รายงานวิจัยฉบับสมบูรณ์

โครงการ การวิเคราะห์โปรตีนในน้ำตาสุนัขปกติและสุนัขที่เป็นโรคตาแห้งและอธิบายลักษณะการตอบสนองของโปรตีนในน้ำตาสุนัขป่วยที่ได้รับการรักษาด้วยยากระตุ้นน้ำตาชนิด cyclosporine

โดย อ.สพ.ญ.ดร.เมทิตา สัสดี

เดือน ปี ที่เสร็จโครงการ เมษายน พ.ศ.2562

สัญญาเลขที่ MRG6080134

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มหาวิทยาลัยเกษตรศาสตร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

Abstract

Project Code : MRG6080134

Project Title : Proteomic analysis of normal and keratoconjunctivitis sicca dog tear film and characterization of responsive proteins in cyclosporine-treated keratoconjunctivitis sicca dogs

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Project Period : 2 years

Dry eye or keratoconjunctivitis sicca (KCS), a deficiency of aqueous layer of the precorneal tear film is most often considered to be immune-mediated dacryoadenitis in dogs. The disease results in symptoms of tear film instability, with potential damage to the ocular surface, similar to the dry eye disease in human. In this study, a comparative proteomic analysis was used to identify altered tear proteins in the KCS dogs and dogs treated with cyclosporine (CsA). Tear samples were collected from dogs of either gender and of any breed or age. The subjects were divided into 3 groups, 1) healthy dogs, KCS dogs and CsA treated KCS. Two-dimensional electrophoresis (2-DE) of tear proteins extracted from the Schirmer's tear test (STT) strips revealed significant alterations in several proteins in the affected dog group. In 2-DE profiled of dog tears, 13 protein spots of differential expression were excised and subjected to protein identified by mass spectrometry. Tear samples from both groups showed similar distribution of major tear proteins in the 2-DE maps, but some proteins appeared altered in the concentrations. These proteins include Rho GDP-dissociation inhibitor 2, lysozyme C, heat shock protein beta-1, keratin, type II cytoskeletal and protein S100-A12. The comparative proteomic analyses of tears from healthy dogs, KCS dogs and CsA treated dogs were first reported. Differential protein expression will expand the knowledge of physiologic characteristics of tear fluid and pathology of KCS in dogs, in addition to explore a novel therapeutic approach to dry eye disease in dogs

Keywords : dog, keratoconjunctivitis sicca, tear protein, cyclosporine

บทคัดย่อ

รหัสโครงการ : MRG6080134

ชื่อโครงการ : การวิเคราะห์โปรตีนในน้ำตาสุนัขปกติและสุนัขที่เป็นโรคตาแห้งและอธิบายลักษณะการตอบสนองของโปรตีนในน้ำตาสุนัขป่วยที่ได้รับการรักษาด้วยยากระตุ้นน้ำตาชนิด cyclosporine

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ระยะเวลาโครงการ : 2 ปี

โรคตาแห้งในสุนัขเป็นความผิดปกติที่เกิดจากภูมิคุ้มกันทำลายต่อมน้ำตา ทำให้เกิดภาวะต่อมน้ำตาอักเสบและสร้างน้ำตาได้น้อยลง เช่นเดียวกับในคนโรคตาแห้งทำให้แผ่นน้ำตาผิดปกติและเกิดความเสียหายอย่างมากขึ้นกับกระจกตาตามมา การศึกษานี้ได้ใช้เทคนิคโปรติโอมิกส์ทำการเปรียบเทียบการเปลี่ยนแปลงของโปรตีนในสุนัขปกติ สุนัขที่เป็นโรคตาแห้งและสุนัขป่วยที่ได้รับการรักษาด้วยยากระตุ้นน้ำตาชนิด cyclosporine โดยทำการเก็บตัวอย่างน้ำตาจากสุนัขไม่จำกัดเพศ สายพันธุ์และช่วงอายุ ทำการแบ่งสุนัขออกเป็นสามกลุ่ม ได้แก่ สุนัขที่มีสุขภาพดี สุนัขที่เป็นโรคตาแห้ง และสุนัขป่วยที่ได้รับการรักษาด้วยยากระตุ้นน้ำตา ทำการสกัดและแยกโปรตีนในน้ำตาจากแผ่นกระดาษกรองวัดน้ำตาด้วยเทคนิค Two-dimensional electrophoresis (2-DE) ซึ่งพบว่ามีการเปลี่ยนแปลงของโปรตีนหลายชนิดอย่างมีนัยสำคัญ จากนั้นคัดเลือกจุดของโปรตีนบนแผ่นเจล 2-DE จำนวน 13 จุดไปใช้ในการะบุชนิดของโปรตีนด้วยเทคนิค Mass spectrometry ผลการทดลองพบว่าชนิดของโปรตีนในแต่ละกลุ่มสุนัขมีการกระจายของโปรตีนหลักในน้ำตาที่คล้ายคลึงกันแต่มีความเข้มข้นของโปรตีนบางชนิดต่างกัน โปรตีนที่ต่างกันได้แก่ Rho GDP-dissociation inhibitor 2, lysozyme C, heat shock protein beta-1, keratin, type II cytoskeletal และ protein S100-A12 การศึกษานี้เป็นการศึกษาแรกที่รายงานการเปรียบเทียบชนิดของโปรตีนที่แสดงออกในน้ำตาของสุนัขปกติ สุนัขที่เป็นโรคตาแห้ง และสุนัขป่วยที่ได้รับการรักษาด้วยยากระตุ้นน้ำตา ซึ่งโปรตีนที่แสดงออกต่างกันนั้นจะช่วยพัฒนาความรู้ด้านสรีรวิทยาของน้ำตาและการเกิดพยาธิสภาพในสัตว์ป่วยโรคตาแห้งได้ นอกจากนี้ยังอาจนำไปสู่ความรู้ใหม่สำหรับวิธีการรักษาโรคตาแห้งในสุนัขอีกด้วย

คำสำคัญ : สุนัข โรคตาแห้ง โปรตีนในน้ำตา ไซโคลสปอริน

Executive Summary

ความสำคัญและที่มาของปัญหา

Researches on the analysis of tears involving the composition and quality of tear film components that reflected the health status of the ocular surface has been established for several years (Zhou and Beuerman, 2012). Keratoconjunctivitis Sicca (KCS) or dry eye afflicts millions of people worldwide and symptoms of the disease are reported by 17-25% of patients visiting ophthalmic clinics (Schaumberg et al., 2003). The disease also occurs in a variety of dog breeds in Thailand including American cocker spaniel, miniature schnauzer, Pekingese, poodle, pug, Samoyed, Shih Tzu, West Highland white terrier, and Yorkshire terrier (Kaswan et al., 1998). KCS is a chronic inflammatory disease caused by the deficiency of the aqueous component of the lacrimal film which promotes conjunctivitis, keratitis and progressive corneal disease including secondary corneal ulcers resulted in risks for vision loss (Maggs et al., 2008). The diagnosis of KCS is based on clinical signs and Schirmer's tear test (STT). In human, there are early efforts tried to use several techniques of tear protein analysis as an objective test for diagnosis of dry eye and other ocular and systemic diseases (Grus et al., 2005). Proteomic, the study of entire set of proteins expressed by a genome, cell, tissue, or organism at a certain time, is used in the tear protein study field. For example, Grus et al. (2005) used surface-enhanced laser desorption ionization-mass spectrometry (SELDI-TOF-MS) to profile tear proteins from patients with dry eyes. As well as, they identified the potential tear biomarker which revealed and increase of inflammatory related proteins and decrease of some proteins (Grus et al., 2005). Human tear fluid is increasingly being used to detect protein biomarkers related to eye diseases (Li et al., 2008B) such as keratoconus (Lema et al., 2010), Graves ophthalmopathy (Matheis et al., 2012), and diabetic retinopathy (Torok et al., 2015). Tear protein profile has been established in dogs by Winiarczyk and coworkers in 2015 (Winiarczyk et al., 2015). There are several applications of tear protein analysis including to develop the potential cancer markers for diagnosis or management of canine cancers (Campos et al., 2008). Proteomic analysis has become an important factor to biomedical research, since it is a valuable means of studying the healthy and diseased eye and correlates with systemic diseases. As it is the potential biomarker, proteomics holds the key for unlocking to the advanced veterinary pathology and diagnosis (Ceciliani et al., 2014).

Although a recent study evaluated dog tear film proteome, no study has systematically investigated and characterization of protein changes in KCS dogs and responsive proteins in KCS dogs treated with topical cyclosporine A, an immunosuppressant drug which is an efficacious medication for KCS in this species. In this study, the tear film collected from normal control and affected dogs are initially examined using proteomic techniques, two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). The analysis of the differentially expressed tear proteins can provide better understand the physiology of dog tear film, pathology of KCS as well as further exploring potential application for treatment of KCS and other ocular surface disorders.

วัตถุประสงค์

1. To investigate the tear proteome profiles of normal dogs
2. To differentiate protein levels in normal and KCS dogs
3. To investigate the tear proteome and protein levels in untreated compared to CsA treated KCS dogs

วิธีทดลอง

1) Subjects and dog tear sampling

This study was approved the use of animal research by the Kasetsart University Research and Development Institute (ACKU59-VTN-005). Tear samples were collected from dogs of either gender and of any breed or age. The subjects were divided into 3 groups; G1 (healthy dogs), G2 (KCS dogs) and G3 (CsA treated KCS dogs). The G1 served as the control group was determined from STT value more than 15 mm/min with no abnormal ocular signs or diseases. The KCS dogs in G2 were diagnosed based on a Schirmer's tear test value $\leq$ 10 mm/min. Dogs in this group were excluded from the study if they had ever been treated previously with topical or systemic CsA or with any of the following drugs within 14 days before the study: topical or systemic corticosteroids, atropine, antihistamines, pilocarpine, or sulfa-containing drugs, essential fatty acids or general anesthetics. Other exclusion criteria included the presence of any systemic disease other than dermatological disorders or of any ocular diseases affecting the ocular surface other than those related to orbital conformation in brachycephalic breeds. Dogs in which KCS was determined to be congenital, secondary to neuropararalysis, to surgery of the nictitans gland, to distemper or to the use of lacrimotoxic drugs, were not included in the study. G3 dogs were all G2 dogs that are first

diagnosed as KCS and then received topical CsA (2% concentration in corn or olive oil) twice-daily for 45 days. In severe cases (STT < 5 mm/min), when considered necessary by the veterinarian, dogs were treated with commercial artificial tears three times a day.

Tear samples were collected from all groups using STT type I. The Schirmer strips were inserted for one to five minutes in the middle of lower eyelid (Figure 1) . Then the strips were placed in elution buffer consisting of 250 ml of lysis buffer with protease inhibitors and stored at -20 ^{o}C.

Figure 1. Tear samples are collected using the Schirmer strips by inserted for one to five minutes in the middle of lower eyelid of the dogs.

2) Tear protein extraction

2.1 Sonicate sample in an ultrasonic bath (incubate on ice) for 15 minutes then vertex 30 seconds.

2.2 Centrifugation the sonicated sample at 14000 rpm at 4 ^{o}C for 20 minutes then carefully take the supernatant into a new 1.5 ml micro tube

2.3 Add 600 µl Methanol into the extracted protein and vortex for 30 seconds.

2.4 Add 150 µl Chloroform and vortex for 30 seconds.

2.5 Add 450 µl sterile distilled water and vortex for 30 seconds then centrifugation at 14000 rpm, 4 ^{o}C for 5 minutes

2.6 Remove aqueous phase and keep the white pellet in the inter-phase then add 1 ml of methanol to wash the pellet and invert the tube.

2.7 Centrifugation at 12000 rpm, 4 ^{o}C for 5 minutes and discard the supernatant

2.8 Open the tube cap and air-dry the pellet for 5 minutes.

2.9 Add 10 µl of lysis buffer and vortex then sonicate sample in ultrasonic bath for 30 minuts.

2.10 Centrifugation at 12000 rpm, 4 ^{o}C for 10 minutes. Take the solubilized protein to a new tube, and keep as a tear protein stock.

3) Determination of tear protein concentration (Bradford's method)

3.1 Prepare the following mixed solutions in triplicates.

Component	Volume (µl)		
	Blank	Standard curve	Sample
Protein sample (diluted X times)	-	-	2
BSA (0.1, 0.2, 0.4, 0.6, 0.8 mg/ml)	-	10	-
MilliQ water	58	48	58
Lysis buffer	2	2	-
Bradford reagent	300	300	300

3.2 Load 180 µl of each reaction solution into a 96-well plate (3 replicates each).

3.3 Determine the absorbance a 595 nm using Spectra Max spectrophotometer.

3.4 Determine protein concentration using standard curve and calculated concentrations.

4) Two-dimensional gel electrophoresis (2-DE)

To identify the altered expression protein levels in KCS dogs, the protein expression profiles of G1 and G2 are compared. To identify the responsive protein after CsA treatment in KCS dogs, the protein expression profiles of to G2 and G3 are compared. Each pooled protein sample are separated through the 2-DE which composed of first dimensional gel electrophoresis (IEF) and second dimensional gel electrophoresis (SDS-PAGE).

4.1 First Dimensional Gel Electrophoresis

1) Prepared the following solution for IEF.

Component	Volume (µl)
Tear protein sample (150 µg)	X
IPG buffer	1.5

1% Bromophenol blue	0.75
Lysis buffer	130 – (X+2)
Total volume	130

2) Centrifuge at 12000 rpm 4 ^{o}C for 5 minutes and transfer 125 µl of prepared sample into 7-cm strip holder (avoid air bubble)

3) Place an IPG strip over the solution (gel facing down), add 500 µl of cover fluid, and place the lid.

4) Rehydrate the protein sample for 12 hours and then run IEF program on Ettan IPGPhor II, using following condition;

Step (V-hour)	Voltage (V)	Times (hour)	Volt-hours
Rehydration step	-	15	-
S1 step and hold	100		100
S2 step and hold	300		200
S3 gradient	1000		300
S4 gradient	3000		4000
S5 gradient	5000		4500
S6 step and hold	5000		3000
S7 step and hold	100	10	-

5) After a completion of IEF, place the gel strip into a freshly prepared solution of 25 mg DTT in 2.5 ml SDS-PAGE equilibration buffer. Shake for 15 minutes for strip equilibration.

6) Replace the DTT solution with a freshly prepared solution of 60 mg IAA in 2.5 ml SDS-PAGE equilibration buffer then shake for 15 minutes.

4.2 Preparation for Second Dimension Gel Electrophoresis (SDS-PAGE)

1) Clean the glass plates and assembly the gel cassette.

2) For 12.5% Acrylamide gel, 1 mm-thick gel, prepare the following solution;

MiliQ water	3.2 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
Acrylamide stock solution (30% acrylamide, 0.8% bis-acrylamide)	4.2 ml

10% SDS	100 ml
10% APS	70 ml
TEMED	5 ml

3) Mix and carefully pipette the mixed solution into the assembled gel cassette and layer with 50% Ethanol on top of the gel.

4) Allow the gel to polymerize for 15-30 minuts. Wash the gel with 1X running buffer before use.

5) Rinse the gel strip with 1 ml of SDS-PAGE running buffer twice and gently insert the strip into the top of SDS-PAGE gel.

6) Load protein marker strip on the anode end of the IEF strip.

7) Add SDS-PAGE running buffer to cover the gel and start the second dimensiton separation at 120 Volts then allow the running of SDS-PAGE separation until the tracking dye has reached the bottom of the gel plate.

8) Gently remove the gel from glass plates. Rinse gel with milliQ water for twice and place in 100 of staining solution containing Colloidal Coomassie Blue G-250 (CBG) for overnight at room temperature.

9) De-stain the gel with several changes of MilluQ water

10) Imagescanner III (GE Healthcare) is used to scan the G250-stained gel images. The protein spots are then detected and analyzed using the ImageMaster 2D software (GE Healthcare). Before matching protein spots across the gels, the percentage of intensity volume (% volume) of each spot is normalized to the total intensity volume of all spots in each gel. The differences in the % volume of each spot between the G1 and G2, and between G2 and G3 groups are then compared with acceptable statistical criteria of the Student's t test. Only the eligible protein spots with at least a 3-fold alteration in the protein expression level in all gels are later subjected to mass spectrometry for protein annotation.

5) Mass spectrometry

The protein spots from 2-DE are destained using 50% acetonitrile in 50 mM ammonium bicarbonate. Protein reduction and alkylation were done using final concentration of 4 mM dithiotreitol and 10 mM iodoacetamide, respectively. Individual gel pieces were done tryptic digestion and

subjected peptide mixture to an Ultimate 3000 nano-LC system (Dionex; Surrey, UK) equipped with a Acclaim PepMap RSLC (Thermo Scientific, Waltham, MA) for peptide separation. Subsequently, a micrOTOF-Q (Bruker Daltonics, Bremen, Germany) is coupled with the LC and online analyzed the eluted peptides. MASCOT search engine 2.2 (Matrix Science, Ltd.) was used for protein identification. The search parameters were set at SwissProt database, one miss cleavage and trypsin digestion. Only proteins above 95% confidence interval were reported in this research.

ผลการทดลอง

1. Study population and ocular examination

Thirty-eight dogs were enrolled into the study (Table 1-3). The mean STT values in healthy dogs (G1), KCS dogs (G2) and CsA treated dogs (G3) were 21.3 ± 0.9 mm/min (n=15), 4.0 ± 0.4 mm/min (n=13) and 16.1 ± 0.6 mm/min (n=10), respectively. Statistically significant different were observed within G1-G2 and G2-G3 groups ($P<0.001$). For G3 groups, dogs responding to CsA treatment were defined as those in which STT values increased to more than 10 mm/min.

Table 1. Breed, gender and of normal dogs included in the pooled tear sample for G1 group

Breed	Sex	Age	STT (mm/min)		IOP (mmHg)		Ocular sign
		(year)	Left	Right	Left	Right	
Mixed	M	5	22	25	16	14	normal
Shih Tzu	M	11	27	23	13	15	normal
Shih Tzu	F	8	16	19	21	23	normal
Poodle	M	4	26	24	18	17	normal
Pomeranian	M	2	17	15	11	12	normal
English Cocker Spaniel	F	6	21	25	12	12	normal
Thai Ridgeback	M	5	17	16	19	17	normal
Chihuahua	F	4	23	20	22	20	normal
Labrador Retriever	F	3	21	19	16	16	normal
Shih Tzu	M	2	25	24	24	22	normal

Jack Russell	M	3	26	23	13	12	normal
Pomeranian	M	5	15	15	17	15	normal
French bulldog	F	2	23	22	16	16	normal
Mixed	F	6	25	24	20	19	normal
Thai Ridgeback	M	8	16	17	13	11	normal

Table 2. Breed, gender and of normal dogs included in the pooled tear sample for G2 group

Breed	**Sex**	**Age**	**STT (mm/min)**		**IOP (mmHg)**		**Ocular sign**
		(year)	**Left**	**Right**	**Left**	**Right**	
Moltese	F	13	2	5	12	11	Corneal pigmentation
English Cocker Spaniel	M	8	3	3	15	17	Corneal pigmentation Mucous ocular discharge
Shih Tzu	F	10	4	2	14	13	Corneal pigmentation
French bulldog	F	5	6	5	16	18	Mucous ocular discharge
Mixed	F	12	7	8	21	20	Corneal edema Corneal vasculization
Mixed	M	7	1	3	15	15	Corneal pigmentation
Shih Tzu	M	9	2	4	11	11	Mucous ocular discharge
Poodle	F	5	5	3	14	13	Corneal pigmentation Mucopurulent ocular discharge

Shih Tzu	M	11	4	7	21	19	Corneal vasculization Mucopurulent ocular discharge
Pomeranian	M	12	6	3	16	16	Corneal pigmentation
English Cocker Spaniel	M	7	5	4	12	11	Corneal pigmentation Mucopurulent ocular discharge
Mixed	F	9	2	2	15	13	Corneal pigmentation Corneal vasculization
English Bulldog	F	8	3	3	13	13	Corneal pigmentation Mucopurulent ocular discharge

Table 3. Breed, gender and of normal dogs included in the pooled tear sample for G3 group

Breed	**Sex**	**Age**	**STT (mm/min)**		**IOP (mmHg)**		**Ocular sign**
		(year)	**Left**	**Right**	**Left**	**Right**	
Poodle	F	5	15	17	11	8	Corneal pigmentation Mucous ocular discharge
English Cocker Spaniel	M	7	22	24	15	13	Corneal pigmentation Corneal edema
Shih Tzu	M	11	19	22	11	11	Corneal pigmentation
English Bulldog	F	8	14	17	17	16	Corneal pigmentation

							Corneal vasculization
Shih Tzu	M	11	19	13	20	18	Normal
Pomeranian	M	12	15	17	22	22	Corneal pigmentation
Pomeranian	F	14	20	18	17	17	Corneal pigmentation Corneal vasculization
Mixed	F	12	15	17	13	14	Corneal pigmentation Corneal edema
Shih Tzu	M	8	16	14	15	15	Normal

2. 2-DE Proteome profiles of tears

To investigate the difference in protein composition of tears among different dog groups, extracted tear samples were analyzed by two-dimensional gel electrophoresis. The volumes of the protein spots were calculated. Representative gels for pools normal dog tears and pooled tears from KCS dogs are shown in Figure 2. Tear samples from both groups showed similar distribution of major tear proteins in the 2-DE maps, but some proteins appeared altered in the concentrations. The majority of the tear proteins appear as large protein spots reflecting their high level of expression. Several protein spots in the 2-DE analysis showed significant differences in protein patterns (Spot no. 1-13). Four spots from healthy dogs (spot no. 1, 2, 3 and 12) and eleven spots from KCS dogs (spot no. 1 and 4-13) were selected and excised to protein identification by mass spectrometry. The identified proteins are shown in Table 4. The spots 1 was verified as Rho GDP-dissociation inhibitor 2. Spots 2 – 7 were identified as lysozyme C protein. Base on the intensity of staining (with Coomassie blue), the amount of Rho GDP-dissociation inhibitor 2 and lysozyme C in KCS dogs may be less than that in healthy dog tears. Many of protein groups display charge heterogeneity, most likely due to posttranslational processing (Campos et al., 2008). Spot no. 9, 10, and 11 in KCS dog tears were identified as keratin protein which were not detected in healthy dogs in the same area. Heat shock protein beta-1 was detected in both groups of dog tears but showed higher intensity of staining in KCS dog tears than the healthy dog tears. Another major tear proteins, protein S100-A12 (spot no. 13), detected at the low molecular weight area was found only in KCS dog tears.

Interestingly, protein identification in tears from dogs received topical CsA twice-daily for 45 days (G3 dog group) showed similar from tears of KCS dogs (Figure 3). Lysozyme C, Rho GDP-dissociation inhibitor 2 and keratin protein were found in the CsA treated dogs with different intensity of protein spot staining.

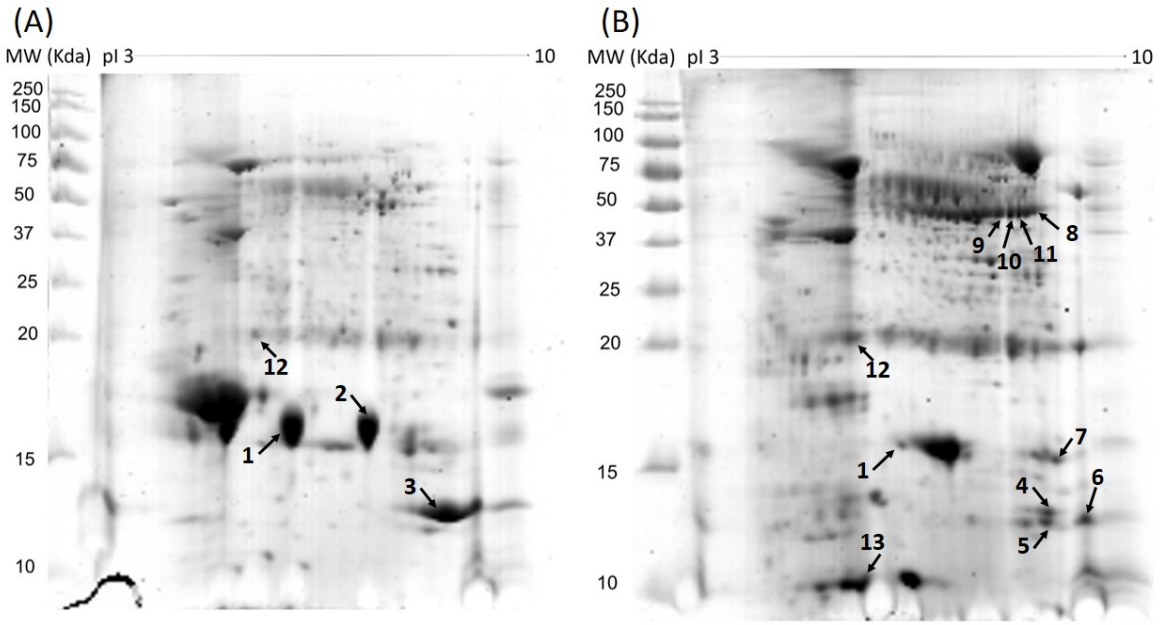

Figure 2. Protein expression profiles in dog tear. 2-DE analysis of 120 µg extracted proteins was resolved by isoelectric focusing at pH 3-10 nonlinear with 7 cm IPG strip, followed by 12.5% SDS-PAGE in comparison between pooled tear protein from healthy dogs (A) and KCS dogs. The Precision Plus Protein^{TM} Unstained Standards (Bio-Rad) was used as a protein marker. The arrows and numbers indicate the altered protein expression between the healthy and KCS dogs.

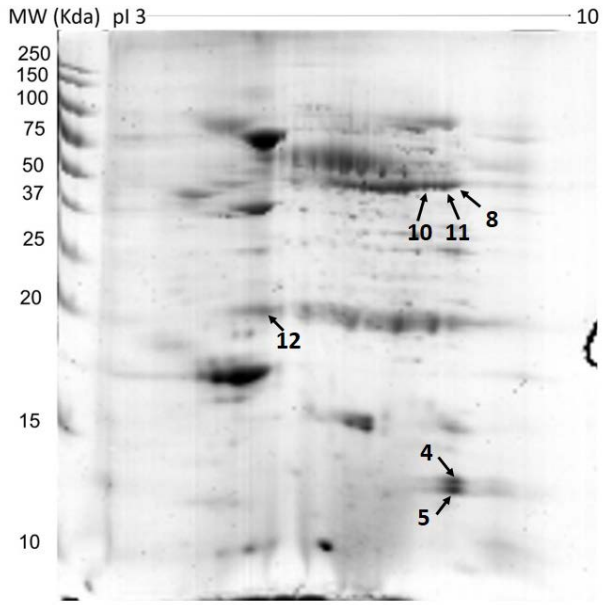

Figure 3. Protein expression profiles in dogs responding to CsA treatment. The Precision Plus Protein™ Unstained Standards (Bio-Rad) was used as a protein marker. The arrows and numbers indicate the altered protein expression between the pooled tear protein from KCS dogs and dogs responding to CsA treatment.

Table 4. Identification of significantly different expressed proteins in dog tears. Mass spectrometry analysis of selected protein spot of healthy and KCS dogs were obtained from 2-D separation.

Spot no.	Identified proteins	Protein score	Mass (kDa)	pI
1	Rho GDP-dissociation inhibitor 2	139	22.7	5.1
2	Lysozyme C	479	14.5	8.6
3	Lysozyme C	2294	14.5	8.6
4	Lysozyme C	1048	14.5	8.6
5	Lysozyme C	1072	14.5	8.6
6	Lysozyme C	642	14.6	9.1
7	Lysozyme C	642	14.6	9.1
8	Lactotransferrin	42	75.9	8.3
9	Keratin, type II cytoskeletal 5	72	61.7	7.6
10	Keratin, type II cytoskeletal 1	836	65.9	8.2
11	Keratin, type II cytoskeletal 6B	132	60.0	8.1

12	Heat shock protein beta-1	133	22.9	6.2
13	Protein S100-A12	36	10.7	5.7

สรุปและวิจารณ์ผลการทดลอง

This is the first study that reports the comparative proteome profile of tears from normal dogs and dry eye dogs. In 2015, canine tear proteome was explored using mass spectrometry which identified 125 proteins in the tear film of six healthy dogs (Winiarczyk et al., 2015). In that report, one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) was used as the first step to separate the tear proteins then analyzed each visualized bands by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) to identify the tear protein. The recent study, two-dimensional gel electrophoresis was used to compare the proteome profile of tears from normal and KCS dogs. Because of different techniques, the variation of tear proteins was identified. Rho GDP-dissociation inhibitor 2 and heat shock protein beta-1 was found in both normal and affected tear dogs but they were not detected previously in dog tears. Rho GDP-dissociation inhibitor 2 is one of the small GTPase proteins which important molecules for linking cell shape and cell-cycle progression. The molecular function of this protein is to prevent the dissociation of GDP from the small GTPase Rho, thereby preventing GTP from binding and their role in both cytoskeletal arrangements and mitogenic signaling (Fugita et al., 2012). The amount of Rho protein in KCS dog tear may be less than that in normal tears. Heat shock protein beta-1 is a small heat shock protein which functions as a molecular chaperone probably maintaining denatured proteins in a folding-competent state. It plays a role in stress resistance and actin organization. This protein was found in all group of tear samples. Protein S100-A12 was detected in KCS dog tear but not in healthy and CsA treated dog tears. This protein previously reported in normal cow tears (Shamsi et al., 2016). S100-A12 plays a prominent role in the regulation of inflammatory processes and immune response. Its proinflammatory activity involves recruitment of leukocytes, promotion of cytokine and chemokine production. The dry eye dogs who treated with topical CsA, an effective treatment for canine KCS, mostly found a decrease in severity of inflammation (Hendrix et al., 2011). Therefore, the S100-A12 protein was not detected in normal and treated dog tears. Keratin type II cytoskeletal proteins were observed in both human and dog tears (Winiarczyk et al., 2015). This study, the keratin type II cytoskeletal were detected significantly in KCS and CsA treated dog tears.

The protein spots of lysozymes were detected vary in the 2-DE map of tear fluid from all dog groups. Lysozyme has important roles in tear fluids. It is manufactured in acinar cells of the lacrimal glands. It has an antibacterial action with hydrolyzing glycosidic bonds, particularly those of certain

gram-positive bacteria, in the bacterial cell walls (Ohashi et al., 2005). The concentration of lysozyme may be less than in KCS dog and CsA treated dog because of low-intensity protein spot staining. This may be indicated that the used of CsA treatment may not improve the concentration of lysozyme in the dog tear. However, this study we collected the tear sample of G3 dogs from the KCS dogs who received CsA for 45 days, the concentration of lysozyme with a longer follow up period was not known.

Proteomic techniques are useful tools in the analysis of tear protein. This study, we selected partials tear protein spots which found different staining from three conditions of dogs for protein identification, it is thus highly the unidentified proteins may have possibly been missed, especially less-abundant tear protein. Our findings revealed that the composition of tear proteins in dry eye dogs were different from that of healthy dogs and may provide further insights into the pathogenesis and novel treatment of this disease.

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