รายงานการวิจัยฉบับสมบูรณ์

โครงการ ศักยภาพในการเปลี่ยนแปลงของเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันนำนมและฟันแท้

โดย

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กุมภาพันธ์ 2556

สัญญาเลขที่ DBG5180009

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ชื่อโครงการ: ศักยภาพในการเปลี่ยนแปลงของเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันน้ำนมและฟันแท้

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บทคัดย่อ

วัตถุประสงค์ : เปรียบเทียบการแบ่งตัว การแสดงออกของยีน การสะสมแร่ธาตุของเซลล์เนื้อเยื่อในโพรงประสาท ฟันน้ำนมและฟันแท้ รวมตลอดถึงการเจริญเติบโต ในโครงร่างทางชีวภาพไคโตซาน

วิธีทดลอง : เซลล์ดังกล่าว (SHED & DPSC) ถูกแยกด้วยวิธีใช้เอ็นไซม์ และความสามารถในการสร้างกลุ่ม (CFU-F) วิเคราะห์การแบ่งตัวของเซลล์ในวันที่ 1 7 และ 14 ด้วยวิธี เอ็มทีที ประเมินการแสดงออกของยีนดีเอสพีพี ในวันที่ 7 และ 15 การสะสมแร่ธาตุประเมินจากการย้อมสี อลิสซาริน เรด ในวันที่ 7 14 21 และ 28 โครงร่างชีวภาพไคโตซาน (2% และ 3%) ผลิตขึ้นเองด้วยวิธีการปั่นเหวี่ยงแล้ววิเคราะห์การบวม การย่อยสลายและการเข้ากันได้กับเซลล์ ตรวจดูการเกาะของเซลล์เอสเอชอีดี และ ดีพีเอสซี ที่เลี้ยงในโครงร่างไคโตซานด้วยเอสอีเอ็ม วิเคราะห์การเจริญเติบโตของเซลล์ในวันที่ 8 15 และ 21 ด้วยวิธี ดับบลิว เอส ที–วัน

ผลการทดลอง : แม้ไม่สามารถวัดการแสดงออกของยีนดีเอสพีพี แต่เซลล์เอสเอชอีดี สามารถแบ่งตัวและสะสมแร่ธาตุได้มากกว่าเซลล์ดีพีเอสซี โครงร่างทางชีวภาพไม่มีพิษ และสามารถช่วยให้เซลล์เจริญเติบโตได้ ความมีชีวิตของเซลล์ทั้งสองชนิดในโครงร่างชีวภาพ 2% ดีกว่าในโครงร่างชีวภาพ 3%

สรุป : โครงร่างชีวภาพไคโตซาน ผลิตด้วยวิธีการใหม่นี้เหมาะกับการเจริญเติบโต และความมีชีวิต ของเอสเอชอีดี และ ดีพีเอสซี

คำหลัก : การแบ่งตัวของเซลล์ (Proliferation), การแสดงออกของยีน (gene expression), การสะสมแร่ธาตุ (mineralization), เอสเอชอีดี (stem cells of human exfoliated deciduous teeth), ดีพีเอสซี (dental pulp stem cell), ซีเอฟยู-เอฟ (colony-forming efficiency)

Project Code: DBG5180009

Project Title: Differentiation potential of dental pulp stem cell derived from deciduous and permanent teeth

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Abstract

Objective: compare the proliferation, genes expression, mineralization of dental pulp cells derived from primary and permanent teeth and their growth in chitosan scaffolds.

Methods: those cells (SHED & DPSC) were isolated by enzyme digestion and analyzed for their colony-forming capacity (CFU-F). The cell proliferation was measured by the MTT assay on day 1, day 7, and day14. The expression of DSPP was investigated at day 7 and day 15. Alizarin Red staining was used to detect mineralized nodule formation of the cells on day 7, 14, 21, and 28. Chitosan scaffolds (2 % & 3 %) were fabricated using our own centrifugation method. They were tested for swelling, degradation and cytocompatibility. SHED and DPSC were cultured in the scaffolds. The cells attachments were examined with SEM. The WST-1assay was performed on day 8, 15 and 21 to assess the cells growth.

Results: although DSPP expression could not be detected from both cells, SHED had a higher proliferation rate and mineralization rate than DPSC. The scaffolds were shown to be non-toxic and could promote the cells growth. The viability of both cells on 2% scaffolds was higher than that of the 3% scaffold group.

Conclusion: chitosan scaffolds fabricated with our novel method were suitable for the growth and survival of SHED and DPSC.

Key words

Proliferation, gene expression, mineralization, SHED (stem cells of human exfoliated deciduous teeth), DPSC (dental pulp stem cell), CFU-F (colony-forming efficiency).

Contents

List of Table

List of Figures

Chapter 1

Introduction

1. Introduction

The findings of human dental pulp stem cell (DPSCs) [1] and stem cells of human exfoliated deciduous teeth (SHED) [2] possible facilitate pulp tissue engineering. Stem cells play an important role that organizes a net-work of inducing the connective tissue to regenerate new tissue [3]. As a carrier, many kinds of scaffolds can be selected for tooth structure engineering. Potential scaffolds have been tested for regeneration of bone, possibly suitable for dental field. However, regenerating pulp/dentin is not the same as regenerating bone. Pulp and dentin in the canal space have their specific locations; therefore, any scaffold system that is osteo-inductive such as hydroxyapatite and tricalcium phosphate is in fact not appropriate for pulp/dentin regeneration [4]. On the other hand, when the pulp tissue engineering applies in vital pulp therapy, the material should possess the ability of anti-microbial and can be shaped easily. The natural materials such as collagen and chitosan have a better biocompatibility, besides their shape can be fabricated optionally. The collagen-based scaffolds were studied with DPSC, and those scaffolds could support cell growth and differentiation *in vivo* [5]. However, the collagen does not have the function of anti-microbial. Collagen scaffolds often lose shape and size because of the rapid degradation when contacted with body fluid or cell-culture medium [6]. Huang reported that pulp cells markedly caused the contraction of collagen. Their data showed that collagen shrank to half of its original size by 3-15 days [7]. In other words, pulp cells

would lose the space to survive when they were carried by collagen scaffolds. Therefore, collagen matrix may not be a suitable scaffold for pulp tissue engineering [7]. Chitosan is a natural polymer from renewable resources, obtained from shell of shellfish, and the wastes of the seafood industry, and it is a deacetylated derivated of chitin. Chitosan scaffolds possess some special properties for use in tissue engineering. First, it can be molded in various forms [8]. In particular, it possesses excellent ability to form porous structures [9]. Regulation of porosity and pore morphology of scaffolds is critical for controlling cell growth and organization within an engineered tissue. The microstructure of porous chitosan scaffolds allows themselves to possess the swelling ability, which attributes to promote the cell attachment, nutrient supply and the 3-D structure maintenance. Second, the cationic nature of chitosan is primarily responsible for electrostatic interactions with other negatively charged molecules. The benefit of this property for tissue engineering is that numbers of cytokines/growth factors can be bound [10]. Another distinct property of chitosan is that it confers considerable antibacterial activity against a broad spectrum of bacteria [11]. Fourth, the degradation rate of the chitosan can be adjusted by distribution of acety1 groups （DD） and molecular weight （Mw) [12]. The degradation rate also affects the degrade ability of a scaffold plays a crucial role on the long-term performance of tissue-engineered cell/material construct because it affect biocompatibility, including cell growth, tissue regeneration, and host response. It has to maintain the mechanical strength until tissue regeneration is almost completed [11]. Therefore, in respect that many advantages of chitosan, the chitosan scaffolds were studied in various tissue engineering applications

such as skin, bone, cartilage, liver, nerve and blood vessel [11]. Accordingly, the three dimensional chitosan scaffolds are possibly suitable for the vital pulp therapy and the restoration of dental structure. Therefore, the understanding of the SHED and DPSC proliferation, mineralization processes， characterization as well as cytocompatibility of scaffolds is necessary.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Cell culture

Primary and permanent teeth were collected under the approved guideline of Ethics Committees of Prince of Songkla University. The human primary exfoliated teeth were collected from 6 to 12-year-old children (n = 6). Consensuses were obtained from the parents. Permanent teeth were obtained from adult (≤ 29 years old, n = 6) impacted third molars and bicuspids extracted due to orthodontic considerations [13]. All of these teeth contained the normal healthy pulp tissue and follow the criteria: verbal history confirming no history of pulpal pain, and both clinical and radiographic examination assuring that these teeth had no caries, no restorations or periodontal disease [14], and no pulpitis.

Cells were isolated by enzyme digestion as described by Gronthos *et al* [1]. Tooth surfaces were cleaned by 70% alcohol and cut around the cementum-enamel junction by using sterilized dental fissure burs to reveal the pulp chamber. The pulp tissue was gently separated and minced. The minced pulp tissues were digested in a mixture of 3 mg/ml collagenase type I and 4 mg/ml dispase (Sigma, St. Louis, Mo., USA) for 30–60 min at 37°C water-bath. Cell suspensions were obtained by passing the digested tissues through a 70–μm cell strainer (Becton/Dickinson, Franklin Lakes, N.J., USA). Single cell suspensions were seeded in the 100mm cultural plates (Nunc, Denmark) containing DMEM (Life Technologies/GIBCO BRL) supplemented with 20%

FBS (Biochrom AG, Germany), 2 mM L-glutamine (Gibco Invitrogen, USA), 100 U/ml penicillin-G, 100 µg/ml streptomycin, 50 U/ml mycostatin and 100 µg/ml kanamycin and maintained under 5% CO_2 at 37°C.

SHED and DPSC were identified and collected as following techniques by assessing their colony-forming efficiency (CFU-F) [15]. The single cells suspensions seeded in the 100 mm cultural plates at day 10 to 12 of culture. The cells aggregated less than 50 cells were scratched and washed away by Phosphate buffered saline (PBS). The other cells with the number of CFU-F colonies (aggregated of ≥50 cells) were evaluated for all unfractionated cell preparations, and then were transferred to the T-75 cultural flasks (TPP, Switzerland). Those cells were continuously passaged at 1:3 ratios when they reached 70~80% confluent.

2.2 Determined the cell proliferation by MTT assay

SHED, DPSC, gingival fibroblast and MG-63 were seeded at the density of 3×10^3 cells/well in 96-well-plates (Nunc) (n = 6). At least three wells without cell were served as a control for the minimum absorbance. After 24 hours, day 7 and day14, the cell proliferation was measured by MTT assay modified from Mosmann *et al* [16]. Absorbance of the colored solution was measured at wavelength 572 nm by the plate reader (Biotrak II, Amersham Biosciences)

2.3 Measured the mineralized nodule formation by Alizarin Red S staining

SHED, DPSC, gingival fibroblast and MG-63 were seeded at the density of 2×10^4 cells /well in 24-well-plates (n = 6). Those cells were cultured with the normal growth media until reaching confluence, and then DMEM 1 ml supplemented with 10%

FBS, 10mM β-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma), 100 μM L-ascorbic acid 2-phosphate (Sigma), 2 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml mycostatin and 100 μg/ml kanamycin was added to each well. The plates were cultured at 37°C in an incubator setting in a humidified atmosphere of 5% CO_2. The cells within normal media were set as control. The media were changed every 2 days. Detecting of mineralization during cell differentiation was performed at day 7, 14, 21 and 28. Alizarin Red S (Nacalai Tesque Inc.) staining was used to detect mineralized nodule formation of the cells [17-19], and the nodule formation was observed under the light microscope (Inverted Nikon TS 100E).

2.4 Fabrication of chitosan scaffolds

Chitosan used in the present work were with deacetylation degree of 85% and molecular weight of 57,000. The fabrication of chitosan scaffolds were modified from several studies. In brief, the methods contained centrifugation, freeze-drying, stabilization [20-24]. Chitosan solutions with concentrations of 2 and 3% (w/v) were prepared by dissolution in 0.2 M acetic acid. Each 10 ml of chitosan solution was injected into 60 ml of 1M NaOH using syringe with No. 22 needles. Fibril-like chitosans were formed. After filtered through sheet cloth, they were placed in 15 ml centrifuge tubes and centrifuged at 3,000 rpm for 5 min, kept at 4 °C for 24 h, frozen at –20°C for 24 h. Then they were stabilized by immersing in 96% alcohol for 1 h, 1 M NaOH for 5 min, and 70 % alcohol for 12 h. Chitosan scaffolds were sectioned into slices with 5 mm diameter and a. 2 mm thickness (for characteristics of chitosan scaffolds studies); b. 1 mm thickness (for cell seeding study). They were submerged into liquid nitrogen for a

few seconds. Those chitosan scaffolds were then placed into 24-well plates and dried at 37 °C for 2 days.

2.5 Swelling test

The swelling study was designed to investigate the ability of water uptake [25] and the dimension changes of chitosan scaffolds. Stimulated body fluid (SBF) was prepared by dissolving appropriate quantities of the precursor chemicals in deionized water, with ion concentrations nearly equal to those of the inorganic constituents of human blood plasma (Tab 2.1) as described [26].

2.5.1 Swelling ratio

Chitosan scaffolds (n=9) were placed in the SBF for 5 minutes. Those specimens were taken out and were weighted by electronic balance after no more dripping water. Swelling ratios were determined by using the following equation:

Swelling ratio = $(W- W_0) / W_0$

W_0 represents initial dry weight and W denotes wet weight of chitosan scaffold.

2.5.2 Dimension changes of chitosan scaffolds

Chitosan scaffolds (n=9) were immersed in the SBF for 21 days and the time of investigation was set at 5 minutes, 7, 14, and 21 days. At each time point, pictures of the specimens were taken using microscope. The diameters of cross-sectional and longitudinal were measured from the pictures using Program Image Frame Work v.0.9.9.

2.6 *In vitro* degradation study

The degradation study was performed by the principle of what chitosan is mainly degraded by lysozyme [27]. The experimental procedures were modified from the previous studies at the intervals of 7, 14 and 21 days [27, 28]. Scaffolds (n=9) were incubated in a pH 7.4 of PBS with 1×10^4 U / ml of lysozyme at 37℃. At a predetermined time interval, the scaffolds were washed with double distilled water and were freeze-dried. The degradable ratio was determined by weight loss from the formula:

Percentage weight loss = $(W_0 - W_t) / W_0$

W_0 denotes the original weight, and W_t represents the weight at each interval.

2.7 Cytotoxicity test

Cytotoxicity test acts as a practical tool for testing the potential toxicity of materials and medical devices. It generally represents the toxicity of test components by cell death or other serious negative effects on cellular functions [29-31]. The extraction and testing procedures were modified form a previous study [32]. The cytotoxicity of leachables of all materials was evaluated using cell culture methods, namely MEM extraction test (72 h) according to ISO/EN 109935 guidelines [33]. In brief, sterilized 2% and 3% chitosan scaffolds was respectively immersed in media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml

mycostatin and 100 μg/ml kanamycin for 24 hours at 37℃. The extraction media of 2% and 3% chitosan scaffolds were respectively collected in the sterilized tubes. SHED, DPSC, gingival fibroblast and MG-63 were seeded at the density of 5×10^4 cells/well in 96-well-plates with the 150 μl of normal growth media 24 hours with 5% CO_2 and at 37°C in order to establish an 80% confluent monolayer. After the removal of normal growth media, the cells of each cell type were seeded with normal growth media, extraction media of 2% and 3% respectively. After 24, 48, 72 hours, the cells were evaluated under a light microscope, and after the microscopic evaluation of 72 hours, the cell viability was performed by MTT assay.

2.8 Cell morphology and viability within the chitosan scaffolds

2.8.1 Cell seeding within scaffold

Before cell seeding, chitosan scaffolds were immersed in 70% alcohol for 1 hour, then thoroughly washed with sterilized distill water and PBS. Those scaffolds were sterilized under ultraviolet light overnight. The sterilized 2% and 3% chitosan scaffolds were placed in the 48-well plates. Each specimen was enriched in 500μl of D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml mycostatin and 100 μg/ml kanamycin for 24 hours at 37℃. SHED, DPSC, gingival fibroblast and MG-63 were loaded with the density of 5×10^4

cells/scaffold and incubated for 3 hours at 37℃ in a humidified atmosphere 5% CO_2.

After 3 hours, the media were changed to phenol red free DMEM with high glucose (Gibco) and the same supplemented substrates as mentioned above.

2.8.2 Cell viability within the scaffolds

The cell viability within the scaffolds was performed by a water-soluble tetrazolium (WST-1) assay (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate). The WST-1 reagent produces a water-soluble formazan rather than the water-insoluble product of the MTT assay [34]. In our pilot study, products of the MTT assay were deposited inside of scaffolds, and the solubilization step was blocked by complex microstructure of scaffolds. The WST-1 assay (Roche, Mannheim, Germany) was performed at day 8, 15 and 21 according to the protocol of manufacturer. Absorbance of formazan was measured at 450 nm with 620 nm as reference and corrected to blank values (scaffolds without cells).

2.8.3 Cell morphology and microstructure of scaffolds detected by SEM (scanning electron microscope)

Specimens were washed with PB (0.1M) buffer and fixed in 2.5% glutaraldehyde (sigma) at room temperature for 2 h. After they had been washed with PB (0.1M) buffer to remove residual glutaraldehyde, the cell-loaded scaffolds were dehydrated through gradient concentration of ethanol. After the specimens were critically point dried and coated with an ultrathin gold layer, they were observed by SEM (JSM-5800LV, JEOL). The specimens without cells were prepared omitted the fixative

part.

2.9 Data analysis

The analyses were performed by using SPSS software (Version 16.0, Standard Software Package Inc., USA). The data were presented as mean ± SD. Difference among groups or difference among time intervals was analyzed using one-way analysis of variance (ANOVA). When a difference was statistically significant at $P < 0.05$, a multiple comparison test was performed. If the variances of the data were equal, the Scheffe method was used. If the variances of the data were not equal, the Dunnette T3 method was used. Significant differences were set at 95% confidence.

Chapter 3

Results

3. Results

3.1 Cell proliferation

As showed in Figure 1, there was no significant difference among the different cell groups on day 1. On day 7, the optical density (OD) of SHED (0.586 ± 0.068) was found to be significantly higher than that of DPSC (0.308 ± 0.019). The OD of MG-63 cell (1.503 ± 0.110) group was significantly higher than those of the other three groups. On day 14, the OD of SHED (0.815 ± 0.056) was still markedly higher than that of DPSC (0.495 ± 0.030), and the OD of gingival fibroblast (0.861 ± 0.074) was significantly higher than that of DPSC. Moreover, MG-63 cell (2.037 ± 0.096) group maintained the highest OD.

3.2 Mineralized nodule formation

There was no nodule formation on day 7 in all groups (Fig. 2). On day 14, some mineralized nodules could be found in the test groups of SHED and MG-63. In contrast, we could not find any mineralized nodule surrounding the cells in the test groups of DPSC and gingival fibroblasts (Fig. 3). On day 21, the nodules were present in every test groups. Many more mineralized nodules could be observed both in the SHED and the MG-63 group compared to the DPSC group. In addition, few mineralized nodules could be found in the gingival fibroblast group (Fig.4). On day 28, a great number of mineralized nodules appeared in the test groups of DPSC and SHED, and the mineralized nodules in SHED groups were still more than in DPSC group. However,

the circumstances for mineralized nodule formation in the MG-63 test group were not different from day 21 (Fig. 5).

3.3 Characterization of chitosan scaffolds

3.3.1 Morphology of chitosan scaffolds

Figure 6 showed the cross sections (Fig. 6A and Fig. 6C) and longitudinal sections (Fig. 6B and Fig. 6D) of chitosan scaffolds. The microstructures of 2% and 3% scaffolds revealed the interconnected micropores. Respectively, the pore sizes of 2% and 3% scaffold were 188.71 ± 51.90 μm and 195.30 ± 67.21 μm, and they were not significantly different ($P > 0.05$, analyzed by non-parametric analysis, Mann-Whitney U test, $n = 9$).

3.3.2 Swelling study

As described in Figure 7, swelling ratios (738.47 ± 18.27) of 3% chitosan scaffolds were significantly lower than that (883.89 ± 20.92) of 2% chitosan scaffolds ($P < 0.05$, analyzed by student t-test, $n = 9$). For further investigation on the dimension changes of chitosan scaffolds, the specimens were immersed in the SBF buffer for 21 days. The longitudinal (Fig 8A) and cross (Fig 8B) sectional sizes of the scaffolds only significantly changed in the first 5 minutes, and the scaffolds maintained their dimension after that.

3.3.3 *In vitro* degradation of 2% and 3% chitosan scaffolds

Comparing to the weight loss of 2% and 3% scaffold on day 7, the weight loss of those scaffolds significantly increased on day 14. Similarly, the weight loss of 2% and 3% scaffolds on day 21 were significantly more than that on day 14 ($P < 0.05$,

analyzed by one-way ANOVA, multiple comparisons, n = 9). However, there were not significantly different between 2% and 3% chitosan scaffolds at each timing point ($P > 0.05$, analyzed by student t-test, n = 9) (Fig. 9)

3.4 Cytocompatibility

3.4.1 Cytotoxicity test

After 24, 48 and 72h, the reaction of cells to the extraction media was evaluated microscopically, and there are not obvious floating cells and changes in cellular morphology were observed in both treatment and control groups. Figure 10 demonstrate the cell viability after cells exposed to the extraction media 2% and 3% for 72 h (one-way ANOVA, multiple comparisons, n=6). DPSC viabilities between the extraction media of 2% (0.680 ± 0.055) and 3% (0.627 ± 0.043) groups were not significant different, but they were significantly higher than cell viability of the normal media control group (0.430 ± 0.044) ($P<0.01$). For SHED and MG-63, cell viabilities of the 2% (SHED: 0.454 ± 0.022, MG-63: 1.239 ± 0.029) groups were significantly higher than those of the 3% (SHED: 0.417 ± 0.016, MG-63: 1.120 ± 0.040) and the control groups (SHED: 0.366 ± 0.021, MG-63: 1.070 ± 0.050) ($P<0.01$)). The viabilities of gingival fibroblast among control (0.623 ± 0.020), 2% (0.644 ± 0.019) and 3% (0.667 ± 0.013) groups were not significantly different.

3.4.2 Morphology of cells seeded in scaffolds

Cells could attach to the bottom (Fig. 11C) and wall (Fig. 11D) of each pore. They spared on the wall, and the pseudopods could be seen clearly (Fig. 11A). When the cells were confluent, they could cross the border of the pores (Fig. 11B) and

connect together.

3.4.3 Cell viability on scaffolds

The viability of DPSC, SHED, gingival fibroblasts and MG-63 in the chitosan scaffolds showed in Table 1. For DPSC, the OD of 2% and 3% scaffold groups were significantly higher than those of control groups on day 8, 15 and 21. Particularly on day 21, the OD of the 2% scaffold group was not only higher than that of the control group but also higher than that of the 3% scaffold group (Fig. 12A). SHED showed similar results as DPSC except that between day 15 and day 21, the OD of the 3% group had a decreasing trend (Fig. 12B). For gingival fibroblasts, the OD of the test group was higher than that of the control group since day 8, and the OD of the 2% scaffold group was higher than the 3% group started from day 15. From day 15 to day 21, the OD of control groups were almost the same, whereas the OD of the 3% scaffold group decreased (Fig. 12C). For MG-63, the OD of the test group was higher than that of control group, but there was no significant difference between the 2% and the 3% scaffold groups at all time-points (Fig.12D).

Chapter 4

Discussion and Conclusion

4. Discussion and Conclusion

Langer and Vacanti reported that the most common approach for engineering biological substitutes is based on living cells, signal molecules, and polymer scaffolds [35]. A functional but challenging approach for dental pulp therapy is the using of tissue engineering techniques [36]. The presence of the unique populations, DPSC and SHED, has been reported [1, 2, 37]. These populations are capable of extensive proliferation and multipotential differentiation. Moreover, the researchers pointed out that SHED is perhaps more immature than previously examined postnatal stromal stem cell populations [2]. Corresponding to previous studies [2, 38], the data in this study showed that the proliferation of SHED are significantly higher than that of DPSC [Figure 1].

Both of DPSC and SHED have the ability to differentiate into odontoblasts [1, 2], and those cells can form mineralized nodules under the calcified condition. Those specific crystalline structures are similar to physiological dentin but different from bone structures [39]. Previous studies demonstrated that the nodule formation can be detected by Alizarin Red S staining from 2 to 8 weeks in differentiated dental pulp cell cultures [1, 39-41]. One study shows mineralization of the rat dental pulp cell begins on day 11 [42]. In order to determine the differentiation ability of DPSC and SHED, the cultural condition of mineralized nodule formation was established by Tsukamato *et al* [43] and the mineralized nodule formation was observed using Alizarin Red S staining. The osteosarcoma cell line, osteoblast-like cell MG-63 [44] which can form the

mineralized nodules under osteogenic condition, was set as positive control. The gingival fibroblasts were set as negative control. The results of this study show that the mineralized nodules appeared from day 14 in test groups of SHED and MG-63 (Fig. 3). However the mineralized nodule formation of DPSC can be found at 21 days (Fig. 4). On day 21 and day 28, the mineralized nodules of SHED groups were more than in DPSC groups (Fig. 4, 5). There are many factors effect on the time of mineralized nodule appearance, such as isolation method, seeding density and donor age [36]. Moreover, culture conditions, the inorganic phosphate source such as the use of KH_2PO_4 enhance mineralization over the use of organic Na-β-glycerophosphate [5]. Under the same isolation method, seeding density and culture condition, “SHED is perhaps more immature than DPSC” [2] might be a reason for these results. Therefore, the results of the current study suggest that the mineralized nodule formation of SHED may be earlier than that of DPSC. In contrast, although the mineralized nodules formation of DPSC appeared late and were not as many as that of SHED, they increased very fast after their appearance. These possibly suggest that SHED maybe possess stronger mineralization ability than DPSC do.

The procedure of chitosan scaffold preparation is easy to handle and the materials are economic. The microstructure such as pore size, shape and distribution, has prominent influence on cell intrusion, proliferation and function in tissue engineering. A previous research shows that the pore diameters of the chitosan scaffolds are might not equal from the edge to the center due to the temperature gradient [21]. For the purpose of making equal pore size distribution in the same cross-section and different

pore size distribution in longitudinal-section, the centrifugal method [24] was applied before the step of freeze-drying. Park *et al* fabricated the chitosan scaffolds by adjusting the centrifugal speed to make the scaffolds with gradually increasing pore size along one direction [24]. Kose *et al* suggest that the average pore size should be at least three times (>100 μm) larger than the size of cells so that a single cell could establish contact with others [45]. Fibroblasts have been demonstrated that they bound to a wide range pore size from 95 to 150 μm and cells would increase its viability with decreasing pore size until no cells could fit into the pores [46]. Another study suggested that porous scaffold microstructure with minimal pore size ranging from 100 to 150 μm was usually required to allow tissue ingrowth [47]. The mean size range of osteoblasts is 10-30 μm [48], and the sizes of those cells in our study were measured using cell counter (Countess, Invitrogen). The viable cell sizes of DPSC, SHED, gingival fibroblast and MG-63 are at the range of 10-40 μm. Therefore, it is reasonable to choose the scaffolds with the mean size bigger than 100 μm. In the pilot study, we measured the pore size in different position of the 15 ml tubes after centrifugal step (Fig. 13). Respectively, the specimens of 2 to 2.5 cm and 3 to 3.5 cm from 2% (Fig. 13A) and 3% (Fig. 13B) chitosan scaffolds were chosen (Fig. 13), and their pore size were respective 188.71 ± 51.90 μm and 195.30 ± 67.21 μm. Next, we examined the swelling ratio and strength of those chitosan scaffolds. The results in Figure 7 show that the chitosan scaffolds possess a high swelling ratio. It could preserve a high volume of water within the porous structure and could further enhance the penetration of cells into the inner area of the scaffolds [25, 49, 50]. Our results agree with the previous study [21] that swelling

ratios of 3% chitosan scaffolds were significantly lower than that of 2% chitosan scaffolds since the pore interconnectivities were relatively lower. Moreover, all of the chitosan scaffolds swelled and could maintain their structure without changing after their swelling [Fig. 8]. These results are possibly due to the higher swelling ratio could maintain three-dimensional structure of the scaffolds [49, 50]. An optimal degradation time of scaffolds is when the cells begin to differentiate. Generally, the cells begin to differentiate when they get confluence. Fast degradation may cause the cells lost the support. Ideally, the rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules [12]. Chitosan is mainly degraded by lysozyme [27] which commonly exists in various human body fluids and tissues [45]. DD, distribution of acety1 groups and Mw (molecular weight) are responsible for the degradation rate of the chitosan [12]. In our study, the chitosan scaffolds significantly degraded in lysozyme solution during 7 to 14 days (Fig. 9). According to our cell proliferation study *in vitro*, both of DPSC and SHED grew fast and reach confluence at about day 7. These results suggest that chitosan scaffolds could support the proliferation of those cells.

We tested cytotoxicity of the chitosan scaffold specimens before cell seeding. Toxicity of chitosan is reported to depend on DD. The chitosan with DD higher than 35% showed low toxicity, while a DD under 35% caused dose dependent toxicity [51]. The DD of the chitosan in our study is 85%, which is assumed to be non-toxic. Figure 10 show that there are not significantly different between test groups and control groups. During or after 3 days cultured, cells in all of the test groups did not show the changes

of cellular morphology and the floating cells. These results demonstrated that the chitosan scaffolds in this study were non-toxic. Moreover, cells of the test groups proliferated more than those of the control groups. During the MEM extraction procedure, some chitosan oligomers maybe released. Those oligomers have been demonstrated to possess biological activities [52]. Extraction media of the test groups which may contain some chitosan oligomers. Those oligomers could stimulate the growth of cells. Similarly, those oligomers affect proliferation of SHED and DPSC positively. Therefore, our studies indicate that those specimens are not only non-toxic but also promote growth of DPSC and SHED. In addition, those cell viabilities toward extraction media 2% groups and 3% groups of SHED and MG-63 are significant different whereas, that of DPSC and gingival fibroblasts are not different. Some *in vivo* study suggested that chitosan may accelerate cell proliferation indirectly [53], possibly through forming polyelectrolyte complexes with serum components such as heparin [53], or potentiating growth factors such as platelet derived growth factor (PDGF) [54]. It is possible that during the extraction media preparation, chitosan bind some factors or nutrients from the media or serum. Therefore, the extraction media 2% groups contain those factors or nutrients more than extraction media 3% groups do. Those factors or nutrients might be suitable to promote the proliferation of SHED and MG-63, but not effect on DPSC and gingival fibroblasts. At the same time, In contrast, SHED viabilities toward extraction media 2% and 3% groups were stimulated by chitosan oligomers but not as high as those of DPSC because of the loss of some favorable factors or nutrients. These results suggest that the culture condition of DPSC and SHED are

possibly somewhat different. When attach on the surface of conventional plastic dishes, DPSC show a typical fibroblastic, spindle-shape to polygonal morphology [39， 40] and SHED present a spherical shape [2].

The cytocompatibility of chitosan has been proved *in vitro* with myocardial, endothelial and epithellial cells, fibroblast, hepatocytes, condrocytes, keratinocytes [55] and periodontal ligament cells [56]. Chitosan contains a large amount of amino groups, which give it high positive charges. It has been demonstrated that all vertebrate cells possess unevenly distributed negative surface charges [57]. Cells could bind tightly within chitosan via electrostatic interaction. Figure 11 exhibits that the cells remained their normal morphology on the scaffolds. Cells spread on the wall of the scaffolds, and cross the pores and reach confluence in the scaffold. These results indicate that the component of the chitosan scaffolds support the cell attachment and the materials were biocompatible [58]. Those scaffolds might be suitable for DPSC and SHED to adhesion and proliferate. The general recommendations for usage of chitosan in tissue engineering are summarized by Inmaculada *et al*: 1) A DD around 85% is good for the cell proliferation and the scaffold structure maintenance; 2) A High Mw assists in prolonging biodegradation of scaffolds [12]. We chose the commercial chitosan product of 85% as mentioned above, a DD around 85% is good for the cell proliferation. Our pilot study showed that during the first week of cell growth, the cell proliferation into the scaffolds was comparable to the control. The results showed that all of the cell types in our study had high viability in chitosan scaffolds. Moreover, the results of our study demonstrated that the proliferation of the cells within scaffold groups were significantly

higher and faster than those of the cells on cultural dish surface groups (Fig. 12). Peng and Zhou suggested when the cells were implanted onto scaffolds, cells needed adhere on surface of scaffold and began to adapt three-dimensional growth environment. Once cells completely adapted with scaffolds, they grew faster than before. Since there was much culture space in three dimensional scaffolds, cells could continue to grow without contacting inhibition [56]. The 2% chitosan scaffolds in our study possess higher swelling ratio than 3% chitosan scaffolds, in other words, 2% chitosan scaffolds could preserve a higher volume of media within the porous structure and could further enhance the penetration of cells into the inner area of the scaffolds than 3% scaffolds do. This may be a possible reason that cell density in 2% scaffolds is significantly higher than that of 3% scaffolds. Notice from Figure 12 and Table 1, MG-63 cell density of 2% scaffolds and 3% scaffolds are not different. Moreover, the 2% chitosan with a higher density of gingival fibroblasts could be found from day 14. At the same time, the densities of SHED and DPSC in 2% scaffolds higher than those of 3% scaffolds could be found on day 21. These results suggest that the concentration of chitosan might affect the cell growth. However, the responses of this affection are different in each cell type. Chatelet *et al* studied the relationship between the cell type and adhesion by comparing between keratinocyte and fibroblasts. They concluded that the type of cell was a factor that also affected the adhesion, being more favorable for fibroblasts which exhibit a more negative charge surface [55]. It indicated that, besides DD, the cell types probably related to cytocompatibility. Accordingly, we demonstrsted that the effects of 2% and 3% chitosan on the proliferation among dental pulp stem cells, gingival

fibroblasts and MG-63 are distinct, and 2% chitosan scaffolds are more suitable for cell growth.

In conclusion, the present study demonstrated that SHED possessed higher proliferation and earlier mineralization ability than DPSC. For characteristics, those scaffolds represent suitable swelling and degradation abilities in mimic body fluid and lysozyme solution respectively, and chitosan oligomers possibly could simulate the DPSC and SHED growth. Moreover, this study suggested that culture condition of DPSC and SHED are possibly somewhat different. However, we do not exactly know how different of dental derived stem cell capacity at different donor ages or at the same age but different dentitions; how distinct between the culture condition of DPSC and SHED; whether chitosan scaffold could bring its antimicrobial ability into dental pulp therapy. Those questions might be expanded by further studies.

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Table legend:

Table 1 Cell viability on scaffolds detect by WST-1 assay:

Means ± *SD* of optical density in different cell and chitosan scaffold groups analyzed by One-way ANOVA, and the statistical significance was accepted at the 0.05 confidence level. (* $P<0.05$ between control and test groups, $^{\#}$ $P<0.05$ within 2% and 3% scaffold groups)

Figure legends:

Figure 1: Cell proliferation detected by MTT assay:

OD (*A*=572 nm) was expressed as a measure of cell proliferation on 1, 7 and 14 days, *n*=6. Errors bars represent means ± *SD*. Data was analyzed by One-way ANOVA, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.01)

Figure 2: Mineralized nodule formation detected by Alizarin Red S staining on day 7.

A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

Figure 3: Mineralized nodule formation detected by Alizarin Red S staining on day 14.

A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

Figure 4: Mineralized nodule formation detected by Alizarin Red S staining on day 21.

A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

Figure 5: Mineralized nodule formation detected by Alizarin Red S staining on day 28.

A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

Figure 6: SEM micrographs showed the microstructures of chitosan scaffolds:

A. Cross section of 2% chitosan scaffolds; B. Longitudinal section of 2% chitosan scaffolds; C. Cross section of 3% chitosan scaffolds; D. Longitudinal section of 3% chitosan scaffolds.

Figure 7: Swelling ratios (738.47 ± 18.27) of 3% chitosan scaffolds were significantly lower than that (883.89 ± 20.92) of 2% chitosan scaffolds ($P < 0.05$, analyzed by student t-test, n = 9).

Figure 8: Dimensional changes of 2% and 3% chitosan scaffolds in longitudinal-section (A) and in cross-section (B).

Figure 9: *In vitro* degradation of chitosan scaffolds, Errors bars represent means ± *SD.* Comparisons among time points: P > 0.05, Data wasanalyzed by student t-test, n = 9; Comparisons between 2% and 3% chitosan scaffolds P < 0.05, analyzed by one-way ANOVA, multiple comparisons, n=9.

Figure 10: Cytotoxicity test by MTT assay:

OD (*A*=572 nm) was expressed as a measure of cell viability after exposure to the extraction media 2% and 3% after 72h, and the cells cultured with normal media were set as control, n = 6. Errors bars represent means ± *SD.* Data was analyzed by One-way ANOVA, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.01)

Figure 11: Scanning electron micrographs of cells cultured in chitosan scaffolds for 16 days.

A. Gingival fibroblasts on the scaffolds; B. Gingival fibroblasts across the border of the pores; C. SHED on the bottom of the pores; D. SHED on the wall of the pores.

Figure 12: OD (*A* = 450 nm) was expressed as a measure of cell (A. DPSC; B. SHED; C. Gingival fibroblasts; D. MG-63 cells) viability in scaffolds on Day 8, 15and 21, *n*=6. Errors bars represent means ± *SD.* Means ± *SD* of optical density in different cell and chitosan scaffold groups analyzed by One-way ANOVA, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.05 between control and test groups, # P<0.05 within 2% and 3% scaffold groups)

Figure 13: Pore size of chitosan scaffolds in different position of 15 ml tube

a. 2% chitosan scaffolds; b. 3% chitosan scaffolds.

Table 1

DPSC			
	Control	2% chitosan	3% chitosan
Day 8	0.777±0.063	1.142±0.112*	1.105±0.095*
Day 15	1.414±0.275	2.286±0.049*	2.062±0.101*
Day 21	1.534±0.018	2.854±0.103*#	2.343±0.212*#
SHED			
Day 8	0.787±0.098	1.365±0.067*	1.603±0.018*
Day 15	1.214±0.129	2.470±0.099*	2.150±0.076*
Day 21	1.453±0.012	2.905±0.066*#	2.121±0.178*#
Gingival fibroblasts			
Day 8	0.957±0.139	1.680±0.138*	1.517±0.157*
Day 15	1.954±0.019	3.140±0.039*#	2.595±0.077*#
Day 21	1.998±0.010	3.681±0.152*#	2.523±0.125*#
MG-63			
Day 8	1.141±0.110	1.488±0.095*	1.650±0.054*
Day 15	1.227±0.048	2.406±0.060*	2.281±0.119*
Day 21	1.748±0.010	3.054±0.032*	2.823±0.089*

Figure 1

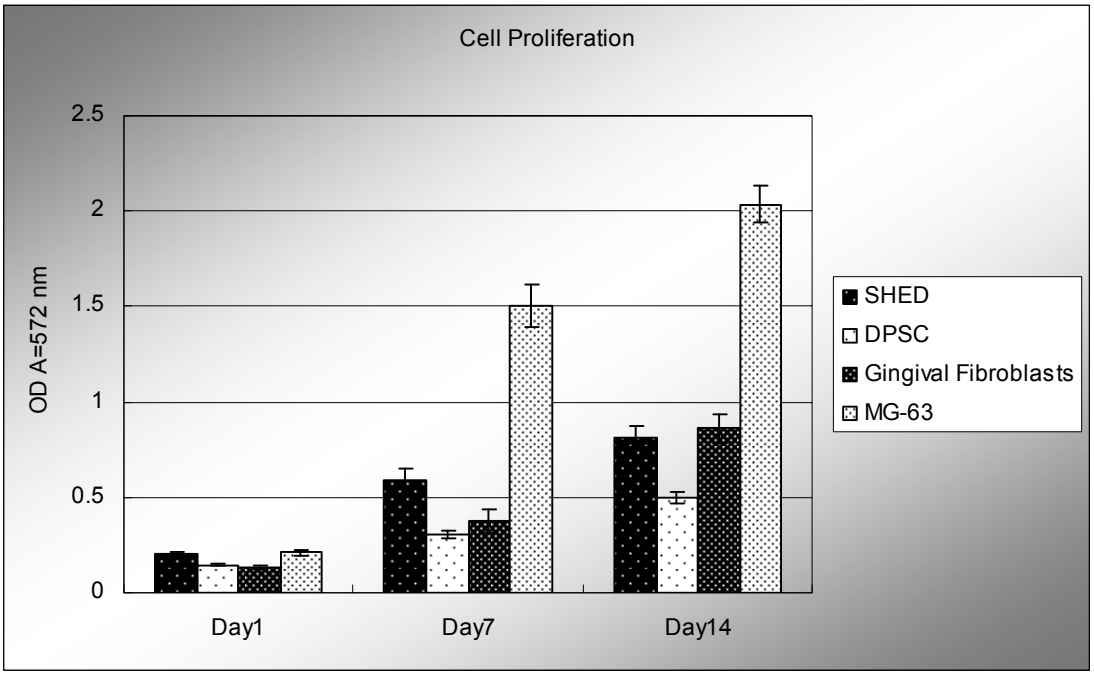

Cell Proliferation
OD A=572 nm
2.5
2
1.5
1
0.5
0
Day1
Day7
Day14
SHED
DPSC
Gingival Fibroblasts
MG-63

Figure 2

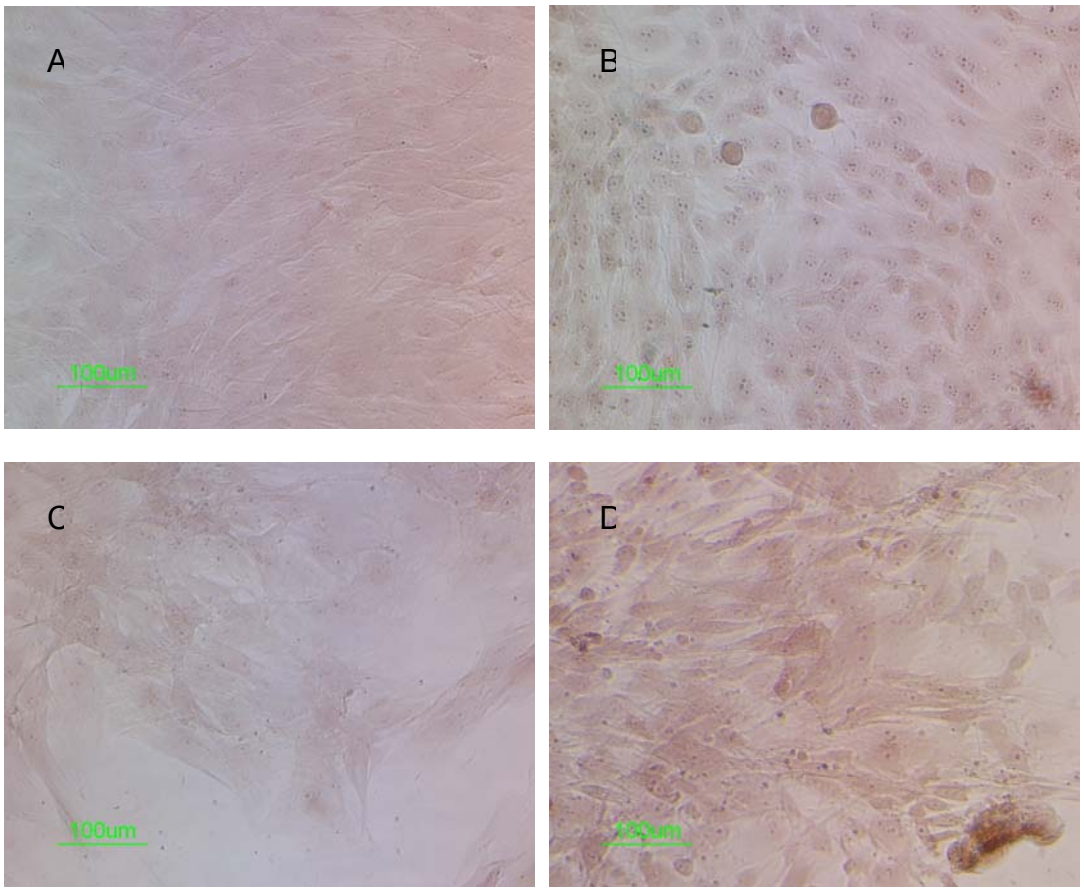


Figure 3

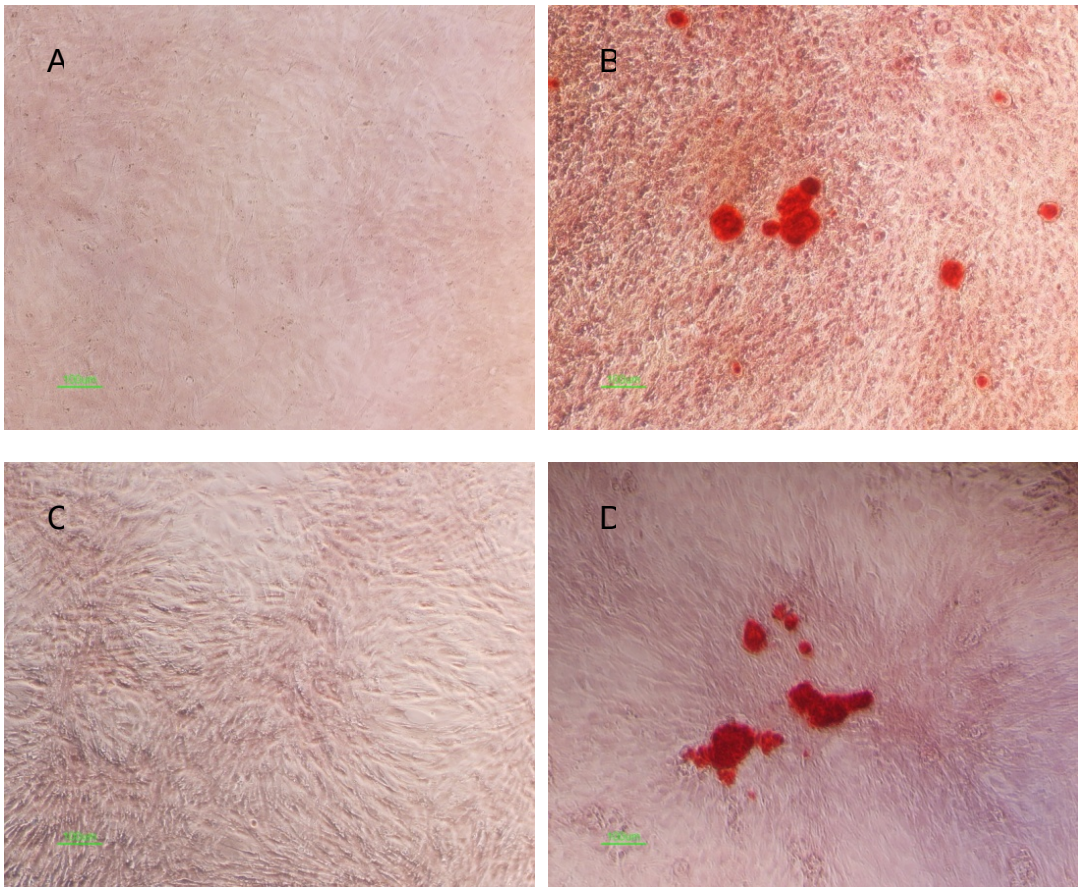


Figure 4

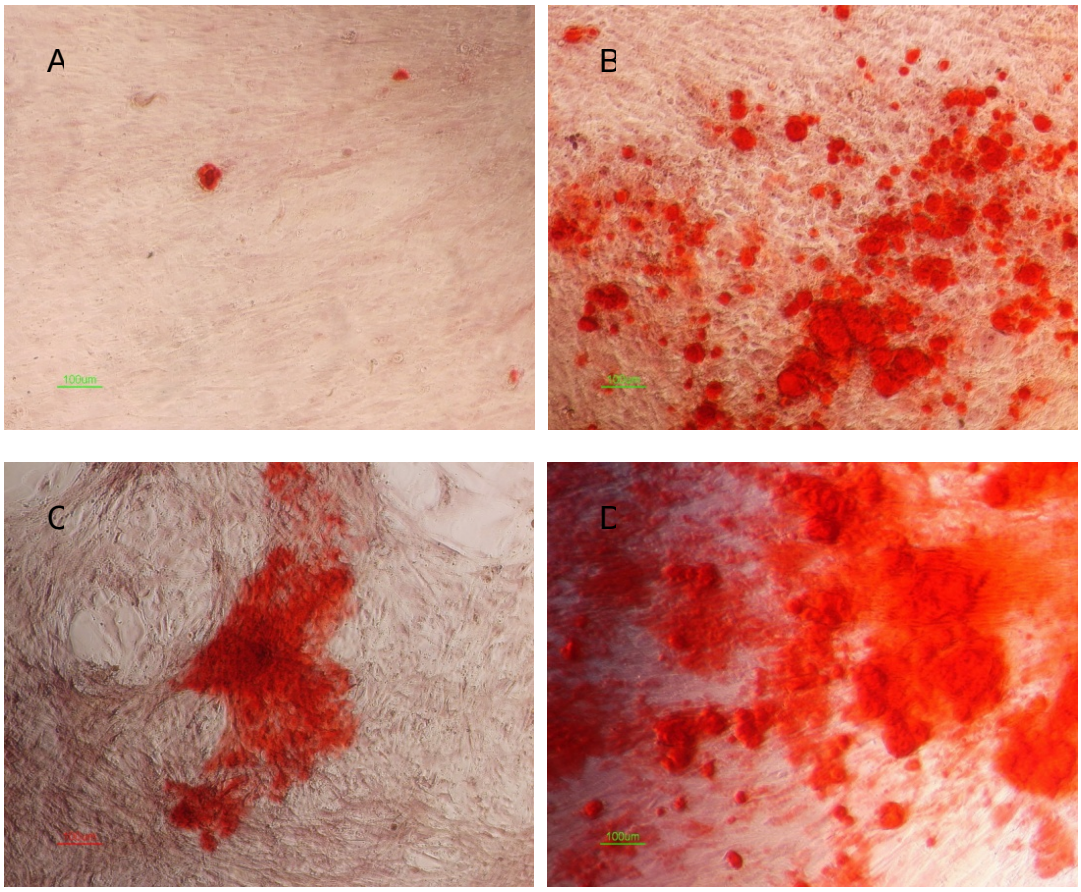


Figure 5

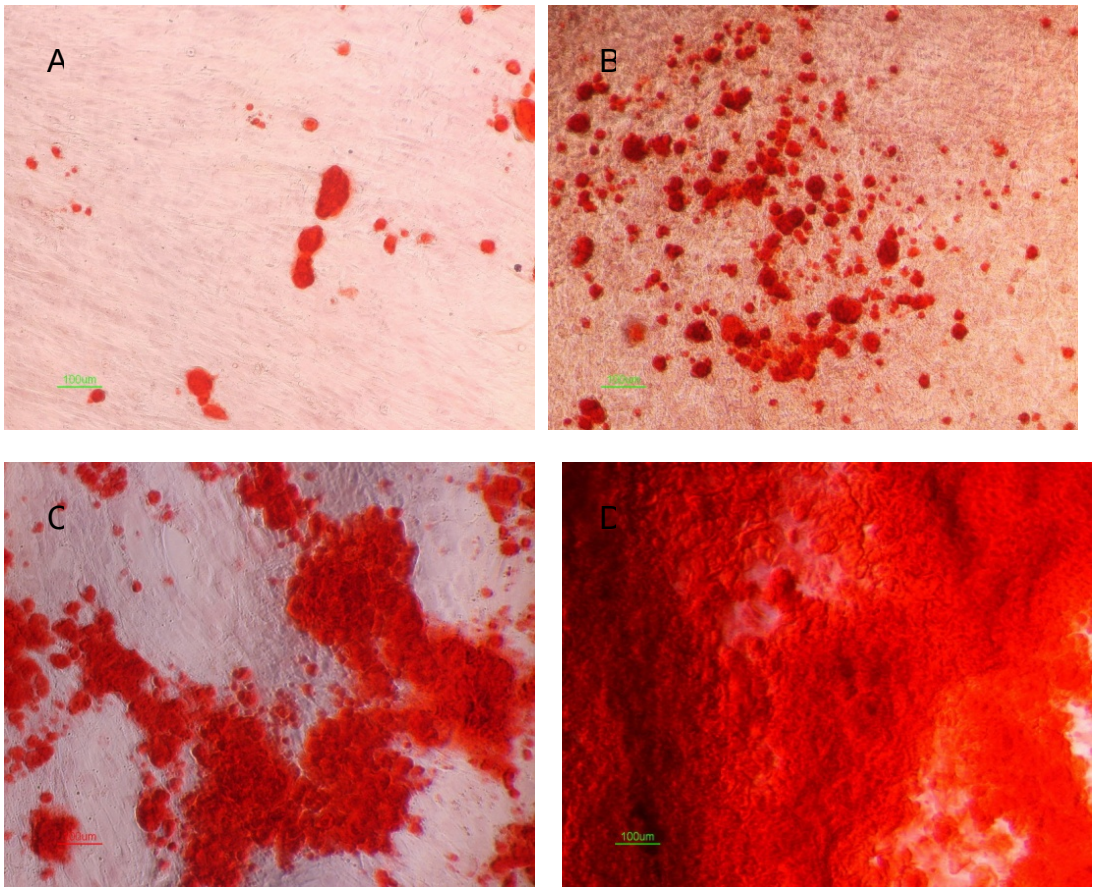


Figure 6

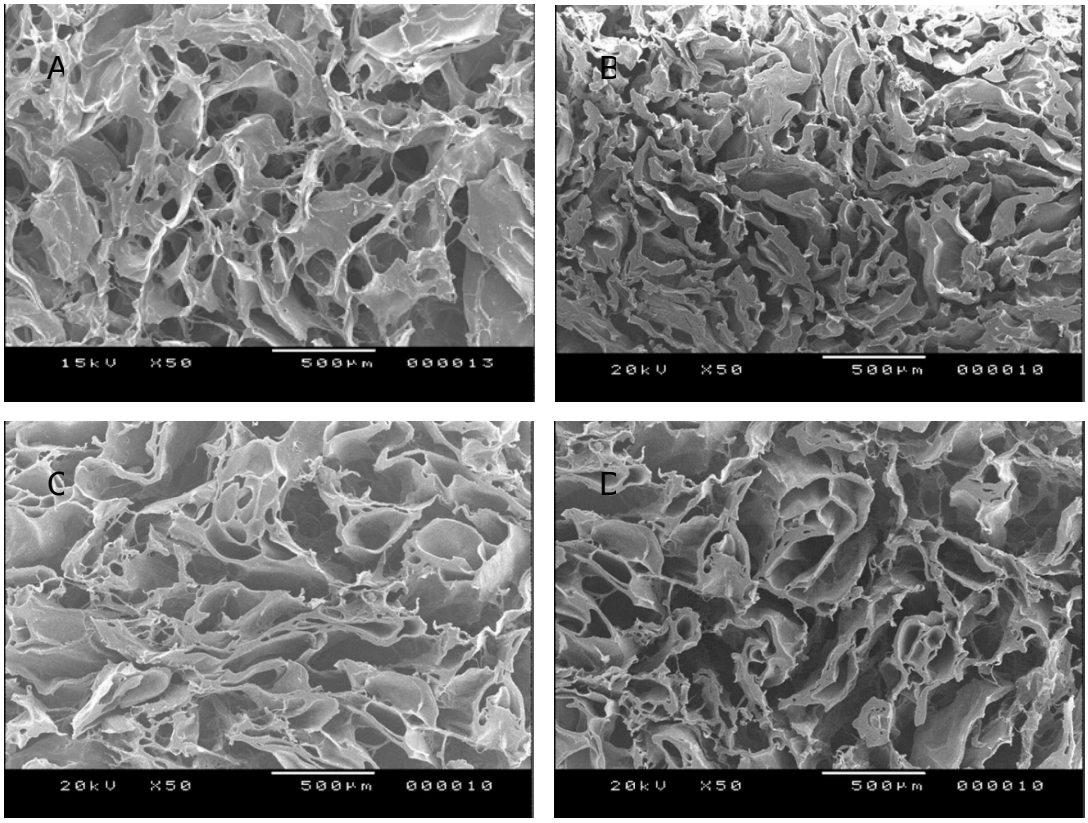

A
15kV X50 500µm 000013
B
20kV X50 500µm 000010
C
20kV X50 500µm 000010
D
20kV X50 500µm 000010

Figure 7

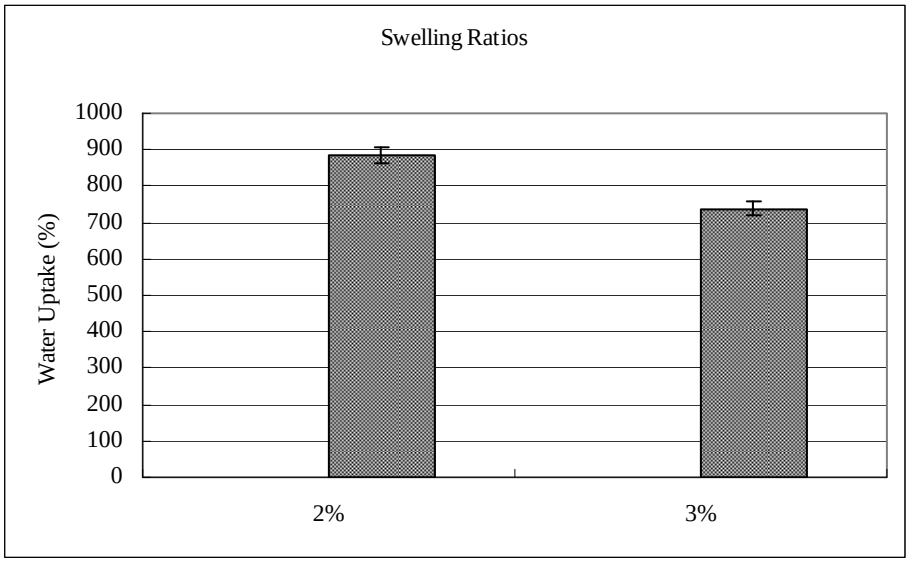

Swelling Ratios
Water Uptake (%)
1000
900
800
700
600
500
400
300
200
100
0
2%
3%

Figure 8

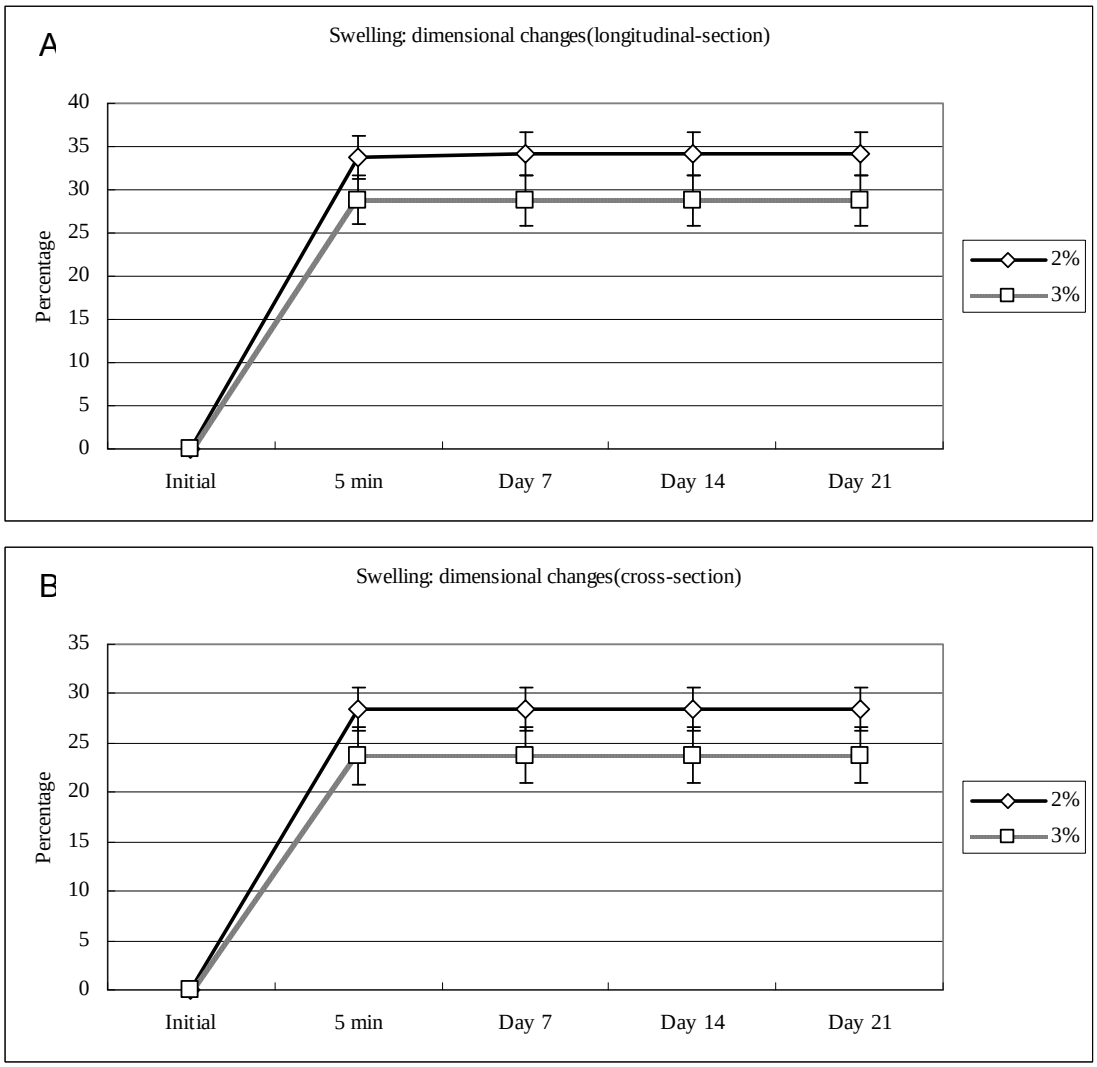

A
Swelling: dimensional changes(longitudinal-section)
Percentage
40
35
30
25
20
15
10
5
0
Initial
5 min
Day 7
Day 14
Day 21
2%
3%
B
Swelling: dimensional changes(cross-section)
Percentage
35
30
25
20
15
10
5
0
Initial
5 min
Day 7
Day 14
Day 21
2%
3%

Figure 9

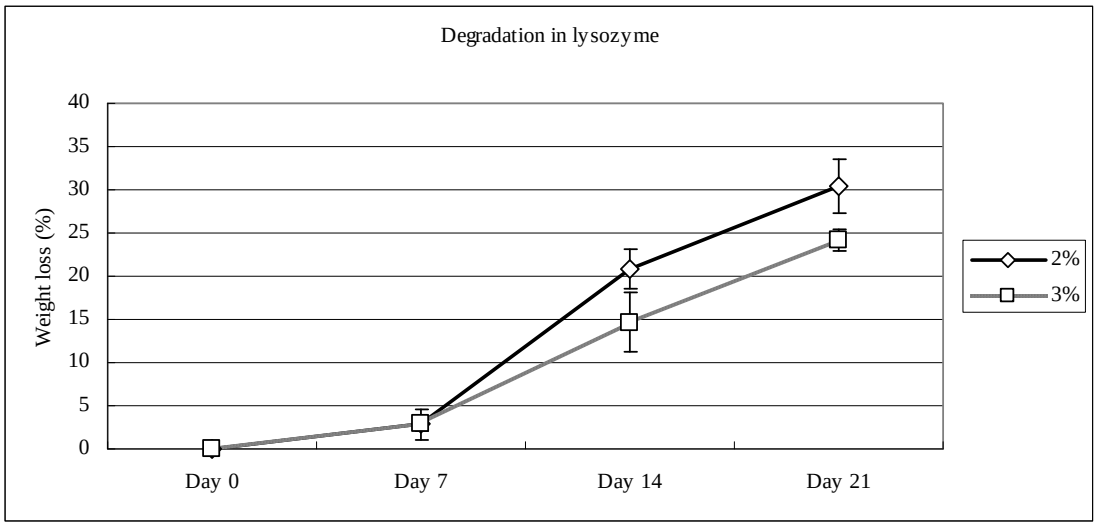

Degradation in lysozyme
Weight loss (%)
40
35
30
25
20
15
10
5
0
Day 0
Day 7
Day 14
Day 21
2%
3%

Figure 10

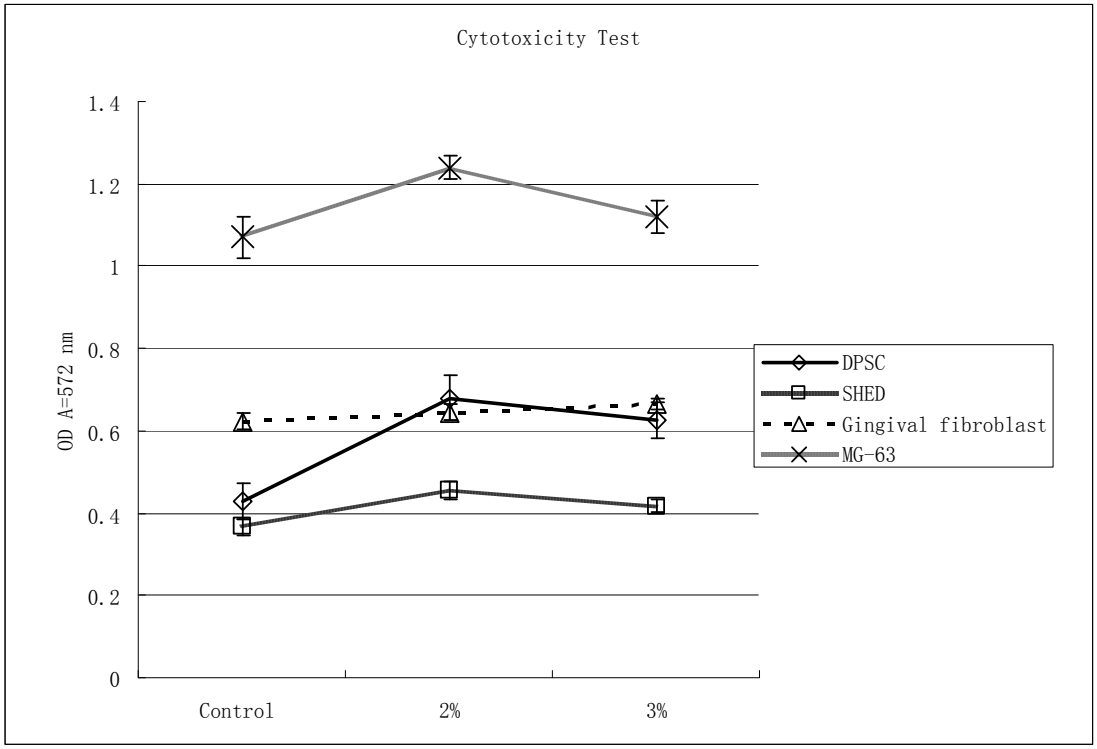

Cytotoxicity Test
OD A=572 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
2%
3%
DPSC
SHED
Gingival fibroblast
MG-63

Figure 11

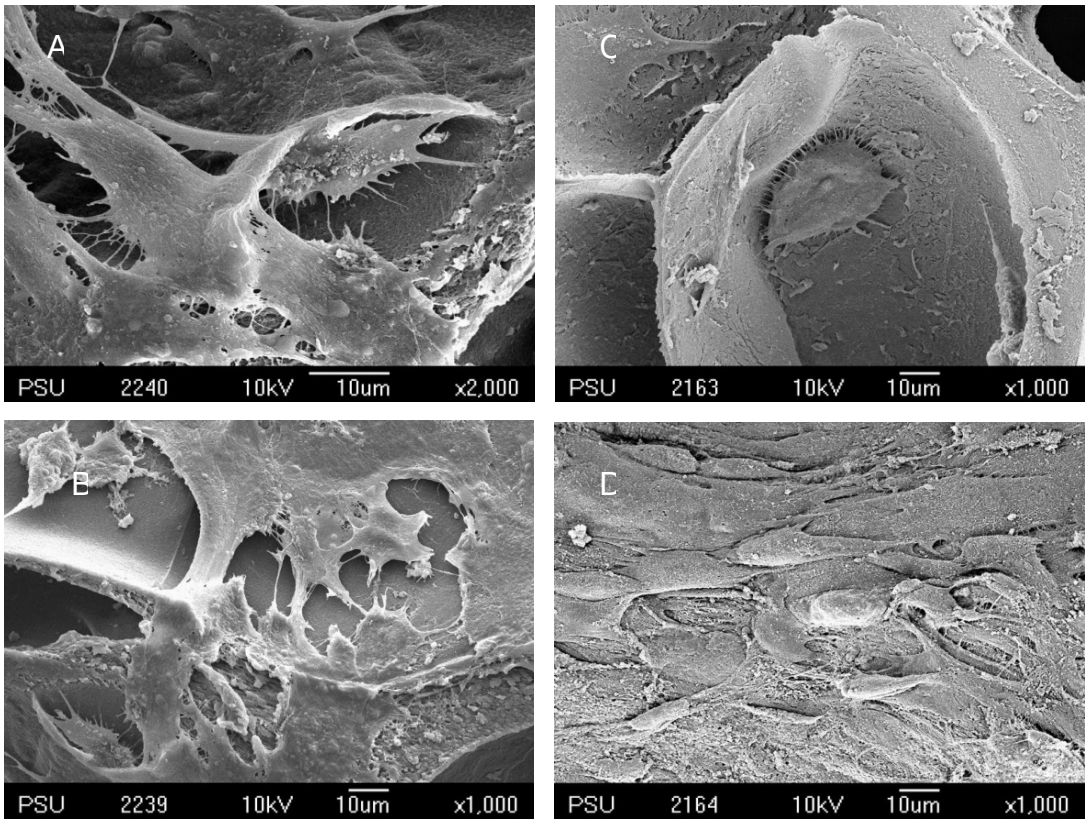
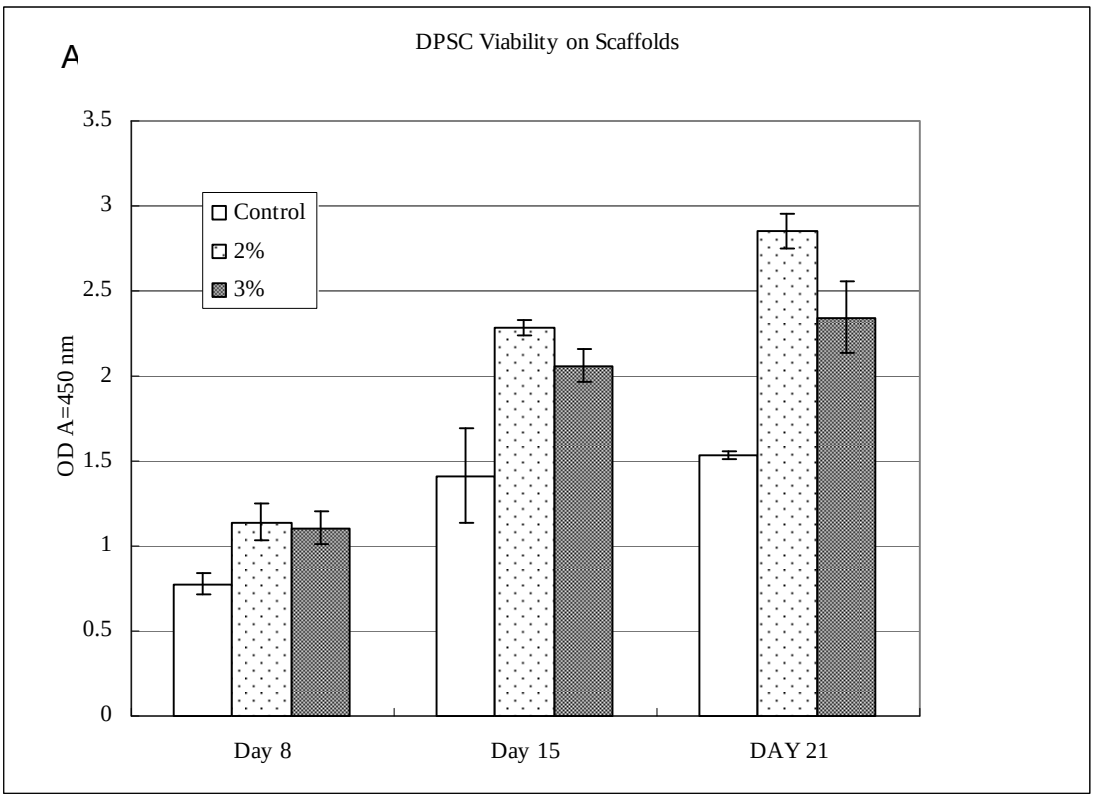
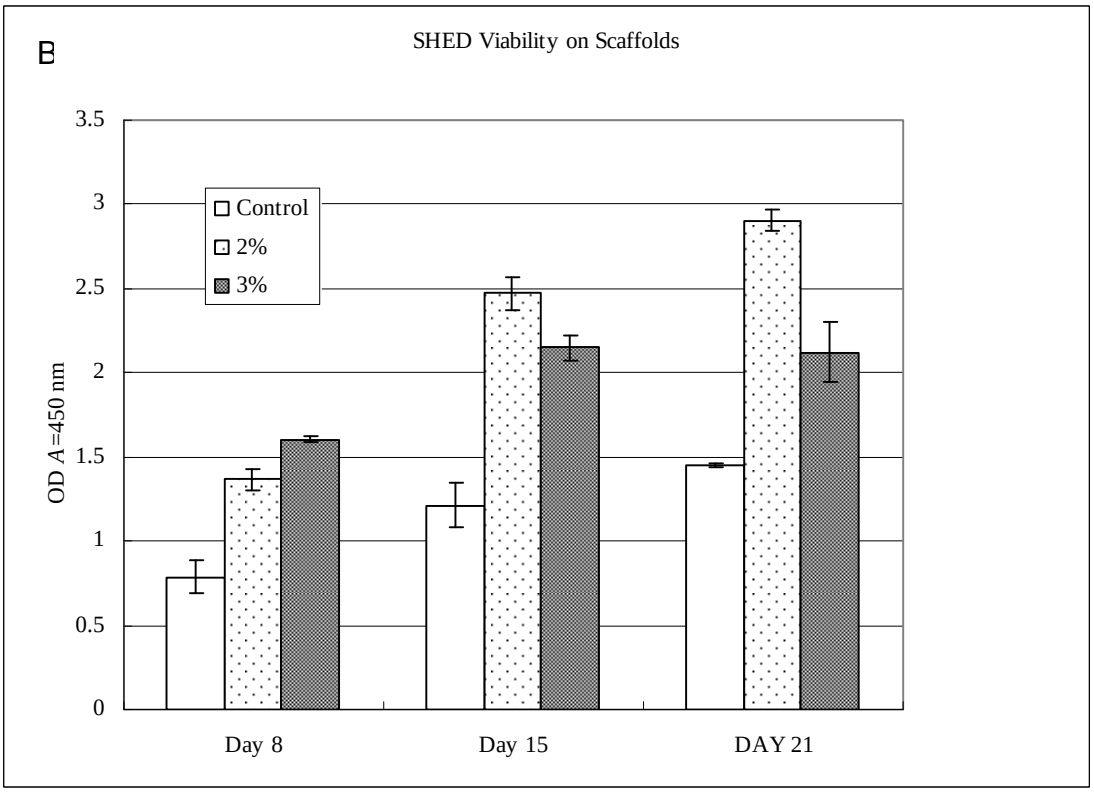
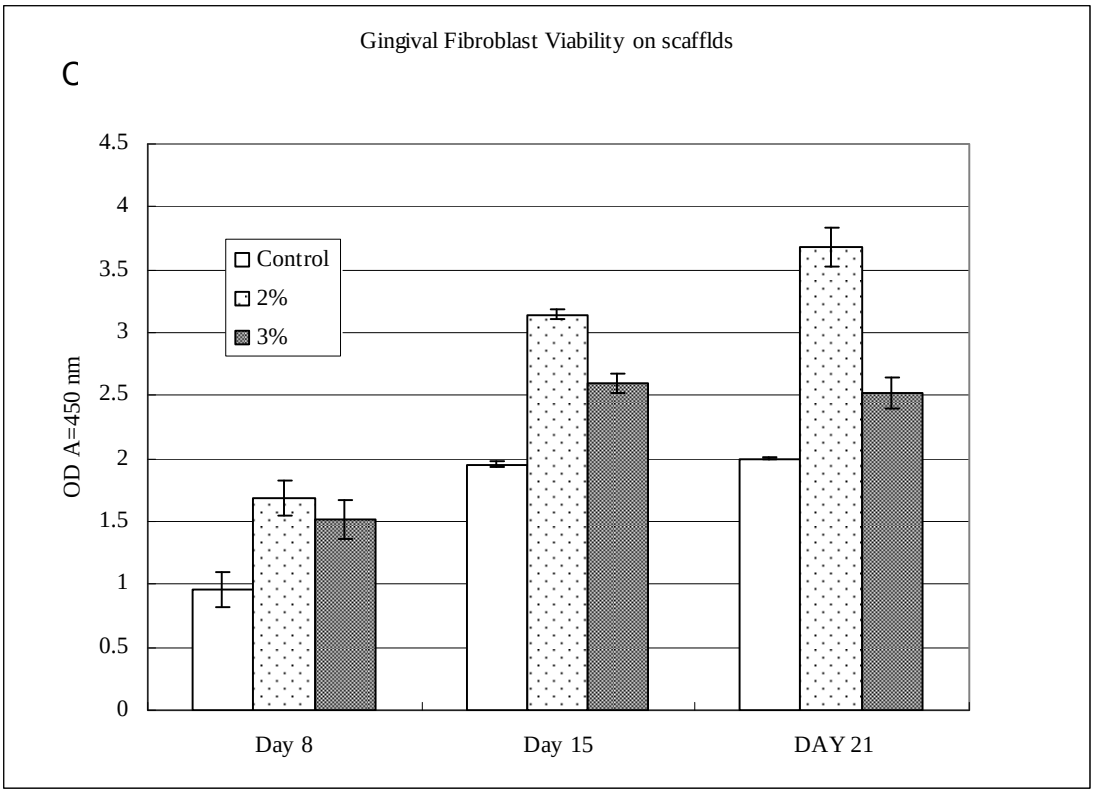

A
PSU 2240 10kV 10um x2,000
C
PSU 2163 10kV 10um x1,000
B
PSU 2239 10kV 10um x1,000
D
PSU 2164 10kV 10um x1,000

Figure 12


A
DPSC Viability on Scaffolds
OD A=450 nm
3.5
3
2.5
2
1.5
1
0.5
0
Control
2%
3%
Day 8
Day 15
DAY 21


B
SHED Viability on Scaffolds
OD A=450 nm
3.5
3
2.5
2
1.5
1
0.5
0
Control
2%
3%
Day 8
Day 15
DAY 21

C


Gingival Fibroblast Viability on scaffolds
OD A=450 nm
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
2%
3%
Day 8
Day 15
DAY 21

D

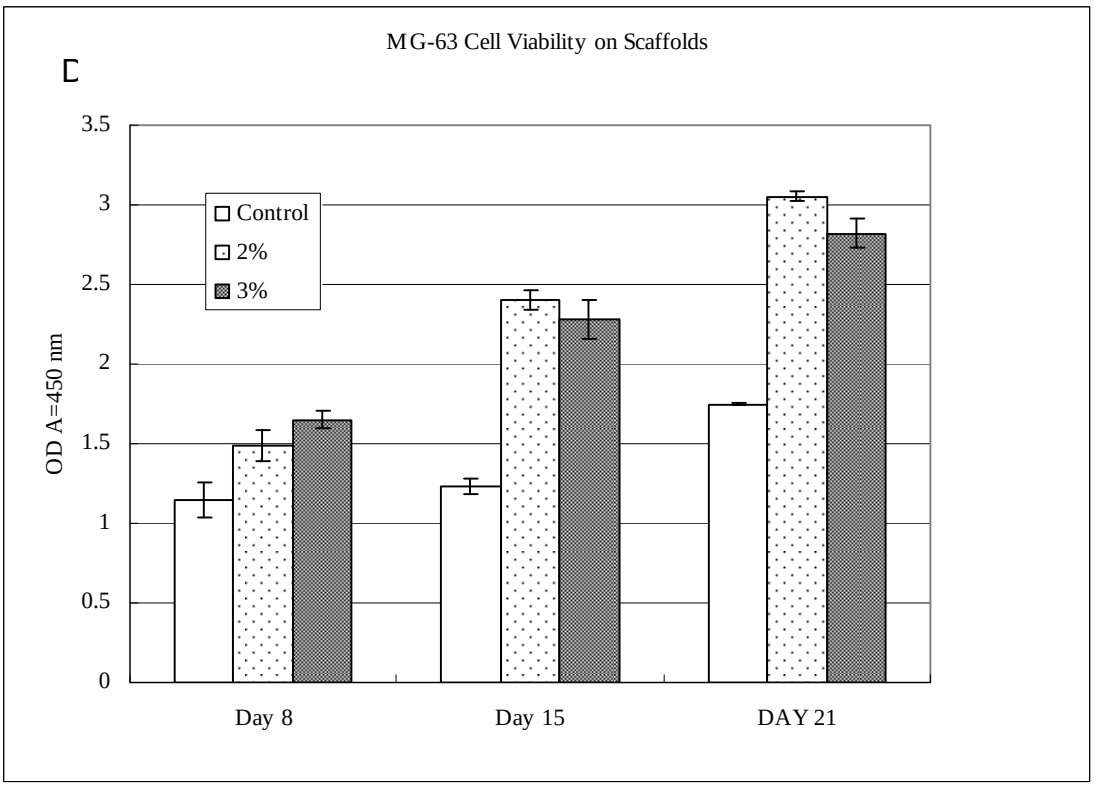
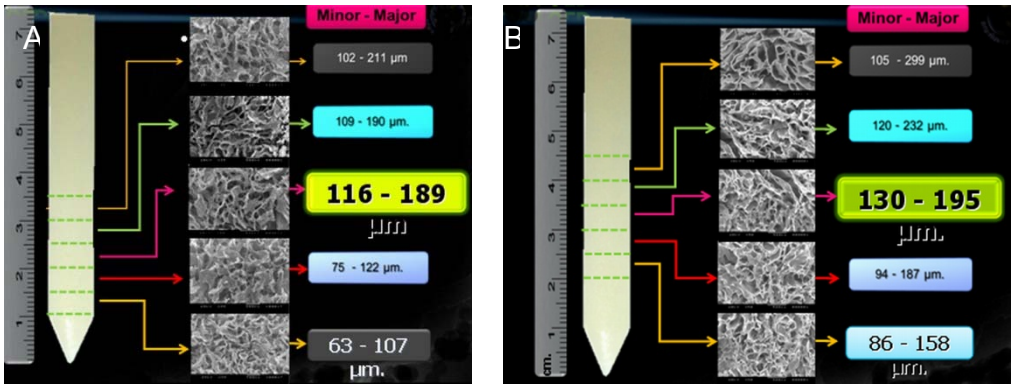

MG-63 Cell Viability on Scaffolds
OD A=450 nm
3.5
3
2.5
2
1.5
1
0.5
0
Control
2%
3%
Day 8
Day 15
DAY 21

Figure 13


A
Minor - Major
102 - 211 µm
109 - 190 µm.
116 - 189
µm.
75 - 122 µm.
63 - 107
µm.
B
Minor - Major
105 - 299 µm.
120 - 232 µm.
130 - 195
µm.
94 - 187 µm.
86 - 158
µm.

Output from this project

1. Pulication: Guan Z, Shi S, Kamolmatyakul S. Proliferation and mineralization ability of dental pulp cells derived from primary and permanent teeth. Songklanakarin J. Sci. Technol. 2011; 33 (2), 129-134.

2. Benefits from this project

2.1 International Collaboration with Malaya

2.1.1 Bi/tri layers cell culture in our novel scaffolds with

Associate Professor Dr. Chai Wen Lin regarding

Department of General Dental Practice and Oral & Maxillofacial (Imaging),

Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia.

2.1.2 Modification of our novel scaffolds using proteins from sea animals with

Dr. Badrul Hisham Yahaya, PhD (Edinburgh) Senior Lecturer Cluster for

Regenerative Medicine Advanced Medical & Dental Institute (AMDI)

Universiti Sains Malaysia, No: 6 Level 1 (Lot 13) Persiaran Seksyen,

4/9 Bandar Putra Bertam, 13200 Kepala Batas, Malasia.

2.2 Implementation of a research unit at Prince of Songkla University: “Stem Cell and Regenerative Dental Medicine” (detail at: http://www.dent.psu.ac.th/stemcell/).

2.3 Achievement of a new M.Sc. student: “Dr. Guan Zheng” who is now working at Biomedical Research Center, The First People’s Hospital of Kunming, Kunming, Yunnan, China 65001.

2.4 Achievement of a new researcher in our research unit: “Dr. Nuttawut Thuasuban” (detail at: http://www.dent.psu.ac.th/stemcell/).

3. Others

3.1 Patent application on June 24, 2010

3.1 Presentation at The International Association of Dental research meetings

3.1.1 Kamolmatyakul S, Guan Z, Shi S, Proliferation and Mineralization of Dental Pulp Cells. J. Dent. Res. 88(special issue): 2146, 2010.

3.1.2 Guan Z, Shi S, Kamolmatyakul S. Chitosan as scaffolds for DPSC and SHED. J. Dent. Res. 88(special issue): 572, 2010.