estimated approximately 1:1,000 per year and its prevalence is 20:100,000 in the population worldwide and higher in Southeast Asia (especially Thailand, Laos, Cambodia, Vietnam, Japan and China) at 26-38 in 10,000.⁽³⁻⁷⁾

There are several etiologies proposed for SUNDS including hormones, weather, food consumption behavior, and genetic factors.⁽⁸⁾ Approximately, 20 - 30% of SUNDS patients have family history and 30% of them have identifiable pathogenic mutations.⁽⁸⁻¹¹⁾ SUNDS is inherited as autosomal dominant pattern.⁽²⁾ Since its phenotypes were previously reported to be similar to Brugada syndrome which is caused by the mutations of the gene encoding for voltage-gated sodium channel type 5, alpha subunit (*SCN5A*), the genetic screening of this gene has been performed in SUNDS patients in various populations.^(3, 11, 12) However, the association between the genetic variations of this gene and Thai SUNDS cadavers is still unknown. This study, therefore, aimed to characterize variations in this gene in Thai cadavers died with SUNDS, which would be beneficial for understanding the etiology of this syndrome in Thai SUNDS cadavers and for the deceased's relatives who might be at risk.

2. Materials and methods

2.1 Sample enrollment

Thai SUNDS cadavers were recruited from the Department of Forensic Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. All the subjects were evaluated for SUNDS criteria; the cadavers who were previously healthy and died during sleep without anatomic and toxicologic causes of death. The exclusion criteria of subjects were the presence of decomposition, formalin injection, severe injury leading to incapability of blood collection, blood transfusion within 2 years⁽¹²⁾, having conditions associated with the genetic variations of *SCN5A* (cardiovascular diseases, etc.) or history of cardiac conduction disorders (any forms of cardiac arrhythmias), having obtained medical treatments of radiotherapy, chemotherapy or bone marrow transplantation, and any genetic diseases. All blood samples were collected from femoral veins of the cadavers. Genomic DNA was then extracted from the blood samples and its quality and quantity were evaluated by Nanodrop[®] (ND-1000, Thermo Scientific, Waltham, MA, USA)⁽¹³⁾.

2.2 DNA analysis

The DNA samples were amplified by polymerase chain reaction (PCR) using 41 pairs of primers spanning all of the 28 coding exons of *SCN5A* gene. The PCR products were sequenced using an automated ABI PRISM 310 genetic analyzer with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) to detect the *SCN5A* genotype. The sequencing data were aligned with *SCN5A* reference sequence by computational programs. All variations were evaluated with dbSNP database. Novel or unclassified variations were assessed for the deleterious effects by using online prediction programs (MutationTaster, PROVEAN and Human Splicing Finder 3.0).

2.3 Statistical analysis

For descriptive analysis, categorical variables are presented as frequency and continuous variables as mean $\pm$ standard deviation and range. The Statistical Package for the Social Sciences (SPSS) software for Windows version 18.0 (PASW[®] Statistics 18, SPSS Inc., IBM Company, Chicago, Illinois, U.S.A.) was used for analysis.

3. Results

3.1 Thai SUNDS cadavers

A total of five Thai SUNDS cadavers were recruited in this study. All of them were males and from Northeastern region of Thailand. The cadavers aged between 20 to 45 years (mean age 33.2 ± 9.3 years). All cases were healthy with normal heart structure and died in summer (April-June). Of these cases, only one had a family history of SUNDS (**Table 1**).

Table 1 The epidemiologic data of Thai SUNDS samples

SUNDS case no.	Sex	Age	Body mass index	Month of death	Resident province	Family history
1	Male	20	32.8	June	Yasothon	Yes
2	Male	30	22.9	April	Surin	No
3	Male	33	25.1	May	Khon Kaen	NA
4	Male	45	20.1	May	Udon Thani	NA
5	Male	38	24.5	May	Surin	NA

NA = Not available

3.2 SCN5A gene screening

Sequence analysis of 41 fragments of SCN5A revealed 11 variations in either homozygous or heterozygous forms (**Figure 1**). All variations were described as polymorphisms, including one non-synonymous variant (rs1805124 in exon 12), three synonymous variants (rs6599230 in exon 2, rs7430407 in exon 17, and rs1805126 in exon 28) and seven variants in 3'UTR (rs7429945, rs41310757, rs4073797, rs4073796, rs11414422, rs41315485 and rs45592631) as shown in **Table 2**. There were two single nucleotide polymorphisms (SNPs), rs7430407 and rs45592631, which showed only homozygous major alleles.

However, of seven variants in 3'UTR, one (rs45592631) showed minor allele frequency less than 0.01 (0.0068), and another two variants (rs11414422 and rs4592631) were unclassified. Therefore, the two variants were further evaluated for the functional effects by three prediction programs and the results showed that they were predicted to be polymorphisms.

Variation	Relevant wild-type	Heterozygous form	Homozygous form
rs6599230 (c.87A> <u>G</u> , p. Ala29=)	C G C A T G G C <u>A</u> G A G A A G 	C G C A T G G C <u>G</u> G A G A A G c. 87A> <u>G</u> 	C G C A T G G C <u>G</u> G A G A A G c. 87A> <u>G</u>
rs1805124 (c.1673A> <u>G</u> , p. His558Arg)	A G C C A C C <u>A</u> C A C A T C 	A G C C A C C <u>G</u> C A C A T C A c. 1673A> <u>G</u> 	Not available
rs7430407 (c.3183A> <u>G</u> , p. Glu1061=)	Not available	Not available	C A A G A A G A <u>G</u> G A T G A G c. 3183A> <u>G</u>
rs1805126 (c.5457T> <u>C</u> , p. Asp1819=)	T T T G C C G A <u>T</u> G C C C T G 	T T T G C C G A <u>C</u> G C C C T G c. 5457T> <u>C</u> 	T T T G C C G A <u>C</u> G C C C T G c. 5457T> <u>C</u>
rs7429945 (c.*123A> <u>G</u>)	G G C C T C <u>A</u> G C C C C G C 	G G C C T C <u>G</u> G C C C C G C c.*123A> <u>G</u> 	G G C C T C <u>G</u> G C C C C G C c.*123A> <u>G</u>
rs41310757 (c.*753C> <u>T</u>)	C G C A G G T <u>C</u> A T A T T G T 	C G C A G G T <u>T</u> A T A T T G T c.*753C> <u>T</u> 	Not available
rs4073797 (c.*962T> <u>A</u>) and rs4073796 (c.*962T> <u>A</u>)	C C A G C C A <u>T</u> C G G A A G 	C C A G C C A <u>A</u> T G G A A G c.*962T> <u>A</u> c.*963C> <u>T</u> 	C C A G C C A <u>A</u> T G G A A G c.*962T> <u>A</u> c.*963C> <u>T</u>
rs11414422 (c.*1453_1454 insA)	T T T T A A C A T A G A G A A 	T T T T A A C A T <u>A</u> A G A G A A c.*1453_1454insA 	T T T T A A C A T <u>A</u> A G A G A A c.*1453_1454insA
rs41315485 (c.*1537T> <u>C</u>)	T C T C C C A <u>T</u> G G A G C C C 	T C T C C C A <u>C</u> G G A G C C C c.*1537T> <u>C</u> 	Not available
rs45592631 (c.*2145_2146 insGAGAAGAGA GTAGGAAAAAG GAGGG)	Not available	Not available	G G G G A G A A G A G A T A G G A A A A A G G A G G G A A G c.*2145_2146ins

Figure 1 Eleven variations of SCN5A identified in 5 Thai SUNDS cadavers. (rs386660270 can be referred to when rs4073797 (c.*962T>A) and rs4073796 (c.*962T>A) were found together.)

Table 2 *SCN5A* variations in Thai SUNDS cadavers

No	Exon	dbSNP ID	Position	Clinical significance	SUNDS cases					MAF/ Minor allele count (1000 genomes)	Ref.
					1	2	3	4	5		
1	2	rs6599230	c.87A> <u>G</u> g.21452A> <u>G</u> p. Ala29=	Benign allele	A/A	A/ <u>G</u>	<u>G</u> / <u>G</u>	<u>G</u> / <u>G</u>	A/A	T=0.2183/1093	(14-16)
2	12	rs1805124	c.1673A> <u>G</u> g.50744A> <u>G</u> p.His558Arg	With pathogenic allele	A/A	A/ <u>G</u>	A/A	A/A	A/A	C=0.2304/1154	(14, 15, 17-21)
3	17	rs7430407	c.3183A> <u>G</u> g.73697A> <u>G</u> p.Glu1061=	Benign allele	<u>G</u> / <u>G</u>	<u>G</u> / <u>G</u>	<u>G</u> / <u>G</u>	<u>G</u> / <u>G</u>	<u>G</u> / <u>G</u>	T=0.0769/385	(14, 15)
4	28	rs1805126	c.5457T> <u>C</u> g.103758T> <u>C</u> p.Asp1819=	Benign allele	C/C	T/ <u>C</u>	T/T	T/ <u>C</u>	T/C	G=0.4924/2466	(14, 15)
5	28	rs7429945	c.*123A> <u>G</u> g.104475A> <u>G</u>	NA	<u>G</u> / <u>G</u>	A/ <u>G</u>	A/A	A/ <u>G</u>	A/ <u>G</u>	C=0.4944/2476	(14, 15)
6	28	rs41310757	c.*753C> <u>T</u> g.105105C> <u>T</u>	NA	C/ <u>T</u>	C/C	C/C	C/C	C/C	A=0.1144/573	(14, 15)
7	28	rs4073797	c.*962T> <u>A</u> g.105314T> <u>A</u>	NA	<u>A</u> / <u>A</u>	T/ <u>A</u>	T/T	T/ <u>A</u>	T/T	T=0.4918/2463	(14, 15, 22)
8	28	rs4073796	c.*963C> <u>T</u> g.105315C> <u>T</u>	NA	<u>T</u> / <u>T</u>	C/ <u>T</u>	C/C	C/ <u>T</u>	C/C	A=0.4940/2474	(14, 15, 22)
9	28	rs11414422	c.*1453_ *1454 <u>insA</u> g.105805_105806 <u>insA</u>	NA	<u>A</u> / <u>A</u>	-/-	-/-	-/ <u>A</u>	-/-	T=0.3169/1587	(14, 15)
10	28	rs41315485	c.*1537T> <u>C</u> g.105889T> <u>C</u>	NA	T/T	T/ <u>C</u>	T/T	T/T	T/ <u>C</u>	G=0.1795/899	(14, 15)
11	28	rs45592631	c.*2145_ *2146 <u>insGAGAAGAG</u> <u>AGTAGGAAAA</u> <u>AGGAGGG</u> g.106497_106498 <u>insGAGAAGAG</u> <u>AGTAGGAAAA</u> <u>AGGAGGG</u>	NA	<u>ins/in</u> <u>s</u>	<u>ins/in</u> <u>s</u>	<u>ins/i</u> <u>ns</u>	<u>ins/in</u> <u>s</u>	<u>ins/in</u> <u>s</u>	=0.0068/34	(14, 15)

MAF = Minor allele frequency

NA = Not available

4. Conclusion and Discussion

To investigate the association between genetic variations of *SCN5A* and SUNDS in Thai cadavers, five subjects were recruited in this study. All were young males from Northeastern region of Thailand where the highest incidence of SUNDS occur⁽²³⁾. All cases died in summer, reflecting the hot weather as one of environmental factors having an effect on the risk of SUNDS. These results were in accordance with the previous study performed by Tungsanga and Sriboonlue⁽²³⁾.

Genetic analysis of *SCN5A* revealed eleven variations. Interestingly, some variations have been described as pathologic variants of cardiac conduction defects even if they revealed heterogeneous effects. A non-synonymous variant (rs1805124) has been reported to modify expression of an arrhythmia causing mutation⁽¹⁷⁾ and associated with Brugada syndrome⁽²⁴⁾. Additionally, this variant and two synonymous variants (rs1805126 and rs6599230) were previously reported to be associated with cardiac conduction by affecting PR and QRS intervals.⁽¹⁶⁾ Recent studies have reported several regulatory SNPs; i.e. rs41310757 (c.*753C>T), rs4073797 (c.*962T>A), rs4073796 (c.*963C>T) and rs41315485 (c.*1537T>C) which affected gene expression by changing miRNA binding site.^(22, 25) For rs7429945 (c.*123A>G), no defects on splicing sites have been demonstrated by bioinformatics analysis. Computational prediction for the functional effects of the three remaining unclassified variants, rs7430407 (p.Glu1061=), rs11414422 (c.*1453_1454insA), and rs45592631 (c.*2145_*2146insGAGAAGAGAGTAGGAAAAGGAGGG) was found to be only neutral polymorphisms. Although there were no pathogenic mutations identified in *SCN5A*, some reported variations in this gene are interesting to be further studied for genetic association with SUNDS in Thai cadavers.

As a preliminary study, this study was able to identify a total of eleven variants in *SCN5A*, most of which would be possibly associated with SUNDS. Further study is needed to confirm the results and elucidate the association of these variations and SUNDS in Thai population.

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BEI01

Storage Cost Reduction : A Case Study of Star Plus Chemical Co., Ltd.

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Abstract

Growth of property market increased up to 69% from 2010 to 2014. Therefore, it becomes necessary for many property related industries to increase storage capacity. This research aim to find the best alternative for minimizing storage cost in the business of Urea Formaldehyde Polymers' manufacturer, "Starplus Chemical Co., Ltd". Urea Formaldehyde Polymers is the main material in making glue that use in furniture industry. Data was collected through qualitative research, such as interview and existing data of the company and then was recommended possible solutions as well as Pros & Cons of these solutions were discussed in this study. Basic financial instrument analysis to diagnosis the best alternative was gathered to evaluate the possibility of the project. The findings revealed that the company should expand their own storage. Although it is a long-term investment and it will reach at break-even point in about 9 years, they can generate income from rental fees.

Keywords : Cost saving, Storage cost, Urea Formaldehyde Polymers, Future Value, Financial instrument

Introduction:

"The real estate market in Thailand tends to grow dramatically", (Kanana Katharangsiporn). Property Focus 2015 | Bangkok Post: property mentioned that, "the property market is improving gradually and housing demand in Bangkok is stable despite of slowing economy". As a result of this trend, the demand for furniture is also in an increasing fashion.

Starplus Chemical Co.,Ltd. supply Urea Formaldehyde Polymers which is main material in glue production to support furniture industry. Starplus is also facing the storage shortage due to the growth of housing industry. According to Mr.Wanchai Chaijirawiat, the Managing Director (MD) of Starplus Chemical Co.,Ltd, the company is in search of the best solution to minimize cost so as to increase its production ability in the near future. Therefore, it is necessary to prepare the solutions to support this expansion.

Research Objectives:

1. To study storage cost structure of the company
2. To compare and analyze all alternatives means
3. To recommend the best solution

Research Question

1. How to handle the cost and control efficiency?
2. What is the best solution to minimize storage cost?

Literature Review

1. Inventory Management

Dimitrios P. Koumanakos (2008) studied the effect of inventory management on firm performance. The methodology of the study was based on data analysis by using ICAP database. The result of the study revealed that investment principles should be concerned in discussion and will be of interest to those in same topics. In





2004, Drew Stapleton, Sanghamitra Pati, Erik Beach and Poomipak Julmanichoti presented the case study about Activity-based Costing (ABC) for logistics and marketing. The methodology is an analytical concept of ABC, how ABC provides many kinds of customers, how ABC can make customer delighted in marketing department. The result of this study focuses on using ABC; the company can control and manage the costs efficiently and effectively.

2. Process Improvement

Anne Webster (1999) presented the continuous improvement improved by utilizing the lessons to define the main components of the approach to continuous improvement. The result of the study revealed that each organization have to develop an approach to continuous improvement, which is suitable for the organization and its chosen mission. Jay Bal (1998) studied the process analysis tools for process improvement. The methodology in this study is about comparison hardware and software features, user features, modeling capabilities, simulation capabilities, analysis capabilities, and integration capabilities. The result of the study revealed that the process analysis tools is more proper for operational rather than strategic process.

3. Cost Analysis

Anni Lidholm and Petri Suomala (2007) presented a case study about learning by costing to discuss life cycle cost management and highlight the practical challenges. The methodology of this paper was conducted in Finnish Defense Forces and developed by a life cycle cost model for a case product. "Operating cost analyses of long-term care facilities," proposed by Thomas Madritsch and David Steixner (2008). The methodology of this study is questionnaire. The result of the research was helpful to establish cost benchmarking and develop it as a strategic planning tool in order to support management in the healthcare sector in the decision-making process.

Research conceptual and framework

The review of theoretical and related literatures suggested the conclusion as; the company should focus on its storage cost because it is one of the important factor to be considered while managing and running business. In order, to minimize the storage cost the company should have an efficient logistic activity. While considering the process improvement or the cost analysis, the company should also compare the pros and cons of the efficient logistic activities and the cost related to it. If the company take into consideration these cost then it might be helpful to make decision in favor of reducing the storage cost.

Research Methodology

- 1) Define the problems: to define the problems by studying the background of the company, product, and raw materials
- 2) Study the possible solutions: to study the possible solutions to minimize the storage cost
- 3) Collect the data: Collect the data by interviewing the management team
- 4) Analyze the influential factors: Analyze the factor that can impact the cost of storage data
- 5) Compare and propose the data: Comparing the guidelines and propose the best way to minimize the cost
- 6) Conclusion: Make a conclusion as a guideline for the company to make a decision in the future

Data Collection

All data were collected from internal company information such as company reports, research papers, case studies done in logistics and related field etc are involved in this study.

Data Analysis

The data collected were analyzed using , first finding the solution for reducing storage cost by studying the company cost structure and the cost of own storage and then comparing with the outsourcing companies cost so as to find the difference if any





Result and Discussion

From the data analysis, it was concluded that the guidelines to solve this problem is to rent the storage at port, rent the third parties storage area and expand the storage area.

Now, if the company uses the first guideline; rent the storage at port, this will affect the cost because the rental fee is high and besides rental fee, the company need to pay others expense (i.e. demurrage and detention free times, relocations fee, etc.) which will cost more money in the end. The estimate rental cost at the port is as below;

Assume that the cargo is 200 containers per lot

Rental for a month:

Day 1 – Day 7 = 125 baht/day/containers = $125 \times 7 \times 200 = 175,000$ baht

Day 8 – Day 14 = 250/day/containers = $250 \times 7 \times 200 = 350,000$ baht

Day 15 onward = 500/day/containers = $500 \times 15 \times 200 = 1,500,000$ baht

Total is 2,025,000 baht per month

Rental for 3 months: $2,025,000 \times 3 = 6,075,000$ baht

Rental for 6 months: $2,025,000 \times 6 = 12,150,000$ baht

From the assumption above, the conclusion is that the company will face a storage cost problem so they will need to find other solutions to minimize cost.

1. Rent the other storage area

Evalon Textile (Thailand) Company Limited is located in Hemraj industrial Park at 335/1 Moo 10 Nonglallork, Bankai, Rayong province. They allow Starplus Chemical Co.,Ltd. to rent the area as storage for the raw material. The distance between the plant is only 50 meters so , this makes it more convenient to transport. The Evalon Textile Co.,Ltd. settled the rental fee at about 65 Baht per Squaremeters and Starplus Chemical Co.,Ltd. want to rent the area for 1000 Squaremeters for storing urea so, the rental fee per month will be 65,000 baht. The urea was imported into the containers by 50 kg bags. There are 400 bags per containers. Then, Starplus Chemical Co.,Ltd. will repackage from 50 kgs bags into one jumbo bag which contains 1 tons to save the area for storage and easy to use in production line.

As a result, the rental fee can be separated as below;

Assume the rental fee by 3 months, 6 months, and 12 months

1 Month	3 Months	6 Months
$65 \times 1000 \times 1 = 65,000$ baht	$65 \times 1000 \times 3 = 195,000$ baht	$65 \times 1000 \times 6 = 390,000$ baht

2. Expand company own warehouse

Table1 The construction cost

Detail	Calculation	Cost
Construction price	$1,600 \times 5,000$	8,000,000
Other expense (i.e. electricity, security system)		1,000,000

From table 1, the construction cost will be equal to $1,600 \times 5,000 = 8,000,000$ Baht plus approximation for other expense such as electricity, security system, air control etc. 1,000,000 baht. Therefore, Starplus Chemical Co., Ltd. needs to invest $8,000,000 + 1,000,000 = 9,000,000$ Baht to expand the storage. Normally, the peak period for stock inventory is 3 months so the company can lease their storage for other company, which needs to rent the space.





Assume the rental fee that the company set up is 65 baht per square meters.

Assumption 1 If the company has no shipment, they can lease full area to other companies

1 Month	3 Months	6 Months
$65 \times 1600 \times 1 = 104,000$ baht	$65 \times 1600 \times 3 = 312,000$ baht	$65 \times 1600 \times 6 = 624,000$ baht

From the table, if the company leases the area for 1 month, they will gain 104,000 baht. If there is no problem about the inventory, they can make money up to 624,000 baht in 6 months.

Assumption 2 if the company have shipment, they can leasing 600 square meters to other companies

1 Month	3 Months	6 Months
$65 \times 600 \times 1 = 39,000$ baht	$65 \times 600 \times 3 = 117,000$ baht	$65 \times 600 \times 6 = 234,000$ baht

From the table, if the company leases some area to other companies, they will gain 39,000 per month. If they have problem about inventory, they still gain up to 234,000 baht in 6 months.

After interviewing the top management, they are planning to expand the factory in the next 5 years to increase the production ability to 100,000 tons per year as the demand of furniture market in Southeast Asia tends to increase, so it is reasonable to expand the area for support the business.

3. Compare cost of the guideline

After collecting the data, the cost of the guideline will be compared as below;

Table2 the comparison of all alternative to solve the storage problem

Guideline	1 month (B1)	3 month (B2)	6 month (B3)
Rent the storage at port (A)	2,025,000 Baht	6,075,000 Baht	12,150,000 Baht
Rent the other storage area	65,000 Baht	195,000 Baht	390,000 Baht
Expand the storage area (C)	The investment for expanding storage will be 9,000,000 Baht		

Table3 Cost saving compared with A

Solution	1 month	3 months	6 months
Rent the third parties storage (B)	1,960,000 baht	5,880,000 baht	11,760,000 baht
Expand company's own warehouse (C)	3,150,000 baht		

From the table 3 if the company chooses the solution B, they can save 1,960,000 baht per month, 5,880,000 baht per 3 months, and 11,760,000 baht per 6 months. On the other hand, if they choose the solution C they can save 3,150,000 baht for rental fee. The breakeven for solution C will be 7 months when compared with the solution A





Table 4 Breakeven for solution when compared with Solution B

Expand company's own warehouse (C)	3 months	6 months
Breakeven compared with (B)	46.2 or about 46 years 2 months	23.07 or about 23 years

Assume that the company rent out all the area for other companies or for own usage. The receivable will be $1600 \times 65 \times 12 = 1,248,000$ baht per year. (When 65 is the assumption rental fee as the same with the solution B). If the company chooses this solution, they can breakeven in 7 years. Moreover, we are deciding to calculate Modified Internal Rate of Return (MIRR) for evaluating the cost and profitability of this project to make the solution reasonable.

The definition of MIRR is while the internal rate of return (IRR) assumes the cash flows from a project are reinvested at the IRR; the modified IRR assumes that positive cash flow are reinvested at the firm's cost of capital, and the initial outlays are financed at the firm's financing cost. Therefore, MIRR more accurately reflects the cost and profitability of a project ("Modified Internal Rate of Return", 2003)

The formula for MIRR is

$$MIRR = \sqrt[n]{\frac{FV(PositiveCashFlows, costofcapital)}{PV(InitialOutlays, FinancingCost)}} - 1$$

Assume that the Minimum Retail Rate (MRR) from the bank is 7.85% per year. So, it will be 0.65% per month. For the rental fee, it may increase 5% per year. We assume that the project will last for 10 years. Therefore, the MIRR of the project can be calculated as follow; the PV will be 10,504,585.24 and the FV will be 26,944,955.50 so the MIRR will be 11.01%. By analyzing the present value of the income streams, we found that it will take about 9 years and 5 months to reach the breakeven with 9,047,685.51 baht.

Therefore, in case that the company have to make a decision for the guideline, if the company is concerned about the storage cost and they are convinced that the shipment will be delayed every year, the company should choose to rent the storage from Evalon Textile (Thailand) Co.,Ltd because the rental fee is cheap and it is very convenient to transport the raw material to their plant. For security, the company can support a guard to stay at Evalon's storage to prevent any damages and lost that may occur. On the other hand, if the company is concerned about long-term investment, they should expand the company storage because they can keep other raw materials and spare parts for company usage or lease to other companies. In terms of cost, they can break even in 7 years so after that they do not need to be concerned about the storage cost or other expenses and the MIRR shows that the return of investment will occur in 9 years and 5 months and the discount rate is 9,047,685.51 baht. So if the company is planning to expand the factory to increase production ability, expanding the storage area should be the best solution to solve this problem.

Conclusion the study

The summary is based on the findings and the discussions relating to the research aim and objectives. This research specifies on finding the reduction of the storage cost. At present, the company chooses to storage shipment at the port when they do not have enough capacity at the plant. This will cost them 2,025,000 baht per month. The findings of this research showed that the company should choose in-between the cost of either to rent the storage at port, rent the storage from other company, or expand the company's store. The result showed that renting the storage from other company is 65,000 baht per month, which will save cost more than storage at port, which is about 1,960,000 baht per month. To compare with expanding the company's storage, the company must invest 9,000,000 baht in the first year and will meet break even in 7 years when compared with renting the third parties' storage. The advantage of renting the storage from other company is that the rental fee is cheapest and it is convenient to relocate back to the plant due to the distance between the companies is only 50 meters, so they can use forklift or small truck to relocate the urea. The disadvantage is that the security is quite low because there is no CCTV and a few security guards, so the cargo can be damaged or lost. The advantage of expanding





the company's store is that they do not need to pay for rental fee and other expenses that may occur in the future. Moreover, they can transport the cargo into their storage as soon as the shipment arrives. Besides, they can gain money from leasing an empty area to other companies. The disadvantage is that they need to invest money to construct the storage first and this is a long-term investment. They also need to be concerned about the other expenses that may occur such as employment, maintenance, and other instruments.

From the reason that was explained above, as the company is planning to expand the plant in the next 5 years in order to support the growth of furniture market so they want to maximize the production ability at 100,000 tons per year. Therefore, the company should be expanding their own storage area to support the amount of the shipment in the near future. Although, the investment is high but it is a guarantee space to support the cargo. Besides, they can rent out the remaining area to other companies to increase profit. Overall, it is the best way to minimize the storage cost in the near future.

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Pussacha Jangsiripornpakorn

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BEI02

Professional Growth and Development among Business Administration Teachers of Selected Universities in Baguio City

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Abstract

The purpose of this study was to evaluate the status of professional growth and development among business administration teachers of selected universities in Baguio City. This study made use of the descriptive research method with the questionnaire as the principal tool for gathering data. All the specific research problems were answered by means of the data gathered through the questionnaire. These were supplemented by informal interviews with the respondents. The findings show that business administration teachers are *much involved* in opportunities for professional growth and development. The findings also show that business administration teachers are *very much motivated* in pursuing professional growth and development activities. The challenges encountered in the pursuit of professional growth and development is *moderately serious*.

Based on the findings of the study, the teachers manifested a greater extent of involvement in individual-led continuing professional development (CPD) activities. This shows that the teachers take initiative and action for their own professional growth. With respect to the hierarchy of needs, the teachers were motivated most by self-actualization needs which is at the apex of the hierarchy. This shows that the teachers are intrinsically motivated to pursue CPD activities and they continue to act and live up to their potential, personal satisfaction and self-fulfilment. The teachers encountered less challenged in individual – led CPD, which shows that they are more inclined to pursue CPD on their own initiative.

Keywords: development, growth, motivation, opportunities, teacher

Background of the Study

Teaching, like all professions, is rooted in the development of its practitioners' individual expertise. The profession of teaching demands that teaching professionals demonstrate professionalism by being intentionally inviting in functioning as teachers. The highest-level teacher is intentionally inviting (Novak & Purkey, 2009). This view reflects the recent developments in how people involved in education view teaching and the teaching profession. Teachers have traditionally been viewed as individuals who existed at the bottom of a bureaucratic and hierarchical system. In this view, teachers were responsible mainly for implementing the plans and designs conceived by upper echelon educational managers. Today, a more professional view of teaching holds that effective teachers believe that they control their own classroom destinies and they demonstrate behaviors related to self-actualization and empowerment. This develops through a strong sense of self-esteem and self-efficacy (McCabe, 2003).

Today's environment requires that teachers keep up with new and ever-changing conditions and challenges and are willing to devote a significant portion of their time to learn new and more effective ways of teaching. As responsible professionals, teachers should grow in the profession of teaching by attaining expertise and by acquiring an expanding repertoire of effective teaching practices (Arends & Kilcher, 2010). Traditionally, training and development was not viewed as an activity that could help companies create value and successfully deal with competitive challenges. It used to be viewed as merely an expense and therefore, must be minimized or avoided. Today, training and development is viewed as a tool for a company to meet competitive challenges.





Training nowadays is not just an option of faculty development but the need of the changing environment. To meet the global challenges, now the universities of Pakistan are focusing on quality assurance in terms of faculty development (Bhatti, 2011). A higher education institution projects a tri-fold function: instruction, research, and extension. As such, a comprehensive and efficacious faculty development program embodies activities that enhance effective instruction, with increasing involvement in action research for improvement of existing practices, and collaborative efforts towards community extension programs.

It is in the above contexts that this study on the professional growth and development of the faculty of business administration schools in Baguio City was conceived.

The underlying concept upon which this study is anchored is the concept of continuing professional development or CPD, also referred to as career development. The main purpose of this study was to determine the status of professional growth and development among business administration teachers of selected universities in Baguio City. Specifically, it sought to find answers to the following problems:

1. What is the level of involvement in the different opportunities for systems-led, individual-led, and profession-led continuing professional development to which business administration teachers responded?
 - 1.1 Are there significant differences in the level of involvement in the different opportunities for systems-led, individual-led, and profession-led continuing professional development to which business administration teachers responded considering the moderator variables?
2. What is the level of motivation of teachers to pursue identified professional growth and development activities?
 - 2.1 Are there significant differences in the level of motivation of teachers to pursue professional growth and development activities considering the moderator variables?
3. What is the degree of seriousness of the challenges encountered by the faculty in the pursuit of professional growth and development?
 - 3.1 Are there significant differences in the perceptions of the respondents regarding the degree of seriousness of the challenges they encountered in the pursuit of professional growth and development considering the moderator variables?

Material and Methods

This descriptive-survey research used questionnaire as its main tool to gather data. The tool was pre-tested with 0.95 overall coefficient, indicating high reliability for the tool. Each statement was measured on a four-point Likert-scale with the following description:

On opportunities for CPD

Weight	Scale	Qualitative Interpretation	Rubrics
4	3.26-4.00	Very Much Involved	Excellent level of involvement; teacher participates very often actively in almost all CPD activities, given the opportunity
3	2.51-3.25	Much Involved	Very satisfactory level of involvement; teacher participates actively in most CPD activities given the opportunity
2	1.76-2.50	Moderately Involved	Moderate level of involvement; teacher participates actively in some CPD activities, given the opportunity
1	1.00-1.75	Not Involved	Poor level of involvement; teacher does not avail/participate in CPD activities





On motivations to pursue CPD

Weight	Scale	Qualitative Interpretation	Rubrics
4	3.26-4.00	Strongly Agree	The teacher is highly motivated and pursues CPD at a very considerable extent.
3	2.51-3.25	Moderately Agree	The teacher is moderately motivated, and pursues CPD at a moderately considerable extent.
2	1.76-2.50	Slightly Agree	The teacher is fairly motivated and pursues CPD at a very minimum extent.
1	1.00-1.75	Disagree	The teacher lacks motivation to pursue CPD.

In scoring the motivational questionnaire, the item numbers that correspond to indicators of the different levels of Maslow's Hierarchy of Needs were grouped together to simplify tabulation of data.

Basic Needs	Safety Needs	Belonging Needs	Esteem/Ego Needs	Self-Actualization Needs
#1	#5	#7	#4	#2
#3	#10	#9	#6	#15
#14	#13	#12	#8	#16
#17	#20	#19	#11	#18

On challenges encountered in CPD

Weight	Scale	Qualitative Interpretation	Rubrics
4	3.26-4.00	Very Serious	The challenge is very considerably extensive.
3	2.51-3.25	Moderately Serious	The challenge is moderately extensive.
2	1.76-2.50	Slightly Serious	The challenge is at a minimum extent.
1	1.00-1.75	Not a Problem	There is no challenge.

The study was conducted in Baguio City during the first semester of school year 2013 to 2014, specifically at Baguio Central University, University of Baguio, University of the Cordilleras, and Saint Louis University schools of business administration. To observe professional ethics, letters (A, B, C, and D) were designated to refer to each university but not according to their order in Table 1.

The population was composed of a total enumeration of teachers handling business administration courses in the four universities mentioned.

Table 1

The Population of the Study (N=41)

School	Total Number of Business Administration Teachers	Participants	Percentage
BCU	7	5	71.43
UB	11	11	100
UC	23	13	56.52
SLU	22	12	54.55
Total	63	41	65.07

Table 1.1

The Population of the Study According to Educational Attainment

School	Bachelor's Degree	Master's Degree	Doctorate Degree	Total
BCU	1	3	1	5
UB	1	10	0	11
UC	1	12	0	13
SLU	2	9	1	12
Total	5	34	2	41



**Table 1.2***The Population of the Study According to Field of Specialization*

School	Management	Finance	Marketing	Others
BCU	2	2	0	1
UB	5	5	1	6
UC	5	5	3	2
SLU	7	5	1	3

*Multiple responses

Table 1.3*The Population of the Study According to Employment Status*

School	Full-Time	Part-Time	Total
BCU	3	2	5
UB	10	1	11
UC	12	1	13
SLU	9	3	12
Total	34	7	41

Table 1.4*The Population of the Study According to Years of Teaching Experience*

School	0	1-5	6-10	11-15	16 and above	Total
BCU	0	3	1	1	0	5
UB	0	3	2	4	2	11
UC	1	8	2	0	2	13
SLU	0	4	3	2	3	12
Total	1	18	8	7	7	41

Table 1.5*The Population of the Study According to Years of Industry Experience*

School	0	1-5	6-10	11-15	16 and above	Total
BCU	1	3	0	1	0	5
UB	1	4	2	0	4	11
UC	2	6	2	2	1	13
SLU	4	2	1	2	3	12
Total	8	15	5	5	8	41

Table 1.6*The Population of the Study According to Subjects Taught*

School	Basic Business	Business Education	Professional Subjects	Practicum
BCU	3	4	3	2
UB	8	7	10	1
UC	13	8	8	2
SLU	10	8	9	5

*Multiple responses

The questionnaires were administered among the teachers handling business administration courses in the four universities concerned. Interviews with some teachers were also conducted to corroborate the answers taken from the questionnaires.

The accomplished questionnaires were retrieved, tallied, collated, and the data were tabulated and interpreted accordingly. Answers to the questions asked during the informal interviews were recorded and used to corroborate the results of the study.





Results and Discussion

Opportunities for Professional Growth and Development Activities Systems-Led CPD

As shown in the table, business administration teachers were *moderately involved* (2.12) in systems-led CPD. The highest rated indicator in this sub-area with a mean of 2.71 is “Institutional lecture, exhibits, poster presentations, and echo-seminars” interpreted as *much involved*. Attendance of teachers in activities that are accessible to them and those that do not require much in terms of money and time is highly probable. Moreover, activities that are compulsory are more likely to be attended by teachers.

Table 2

Extent to which Business Administration Teachers were involved in Opportunities for Systems-Led Continuing Professional Development (N=41)

Indicators	Mean	Interpretation
1.1 Government-sponsored conferences and workshops	1.88	Moderately Involved
1.2 Sabbatical leave	1.15	Not Involved
1.3 Participation in educational tour	1.88	Moderately Involved
1.4 Institution-sponsored graduate studies	2.37	Moderately Involved
1.5 Institutional lecture, exhibits, poster presentations, and echo-seminars	2.71	Much Involved
1.6 Industry immersion	2.02	Moderately Involved
1.7 Invitation to become resource speaker	2.17	Moderately Involved
1.8 Adviser / Consultant in academic projects	2.56	Much Involved
1.9 Panel member / judge in academic interactions	2.51	Much Involved
1.10 Writing and publishing articles / treatises	1.98	Moderately Involved
Area Mean	2.12	Moderately Involved

“Sabbatical leave” has the lowest weighted mean of 1.15. This is interpreted as *not involved*. This means that the teachers have not availed of a sabbatical leave. Of the 41 respondents, only 2 gave a rating of 4 which means that they are very much involved. The rest of the 39 respondents gave a rating of 1 which means that they are not involved or that they have not availed of sabbatical leave. Sabbatical leaves are offered to encourage academic and institutional revitalization by providing sustained time for study or research or other creative activities. There is also the phenomenon that, when the teacher who goes on sabbatical leave comes back, his/her position/item is already occupied by someone else. Also, there is the “unwritten policy” that sabbatical leave grants are awarded to faculty on a “case-to-case” basis.

Universities A, C, and D have a provision for sabbatical leave as indicated in their respective faculty manuals. In an informal interview with personnel from University B’s Human Resource Department, they said that the university also has a provision for sabbatical leave. The low or non-involvement of faculty members is due to the lack of information regarding this benefit. Several members of the faculty of University C held that they never heard of this benefit and if anyone from the university was ever granted a sabbatical leave.





Individual-Led CPD

Table 3

Extent to which Business Administration Teachers were involved in Opportunities for Individual-Led Continuing Professional Development (N=41)

Indicators	Mean	Interpretation
2.1 Pursuing graduate studies	3.07	Much Involved
2.2 Professional readings, reflections; independent, on-line studies; keeping professional diaries, journals, professional portfolio	3.10	Much Involved
2.3 Preparing instructional modules/materials	3.39	Very Much Involved
2.4 Conducting individual action researches and publishing outputs	2.15	Moderately Involved
2.5 Attending meetings, symposia, public lectures, serving as resource speaker/facilitator	2.93	Much Involved
Area Mean	2.93	Much Involved

The highest rated indicator is “Preparing instructional modules/materials” with a mean of 3.39. This is interpreted as *very much involved*. One of the responsibilities of a teacher is to prepare instructional materials that will enhance the teaching-learning process, and modules to facilitate self-directed and self-paced learning. But this is not an easy task. To confidently adjust instruction in response to students’ needs and inquiries, teachers need to develop a certain depth and breadth of knowledge about their subject matter.

On the other hand, the lowest indicator is “Conducting individual action researches and publishing outputs.” It has a weighted mean of 2.15, interpreted as *moderately involved*. The finding implies that many teachers have an aversion to conducting researches. Even if release time and monetary rewards are guaranteed, there are still very few teachers who conduct action researches.

Profession-Led CPD

Table 4

Extent to which Business Administration Teachers were involved in Opportunities for Profession-Led Continuing Professional Development (N=41)

Indicators	Mean	Interpretation
3.1 In-service training workshops sponsored by associations	2.83	Much Involved
3.2 Crash courses on specialized fields	2.83	Much Involved
3.3 Accreditation and career advancement courses	2.49	Moderately Involved
3.4 Conducting collaborative researches and publishing inputs	1.95	Moderately Involved
3.5 Attending conferences, assemblies, seminars sponsored by organizations; serving as resource speaker/facilitator	2.71	Much Involved
Area Mean	2.56	Much Involved

As shown in the table, “In-service training workshops sponsored by associations” and “Crash courses on specialized fields” top the list with means of 2.83, interpreted as *much involved*. Attendance in in-service training activities is expected since such activities cover the basic training initially required to carry out the job.

On the other hand, the lowest indicator is “Conducting collaborative researches and publishing inputs”. It has a weighted mean of 1.95, interpreted as *moderately involved*. Conducting researches require considerable time, effort and money. These can discourage teachers to engage in research activities. There is a similarity between this indicator and another indicator under individual-led CPD which is “Conducting individual action researches and publishing outputs.” It has a weighted mean of 2.15, interpreted as *moderately involved*. These two related findings indicate that many of the respondents have an aversion towards research whether individual or collaborative. As earlier pointed out, the moderate level of involvement of the business administration teachers in action researches and publishing outputs is rooted in the teachers’ inadequately developed skill in conducting action researches. Many teachers claim they lack the time to do research. The answer to this is: “There is always enough time, if people make the best use of time.” In doing research, time management is essential. Lack of funding is another area of concern. Not that there is no money for research but the policy on how such is allocated and released can be discouraging to teachers.





Table 5

Summary of the Extent to which Respondents were Involved in Opportunities for CPD Regardless of Profile

CPD Model	Mean	Interpretation
Systems-Led	2.12	Moderately Involved
Individual-Led	2.93	Much Involved
Profession-Led	2.56	Much Involved
Overall Mean	2.54	Much Involved

Based on the data presented, the extent to which respondents were involved in opportunities for CPD regardless of profile is rated 2.54 interpreted as *much involved*.

Among the three models, individual-led CPD has the highest mean. This model has a mean of 2.93 which means that the respondents are *much involved*. The result indicates that the teachers are intrinsically motivated to participate in continuing professional development activities. This means that given the right amount of resources to be more actively involved, they will be more enthusiastic about CPD.

This corroborates the findings of Karaaslan (2003) which presents that 51% agree that teachers should take the initiative and action for their own professional development. Teachers cannot ignore their development because they should be models for their students as enthusiastic, life-long learners.

2. Significant Differences in the Level of Involvement on the Different Opportunities for Systems-Led, Individual-Led, and Profession-Led CPD to which Business Administration Teachers Responded Considering the Moderator Variables

Educational Attainment

Table 6

Summary of the Extent to which Respondents were Involved in Opportunities for CPD according to Educational Attainment

CPD Model	BS	MA/MS	PhD/EdD
Systems-Led	1.78 (MoI)	2.19 (MoI)	1.80 (MoI)
Individual-Led	2.64 (MuI)	3.00 (MuI)	2.50 (MoI)
Profession-Led	1.96 (MoI)	2.67 (MuI)	2.20 (MoI)
Overall Mean	2.13 (MoI)	2.62 (MuI)	2.17 (MoI)

Legend: MoI – (Moderately Involved); MuI – (Much Involved)

The data shows that those with master's degree are *much involved* in opportunities for CPD while both bachelor's degree and doctorate degree holders are *moderately involved* in opportunities for CPD. Despite the similarity in results, it is important to note that bachelor's degree holders are *much involved* in individual-led CPD. Teachers who are bachelor's and master's degree holders who are aiming at career advancement and personal growth incessantly take the initiative to participate in as many CPD activities as possible.

The F-stat (5.04) is more than the critical value (3.16) and the p-value (0.0096) is lesser than the alpha (0.05). Thus, the null hypothesis is rejected. Therefore, there are significant differences in the level of involvement on the different opportunities for systems-led, individual-led, and profession-led CPD considering educational attainment. This is an indication that educational attainment influences the involvement of business administration teachers in CPD.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for the Master's Degree group (M=2.62, SD=0.5723) was significantly different from Bachelor's Degree group (M=2.13, SD=0.5529) and the Doctorate Degree group (M=2.17, SD=0.4375). The other groups did not differ significantly from one another. This means that the Bachelor's Degree group does not differ significantly from the Doctorate group.





Field of Specialization

Table 7

Summary of the Extent to which Respondents were Involved in Opportunities for CPD according to Field of Specialization

CPD Model	Management	Finance	Marketing	Others
Systems-Led	2.03 (MoI)	2.22 (MoI)	2.70 (MuI)	1.95 (MoI)
Individual-Led	2.79 (MuI)	2.82 (MuI)	3.40 (VMI)	2.90 (MuI)
Profession-Led	2.26 (MoI)	2.66 (MuI)	2.76 (MuI)	2.72 (MuI)
Overall Mean	2.36 (MoI)	2.57 (MuI)	2.95 (MuI)	2.52 (MuI)

Legend: MoI – (Moderately Involved); MuI – (Much Involved); VMI – (Very Much Involved)

The table shows that those whose field of specialization is Management or includes Management has the lowest mean which is 2.36, which means that they are *moderately involved* in opportunities for CPD. All the rest are *much involved*. Since the implementation of CMO 39 series of 2006, much of the subjects discussed in most CPD activities were focused on specialized courses. Because of this, it was not easy for those who specialize in Management to fit in. Even professional organizations that offer CPD activities cater to a specific group of teachers who teach specialized courses.

The F-stat (4.31) is more than the critical value (2.72) and the p-value (0.0073) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of involvement on the different opportunities for systems-led, individual-led, and profession-led continuing professional development to which business administration teachers responded considering field of specialization.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for Marketing (M=2.95, SD=0.5785) was significantly different from Management (M=2.36, SD=0.4938) and the "Others" group (M=2.52, SD=0.675) while the other groups did not differ significantly from one another. This implies that business administration teachers who specialize in Marketing participate actively in most CPD activities given the opportunity.

Employment Status

Table 8

Summary of the Extent to which Respondents were Involved in Opportunities for CPD according to Employment Status

CPD Model	Full Time	Part-Time
Systems-Led	2.11 (MoI)	2.19 (MoI)
Individual-Led	2.96 (MuI)	2.77 (MuI)
Profession-Led	2.62 (MuI)	2.26 (MoI)
Overall Mean	2.56 (MuI)	2.41 (MoI)

Legend: MoI – (Moderately Involved); MuI – (Much Involved)

As shown in the table, full time faculty members are *much involved* while part-time faculty members are *moderately involved* in opportunities for CPD. There are more opportunities for CPD available to full time faculty members. Unless the part-time faculty members are given these opportunities in the company where they work full time, they will have to take the initiative to do this on their own. In terms of profession-led CPD, where full time faculty members are *much involved* while part-time faculty members are *moderately involved*, Hoban (2002) points out that teachers potentially have access to a wide network of resources for developing teaching (colleagues, courses, online resources, etc.) and need to learn to exploit these.

The F-stat (0.40) is less than the critical value (4.10) and the p-value (0.5308) is greater than the alpha (0.05). In this case, the null hypothesis is not rejected. Therefore, there are no significant differences in the level of involvement on the different opportunities for systems-led, individual-led, and profession-led continuing





professional development to which business administration teachers responded considering employment status. This suggests that regardless of employment status, business administration teachers involve themselves in professional growth and development activities. It is however noted that, in the overall mean, the full-time teachers registered a higher mean (2.56, much involved), than the part-time teachers, with a mean of 2.41 (moderately involved).

Years of Teaching Experience

Table 9

Summary of the Extent to which Respondents were Involved in Opportunities for CPD according to Years of Teaching Experience

CPD Model	0	1-5	6-10	11-15	16 and above
Systems-Led	2.50 (MoI)	2.29 (MoI)	1.99 (MoI)	1.80 (MoI)	2.10 (MoI)
Individual-Led	3.40 (VMI)	2.97 (MuI)	2.98 (MuI)	2.71 (MuI)	2.91 (MuI)
Profession-Led	1.60 (NI)	2.60 (MuI)	2.40 (MoI)	2.66 (MuI)	2.69 (MuI)
Overall Mean	2.50 (MoI)	2.62 (MuI)	2.45 (MoI)	2.39 (MoI)	2.57 (MuI)

Legend: NI – Not Involved; MoI – (Moderately Involved); MuI – (Much Involved); VMI – (Very Much Involved)

The table shows that the group with the highest mean (2.62) is the one with 1 to 5 years of teaching experience. This means that they are *much involved* in opportunities for CPD.

It is worth to note that those with less than one year of teaching experience are *very much involved* in individual-led CPD.

The group with the lowest mean (2.39) is the one with 11 to 15 years of teaching experience. These are teachers who are usually experiencing mid-career crisis or work-life management dilemmas that adversely affect their commitment to professional growth and development. After this stage, involvement in CPD activities starts to pick up. As can be seen on the table, the overall mean of the extent to which respondents were involved in opportunities for CPD is 2.57 for teachers with 16 and more years of teaching experience. This is an indication that teachers reach a point of reassessment and renewal.

The F-stat (0.47) is less than the critical value (2.47) and the p-value (0.7570) is greater than the alpha (0.05). In this case, the null hypothesis is not rejected. Therefore, there are no significant differences in the level of involvement on the different opportunities for systems-led, individual-led, and profession-led continuing professional development to which business administration teachers responded considering years of teaching experience. It can be deduced from this finding that teachers, whether novice or expert, need continuous training.

Years of Industry Experience

Table 10

Summary of the Extent to which Respondents were Involved in Opportunities for CPD according to Years of Industry Experience

CPD Model	0	1-5	6-10	11-15	16 and above
Systems-Led	1.70 (NI)	2.37 (MoI)	2.20 (MoI)	1.88 (MoI)	2.19 (MoI)
Individual-Led	2.70 (MuI)	3.23 (MuI)	2.80 (MuI)	2.64 (MuI)	2.85 (MuI)
Profession-Led	2.23 (MoI)	2.81 (MuI)	2.88 (MuI)	2.44 (MoI)	2.30 (MoI)
Overall Mean	2.21 (MoI)	2.80 (MuI)	2.63 (MuI)	2.31 (MoI)	2.45 (MoI)

Legend: NI – Not Involved; MoI – (Moderately Involved); MuI – (Much Involved)





The table shows that with a mean of 2.80, those with one to five years experience in the industry are *much involved* in opportunities for CPD. On the other hand, those with less than one year experience in the industry are *not involved* (1.70) in systems-led CPD. Teachers who did not have any industry experience and are new in the teaching profession are just checking out options and are therefore avoiding commitments. Limited opportunities to new teachers also lead to less involvement in CPD.

Among all the CPD models, the individual-led is the one that are much involved in by teachers regardless of years of industry experience. This shows that whether they have had long industry experience or none, the interest of teachers to be involved in CPD springs from within.

According to years of industry experience, 1-5 and 6-10 groups were much more involved in CPD activities. This means tend to indicate that, except for the zero group of industry experience, the longer the business teachers stay in the industry, the less they become involved in CPD activities. The means of the 1-5 and 6-10 industry experience groups indicate much involvement; whereas, they means of the 11-15 and 16 and more industry experience groups indicate moderate involvement. The more experienced groups are the older groups. The involvement of older people tends to diminish because the opportunities for CPD favor the younger employees compared to the older ones.

The F-stat (3.15) is more than the critical value (2.47) and the p-value (0.0176) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of involvement on the different opportunities for systems-led, individual-led, and profession-led continuing professional development to which business administration teachers responded considering years of industry experience.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for the 0 group (M=2.21, SD=0.5997) was significantly different from the 1-5 years group (M=2.80, SD=0.6078) while the other groups did not differ significantly from one another.

The different companies where these teachers were once connected or are still connected with have different CPD opportunities for their employees. These, plus those offered by the academe have led to such differences on how the respondents regard opportunities for CPD. For instance, one of the respondents from University C mentioned that there is an abundance of local and international seminars and conferences available to most employees of the company where he used to work and was disappointed not to see the same in the academe.

Subjects Taught

Based on the data presented, those who are teaching subjects classified as professional courses have rated their involvement in opportunities for CPD 2.60, interpreted as *much involved*. Most of those who teach professional courses have more reasons to get involved in CPD activities considering that topics and themes of many seminars and conferences relate to most of the major subjects under the different major fields of specialization under the Business Administration program.

Table 11

Summary of the Extent to which Respondents were Involved in Opportunities for CPD according to Subjects Taught

CPD Model	Basic Business	Business Education	Professional Courses	Practicum
Systems-Led	2.10 (MoI)	2.13 (MoI)	2.22 (MoI)	2.00 (MoI)
Individual-Led	2.86 (MuI)	2.90 (MuI)	2.92 (MuI)	2.60 (MuI)
Profession-Led	2.45 (MoI)	2.61 (MuI)	2.65 (MuI)	2.50 (MoI)
Overall Mean	2.47 (MoI)	2.55 (MuI)	2.60 (MuI)	2.37 (MoI)

Legend: MoI – (Moderately Involved); MuI – (Much Involved)

One respondent from University C teaching basic business core subjects mentioned that though there are opportunities for CPD, the topics are not related to the subjects she teaches. This is also true with Practicum teachers. There are very few relevant CPD activities available related to the subject. This means that even if there are opportunities for CPD, if the activities do not suit the needs of the prospective participants, attendance or involvement is not guaranteed. This emphasizes what Bruner (as cited in Weibell, 2011) suggested; that the best stimulus to learning is an “interest in the material to be learned”, and the best way to create interest in the





subject is to “render it worth knowing, which means to make the knowledge gained usable in one’s thinking beyond the situation in which it has occurred”.

The F-stat (0.62) is less than the critical value (2.72) and the p-value (0.6031) is greater than the alpha (0.05). The null hypothesis is not rejected. Therefore, there are no significant differences in the level of involvement on the different opportunities for systems-led, individual-led, and profession-led continuing professional development to which business administration teachers responded considering subjects taught. This implies that regardless of the subjects handled by business administration teachers have training needs that must be satisfied. There is a need for them to undergo training so that they will be updated on current industry practices. This supports Scott’s (2003) article where he mentioned that business education teachers are obligated to remain current in the field or suffer obsolescence.

3. Motivations to Pursue Continuing Professional Development

The data gathered to address problem number two are presented in table 12. The needs are arranged in a hierarchy of prepotency. The order according to Abraham Maslow was followed.

Table 12

Level of Motivation to Pursue Professional Growth and Development (N=41)

Needs	Mean	Interpretation
Basic		
1. Salary increase	3.20	Moderately Motivated
3. Provision of latest technology equipment so I can update my teaching strategies	3.29	Very Much Motivated
14. Automatic wage/salary adjustment/other benefits	3.05	Moderately Motivated
17. Provision of better working conditions that promote my efficiency and effectiveness	3.37	Very Much Motivated
Area Mean	3.23	Moderately Motivated
Safety		
5. Job security enhancement	3.15	Moderately Motivated
10. Maintenance of job security and ability to compete in the job arena	3.27	Very Much Motivated
13. Prospective promotion in the job/upgraded rank/position	2.98	Moderately Motivated
20. Encouragement given to participate in CPD planning and organizing	3.29	Very Much Motivated
Area Mean	3.17	Moderately Motivated
Belonging		
7. Socialization enhancement	3.27	Moderately Motivated
9. Encouragement to maintain membership in professional societies and/or attendance in seminars and symposium	3.15	Moderately Motivated
12. Encouragement of friendly, cooperative working relationships by supervisor/facilitators	3.32	Very Much Motivated
19. Supervisors / facilitators who empathize with me and manifest their feelings for teachers rather than simply treating them as “inanimate tools”	3.22	Moderately Motivated
Area Mean	3.24	Moderately Motivated
Esteem / Ego		
4. Recognition given for attending CPD Activities	2.88	Moderately Motivated
6. Feedback on strategies information	3.22	Moderately Motivated
8. Empowerment provided to decide and set my own goals and review my performance	3.39	Very Much Motivated
11. Continuing pursuit of the tradition of excellence	3.51	Very Much Motivated





Area Mean	3.25	Moderately Motivated
Self-Actualization		
2. The self-fulfillment afforded by CPD	3.51	Very Much Motivated
15. Opportunity to do what I do best	3.49	Very Much Motivated
16. Opportunity to be truly creative	3.46	Very Much Motivated
18. Opportunity to attain self-development	3.66	Very Much Motivated
Area Mean	3.53	Very Much Motivated
Overall Mean	3.28	Very Much Motivated

Of the five levels of Maslow's Hierarchy of Needs, Self-Actualization has the highest area mean, 3.53, which is interpreted as *very much motivated*.

Significant Differences in the Level of Motivation to Pursue Professional Growth and Development Considering the Moderator Variables

Educational Attainment

Table 13

Summary of the Level of Motivation to Pursue Professional Growth and Development according to Educational Attainment

Needs	BS	MA/MS	PhD/EdD
Basic	3.10 (MM)	3.35 (VMM)	1.50 (NM)
Security	3.00 (MM)	3.29 (VMM)	1.50 (NM)
Belongingness	2.85 (MM)	3.38 (VMM)	1.75 (NM)
Esteem/Ego	2.85 (MM)	3.38 (VMM)	2.00 (SM)
Self-Actualization	3.25 (MM)	3.63 (VMM)	2.50 (SM)
Overall Mean	3.01 (MM)	3.41 (VMM)	1.85 (SM)

As presented in table 13, those who have master's degree are *very much motivated* (3.41) to pursue professional growth and development and that the highest rated need that motivates them is self-actualization with a mean of 3.63 interpreted as *very much motivated*.

On the other hand, those with doctorate degrees are *slightly motivated* (1.85) to pursue professional growth and development. Teachers who have attained the highest possible academic degree have reached a point where their interests shift from professional growth and development to something more personal. In Maslow's hierarchy of needs, they have attained self-actualization. As such, they are regarded as trainers/mentors more than being trainees or mentees.

The F-stat (80.10) is more than the critical value (3.16) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of motivation to pursue professional growth and development considering educational attainment.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for Bachelor's Degree (M=3.01, SD=0.2864) was significantly different from the Master's Degree (M=3.41, SD=0.1924) and Doctorate Degree (M=1.85, SD=0.6091). Similarly, the mean score for Master's Degree was significantly different from the Doctorate Degree. This shows that the attainment of different levels of education can influence the motivations of people.





Field of Specialization

Table 14

Summary of the Level of Motivation to Pursue Professional Growth and Development according to Field of Specialization

Needs	Management	Finance	Marketing	Others
Basic	2.87 (MM)	3.25 (MM)	3.40 (VMM)	3.40 (VMM)
Security	2.76 (MM)	3.32 (MM)	3.55 (VMM)	3.15 (MM)
Belongingness	2.86 (MM)	3.29 (VMM)	3.55 (VMM)	3.27 (VMM)
Esteem/Ego	2.97 (MM)	3.29 (VMM)	3.80 (VMM)	3.25 (MM)
Self-Actualization	3.28 (VMM)	3.59 (VMM)	4.00 (VMM)	3.67 (VMM)
Overall Mean	2.95 (MM)	3.35 (VMM)	3.66 (VMM)	3.35 (VMM)

Legend: MM – Moderately Motivated; VMM – Very Much Motivated

Based on the data presented on table 14, regardless of field of specialization, business administration teachers are *very much motivated* by self-actualization needs.

One of the characteristics of self-actualizing people is that they are seeking peak experiences that leave a lasting impression (“Maslow’s hierarchy of needs theory,” 2012). Teachers, whose fields of specialization are marketing (3.66), finance (3.35), and “others” (3.35), are *very much motivated* to pursue professional growth and development.

On the other hand, respondents whose field of specialization is management or includes management are only *moderately motivated* (2.95) to pursue professional growth and development. This is on account of the nature of the management field where managers tend to exert influence on others more than they being influenced by others in CPD activities.

These findings accentuate the result on the extent to which respondents were involved in opportunities for CPD according to field of specialization.

The F-stat (24.61) is more than the critical value (2.72) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of motivation to pursue professional growth and development considering field of specialization.

Post ANOVA comparisons using the Tukey’s HSD test indicated that all the comparisons were significantly different from each other. Only the mean score for Management (M=2.95, SD=0.0469) and “Others” (M=3.35, SD=0.0582) was not significantly different.

Employment Status

Table 15

Summary of the Level of Motivation to Pursue Professional Growth and Development according to Employment Status

Needs	Full Time	Part-Time
Basic	3.32 (VMM)	2.75 (MM)
Security	3.24 (MM)	2.86 (MM)
Belongingness	3.33 (VMM)	2.79 (MM)
Esteem/Ego	3.35 (VMM)	2.79 (MM)
Self-Actualization	3.60 (VMM)	3.18 (MM)
Overall Mean	3.37 (VMM)	2.87 (MM)

Legend: MM – Moderately Motivated; VMM – Very Much Motivated





Based on the data presented in table 15 and the computed mean (3.37), full-time teachers are *very much motivated* to pursue professional growth and development. Full-time teachers spend most of their working hours in the school. Part-time teachers on the other hand, spend much of their time in their full-time jobs. For full-time teachers, the academe is the only avenue for them to take advantage of all possible opportunities for CPD to satisfy their needs.

The F-stat (34.92) is more than the critical value (4.10) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of motivation to pursue professional growth and development considering employment status. Full-time teachers who depend on their teaching job as their sole source of income would take advantage of all available opportunities to protect their job to afford them their basic needs and satisfy their other needs such as belongingness, esteem, and self-actualization.

Years of Teaching Experience

Table 16

Summary of the Level of Motivation to Pursue Professional Growth and Development according to Years of Teaching Experience

Needs	0	1-5	6-10	11-15	16 and above
Basic	3.75 (VMM)	3.22 (MM)	3.03 (MM)	3.00 (MM)	3.61 (VMM)
Security	4.00 (VMM)	3.31 (VMM)	3.00 (MM)	2.71 (MM)	3.36 (VMM)
Belonging	3.25 (MM)	3.35 (VMM)	3.00 (MM)	2.93 (MM)	3.54 (VMM)
Esteem/Ego	4.00 (VMM)	3.31 (VMM)	3.13 (MM)	3.00 (MM)	3.39 (VMM)
Self-Actualization	4.00 (VMM)	3.67 (VMM)	3.28 (VMM)	3.18 (MM)	3.75 (VMM)
Overall Mean	3.80 (VMM)	3.37 (VMM)	3.09 (MM)	2.96 (MM)	3.53 (VMM)

Legend: MM – Moderately Motivated; VMM – Very Much Motivated

Results show that in terms of years of teaching experience, the highest mean among the five groups is that of those with less than one year of teaching experience.

The mean of 3.80 is interpreted as *very much motivated*. During the first stages of their career, teachers go through easy or painful beginnings to their career and they present initial commitment and enthusiasm (Karaaslan, 2003).

The two groups with the lowest means are those who had teaching experience of 6 to 10 and 11 to 15 years. The former has a mean of 3.09 and the latter has a mean of 2.96, interpreted as *moderately motivated*. Teachers who have served the university this long are already tenured and secured in their positions. As such, job security enhancement and promotion do not motivate them that much.

The F-stat (23.34) is more than the critical value (2.47) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of motivation to pursue professional growth and development considering years of teaching experience. This shows that teachers at different stages of their careers may experience, feel, think, and act differently. Those who are new in any position are likely to feel the highest positive regard for the job. They are motivated and excited about their new work. This period is characterized by high motivation and excitement. Those who have been teaching for 16 years and more start focusing on their lives beyond the classroom as they approach retirement while at the same time making their remaining years in the academe meaningful and significant.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for 1 to 5 years ($M=3.37$, $SD=0.244$) was not significantly different from the 16 years and above group ($M=3.53$, $SD=0.2162$). Likewise, the mean score for 6 to 10 years ($M=3.09$, $SD=0.2155$) was not significantly different from 11 to 15





years group ($M=2.96$, $SD=0.2162$). All the other groups differed significantly from one another. The significant differences lie in the zero experience group which caused the biggest difference. This implies that motivation to pursue CPD is greatest among the zero experience groups. Being new to the teaching profession, this group naturally is very much motivated to prove that they can tackle their responsibilities as teachers and are very eager to learn about the science and art of teaching. Beginning teachers are generally observed to start the process of becoming effective teachers by learning to access the knowledge base on teaching, accumulating the wisdom of effective practice, and reflecting on their experiences (Arends, 2012). Another reason why the zero experience group is very much motivated to pursue CPD is the regard for tenure and job security. In Maslow's hierarchy of needs, the need to feel secure in the job (tenure) is one of the early goals targeted by new employees who are still under probationary status and are still working toward tenure status.

Years of Industry Experience

Table 17

Summary of the Level of Motivation to Pursue Professional Growth and Development according to Years of Industry Experience

Needs	0	1-5	6-10	11-15	16 and above
Basic	3.22(MM)	3.30(VMM)	3.60(VMM)	2.85(MM)	3.09(MM)
Security	3.19(MM)	3.33(VMM)	3.45(VMM)	2.70(MM)	2.97(MM)
Belonging	3.09(MM)	3.42(VMM)	3.40(VMM)	2.85(MM)	3.19(MM)
Esteem/Ego	3.13(MM)	3.40(VMM)	3.45(VMM)	2.85(MM)	3.22(MM)
Self-Actualization	3.44(VMM)	3.68(VMM)	3.85(VMM)	3.20(MM)	3.34(VMM)
Overall Mean	3.21(MM)	3.43(VMM)	3.55(VMM)	2.89(MM)	3.16(MM)

Legend: MM – Moderately Motivated; VMM – Very Much Motivated

Based on the table, the motivation to pursue professional growth and development based on the hierarchy of needs of Maslow is highest among business administration teachers who have had 6 to 10 years of industry experience. The mean of 3.55 is interpreted as *very much motivated*. These teachers have reached the peak of their career. They have reached a stage characterized by financial rewards and recognition of their performance. The teachers who have had 11 to 15 years of industry experience show that they are *moderately motivated* (2.89) to pursue professional growth and development. The level of motivation decreases when they reach this point in their career where people often make a major reassessment of their progress relative to their original career ambitions and goals. At this point, there will be decreased organizational commitment, involvement, satisfaction, and performance relative to their earlier years in their respective careers.

The F-stat (18.35) is more than the critical value (2.47) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the level of motivation to pursue professional growth and development considering years of industry experience.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for 0 years ($M=3.21$, $SD=0.2224$) was not significantly different from the 1 to 5 years group ($M=3.43$, $SD=0.2215$) and from the 16 years and above group ($M=3.16$, $SD=0.2439$). Likewise, the mean score for 1 to 5 years ($M=3.43$, $SD=0.2215$) was not significantly different from the 6 to 10 years group ($M=3.55$, $SD=0.3426$). All the other groups differed significantly from one another.





Subjects Taught

Table 18

Summary of the Level of Motivation to Pursue Professional Growth and Development according to Subjects Taught

Needs	Basic Business	Business Education	Professional Courses	Practicum
Basic	3.15(MM)	3.25(MM)	3.20(MM)	3.23(MM)
Security	3.12(MM)	3.14(MM)	3.13(MM)	3.05(MM)
Belonging	3.15(MM)	3.23(MM)	3.28(VMM)	3.25(MM)
Esteem/Ego	3.21(MM)	3.29(VMM)	3.28(VMM)	3.15(MM)
Self-Actualization	3.47(VMM)	3.58(VMM)	3.50(VMM)	3.30(VMM)
Overall Mean	3.22(MM)	3.30(VMM)	3.28(VMM)	3.20(MM)

Legend: NM – Not Motivated; SM – Slightly Motivated; MM – Moderately Motivated; VMM – Very Much Motivated

The table shows that the level of motivation to pursue professional growth and development considering subjects taught is highest among those teaching business education core subjects with a mean of 3.30, interpreted as *very much motivated*. This is followed by those teaching professional subjects with a mean of 3.28 which is likewise interpreted as *very much motivated*.

As shown in table 18, respondents strongly agree that self-actualization needs drive them to pursue professional growth and development. Each and every group is *very much motivated* by this specific need. This shows the strong desire among business administration teachers, regardless of the subjects they teach to achieve self-actualization.

The F-stat (1.20) is less than the critical value (2.72) and the p-value (0.3168) is greater than the alpha (0.05). In this case, the null hypothesis is not rejected. Therefore, there are no significant differences in the level of motivation to pursue professional growth and development considering subjects taught.

4. Challenges that Impinge upon the Pursuit of Professional Growth and Development

Systems-Led CPD

Table 19

Degree of Seriousness of Problems Encountered in Pursuing Systems-Led CPD (N=41)

Indicators	Mean	Interpretation
1.1 Sufficiency of time to pursue CPD activities	3.05	Moderately Serious
1.2 Availability/adequacy of funds	3.15	Moderately Serious
1.3 Administrative support	3.07	Moderately Serious
1.4 CPD viewed by administrators, policy-makers and school heads as an expensive fringe benefit	2.95	Moderately Serious
1.5 Adequacy of systematic programs of CPD	3.07	Moderately Serious
Area Mean	3.06	Moderately Serious

Of the indicators under systems-led, “Availability / adequacy of funds” had the highest weighted mean of 3.15 which means that the respondents find this *moderately serious*. An informal interview with some teachers from the different universities in Baguio City revealed that many of them are not given that much opportunity to participate in professional growth and development activities. Some claim that they were expected





to engage in development activities individually, drawing upon their own resources to pursue the learning they would need to achieve their growth goals.

In a study by Wan and Lam (2010), 10.3% of their respondents perceived financial factor as one that inhibits continuing professional development. Similarly, Howell (2007) found out in her study that financial constraints affected the amount of time and the type of professional development that respondents selected.

Individual-Led CPD

Table 20

Degree of Seriousness of Problems Encountered in Pursuing Individual-Led CPD (N=41)

Indicators	Mean	Interpretation
2.1 Work-load versus graduate course load	3.07	Moderately Serious
2.2 Funding	3.07	Moderately Serious
2.3 Time factor	3.12	Moderately Serious
2.4 Clarity of policies in connection with CPD participation	3.07	Moderately Serious
2.5 Provision for echoing programs	2.66	Moderately Serious
2.6 Meaningfulness of CPD activities to hold participants' interest	2.78	Moderately Serious
2.7 Variety of opportunities for greater number of faculty to undergo CPD activities	2.90	Moderately Serious
2.8 Equality of opportunity to undergo CPD activities	2.85	Moderately Serious
2.9 Opportunities to conduct researches	2.88	Moderately Serious
2.10 "Anointed Ones" syndrome (favored staff to undergo CPD)	2.63	Moderately Serious
Area Mean	2.90	Moderately Serious

Results show that the problem rated the highest is "Time factor". This has a mean of 3.12 which is interpreted as *moderately serious*. Teachers, researchers and policy-makers consistently indicate the greatest challenge to implementing effective professional development is lack of time (Abdal-Haqq, 1996)

One respondent from University D mentioned that since they have to report six times a week, there really is not much time for CPD since chores and errands can be done only during Sundays.

Most CPD activities are held during weekends because school administrators do not want that contact hours with students be sacrificed. If this is the case, many target participants may not want to attend even if it is compulsory because they have other appointments or because they would rather spend this time with their family.

A respondent from University B shared that if they attend any CPD activity which means that they will be missing their class, they have to conduct a make-up class. And since this means extra hours have to be committed for that make-up class, they would rather go to class than attend any seminar or other related activities.

Graduate school classes are held if not during weekdays in the evening, during weekends. The schedule of graduate school classes may not be appealing and may discourage teachers from enrolling.

Profession-Led CPD

Table 21

Degree of Seriousness of Problems Encountered in Pursuing Profession-Led CPD (N=41)

Indicators	Mean	Interpretation
3.1 Organizing CPD programs that embody structural components of reform type, duration, and collective participation.	2.90	Moderately Serious
3.2 Ability of one-shot-in-service workshops delivering pre-packaged information to accomplish the goals of effective CPD programs.	2.95	Moderately Serious
3.3 Adequacy of CPD programs that are rooted in practice, research-based, collaborative, and long-term.	2.90	Moderately Serious
3.4 Availability and adequacy of resources.	3.15	Moderately Serious
3.5 Breadth of coverage, membership and participation in CPD activities sponsored by professional organizations.	3.02	Moderately Serious
Area Mean	2.99	Moderately Serious





Challenges encountered in pursuing profession-led CPD activities have an area mean of 2.99. This is interpreted as *moderately serious*.

The table shows that the problem on the “availability and adequacy of resources” is perceived as *moderately serious* (3.15) by the respondents. It has the highest weighted mean among all indicators. Funding, though essential in any reform or effective professional development, is a major challenge faced by teachers and educators. A respondent from University C mentioned that the lack of funds and the delayed release of such discouraged her from attending seminars especially those held outside Baguio City.

Table 22

Summary of the Degree of Seriousness of Problems Encountered in Pursuing CPD Regardless of Profile

CPD Model	Mean	Interpretation
Systems-Led	3.06	Moderately Serious
Individual-Led	2.90	Moderately Serious
Profession-Led	2.99	Moderately Serious
Overall Mean	2.98	Moderately Serious

Challenges encountered in pursuing systems-led continuing professional development have an area mean of 3.06 interpreted as *moderately serious*. This has the highest area mean compared to individual-led continuing professional development and profession-led continuing professional development.

Systems-led continuing professional development manifested the most challenges in relation to professional development of teachers. The most challenging feature in systems-led CPD is funding. This dovetails with the extent of involvement in CPD activities where the teachers were least involved. Inadequacy or unavailability of funds often restricts the participation of teachers in systems-led CPD activities. Also, inadequacy or lack of systematic, regular CPD programs hampered the pursuit of CPD by the teachers. As pointed out by Skinner (2010), the lack or inadequacy of regular and systematic CPD programs in school organizations/institutions cause teachers to be demotivated. This is so because systems-led CPD is often linked to a ladder of promotions, further qualifications and enhancement. Individual-led CPD challenges have the lowest mean of 2.90 (*moderately serious*). This dovetails with the extent of involvement in CPD activities, which the teachers were most involved.

5. Significant Differences in the Degree of Seriousness of the Challenges that Impinge upon the Pursuit of Professional Growth and Development Considering the Moderator Variables

Educational Attainment

Table 23

Summary of the Degree to which the Challenges Impinge upon the Pursuit of Professional Growth and Development Considering Educational Attainment

CPD Model	BS	MA/MS	PhD/EdD
Systems-Led	2.76 (MoS)	3.05 (MoS)	3.90 (VS)
Individual-Led	2.52 (MoS)	2.91 (MoS)	3.85 (VS)
Profession-Led	2.40 (MoS)	3.01 (MoS)	4.00 (VS)
Overall Mean	2.56 (MoS)	2.99 (MoS)	3.92 (VS)

Legend: MoS – Moderately Serious; VS – Very Serious





The degree to which the challenges impinge upon the pursuit of professional growth and development was rated 3.92, interpreted as *very serious*, by doctorate degree holders. On the other hand, the bachelor's degree holders and master's degree holders rated the challenges in pursuing professional growth and development as *moderately serious*. This shows that the higher educational attainment one has the higher their standards become. They become keener even on the smallest details. Doctorate degree holders, having attained the highest educational attainment, encounter more serious challenges particularly in system-led CPD activities, specifically along the organization of CPD programs. This could be the reason why they are slightly motivated and moderately involved in CPD activities.

The F-stat (188.89) is more than the critical value (4.10) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the degree of the seriousness of the challenges that impinge upon the pursuit of professional growth and development considering educational attainment.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean scores for the bachelor's degree (M=2.56, SD=0.214), master's degree (M=2.99, SD=0.1612), and doctorate degree (M=3.92, SD=0.2616) groups were significantly different from each other. This indicates that educational attainment has a potent impact on how the respondents perceive the degree of seriousness of the problems encountered in the pursuit of CPD.

Table 24 presents the summary of the degree to which the challenges impinge upon the pursuit of professional growth and development considering field of specialization.

Table 24

Summary of the Degree to which the Challenges Impinge upon the Pursuit of Professional Growth and Development Considering Field of Specialization

CPD Model	Management	Finance	Marketing	Others
Systems-Led	2.97 (MoS)	2.81 (MoS)	3.36 (VS)	2.93 (MoS)
Individual-Led	2.82 (MoS)	2.69 (MoS)	2.94 (MoS)	2.69 (MoS)
Profession-Led	2.88 (MoS)	2.68 (MoS)	3.04 (MoS)	2.97 (MoS)
Overall Mean	2.89 (MoS)	2.73 (MoS)	3.11 (MoS)	2.86 (MoS)

Legend: MoS – Moderately Serious; VS – Very Serious

As shown on table 24, all the respondents perceive the challenges that impinge upon the pursuit of professional growth and development as *moderately serious*.

Those whose field of specialization is marketing or includes marketing rated the challenges they encounter 3.11, interpreted as *moderately serious*.

It is worthwhile to note that even if the marketing group had the highest rating in terms of the degree to which challenges impinge upon the pursuit of professional growth and development, they also have the highest mean in terms of involvement in CPD. This suggests that despite of the challenges they encounter, the marketing group still engage in CPD activities.

The F-stat (7.53) is more than the critical value (2.72) and the p-value (0.0002) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the degree of challenges that impinge upon the pursuit of professional growth and development considering field of specialization.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for the Management group (M=2.89, SD=0.1731) was significantly different from the Marketing group (M=3.11, SD=0.357). Likewise, the Marketing group was significantly different from the "Others" group (M=2.86, SD=0.1821). All the other groups did not differ significantly from each other.





Employment Status

Table 25

Summary of the Degree to which Challenges Impinge upon the Pursuit of Professional Growth and Development Considering Field of Specialization Employment Status

CPD Model	Full Time	Part-Time
Systems-Led	3.16 (MoS)	2.54 (MoS)
Individual-Led	3.04 (MoS)	2.26 (SS)
Profession-Led	3.14 (MoS)	2.23 (SS)
Overall Mean	3.11 (MoS)	2.34 (SS)

Legend: SS – Slightly Serious; MoS – Moderately Serious

The table shows that the full-time business administration teachers find the problems they encounter in the pursuit of professional growth and development as *moderately serious*. On the other hand, part-time business administration teachers find the problems *slightly serious*. Most CPD opportunities are provided to full-time teachers. In most cases, part-time teachers do not have the same access to CPD activities as full-time teachers are afforded. Moreover, professional development activities for part-time teachers are quite limited. Time constraints as well as organizational policies are often cited as drawbacks.

The F-stat (204.31) is more than the critical value (3.16) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, we reject the hypothesis. Therefore, there are significant differences in the degree of challenges that impinge upon the pursuit of professional growth and development considering employment status.

Years of Teaching Experience

Table 26

Summary of the Degree to which Challenges Impinge upon the Pursuit of Professional Growth and Development Considering Years of Teaching Experience

CPD Model	0	1-5	6-10	11-15	16 and above
Systems-Led	2.00 (SS)	3.19 (MoS)	3.13 (MoS)	3.03 (MoS)	2.83 (MoS)
Individual-Led	1.00 (NS)	3.01 (MoS)	3.20 (MoS)	2.93 (MoS)	2.56 (MoS)
Profession-Led	1.00 (NS)	2.96 (MoS)	3.35 (VS)	3.00 (MoS)	2.91 (MoS)
Overall Mean	1.33 (NS)	3.05 (MoS)	3.23 (MoS)	2.99 (MoS)	2.77 (MoS)

Legend: NS – Not Serious; SS – Slightly Serious; MoS – Moderately Serious

The data presented on table 41 shows that those who have been teaching for 1 to 5 years, 6-10 years, 11-15 years, and those with 16 and more years of teaching experience consider the problems *moderately serious*.

Teachers who have been in the academe for a considerable period of time have been exposed to issues concerning CPD. They have been witnesses to the shortcomings of the institution they belong to and are therefore aware of the challenges that hinder them from achieving professional growth and development. These teachers have personally experienced problems associated with their pursuit of continuing professional development.

The F-stat (95.95) is more than the critical value (2.47) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the degree of challenges that impinge upon the pursuit of professional growth and development considering years of teaching experience.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for 0 years (M=1.33, SD=0.6387) was significantly different from the 1 to 5 years group (M=3.05, SD=0.2121), the 6 to 10 years group (M=3.23, SD=0.2102), the 11 to 15 years group (M=2.99, SD=0.2983), and the 16 years and above





group ($M=2.77$, $SD=0.2777$). Likewise, the mean score of the 1 to 5 years group was significantly different from the 16 years and above group. Also, the mean score of the 6 to 10 years group was significantly different from the 16 years and above group. All the other groups did not differ significantly from each other.

Years of Industry Experience

Table 27

Summary of the Degree to which Challenges Impinge upon the Pursuit of Professional Growth and Development Considering Years of Industry Experience

CPD Model	0	1-5	6-10	11-15	16 and above
Systems-Led	2.98 (MoS)	3.39 (VS)	2.12 (SS)	3.68 (VS)	2.73 (MoS)
Individual-Led	2.88 (MoS)	3.31 (VS)	2.08 (SS)	3.28 (VS)	2.45 (SS)
Profession-Led	3.08 (MoS)	3.36 (VS)	2.36 (SS)	3.24 (MoS)	2.43 (SS)
Overall Mean	2.98 (MoS)	3.35 (VS)	2.19 (SS)	3.40 (VS)	2.53 (MoS)

Legend: SS – Slightly Serious; MoS – Moderately Serious; VS – Very Serious

The table shows that those with industry experience of 11 to 15 years consider the challenges that impinge upon the pursuit of professional growth and development as *very serious*. When problems encountered in pursuing professional growth and development become serious, this can discourage involvement.

The F-stat (79.16) is more than the critical value (2.47) and the p-value (0.0000) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the degree of challenges that impinge upon the pursuit of professional growth and development considering years of industry experience.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for 1 to 5 years group ($M=3.35$, $SD=0.1846$) was not significantly different from the 11 to 15 years group ($M=3.40$, $SD=0.2922$). All the other groups differed significantly from each other.

Subjects Taught

Table 28

Summary of the Degree to which Challenges Impinge upon the Pursuit of Professional Growth and Development Considering Subjects Taught

CPD Model	Basic Business	Business Education	Professional Courses	Practicum
Systems-Led	3.16 (MoS)	2.96 (MoS)	3.15 (MoS)	2.96 (MoS)
Individual-Led	2.99 (MoS)	2.89 (MoS)	3.00 (MoS)	2.81 (MoS)
Profession-Led	3.04 (MoS)	2.93 (MoS)	3.07 (MoS)	3.02 (MoS)
Overall Mean	3.06 (MoS)	2.92 (MoS)	3.07 (MoS)	2.93 (MoS)

Legend: MoS – Moderately Serious

As shown in table 28, business administration teachers find the challenges *moderately serious*. The highest computed overall mean is that of the teachers teaching professional courses (3.07) then by those teaching basic business core subjects (3.06) followed by those teaching practicum (2.93) and lastly, by those teaching business education subjects (2.92). Professional courses lay the fundamental concepts that every business administration student has to know in the field of business which means that teachers need the most training in this area. However, teachers express high levels of dissatisfaction with CPD events that do not meet their needs or failed to live up to their expectations. This dovetails with the extent of involvement in CPD activities where teachers handling professional courses are much involved.





The F-stat (4.73) is more than the critical value (2.72) and the p-value (0.0044) is lesser than the alpha (0.05). In this case, the null hypothesis is rejected. Therefore, there are significant differences in the degree of challenges that impinge upon the pursuit of professional growth and development considering subjects taught. Regardless of subjects taught, teachers today have a duty not just of instilling the concepts, skills, and appreciation of learning; teachers today, but also to manifest the characteristics of lifelong learning themselves and of applying them throughout the course of their own professional lives.

Post ANOVA comparisons using the Tukey's HSD test indicated that the mean score for the Basic Business group (M=3.06, SD=0.1729) was significantly different from the Practicum group (M=2.93, SD=0.1974). Likewise, the mean score for the Professional Courses group (M=3.07, SD=0.1593) was significantly different from the Practicum group. All the other groups do not differ significantly from each other.

6. Status of Professional Growth and Development among Business Administration Teachers of Selected Universities in Baguio City

Business administration teachers are *much involved* in the different opportunities for CPD. Specifically, they are *moderately involved* in systems-led CPD and are *much involved* in individual-led and profession-led CPD.

There are significant differences in the level of involvement of business administration teachers in CPD considering educational attainment, field of specialization, and years of industry experience influenced.

In contrast, there are no significant differences in the level of involvement of business administration teachers in CPD according to employment status, years of teaching experience, and subjects taught.

Business administration teachers are *very much motivated* in pursuing professional growth and development activities. Specifically, they are *moderately motivated* by basic, safety, belongingness, and esteem/ego needs and are *very much motivated* by self-actualization needs.

While there are no significant differences in the level of motivation according to subjects taught, there are significant differences in the level of motivation considering educational attainment, field of specialization, employment status, years of teaching experience, and years of industry experience.

Business administration teachers find the challenges that impinge upon the pursuit of professional growth and development moderately serious.

There are significant differences in the perceptions of the respondents regarding the degree of seriousness of the challenges encountered in the pursuit of professional growth and development considering the moderator variables.

Conclusion

The teachers manifested a greater extent of involvement in individual-led CPD activities. This shows that the teachers take initiative and action for their own professional growth.

Educational attainment, field of specialization, and years of industry experience influenced the extent of involvement in the CPD activities. On the other hand, employment status, years of teaching experience, and subjects taught did not influence extent of involvement in CPD activities. With respect to the hierarchy of needs, the teachers were motivated most by self-actualization needs which is at the apex of the hierarchy. This shows that the teachers are intrinsically motivated to pursue CPD activities and they continue to act and live up to their potential, personal satisfaction and self-fulfillment.

Educational attainment, field of specialization, employment status, years of teaching experience, and years of industry experience influenced motivation to pursue CPD activities. Subjects taught did not influence motivation to pursue CPD.

The teachers encountered less challenges in individual – led CPD, which shows that they are more inclined to pursue CPD on their own initiative.

Educational attainment, field of specialization, employment status, years of teaching experience, years of industry experience, and subjects taught influenced degree of seriousness of challenges.

Based on the conclusions, the following are recommended for consideration:

1. More opportunities for CPD should be provided by educational institutions in a more systematically planned and implemented CPD program.
2. Make CPD flexible, personalized, contextualized, and connected to real, tangible rewards such as incentives and recognition.





3. Teachers' professional growth and development programs must be systematically researched, planned, supported, and funded to guarantee effectiveness.
4. Encourage the conduct of multi-disciplinary research among business administration teachers.
5. Research must be included as a component in the evaluation tool for teachers.
6. The definition and implementing guidelines related to continuing professional development must be communicated clearly and succinctly to all teachers.
7. The model is strongly recommended for adoption and implementation. **(available upon request)**
8. A copy of the research will be provided to universities concerned upon request.
9. Further research should be done on the same topic to verify, amplify, or negate the findings of this study.

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EDI01

Developing and Validating a DC Motor Multiple Choice Questionnaire for High School Bhutanese Students

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Abstract

Although a DC motor has been used to teach the concept of electromagnetism to high school students, there is a lack of report on students' conceptual understanding about the working of a DC motor. This paper presents the development of a multiple choice questionnaire (MCQ) that can probe students' conceptual understanding of a DC motor. The first questionnaire, which composed of 8 open-ended questions, was designed by the researchers to find Bhutanese students' understanding about the parts and working of a DC motor including the purpose of a split ring, cause of rotation of the armature, and so on. After validation of these questions by three Bhutanese physics teachers, responses were gathered from 80 Grade 11 students from one of the schools in Eastern Bhutan. The data were analyzed by grouping similar ideas which was then used as alternatives for the MCQ. The difficulty and discrimination index of the questionnaire was found by implementing the MCQ to 130 Grade 10 Bhutanese students. The difficulty index ranged between 0.4 and 0.7 which showed that the items were not very difficult. The discrimination index of all items, except item 3, 4i, 5, and 6, ranged between 0.4 – 1.0 showed that they can separate high and low achievers.

Keywords : Bhutanese students, conceptual understanding, DC motor questionnaire

Introduction

Physics Education Research (PER) focuses on the importance of understanding the ways students learn and their ideas about a physical concept (Redish, 1994, 1999; McDermott, 2001). One of the important concepts covered in physics is about electromagnetism.

The discovery of the connection between electricity and magnetism (electromagnetism), the flow of current caused magnetic effects, by Oersted in 1820 has resulted in the development of many world-changing technologies. However, there were many researchers who reported the difficulty of students to learn the concept of electromagnetism due to its abstract nature and they also recommended teaching this concept by relating it to everyday examples (Rosenbaum, 1990; Leppävirta, Kettunen, & Sihvola, 2011). A DC motor is one of the simple example that works on electromagnetism. It converts electrical energy to mechanical energy and is used in many everyday appliances such as fans, hair dryers, etc.

Upon review of literatures and teaching resources (Wainwright, 2002; National Geographic Society, Ezrailson, Zike, & Zorn, 2002; Yap & MacIsaac, 2006) for tests to find students' understanding about a DC motor, none was found. Since electromagnetism is related to electricity and magnetism, literatures on the tests developed to measure students' conceptual understanding in these concepts such as the Conceptual Survey of Electricity and Magnetism (CSEM) (Maloney, Kuma, Hieggelke, and Heuvelen, 2001) and the Brief Electricity and Magnetism Assessment (BEMA) tool (as cited in Ding et al., 2006) were reviewed to find if any of the questions were based on a DC motor. Although some questions were asked about magnetic force in both CSEM





and BEMA, no questions were found about a DC motor. In 2006, Saglam & Millar developed a diagnostic test to find students' understanding of electromagnetism by focusing on concepts such as magnetic field, magnetic force and electromagnetic induction. They purposefully excluded the topic on a d.c. motor because it was easier for them to diagnose students' understanding of electromagnetism with direct questions. However, one of the result they found was that students faced difficulty in questions which required the application of the left-hand rule such as the direction of the field or current. The left-hand rule is usually used for identifying the direction of current, external magnetic field and force in the arms of the armature of a DC motor (Sethi, 2006).

From these literature reviews, it could be concluded that a DC motor has not been used as a means to find students' understanding about electromagnetism. In addition, all the diagnostic tests that were based on electricity and magnetism or electromagnetism were too complicated for high school students.

In the Bhutanese physics curriculum, students are introduced to the concept of electromagnetism and DC motor in Grade 10. This concept is learnt in depth upon reaching Grade 12 and undergraduate engineering courses. Since basic conceptual understanding of electromagnetism is necessary for higher Grades, there was a need to determine and assess students' ideas when they were in Grade 10. (CAPSD, 2008)

All this directed us to develop a test that could determine Grade 10 Bhutanese students' understanding about a DC motor which would then help us diagnose their understanding about electromagnetism.

Research objectives

The objectives of the research were to:

- 1) develop a multiple choice questionnaire (MCQ) to diagnose students' conceptual understanding about the parts and working of a DC motor, and
- 2) determine the item difficulty and discrimination indices of the developed MCQ.

Significance of the study

The results would be useful for teachers to know students' understanding about a DC motor and electromagnetism. This could be used as a guide to design the teaching module that could help students improve their understanding.

Method

1. Research design

This research was carried out as a survey. In order to fulfill the two objectives, the first part of the survey was carried out to collect responses from open and closed-ended questions. Based on these responses, the alternatives for an MCQ can be designed. This method of designing alternatives was adapted from a study by Engelhardt & Beichner (2004) which was used to design their test called DIRECT (Determining and Interpreting Resistive Electric Circuit Concepts Test). The details of the development of the questionnaire are given below.

2. Development of the questionnaire

The development was started with a paper-and-pencil test comprising of 10 open-ended questions based on the physics syllabus prescribed by the Curriculum and Professional Support Division (CAPSD) under the Ministry of Education of Bhutan (CAPSD, 2008). These questions were designed by the researchers and were validated by three Bhutanese teachers who had at least 5 years of experience in teaching physics. Then, 2 questions were found to be irrelevant and some questions were modified to be close-ended. Finally, the modified version comprised of 8 questions with both open and close-ended questions (see Appendix A).

This questionnaire was introduced to 80 Grade 11 Bhutanese students from one of the schools in Eastern Bhutan by their teacher. These participants were those majoring in science and had already studied about DC motor in the previous academic year. They were informed that this test would have no effect on their grade reports and no monetary incentives were given to them. Then, those of students' common responses were coded and grouped together to form alternatives for the MCQ.





Since it was found that all of the students' responses to question 1 were correct, this question was not included in the MCQ. In addition, questions 6 and 7 were combined to be one question. Finally, only 6 questions, one of them consisted of seven sub-questions, were included in the MCQ (see Appendix B). The number of alternatives per question varied because the alternatives were based on students' responses.

3. Implementation of the MCQ

The MCQ was introduced to 130 Grade 10 students from two schools in Eastern Bhutan. These students had already learnt about a DC motor 3 months before the implementation. They took about 15 minutes to complete the questionnaire.

Analysis of the multiple choice questionnaire

The quality of the questionnaire was determined in terms of the item difficulty and item discrimination. Students' responses to the questionnaire were analyzed by scoring +1 for correct and 0 for incorrect answers. Based on this scoring, the details of the analysis are given as follows:-

1. Difficulty index(P)

A difficulty index (*P*) shows the level of difficulty of a question and is calculated by comparing the ratio between the number of students who gave a correct answer to the total number of students who attempted the question (Ding et al., 2006).

If the value of difficulty index is high, it means that there are more number of students who have correctly answered the question which indicated that the question was easier for the students (Ding et al., 2006; Maloney et al., 2001). The appropriate value of the difficulty index suggested by Doran (1980) should be in the range between 0.3 to 0.9.

The difficulty index for each question is given below in Figure 1.

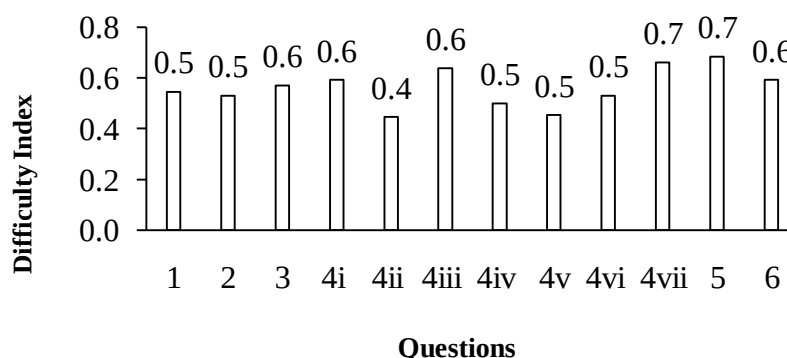


Figure 1: Difficulty index of MCQ

2. Discrimination index (D)

The discrimination index (*D*) shows how well a question can differentiate between high and low achievers. In other words, it can be said that students scoring high on the test as a whole (high achievers) can also score higher than low achievers in a particular question (Maloney et al., 2001).

The discrimination index for each question (see Figure 2) was calculated by first sorting students in order of total scores, highest–lowest. Then three groups were formed to get the high, average, and low achievers groups. After that, the following expression was used to calculate the discrimination index.

$$\frac{N_U - N_L}{(N_U + N_L)/2}$$

where N_U = number of students answering correctly in the **top** 27% of the overall score range (high achievers group)

N_L = number of students answering correctly in the **low** 27% of the overall score range (low achievers group)



The discrimination index value ranges from -1 to 1. Maloney et al. (2001) suggested that the lowest acceptable value was 0.2 while Doran (1980) recommended a good discrimination index had a value ≥ 0.3 .

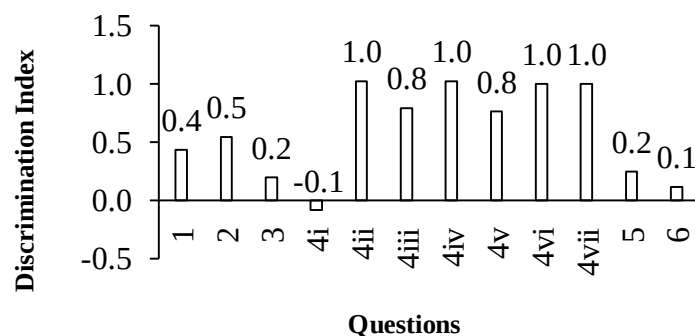


Figure 2: Discrimination index of the MCQ

Result

The value of difficulty indices for this MCQ ranged from 0.4 to 0.7 which mean that difficulty of the questions were not very high and it was acceptable.

In addition, the discrimination index of items were between 0.4 – 1.0 except item 3, 4i, 5, and 6. This mean that the items could be used to separate high and low achievers. This is because the discrimination index of items 3, 4i, 5, and 6 were low (– 0.1 to 0.2) and these items need to be revised.

In a research carried out by Pande, Pande, Parate, Nikam, & Agrekar (2013) to determine how well their MCQ was able to discriminate the students' performance, they found their discrimination index range from – 0.54 to 0.8. The authors used a different range to discriminate their items (negative: defective item/wrong key; < than 0 – 0.19: poor discrimination; between 0.2 – 0.29: acceptable discrimination; between 0.3 – 0.39: good discrimination; >than 0.4: excellent discrimination). Nevertheless, their test item showed a 4% of negative discrimination. According to their guidelines, the negative discrimination index indicates a defective item or a wrong key, which they mention as in need of revision. (Pande et al., 2013)

Conclusion

From the difficulty index we can conclude that the items were not very difficult and these questions were suitable for the participants.

The discrimination index for item 4i was found to be negative whereas it was < 0.3 for items 3, 5 and 6 when compared to the acceptable range of ≥ 0.3 (Doran, 1980).

According to Ding et al. (2006), items with negative discrimination index must be removed. However, since this item is an important concept to be considered while diagnosing students' conceptual understanding about the working of a DC motor, it would be inappropriate to remove from the test. Nevertheless, it can be revised and improved as suggested by Pande et al. (2013).

The result can help teachers find students' conceptual understanding about a DC motor and electromagnetism in detail. This findings will be used to design a learning unit on a DC motor to teach about electromagnetism.

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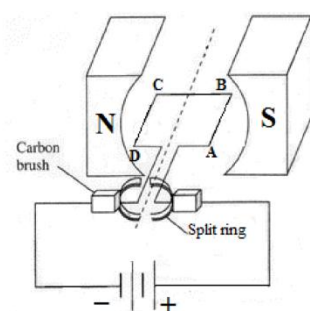




Appendix A

Open-ended questionnaire

1. Can a single loop of an armature rotate? Explain in brief.
2. Why is split/slip ring used in a DC motor?
3. How does the flow of current in the armature make it rotate?
4. If you change the shape of the armature, say from rectangular to circular, do you think it will still rotate? Explain in brief.
5. Use the diagram given below to answer the following questions.



Modified from: <http://lmstuition.weebly.com/uploads/9/5/0/4/9504897/9171408.jpg?302>

- a) The direction of magnetic field of the permanent magnet is from ...pole to pole
 - b) In the arm AB, the direction of:
Current is from point..... to point
Force is.....
 - c) In the arm BC, the direction of:
Current is from point..... to point
Force is.....
 - d) In the arm CD, the direction of:
Current is from point..... to point
Force is.....
6. Which rule did you use to identify the direction of forces in the above diagram?
 7. What does the thumb, index finger and middle finger represent in the rule you stated in question 6?
Thumb represents.....
Index finger represents.....
Middle finger represents.....
 8. How can you increase the rotational speed of the armature? Explain in brief.





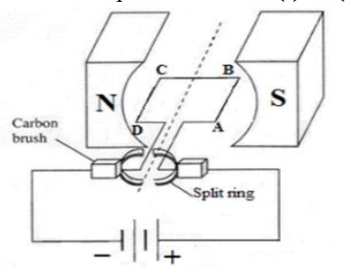
Appendix B

Multiple Choice Questionnaires on DC motor

DIRECTIONS:

Given below are SIX multiple choice questions. You have 10-15 minutes to CAREFULLY READ and put a tick mark [✓] in the box provided near the answer that you think is the best answer.

1. What is the reason for using split/slip rings rather than a complete ring in a DC motor?
 - ☐ To let current flow in the armature, so that it can rotate in one direction.
 - ☐ To let the armature rotate faster since the weight of a split ring is lesser than that of a complete ring.
 - ☐ A split ring can be made using less material compared to a complete ring, thus reducing the cost of production.
 - ☐ A split ring splits the positive and negative charge so that they cannot combine.
2. When current is passed through an armature that is placed near a permanent magnet, it rotates because
 - ☐ The forces of the current will pressure the motor to move which will make it rotate.
 - ☐ Current causes a magnetic field to develop around the armature, which develops force to rotate.
 - ☐ When the current flows from the opposite pole to another pole, the armature strikes with each other and lead to rotation of armature.
 - ☐ Positive and negative charges passes in the opposite direction to the motor and this different direction of flow of charges make motor to rotate.
3. Which sentence about the shape of the armature is correct?
 - ☐ Only the rectangular shaped armature will rotate because we can apply left hand rule.
 - ☐ Only the circular shaped armature will rotate because all things that are circular will rotate.
 - ☐ Both the armatures will rotate because shape does not affect the rotation as long as current and magnetic field is present.
4. Use the diagram given below to answer questions from (i) to (vii).



Modified from: <http://lmstuition.weebly.com/uploads/9/5/0/4/9504897/9171408.jpg?302>

- (i) The direction of magnetic field of the permanent magnet given in the above diagram is from
- ☐ N – S
 - ☐ S – N





- (ii) The direction of current in arm AB of the armature ABCD is
- ☐ A to B
 - ☐ B to A
 - ☐ No current
- (iii) The direction of force in the arm AB is
- ☐ Upwards
 - ☐ Downwards
 - ☐ No force
- (iv) The direction of current in arm BC of the armature ABCD is
- ☐ B to C
 - ☐ C to B
 - ☐ No current
- (v) The direction of force in the arm BC is
- ☐ Upwards
 - ☐ Downwards
 - ☐ No force
- (vi) The direction of current in arm CD of the armature ABCD is
- ☐ C to D
 - ☐ D to C
 - ☐ No current
- (vii) The direction of force in the arm CD is
- ☐ Upwards
 - ☐ Downwards
 - ☐ No force
5. Looking at the diagram given in question 4, which rule did you use to identify the direction of forces in the arms AB, BC, and CD in the armature ABCD?
- ☐ Left hand rule
 - ☐ Right hand rule
6. All of the following are ways to increase the rotational speed of the armature EXCEPT:
- ☐ Increasing the current in the coil
 - ☐ Increasing the intensity of the magnetic field
 - ☐ Increasing the number of turns of the coil
 - ☐ Increasing the area of the coil





EDI02

Modelling Science Learning Through Language

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Abstract

Language facilitates students' science learning by providing a system for thinking and constructing understanding, as well as an avenue for communicating students' ideas. In a multilingual setting as in this study, however, the choice of language of instruction in education is a concern and often poses a challenge to both teachers and policy makers. Thus, this study aimed to provide a basis in developing a language model in teaching and learning tertiary level science courses. This study employed a quasi-experimental design to determine the academic achievements of two college science classes where one was taught using monolingual English instruction (control group), while the other (experimental group) was taught using bilingual instruction (English and Filipino) in one of the top universities in Baguio City, about 400 km north of Manila, Philippines. Filipino has been acknowledged as the national lingua franca in the Philippines while English has been referred to as the International lingua franca or second language. Multiple regression and path analysis in the experimental group showed that English and Filipino proficiencies have direct effects on the academic performance. The significant positive correlation between the academic performance of the experimental group and the proficiencies in Filipino and English proved that language plays an important role in learning science. The significant positive correlation between academic performance and English language proficiency of the control group further confirms the relationship that to achieve high academic performance in the course, students must possess high proficiency in the language used in instruction.

Keywords: Academic performance, language proficiency, language model in teaching and learning, science

Introduction

The ways in which children acquire language and construct knowledge in non-school environments and the dynamic relationship with what they are taught in school is maximally relevant to school learning. Children who begin school without having been socialized to conventional forms of school communication may experience communicative failures that are interpreted as lack of aptitude and intelligence (Heath, 1983). Studies of language minority students demonstrate how the routine communicative expectations of language majority teachers can be misinterpreted because of lack of teacher's understanding of the cultural norms and practices in their students' out-of-school lives (Au & Mason, 1983; Erickson & Mohatt, 1982). The interaction of culture, language, and schooling has been a productive focus of research in a number of disciplines. A great deal is known, for example, about how and why differences in cultures and languages of school and home can be problematic for students (Au & Mason, 1983). Even among speakers of the same languages, problems may arise if the home register does not match the privileged formal register of schools.

Kelly (2000) summarized the importance of viewing education through language and social processes by three primary observations. First, teaching and learning occur through processes constructed through discourse and interaction. Discourse consists of all other things that help us make sense of language. These are different ways of thinking, acting, interacting, valuing, feeling, believing, and using symbols, tools and objects. Second, student access to science is accomplished through engagement in the social and symbolic worlds comprising the knowledge and practices of specialized communities. Third, disciplinary knowledge is





constructed, framed, portrayed, communicated, and assessed through language, and thus understanding the epistemological base of science and inquiry requires attention to the uses of language.

Language plays an important role in mediating students' science learning by providing a system for thinking and constructing understanding, as well as means for communicating their ideas (Lee, 2001). It is a mental tool that facilitates development. Thus, language frames one's thinking and this thinking is then carried out through the use of oral and written language (Fain & Horn, 2006). Gee (1996) advocated the term *discourse* as an integrated way of being, valuing, thinking, feeling and acting that can be used to identify oneself as a member of a socially meaningful group. Learning science involves students making sense of nature and naturally occurring events in a sociocultural context (Solomon, 1987; Vygotsky, 1987). Students must use their prior knowledge, sensor experiences, information sources, and social interactions to personally construct understandings of science ideas. Discourse, social practice, and the science content all influence this meaning-making process. Thus, science instruction needs to build a learning community where students' discourses will be recognized and border crossings between discourses facilitated in the process of learning science (Huang, 2006).

A milestone for discourse studies in science education is Lemke's (1990) *Talking Science: Language, Learning, and Values*. Lemke applied a social perspective to classroom discourse in a set of studies of secondary science classrooms. Lemke's work began with the premise that science lessons are social activities constructed through human action. These human actions occur with the contingencies of the moment and are generally constrained by activity structures and norms for interaction. Lemke's analysis identified both thematic and organizational patterns in science dialogues in classrooms. The thematic patterns represented the particular semantic relationships comprising the scientific knowledge discussed. This was often in the form of propositional knowledge, controlled by the teacher, with little opportunity for students to take initiatives within the conversations. Through the analysis of the organizational and thematic patterns and their relationship, Lemke established his central thesis that learning science is learning how to talk science – that is, observing, describing, comparing, classifying, discussing, questioning, challenging, generalizing, and reporting, among other ways of talking science.

Education for All means a quality education for all. This entails considering the various cultural and linguistic contexts that exist in contemporary societies. The Universal Declaration on Cultural Diversity, adopted in 2001, touches upon the importance of languages for the promotion of cultural diversity. Article 6 of the Action Plan for the implementation of the Declaration defines the role that languages should play in the field of education including respect for the mother tongue, linguistic diversity at all levels of education and the promotion of multilingualism from an early age.

In a multilingual setting as in this study, however, the choice of language of instruction in education is a concern and often poses a challenge to both teachers and policy makers. Thus, this study aimed to provide a basis in developing a language model in teaching and learning tertiary level organic chemistry.

Methodology

Research Locale

This study was conducted in one of the major universities in Baguio City, the summer capital of the Philippines, about 400 km north of Manila. Baguio City is home to many immigrants from other parts of the country. Aside from Ilokano, Filipino is the lingua franca in Baguio City. The English and Filipino languages have assumed different roles in Philippine society. Filipino has been acknowledged as the national lingua franca while English has been referred to as the International lingua franca or second language. Thus, Filipino (as lingua franca) is taught starting grade school and widely used and understood in the Philippines.

The Sample/Respondents

The sample of the study included two comparable science classes. Both classes were handled by the researcher-science teacher. One class was exposed to bilingual teaching (experimental group) while another class was randomly designated to be taught using the conventional way of teaching or using English as medium of instruction (control group).





Teaching approaches

The control and experimental groups were taught the same topics for one semester following the same syllabus. Both classes were taught for 60 minutes, three days or three hours each week, for 18 weeks or a total of 54 hours in one semester. The content of instructional and evaluation materials were parallel in both classes following the University calendar. Participatory learning was promoted in both classes during classroom discussions. The techniques include: graded recitations, board works, paired discussion, group reports and quizzes.

Experimental Group

The evaluation materials used in the experimental group were expressed in both English and Filipino and students were allowed to express their answers either in English, Filipino or both particularly in the essay questions. The medium of instruction and learning in the experimental group was bilingual. In which case, additive bilingual instruction was employed, wherein the Filipino language was not removed as a medium of instruction nor used for less than 50 percent of the class hour throughout the semester, except during administration of survey questionnaires, quizzes and examinations. Scientific terms are usually remained to be expressed as such. During classroom activities and discussions, students were encouraged to express their ideas in English, Filipino or in both languages.

Control Group

The English language was the medium of instruction in the control group. Students were highly encouraged to speak in English inside the classroom more especially that there were foreign students in the class. All instructional and evaluation materials used as indicated in the syllabus were in English similar in content to what was given to the other group. Strategies and activities employed in the control group were also done in the experimental group except for the language used in teaching and learning.

Results and Discussion

Academic Achievement

“Often teachers equate lack of adequate proficiency in the language of instruction with laziness, lack of intelligence or an uncooperative attitude on the part of the students. This type of attitude can seriously inhibit students and create anxiety related to language learning and learning in general. To avoid being shamed in front of their classmates, some students refrain from volunteering to answer in class.” (Alidou & Brock-Utne, 2006)

Guided by the circumstance just presented, the researcher carried out this study to address related language issues encountered by Filipino students. The results of the proficiency tests in English and Filipino showed that the levels of proficiency of the students in the two languages were high although the level of proficiency in the English language was not sufficient to achieve high content knowledge of the course taught in English. The data gathered in this study unveiled that the use of bilingual instruction was more effective in facilitating the teaching and learning science than the monolingual English instruction. For students who were not proficient in the English language, the freedom to communicate their answers in Filipino helped them convey their scientific learning better as they were more confident in their own language. Bilingual students participated more often in classroom discussions and demonstrated greater self-confidence and higher motivation, as also established by ADAE (1996), Dalby (1985) and Dutcher (1995). The use of Filipino language allowed the student to express their full range of knowledge and experience and demonstrated their competence. This is supported by pedagogical approaches like those of Piaget and Vygotsky and is productive for learning (Richardson, 2001).

One of the advantages of using both English and Filipino in assessment and evaluation in the experimental group realized in this study was that a student who was not proficient in English but proficient in the latter, was able to express her/his ideas confidently and clearly in the said language. Although the scoring rubrics utilized for the essay questions focused solely on students' achievement with respect to the topic being assessed and not on their ability to write well, students needed to communicate in a manner that will be clear to those scoring their responses. Comparing the responses of one student from the experimental group and another from the control group who had comparable proficiencies in the English and Filipino languages, it was noted that the responses of the student from the experimental group were more comprehensible than the responses





expressed in English by a student from the control group. Undoubtedly, language used in examinations has a great impact on students' performance in examinations. Several studies proved (Kalole, 2004; Makelela, 2005) that in all subject disciplines, students' scores reflect their proficiency in the language used in the examination and that difficulties with, for example, English have a negative effect on results. Wilmot (2003) tested students who gave average performance with mathematics examinations in English. Using the same examination but asking them to answer in the mother tongue, he found that students' level of knowledge was better than in the test conducted in English. Students whose language competencies do not correspond to their knowledge in the subject matter are thus disadvantaged, a fact that will also continue to be reflected in international comparative studies such as TIMSS (Fredua-Kwarteng & Francis Ahia, 2004).

The academic performances (AP) of the control and experimental groups in science were determined through the scores of the students from the seven quizzes and three examinations administered throughout the semester. The total raw scores were converted to percentage values and the mean of each group was analyzed. The data gathered at the end of the semester produced a mean rating of 65.51 (High) by the experimental group and 62.98 (High) by the control group. Although both the experimental and control groups were quite proficient in the English language, students' level of proficiency was not sufficient to use the English language alone to learn subject content as also confirmed by William (1998). International research shows that students learn simple conversational skills in a new language quickly, that is, within one to two years. However, even in well-resourced environments, it takes at least six years to learn abstract academic language skills that characterize teaching and learning (Alidou *et al.*, 2006; Cummins, 2000). What is important to note here is that, while second-language learners may pick up oral proficiency in basic interpersonal communicative skills (BICS) in their new language in as little as two years, it may take up to seven years to acquire the decontextualized language skills or cognitive academic language proficiency (CALP) necessary to function successfully in a second-language classroom (Cummins 1979; Tucker, 1999).

Correlation and Regression Analyses (Experimental Group)

Path analyses were carried out in this study to decompose correlations into different pieces for interpretations of effects. In the model shown in Figure 1, academic performance has paths to it from English proficiency and Filipino proficiency. The numbers on the arrows in Figure 1 are called path coefficients, indicating on the strengths and direction of the relationship. In the model, a single-headed arrow points from English proficiency and Filipino proficiency to academic performance. A double-headed, curved arrow indicates that variables are merely correlated; no causal relations are assumed. This kind of arrow is seen between the variables English proficiency and Filipino proficiency, preferred language in learning and teaching. The causal model implies that English and Filipino proficiencies have direct effects on the academic performance of the experimental group. Preferred language in teaching and learning science has no direct effect upon academic performance, but has an indirect effect through Filipino proficiency. Bilingual preference in teaching is positively correlated to proficiency in the Filipino language while preference in the English language in teaching is negatively correlated to Filipino proficiency. Learning in bilingual mode is also correlated to Filipino proficiency. Proficiency in the English language is correlated to the proficiency in the Filipino language.

The positive correlation between English and Filipino proficiency is consistent with the "developmental interdependence hypothesis" by Cummins (1981). This hypothesis suggested that bilingual students' first and second languages acted on each other. It asserted that students' level of competence in one language was a function of his or her competence in the other language. Thus, exposure in one language contributed to the deeper conceptual and linguistic development of the other language. Ocampo (2002) also concluded that although literacy development in English and Filipino seems to progress at different rates, underlying skills in literacy show a high degree of cross-language interdependence.



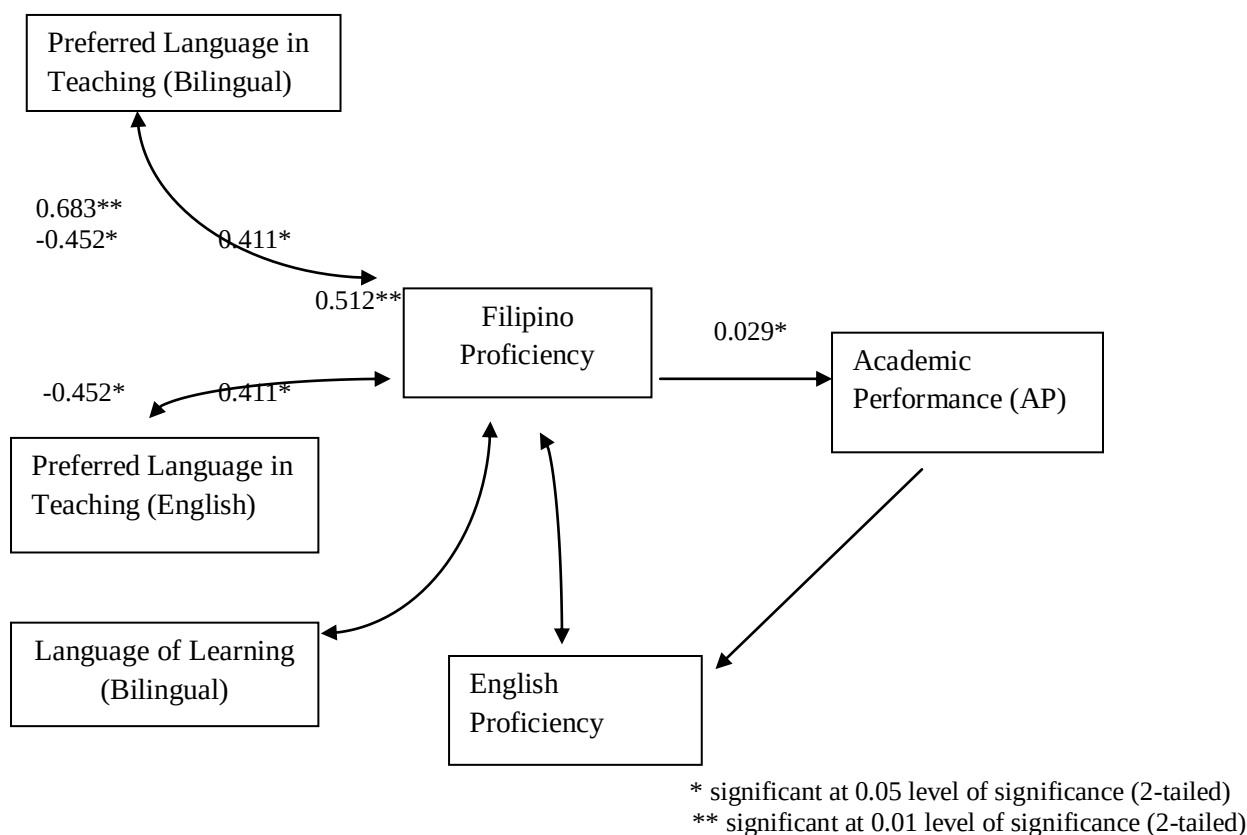


Figure 1. Path Analysis of the Factors Affecting the Academic Performance of the Experimental Group

Correlation and Regression Analyses (Control Group)

Correlation analysis revealed a positive correlation between AP of the control group and English proficiency. A causal model was established based on the multiple regression analyses and multiple correlation analysis as shown in Figure 2.

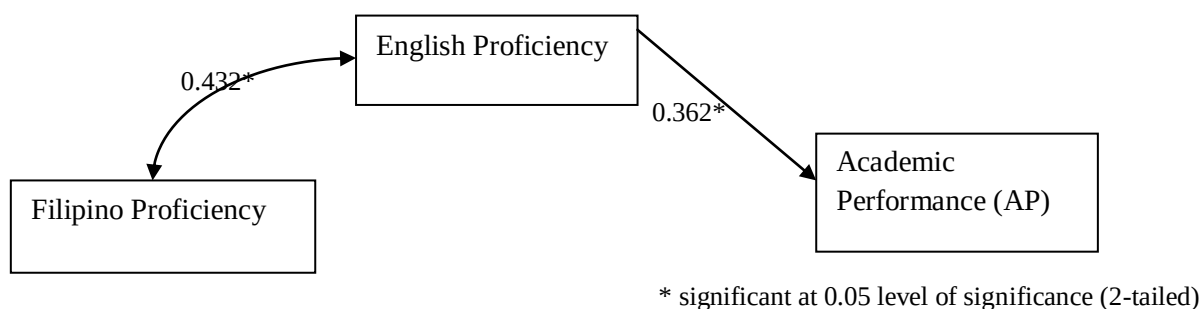


Figure 2. Path Analysis of the Factors Affecting the Academic Performance of the Control Group

The causal model implies that English proficiency has a direct effect on the academic performance of the control group. Meanwhile, Filipino proficiency has no direct effect upon academic performance, but has an indirect effect through English proficiency. English proficiency is positively correlated to proficiency in the Filipino language. The results imply that in order to attain higher academic achievement in monolingual English instruction, proficiency in the same language is very important. The Filipino proficiency has no direct effect on





academic achievement thus making the proficiency in the national language of the Philippines less significant compared to its effect on bilingual instruction. But still the positive correlation between English and Filipino proficiency is consistent with the “developmental interdependence hypothesis” by Cummins (1981).

Conclusions and Recommendations

The significant positive correlation between the academic performance of the experimental group and the proficiencies in Filipino and English proved that language plays an important role in learning science. In particular, the relationship implies that students who have high proficiencies in English and Filipino also have high academic performance in the course. The significant positive correlation between academic performance and English language proficiency of the control group further confirms previously discussed relationship that to achieve high academic performance in the course, students must possess high proficiency in the language used in instruction. Thus, for students in the monolingual English instruction, attainment of high academic performance in science necessitates high proficiency in the English language. Based on the findings of the study, the Commission on Higher Education (CHED) of the Philippines may develop a dual language program in Baguio City employing English and Filipino languages in teaching tertiary level science subjects.

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EDI03

Development of Learning Management Based on Applying Metacognition and Multiple Intelligences Theory, Life and Environment to Promote Critical Thinking and Self-Directed Learning of Prathomsuksa 6 Students

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Abstract

The purposes of this research were : 1) to develop learning management based on applied of Metacognition strategy and Multiple intelligences approach to promote on critical thinking and self-directed learning in science learning for Prathomsuksa 6 students, 2) to study outcomes of learning management based on applied of Metacognition strategy and Multiple intelligences approach in science learning were as : 2.1) to study the efficiency of learning management based on applied of Metacognition strategy and Multiple intelligences approach active learning approach in science for Prathomsuksa 6 level, 2.2) to compare critical thinking of students, between before and after learning based on that approach, and 2.3) to compare self-directed learning of students, between before and after learning based on that approach. This study provided based on Research and Development approach for 3 phase, the first phase was studying for the preliminary problems and needs concerned with science learning management by using questionnaire with 90 students, interviewing of 2 teachers and 10 students who were concerned in learning management, including studying for strategy, theory about metacognition and the multiple intelligences to synthesize learning management to promote on critical thinking and self-directed learning in science.

Then presented learning management to the experts for assessing the quality and appropriateness before implementing. For the second phase was trying-out learning management with 32 students in Choomchon Pon-ngamponsawhang school, and revised for inappropriate issues to be better elements. Lastly, the third phase was implementing for learning management with 23 students in Ban Nonmuang school, Kosumpisai District, Mahasarakham Province. The research instruments were comprised of : 1) questionnaire for the students about problems and current state of leaning management , 2) interviewing forms of the problems and needs in learning management with teachers and the students, 3) plans for learning management based on applied of Metacognition strategy and Multiple intelligences approach in science for 6 plans, with 16 hours 4) students' learning achievement test with 25 items, 5) the 25 items test of critical thinking, and 6) the 15 item scales of self-directed learning. The statistics used for analyzing data were percentage, mean, standard deviation, and t-test (Dependent sample) was employed for testing hypotheses.

The results of the study were as follows :

1. The preliminary information in problems and needs of learning management in science for Prathomsuksa 6 level found that the students pertained in problems and needs to promote of higher critical thinking and self-directed learning. Additionally, the results of developing learning management based on applied of Metacognition strategy and Multiple intelligences approach in science learning strategy. Which was composed of 5 steps, they were : 1) Engagement step, 2) Exploration and confronting the problems, 3)





Analyzing and connecting of knowledge, 4) Synthesizing in gaining of knowledge, and 5) Concluding and affirming of knowledge. Whereas, the result of assessment for the quality and appropriateness of learning management from the experts were at a high to highest level for each plan, and for overall of most plans was at a high level.

2. The learning management based on applied of Metacognition strategy and Multiple intelligences approach, in science learning for Prathomsuksa 6 level, had the efficiencies of 83.86/76.17, which obtained from achievement score.

3. The students who learned based on applied of Metacognition strategy and Multiple intelligences approach showed higher of critical thinking than before learning, at the .05 level of significance.

4. The students who learned based on applied of Metacognition strategy and Multiple intelligences approach showed higher of self-directed learning than before learning, at the .05 level of significance.

Keyword : Development of Learning Management Based on Applied of Metacognition Strategy and Multiple Intelligences Approach, Critical Thinking, Self-directed Learning

Introduction:

Science Subject played an important role in developing the country to be advanced rapidly, and using Science and Technology as a major instrument for different aspects of development. As a result, the country was firm and progressed, people could develop their thinking regarding to Reasoning Thinking, Creative Thinking, Analytical Thinking, Critical Thinking, skill in searching for knowledge, problem solving for developing their work systematically. So, it was necessary to enhance the students to be transferred Science Knowledge. Besides, Science was also an instrument for helping people to have better quality of life as well as facilitating the people's life and work. (Department of Curriculum and Instruction Development, 2011: 1) According to Self assessment in quality and learning achievement of Ban-none-mung-song-kon School (2003: 24) in 2013 academic year, found that Pratomsuksa 6 Students still had problem in studying Science Subject regarding to their Learning Achievement as well as Critical Thinking which didn't accomplish the specified goal of school. The quality of these aspects needed to be developed in higher level in every context of teachers' instructional management in school so that the school would be more successful in Educational Management.

Critical Thinking was a higher order of thinking, and important thinking skill for livelihood in society. It was a kind of thinking to be based on knowledge, data collecting from various experiences, and organized for solving one's life problem. Therefore, the persons who would be able to live happily in current society, had to have Critical Thinking so that they would be survived from different problems and obstacles quickly. (Amonwich Nakontap, 2006 : 11) The Critical Thinking was important for every one in society. Since it would be administered as an instrument for making decision with the occurred information in various situations. Consequently, the broader world view would be happened. Critical Thinking could help people to search for problem as well as problem solving technique by themselves. To enhance the students' Critical Thinking, they had to experience in simple level of thinking before including the preliminary thinking and intermediate thinking without complex steps of thinking. It should be skill to be used in one's real life as well as learning different subjects such as listening, reading, asking, explaining, observing, classification, comparison, and conclusion. Combining these skills, the higher order of thinking would be occurred. The Critical Thinking could be developed when the students participated in activity by themselves in group or pair. The students would have direct experience in thinking. Moreover, they would be practiced in expressing their opinion, being careful, viewing the mistake, and investigating their unsystematic thinking. (Prapansiri Susaorach, 2008: 186-195)





Self-directed in learning, was one's Metacognition Learning Ability which would help the students to have intellectual development by knowing how to use and applying their knowledge in new situation which would help them to understand it better. Furthermore, they would have learning experience in promoting the development of Self-directed in learning. Besides, it was also an important practice which would lead to be life lone learners as well as competent problem solvers in every situation. When the students faced with new problem or situation they never experienced to solve the problem successfully before. They had to learn from those situations in order to find the practice method, information searching, and apply the prior knowledge with the new one in practice. (Nirad Chantarchit, 2007: 118-124) The Self-directed Learning was a method for promoting the students to create their concept and competency in participation in Problem-based activity. The students' knowledge creation would encourage their responsibility in learning. They had to learn how to explain and cope with what they had to learn. (Howard 2002: 41) The utility of learning included the management of various environmental conditions for facilitating the students in participatory activity for thinking and discussion in order to lead to decision making and opinion with persistence in Self-directed in learning, administering in knowledge integration and transfer of learning, and organizing the environment to facilitate the experts advice and students' participation in constructing the body of knowledge.

The Metacognition Strategy, was a method leading to the persons' competency in controlling and monitoring the intellectual process or thought process, being aware of work and able to use the working strategy until it was perfectly finished. According to research studies in Metacognition Strategy for learning development, found that the students had high learning achievement and could develop interaction with new information quickly by associating the new knowledge with the former one very well. In addition, they could have self-directed in developing comprehension in knowledge content intensively. (Oxford 1990: 1) The Metacognition Strategy could help students in learning because the students who were aware of Metacognition Knowledge, would be able to use the Metacognition Strategy in promoting the learning plan efficiently as well as investigating their own learning whether it would accomplish the specified goal. It was a guideline for helping the students to develop the higher order of thinking, and giving an importance for students' learning process by themselves systematically through higher order of thinking until it was successful by using the activity in determining the expected goal to be achieved, designing and planning the studying to be achieved in aligned with the investigation and monitoring the Self-directed under condition of acceptance and understanding in their own concept valuably. In addition, they could have Self-directed in learning successfully. Besides, Metacognition had an influence on people's belief in their learning attitude which could affect the students' behavior and help them to be able to control and evaluate their own thought successfully. (Tidsana Kammanee, 2011: 69)

The application of Metacognition would promote the students to be able to view and comprehend the problem situation in various dimensions, and think about how to search for answer by various dimensions as well. Consequently, the design of learning activity in each context or situation to be expanded its boundary broadly. The Metacognition Learning was interesting and facilitating the students' learning in every context. (Tidsana Kammanee, 2002: 89-90) The people's important intelligence consisted of the creative intelligence, analytical intelligence, and practical intelligence whereas the Problem-based learning management was to create the students' challenging in facing the problem as well as be organized the incidence by applying one's knowledge experience with the searching for answer. (Ong & Borich 2006: 69)

According to the above reasons and significance, the researcher as a practitioner in learning activity of Science Subject for Pratomsuksa 6 Students, Ban-nong-muang-song-kon School, viewed that Pratomsuksa 6 Students faced with the problem in their learning achievement associated with Critical thinking as well as Self-directed in learning which were important factors promoting the students to learn Science successfully. So, the researcher was interested in implementing the development of Critical thinking as well as characteristic of Self-directed in Science learning to be more successfully and efficiently through R & D process by applying the Metacognition Strategy and Multiple Intelligent Theory, in addition to Learning Management Design in order to accomplish research findings with discovery in efficient Learning Management Process which could be modified





as Learning Management Guidelines for students in part related to Science Subject to be successful as well as information technology for those who were interested in the above approaches and research findings in order to conduct additional research and apply in Science Learning Situation to be more efficient.

Research Objectives:

1. To develop Learning Management by applying Metacognition Strategy and Multiple Intelligent Theory, titled “Life and Environment” in Science Subject for Pratomsuksa 6 Students.
2. To study the findings of Learning Management by applying Metacognition Strategy and Multiple Intelligent Theory, titled “Life and Environment” in Science Subject for Pratomsuksa 6 Students as follows:
 - 2.1 To study the efficiency of Learning Management by applying Metacognition Strategy and Multiple Intelligent Theory, titled “Life and Environment” in Science Subject for Pratomsuksa 6 Students.
 - 2.2 To compare Critical Thinking of Pratomsuksa 6 Students who obtained Learning Management by applying Metacognition Strategy and Multiple Intelligent Theory between pretest and posttest.

Research Hypothesis:

1. The students who obtained Learning Management by applying Metacognition Strategy and Multiple Intelligent Theory, had critical thinking in higher level after studying.
2. The students who obtained Learning Management by applying Metacognition Strategy and Multiple Intelligent Theory, had self-directed in higher level after studying.

Delimitation of the study:

In this study, the researcher determined the delimitation of study as follows:

1. The research design of this study implemented based on 3 Phases of Research and Development framework as follows:
 - 1.1 The basic information of basic information in the problems, conditions, and needs as well as theoretical approach of Multiple Intelligence and Metacognition Strategy were studied and synthesized for designing. In addition, the basic information was synthesized for designing the Critical Thinking, and Self-directed. Later on, it was investigated its quality and propriety by the experts.
 - 1.2 The designed Learning Management from Phase 1, was tried out with Pratomsuksa 6 Students. Furthermore, the incomplete issues were improved to be higher quality.
 - 1.3 The Learning Management was administered with Pratomsuksa 3 Students who were the samples. The quality of Learning Management was reported.
2. The Population, sample, and target group of this study consisted of:
 - 2.1 Population included 143 Pratomsuksa 6 Students from 5 classes of Ban-none-muang-song-kon School and Choomchon-pone-ngam-pone-sawang School, Ko-soom-pisai District, Mahasarakam Province during 2014 academic year.
 - 2.2 The samples, 90 students from 3 classrooms, 2 schools who were asked their opinion on the Science Learning Activity in class whether it was interesting or not, and the Science Learning Activity as well as knowledge and competency in Science they wanted to be useful for them by their teachers.
 - 2.3 The target group, 60 Pratomsuksa 6 Students were observed their learning behavior in Science Class. They were studying in Ban-none-muang-song-kon School and Choomchon-pone-ngam-pone-sawang School, Ko-soom-pisai District.
 - 2.4 The target group, 60 Pratomsuksa 6 Students were asked the strength and weakness of Science Learning Activity they wanted to be remained and improved as well as utility they expected to be received from Science Learning from Ban-none-muang-song-kon School and Choomchon-pone-ngam-pone-sawang School.
 - 2.5 The target group, 2 Science Teachers, 1 from each school who were interviewed their classroom learning associated to learning quality according to the goal as well as condition which the students might not accomplish in-learning based on the indicators of curriculum especially the Critical Thinking and





Self-directed in learning.

3. Delimitation of content, the content using in this study was content according to Science Learning Activity Plan, Critical Thinking, and Self-directed of Pratomsuksa 6 Students for practicing their Critical Thinking and Self-directed in learning, included the content in Learning Activity Management titled “Substance in Daily Life,” of Pratomsuksa 6 Students.

4. Duration of study, was started from the second semester of 2014 academic year in January to March 2015.

5. Studied Variables

5.1 Independent Variable included Learning Management by combining the Metacognition Strategy and Multiple Intelligent Theory.

5.2 Dependent Variable included the Critical Thinking and Self-directed.

Data Analysis:

The researcher analyzed the findings of experiment based on research objective by using the Computer Program for data analysis as follows:

1) The quality of Learning Management Process was analyzed by applying the Metacognition Strategy and Multiple Intelligent Theory in to Science Learning of Pratomsuksa 6 Students by calculating the Mean, and Standard Deviation of the experts’ scores in evaluating the quality and propriety. 2)

The efficiency of Learning Management was analyzed by applying the Metacognition Strategy and Multiple Intelligent Theory based on criterion 75/75 by using the formula E_1 / E_2 from the score during studying, and posttest learning achievement score of 23 sample students from Ban-none-muang-song-kon School.

3) The sample students’ pretest and posttest Mean Scores of Critical Thinking, were analyzed in order to compare by t-test (Dependent Samples).

4) The pretest and posttest Mean Scores of Self-directed, were analyzed in order to compare by t-test (Dependent Samples).

Conclusions:

The research findings of development in Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory titled “Life and Environment,” in Science Learning of Pratomsuksa 6 Students, could be concluded as follows:

1. The findings of development in Learning Management by applying the Metacognition Strategy

and Multiple Intelligent Theory titled “Life and Environment,” in Science Learning of Pratomsuksa 6 Students, the quality was found. Since the researcher studied the situation, problem, and basic information of Learning Management Practice by applying the Metacognition Strategy and Multiple Intelligent Theory in Science Subject of Pratomsuksa 6 in both of teachers and students, the basic approach and steps of Learning Management for designing the Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, in Science Subject of Pratomsuksa 6, and presented the experts to investigate the quality and propriety. The findings were as follows:

1.1 Pratomsuksa 6 Student Group of Ban-none-muang-song-kon School still had problem in learning situation and Learning Management Readiness with quality. Moreover, they wanted to enhance their Critical Thinking and Self-directed in higher level.

1.2 The Experts’ quality evaluation in Learning Management Plan by applying the

Metacognition Strategy and Multiple Intelligent Theory, the evaluative findings of each plan ranged from “High” to “The Highest” level, the Mean Values between 4.42-4.62. Besides, the overall finding of quality evaluation was in “High” level, the Mean Value was = 4.49.

2. The findings of Learning Management Plan by applying the Metacognition Strategy and Multiple Intelligent Theory in Science Subject of Pratomsuksa 6, were as follows:





2.1 The Learning Management Plan by applying the Metacognition Strategy and Multiple Intelligent Theory in Science Subject of Pratomsuksa 6, included the efficiency 83.86/76.17 which was higher than the specified criterion as 75/75.

2.2 The students obtained Learning Management Plan by applying the Metacognition Strategy and Multiple Intelligent Theory, had the higher level of posttest Critical Thinking than the pretest at .05 significant level.

2.3 The students obtained Learning Management Plan by applying the Metacognition Strategy and Multiple Intelligent Theory, had the higher level of posttest Self-directed than the pretest at .05 significant level.

Discussions

According to the research findings for developing the Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, titled “Life and Environment,” in Science Learning of Pratomsuksa 6 Students, there were important issues to be discussed as follows:

1. The development of Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, titled “Life and Environment,” in Science Learning of Pratomsuksa 6 Students, the development of development on the basis of context in problem and need of Ban-none-muang-song-kon School, was implemented. It might be due to the nature of subject which was difficult learning context. As a result, the most of Primary School Students still had low level of learning achievement score as well as competency in searching for knowledge or problem solving in rather “Low” level. It was supported by the evaluative findings in students’ quality in each academic year of The Office of National Testing throughout the country, was in rather “Low” level. Since in former year, 2013 academic year, Pratomsuksa 6 Students had their learning achievement in Science Subject = 30.27. (The Office of National Testing, 2013: 2) After the design of learning activity by applying the Metacognition Strategy and

2. Multiple Intelligent Theory which consisted of 5 steps of learning Activity including: (1) the Engagement step, (2) Exploration and confronting the problems, (3) Analyzing and connecting of knowledge, (4) Synthesizing in gaining of knowledge, and (5) Concluding and affirming of knowledge consisted of 6 plans, duration of learning was 16 hours, were presented to the experts to evaluate the quality and propriety, found that the evaluative findings of each plan were ranged from 4.42-4.62, the quality were in “High” level to “The Highest” level. Besides, the overall Mean Value of of each plan was = 4.49 which was in “High” level. It might be because the Learning Activity in the plans were deigned by using the Metacognition Strategy focusing on the students to face the problem situation as well as plan to search for the answer by themselves systematically and reliably in aligned with Critical Thinking and reviewing what they had practiced whether it was accomplished or not, and what would be the cause. They could adjust their practice guidelines by themselves until it was ascertained that their work task could accomplish the expected goal or there would have some weak points or obstacles or not. They encourage their students to think with awareness as well as pay attention to and be persistent for the practice to be successful as being expected. It was congruent with condition of their own capacity. In addition, the Learning Management of this study focused on the students to have opportunity in learning in group to help each other, and collaborate for participating in activity to be successful. When the students viewed that the content was difficult and complex, they would be explained by their fiends in the same group in order to perceive and comprehend simultaneously. Moreover, the students would be suggested how to solve the problem relevant to the issue they want to achieve, and evaluate their success based on collaborative goal.

It was congruent with opinion of Otero (2009: 47) that the Metacognition was an approach supporting the students to think and consider in order to search for answer or participate in activity to be successful by using the process to accomplish goal systematically through the questions enhancing students to monitor and control themselves in creating their comprehension by considering information and news associating with expected goal. Furthermore, to be leader of learning, the students had to stimulate themselves to be successful in studying with expected condition for being aware of learning of themselves, consider and investigate their need to design and





develop strategy for learning to be congruent with their need.

Then, the designed learning strategy would be practiced in order to achieve goal. Kluwe (1982: cited in Hacker et.al., 2009: 1) determined the components of learning process for promoting the efficient learning as Self-awareness, Self-determination, and Self-direction. The students would be leaders and initiated thinking in order to create the understanding in themselves and the other environments. Later on, they would control their thought and behavior as well as monitor and investigate the others to be occurred. Self understanding was a component of Self-concept to perceive and understand the condition of themselves in many aspects based on structure of Sociology, Psychology, and Philosophy. For the structure of Self, the monitoring and investigation in one's comprehension in thinking and thoughtfulness condition. The major components of Metacognition could be applied with every kind of task the students wanted to practice including: (1) knowledge and belief of oneself, (2) investigation of thoughtfulness, (3) monitoring and directing one's own thought. (Hacker et.al., 2009: 2) According to these factors, it affected the pattern designing in Learning Management which applied the learning management approach by applying the Metacognition Strategy and Multiple Intelligent Theory, titled "Life and Environment," in Science Learning of Pratomsuksa 6 Students, which was efficient and useful for students as shown in this study.

3. The development of Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, titled "Life and Environment," in Science Learning for promoting Critical Thinking and Self-directed in Pratomsuksa 6, the Efficiency was = 83.86/76.17 which was higher than the specified criterion. It indicated that students had score during their studying from worksheet and quiz after 6 plans, for 83.86. In addition, the had their posttest score from the Learning Achievement Test = 76.17%. It was indicated that the Lesson Plan by applying the Metacognition Strategy and Multiple Intelligent Theory, developed by the researcher, was efficient. It might be because the Lesson Plan by applying the Metacognition Strategy and Multiple Intelligent Theory titled "Life and Environment," in Science Subject of Pratomsuksa 6 Students, through the design process of learning activity in the designed plan by using the Metacognition Strategy focusing on the students to face problem situation as well as plan to search for the answer by themselves systematically and reliably in aligned with Critical Thinking and reviewing what they had practiced whether it was accomplished or not, and what would be the cause. They could adjust their practice guidelines by themselves until it was ascertained that their work task could accomplish the expected goal or there would have some weak points or obstacles or not. They encourage their students to think with awareness as well as pay attention to and be persistent for the practice to be successful as being expected. It was congruent with condition of their own capacity. In addition, the Learning Management of this study focused on the students to have opportunity in learning in group to help each other, and collaborate for participating in activity to be successful.

When the students viewed that the content was difficult and complex, they would be explained by their friends in the same group in order to perceive and comprehend simultaneously. Moreover, the students would be suggested how to solve the problem relevant to the issue they want to achieve, and evaluate their success based on collaborative goal. It was congruent with opinion of Otero (2009: 47) that the Metacognition was an approach supporting the students to think and consider in order to search for answer or participate in activity to be successful by using the process to accomplish goal systematically through the questions enhancing students to monitor and control themselves in creating their comprehension by considering information and news associating with expected goal. Furthermore, to be leader of learning, the students had to stimulate themselves to be successful in studying with expected condition for being aware of learning of themselves, consider and investigate their need to design and develop strategy for learning to be congruent with their need. Then, the designed learning strategy was practiced to accomplish goal. Carr and Ogle (1987: 626-631) explained the major principle of Critical Thinking that the Learning Activity through Critical Thinking based on Metacognition Strategy, this kind of learning activity associated theory of prior knowledge with learning. Consequently, the students had their thinking process systematically. They made plan, monitor, control, investigate, and evaluate their thinking process. They knew how to select the strategy or guideline for learning in different contexts appropriately. It would be seen that it was Student-centered. Besides, it was a process





indicating the Critical Thinking by concluding one's own performance as well as discussing with friends in group in order to develop one's Critical Thinking efficiently. The Critical Thinking was focused on so that the students would be able to conclude at the end after finishing the instructional activity management.

Consequently, the students were able to accomplish goal of Critical Thinking and Self-directed in learning. According to these conditions and factors, it affected the Lesson Plan by applying the Metacognition Strategy and Multiple Intelligent Theory titled "Life and Environment," in Science Subject of Pratomsuksa 6 Students, to be efficient as shown in this study.

4. The students taught by Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, had higher level of posttest Critical Thinking than the pretest at .05 significant level.

The hypothesis was retained. It might be due to the Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, was valuable and useful for students' Science Learning regarding to their Learning Achievement, Critical Thinking, and Self-directed in learning. So, the posttest was in higher level than the pretest. It could be viewed that the persons taught by Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, would enhance the students who were good at the Metacognition Strategy, to have 2 preliminary capacities including: (1) Knowledge about cognition or Self-understanding which would consist of one's knowledge in what one knew or did not know as well as how to know it. The Inquiry process guidelines might be used to promote the persons' learning to be successful, and (2) Capacity and improving the cognition or Self-regulation which was one's competency in planning, monitoring, and investigating the progress as well as reflecting in order to find the method for improvement their work to be better. (Ann Brown 1987: cited in White et. al., 2009: 176) Moreover, Brown et. al., (1983; cited in McKeown & Beck 2009; 7) explained the basic factors of Learning Management that there were 4 factors through Metacognition Strategy including: (1) the factor of information report using the persons' language statement and thought process because they could present the information in what they knew through the quality method, (2) the factor of condition or rationale for controlling the administration and management or Executive Control being obtained from Information Processing Model. This model consisted of major collection unit which could control the persons' action including: Planning, Evaluation, Controlling, Monitoring and Revising and Improving, (3) the factor of Self-regulation which was one's process in paying attention to one's own action in suggesting and adjusting one's own practice to be accurate and consistency, and (4) the factor of controlling in other external conditions which was to transfer the others' controlling and monitoring into oneself.

5. The students taught by Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, had higher level of posttest Self-directed learning than the pretest at .05 significant level. The hypothesis was retained. It might be due to the researcher synthesized the major rationale in order to design the learning activity. Furthermore, the major skill to be developed in students for successful self-control and self-monitoring in learning, were competencies in investigating, following up, an controlling one's behavior, feeling, emotion or thought in order to be used as alternatives in combining to be relevant to one's need during the facing situation which consisted of the competency in inhibiting or obstructing the response to their first situation in order to create feeling of irrelevant problem and obstacle, and persist in work to be practiced for their successful learning.(Cook & Cook 2010: 352) Therefore, Pratomsuksa 6 students taught by Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, had higher level of posttest Critical Thinking Ability and Self-directed in learning, than the pretest at .05 significant level. The findings of this study were conditions facilitating the promotion of Self-directed in higher level. It was supported by research findings of Ogle (1986; cited in Martinez 2004 : 6) who believed that the Learning Activity by applying the Metacognition Strategy and Multiple Intelligent Theory, was the learning which could help students to learn by practicing in order to analyze and understand the knowledge issues in what the students had learned or known before in part relating to what to be learned in this study. Moreover, they also considered what they wanted to learn further continuously which was based on reading to support them in learning new things. Finally, they would investigate what they had learned later by questioning themselves in order to discuss in large group or small group. This learning process would provide opportunity and freedom to think, express





ones' opinion, and be more independent. In addition, it stimulated the students to be more eager to learn, understand, and see the value of their learning. Furthermore, it was condition affecting the students' increased Self-directed in learning as shown in this study.

Recommendations:

1. General Recommendations:

1.1 The teachers should administer the Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, in Science Subject because the Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, could be able to develop the students' learning in both of Critical Thinking and Self-directed in learning.

1.2 The teachers should administer the Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory. They should prepare readiness in their role by studying the handbook of Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, in details before providing the Learning Management.

1.3 The Learning Management by applying the Metacognition Strategy and Multiple Intelligent Theory, in each step, the teachers should provide students' opportunity in practicing by themselves every time.

1.4 The teachers should administer the appropriate reinforcement in each step of Learning Management during the step of classroom presentation for stimulating the students to develop their potentiality with full potential, and be proud of their own performance.

Recommendations for future research:

2.1 This research design should be used with other levels of students such as Upper Secondary School or Higher Education etc.

2.2 The Learning Management Model by applying the Metacognition Strategy and Multiple Intelligent Theory, should be used to develop other skills such as Science Process Skill, Creativity etc.

2.3 The Learning Management. Model by applying the Metacognition Strategy and Multiple Intelligent Theory, should be used with the other Learning Substances.

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EDI 04

Development of Learning Process Based on Constructivist Theory Combined with Problem-Based Learning to Enhance Academic Achievement and Critical Thinking of Mathayomsuksa 2 Students, in Work, Career and Technology Subject

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Abstract

The purposes of this study were : 1) to develop learning management based on Constructivist theory combined with Problem-Based Learning to enhance academic achievement and critical thinking of Mathayomsuksa 2 students in Work, Career and Technology Subject, 2) to study for the outcomes of learning management based on Constructivist theory combined with Problem-Based Learning, which were composed of : 2.1) to find the efficiency of learning management, with the required establishment of 75/75, 2.2) to compare academic achievement of students, between before and after learning based on that approach, and 2.3) to compare critical thinking of students, between before and after learning based on that approach. This study was provided on Research and Development methodology for 3 phases in the academic year of 2015, those were : The first phase was studied for the preliminary problems and needs concerned with learning management in Work, Career and Technology content, by using the questionnaire, interviewing, and observing activities in class. Including reviewed for the principles and concerned theories for synthesizing of learning management strategy with Constructivist theory and Problem-Based Learning to enhance academic achievement and critical thinking of students. Then proposed for the experts to evaluate the appropriateness of learning management before trying-out and implement with students in the second and the third phase respectively. The research instruments were comprised of :

1) questionnaire for the students replying about current state of leaning management , 2) interviewing forms for teachers and students about problems and needs of learning management, 3) observing form for classroom learning activities, 4) plans for learning management based on Constructivist theory and Problem-Based Learning for 8 plans, with 16 hours, 5) the academic achievement test, with 25 items, 6) 25 item test of critical thinking. The statistics used for analyzing data were percentage, mean, standard deviation, and t-test (Dependent sample) was employed for testing hypotheses.

The results of study were as follows :

1. The results of study for preliminary information of current state and problems about learning management for Work, Career and Technology content in concerned school, were found that students pertained in problems and needs to improve of higher academic achievement and critical thinking abilities. The author synthesized and designed for learning management based on Constructivist theory and Problem-Based learning to promote achievement and critical thinking of learners, which composed of 5 steps were as: 1) Engagement in learning, 2) Presentation of learning activities, 3) Explanation for concept learning 4) Practice and application, and 5) Assessment of learning. Additionally, assessing for quality and appropriateness of learning management plans by experts were at a high to highest level for each plan, and was at a high level for overall of plans.





2. The outcomes of learning management based on Constructivist theory and Problem-Based learning to promote achievement and critical thinking of learners, in Work, Career and Technology content were as :

2.1 The learning management based on Constructivist theory and Problem-Based learning, had the efficiency of 81.51/79.88, which was higher than the criterion establishment of 75/75.

2.2 The students who learned based on Constructivist theory and Problem-Based learning showed higher of achievement in Work, Career and Technology content than before learning at the .05 level of significance.

2.3 The students who learned based on Constructivist theory and Problem-Based learning showed higher of critical thinking than before learning, at the .05 level of significance.

Keywords: Development of Learning Process Based on Constructivist Theory Combined with Problem-Based Learning, Academic Achievement, Critical Thinking

Introduction

Learning Management in Work, career, ad Technology Subject for lower Secondary School Students in Prapariyattidharma School, General Education Division, focused on the students to search for knowledge, competency, and fact to be foundation in constructing the skill for earning one' living as well as livelihood based on Core Curriculum of Basic Education 2008 which specified the students' capacity in critical thinking and life skill by applying their skills and process in daily life. In addition, the Work, Career, and Technology Learning Substance emphasized on the students' necessary basic skill for livelihood, competency in working by creativity in order to earn their living as well as be able to compete in society. The objective was to apply their knowledge in working. Furthermore, Matayomsuksa 2 Students' quality was specified that they would be able to learn by comprehending their work and improving each step of their work, obtaining management skill and systematic collaborative working, being able to select the electrical equipment and technology in daily life, and solve the problem appropriately and valuably .

(Bureau of Academic Affairs and Educational Standards, 2009: 68)

According to experience in organizing the Learning Management of Work, Career, and Technology Learning Substance of Trachu-pittayakom School, Muang District, Khon Kaen Province, the researcher as a persons being responsible for of Work, Career, and Technology Learning Substance, found that most of Learning Management of Work, Career, and Technology Learning Substance» was in “Low” level. The problem was caused by teachers who did not design various developmental techniques for Learning Management, most of learning management was performed by lecturing, the picture learning media was used, the questions were used for conversation, the learning was provided based on different printed materials such as books, magazines, and other documents etc. These activities would not help students to achieve the school goal in learning achievement of Work, Career, and Technology Learning Substance. According to students' learning evaluation by The Achievement Test, was also seen that the students lacked of intensive comprehension in subject content of lesson as well as competency in considering for conclusion of knowledge information and making decision for selecting to practice or apply their knowledge in appropriate way in order to be useful for students themselves and the others as it should be. (Trachu-pittayakom School, 2012: 71-72) Therefore, the Learning Management in Work, Career, and Technology Subject for Secondary School Students, the students should be encouraged to have opportunity to practice and construct body of knowledge by themselves through the problem situation based on subject content in the lesson. The researcher as a teacher teaching Work, Career, and Technology Learning Subject for Matayomsuksa 2, studied guidelines for developing the learning activities in Work, Career, and Technology Learning Substance to be congruent with context and goal of Learning Management for enhancing the Students' Learning Achievement and Critical Thinking by applying the





Constructivist Theory combined with Problem-Based Learning, in order to design the quality Learning Management which would affect the students' learning based on the specified goal.

Since the Learning Activity Management based on Constructivism, was a learning management according to framework of Cognitive Theory which paid attention to students' interest and responsibility in their learning through the activities in Self-questioning and Discovery under theory of study in learning incidence associated with students' life, being supported to learn basic knowledge which was necessary and sufficient to be useful in searching for new knowledge and comprehension. Constructivist believed that the learning would be occurred when the students practiced for organizing their cognitive structure for developing the experience pattern inside them. They would respond the experience perception by constructing the knowledge framework in their mind called cognitive structure regarding to the meaning and comprehension related to students' life. The students would face with problem situation, search for problem solving technique through critical thinking, considering, and solving the problem by themselves as well as participating in solving the problem in group. (Woolfolk. 2010 : 312) Moreover, the Learning Management by Problem-Based Learning was an important characteristic of learning activity related to the problem or question and knowledge searching which promoted the students to try in studying and searching for answer by Inquiry Method or activities the students raised the problem issues they wanted to explore and search for answer as well as select the appropriate and valuable problem solving technique through the students' real practice or the activity that the teachers managed the learning situation being opposite with the learning model focusing on the content, the teachers organized the Learning Management. The Problem-Based Learning Activity needed the teachers to be facilitators for students in organizing the problems without being the problem solvers. (Nirad Chantarachit, 2010: 118) .

According to the above reasons and significance, the researcher as the instructional manager in Work, Career, and Technology Subject titled "System Software and Applied Software," for Matayomsuksa 2 Students, Prapariyattidharma School, General Education Division, viewed that Matayomsuksa 2 Students in this group were facing with problem in low level of learning achievement or academic achievement which was associated with Critical Thinking as an important factor supporting the students to learn Work, Career, and Technology Subject successfully. Therefore, the researcher was interested in developing the Students' Learning Achievement and Critical Thinking in Work, Career, and Technology Subject to be achieved and successful in higher level through R & D Process by applying the Constructivist Theory combined with Problem-Based Learning for designing the Learning Management in order to find the discovery of Efficient Learning Management Process which would be able to adjust as a successful guideline in Learning Management for Students in part of Work, Career, and Technology Subject, and information technology for the persons who were interested in administering the approach and research findings for conducting the research as well as applying in learning situation to be more efficient further.

Research Objectives:

1. To develop the Efficient Learning Management by applying Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, Matayomsuksa 2.

2. To study the findings of Learning Management by applying Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, Matayomsuksa 2 as follows:

2.1 To study the efficiency of Learning Management by applying Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, Matayomsuksa 2 based on criterion 75/75.

2.2 To compare the learning achievement or academic achievement of Matayomsuksa 2 Students taught by Learning Management by applying Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, between pretest and posttest.





Research Hypothesis:

The students taught by Learning Management taught by applying Constructivist Theory combined with Problem-Based Learning, had higher level of posttest learning achievement than the pretest.

Delimitation of the study:

1. Population and Sample

Phase 1:

1) The population were 99 Matayosuksa 2 Students, Trachu-pittayakom School, and Chantana-wittayakom School, Muang Khon Kaen District, Khon Kaen Province, from 3 classes.

2) The samples of 66 students were asked their opinion on learning activity titled “Fundamental of Electric and Electronic,” Work, Career, and Technology Subject regarding to their interesting class and uninteresting class as well as their opinion on Fundamental of Electric and Electronic, and the findings of basic knowledge and competency in Fundamental of Electric and Electronic the teachers expected to utilize 2 classes of students from 2 schools.

3) The target group of 66 students who were observed their learning behavior in Work, Career, and Technology Subject from 2 classes, 1 class from each school.

4) The target group of students who were interviewed the strength and weak point of learning activity in Work, Career, and Technology Subject, they wanted to be remained the same and improved, and usefulness they expected to be received.

5) The target group of 2 teachers in Work, Career, and Technology Subject, were observed their classroom learning activity leading to quality learning achievement as the goal, the condition which the students could not achieve and be successful in learning based on indicator of curriculum especially the competency in Learning Achievement and Critical Thinking, 1 teacher from each school.

The researcher would use the data obtained from basic analysis, to design the Learning Management for details in steps of learning activity in Work, Career, and Technology Subject, Matayosuksa 2, to cover the content titled “System Software and Applied Software,” to provide the students’ Learning Management in order to accomplish the Learning Achievement and Critical Thinking by designing the association with students’ learning context and condition, and presenting the advisor and experts to investigate the quality and validity of Learning Management Process by applying the Constructivist Theory combined with Problem-Based Learning in learning activities of Work, Career, and Technology Subject, and establishing the handbook of designed learning activities in order to used as guidelines in learning management for students further.

Phase 2:

The Learning Process or Learning Management in of Work, Career, and Technology Subject, was administered by applying the Constructivist Theory combined with Problem-Based Learning for enhancing the Learning Achievement and Critical Thinking. It was tried out with Matayosuksa 2 Students in school in order to associate with the students’ learning context. Then, it was presented the advisor and experts to investigate quality and validity of Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in learning activities of Work, Career, and Technology Subject, 29 Matayosuksa 2 Students, Trachu-pittayakom School, 2014 academic year, from 1 classroom. The tried out findings in the related issues to be improved and revised so that it would be more precise, appropriate, and complete.

Population were 99 Matayosuksa 2 Students, Trachu-pittayakom School, and Chantana-wittayakom School, Muang Khon Kaen District, Khon Kaen Province, in 2014 academic year, from 3 classes. The samples of were 37 students Chantana-wittayakom School, Muang Khon Kaen District, Khon Kaen Province, in 2014 academic year, from 1 class.

The researcher would bring the tried out findings to improve and revised the inappropriate issues and precise to cover and more complete especially in the attractive activity, the difficulty of content, the example of lesson, and duration of activity before using with the samples in implementation in Phase 3 of the study.

Phase 3:

The Learning Management was administered with students to study the efficiency and compare the





learning performance in Critical Thinking between pretest and posttest through the Learning Management by applying the Constructivist Theory combined with Problem-Based Learning of Work, Career, and Technology Subject, Matayosuksa 2.

Population were 99 Matayosuksa 2 Students, Trachu-pittayakom School, and Chantana-wittayakom School, Muang Khon Kaen District, Khon Kaen Province, in 2014 academic year, from 3 classes.

The samples of were 29 students Chantana-wittayakom School, Muang Khon Kaen District, Khon Kaen Province, in 2014 academic year, from 1 class.

The researcher would analyze the finding from Phase 3 and report it based on objective including: the evaluative findings of experts' quality investigation in Learning Management, the study of efficiency in Learning Management by applying the Constructivist Theory combined with Problem-Based Learning, the comparison of students' Learning Achievement and Critical Thinking between pretest and posttest of Learning Management by applying the Constructivist Theory and Problem-Based Learning.

1. Content

Work, Career, and Technology Subject, titled "Fundamental of Electric and Electronics."

2. Variables

2.1 Independent Variable was the Learning Management by applying the Constructivist Theory combined with Problem-Based Learning.

2.2 Dependent Variables were the Learning Achievement and Critical Thinking.

3. Duration

The second semester of 2014 academic year.

Data Analysis:

After the researcher implemented it based on schedule, the kept and collected data were analyzed as follows:

1. The analysis of evaluative findings from Lesson Plans.

The researcher analyzed the Propriety of the Quality Evaluation Form of Lesson Plan from the Experts by using the statistic to calculate the Mean and Standard Deviation. For the analysis, the Mean Value was compared with evaluative criterion as follows:

The Mean Value 4.51–5.00 referred to "The Highest" level of Propriety.

The Mean Value 3.51–4.50 referred to "High" level of Propriety.

The Mean Value 2.51–3.50 referred to "Moderate" level of Propriety.

The Mean Value 1.51–2.50 referred to "Low" level of Propriety.

The Mean Value 1.00–1.50 referred to "The Lowest" level of Propriety.

The Mean Value of experts' opinion of this study, the Mean Value over 3.51 was administered, and Standard Deviation not over 1.00 was used.

2. The Efficiency of Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject for enhancing Maayosuksa 2 Students' Learning Achievement and Critical Thinking, was studied by using the E_1/E_2 Formula.





3. The Comparative Analysis of Learning Achievement

The researcher calculated the pretest and posttest scores of 29 students from teaching by worksheet through t-test dependent statistic.

4. The Comparative Analysis of Critical Thinking

The researcher calculated the pretest and posttest scores of 29 students from teaching by worksheet through t-test dependent statistic.

Conclusions:

The findings of development in Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject for enhancing Matayomsuksa 2 Students' Learning Achievement and Critical Thinking, were concluded as follows:

1. The findings of development in Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject for enhancing Matayomsuksa 2 Students' Learning Achievement and Critical Thinking, were concluded as follows:

1.1 The situation, problem, and basic information of learning practice in SWork, Career, and Technology Subject, found that the students were not courage to act, express their opinion, ask their teachers. As a result, the findings of learning activity for Critical Thinking was rather difficult. In addition, the students lacked of their need to access the subject content.

1.2 The evaluative findings of Learning Management by applying the Constructivist Theory combined with Problem-Based Learning by all of 5 experts. The evaluative findings of each plan, was = 4.43–4.62. The overall Mean was = 4.49. The quality was in “High” level of Propriety.

2. The development of Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject for enhancing Matayomsuksa 2 Students, the Efficiency was = 81.51/79.88 which was higher than the specified criterion 75/75.

3. Matayomsuksa 2 Studnets taught by Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, had higher level of posttest Mean Score than the Pretest Mean Score at .05 significant level.

4. Matayomsuksa 2 Students taught by in Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, had higher level of Mean Score in Critical Thinking than the pretest at ,05 significant level.

Discussions:

According to research findings for developing Learning Management by applying the Constructivist Theory combine with Problem-Based Learning in Work, Career, and Technology Subject for enhancing Matayomsuksa 2 Students' Learning Achievement and Critical Thinking, there were important issues to be discussed as follows:

1. The development of Learning Management by applying the Constructivist Theory combine with Problem-Based Learning in Work, Career, and Technology Subject, Matayomsuksa 2, according to study of problem situation and basic information of development in Learning Management, found that there were problem of Critical Thinking. In addition, the students' knowledge an competency in problem solving in Work, Career, an Technology Subject had to be improved and developed into higher level. The researcher studied





Theoretical Approach for supporting the Learning Management to be successful, and viewed that the Constructivist Theory was an alternative of quality approach being able to adjust for using in the learning context as well as basic in designing the Learning Activity in Work, Career, and Technology Subject for enhancing Matayomsuksa 2 Students' Academic Achievement and Critical Thinking. The developed Learning Process of this study consisted of 5 Steps including: 1) Introduction, 2) Activity, 3) Concept, 4) Skill Practice and Knowledge Application, and 5) Evaluation. When the Learning Management Process was presented to experts for being evaluated its quality and propriety, found that the learning process included the quality and propriety to be used for Learning Management in Work, Career, and Technology Subject of Matayomsuksa 2 Students. The evaluative findings in each plan were ranged from "High" level to "The Highest" level. The Mean Values were 4.43-4.52. Moreover, the overall evaluative finding was = 4.49. It might be due to the Learning Activities were designed on the basis of Constructivist Theory combined with Problem-Based Learning focusing on the students to solve the problem by themselves, the teachers provided appropriate environmental experience for their students so that the students would use their intelligence and practice their problem solving ability critically as well as associating their thought and problem solving with member group and sharing.

It was supported by Ampon Makaanong's (2003: 6) statement that the instructional management focusing on student-centered, this theory emphasized on the knowledge to be constructed by students who used their prior knowledge and experience as a foundation in constructing the new knowledge. Learning was occurred inside the students and interacted with outside environment. Each student would construct knowledge through different methods. Consequently, the teaching guideline of this theory was emphasized on learning activity management enhancing the students to communicate as well as interact with their friends. The teachers supported the students to use their knowledge and consider what they obtained from discussions. They had responsibility in providing appropriate learning environment, setting the challenging questions, and helping the students to construct knowledge by themselves. Tidsana Kammanee (1999: 9-10) stated the knowledge creation approach based on Constructivist Theory that the knowledge was constructed by human beings which could be changed and developed to be grown up and progressed continuously depended on the persons' process in developing internal knowledge structure as well as perceiving the surrounded things. According to the above condition and factor, it caused the quality learning development in Work, Career, and Technology Subject based on all 5 steps of learning activity as shown in this study.

2. The development of Learning Management by applying the Constructivist Theory combined with Problem-Based Learning, in order to study the Efficiency of Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, Matayomsuksa 2, the Efficiency was = 81.51/79.88 which was higher than the specified criterion. It might be because the pattern of learning activity management based on Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject was emphasized on the students' critical thinking in solving the occurred problem. The teachers set the problems for their students to brainstorm in problem solving critically, practice their thinking process, analyze the problem, and solve the problem in group. The students were stimulated in searching for the answer in problem solving. It was congruent with Tidsana Kammanee's (2007: 137-138) definition "the instructional management by using Problem-Based Learning is to organize the instructional situation by using the problem as instrument for helping the students to learn according to the goal, the teachers might lead their students to face with real problem situation or the teachers might provide situation for their students to face with problem and practice the process in analyzing the problem as well as solving the problem in group, the students would understand those problems as well as be curious to learn and have thinking process skill and problem solving process." Albanese and Mitchell (1993 ; cited in Rachanewan Sooksena, 2007 : 45) defined the problem solving in Problem-Based Learning Model was to bring the problems to be starting point of learning process without expecting the students' knowledge. The problems would be stimulators the necessary learning into question with no answer which would lead to be searched later. The teaching by Problem-Based Learning had unique characteristic in using the problem as a major material for students to learn their problem solving skill as well as enhance their knowledge in clinical science. These





conditions would lead to the students' meaningful learning as well as searching for various problem solving techniques and new creative knowledge as shown in the research findings of this study.

1. Matayomsuksa 2 Students taught by Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, Matayomsuksa 2, had higher level of posttest Mean Score in Critical Thinking than the pretest at .05 significant level. The hypothesis was retained that the students taught Constructivist Theory combined with Problem-Based Learning, would have higher level of posttest Mean Score in Academic Achievement and Critical Thinking. It might be because the Learning Management by applying the Constructivist Theory combined with Problem-Based Learning for developing the Academic Achievement and Critical Thinking, was focused on students to search for knowledge by themselves from environment constructed by the teachers, in appropriate environment facilitating the analytical thinking, brainstorming, and critical thinking in decision making, direct management with experience, development of students' critical thinking for being applied in their real life as well as stimulation by questioning the students to think so that they would be alert and respond eagerly throughout the time, and they would construct body of knowledge by themselves in what they had learned, thing process, discussion, presentation of guidelines for their own problem solving. It was supported by Nirad Chatarachit's (2010: 267-269) definition of Learning Process by Problem-Based Learning, the major factors affecting the learning process as well as collaboration of small group members under the role of group supervisors were as follows: 1) the role of problem since the problem was stimulus using for stimulating the students' learning process which should be provided clearly without complexity, 2) process of knowledge construction, was the process the students tried to understand, find the explanation the cause and effect of phenomenon or incidence related to the problem. Then discussion in problem issue would enhance the students to be able to synthesize the body of knowledge for explaining the phenomenon or incidence of problem they were faced with. It was to construct concept or conclusions from the findings of problem solving on the basis of former knowledge. In addition, when the students used their common sense and logical thinking, they would be able to find the explanation the phenomenon which would lead to the construction for new knowledge under condition of problem. Furthermore, since there were individual differences in each student, it would cause the students to have different knowledge and thought. The cooperation, discussion, and analysis of problem by each student would lead to new and intensive knowledge, 3) the collaborative effort was a technique each student helped each other in making the issues to be clear. It was a major technique or activity of students taught by Problem-Based Learning, 4) the intrinsic motivation to learn, in students' discussions, when it was found that the questions in some issues were not able to be searched for answers or some alternatives could not be practiced, and the conclusion that they did not have precise idea in explaining the incidence. When the persons knew the gap between what they understood and what they did not understand, they would have the intrinsic motivation to learn. D.W. Johnson and Johnson conducted the study and found that the group discussion was a good instrument for helping students to perceive the gap between what they had known, and what they did not know. It would motivate the students to study and search for more information which would be an activity leading to self-directed learning by supporting the students to have their opportunity in searching for knowledge by themselves in order to obtain precise knowledge and comprehension in each issue from various learning sources. According to these conditions, consequently, Matayomsuksa 2 Students taught by Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, obtained increased Academic Achievement as well as Critical Thinking as indicated in research findings of this study.

Recommendations:

1. General Recommendations

1.1 The teachers teaching Work, Career, and Technology Subject for Matayomsuksa 2, were able to administer the Lesson Plan by applying the Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in their learning and teaching. Since it was the Efficient Lesson Plan with systematic steps, and investigated the quality by experts. It was the Lesson Plan enhancing the students'





Learning Achievement and Critical Thinking in higher level. The persons who wanted to use it, should study the approach, theory, step, and activity management technique to be appropriate with students.

1.2 The Learning Management by applying Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, the teachers should study the students' individual differences, for instance, to observe the sample students' behavior, to study the classroom context and environmental condition etc. so that there would be no problem during the Learning Management by applying Constructivist Theory combined with Problem-Based Learning.

1.3 The easy accessed media and facilitated comprehension in what would be learned for Learning Management, for instance, the concrete and touchable media, should be administered.

1.4 The climate and environment facilitating the students to interact with their group Members, should be created in order to provide the opportunity for every student to participate in activity.

1.5 The relaxed environment, for instance, the rhythm activity before participating in group as an ice breaker, should be developed for students to learn social interaction, and how to adjust themselves with their friends in group.

1. Recommendations for future research :

1.1 The Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, developed by the researcher, should be developed with various teaching methods or integrated with the other Learning Substances.

1.2 The Learning Management by applying the Constructivist Theory combined with Problem-Based Learning in Work, Career, and Technology Subject, should be tried out with various students in order to obtain the newer and more extensive conclusions of research findings.

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Evaluation of Bachelor Degree of Electrical Engineering Curriculum At Vongchavalitkul University in the Academic Year 2014

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Abstract

This evaluation of Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University in the academic year 2014 was quantitative and qualitative research. The number of samples used in this research was a total of 53 people, including 5 faculty members, 6 guest teachers and other department instructors, 32 current students, and 10 expected graduates. The sample group was selected by stratified random sampling. The research instruments consisted of structured interview and questionnaires. The statistics used in this research comprised of frequency, percentage, mean, and standard deviation, 4 editions with the of reliability of the instruments were .86,.88,.88 and .87 respectively.

The results were shown as follows :

The overall evaluation results of context were at the high level. The faculty members at the Faculty of Electrical Engineering, the guest teachers, other department instructors, and current students thought that the appropriateness of curriculum objectives and structure were at high level. The compliance of curriculum and environment, the compliance of curriculum and vision/aspiration of the university, and the compliance of curriculum and the needs of students and society were at high level.

The overall evaluation results of input were at high level. Faculty members, guest teachers and other department instructors thought that the appropriateness of structure and contents was at high level, which was in line with the opinion of current students. The appropriateness of teachers was at high level while current students thought that it was at highest level. The appropriateness of personnel was at high level, which was in line with the opinion of current students. The appropriateness of students was at medium level while current students thought that it was at high level. The appropriateness of educational materials was at medium level while current students thought that it was at high level. The appropriateness of premises and facilities was at high level, which was in line with the opinion of current students.

The overall evaluation results of process were at high level. Faculty members, guest teachers and other department instructors thought that the appropriateness of learning management process, evaluation process, and curriculum activity setup process was at high level, which was in line with the opinion of current students.

The overall evaluation results of product were at high level. Expected graduates thought that characteristics of graduates, academic achievements, and non-academic achievements were at high level, which was in line with the opinion of faculty members, guest teachers and other department instructors.

Keywords: Evaluation, Bachelor Degree of Electrical Engineering, Curriculum, Vongchavalitkul University, Academic year 2014.



**Background and the importance of issues**

Thai Qualifications Framework for Higher Education: (TQF:HEd) refers to a framework that shows the system of higher education qualifications of the country which consists of level of education, division of subject, a link from lower to higher education level, standard of learning outcomes of each education level which increases as per each education level, curriculum characteristics of each education level, amount of learning that is consistent with time required, opportunity to transfer credits from experiences gained in order to promote lifelong learning as well as a mechanism to ensure the implementation effectiveness of the National Qualifications Framework for higher education of institutions to produce graduates that meet the standard of learning outcomes.

The key principles of the National Qualifications Framework for higher education 1) are based on the compliance with the National Education Act of 1999 and the amendment (No.2) of 2002, as well as national educational standards and higher education standards. TQF:HEd aims to be the instrumental in bringing the policy of quality development and standard of education as stipulated in the National Education Act, national education standards, and higher education standards into practice in higher education institutions since TQF:HEd has clear guidelines in curriculum development, teaching strategy adaptation, student learning as well as learning evaluation to ensure that graduates will meet the expected standard of learning outcomes 2) focus on the learning outcomes of the graduates, which is the minimum standard of quality in order to assure the quality of graduates and to communicate with organizations and people involved to certify the production process of producing graduates by beginning with products and results of the educational management, which is to determine the learning outcomes of the expected graduates and then to consider the other elements involving in the process of learning management that will encourage graduates to achieve expected learning outcomes consistently and systematically 3) seek to codify rules and announcements which have already been implemented and link them together so that people involved can have a clear understanding of definition and standards of educational management in each qualification or a degree pre-reach education level 4) aim to promote a qualification or a degree of higher education in any institution in Thailand to be acceptable and comparable to higher education institutions domestically and internationally since TQF:HEd will help to determine the standards of educational management at all stages in a systematic way by giving the opportunities of higher education institutions to arrange a variety of courses and the educational processes but can still keep the final product which is the quality of graduates to meet the expected standard of learning outcomes, to work happily and proudly, and to be good citizens to help strengthen capabilities in developing Thailand.

In the academic year 2012, the Faculty of Engineering at Vongchavalitkul University was managed courses by keeping the principle of maintaining the quality of education as a priority and emphasizing in the process of educational quality assurance which consists of well planning, curriculum implementation, performance evaluation, and quality development, as an integrated curriculum development, in order to let students of the university change their behaviors and develop themselves from their experiences gained during the study. The university defined expected learning outcomes from graduates with at least five areas: (1) Ethical and Moral Development means to develop habits of acting ethically and responsibly in personal and public life in ways that are consistent with high moral standards and to develop ability to resolve value conflicts through application of a consistent system of values. In addition, Vongchavalitkul University gives priority to ethics and morality as the motto of the university is "Ethics lead wisdom", (2) Knowledge refers to the ability to understand, recall, present information, and analyze facts, principles, theories and processes as well as ability in self-learning, (3) Cognitive Skill refers to the ability to analyze situations and apply conceptual understanding of principles and theories in critical thinking and creative problems solving when faced with unanticipated new situations, (4) Interpersonal Skills and Responsibility refer to the ability to work effectively in groups, to exercise leadership, to accept personal and social responsibilities, and to plan and take





responsibility for self-learning, (5) Numerical Analysis, Communication and Information Technology Skills refer to the ability of numerical analysis, the ability to use basic mathematical and statistical techniques, communicate effectively in oral and written forms, and use information and communications technology.

In the academic year of 2014, the Faculty of Engineering at Vongchavalitkul University would like to gather information of curriculum management in Engineering, totally 5 curriculums, including Bachelor Degree of Mechanical Engineering Curriculum, Bachelor Degree of Civil Engineering Curriculum, Bachelor Degree of Electrical Engineering Curriculum, Bachelor Degree of Mechatronics Engineering Curriculum, and Bachelor Degree of Computer Science Curriculum, which will give the ideas of courses, objectives, structures and contents of the curriculums to check whether it conforms with environmental contexts as well as problems and needs, whether it is appropriate with the target audiences, whether it can meet the needs of societies and students, whether the amount of credits and curriculum, contents, and other factors related to the curriculum, including personnel, students, equipment and learning materials, premises and facilities, budget, class conditions, activities, evaluation and assessment are appropriate, whether the process or curriculum implementation activities in actual conditions have problems, and whether students have characteristics that meet curriculum objectives. All of those results can be determined by the academic achievement of students as well as the curriculum evaluation both academic achievements, which are skills and knowledge in classes, and non-academic achievements, which are personality, responsibility, unity, integrity, etc. According to such requirements, the Faculty of Engineering at Vongchavalitkul University had conducted the research to evaluate Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University in the academic year 2014.

Objectives

1. To assess the curriculum quality of Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University to consider the consistency, the appropriateness, and the quality of curriculum as well as the ability to achieve expected goals and to meet the needs of society and implementation
2. To study problems and challenges in curriculum management in order to improve the curriculum management system, monitoring system, and learning management system to be more effective
3. To study the quality of graduates

Methodology

To reach the objectives, the research methodology was set as follows:

1. Population

Population consisted of faculty members, guest teachers and other department instructors, expected graduates, and current students in the Faculty of Electrical Engineering at Vongchavalitkul University, totally 53 people.

2. Sample group

The size of sample was determined by Krejcie, R.V. & Morgan D.W. Table 1 with stratified random sampling method. The number of samples used in this research was a total of 53 people, including 5 faculty members, 6 guest teachers and other department instructors, 32 current students, and 10 expected graduates.





Table 1. Population and Sample Group

Status	Populations					Sample Groups				
	Faculty members	Guest teachers and other department instructors	Current students	Expected graduates	Total	Faculty members	Guest teachers and other department instructors	Current students	Expected graduates	Total
B.Eng.Electrical Engineering	5	6	32	10	53	5	6	32	10	53
Total	5	6	32	10	53	5	6	32	10	53

3. Instruments

Instruments used in this research were the structured interview and questionnaires which were divided into group of population as follows:

1. Structured interview for faculty members

The questionnaires consisted of 3 sections

Section 1: Questions on general information of data providers

Section 2: Questions on curriculum in 4 different areas, including Context (objectives, structure, aspiration, philosophy, appropriateness with society, students, and consistency), Input (contents, personnel, students, educational materials, premised and facilities), Process (learning management, evaluation, extracurricular activities), and Product (academic achievement, satisfaction, and other perspectives)

Section 3: Questions on curriculum problems and suggestion

2. Questionnaires

Questionnaires in this section have been divided into 3 sections as follows:

Section 1: Questions on general information of data providers

Section 2: Questions on curriculum evaluation and quality of current students/graduates

Section 3: Open-ended questions for suggestion/opinions

1) Questionnaires for guest teachers and other department instructors

Questionnaires in this section have been divided into 3 sections as follows:

Section 1: Questions on general information of data providers in checklist form such as gender, age, highest level of education, working experiences, and academic position

Section 2: Questions on curriculum evaluation in 3 areas, including Input (teachers, personnel, students, educational materials, premised and facilities), Process (learning management, evaluation, extracurricular activities), and Product (academic and non-academic achievements)

Section 3: Open-ended questions on curriculum problems and suggestions

2) Questionnaires for current students

Questionnaires in this section have been divided into 3 sections as follows:

Section 1: Questions on general information of data providers in checklist form such as gender, age, year of study, grade point average (GPA), motivation to choose this area of study





Section 2: Questions on curriculum in 3 areas, including Context (policy and university development plan, aspiration, philosophy, objectives, content structure), Input (teachers, personnel, students, educational materials, premises and facilities), and Process (learning management, evaluation, extracurricular activities)

Section 3: Open-ended questions on curriculum problems and suggestions

3) Questionnaires for teachers on characteristics of graduates

Questionnaires in this section have been divided into 3 sections as follows:

Section 1: Questions on general information of data providers in checklist form such as gender, age, grade point average (GPA), period of study, occupation/position, motivation to choose this area of study

Section 2: Questions on curriculum in 3 areas, including Context (policy and university development plan, aspiration, philosophy, objectives, content structure), Input (teachers, personnel, students, educational materials, premises and facilities), and Process (learning management, evaluation, extracurricular activities)

Section 3: Open-ended questions on curriculum problems and suggestions

4. Instrumental Development

4.1 Conduct documentary research from related documents

4.2 Synthesize the data from item 4.1 to come up with the list of variables needed in order to develop questionnaires and structured interview

4.3 Send questionnaires and structured interview to four experts to check the validation of contents and the appropriateness of instruments by using Index of Item Objective Congruence and then improve validated instruments for data collection in the next steps

5. Variables

5.1 Questions on general information of data providers in checklist form such as gender, age, highest level of education, position, working experiences, academic position, etc.

5.2 Data related to the curriculum

5.2.1 Context: policy, philosophy, objectives, structures content

5.2.2 Input: professors, students, personnel, educational materials, premises and facilities

5.2.3 Process: learning management, evaluation, extracurricular activities

5.2.4 Product: academic achievement (skills and knowledge), non-academic achievement (personality, satisfaction, etc.)

5.2.5 Problems and suggestions of curriculum

6. Data collection

The researcher collected data based on research materials as the following details

1. The researcher coordinated with data providers who were faculty members to make an appointment for the structured interview

2. The researcher coordinated with data providers for interviews and questionnaires. Samples were a total of 53 people, including

2.1 5 Faculty members

2.2 6 Guest teachers and other department instructors

2.3 32 Current students

2.4 10 Expected graduates





7. Data analysis and Statistics used

1. Content analysis was used for data from the interview, descriptive data, or qualitative data
2. Statistics used for quantitative data were as follows:
 - 2.1 Frequency
 - 2.2 Percentage
 - 2.3 Mean
 - 2.4 Standard deviation

8. Interpretation

Criteria for determining mean were as follows:

Mean	4.51 - 5.00	Curriculum appropriateness at the highest level
Mean	3.51 - 4.50	Curriculum appropriateness at a high level
Mean	2.51 - 3.50	Curriculum appropriateness at a medium level
Mean	1.51 - 2.50	Curriculum appropriateness at a low level
Mean	1.00 - 1.50	Curriculum appropriateness at the lowest level

Results

1. The evaluation results of context:

1.1 In curriculum objectives and structure, faculty members at the Faculty of Engineering in Electrical Engineering, guest teachers and other department instructors, and current students thought that the appropriateness of curriculum objectives and structure were at a high level.

1.2 In compliance with the environment, faculty members at the Faculty of Engineering in Electrical Engineering, guest teachers and other department instructors, and current students thought that the compliance of curriculum and environment were at a high level.

1.3 In compliance with philosophy of the university, faculty members at the Faculty of Engineering in Electrical Engineering, guest teachers and other department instructors, and current students thought that the compliance of curriculum and vision/aspiration of the university were at a high level.

1.4 In compliance with the needs of students and society, faculty members at the Faculty of Engineering in Electrical Engineering, guest teachers and other department instructors, and current students thought that the compliance of curriculum and the needs of students and society were at a high level.

2. The evaluation results of input:

The overall evaluation results of input were at a high level. When considering in each area, the details were as follows:

2.1 Structure and contents: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of structure and contents was at a high level, which was in line with the opinion of current students.

2.2 Teachers: the results show that faculty members, guest teachers and other department

2.3 instructors thought that the appropriateness of teachers was at a high level while current students thought that it was at the highest level.

2.4 Personnel: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of personnel was at a high level, which was in line with the opinion of current students.

2.5 Students: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of students was at a medium level while current students thought that it was at a high level.





2.6 Educational materials: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of educational materials was at a medium level while current students thought that it was at a high level.

2.7 Premises and facilities: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of premises and facilities was at a high level, which was in line with the opinion of current students.

3. The evaluation results of process:

The overall evaluation results of process were at a high level. When considering in each area, the details were as follows:

3.1 Learning management process: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of learning management process was at a high level, which was in line with the opinion of current students.

3.2 Evaluation process: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of evaluation process was at a high level, which was in line with the opinion of current students.

3.3 Curriculum activity setup process: the results show that faculty members, guest teachers and other department instructors thought that the appropriateness of curriculum activity setup process was at a high level, which was in line with the opinion of current students.

4. The evaluation results of product:

The overall evaluation results of product were at a high level. When considering in each area, the details were as follows:

4.1 Characteristics of graduates: expected graduates thought that characteristics of graduates were at a high level, which was in line with the opinion of faculty members, guest teachers and other department instructors.

4.2 Academic achievements: expected graduates thought that academic achievements were at a high level. However, faculty members, guest teachers and other department instructors thought that academic achievement were at a medium level.

4.3 Non-academic achievements: expected graduates thought that non-academic achievements were at a high level.

Discussion

1. The Quality of Curriculum

1.1 The overall context evaluation of Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University in the academic year 2014 was appropriate at a high level which was in line with the appropriateness of curriculum objectives and structure, the compliance of curriculum and environment, the compliance of curriculum and philosophy of the faculty and university, and the compliance of curriculum and the needs of students. In addition, the curriculum objectives and structure facilitate and promote student learning to gain knowledge and skills together with ethics, which was in line with the research results of Pratana Kowitayangkul (2000), who conducted the research on Curriculum Evaluation of Master of Education in Academic research and statistics education at Chiang Mai University by using CIPP Model, which found that context evaluation in curriculum objectives and structures were at a high level. In addition, the results were also in line with the research results of Sanong Kaewkerd (2002), who studied on Curriculum Evaluation of General Education at Rajabhat Universities in the northeastern area of Thailand, which found that context evaluation in curriculum objectives were at a high level.

1.2 The overall input evaluation of Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University in the academic year 2014 was appropriate at a high level. The appropriateness of structure and contents as well as teachers were at a high level, which was in line





with the research results of PratanaKowitayangkul (2000), who conducted the research on Curriculum Evaluation of Master of Education in Academic research and statistics education at Chiang Mai University by using CIPP Model, which found that input evaluation in characteristics of teachers were at a high level and above standard. On the other hand, SanongKaewkerd (2002), who studied on Curriculum Evaluation of General Education at Rajabhat Universities in the northeastern area of Thailand by using CIPP Model, which found that input evaluation in credits, contents, teachers, and curriculum committee were at a medium level.

Input evaluation: students were ready with knowledge and experiences at a medium level. Educational materials and premises and facilities were at a high level, which was in line with SanongKaewkerd (2002), who studied on Curriculum Evaluation of General Education at Rajabhat Universities in the northeastern area of Thailand, which found that input evaluation in premises and facilities were at a high level.

1.3 The overall process evaluation of Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University in the academic year 2014 was appropriate at a high level. The appropriateness of learning management process, evaluation process, and curriculum activity setup process were at a high level, which was in line with the research results of PratanaKowitayangkul (2000), who conducted the research on Curriculum Evaluation of Master of Education in Academic research and statistics education at Chiang Mai University by using CIPP Model, which found that process evaluation were at a high level. In addition, the results were in line with SanongKaewkerd (2002), who studied on Curriculum Evaluation of General Education at Rajabhat Universities in the northeastern area of Thailand, which found that learning management process, evaluation process, and curriculum management process were at a high level.

1.4 The overall product evaluation of Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University in the academic year 2014 was appropriate at a high level, which was in line with the research results of SanongKaewkerd (2002), who studied on Curriculum Evaluation of General Education at Rajabhat Universities in the northeastern area of Thailand by using CIPP Model, which found that the quality of graduates was at a high level.

2. The Quality of Graduates

The overall evaluation results of product were at a high level. Expected graduates thought that characteristics of graduates, academic achievements, and non-academic achievements were at a high level, which was in line with the opinion of faculty members, guest teachers and other department instructors

3. Problems, Challenges, and Suggestion

1. For basic courses such as mathematics, physics, calculus, and skills in engineering calculation, the instructors should refresh and pretest knowledge of students in order to categorize group of students who lack of knowledge and teach them specifically outside classes or teach prerequisite courses.

2. For engineering courses, especially in Electrical engineering, the instructors should assign additional topics for student to research from international texts, articles, or journals in order to practice research and English skills.

3. The employers expect graduates to immediately work without job coaching through Cooperative Education Program to let students work as staff in companies. Students will get salaries and benefits from the companies, so students may have the opportunity to work immediately after graduation. Therefore, curriculum development should provide such a course as an alternative to students.

4. Educational materials were not update, so students had lack of interest in learning. The amount was not enough to support student to manage courses as planned. And, the modernization of textbooks for research should be improved urgently.

5. The tuition cost is high, so some students have to drop classes in some semesters.





Therefore, the university may reduce tuition cost to let students who face financial problems graduate in time according to the curriculum structure. In addition, prospective students may make decisions easier to join the course. As a result, a number of students in the course may be increased to increase the economy of scale of the faculty as well.

6. The faculty should provide an atmosphere for learning, especially facilities such as tables, chairs, benches, and peaceful environment to encourage students to research while waiting for the class or after class.

7. The faculty should support and develop professors in terms of further study in doctoral degree and encourage them to do research for academic position

8. Graduates thought that students have lack of research, knowledge, and experiences conducive to work.

Recommendation

1. Recommendation for curriculum improvement

According to the research results above, the recommendations were as follows:

1.1 The faculty should improve the educational materials to be update at all time in order to keep up with modern technology at the present age and should have enough amounts of materials to support all students.

1.2 The faculty should support and develop professors in terms of further study in doctoral degree and encourage them to do research for academic positions.

1.3 The faculty should add Cooperative Education Program to let students work as staff in companies. Students will get salaries and benefits from the companies, so students may have the opportunity to work immediately after graduation. Therefore, curriculum development should provide such a course as an alternative to students.

1.4 According to expected graduates, students lack of research skills, knowledge, and experiences conducive to professional works. In addition, they still have insufficient knowledge to further knowledge or innovations; therefore, the faculty should add and improve electrical engineering project courses for students to develop those skills.

1.5 The instructors should refresh and pretest knowledge of students in the basic courses such as mathematics, physics, calculus, and skills in engineering calculation in order to categorize group of students who lack of knowledge and teach them specifically outside classes or teach prerequisite courses.

1.6 The tuition cost is high, so some students have to drop classes in some semesters. Therefore, the university may reduce tuition cost to let students who face financial problems graduate in time according to the curriculum structure. In addition, prospective students may make decisions easier to join the course. As a result, a number of students in the course may be increased to increase the economy of scale of the faculty as well.

2. Recommendation for further research

2.1 There should be in-depth study on curriculum structure, courses, and contents.

2.2 There should be a comparative study between Bachelor Degree of Electrical Engineering Curriculum at Vongchavalitkul University and at other public and private universities.

2.3 There should be a study on attitudes regarding to curriculum products based on the perspective of current students, graduates, and employers in order to compare the information.





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EDI06

Factors Influencing Students' Learning Achievement on Financial Accounting Subject: A Case Study of Non-major Accounting Students of Rangsit University, Thailand

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Abstract

The purpose of this study is to investigate the factors that influence the students' achievement on financial accounting subject of the Faculty of Accountancy, Rangsit University, Thailand. This study uses questionnaire as a means to collect data from non-major accounting students. Time period in data collection was after completing the course in December 2014. The questionnaire were collected from 289 students who registered in financial accounting course. This study was employed two steps of analysis. Firstly, exploratory factor analysis was used to evaluate 34 variables. In this step, the result shows that 34 variables were classified into nine factors namely: 1) the preparation before classes 2) the attention of the instructor 3) the attitude toward the course 4) the lack of attention to class 5) the intention to study 6) the attitude toward the instructor 7) the pattern of examination 8) the lessons revision after classes and 9) the attitude of the students toward themselves. Secondly, multiple regression analysis was employed to test the correlation between factor 1-9 and the students' achievement. The result points that 4 factors have positively effect to the students' achievement which consists of factor 2 (the attention of the instructor), factor 5 (the intention to study), factor 8 (the lessons revision after classes), and factor 9 (the attitude of the students toward themselves), while, factor 4 (the lack of attention to class) have negatively effect to the students' achievement. Interestingly, preparation before classes and other factors have zero correlations to the students' achievement.

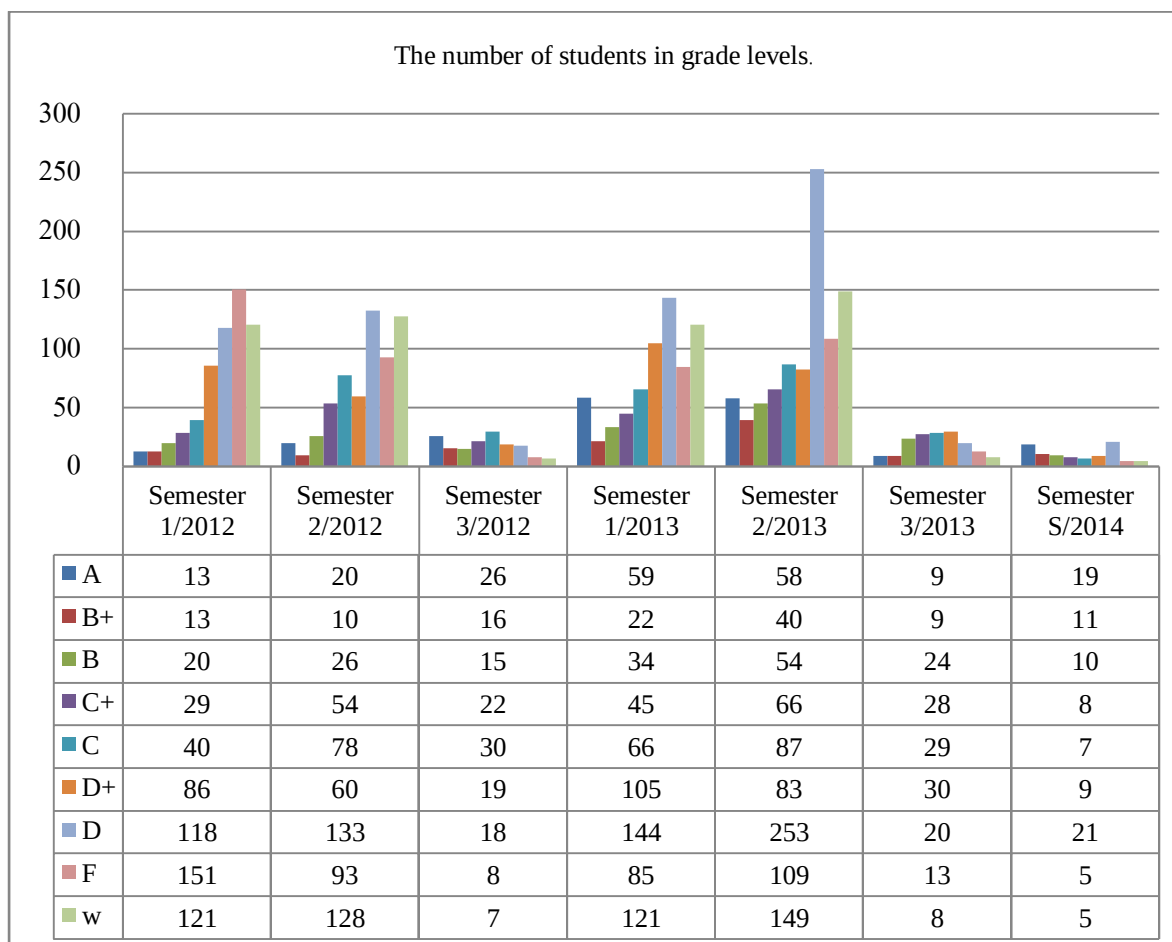
Keywords: learning achievement, attention, attitude, instructor, examination

Introduction

An education is an important factor in creating the quality of life and it's the foundation of creating knowledge, idea, ethics, and how to work with others. An education also has a relationship in development of human resources and development of country. To achieve this expectations we have to develop the quality of people along with it, especially the development of education into higher education level. So the learner will have ethics, skills, and know how to responsibility to society. There are 3 equilibrium in creating a strong Thai society such as the quality of society, the society of knowledge, and the harmony society. National Education Plan (2002-2015) is a plan to develop education to be a foundation in creating quality of life. The developing in quality of teaching are defined at the main plan out of 9 plans that has been planned. To focusing on creating the process of teaching with a wide variety ways of learning such as using technology and social media in teaching. Chidchanok Sengwirot (2011)

From the number of Rangsit University students that are registered in financial accounts (ACC100) course from 2012 to 2014 in database system (RSU.11) can be displayed as the following table.





From the experience in teaching financial accounting consider that most students have learning achievement in low level. Maybe because most of students think this subject is hard, you have to work with numeric data, and need attention to learn. So this may cause students discouraged and make them not succeed. If students have good attitude toward subject is likely to cause students intend to study, learn, and have a good score.

The objectives of research

1. Find a relationship and factors that influence students learning achievement in financial accounts (ACC100) subject, Rangsit University.
2. To provide information that can be used as a guide in development of teaching later. After found out what factors have the most effect on students learning achievement in financial accounts (ACC100) subject, Rangsit University.

1. Materials and Methods

Literature Review

Good (1973) has defined, achievement as accomplishment or knowledge attitude or skill developed in school subject usually designed by test scores or by teacher or both. Consequently, academic achievement could be defined as self-perception and self-evaluation of one's objective success. Bloom (1976) has defined, 3 things that affect the learning achievement: 1. Cognitive entry behaviors or a skills and knowledge that learner already





know. 2. Affective entry characteristics means the motivation that make a learner want to study such as the intention to study, the attitude toward course, and self-achievement. 3. Quality of instruction means the performance of teaching that affects learner.

In addition to the study there are many variables that influence students' achievement. Buaphan Phusahut (2006) and Nawarat Pratumtra (2003) found that a factors that are associated with student learning achievement in science is the attitude toward the course of science , motivation, and classroom environment. Mintra Singhanat (2009) found that variable that hasan influence in family can pass-through and affects students learning achievement include the intention to study, the attitude of family toward the course, and the attitude toward themselves. Von Secker (2004) found that variables that have a positive relationship with students learning achievement include the attitude toward the science course, parent's education level, and family environment . Koutsoulis & Campbell (2001) found that variables that indirectly affect students learning achievement in science and math subject include family income, support from family, and pressure in family by using path analysis can make accuracy in data structure and result. The research shows that the variables in school include quality of teacher teaching and relationship with student. The variable in family include relationship with family and the support from family. The variables in students include an attitude toward the science course and homework has an affect in student learning achievement in science.

Pongpant Keatpituk (1995) said that the preparation before classes and the intention to study means behaviors that show satisfaction and focus on study and knowledge. This behavior include the intention to study, attention in class, planning, hardworking, and responsibility. House(2002a) found that total number of hours per week that is used in study review , and doing homework. It's an important forecast of student learning achievement in science subject. A students who have received the higher score are likely to take hours per week in the classroom more than students who have received lower score.

Research Methodology

In this study using the survey method (Questionnaire) to study about students' learning achievement. To get all the variables in this research covered a research method has been defined as follows:

Step 1. Build and testing a tool by searching for a relating research, theory, document, and reference that is associated with the study. And then trying a trial data with the 30 sample before collect the real data.

Step 2. The simple of this research is students that enrolled in financial accounting (ACC100) of Faculty of Accountancy, at Rangsit University.

Step 3. Factor analysis for a variables that effect student's learning achievement. A variables that found will be used to analysis the regression coefficient of independent variables for forecast in students learning achievement in step 4.

Step 4 Evaluate the analysis of regression coefficient of independent variables for forecast in students learning achievement from step 2.

1. Population and Sampling

The population used in the study is the students that enrolled in financial accounting (ACC100) semester 1, academic year 2014. In this study, using Taro Yamane method by defining sampling error at the confidence level of 95% or equal to 0.05. In the Semester 1, academic year 2014 there are 935 students that registered in the course and 684 students entering for final exam test. The calculation found that the appropriately size of sampling is 252 people. In this study will determine the size of sampling with minimum of 253 people.





2. Variable in the study

This research using a primary data from the questionnaires. A tool that is used to collect data is a questionnaires that the Researcher created by study documents, theory and reference that is associated with the study. And testing a relationship by using factor analysis before apply into sampling. A structure of question is closed ended questions are just that: direct questions that are for specific pieces of information from sampling. The content of the questionnaire is divided into 3 sections as follows (details in the appendix)

Section 1. The general information about students including 4 questions as following:

Nominal scale question include gender, course, and major.

Ratio scale question include the accumulated (GPA) and how many times students has registered in financial accounting.

Section 2, the survey that influencing students' learning achievement in financial accounting (ACC100). By using rating scale from principle of Likert Scale and Build Interval Scale. There are 5 choice of level score to selection as follows: Strongly Agree (5), Agree(4), Neutral(3), Disagree(2), and Strongly disagree(1).

3. The Test Research Tools

To verify tools and data that is used in this research by finding a reliability from Cronbach's Alpha method. The results found that all the questions have a related value of reliability. It's depend on a relationship between a questions or a number of questions which will affect the value of Coefficient of Cronbach's Alpha. However, the value of reliability should not be less than 0.50. The Researcher has improve questionnaire that was collected by using pilot-test to display the value of reliability with 30 sampling and analyze the value of Cronbach's Alpha. There are variable that affect the value of Coefficient of Cronbach's Alpha less than 0.5. So the Researcher has to take off some variable that make a lower value in Coefficient of Cronbach's Alpha. The test results shown in the table 3.

Table 3 The value of reliability with Cronbach's Alpha method.

Variables	Cronbach's Alpha.
The preparation before classes	0.820
The attention of the instructor	0.838
The attitude toward the course	0.804
The lack of attention to class	0.693
The intention to study	0.536
The attitude toward the instructor	0.727
The pattern of examination	0.804
The lessons revision after classes	0.560
The attitude of the students toward themselves	0.536

4. The statistics that are used in data analysis

1. Descriptive statistics are used to describe the basic features of the data in a study such as percentage, frequency, and average.

2. Inferential statistics infer from sample to population. This research use statistics hypothesis testing as follows:

2.1 Factor analysis help create factor from different variable and make it into same factor by putting a relative variable into one factor. This research use Exploratory Factor Analysis (EFA) with principal component to bring a results of analysis use in multiples regression analysis.

2.2 Multiple Regression Analysis by using stepwise method as it is a mix between forward and backward method by considering the independent variables that have the most relationship





to the Y into equation consider from simple correlation between independent variable with the higher Y, then using fit model and selection new independent variables into equation. (like the forward method). In analysis the model if a variable that were selected does not have a relationship to the Y (see from the partial t-test) will take that variable off from the equation. In this research has linear regression equation to describe factors or the influence of variables that affect the learning achievement as follows:

$$\begin{aligned} \text{Achievement in learning} = & \alpha + \beta_1 (\text{the preparation before classes}) + \beta_2 (\text{the attention of the instructor}) \\ & + \beta_3 (\text{the attitude toward the course}) - \beta_4 (\text{the lack of attention to class}) + \beta_5 \\ & (\text{the intention to study}) + \beta_6 (\text{the attitude toward the instructor}) + \beta_7 (\text{the} \\ & \text{pattern of examination}) + \beta_8 (\text{the lessons revision after classes}) + \beta_9 (\text{the} \\ & \text{attitude of the students toward themselves}) \end{aligned}$$

Results

In the study of the factors that influence the students' achievement on Financial Accounting subject of Faculty of Accountancy, at Rangsit University. The Researcher uses questionnaire as a means to collect data from a group of students who takes final exam test in financial accounting (ACC100). There are only 289 out of 400 questionnaires were complete about 72.25 percentage. Divide the analysis as the following: (1) Analysis basic information of sampling. (2) Analysis element of factor analysis factors related to the study. (3) Analysis a relationship of factors that influence students' achievement. By analyzing the linear regression (multiples regression analysis).

1. Analysis basic Information of the sampling

The basic information including gender, major, and how many times that students register in financial accounting (ACC100) distribution by the following:

A table of 1 Percent and number identified by the individual factors.

	Individual Factors	Number of people	Percentage
Gender	Male	120	41.5
	Female	169	58.5
Level of study	1 year	131	45.3
	2 year	126	43.6
	3 year	19	6.6
	4 year	13	4.5
Major	Management	48	16.6
	Financial and Investment	11	3.8
	Industrial Management	12	4.2
	Marketing	33	11.4
	Computer Information Systems	14	4.8
	Human Resources Management	3	1.0
	Logistic Management	87	30.1
	Electronic Business Management	1	0.3
	Retail Business Management	17	5.9
	Aviation Business Management	63	21.8
The number of times that students register for ACC100	First Time	259	89.6
	Second Time	24	8.3
	Third Time	6	2.1





Total	289	100

2 Factor Analysis(Factors related to the study)

There are 45 variables factor that related to the study . In table 4.2 displays the value of KMO (Kaiser-Meyer-Olkin) and value of Bartlett's test of Sphericity of all the variables.

KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.846
Approx. Chi-Square		5523.607
Bartlett's Test of Sphericity	df	990
	Sig.	.000

From an analysis the value of KMO (Kaiser-Meyer-Olkin) as shown in the table 4.2 the value is equal to 0.846 which close to value 1, can conclude that there is a relationship in variables. To consider the Bartlett's Test of Sphericity for verify a relationship of all 45 variables, which enumerate by Chi-Square and the value of Chi-Square = 5523.607, sig. = 0.000 which has a value less than 0.05. Therefore we can conclude that there is a relationship in variables. To analyze the elements in the next steps by considering the weight of the element or a factor loading that has the coefficient more than 0.4 and higher. We find that the weight in each of variable can be classified into 9 elements. And to identify the meaning of 9 elements (factor meaning) the Researcher define meaning that is consistent with the theory of the study as follows:

Factors 1 is the preparation before classes such as study before the lesson, a participate in the classroom, and take a free time doing a research in the library or using Internet access for knowledge.

Factors 2 is the attention of the instructor such as how loud the instructor voice is does he/she speaks clearly. A teacher or instructor giving homework after each lesson and giving a chance or opportunities for students to ask and comment. Instructor having free time for students to come and talk with. Having a Line application group will help make students interested in studying and homework.

Factors 3 is the attitude toward the course. And financial accounting (ACC100) subject should be held in undergraduate class because this subject is very ideas challenge. It will help the learner become more organize. And this subject can also be applied in daily life and it's basic of learning about business.

Factors 4 is the lack of attention to class such as fall to sleep during class, and talk to a friend while teacher is teaching.

Factors 5 is the intention to study such as not doing another assignment during class and listen while teacher is teaching.

Factors 6 is the attitude toward the instructor of how he/she is teaching does he/she is teaching regularly. The instructor has the skills to convey the knowledge to students. Instructor can using Facebook and Line application to contact with students.

Factors 7 is the pattern of examination such as taking a test by using Internet access which is more easy and convenience.

Factors 8 is the lessons revision after classes includes a review of content by its own or with friends.

Factors 9 is the attitude of the students toward themselves with a set of goals in the classroom.





3 Analysis a relationship of factors that influence the students' achievement. By analyzing the multiples regression analysis

In the measurement of the factors that influence the students' achievement in financial accounts (ACC100) of the Faculty of Accountancy, Rangsit University. Use the linear regression line as a tool to describe the influence of the variables by the framework that shows the relationship as the equation below. To select the appropriate variable into the model using the stepwise regression analysis by consider the independent variables that have a relationship with students' achievement in financial accounts (ACC100) of the Faculty of Accountancy, Rangsit University. From an analysis of the above as a table 4.3 show the variable that is put in the regression equation found that variable that influence students' learning achievement is the attitude of the students toward themselves, the intention to study, the lack of attention to class, the attention of the instructor, and the lessons revision after classes. And the variable that not have a relationship with the students' learning achievement is the attitude toward the course, the attitude toward the instructor, and the pattern of examination.

Table 4 The conditions of regression equation test level and the relationship of the variables

Model Summary^f

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.293 ^a	.086	.082	15.4621	
2	.383 ^b	.147	.141	14.9598	
3	.432 ^c	.187	.178	14.6316	
4	.455 ^d	.207	.196	14.4762	
5	.476 ^e	.227	.213	14.3194	1.831

a. Predictors: (Constant), REGR factor score 9 for analysis 2

b. Predictors: (Constant), REGR factor score 9 for analysis 2, REGR factor score 5 for analysis 2

c. Predictors: (Constant), REGR factor score 9 for analysis 2, REGR factor score 5 for analysis 2, REGR factor score 4 for analysis 2

d. Predictors: (Constant), REGR factor score 9 for analysis 2, REGR factor score 5 for analysis 2, REGR factor score 4 for analysis 2, REGR factor score 2 for analysis 2

e. Predictors: (Constant), REGR factor score 9 for analysis 2, REGR factor score 5 for analysis 2, REGR factor score 4 for analysis 2, REGR factor score 2 for analysis 2, REGR factor score 8 for analysis 2

f. Dependent Variable: Score

From a results of analysis in table 4.3 found that there are only 5 independent variable. As shown in model 5 that influence students' learning achievement and coefficient of determination (R^2) = 0.227 or 22.7 percentage. A variable that contains on the attitude of the students toward themselves, the intention to study, the lack of attention to class, the attention of the instructor, and the lessons revision after classes can describe a changes in students' learning achievement by 22.7 % and 77.3% are from another influence variables. In addition, the Durbin-Watson statistics, which used to test the independence of deviation value according to conditions of a regression analysis. The Durbin-Watson value = 1.831 more than 1.5 can indicate that the deviation value are independent.





Table 5 The coefficient of regression equation

Coefficients^a

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations			Collinearity Statistics	
	B	Std. Error	Beta			Zero-order	Partial	Part	Tolerance	VIF
1	(Constant)	60.151	.913	65.904	.000					
	REGR factor score 9 for analysis 2	4.728	.916	.293	5.164	.000	.293	.293	1.000	1.000
2	(Constant)	60.151	.883	68.117	.000					
	REGR factor score 9 for analysis 2	4.728	.886	.293	5.338	.000	.293	.302	1.000	1.000
	REGR factor score 5 for analysis 2	3.994	.883	.248	4.523	.000	.248	.259	1.000	1.000
3	(Constant)	60.145	.864	69.637	.000					
	REGR factor score 9 for analysis 2	4.720	.866	.292	5.448	.000	.293	.308	1.000	1.000
	REGR factor score 5 for analysis 2	3.994	.864	.248	4.624	.000	.248	.265	1.000	1.000
	REGR factor score 4 for analysis 2	-3.220	.864	-.200	-3.726	.000	-.201	-.216	1.000	1.000





Table 6 The coefficient of regression equation(continued)

Coefficients^a

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations			Collinearity Statistics	
	B	Std. Error	Beta			Zero-order	Partial	Part	Tolerance	VIF
4	(Constant)	60.143	.855		70.383	.000				
	REGR factor score 9 for analysis 2	4.718	.857	.292	5.504	.000	.293	.311	.292	1.000
	REGR factor score 5 for analysis 2	3.994	.855	.248	4.674	.000	.248	.268	.248	1.000
	REGR factor score 4 for analysis 2	-3.221	.855	-.200	-3.767	.000	-.201	-.219	-.200	1.000
	REGR factor score 2 for analysis 2	2.278	.855	.141	2.666	.008	.142	.157	.141	1.000
5	(Constant)	60.147	.845		71.158	.000				
	REGR factor score 9 for analysis 2	4.723	.848	.292	5.570	.000	.293	.315	.292	1.000
	REGR factor score 5 for analysis 2	3.994	.845	.248	4.725	.000	.248	.271	.248	1.000
	REGR factor score 4 for analysis 2	-3.219	.846	-.200	-3.806	.000	-.201	-.221	-.200	1.000
	REGR factor score 2 for analysis 2	2.277	.845	.141	2.694	.007	.142	.159	.141	1.000
	REGR factor score 8 for analysis 2	2.270	.846	.141	2.685	.008	.140	.158	.141	1.000

A. dependent variable: score

From the table 4.4 if we put all independent variables ; the attitude of the students toward themselves, the intention to study, the lack of attention to class, the attention of the instructor, and the lessons revision after classes into the model to explain the influence of independent variable that has an effect to dependent variable (achievement in learning) will getan equation model as follows:

$$\text{Achievement in Learning} = 60.147 + 4.723 (\text{the attitude of the students toward themselves}) + 3.994 (\text{the intention to study}) + (-3.219) (\text{the lack of attention to class}) + 2.277 (\text{the attention of the instructor}) + 2.270 (\text{the lessons revision after classes}).$$





From the model found that the coefficient of the attitude to themselves has the higher value ($\beta = 4.723$), the intention to study ($\beta = 3.994$), the lack of attention to class ($\beta = -3.219$), the attention of the instructor ($\beta = 2.277$), and (the lessons revision after classes ($\beta = 2.270$) respectively.

A summary of the hypothesis testing

From a results of relationship between a variable in students' learning achievement. The hypothesis create a model with regression equation multiples found that there are only 5 out of 9 hypothesis that has an effect in students' learning achievement as following:

H₂: The attention of the instructor.

H₄: The lack of attention to class.

H₅: The intention to study.

H₈: The lessons revision after classes.

H₉: The attitude of the students toward themselves.

Discussion

From the results of hypothesis testing can be discuss as follows: The test results found that the second hypothesis or the attention of the instructor. An instructor must explain and speak clearly while teaching and giving an assignment or homework to students. And giving an opportunity for students to ask a questions during class or outside class. And also using social media, such as creating a group LINE to contact with students.

The test results found that the fourth hypothesis or the lack of attention to class. The lack of interest or a lack of attention such as talk to a friends while instructor is teaching or sleep during class. It will make the negative way in students' learning achievement and get a lower in test score.

The test results found that the fifth hypothesis or the intention to study. If students play attention while the instructor is teaching and not doing another work assignment during class just focus and listen to the instructor, it will make students understand better and get a good test score.

The test results found that the eighth hypothesis or the lessons revision after classes. If students spend time to review or revision a content after class by its own or with a group of friends, it will affect the result in students' learning achievement in positive way. So when it is time to take a test students will be able to do it.

The test results found that the ninth hypothesis or the attitude of the students toward themselves. A students need to create their own positive attitude and mind. For example set a goals in a classroom and always thought that, i'm going to graduate. If students have good attitude toward themselves in a positive way it will affect the result in students' learning achievement, which mean a better and higher test score.

Conclusions

This research using the questionnaire as a means to collect data from students who registered in Financial Accounting (ACC100) course of the Faculty of Accountancy, Rangsit University. A data has been collected after the final exam period. In addition of collecting data has a factor that cannot control such as mood and feeling of a group of simple. Another restrictions is a variable that is associated with students' learning achievement by using a results from the review of literature and apply it to a theory of study, which make a variables not clearly explain. This may cause an error or deviation (response bias).





Suggestions for the future research as follows:

1) This research is the measurement of students' learning achievement. By using exam results in Financial Accounting (ACC100) course of the Faculty of Accountancy, Rangsit University. This research is doing without knowing a behavior of students during the semester. So in the future of research we'll have to use experimental research, such as let students take a test in the beginning of semester, during semester, and at the end of semester.

2) This research is only focus on students who enrollment and register in Financial Accounting (ACC100) course of the Faculty of Accountancy, Rangsit University in first semester of school year. In each semester there are different students from different major and sector that enrolled in the courses. In future of research may have to compare a results of each semester with the same technique of teaching and see if the result come out different from the previous semester or not.

3) This research show that there is another factor that has not been considered in this study such as the factors about students. For example the personal problems and family financial problems. These factors may have an effect in students' learning achievement.

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EDI07

The Phenomenon on the 19th Dhamma Discipline At WatpaDhammaPraditJedisimun, Ubonratchathanee province

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Abstract

The objectives of this mixed research were to study 1) the level of satisfaction of the participants towards the project management on the 19th Dhamma Discipline Seminar of the Buddhist Network for Protection the Dhamma Discipline At Wat Pra Dhamma Pradit Jedisimun, Ubonratchathanee province and 2) the phenomenon of the 19th Dhamma Discipline Seminar. Collecting data by quantitative and qualitative methods, the purposive sample and the key informants were 34 monks, 10 novices from 18 provinces around Thailand and 62 men and women who joined this event, a totally of 106 persons. Collecting qualitative data by using the participative observation and in-depthed interview. The questionnaire was used for quantitative data. Using triangulation method for examine the validity and analyzed them by descriptive analysis.

The results :

1. The satisfaction of the participants towards this project was at “much” level ($\bar{x} = 3.96$) as a whole from 17 aspects. The 3 highest aspects were the lecturers who taught and gave the advisement on the Vinaya for the monks and the Ubasok and Ubasika; the food for the monks, and the utilization following the project purposive, and the lowest aspect was the staying places for the monks aspect ($\bar{x} = 3.34$ = the middle level). For the whole successful image of this project was at the “much” level ($\bar{x} = 4.11$)

2. The satisfaction according to the opinions of the monks who joined the seminar project was at the “much” level ($\bar{x} = 3.88$) as a whole. The 4 highest aspects were the food aspect for the monks, the lecturers who taught and advised the monks in the seminar rooms, and the food service “Rong Than” for the people and the utilization or the benefit of the project success.

3. The phenomenon of this situation and the advisement found that :

3.1 The participation from the officials, the private section or the government organizations for the raised improvement on the protection or preserve the Buddhist religion there should be more truly interested and took more actions or activities on any marvelous or good model or the tradition value Thai culture, especially like this project continuously, formally and systemly so as to empower the morality of the team management and the people who joined and trained on the teaching of the Bhuddha in order to preserve and protect the Buddhist religion forever.

3.2 There should be continued this protect in order to do or practice or train the mindselves following the Pali Canon as the network team manager tried to set and organized the 19th value project in Ubonrathanee province, Thailand. There were about 300 monks, 100 novices and 300 people, totally about 700 people joined the activities for listening to the Bhuddha’s teachings, the Vinaya from the Tripitaka. That was very wonderful phenomenon that showed the Bhiddhist power of trust, intellectual and the respect sincerely for the Bhuddha’s teachings.

3.3 The important problems or the obstacles happened and solved be should them in the future. There were not enough; 1) the staying places for the monks and the people who joined the project, 2) the rest rooms, 3) the water service and how to manage and organize the project successfully by the team project managers so there should be improved the strong team because there were a lot of people stayed at Wat Kho Don Tard Hai for 7 days and surely there were many things for solving . However, everything passed quite completely.

3.4 The outstanding image of the 19th seminar at Wat Kho Don Tard Hai;

3.4.1 The wonderful, peaceful land surrounded with Mae Num Moon and the nature forest for 70 Rais. It suited for the monks who preferred to study the Vinaya.

3.4.2 There were many trustful monks and Four Assemblies who believed on the Tripitaka and behaved themselves by Vinaya of the Buddha came to join this event together once a year and the 20th seminar project will be set at Wat Prathat Nong Sam Mun, Chaiyaphum Province, Thailand during the 4th-10th July, 2016. They were the same believable and trustful on the Buddha.



4. The guidelines for development; the monks, novices and people in nearby community should be more interested and join the seminar at the full time in every subject, in order to gain more knowledge about the Buddha's teaching. The academic team should increase the ability on presentation and focus on the Tripitaka, build the faithful, the conscious mind, the canon, the meditation and the wisdom to the Buddhist and the participants for the world society, in the future.

Keywords: phenomenon, Dhamma Discipline, Buddhist Network, the Pali Canon, Vinaya Seminar

Introduction

The term Buddhadasana 'Buddhism's real signification refers to ' the Teaching of the Buddha'. To maintain Buddhism is to preserve the word of the Buddha. By 'the word of the Buddha' are meant the Doctrine (Dhamma) and Discipline (Vinaya). It made known to all Buddhists that the Doctrine and Discipline would take his place. The Vinayapitaka is the collection of monastic rules laid down for the monks. Buddhists can qualify as worthy custodians of the Teaching which to learn and understand the authentic Doctrine and Discipline in the first place. So in this sense, the Pali Canon is the guiding principle for the Four Assemblies and must exist alongside them, providing the basic for their becoming worthy custodians of the Teaching

The Vinayapitaka as the collection of the monastic rules for monks, including the rules of the Patimokkha, constitutes the Discipline or sila 'morality' the training and development of bodily and verbal behavior. The importance of the Pali Canon can be summarized as follows: 1) The Pali Canon is where the Teacher of all Buddhists resides, as the Doctrine and Discipline, which the Buddha said would be his successor after his Final Nibbana, are enshrined in it, 2) Any Teachings or saying claimed to be Buddhist Teachings must be in compliance with the Doctrine and Discipline that come in the Pali Canon. However, it is rather surprising and disheartening that people nowadays do not seem to understand what the Pali Canon is, why it should be preserved and protected, why it should be employed as the standards or criteria for judging what constitutes the Dhamma and Vinaya, or in other words what constitutes the Buddha's teachings (P.A. Payutto, 2558).

Lastly, we should therefore be alerted to the threat and join forces to tackle it by promoting proper practice based on the true teachings, which we must help preserve in the pristine state. In fact, it is high time for Buddhists to be rehabilitated, directed back to the course of Dhamma and Vinaya, and take up a serious study of the Pali Canon once again. Because of the most important how to preserve and protect the "Vinaya" of the Four Assemblies, this network organization has been organizing the 19th the "Vinaya Seminar" for the local assemblies at Wat Koh Don Tard Hai, Ubonratchathane province during 16-21 July, 2015. This project has been setting by the team managing committee and the Buddhist local people, who interested in these activities all parts of Thailand. So this phenomenon was very interesting and very useful for the Buddhist people to learn and practice themselves to improve their mind according to the Teaching of the Buddha.

For this research, we used the mixed quantitative and qualitative research for collecting data in order to study the satisfaction of the participants by using the questionnaires and the semi structured interview for in-depth interview the monks, the committee the Ubasok and Ubasika who participated the event in order to know the opinions about the phenomenon and the guidelines for improving the next continuously project that will be occurred next year at Wat Prathad Nongsammun, Chaiyaphom province.

The objectives :

1. To study the satisfaction of participants towards the project management.
2. To study the phenomenon and the guidelines of "the 19th Vinaya Seminar of the Buddhist Four Assemblies who protect the Dhamma Vinaya Network" At Wat Pa Don Dhamma Pradit Jedisimun, Ubonratchathane province.

The research design :

1. The purposive participants and the key informants :
 - 1.1 The 34 monks and the 10 novices who were the network committee and monks who were the leaders of the temples from 18 provinces; Chantaburi, Chachungsua, Roi-Ed, Ubonratchathane, Prachubkirikan, Samutprakran, Udonthani, Laos, Bangkok, Khon Kaen, Chaiyaphom, Mahasarakham, Nakornratchasima, Buriram, Chiangrai, Panna Nakhonsithumarat and Songkla provinces.
 - 1.2 The 62 men, and women who joined this project.
2. The research instruments :
 - 2.1 The questionnaire for quantitative data
 - 2.2 The semi-structured interview for qualitative data
3. Collecting data and statistical analysis :



- 3.1 For collecting quantitative data in order to answer the first objective for this research using the questionnaire and using $\bar{x}$ and S.D. for statistical analysis.
- 3.2 For qualitative data collecting, using the semi-structured interview, in-depth – interview, formal and informal observation the situations or the activities happened in this phenomenon At Wat Koa Don Tard Hai
4. The team researchers:
 - 4.1 Dr. Kularb Purisarn the researcher
 - 4.2 Assoc. Narumon Sissu - phan - the assistant researcher
 - 4.3 The 7 Master Students from CAS. the assistant researchers
5. Field Study during 16-22 July, 2015, and the researcher manages joined the Meeting for preparing this project on the 30th May, 2015 at Sarinthip Company, Nothaburi province for collecting data by indepted interview the secretaries of this project and could get the information how they prepared the activities for the monks and the people who joined the project.
6. For doing the research used for 5 months; during May till September 2015.

Research Results and Discussion

1. The satisfaction of the participants towards this project was at “much” level ($\bar{x} = 3.96$) as a whole from 17 aspects. The 3 highest aspects were the lecturers who taught and gave the advisement on the Vinaya for the monks and the Ubasok and Ubasika; the food for the monks, and the utilization following the project purposive, and the lowest aspect was the staying places for the monks aspect ($\bar{x} = 3.34$ = the middle level). For the whole successful image of this project was at the “much” level ($\bar{x} = 4.11$)

2. The satisfaction according to the opinions of the monks who joined the seminar project was at the “much” level ($\bar{x} = 3.88$) as a whole. The 4 highest aspects were the food aspect for the monks, the lecturers who taught and advised the monks in the seminar rooms, and the food service “Rong Than” for the people and the utilization or the benefit of the project success.

3. The results from the qualitative study by descriptive analysis on the phenomenon of this situation and the important issues from the opinions of the monks and the key informants could conclude on the dimensions as followings:

3.1 The participation from the officials, the private section or the government organizations for the raised improvement on the protection or preserve the Buddhist religion there should be more truly interested and took more actions or activities on any marvelous or good model or the tradition value Thai culture, especially like this project continuously, formally and systemly so as to empower the morality of the team management and the people who joined and trained on the teaching of the Bhuddha in order to preserve and protect the Buddhist religion forever.

3.2 There should be continued this protect in order to do or practice or train the mindselves following the Pali Canon as the network team manager tried to set and organized the 19th value project in Ubonrathanee province, Thailand. There were about 300 monks, 100 novices and 300 people, totally about 700 people joined the activities for listening to the Bhuddha’s teachings, the Vinaya from the Tripitaka. That was very wonderful phenomenon that showed the Bhiddhist power of trust, intellectual and the respect sincerely for the Bhuddha’s teachings.

3.3 The important problems or the obstacles happened and solved be should them in the future. There were not enough; 1) the staying places for the monks and the people who joined the project, 2) the rest rooms, 3) the water service and how to manage and organize the project successfully by the team project managers so there should be improved the strong team because there were a lot of people stayed at Wat Kho Don Tard Hai for 7 days and surely there were many things for solving . However, everything passed quite completely.

3.4 The outstanding image of the 19th seminar at Wat Kho Don Tard Hai;

3.4.1 The wonderful, peaceful land surrounded with Mae Num Moon and the nature forest for 70 Rais. It suited for the monks who preferred to study the Vinaya.

3.4.2 There were many trustful monks and Four Assemblies who believed on the Tripitaka and behaved themselves by Vinaya of the Buddha came to join this event together once a year and the 20th seminar project will be set at Wat Prathat Nong Sam Mun, Chaiyaphum Province, Thailand during the 4th-10th July, 2016. They were the same believable and trustful on the Buddha.

The research results got along with the study of Kularb Purisarn (2014) on the title was “ The effectiveness Buddhist network management model and the phenomenon of the 18th Buddhist Discipline Seminar at Sawangphochai Temple, Mahasarkham province in 2557 B.E” It showed that The satisfaction of the seminar management was at the much level as a whole ($\bar{x} = 4.10$). The highest aspect was the welfare and food, the lower aspects were the lecturers, the utilization and the whole seminar actives. The lowest aspect was the



staying places for the participants ($\bar{x} = 3.70$) and the phenomenon of the 18th seminar found that the management of this project had been succeeded at high level. The contents were about the Buddhist Discipline and particples which were very useful. There were at least 500 teachers, students, and people from the communities came to join the seminar beside the monks and the novices.

In addition Prathepmeti (2534) and Prathamapidok (2558) focused on the concept like these “the standard about the Buddhist people should believed on the behavior of themselves, “Kharm”. They should be trusted on Sila, Smathi and Panya and study the Tripitaka clearly.

However the results of Khamhang Wisutrangkul (2557) studied on the “Analyzing the understanding the Buddhist principles of the monks in Khon Kaen” ; it found that the monks interested the principles at the middle level as there should be alerted on studying Pra Tripika directly.

The Advisements from the research’s results :

1. The network management committee should prepare and set the Seminar on the Vinaya like this phenomenon in the future in order to protect the religion sustainable for a long time.

2. The government and the Buddhist officials of Thailand should pay attainion and support the good activities formally and systemly in order to improve the ethical mind of people.

3. For the next researches, there should be studied on these topics; the ethical leadership of the monks’ model, the effectiveness management of the team network for protection Buddhist Region by using the mixed methods, quantitative and qualitative research in order to get new knowledge for developing on this field.

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EDI08

Model of cademic Administration of Basic Education under The Khon Kaen Primary Educational Service Area Office 5.

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Abstract

The research aims 1) to study the current condition and problem in academic administration of primary schools under Khon Kaen Primary Educational Service Area Office 5 2) to improve the academic administration patterns for primary schools under Khon Kaen Primary Educational Service Area office 5.

The research conducted in two stages which consisted of five steps. Stage 1 Step 1 was to study relevant documents and researches in order to create a framework. Stage 1 Step 2 was to ask about current problems in academic administration of primary schools under Khon Kaen Primary Educational Service Area Office 5 using Simple Random Sampling of 306 samples including 153 school administrators and 153 academic teachers. Stage 1 Step 3 was to interview Purposive Sampling including a administrators from the ideal schools. Stage 2 Step 4 was to draft the academic administration patterns for primary schools. Stage 2 Step 5 was to confirm the suitability and possibility of the patterns using Focus Group Discussion. The group used Purposive to choose 9 experts. The tools used to gather data were questionnaire, interview, record, and evaluation on statistics, e.g. percentage, mean and standard deviation which were used to analyze data

The results showed that

1. The condition of academic administration of primary schools under Khon Kaen Primary Educational Service Area Office 5 in overall was at high level. The problem in academic administration of primary schools under Khon Kaen Primary Educational Service Area Office 5 in both overall and individual aspect were at moderate level.

2. The academic administration patterns of primary schools under Khon Kaen Primary Educational Service Area Office 5 consisted of 9 elements 46 indicators including 5 indicators on curriculum developments 46 indicators including process development , 4 indicators on internal supervision development, 8 indicators of internal quality assurance, 5 indicators on research and education quality development, 5 indicators on teaching development, 5 indicators on evaluation, 5 indicators on learning resource support and development and 4 indicators on academically strong community encouragement. And the result of the academic administration patterns of primary schools under Khon Kaen Primary Educational Service Area Office 5 evaluation was that the patterns were suitable and possible at high level.

Keywords: Academic Administration , Primary School





Preface

National Education Act of 2542, as amended (No. 2) Act of 2545, Section 36 (Commission of National Education. 2550: 24), specified that the education is the process of learning in order to prosper the people and society; by knowledge delivery, training, cultural continuance, academic progress creation, and knowledge creation from educational society arrangement and supportive factors for people's lifelong learning. Education management must be to improve Thai people to be complete human being with perfect body, mind, consciousness, intellect, knowledge, moral, ethics, and culture so that they can happily live with others. Therefore, the school and administrators must willingly and devotedly focus on the important of mission. Academic is considered to be the heart among other aspect education administration. It supports and encourages learners to learn efficiently as specified in the purpose of education (Samarn Assawabhumi. 2549: 239).

The school administrators, as key person, must be the academic leader because the school quality is judged by academic performance; which manages every activity relating to developing efficient learning and teaching process for learner's maximum benefits (Preeyaporn Wonganutraroj.2546:3). The desire for quality and effectiveness exists in every category either business or education. According to the Office of the Education council (ONEC) education management in the Economic and Social Development Plan 10(year 2550-2554), it concluded that the Thai student education accomplishment from the 4 major subjects (Thai, English, Mathematics and Science) has been under 50 percent continuously. Mean while, the evaluation in quality of education in international competitiveness showed that Thailand was behind Malaysia And India. Therefore, the quality of education must be improved urgently. (Economic and Social Development Plan. 2555: 58). Also the second external school evaluation, at primary education level, by the ONESQA (Office of National Educational Standard Quality Assessment 2549-2553) found that 24,901 primary schools, under Primary Education Office, of 30,284 schools were accredited by ONESQA (82.22%),and the rest 5,383(17.78%) Schools were not accredited (ONESQA. 2553: 19 -24). From the mentioned report, it showed that education management of schools under Primary Education Office countrywide was critical. Most schools lacked of knowledge and understanding in managing qualified education to fulfill the papers of education under the National Education Act 2542, Amended No.2,2545. As school administrators lacked of interest in academic administration, it led to lack of quality learners, and teachers, in conducting child-centered teaching pattern (Tipawadee Meksawan.2541:20)

Academic Administration is the key element of the schools because the main purpose of the schools is to educate and train youth to be future national good citizen(Uthai Boonnprasert. 2540:62) It is said that the important task of schools is academic task. If school failed academically, it would be considered fall down. On the other hand, the schools with academic success should be honored as effective schools. For education management, Khon Kaen Primary Educational Service Area Office 5 found out that academic proceeding improved education quality to meet the education quality evaluation by ONESQA. The result of third external schools quality evaluation by ONESQA. The result of third external schools quality evaluation, primary schools under Khon Kaen Primary Educational Service Area Office 5 from 161 school, 135 schools passed, 3 medium schools and 23 small school failed (Supervising and Monitoring Group.2553). According to the learning accomplishment test(year 2556), the average results of Third Graders were Thai at 49.23%, Mathematics at 43.39%, science at 39.91. The average results of Sixth Graders were Thai at 42.47%, Mathematics at 36.75%, science at 39.54 and English at 35.80. These were under standard and must be corrected (Khon Kaen Primary Educational Service Area Office 5.2557:13)

The research, as educationist, were interested in doing research in order to find out academic administration patterns for primary schools under Khon Kaen Primary Educational Service Area Office 5 to use as a tool to improve nations primary education and offer as a way of effective academic administration. It would lead to the stable academic improvement in the future.

Research Objectives

1. To study current condition and problems in academic administration of primary schools under Khon Kaen Primary Educational Service Area Office 5





2. To improve the academic administration patterns of primary school under Khon Kaen Primary Educational Service Area Office 5

Scope of Research

1. Scope of Content

Researcher studied the scope and duties of academic administration of educationist and related organization responsible for education. To conclude, the scope of academic administration of primary schools divided into 9 aspects: 1. improve and support resources, 2. Supervise internally, 3. improve curriculum, 4. improve teaching process, 5. improve learning process, 6. assure internal quality process for quality assurance within education, research and development, quality education 7. research and improve education quality 8. evaluate 9. encourage the stronger academic community.

2. Scope of population and sample

2.1 Stage 1 was survey research to study the current condition and problems in academic administration in primary schools under Khon Kaen Primary Educational Service Area Office 5

2.1.1 Population were consist of 258 school administrators and 258 academic teachers from 258 under Khon Kaen Primary Educational Service Area Office 5.

2.1.2 Samples group consisted of 153 schools under Khon Kaen Primary Educational Service Area Office 5. The sample were chosen by Sample Random Sampling. The sample size were specified by comparing the number from Krejcie and Morgan (Boonchom Srisaard. 2545: 43

2.1.3 Research studies the concept schools, using Purposive Sampling including 9 administrators from concept.

2.2 Stage 2 was to improve academic administration pattern of primary school under Khon Kaen Primary Educational Service Area Office 5. This stage used Focus Group Discussion. The group consisted of school administrators, school academic associate administrators or head of academic department concept schools. They were the best practice schools under Khon Kaen Primary Educational Service Area Office 5. Moreover, the groups included 3 educationists from 3 government universities and 3 educationist from 3 private universities from Purposive Sampling.

Research Methodology

The research was divided into two stages with steps as follow:

Stage 1 was to study the current condition and problem in academic administration in primary schools under Khon Kaen Primary Educational Service Area Office 5.

Step 1 was to study relevant documents and researches relating to the scope of academic administration and create academic administration framework for primary school under Khon Kaen Primary Educational Service Area Office 5.

Step 2 was to create a tool which is a five-scale questionnaire asking about the current conditions and the problems in academic administration in primary school under Khon Kaen Primary Educational Service Area Office 5.

Step 3 interviewed the academic management in primary school under Khon Kaen Primary Educational Service Area Office 5 by using analyzed data from a study in the second step and established a structured interview to interview the academic management plan of 9 members from the Best Practice schools selected by purposive sampling method.

Stage 2 was the form of academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5

Step 4 was to draft an academic management policy for the basic education institutes. The



researchers would use data obtained from studies conducted in the first stage to improve the management policy of the academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5

Step 5 was to ensure the possibility of the system by using focus group discussion consist of three representatives from school executive administrators, deputy school executive administrators or Best Practice schools head academic administration, and academician in basic education and academician in higher education institutions both public and private. Totaled of 9 personnel were selected by a purposive sampling method.

Tools Used in the Research

1. Questionnaire about current conditions, problems in academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5. Questionnaire was in a form of check list, completion, and a rating scale. Questionnaire has a item discrimination power of 0.38 to 0.94. The Analysis for the reliability rate of the questionnaire was by a coefficient alpha method as used by Cronbach (1970: 161, quoted in Boonchom Srisaard 2545: 7-9) The questionnaire were found to have the confidence of 0.84

2. The researchers structured the interviews according to the concept of academic administration from the nine current conditions and problems in academic administration in basic education.

3. Data collection was created by the nine theories of academic managements to use in a divided group discussion.

4. The evaluation model is a five-level evaluation scale used to evaluate the suitability and feasibility of the model academic management of basic education and make recommendations for improvement to obtain a complete form.

Data Collection

Data were collected from questionnaires. The researchers sent questionnaires to samples in the mail and returned by mail. The questionnaires that were delivered by hand were returned with an interview by an assistant researcher. Small group discussion's data were collected and conducted by researchers and research assistants.

Data analysis

1. Questionnaire analyzed the current situation and problems of academic administration of basic education by analyzing the standard deviation then compare it with the criteria Interpretation

2. Questionnaire analyze the data by synthesizing key points from the interview to summarize the key points to be used as an element of style.

3. Assessment form Based on the analysis assessing the patterns of academic administration of basic education on average, standard deviation then compare it with the criteria of interpretation.

The statistics used for data analysis.

1. The statistics used to determine the quality of the tools.

2.1 Percentage

2.2 Arithmetic Mean

2.3 Standard Deviation

Research results

The results showed that

1. Results of the study of the condition and problems in the academic management of basic education schools under Khon Kaen Primary Educational Service Area Office 5 found that school administrators and academic teachers having opinion on the state of academic administration of basic education under Khon Kaen





Primary Educational Service Area Office 5 overall at a high level. The problems of academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5 overall are at a moderate level.

2. The result of development of academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5 found that form the academic management of basic education under Khon Kaen Primary Educational Service Area Office 5 consists of nine elements and 46 indicators, including 5 indicators for the development of school curriculum, 5 indicators on developed learning process, 4 indicators on internal communication, 8 indicators on quality insurance in the schools, 5 indicators on research and development, and 5 indicators on assessment and evaluation, 5 indicators for development and promote learning, and 4 indicators for developing the community to be strong academically.

3. Assessment of Academic Administration forms of basic education under Khon Kaen Primary Educational Service Area Office 5 found that the academic management form of basic education under Khon Kaen Primary Educational Service Area Office 5 has the overall rate at a high level.

Discussions

The result of the study of current condition of the academic administration of basic education overall is in the high level. This is because the academic management is under the ministry of education set out in the Education Act of 2542, as amended (No. 2) Act 2545, section 36 requiring that education is a learning process to the growth of individuals and society by the transfer of knowledge, training, cultural heritage and the creation of academic progress arising from the social environment of learning and supporting factors for individual learning continues throughout life. Education must be for the people of Thailand to be completed in body, mind, consciousness, intelligence, knowledge and ethics, morality and culture in life and the people can coexist with others happily with academic work at the heart of the school because the main task of education, the academic work related to the course the education program, the teaching, and learning And improving the quality of the students to meet the expectations of the National Education Act BE 2542 as Kamon Bhuprasert. (2545: 6) says the academic administration. School administrators absolutely necessary to have a special care for academic administration. The need to focus on this mission with commitment and dedication is important to the school administrators, teachers and academic opinion on the state of academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5 overall at a high level. This is consistent with the research of teaching loyal Boonperm Sornpakdee(2554: 98) has researched the development of academic administration, the principles of working as a team in the School Wat Pho Uttaradit municipality found that the performance evaluation model of academic administration is at a high level.

1. Problems of Academic Administration of basic education under Khon Kaen Primary Educational Service Area Office 5 overall in the medium. This is because Academic Administration is the main task of education. This is consistent with the crook of modest Pichai Sangaimjit (2542: 54-55) at the Academic Administration that the primary duty of all educational institutions to educate students in academic education lay the foundation. People Development School administrators must realize the importance of academic work. Understand the system and the scope of work involved. Whether planning an academic career. The performance on the course. Academic Administration Extracurricular activities for the students to develop their own experience. The system supervision, monitoring and academic development. The importance of such job fairs. Enabling administrators to focus and develop teachers' knowledge, understanding and skills in the management of academic administration in order to achieve maximum performance per student. From the above reasons, the school administrators. And teachers were at a moderate level. Consistent with the findings of Breda Guest colored Chorratda Kestong (2550: 92) has proposed guidelines for research Academic Administration smaller. Uthai Thani Educational Service Area Office found that the administration of a small academic school. The overall moderate.

2. Forms of academic administration of basic education under Khon Kaen Primary Educational Service Area Office 5 consists of 9 elements of the 46 indicators, including curriculum development. Development of the learning process The internal supervision Quality assurance in education. Research and development of quality education. The development of teaching and learning. Measurement and Evaluation Develop and promote learning. And encouraging the academic community is strengthened. The researchers have developed a model of





academic administration, said based on the concept of the scope and mission of academic administration, academic and educational authorities such as the Ministry of Education (2550: 29-51). and many others, which are much more reliable. Then, through a process developed by discussion groups (Focus group Discussion) from chief executives, scholars and academics studying in higher education, both public and private. These are the people who have the knowledge, skills and understanding in academic administration as well. The individual elements of conformity assessment, as well as the suitability and feasibility of the model of academic administration. And propose ideas to improve and develop until the indicator in the form of academic administration for basic education that is appropriate and possible utilization in basic education. Consistent with Parichat Chomchun(2555: 287-288) has researched forms of academic administration with community participation. Effective in Study Under the jurisdiction of primary education. Found that the main components of Academic Administration has seven components: 1) curriculum development and curriculum to use 2) the development of the learning process, 3) developing innovative media and educational technology 4) planning and development to promote academic. 5) the development of learning 6) Supervision and 7) measurement and evaluation. And is also consistent with Wipa Tongngum(2554: 283-289) has researched forms of academic administration of the schools under the Bangkok Metropolitan Administration found that the composition of the technical schools under the Bangkok consists of six components: 1) quality assurance. Study 2) develop learning potential of students 3), the Supervision 4) Education 5) participation in education and 6) the medium of instruction.

3. The results management model. Research of Basic Education found.

Overall and all have the right and the possibility at a high level. This may be due The Development of Academic Administration of basic education in this study were consistent with the concept of developing a form of Saman Assawapum(2549: 15), which describes the development of that form. To develop a model that starts from the principle. Basic information and knowledge on which to build. The results that have come to define the concept arm. And draft form Then design and offer new ways to get the better form. Then seek the confidence of the model by a qualified person. Finally, there must be an assessment of the suitability and feasibility of the model. In this study, the research team has developed the model by studying the concepts in question and interviews from those involved to be prepared in draft form. And also through focus groups of people who are directly involved in academic administration. And to improve the model with reasonable accuracy and can be applied to practical use. In addition, experts are assessing the suitability and feasibility of the model academic management of basic education. Is appropriate and possible in high level. Consistent with the findings of Parichat Chuchom(2555: 123) found that the pattern of academic administration, with the effective participation of the community in education. Under the jurisdiction of primary education. Is appropriate and possible in high level. And is also consistent with the findings of Kampi Sudtae (2553: 181-184), researchers have developed a model of academic administration for small schools that evaluated the feasibility of appropriate and useful. the overall style and the highest level in all aspects.

Suggestion

1. General Recommendations

1.1 Administrators under Khon Kaen Primary Educational Service Area Office 5 should take the forms of academic administration to academic administration in the schools. Follow the indicator in the form of academic management to optimize the management of academic performance.

1.2 Educational Service Area Office Should promote academic administration which is already good to make it even better. They should study the details of each component and the operators choose. Variations in academic administration to school can be a real school improvement. At the same time, it should support the elements are still problems in practice, such as research, in-class activities or teaching a higher quality by training teachers to be knowledgeable in such matters and supervising the research results of ongoing teacher.

1.3 The management of basic education. Department of basic education should take the form of academic administration applied in academic administration in the schools. By order of importance, each element is appropriate with regard to the context of the school and the policy of both creating knowledge and





understanding among all concerned parties and are assessed periodically to be used to improve school quality to standard.

2. Suggestions for further research.

2.1 should study experiments of using the model of academic administration, monitor the quality of the academic management model is built.

2.2 Each element should research both qualitative and quantitative

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HEI01

Genetic polymorphism of Apolipoprotein AI (ApoAI) gene in Thai people with Down's syndrome

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Abstract

Down's syndrome (DS) is one of the genetic disorders that associated with mental retardation and other health problems including heart disease, cancer and neurological disorder. From the previous studies, it has been suggested that people with DS have higher tendency to develop Alzheimer's disease (AD). Apolipoprotein AI (ApoAI) is an apolipoprotein that encoded by *APOA1* gene. The impact of polymorphisms of *APOA1* gene on cognitive decline and onset of AD has been suggested; however, the latter impact still has controversy. We herein investigated the polymorphisms of *APOA1* gene in Thai DS patients with aim to evaluate the risk to develop diseases and impact of ApoAI in this group of people. The genomic DNA was extracted from the whole blood (20 DS and 35 healthy subjects), and it was used to generate *APOA1* gene fragment covering promoter and the first intron by polymerase chain reaction (PCR). The pattern of the DNA fragments generated after the digestion with *MspI* showed that the frequency of G allele at the position -75bp in *APOA1* promoter was higher in the healthy subjects (72%) than in the DS (60%), whereas, the frequency of A allele was higher in DS patients (40%) than in the healthy subjects (27%). However, the frequency of C/T substitution at the position +83 bp in the first intron of *APOA1* that found in the healthy subjects was not significantly different from that observed in the DS. These indicated to impact of the G/A substitution at position -75 bp, but not the C/T substitution at position +83 bp, on the risk to develop AD and other related diseases in Thai DS.

Keywords: ApolipoproteinAI, Down's syndrome, Alzheimer's disease

Introduction

The presence of a complete or partial copy of chromosome 21 (Hsa21)¹ is the major causative factor of Down's syndrome (DS) since the excess of the genetic material leads to dysregulation of the certain gene. DS is the most common genetic with an incidence of ~1/600-1000 live births². Usually, it leads to mental retardation and other health problems including congenital heart disease, leukemia, thyroid disorders³. In addition, many studies suggested that people with DS have a high risk to develop Alzheimer's disease (AD)^{6,7}.

Apolipoprotein AI (ApoA1) is a major apolipoprotein that found as the major component of high-density lipoprotein (HDL) and is principally involved in reverse cholesterol transport⁶. It is coded by *ApoAI* gene which locates on chromosome 11q23-q24⁶ and encodes the mature mRNA consisting of 804 nucleotides⁹. It has been supported that the screening for genetic polymorphism, the variation of gene that abundant and highly distribute in all genome¹⁵, of an individual enables the detection of genetic predisposition to a disease and, subsequently, pre-diagnosis and early protection¹⁶. Although, several reports revealed the influences of the genetic polymorphisms in the non-coding sequence of *ApoA1* gene on metabolism of cholesterol, high risk to develop some diseases including myocardial infarction¹¹, coronary disease¹², gallstone disease¹³, and breast cancer¹⁴, in addition, AD¹⁰ particularly in DS patients¹⁷. However, from our best knowledge, none has been reported in Thai DS. Therefore, in this study, two polymorphisms of *ApoA1* gene, i.e. guanine to adenine (G/A)



substitution at the position -75 bp of the gene promoter and cytosine to thymine (C/T) substitution at the position +83 bp in the first intron of the gene, which have been reported for the association with the risk to develop AD⁶, were explored with aim to evaluate their association and risk in Thai DS.

Methods

1. Subjects

Twenty DS and 35 non-DS (healthy subject), under 18 years of age, were recruited at Rajanukul Institute, Bangkok, Thailand, after obtaining family informed consent. Recruitment, informed consent, and study procedures were approved by the Ethical Review Committee for Research in Human Subjects, Rajanukul Institute, Department of Mental Health, Ministry of Public Health, Thailand. Blood withdrawing was performed by a standard protocol, and the blood samples were collected into EDTA-containing tubes. Genomic DNA was extracted from blood leukocytes.

2. Genomic DNA Isolation

The buffy coat layer containing leukocytes was separated from the whole blood by centrifugation at 3000 rpm for 10 min, at 4°C. Then, genomic DNA was purified using genomic DNA mini kit (Geneaid) and followed the protocol explained by the company. Purity of the DNA was determined by electrophoresis on 0.8% agarose gel prior to aliquot and storage at 4°C until used.

3. Amplification of *ApoA1* gene fragment by PCR and purification

The reaction mixture (20 µl) comprised purified genomic DNA (as a template), PCR buffer (20 mM Tris-HCl, pH 8.4 containing 50 mM KCl), 50 mM MgCl₂, 10 mM dNTPs, 2.5 units of Taq polymerase, and 10 pmoles of specific primer pair (Table 1). The amplification was carried out for 35 cycles of 95°C for 30 sec, 57°C for 30 sec, and at 72°C for 1 min. The final elongation was performed at 72°C for 5 min. The product was then purified and concentrated using Gel/PCR DNA fragment extraction kit (Geneaid), and its purity was determined by spectrometry and electrophoresis on 1.5% agarose gel.

Table 1 The oligonucleotide primer pair used for the amplification of *ApoA1* gene fragment

Type of primer	Primer Sequence (5' → 3')
Forward	GGGACAGAGCTGATCCTTGAAC
Reverse	GGGGACACCTACCCGTCAG

4. Restriction fragment length polymorphism (RFLP) of *ApoA1* DNA fragment

The purified PCR product (400 ng) was digested with restriction enzyme MspI at 37°C, overnight. Then, the DNA fragments were separated by electrophoresis on 20% polyacrylamide gel (prepared from 40% acrylamide stock solution containing acrylamide : bis-acrylamide at ratio of 38g : 2g). The DNA separation was performed in TBE, pH 8.3, at a constant voltage of 150 volts with a cooling system. Thereafter, gel was subjected to silver staining to visualize the DNA bands and their pattern of distribution on gel.

5. Statistical analysis

The frequency of genotypes (GG, GA, AA) of the G/A substitution at the position -75 bp and the frequency of genotypes (++ , +-, --) of the C/T substitution at the position +83 bp were assessed by chi square (χ^2) test. To determine the allelic frequency, Hardy Weinberg equations ($p + q = 1$ and $p^2 + q^2 + 2pq = 1$, where p and q are the frequencies of each allele at the particular locus) were applied. The result is statistically significant when p value of ANOVA test is equal or less than 0.05.

6. Determination of DNA concentration and purity

The concentration of DNA was determined by spectrometry. The absorbance of DNA sample was measured at 260 nm, and it was used to calculate a concentration according to the conversion factor i.e. 1 optical density (O.D.) at 260 nm is equal to the DNA concentration of 50 µg/ml. To evaluate the purity of DNA, the ratio of the absorbance at 260 nm to the absorbance at 280nm was applied. The DNA with A260/A280 ratio of 1.7–2.0 was accepted for sufficient quality to process.



Results

After amplification by PCR, a single band of DNA corresponding to 433 bp fragment of *ApoA1* gene was observed on the agarose gel (Fig. 1). After the digestion with *MspI*, 4-6 small fragments were detected on polyacrylamide gel, in addition, sizes of the DNA fragments generated from *MspI* digestion are specific for each genotype. Only 3 polymorphisms at the position -75 bp, i.e. GG, GA, and AA, and 3 polymorphisms at the position +83, i.e. ++, +-, and -- were detected in all of the studied DS and healthy subjects (Fig 2).



Fig. 1 ApoA1 gene fragment amplified by PCR

Genomic DNA was purified from leukocytes and aliquot (150 ng) of the purified DNA was used as template to amplify the *ApoA1* gene fragment by PCR in the presence of specific primer pair. Thereafter, the PCR product was purified and analyzed by electrophoresis on 1.5% agarose gel in the presence of ethidium bromide. The DNA bands were detected under UV. M, 100 bp ladder; 1-7, *ApoA1* gene (433 bp) amplified from the genomic DNA isolated from individuals.

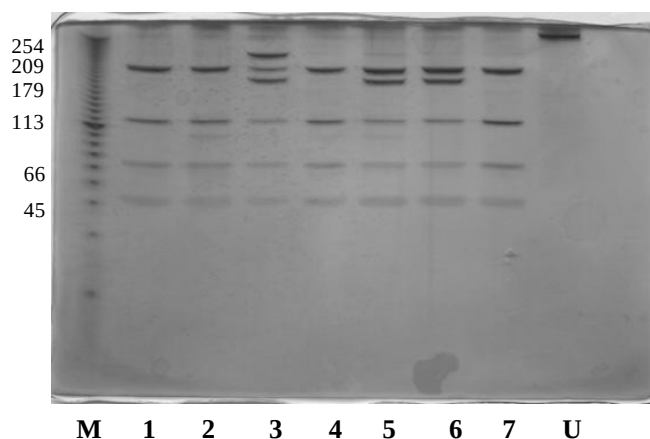


Fig. 2 DNA pattern on polyacrylamide gel of ApoA1 gene fragment after digestion with MspI

Aliquot of purified PCR product (100 ng) was digested with 4 units of *MspI* at 37°C, overnight. Then, the reaction mixture was analyzed by electrophoresis on 20% polyacrylamide gel under cold condition. M, 10 bp ladder; 1-2, GG/++ polymorphism contains fragments 45, 66, 113 and 209 bp; 3, GG/+- polymorphism contains fragments 45, 66, 113, 179, 209 and 254 bp; 4, GG/++ polymorphism contains fragments 45, 66, 113 and 209 bp; 5-6, GA/++ polymorphism contains fragments 45, 66, 113, 179 and 209 bp; 7, GG/++ polymorphism contains fragments 45, 66, 113 and 209 bp; U, negative control.



Prevalance of ApoAI polymorphisms at position -75 bp

The distribution of genotypes and allele frequency of G/A substitution at the position -75 bp in ApoA1 gene were shown in Table 2. The GG genotype was found in 4 DS and 16 healthy subjects, whereas, GA genotype was found in 16 DS and 19 healthy subjects. However, AA genotype was not found in either group. The frequency of G allele was higher (72%) in the healthy subjects than in the DS (60%). In contrast, the frequency of A allele was higher in DS (40%) than in the healthy subjects (27%).

Prevalance of ApoAI polymorphisms at +83 bp

The distribution of genotypes and allele frequency of ApoAI (C/T substitution at position +83) polymorphism were shown in Table 3. The homozygous CC (++) at position +83 was found in 7 DS and 13 healthy subjects. In addition, heterozygous CT (+-) at position +83 was detected in 13 DS and 22 healthy subjects. However, homozygous TT was not found in either group. The frequency of C allele in DS was 67.5%, whereas, that in the healthy subjects was 68%. Moreover, the frequency of T allele in DS was 32.5%, and that in the healthy subjects was 32%.

Table 2 Distribution of genotype and allele frequency of ApoA1 (G/A substitution at the position -75) polymorphism in the studied DS and control groups

Subject	N	Allele Frequency		Genotype			χ^2	degree of freedom (df)	p value
		G	A	GG (%)	GA (%)	AA (%)			
DS	20	0.6	0.4	4(20)	16(80)	0	1.28	1	0.74
Healthy	35	0.72	0.27	16(45.71)	19(54.28)	0			

Table 3 Distribution of genotype and allele frequency of ApoA1 (C/T substitution at the position +83) polymorphism in the studied DS and control groups

Subject	N	Allele Frequency		Genotype			χ^2	degree of freedom (df)	p value
		+	-	++ (%)	+ - (%)	-- (%)			
DS	20	0.675	0.325	7(35)	13(65)	0	1.56	1	0.79
Healthy	35	0.68	0.32	13(37.14)	22(62.85)	0			

Discussion

Our investigation was focused on evaluation of the substitution in ApoA1 gene at position -75 bp in the promoter region and at position +83 bp in the first intron on risk to develop AD in Thai DS. Since GG genotype at position -75 bp was found more frequently in the healthy subjects than in DS, and GA genotype was found more in DS than in the healthy subjects, which led to high frequency of G allele in the healthy subject and high frequency of A allele in DS. This could imply to more effect of A allele than G allele on the risk to develop AD in the Thai DS. Since the G-75A polymorphism of ApoA1 gene is associated with diseases such as coronary artery disease¹¹. Therefore, it is conceivable to the high risk to develop coronary artery disease in Thai DS. The frequency of ++ genotype was detected higher in the healthy subjects (37.14%) than in the DS (35%). In the contrary, the frequency of +- genotype was detected higher in the DS (65%) than in the healthy subjects (62.85%). However, the prevalence of ++ and +- genotypes, and frequency of + and - alleles in these two groups were not significantly different. These suggested to less impact of + and - allele on the risk to develop AD in the Thai DS. In conclusion, our results indicated that the G/A substitution at position -75 bp in ApoA1 promoter in comparing with the C/T substitution at position +83 bp in intron 1 of the gene plays more impact on the risk to develop AD in Thai DS. In addition, these results well agree with the previous reports on the influence of the substitution at position -75 bp on early onset and the risk for AD⁶.





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HEI02

Effect of RNAi knockdown of the Fatty Acyl-CoA Dehydrogenases on Mitochondrial Supercomplex Integrity in *C. elegans*

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Abstract

Physical interaction between enzymes in the fatty acid oxidation (FAO) and the mitochondrial respiratory chain (MRC) has been demonstrated mammal. It has also been shown that this interaction enhanced the function of the MRC. However, whether such physical interaction is required for the formation and function of the mitochondrial respiratory supercomplexes remains unknown. We addressed this question by using the nematode *C. elegans* as a study model. Wild type *C. elegans* was subjected to RNAi knockdown using dsRNA-expressing *E. coli*. The target genes for RNAi knockdown included *acdH-2*, *acdH-4* (short-chain acyl-CoA dehydrogenase or SCAD); *acdH-7*, *acdH-8*, *acdH-10* (medium-chain acyl-CoA dehydrogenase or MCAD); *acdH-5* (long-chain acyl-CoA dehydrogenase or LCAD); and *acdH-11*, *acdH-12* (very long-chain acyl-CoA dehydrogenase or VLCAD). Lifespans of the RNAi knockdown worms were scored. Formation and function of the mitochondrial supercomplexes were investigated by blue native gel (BNG) electrophoresis and enzymatic assay, respectively. We found that all RNAi-knockdown worms except *acdH-12* exhibited shortened lifespan compared to wild type. The MRC function of the RNAi-knockdown worms was not significantly different from that of wild type. However, alteration in formation of the mitochondrial supercomplex in RNAi-knockdown was detected. The result suggested that the acyl-CoA dehydrogenases may be required for mitochondrial supercomplex formation in *C. elegans*.

Keywords: mitochondrial supercomplex, acyl-coA dehydrogenase, *C. elegans*

Introduction

The MRC is composed of four multimeric enzyme complexes (complexes I - IV) and ATP synthase complex (complex V). These enzyme complexes assemble into large molecules called mitochondrial supercomplexes or respirasomes. Supercomplex is regarded as a functional entity of the MRC. It is conserved in many eukaryotes including yeast, plants and animals. Supercomplex consists of two or more different respiratory complexes (Acin-Perez et al., 2004; Acin-Perez, Fernandez-Silva, Peleato, Perez-Martos, & Enriquez, 2008).



Mitochondrial FAO takes place in the mitochondrial matrix. There are four steps of FAO, which break down fatty acid, ultimately leading to energy production. The acyl-CoA dehydrogenase (ACAD) is the enzyme at the first step of FAO. It makes a double bond into a carboxylic acid between α and β carbons. ACAD requires FAD as a cofactor, which will then transfer electrons into the ETC (Lehninger, Nelson, & Cox, 2005).

Interestingly, the evidence for physical interaction of FAO enzymes and the respiratory complexes in mammal has been reported (Wang, Mohsen, Mihalik, Goetzman, & Vockley, 2010). Mitochondrial supercomplexes-containing fractions isolated from BNG could be activated in a presence of palmitoyl-CoA, a substrate in FAO. It was then suggested that physical interaction between the ACADs and the mitochondrial supercomplexes could facilitate electron transfer through the electron transport chain system.

However, little is known whether such physical interaction is required for the mitochondrial supercomplex integrity, i.e. formation and function. Intriguingly, this interaction has been found to be conserved in *C. elegans* (Suthammarak, Yang, Morgan, & Sedensky, 2009). Our preliminary analysis revealed that MCAD and mitochondrial supercomplexes were detected as co-migrating entities as shown by BNG technique. Therefore, we proposed a research project to address this question using the nematode *C. elegans* as a study model. The ACADs in *C. elegans* will be knockdown using a well-established RNAi-knockdown technique (Fire et al., 1998; Kamath & Ahringer, 2003). We hypothesize that physical interaction between ACADs and mitochondrial supercomplexes is required for maintaining mitochondrial supercomplexes integrity in *C. elegans*.

Research design

Wild type *C. elegans* was subjected to RNAi knockdown by feeding on dsRNA-expressing *E. coli*. The target genes for RNAi knockdown included the acyl-CoA dehydrogenases as mentioned in materials and methods. Lifespans of the RNAi knockdown worms were scored. Formation and function of the mitochondrial supercomplexes were investigated by blue native gel (BNG) electrophoresis and enzymatic assay, respectively. Data were analyzed in comparison to the normal control which was wild type *C. elegans* fed on *E. coli* strain OP50 or K12.

Materials and methods

Worm culture

Wild type *C. elegans* was obtained from Caenorhabditis Genetics Center (Minnesota, USA). Worms were cultured on Nematode Growth Media (NGM) agar plates and transferred to liquid culture for a large amount of worms. RNAi knockdown in *C. elegans* was performed by feeding wild type animals dsRNA-expressing *E. coli*. The knockdown genes included *acdh-2*, *acdh-4* (SCAD); *acdh-7*, *acdh-8*, *acdh-10* (MCAD); *acdh-5* (LCAD); and *acdh-11*, *acdh-12* (VLCAD).

Lifespan Study

Wild-type *C. elegans* were fed on either *E. coli* strain OP50 as a control, or RNAi bacteria to knockdown the acyl-CoA dehydrogenases as mentioned earlier. Worms were counted everyday to score live animals. Data were analyzed by Log-Rank survival analysis performed in Aging Research (Yang et al., 2011).



Mitochondrial isolation

All preparations were performed at 4 °C. Clean worms were suspended in MSM-E (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EDTA, pH 7.4). A glass tissue grinder with a Teflon pestle was used for initial rupture: 3 strokes at 400 RPM. 5 mg/g (of fresh weight worms) proteinase type XXVII was added to the homogenate and stirred on ice for 10 min. The mixture was further homogenized in a glass tissue grinder with a Teflon pestle for 10 strokes. After adding equal volume of MSM-E containing 0.4% BSA, the homogenate was centrifuged at 500g, 10 min, 4°C. The supernatant was removed and re-centrifuged at 7000g, 10 min, 4°C. The mitochondrial pellet was re-suspended in MSM-E and washed twice by centrifugation at 7000g, 10 min, 4 °C. The final mitochondria pellet was re-suspended in MSM. Total protein was determined by the Lowry assay as described previously (Lowry, Rosebrough, Farr, & Randall, 1951).

Electron Transport Activity Assay

Function of the mitochondrial supercomplexes was be investigated by the ETC assay. The assays included CI; rotenone-sensitive NADH-decylubiquinone oxidoreductase, NFR; NADH-ferricyanide reductase, CII without DQ; malonate-sensitive succinate-DCPIP oxidoreductase, CII with DQ; malonate-sensitive succinate-DQ-DCPIP oxidoreductase, SCR; antimycin A-sensitive succinate-cytochrome c oxidoreductase, and CIV; KCN-sensitive cytochrome c reductase as described previously (Janssen et al., 2007).

Blue native gel electrophoresis (BNG)

Formation of the supercomplex was be determined by BNG as previously described (Wittig, Braun, & Schagger, 2006). We have performed a BNG on mitochondria isolated from the knockdown strains along with the wild type.

Result

Lifespan study was investigated as a parameter for phenotypic changes followed RNAi knockdown. It was shown that all RNAi-knockdown strains, except *acdh-12*, exhibited significantly shortened lifespan in comparison to wild type (Fig. 1). Function of the MRC of RNAi-knockdown strains and wild type as determined by ETC assay showed no significant difference (Table 1). However, the alteration of mitochondrial supercomplex formation was detected in RNAi-knockdown worms. The BNG showed that supercomplex I:III in several RNAi-knockdown strains appeared to be more pronounced than that in wild type. This indicated the reverse supercomplex I:III:IV to I:III ratio (Fig. 2).

Discussion and Conclusion

RNAi knockdown of acyl-CoA dehydrogenases in *C. elegans* resulted in significantly shortened lifespan. The alteration of mitochondrial supercomplex formation was detected although the function of the MRC was not affected by RNAi knockdown. Alteration in mitochondrial supercomplex formation observed in acyl-CoA dehydrogenase deficient *C. elegans* indicated the physical interaction of the enzymes in FAO and MRC similar to mammals (Wang et al., 2010). Moreover, such interaction was also required for the formation of the mitochondrial supercomplex in *C. elegans*. However, more knockdown experiments are needed to obtain more conclusive result.



Figures and Figure Legends

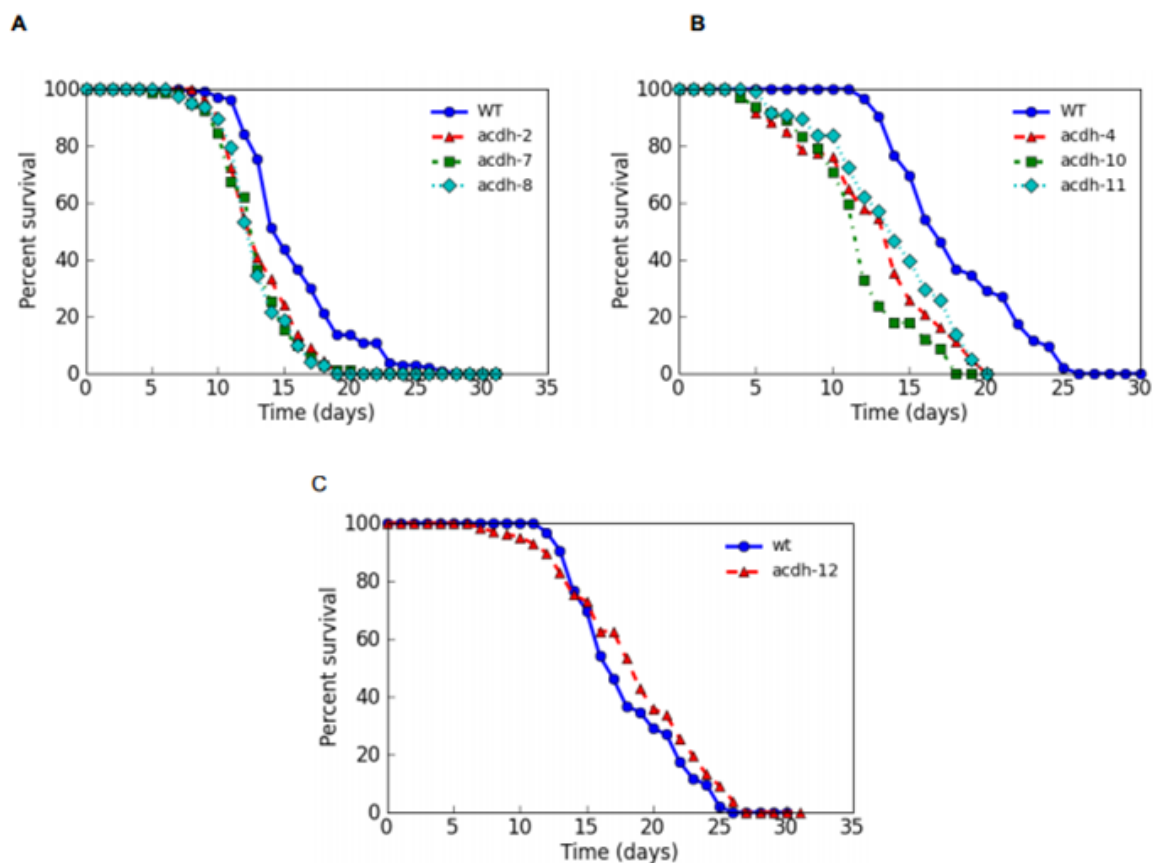


Figure 1. Lifespan of ACAD-knockdown worms. The median lifespan of all ACAD-knockdown worms, except *acdH-12*, were significantly shortened compared to wild type animals (WT).



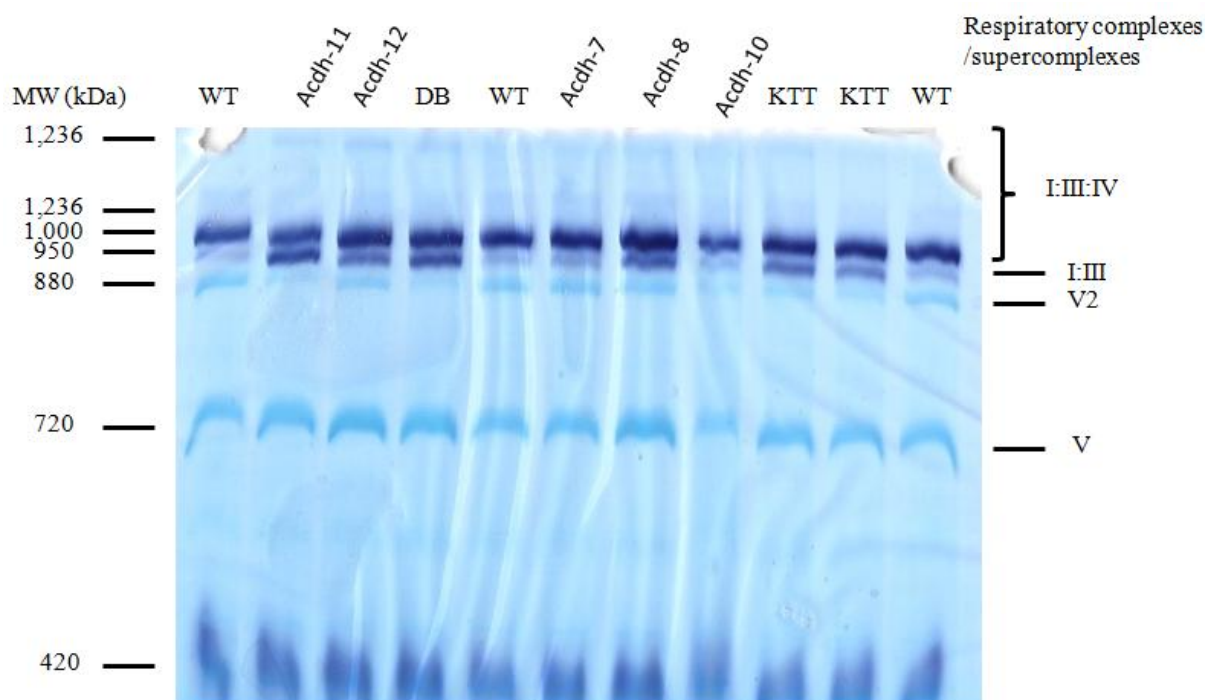


Figure 2. Formation of the mitochondrial supercomplexes. Mitochondrial proteins from the wild type (WT) and the knockdown animals (*acd**h*-11, *acd**h*-12, double VLCADs knockdown of *acd**h*-11 and *acd**h*-12 (DB), *acd**h*-7, *acd**h*-8, *acd**h*-10 and triple MCADs knockdown of *acd**h*-7, *acd**h*-8, and *acd**h*-10 (KTT) were solubilized in digitonin before loading onto a 3.5-12% native gradient polyacrylamide gel for electrophoretic separation. After electrophoresis was complete, the gel was subjected to complex I-in gel staining as shown. The respiratory complexes and supercomplexes were shown as indicated. *I:III:IV*; supercomplex *I:III:IV*, *I:III*; supercomplex *I:III*, *V*₂; dimeric complex V, *V*; monomeric complex V.



ETC assay	RNAi knockdown strains							
	WT (n = 3)	<i>acdH-7</i>	<i>acdH-8</i> (n = 1)	<i>acdH-10</i>	Triple MCADs knockdown) <i>acdH-7</i> ; <i>acdH-8</i> ; <i>acdH-10</i> (n = 1)	<i>acdH-11</i> (n = 2)	<i>acdH-12</i> (n = 2)	Double VL CADs knockdown <i>acdH-11</i> ; <i>acdH-12</i> (n = 1)
CI	207 (114.9)	n/a	133.9 (n/a)	n/a	169.1 (n/a)	226.6 (n/a)	n/a	n/a
NFR	1481.8 (275.2)	n/a	1583.6 (n/a)	n/a	1307.0 (n/a)	1517.5 (170.8)	1259.7 (156.1)	1170.1 (n/a)
NCR	678.0 (263.0)	n/a	n/a	n/a	495.2 (n/a)	n/a	458.4 (272.2)	458.4 (n/a)
CII w/o DQ	35.5 (16.1)	n/a	40.8 (n/a)	n/a	0 (n/a)	26.8 (0.5)	36.9 (14.1)	30.0 (n/a)
CII w/ DQ	50.9 (18.8)	n/a	41.9 (n/a)	n/a	0 (n/a)	34.5 (0.3)	51.2 (20.6)	43.3 (n/a)
SCR	209.3 (55.3)	n/a	181.0 (n/a)	n/a	162.6 (n/a)	n/a	188.7 (105.1)	188.7 (n/a)
CIII	582.3 (146.4)	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CIV	3953.2 (391.6)	n/a	3013.1 (n/a)	n/a	4844.7 (n/a)	4035.1 (370.6)	3860.0 (663.2)	4328.9 (0.0)

Table 1. Function of the MRC. Respiratory chain enzyme activities of the wild type (WT), and the RNAi-knockdown worms were determined by spectrophotometric analysis. The assays included CI; rotenone sensitive NADH-decylubiquinone oxidoreductase, NFR; NADH-ferricyanide reductase, CII without DQ; malonate-sensitive succinate-DCPIP oxidoreductase, CII with DQ; malonate-sensitive succinate-DQ-DCPIP oxidoreductase, SCR; antimycin A-sensitive succinate-cytochrome c oxidoreductase, CIII; antimycin A-sensitive decylubiquinol-cytochrome c oxidoreductase, and CIV; KCN-sensitive cytochrome c reductase. The activities (mmol substrate/min/mg protein) were reported as mean and S.D. (in the parentheses). n/a; not available.

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STI01

LCA of Business-related Garbage Recycling with the Experiment of Cover Material MethodYuxue Zhang¹, Ami Kudo¹, Kayoko Kondo^{1*}, Ikuyo Kikusawa¹, Yasushi Matsufuji²¹Kyushu University, Japan ² Fukuoka University, Japan

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Abstract

In Japan, the garbage recycling rate in small companies is not so improved. It is difficult for small companies to separate garbage elaborately and too much low quality garbage is generated. Therefore, we need to make a recycling system considering not only the high quality garbage of big companies but also the low quality garbage of small companies. We did LCA analysis on five scenarios of garbage disposals. Our result showed that methane fermentation method gained the highest value in LCA followed by composting, cover material and ecofeed, and the worst was incineration. Among them, cover material is the only method which can recycle low quality garbage, and should be introduced to local recycling system.

Keywords: Cover material, LCA, Ecofeed, Methane fermentation, Composting,

Introduction

Now high quality garbage is necessary for recycling system, and there is a large amount of low quality garbage which is currently incinerated (e-Stat Japan, 2012). Therefore, the goal of this study is to examine five disposal methods including a method of using un-recyclable garbage as cover material, and to show which could be effective to promote garbage recycling and to reduce environmental load. We evaluated every method by Life Cycle Assessment. Life Cycle Assessment (LCA) is a method to assess the environmental impact of products including obtaining material, processing, use, disposal and recycling (Takasaki.H, 2000) (Hirai.Y&Murata.M&Sakai.S,2002) (Liu.Y&Kondo.K, 2008).

Methods**1 outline of LCA scenario**

Table 1 Amount of waste composition in Fukuoka City

Population		1,463,743 people	2010 census in Japan
Area		340.6 km ²	
Combustible waste		628,280 t/year	2012 actual value in model city
Breakdown	Household garbage (43%)	271,90 t/year	
	business-related garbage (38%)	239,721 t/year	
	Garbage waste(29%)	184,086 t/year	





Table 2 System boundaries of 5 evaluated scenarios

Scenario	Process
1. Incineration	A→B→G→I
2. Ecofeed	A→C→G
3. Methane fermentation	A→D→E→G→H
4. Composting	A→E→G→H
5. Using Unrecyclable Garbage as Auxiliary Cover Material	A→F→G→I

A: Collection B: Incineration C: Ecofeed D: Methane fermentation E: Composting
 F: Using Unrecyclable Garbage as Auxiliary Cover Material G: Transport H: Farmland I: Landfill

Table 3 Process conditions

Collection process		
Superimposed load		4 t/car
Collection distance		Each recycling facility adjacent to each incineration facility
Light oil fuel consumption		2.396 km/L
Transport process		
Superimposed load		4 t/car
Transport distance	Landfill	between each incineration and each landfill (3.3 km, 23 km, 11 km, 10 km)
	Farmland	10 km
	Cattle farm	20 km
Light oil fuel consumption		5 km/L
Incineration process		
Methods		all continuous stoker furnace
Complete combustion efficiency		98.9%
Generating efficiency		13.5 % (average of each incineration)
NO ₂ discharge control (non-catalytic)		0.71 kg/t
After heat use		Power-generating (captive use, electric power selling) and supply (conversion to kerosene)
Lower heat value of Kerosene		8900 kcal/L
Heat recovery rate		10%
Amount of kerosene (each Incineration)		1.75 L/t-Combustible waste
Power of access to electricity (each)		3.75 kwh/t
Power-generating capacity (each)		418 kwh/t
Lower heat value of non garbage waste		488 kwh/t
Ecofeed process		
Methods		Steam heating and drying
capacity to manage		9.6 t/day
amount of production		2.3 t/day
Consumption of Natural gas		679 m ³ /day
power consumption		1418 kwh/day
Methane fermentation process		
Methods		Horizontal tank of continuous extrusion flow (Dry-type, thermophilic fermentation)
Methane yield		73.2 Nm ³ /t
Lower heat value of Methane		8550 kcal/Nm ³
Generating efficiency		30%
Consumed power		80 kwh/t
Amount of fermentation residue		Calculated by elemental composition of garbage. Emissions of N ₂ O due to de-water separation process from the residue, calculated by multiplying the conversion rate of N ₂ to the discharge to the inflow amount of nitrogen to the wastewater treatment facilities (0.1%).
Water content ratio of dewatered residue at the time of gas combustion		62% 10 g-NO ₂ /t 2.5 g-SO ₂ /t
Transport of fermentation residue		Methane fermentation facility adjacent to the composting facility.





Composting process	
Methods	Bottle type scoop formula
capacity to manage	17 t/day
power consumption	50 kwh/t
Methane ratio in a gas	5%
Conversion ratio of nitrogen to dinitrogen monoxide	1.25%
Water content ratio of compost	30%
Degradation rate of garbage waste	50%
Degradation rate of fermentation residue	15%
Using Unrecyclable Garbage as Auxiliary Cover Material process	
Methods	piled compost by a wheel loader
Capacity to manage	32 t/day
Light oil fuel consumption	0.46 L/t
Environmental load of Composting	equivalence of Composting process
Landfill process	
Coefficient of volumetric of incineration ash	0.588 m ³ /t
Light oil fuel consumption	0.736 L/t
power consumption (Leachate treatment)	6.38 kwh/t
Farmland process	
Fertilizer manufacture	3.1 kg- CO ₂ /kg-N 0.176 g-N ₂ O/kg- N 5.98 g-NO ₂ /kg-N 3.32 g-SO ₂ /kg-N
Compute methods about facilities	
gross margin rate	construction industry : 18 % manufacturing : 24.8 %

Amount of environmental load (kg/t-Combustible waste • year)

$$= \text{facilities cost (yen)} \div \text{depreciation period (year)} \times \text{Emission factor}(\text{CO}_2 \cdot \text{NO}_2 \cdot \text{SO}_2)(\text{kg/yen})$$

$$\div \text{Capacity to manage (kg/t-Combustible waste • year)}$$

Table 4 Characteristics of waste

	Combustible waste	Non food waste	Food waste
Water content	42.47%	21.90%	80.93%
Ash	5.80%	5.94%	2.84%
Combustible content	51.73%	72.16%	16.23%
Lower heat value	2133 kcal/kg	3108 kcal/kg	282 kcal/kg

Table 5 Environmental emission unit for various energy sources

Substances	Unit	Emission factors		
		CO ₂	NOx	SOx
Electric power	kg/kwh	0.612	0.0003	0.0002
Heavy oil	kg/L	2.71	-	-
Kerosene	kg/L	2.49	-	-
Light oil	kg-CO ₂ /L	2.58	4.74	0.95
Natural gas	kg-CO ₂ /1,000Nm ³	2220	-	-

Table 6 Specification of process facilities

	Purchase price(yen)	The period of durability(yen)	Treatment scale
Incineration facility	24,400,000,000	30	750 t/day
Landfill	21,222,515,000	30	3400,000 t/30 years
Leachate treatment facility	4,798,272,000	30	3400,000 t/30 years
Composting facility	460,476,000	30	24 t/day
The methane institution fermentation	3,279,297,000	30	55 t/day
Wheel loader	8,800,000 /car	10	31360 t/year
Truck	5,000,000 /car	10	4 t/car
Garbage truck	10,000,000 /car	10	4 t/car

Table 7 Emission factors of Facilities (3EID)

3EID departments	Facilities	Emission factors		
		Mg-CO ₂ /million yen	kg-NOx /million yen	kg-SOx /million yen
Disposal of waste (public)	Incineration facility	10.884	15.001	2.587
Waterway, sewer, other public works	Landfill	3.650	9.696	2.352
Sewer	Leachate treatment facility	5.994	6.222	14.333
Other general industrial machinery and device	Composting facility	3.592	4.947	2.926
Other general industrial machinery and device	Methane fermentation facility	3.592	4.947	2.926
Steam generator	Ecofeed facility	2.967	4.458	2.522
Non-home building (non-wooden construction)	Ecofeed building	3.021	6.014	2.206
Transporting machine	Wheel loader	3.102	4.321	2.508
Truck, bus, other automobile	4t truck, 4t garbage truck	2.843	4.724	2.924

The site of scenario simulation is Fukuoka City in Japan. Fukuoka City is a commercial city with a population of 1.5 million and its business-related garbage accounts for 38% of the total amount of 628,280 tons per year. (Table1)(Environment station, Japan 2013).The scenario of LCA is configured under 5 kinds of garbage treatment scheme; incineration, ecofeed, methane fermentation, compost and cover material (Table 2). Ecofeed is livestock feed produced from garbage. Cover material is a garbage recycling method which Matsufuji laboratory of Fukuoka University developed: Low quality garbage is utilized to cover open landfills so that it helps decomposing of land filled waste (Matsufuji.Y&Tchifuji.A, 2005) (Tchifuji.A&Matsufuji.Y, 2009). We conducted the environmental impact assessment which synthetically evaluates the impact of global warming (CO₂, N₂O, CH₄), acidification (NOx, SOx) and final dumping place (landfill consumption).We compared the environmental impact of five garbage treatments with the unit of 1 ton combustible garbage.



System description

The energy consumption of a series of waste treatments process is described in Table 3 to 7. Table 3 is the process conditions when we evaluate every method. The data on waste collection trucks, transportation distance and fuel consumption is shown in 'collection process'. In 'incineration process', the amount of electricity productivity is calculated by multiplying generation efficiency by the lower heating value of every garbage. Facilities construction and the production of the trucks for collection and transportation and loading shovels in landfills are all taken into account. Table 4 shows that the lower heating value of combustible garbage without garbage will reach 1.5 times higher than that of general combustible garbage (Takatsuki.H, 2000) (Tahara.K,&Inaba. A, &Sakane.Y, &Kojima.T, 2004) (Francesco.C,&Silvia.B,&Sergio.U, 2008). That is the reason to separate garbage with high water content from the combustible garbage. Table 5 indicates the emission factors of energy sources (Ministry of Environment Government, Japan, 2005). Table 6 shows the cost of processing facilities, machineries and vehicles, the depreciation, and treatment capacity. Table 7 shows the emission factors including CO₂, NO_x and SO_x of the construction of facilities according to Embodied Energy and Emission Intensity Data for Japan (3EID) (National Institute For Environmental Studies, Japan, 1995). The assessment was conducted with Life-cycle Impact assessment Method based on Endpoint modeling (LIME2) issued by the Agency of Industrial Science and Technology (Itsubo.N,&Inaba.A, 2010).

Assessment result

1. characterization Assessment of LCA

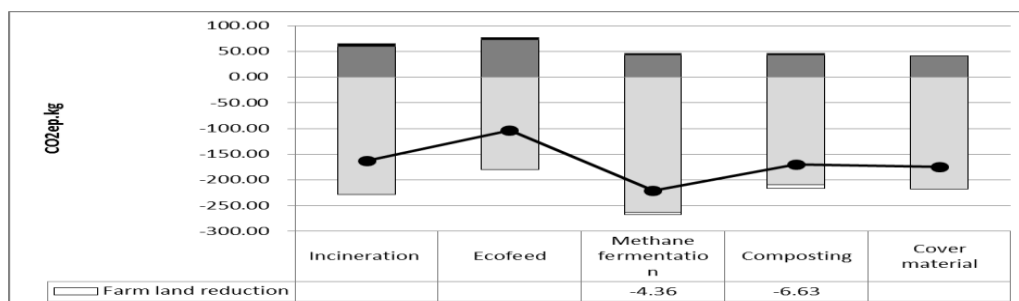


Figure 1 Characterization assessment of global warming

Characterization assessment is conducted in order to know environmental impact on global warming, acidification and final dumping place of five scenarios respectively. We converted the environmental load items of Life Cycle Inventory to single index of three categories of characterization by using assessment coefficient of LIME2

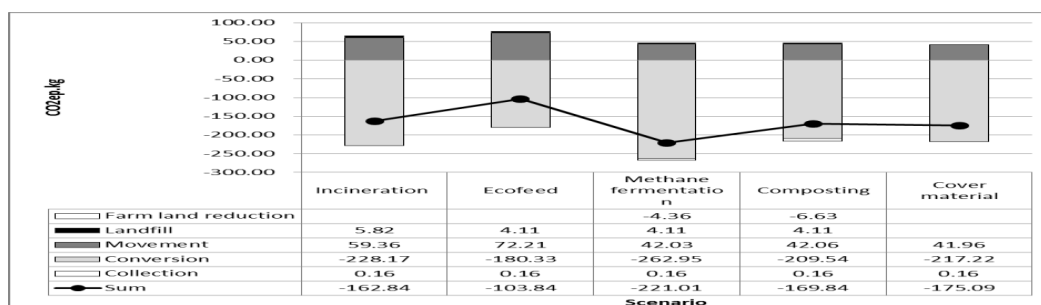


Figure 2 Characterization assessment of acidification



In the matter of ‘global warming’, the load of methane fermentation is the lowest, followed by the cover material scenario, the composting scenario and the ecofeed process scenario (Figure 1). In the four recycling scenarios (ecofeed, methane fermentation, composting and cover material), the energy recovered from incineration becomes higher with the proper separation. However, it was also found that the environmental load of ecofeed process scenario is even larger than the incineration scenario due to the large energy consumption in transportation.

Regarding ‘acidification’, the environmental load of ecofeed process scenario is largest because of the transportation and oil consumption of long distance. The cover material scenario has the lowest environmental load (Figure 2).

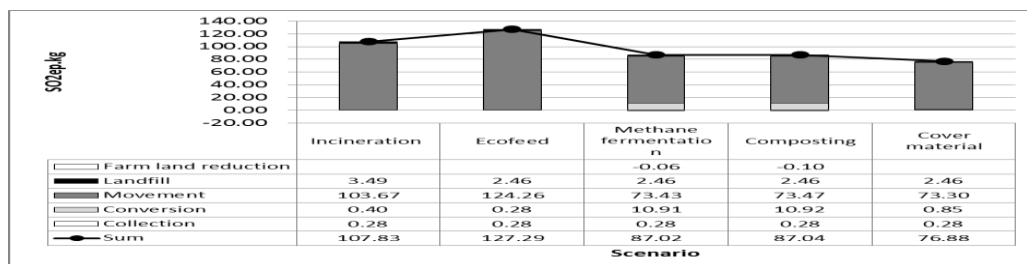


Figure 3 Characterization assessment of landfill

In respect of ‘final dumping place’, the environmental load of the recycling scenarios is lower in general, as the burying quantity of garbage is decreased after the separation (Figure 3). While it takes 150 years to recover the landfill in the business-as-usual (BAU) scenario, it shortens to 50 years in the cover material scenarios the experimental result indicates that layered soil was decomposed 3 times faster with the action of the microbe of the garbage compost.

2. integration assessment of LCA

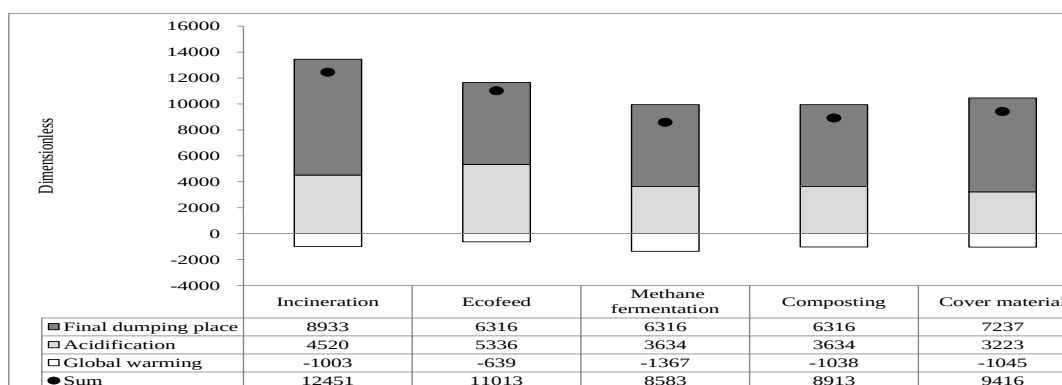


Figure 4 Integration assessment of basic version

Based on the results of Characterization assessment we did the integration assessment of five scenarios. We multiply the characterization results by integration coefficient of each category and then make a sum.

The environmental load generated by the landfill (landfill consumption) is the highest. The methane fermentation scenario is the best, followed by the composting scenario, the cover material scenario and the ecofeed scenario (Figure 4).





Conclusion

This research discussed the LCA of five kinds of garbage treatments. Through the results of integration assessment, methane fermentation method gained the highest value in LCA followed by composting, cover material and ecofeed method, and the worst was incineration. But it is difficult to obligate small companies to separate garbage carefully for composting and methane fermentation, of which the good quality of garbage is necessary. With the cover material method, however, it does not require companies to separate garbage elaborately. Besides, cover material also has a low environmental load especially in acidification.

Importantly, the method of using unrecyclable garbage as cover material is introduced in LCA for the first time. This method enables companies with a minimal effort (intention) of sorting waste to start their recycling activities, which will help to reduce the environmental load. It is hard to improve the garbage recycling rate of small-scale businesses without using unrecyclable garbage as cover material. Also, it is significant to establish the facility to low quality garbage.

It is necessary to establish the local recycling system which includes various recycling agencies, corporations that generate garbage and government sectors. The remaining issue is to create a network connecting the corporations and recycling agencies. This research will contribute to the further development of cover material technology and improve the food garbage recycling of small-scale companies

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