Comparisons of Proliferation, Mineralization and Cytocompatibility toward Chitosan Scaffolds between SHED and DPSC

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ABSTRACT

Introduction: In order to obtain a functional vital pulp therapy, dental pulp stem cells could play an important role to organize a net work of inducing the connective tissue for new tissue regeneration. As a carrier, scaffolds sould support cell adhesion, proliferation and differentiation. The aims of this study were to compare proliferation and mineralization ability between stem cells of human exfoliated deciduous teeth (SHED) and human dental pulp stem cells (DPSC) as well as to evaluate cellular response and cytocompatibility of these cells toward chitosan scaffold. **Materials and Methods:** Primary and permanent teeth were collected using appropriate criteria. Cells were isolated by enzyme digestion. Single cell suspensions were seeded in the 100mm cultural plates containing D-MEM supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml mycostatin and 100 μg/ml kanamycin. SHED and DPSC were initially identified and collected by assessing their colony-forming efficiency. The cell proliferation was measured by MTT assay on day 1^{st}, day $7^{th,}$ and day14^{th}. For mineralization test, cells were maintained in growth media supplemented with 10mM β-glycerophosphate, 10^{-8} M dexamethasone, 100 μM L-ascorbic acid 2-phosphate. Alizarin Red S staining was used to detect mineralized nodule formation of the cells on day 7^{th}, 14^{th}, 21^{st} and 28^{th}. Chitosan scaffolds were fabricated and sterilized. Indirect cytotoxicity test was performed according to ISO/EN 10993-5 guidline. WST-1 assay was carried out to investigate the cell viability in chitosan scaffolds. The data were analyzed using one-way analysis of variance (ANOVA). Significant differences were set at 95% confidence. **Results:** Proliferation of SHED was significantly higher than that of DPSC in all periods of the experiment. Upon cultured cells in mineralization inducing media, the mineralized nodules appeared as early as day 14^{th} in SHED and MG-63, whereas those of DPSC can be found at day 21^{st}. On day 21^{st} and day 28^{th}, the mineralized nodules of SHED group were more than those in DPSC group. Mineralized

nodule formation in DPSC group appeared later and less than those of SHED. However, mineralized nodules in DPSC group increased very fast after their appearance. For the cytotoxicity test, cell viability among test groups is significantly higher than those of control groups in SHED, DPSC and MG-63 but not in gingival fibroblast. Moreover cell proliferation rates were significantly higher when adhered on chitosan scaffolds compared to those on cultural dishes.

Conclusion: SHED had higher proliferation rate and mineralization when compared to DPSC. These cells were able to attach and growth in three-dimensional chitosan scaffolds. These result suggested that the combination of SHED and DPSC with chitosan scaffolds might be a candidate strategy for vital pulp treatment.

Keywords: SHED, DPSC, Proliferation, Mineralization, Chitosan Scafold.

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μg	=	Microgram
μl	=	Microliter
μM	=	Micromolar
μm	=	Micrometer
°C	=	Degree celcius
%	=	Percent
3-D	=	Three-dimensional
ANOVA	=	One-way analysis of variance
$Ca(OH)_2$	=	Calcium hydroxide
CFU-F	=	Colony-forming efficiency
cm	=	Centimeter
DD	=	Degree of deacetylation
D-MEM	=	Dulbecco's Modified Eagel Media
DMSO	=	Dimethyl sulfoxide
DPSC	=	Human dental pulp stem cell
et al.	=	And (the) other people
FBS	=	Fetal bovine serum
FC	=	Formocresol
g	=	Gram
HMDS	=	Hexamethyldisilazane
kD	=	kilo Dalton
L	=	Liter
M	=	Molar
mg	=	Milligram
ml	=	Milliliter
mm	=	Millimeter
MTA	=	Mineral trioxide aggregate
MTT	=	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide
Mw	=	Molecular weight

NaOH	=	Sodium hydroxide
nm	=	Nanometer
OD	=	Optical density
PBS	=	Phosphate buffer saline
PB	=	Phosphate buffer
PH	=	Pondus hydrogenii
rmp	=	Round per minute
SD	=	Standard deviation
SEM	=	Scanning electron microscope
SHED	=	Stem cells of human exfoliated deciduous teeth
UV	=	Ultraviolet
WST-1	=	Water-soluble tetrazolium

CHAPTER 1

INTRODUCTION

Introduction

The vital pulp therapy was applied to numerous of patients in dental clinics due to operative exposure, traumatic injury and dental caries. Many medicaments are used such as formocresol, ferric sulfate, gluteraldehyde, calcium hydroxide and mineral trioxide aggregate (MTA). Formocresol and calcium hydroxide are respectively considered as standard pulpotomy agents for primary and permanent teeth in many countries[1]. However, the toxicity of formocresol is concern for its usage in dental treatment[2, 3]. Moreover, calcium hydroxide was observed to stimulate internal resorption than dentine formation[4]. Ferric sulfate has been recommended as an alternative treatment to formocresol, even though ferric sulfate has no fixative effect[5]. Feifal *et al* question the rationale for glutaraldehyde as an alternative to formocresol[6]. Furthermore, the applications of MTA for primary and young permanent teeth therapy are still controvertible[7]. In addition, formocresol and glutaraldehyde pulpotomy only eradicate and inhibit the inflammation in the coronal pulp to prevent further inflammation of the pulp tissue. The mechanisms of ferric sulfate, calcium hydroxide and MTA are as the barrier between the vital pulp and the outside environment. All current treatment agents do not promote the true healing and regeneration of dental pulp tissues. In other words, the conventional treatments of vital pulp therapy for primary and young permanent teeth are still far away from perfection.

As a hypothesis, the vital pulp therapy is accomplished by forming a new functional dentin-pulp complex. The recent achievements of tissue engineering research provide the possibility of the hypothesis. Langer and Vacanti reported that the most common approach for engineering biological substitutes is based on living cells, signal molecules, and polymer scaffolds[8]. The tissue engineering techniques can be possibly applied in vital pulp therapy. Those techniques include the application of a scaffold on an open pulp[9, 10]. As a carrier, many kinds of scaffolds can be selected for tissue engineering, and scaffolds could support cell adhesion, proliferation and differentiation. Stem cells play an important role that organizes a net

work of inducing the connective tissue to regenerate new tissue[11]. The findings of human dental pulp stem cell (DPSCs)[12] and stem cells of human exfoliated deciduous teeth (SHED)[13] possible facilitate the dentin-pulp complex formation. However, they have some differences. DPSC are able to regenerate a dentin-pulp-like complex[12], and Miura *et al* indicated that SHED is perhaps more immature[13]. Therefore, before the using of this potential clinical application, the understanding of the cell proliferation, differentiation processes as well as the characteristics of scaffold is indispensable.

Literature review

1.1 Vital pulp therapy

When the dental pulp is exposed by operative exposure, traumatic injury and caries, the objective of vital pulp therapy is to maintain the vitality of the pulp. For primary and permanent teeth, the direct capping is valid for small mechanical or traumatic pulpal exposures, and the pulpotomy is indicated when the infected coronal pulpal tissue can be amputated and the remaining radicular tissue is judged to be vital, or affected but still vital, by clinical and radiographic criteria. Moreover, the apexogenesis is indicated for traumatized or pulpally involved, vital permanent teeth when the root apex is incompletely formed[14]. No matter what kind of medicaments is used, once the apex of the young permanent teeth is closed, a conventional root-canal therapy should be performed[1].

Formocresol is the most popular pulp dressing material for pulpotomy treatment in primary teeth. However the use of formocresol is decreasing due to its toxic effect[15]. The formaldehyde is an ingredient of Buckley's formocresol is classified as carcinogenic to human[3]. In an attempt to further reduce formocresol toxicity, glutaraldehyde was advocated as an alternative because it is a true cross-linking fixative[16], but the glutaraldehyde also has the toxicity including the ability to induce allergic contact dermatitis[17]. Ferric sulfate has been proposed as a substitute for formocresol, and its success rates are comparable to those of formocresol[18, 19]. Nevertheless, it dose not stimulate reparative dentine; it has no fixative effect[5]; it dose not improve pulp response compared to formocresol[20, 21]. Calcium hydroxide is the standard for immature permanent teeth. Under optimal conditions it can stimulate

a dentine bridge, but it is not ordinarily used for that purpose in a pulpotomy procedure on permanent teeth. Instead, it serves to maintain the vitality of radicular tissue until apexogenesis is complete. The negative impact of calcium hydroxide in a classic pulpotomy may result from too little pulp tissue confined with too much chemical[1]. Calcium hydroxide pulpotomy has fallen from favor because of the high incidence of internal resorption[4], leading to inflammation and necrosis of the pulp tissue[22].

MTA was introduced to endodontics by Torabinejad *et al* in 1993[23]. More recently, MTA has been used as a pulpotomy medicament in primary molar and it was found to be a better material[24-27]. Srinvasan *et al* reviewed the MTA usage in pediatric dentistry. Table 1 shows the evidence from human clinical studies for the clinical applications of MTA. The table 1 exhibits that the using of MTA in pulpotomy for primary teeth and in pulp- capping for permanent teeth is with highly successful rate in long-term study. However, its clinical success outcomes are less than 75% in pulpotomy for permanent teeth[7]. Similarly to calcium hydroxide, the mechanism of MTA is the stimulation of dentine bridge formation in order to protect the vital pulp tissue. Those medicaments can not promote a functional dentin-pulp complex formation. The future vital pulp therapies require the techniques and materials with the abilities of antimicrobial, not only the maintenance of the pulpal vitality, but also the formation of the dentin-pulp complex.

Table 1 Evidences from human clinical studies for the clinical applications of MTA

Clinical application	Studies	Evidence
Pulp capping - permanent teeth	Prospective studies comparing MTA to calcium hydroxide $Ca(OH)_2$	Studies in teeth capped after mechanical pulp exposures (Validation by histopathology)
	-Aeinehchi *et al*. 2003	Thicker dentinal bridge, less pulpal inflammation with MTA
	-Iwamoto *et al*. 2006	No significant difference between MTA and $Ca(OH)_2$
	-Nair *et al*. 2008	Thicker dentinal bridge, less inflammation with MTA

Clinical application	Studies	Evidence
	-Accorinte Mde L *et al*. 2008	Initial healing is better with MTA; subsequent healing similar in MTA and Ca $(OH)_2$
	MTA-observational study	Study in teeth capped after carious pulpal exposures: Clinical success outcomes
	-Bogen *et al.* 2008	97.6% of the sample showed favourable outcomes; all immature teeth showed subsequent complete root formation
Pulpotomy - permanent teeth	MTA-observational study	Studies in teeth with pulpal exposures due to caries/fractures Clinical success outcomes
	-Barrieshi-Nusair and Qudeimat. 2006	< 75% of the sample in both studies showed favourable outcomes
	-Witherspoon *et al.* 2006	
	Prospective studies comparing MTA to formocresol	
	-El-Meligy and Avery 2006	Similar clinical and radiographic outcomes
Pulpotomy - primary teeth	Prospective studies comparing MTA to formocresol (FC)	Studies in teeth with carious pulpal exposures Clinical success outcomes
	-Eidelman *et al.* 2001	Grey: > 90% over 6-30 months
	-Agamy *et al.* 2004	Grey: 100% over 1 year. White: 84.2% over 1 year
	-Holan *et al.* 2005	Grey: 97% over 4-72 months
	-Farsi *et al.* 2005	Grey: 100% over 2 years
	-Aeinehchi *et al*. 2007	Grey: 100% over 6 months
	-Noorollahian. 2008	White: 100% over 2 years
	Prospective longitudinal studies	

Clinical application	Studies	Evidence
	-Maroto *et al.* 2005	Grey: 100% over 6 months
	-Maroto *et al.* 2007	White: 100% over 42 months
	Prospective study comparing MTA to $Ca(OH)_2$	
	-Percinoto *et al.* 2006	Grey: 95 % over 1 year
	Prospective study comparing MTA to FC and $Ca(OH)_2$	
	-Moretti *et al.* 2008	Grey: 100% over 2 years
Pulp Capping - Primary teeth	Prospective study comparing MTA to $Ca(OH)_2$	Study in teeth with pulpal exposures due to caries
	-Tuna and Olmez. 2007	Clinical and radiographic outcomes were comparable for MTA and $Ca(OH)_2$ Histological validation recommended.

1.2 Stem cell

Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation[28, 29]. Pluripotent stem cells are isolated from the embryonic inner cell mass. In the adult organism, most tissues generally maintain a small subpopulation of cells in a stem-cell pool with self-replication and generate more committed progenitors through differentiation along multiple lineages[30]. Such cells usually remain quiescent within the adult tissue; however, they may respond to tissue injury and play an integral role in the tissue repair processes[22]. The stem cells are also commonly subdivided according to their plasticity (Table 2)[31].

Table 2 Types of stem cells

Stem cell type	Cell plasticity	Source of stem cell
Totipotent	Each cell can develop into a new individual	Cells from early (1–3 days) embryos

Stem cell type	Cell plasticity	Source of stem cell
Pluripotent	Cells can form any (over 200) cell types	Some cells of blastocyst (5–14 days)
Multipotent	Cells differentiated, but can form a number of other tissues	Fetal tissue, cord blood, and postnatal stem cells including dental pulp stem cells

The presence of a unique population, dental pulp stem cells (DPSC), has been reported by Gronthos *et al* [12, 32]. The possible role for DPSC in regeneration is demonstrated by their in vitro differentiation into odontoblast-like cells and deposition of mineralized deposits after treatment with dentine matrix extracts in association with a mineralization supplement of β-glycerophosphate and ascorbic acid[33]. These studies have demonstrated that the DPSC is able to regenerate a dentin-pulp-like complex by its differentiation into odontoblast-like cells, which formed the dentine matrix with some tubular features *in vivo*[12]. Furthermore, these cells showed the capacity of high proliferative potential, self-renewal and multi-lineage differentiation alike a novel adult stem cell[32]. Under an adipogenic inductive condition, DPSC differentiated into adipocyte-like cells that were positive to Oil red O, and expressed PPARγ2 and lipoprotein lipase (LPL); those cells also can differentiate into neuron-like and glial-like cells by expressing both nestin, an early marker of neural precursor cells, and glial fibrillary acid protein (GFAP), an antigen characteristic of glial cells[12]. Nosrat *et al* proposed the DPSCs with neuronal differentiation capacity that might provide additional benefits when grafted into central nervous system (CNS)[34]. The other potential mesenchymal stem cell population derived from human baby teeth has also identified and be named stem cell from human exfoliated deciduous SHED. SHED is 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, and they suggested that primary teeth are possibly an ideal source of stem cell for restoration of damaged tooth structure, bone regeneration, and therapy of neural tissue injury or degenerative disease[13]. A practical ability of SHED is a significant amount of bone formation *in vivo*, providing a possibility for alveolar and orofacial bone defect[13, 35, 36].

The protocol of DPSC isolation is described by Liu *et al*. The similar protocol can be used for SHED isolation. Briefly, the enzymatic digestion is performed on isolating cells

from the pulp tissue, and the cells are seeded in a low density that is gained by obtaining the single-cell suspensions from filtration method[33]. Although a research indicated that DPSC can be isolated from donors at 30 to 45 years of age[37], most studies selected the donors at ages less than 30 years since the influence of the donor age might have an effect on the culture population and content of stem/progenitor cells[38]. In addition, DPSC is demonstrated that those cells can be cryopreservation, and their growth and differentiation potential will not be changed[39].

The initial identification of DPSC and SHED are performed by assessing their colony-forming efficiency (CFU-F). The cells with the number of CFU-F colonies (aggregated of $\geq$50 cells) were evaluated for all unfractionated cell preparations[40].

SHED and DPSC express the perivascular cell surface marker STRO-1 and CD146, but do not react with the hematopoietic markers CD14 (monocyte/macrophage), CD45 (leucocyte) or CD34 (hematopoietic stem cells/endothelium). These results indicated the pulp stem cells located in the perivascular niche in their respective tissues[40, 41]. A common expression pattern profile of is summarized that CD 44, CD 106, α-smooth muscle actin, 3G5, BMPs, alkaline phosphatase, Type-I collagen, osteonectin, osteopontin, osteocalcin, bone siaoloprotein and Type-III collagen can be found from DPSC and SHED[40]. Moreover, stem cells of the non-exfoliated deciduous teeth have been demonstrated that they can express embryonic stem cell markers Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. The researchers believe that those cells have a broad therapeutic potential, including treatment of neurotrauma, myocardial infarct, connective tissue damage and more[42].

It was discovered that dental pulp cells might possess a unique ability to form specific crystalline structures in mineralized nodules, similar to physiological dentin but different from bone structures[43]. Mineralized nodule is deposited when SHED and DPSC terminally differentiate into the odontoblasts under the special condition media *in vitro*[12, 32, 13]. Dentin sialoprotein (DSP) has been used as a late marker for odontoblast formation. Type I collagen is upregulated as the cells differentiate and osteonectin is expressed in young odontoblasts. Dentin matrix acidic phosphoprotein (DMP1) is expressed by differentiating odontoblasts[44]. DSPP is dentin-specific gene and is expressed mostly by odontoblast cells[45]. It encodes two major tooth matrix proteins DSP[46] and dentin phosphoprotein (DPP)[47]. DSP and DPP are considered to arise from a single transcript, the product of which is cleaved into the two proteins[48]. They are co-expressed during tooth development and are expressed in preodontoblasts and odontoblasts

throughout dentine matrix production[49]. The expression of DSPP occurs after the formation of non-collagenous predentine matrix and can be found in odontoblast throughout the dentinogenesis [12, 45]. Runx2 is a master transcription factor of bone and plays a role in all stages of bone formation. It is essential for the initial commitment of mesenchymal cells to the osteoblastic lineage and also controls the proliferation, differentiation, and maintenance of these cells[50]. Osteoblasts and odontoblasts share several similarities, including the expression of similar genes[51]. Multiple RUNX2-binding sites have been identified in the regulatory elements of the mouse DSPP gene[52, 53]. RUNX2 increased DSPP expression in immature odontoblasts, but down-regulated expression in more mature cells, showing that the effect of RUNX2 is dependent on the state of differentiation of the target cell[54]. Runx2 is up-regulated in early odontoblasts[44], showing that levels of RUNX2 are necessary at this stage[51]. In contrast, Runx2 expression is remarkably low or undetectable in differentiated odontoblasts[55, 56].

1.3 Pulp tissue engineering

Mooney *et al* studied the dental pulp fibroblast combined with polyglycolic acid (PGA) could develop into a tissue with similar cellularity to normal pulp[57]. Other scaffolds, including a spongeous collagen, a porous ceramic, and a fibrous titanium mesh, can support the attachment, growth, and differentiation of dental pulp stem cells in vitro, and when such constructs were implanted in vivo, the cells organized into a well-vascularized tissue that expressed DSPP[9]. Goncalves *et al* established a model system which was useful for evaluation of the effect of angiogenic factors on the process of revascularization of severed human dental pulp[58]. Later on, researchers have successfully used SHED with this model system to produce tissues that closely resemble physiologic dental pulp tissue with vascularization, both in terms of architecture and cellularity[59]: They assess morphological characteristics of the tissues produced from SHED. Biodegradable scaffolds within the human tooth slices were prepared, which were seeded with SHED and introduced into immunodeficient mice. Moreover, they demonstrated that SHED could differentiate into blood vessel-forming cells which contribute to vascularization[59]. Recently, researchers use the same model system to study DPSC and stem/progenitor cells from apical papilla (SCAP) for pulp tissue regeneration[60]. Their study is the first *in vivo* evidence demonstrating *de novo* synthesis of vascularized human pulp-/dentin-like tissues in an emptied

human root canal: root canal space was filled entirely by a pulp-like tissue with well-established vascularity. In addition, a continuous layer of dentin-like tissue was deposited onto the canal dentinal wall. This dentin-like structure appeared to be produced by a layer of newly formed odontoblast-like cells expressing DSPP, BSP, alkaline phosphatase, and CD105. These cells in regenerated pulp-like tissue reacted positively to anti-human mitochondria antibodies, indicating their human origin[60]. Moreover, regeneration of a small portion of pulp after pulpotomy using a collagen matrix appears to be successful as demonstrated in a dog study model[61]

1.4 Scaffolds

Scaffold is another key role of tissue engineering. An appropriate scaffold material should demonstrate biocompatibility, non-toxicity and proper physical as well as mechanical strength. The scaffold may be implanted alone to induce host cell migration to the wound site and initiate tissue regeneration, or it may serve as a carrier to provide optimal conditions for cell adhesion, migration, proliferation and differentiation[62]. In addition, suitable scaffolds for dental restoration require the biomechanical features including tensile, compressive and flexural strength. Further, material of implantation should be replaced by regenerated tissue matrix contemporaneous to the scaffold degradation without any volume loss. Potential scaffolds have been tested for regeneration of bone, possibly suitable for dental field, composed of natural or synthetic polymers and can be divided in three groups: (1) natural organic materials, (2) synthetic organic materials, such as alginate, polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers, (3) hydroxyapatite (HA), tricalcium phosphate (TCP), and compositions of silicate, phosphate glasses and bioglasses[38]. 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. This approach would likely generate scattered calcified tissue in the entire canal space[125]. Considering the demands of 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*[9]. However, the collagen does not have the

function of anti-microbial. Collagen scaffold often lose shape and size because of the rapid degradation when contacted with body fluid or cell-culture medium[63]. 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[59]. In other words, pulp cell would lose the space to survive when they were carried by collagen scaffold. Therefore, collagen matrix alone may not be a suitable scaffold for pulp tissue engineering[64].

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. The basic knowledge of chitosan is summarized in many reviews[65, 66]. In brief, chitosan is a linear polysaccharide, composed of glucosamine (deacetylated unit) and N-acetyl glucosamine (acetylated unit) linked by β (1–4) glycosidic bonds. The content of glucosamine is called as the degree of deacetylation (DD). Depending on the source and preparation procedure, its molecular weight (Mw) may range from 300 to over 1000 kD with a DD from 30% to 95%[65]. Commercially available preparations have degrees of deacetylation ranging from 50 to 90%[66]. Chitosan is normally insoluble in aqueous solution above PH 7 in crystalline form. However, in dilute acids (PH<6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule [66].

Chitosan scaffolds possess some special properties for use in tissue engineering. First, it can be molded in various forms[67]. In particular, it possesses excellent ability to form porous structures[68]. 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 them to possess the swelling ability, which attributes to promote the cell attachment, nutrient supply and the 3-D structure maintenance. Sufficient nutrient supply as well as removal of metabolic waste-products, guided by the porosity, interconnectivity and pore size is of great interest to ensure the viability and migration of dental cells[38]. Therefore, the chitosan scaffolds seem to be a good choice for carry the dental cells. Second, the cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules. The benefit of this property for tissue engineering is that numbers of cytokines/growth factors can be bound[65]. Another distinct property of chitosan is that it confers considerable antibacterial activity against a broad spectrum of bacteria[69]. Accordingly, chitosan has been blended with other polymers due to this property[70].

Fourth, The degrade ability of a scaffold plays a crucial role on the long-term performance of tissue-engineered cell/material construct because it affect many cellular process, including cell growth, tissue regeneration, and host response. It has to maintain the mechanical strength until tissue regeneration is almost completed[69]. The degradation rate also affects the biocompatibility since very fast rates of degradation will produce an accumulation of the amino sugars and produce an inflammatory response[71]. Lysozyme is the primary enzyme responsible for *in vivo* degradation of chitosan, which appears to target acetylated residues[71].

Aranaz *et al* summarized other properties of chitosan such as analgesic, antitumor, hemostatic and antioxidant ability. However, some properties such as antitumor ability are of controversial[72]. They also listed the relationship between chitosan biological properties and their characteristics (Table 3)[72]: 1) The final degradation products are biocompatible chitosan oligosaccharides of variable length. The degradation rate is inversely related to the degree of crystallinity which is controlled mainly by the DD. Highly DD exhibit relatively a low degradation rate and may last several months *in vivo*[69]. 2) Toxicity of chitosan is reported to depend on DD, and Mw of chitosan did not influence toxicity[68]. 3) A high DD with solid-state chitosan is more haemostatic[74]. 4) The main analgesic effect of chitosan is the absorption of proton ions, bradykinin, one of the main substances related to pain released in the inflammatory area. It depends on the free primary amino groups that chitosan have[72]. 5) The chitosan oligomers with Mw from 8 to 5 kDa showed good antibacterial activities from the study of Liu et al[75]. 6) chitosan with higher DD exhibited the highest scavenging activity[76], and it with low Mw can reduce radical scavenging effect and ion-chelating potency[77].

Table 3 Relationship between chitosan biological properties and their characteristics

Property	Characteristic
Biodegradability	DD, distribution of acetyl groups, Mw
Biocompatibility	DD
Hemostatic	DD, Mw
Analgesic	DD
Adsorption enhancer	DD

Property	Characteristic
Antimicrobian	Mw
Antioxidant	DD, Mw

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[69]. 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 as well as the cytocompatibility of scaffold is necessary.

Aims of the study

The aims of this study were to compare the ability of proliferation and mineralization between SHED and DPSC. Cytocompatibility between those fabricated chitosan scaffold and the cells were also studied.

Hypothesis

We hypothesized that

1. SHED would possess the higher ability of proliferation and mineralization than DPSC.
2. Those fabricated chitosan scaffolds would be non-toxic to the cells.
3. The 3 - D chitosan scaffolds would support and promote the cell growth compare to cell seeding in culture dish.

Objectives of the study

The objectives of this study were to

1. Compare the cell growth between SHED and DPSC.
2. Compare mineralization between SHED and DPSC.

3. Test the cytotoxicity of those fabricated chitosan scaffolds.
4. Observe the microstructure of chitosan scaffolds and the cell morphology in those scaffolds.
5. Investigate the cell growth in the chitosan scaffolds.

CHAPTER 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 ($\leq$ 29 years old, n = 6) impacted third molars and bicuspids extracted due to orthodontic considerations[78]. All of these teeth should contained the normal health 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[79], and no pulpitis.

Cells were isolated by enzyme digestion as described by Gronthos *et al*[12]. 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 by No. 22 blades. 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 Dulbecco's Modified Eagel Media (D-MEM, Life Technologies/GIBCO BRL) supplemented with 20% fetal bovine serum (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)[40]. 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 $\geq$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) for 18 wells of each cell type (n = 6). At least three wells without cell were served as a control for the minimum absorbance. DMEM 150 μl was added to each well 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. The plates were cultured at 37°C in an incubator setting in a humidified atmosphere of 5% CO_2. The cultural media were changed every 2 days. After 24 hours, day $7^{th,}$ and day14^{th}, the cell proliferation was measured by MTT assay modified from Mosmann *et al* [80].

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) (Sigma) assay was used as a measure of relative cell viability. It is based on the ability to produce purple formazan in the mitochondria of living cells. The insoluble formazan product usually dissolved into a colored solution by the solubilization solution, dimethyl sulfoxide (DMSO) (Amresco, USA). The absorbance of this colored solution can be quantified by measuring at a certain wavelength by a spectrophotometer. Therefore, the absorbance values can be correlated to the cell number.

The MTT solution (5mg/ml) was prepared by:

1) Weighted MTT powder 0.5 g
2) Dissolved the powder by 100 ml of PBS solution
3) Filtrated the MTT solution

At time interval, The MTT assay of each well was performed by the following steps:

1) Removed the media and washed by PBS twice
2) Added 100 μl of media and 10 μl of MTT solution
3) Incubated under 5% at 37°C for 4 hours
4) Removed the MTT solution
5) Added 100 μl of the DMSO to dissolve the crystals of formazan product
6) Incubated and avoided from light for 15 minutes at room temperature

7) Mixed by micropipette several times
8) Measured the absorbance of the colored solution 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 for 36 wells of each cell type (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 (18 wells of each cell type). 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 (18 wells of each of each cell type). The media were changed every 2 days. Detecting of mineralization during cell differentiation was performed at day 7^{th}, 14^{th}, 21^{st} and 28^{th}.

Alizarin Red S (Nacalai Tesque Inc.) staining was used to detect mineralized nodule formation of the cells[81-83], and the nodule formation was observed under the light microscope (Inverted Nikon TS 100E). The principle of Alizarin Red S staining is that the calcium forms an alizarin red S-calcium complex in a chelation process.

Preparation of Alizarin Red Solution:

Alizarin Red powder: 0.5 g

PBS: 100 ml

1) Mixed the solution
2) Adjusted the pH to 4.1-4.3 (the pH is critical)

In each well of the 24-well-plates, the experiment was performed as the following steps:

1) Removed the media
2) Washed twice with PBS
3) Fixed by 500 μl of 10% formalin (room temperature, 15 minutes)
4) Rinsed twice (5-10 minutes each) with double distill water
5) Stained with 500 μl of Alizarin Red S solution for 20 minutes

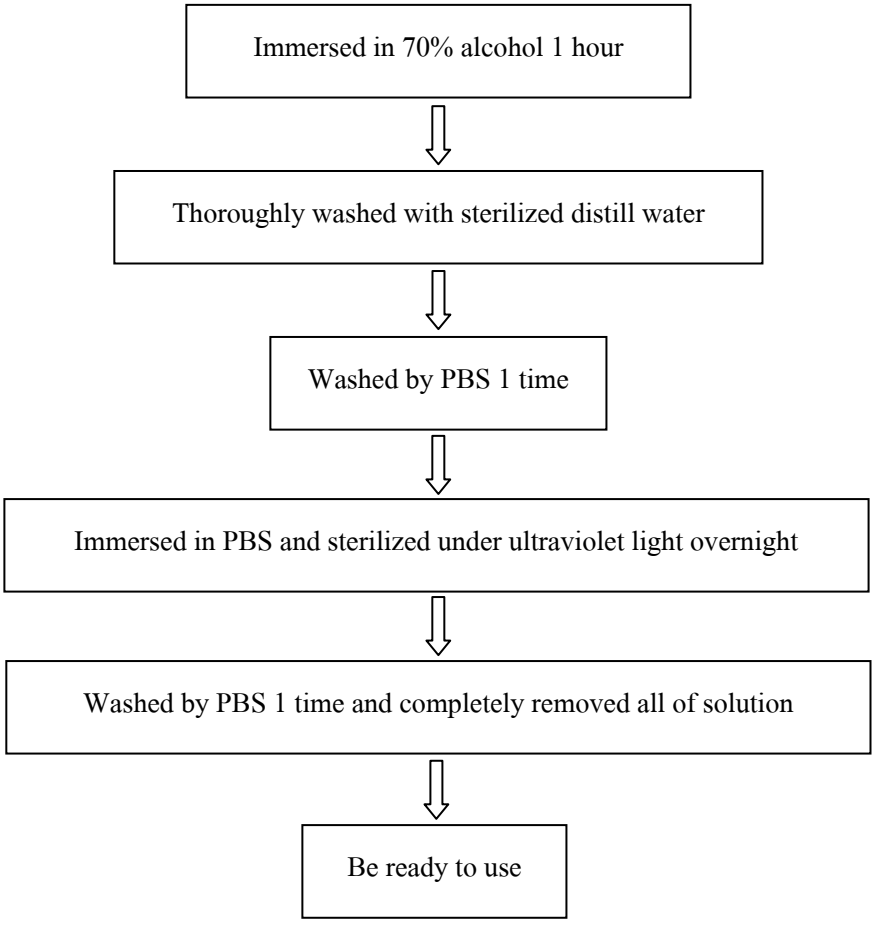
6) Washed several times by double distill water to remove the remaining stain.
7) Added 1 ml water for preventing the cells from drying
8) Examined under the microscope (Nikon, Eclipse TE2000-U, Digital camera DXM 1200) for visual inspection and took pictures.

2.4 Preparation of chitosan scaffolds

2.4.1 Fabrication of chitosan scaffolds

The 2% and 3% chitosan scaffolds were fabricated by the undergraduate students for their junior project.

2.4.2 Sterilization of prepared chitosan scaffold:



2.5 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[84-86]. The test usually is performed in direct and indirect contact tests base on different purpose[87, 88].

The extraction and testing procedures were modified form a previous study[89]. 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[90]. The evaluation of cytotoxicity was refered to Table 4 and Table 5. the scores correspondent to these four quantitative and qualitative parameters are combined, resulting in a response index ranging from 0 to 8, and the criterion is then used to classify the reactivity of the materials[89].

Table 4 Quantitative and qualitative scores used in the cytotoxiciy tests.

Score	Confluence	Floating cells	Change of cellular morphology	Inhibition of cell growth
0	100%	0%	No changes during test period	0-10%
1	90-100%	0-5%	Slight changes, few cells affected	10-30%
2	60-99%	5-10%	Mild changes, some cells round/spindle shaped	30-50%
3	30-60%	10-20%	Moderate changes, many cells round/spindle shaped	50-70%
4	0-30%	>20%	Severe changes, about all cells show morphological changes	70-100%

Table 5 Cytotoxicity index (0-8)

Cytotoxicity index	Reactivity
0-1	none
1-3	slightly toxic

Cytotoxicity index	Reactivity
3-5	mildly toxic
5-7	moderately toxic
7-8	severely toxic

2.5.1 Extraction procedure:

1) The total volume of the media using in test groups was calculated to be 10.8 ml.
2) 11 pieces (2mm thick, 5mm in dimeter) of sterilized 2% and 3% chitosan scaffolds was respectively immersed in the 11 ml 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°C.
3) The extraction media of 2% and 3% chitosan scaffolds were respectively collected in the sterilized tubes.

2.5.2 Test culture and evaluation:

1) SHED, DPSC, gingival fibroblast and MG-63 were seeded at the density of 5×10^4 cells/well in 96-well-plates for 54 wells of each cell type(n = 6) 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.
2) After the removal of normal growth media, the extraction media of 2% and 3% were added into each testing well for 18 wells of each cell type in each concentration.
3) The cells were seeded with 150 μl of normal growth media as the control (18 wells of each cell type).
4) After 24, 48, 72 hours, the cells were evaluated under a light microscope.
5) After the microscopic evaluation of 72 hours, the cell viability was performed by MTT assay as described above in section 2.2.

2.6 Cell morphology and viability in chitosan scaffolds

2.6.1 Cell seeding into scaffolds

1) 348 pieces (2 mm thick, 5mm in diameter) sterilized chitosan scaffolds were placed in the 48-well plates for 72 wells in each concentration.
2) Each specimen was immersed 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°C.
3) The media were removed completely.
4) SHED, DPSC, gingival fibroblast and MG-63 were trypsinised and centrifuged to get the approximate cell density at 5×10^4 cells in 50 μl media.
5) Each 50 μl media with 5×10^4 cells were loaded onto the scaffolds and incubated for 3 hours at 37°C in a humidified atmosphere 5% CO_2.
6) After 3 hours, D-MEM (DMEM High glucose and phenol red free, Gibco) was added to these scaffold for 500μl/well 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.

2.6.2 Cell viability in the scaffolds

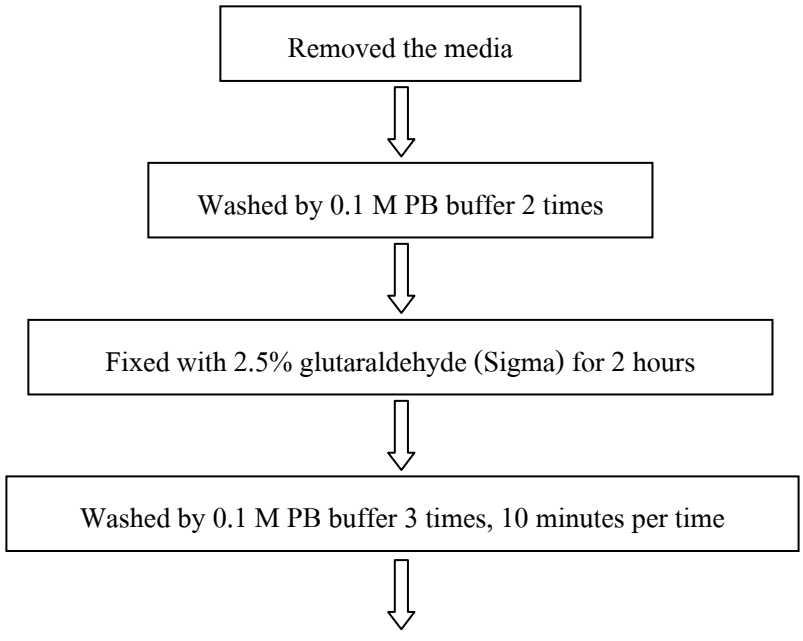
SHED, DPSC, gingival fibroblast and MG-63 seeding within the scaffolds was carried out as described above in section of 2.6.1. Each 50 μl media with 5×10^4 different type of cells were seeded onto the surface of the wells as control for 18 wells. The cell viability within the scaffolds was performed by a water-soluble tetrazolium (WST-1) assay (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate). This assay is based on the finding that living cells are capable of cleaving slightly red colored WST salt to dark red colored formazan by mitochondrial dehydrogenases[91]. Moreover, the WST-1 reaction product can be quantified in 0.5 to 4 hours without an additional solubilization step. The WST-1 reagent produces a water-soluble formazan rather than the water-insoluble product of the MTT assay[92]. In our pilot study,

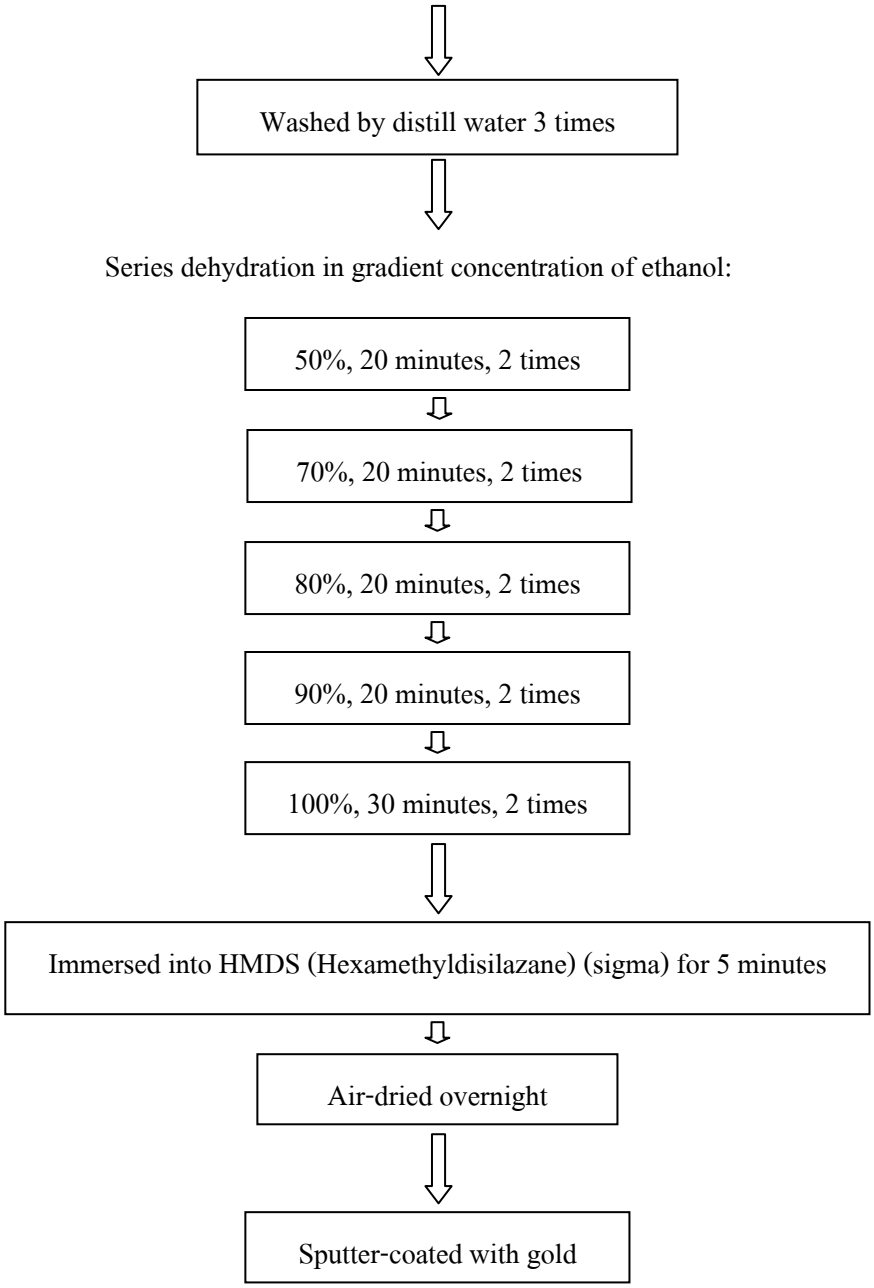
products of the MTT assay were deposited inside of scaffolds, and the solubilization step was blocked by complex microstructure of scaffolds. Therefore, the MTT assay was substituted by WST-1 assay to detect the cell viability within the porous scaffolds. The WST-1 assay (Roche, Mannheim, Germany) was performed at day 8^{th}, 15^{th} and 21^{st} according to the protocol of manufacturer.

1) After media removal, the cells within scaffolds were thoroughly washed by PBS to remove the remaining phenol red.
2) Every wells were added 300 ml of serum and phenol red free media supplemented with, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml mycostatin and 100 μg/ml kanamycin.
3) 30 μl of WST-1 kit was added into each well.
4) Absorption was measured at 450 nm with 620 nm as reference and corrected to blank values (scaffolds without cells).

2.6.3 Cell morphology and microstructure of scaffolds detected by SEM (scanning electron microscope) (JSM-5800LV, JEOL)

The specimens with cells were prepared for SEM according to the following protocol, and the specimens without cells were prepared omitted the fixative part:





2.7 Data analyses

1. Every experiment was performed triplicately.
2. The data of cell proliferation (MTT), cytotoxicity (MTT) and cell viability in scaffold were tested for normal distribution and homogeneity. Those data were presented as mean $\pm$ 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.

3. Mineralization, microstructure of chitosan scaffolds and morphology of the cell seeding in scaffolds were descriptively described.

The analyses were performed on a personal laptop using commercial SPSS software (Version 16.0, Standard Software Package Inc., USA).

CHAPTER 3

RESULTS

3.1 Cell proliferation was determined using the MTT assay

MTT assay was used to assess cell proliferation on day 1st, 7th and 14th (Fig. 1). There was no significant difference among the different cell groups on day 1.

On day 7th the optical density (OD) of SHED was found to be significantly higher than that of DPSC. The optical density of MG-63 cell group was significantly higher than those of the other three groups.

The OD of SHED was still markedly higher than that of DPSC on day 14th. Optical density of gingival fibroblast was significantly higher than that of DPSC. Moreover, MG-63 cell group maintained the highest optical density.

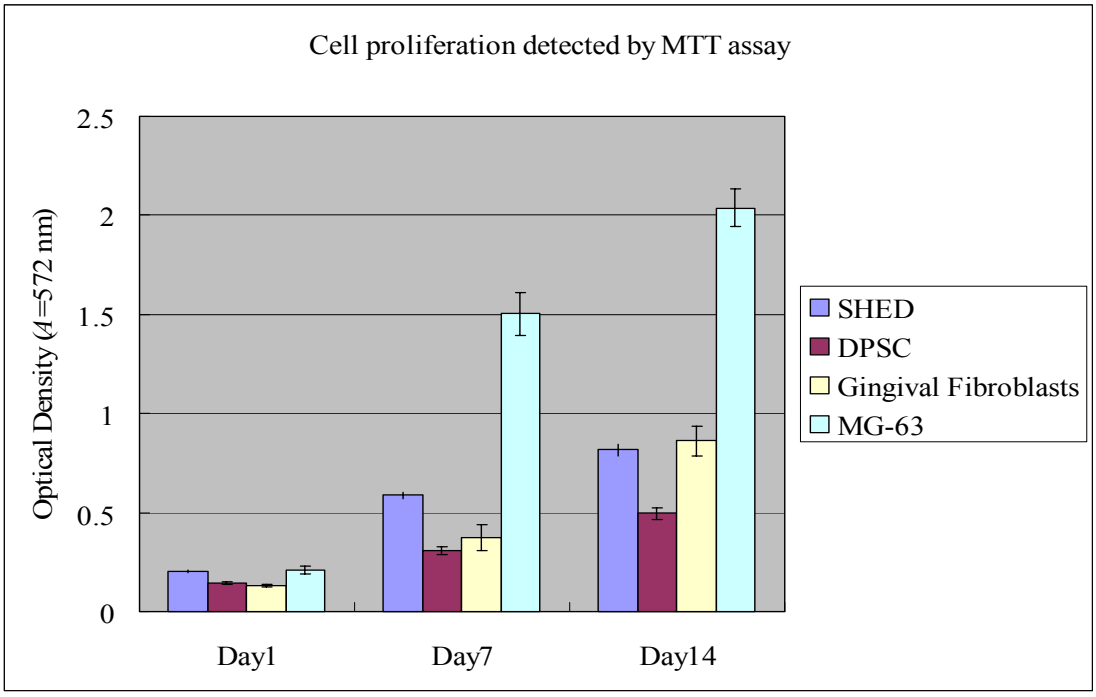


Figure 1 Cell proliferation detected by MTT assay:

Optical density (A=572 nm) was expressed as a measure of cell proliferation on 1, 7 and 14 days, n=6. Errors bars represent means ± SD.

Table 6 Cell proliferation detected by MTT assay on day 7th:

Means ± *SD* of optical density in different cell groups analyzed by One-way ANOVA on day 7th, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.01)

	SHED 0.586±0.068	DPSC 0.308±0.019	Gingival Fibroblasts 0.374±0.064	MG-63 1.503±0.110
SHED 0.586±0.068	\	*	-	*
DPSC 0.308±0.019	*	\	-	*
Gingival Fibroblasts 0.374±0.064	-	-	\	*
MG-63 1.503±0.110	*	*	*	\

Table 7 Cell proliferation detected by MTT assay on day 14th:

Means ± *SD* of optical density in different cell groups analyzed by One-way ANOVA on day 14th, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.01)

	SHED 0.815±0.056	DPSC 0.495±0.030	Gingival Fibroblasts 0.861±0.074	MG-63 2.037±0.096
SHED 0.815±0.056	\	*	-	*
DPSC 0.495±0.030	*	\	*	*
Gingival Fibroblasts 0.861±0.074	-	*	\	*
MG-63 2.037±0.096	*	*	*	\

3.2 Mineralized nodule formation was detected by Alizarin Red S staining

The Alizarin red S staining was performed on day 7^{th}, 14^{th}, 21^{st} and 28^{th} to examine the nodule formation. SHED formed more mineralized nodules than DPSC in all periods of the experiment.

There was no nodule formation on day 7^{th} in all groups (Fig. 2).

On day 14^{th}, some mineralized nodules could be found in the test groups of SHED and MG-63. In contrast, we could not find any true mineralized nodule surrounding the cells in the test groups of DPSC and gingival fibroblasts (Fig. 3). There was no mineralized nodule formation in control groups.

On day 21^{st}, the nodules were present in every test group. 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). Besides, a small number of mineralized nodules could be seen in some wells of control groups.

On day 28^{th}, 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^{st} (Fig. 5). Similarly to day 21^{st}, the mineralized nodules could be found in the gingival fibroblast test group and in control groups of SHED, DPSC, gingival fibroblast and MG-63.

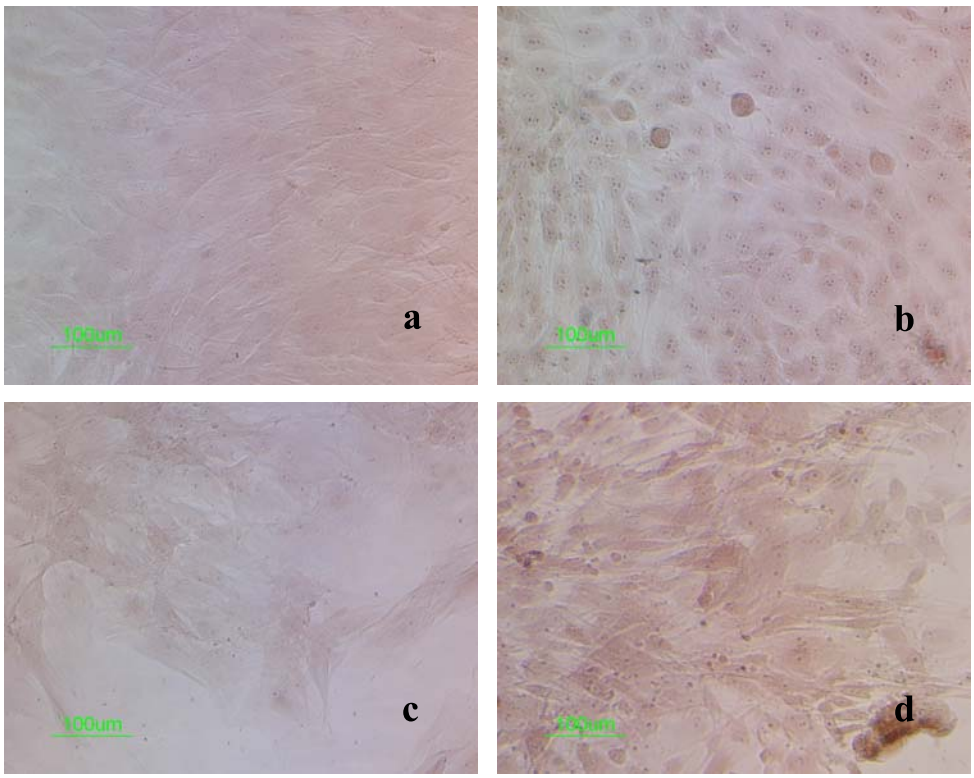


Figure 2 Mineralized nodule formation detected by Alizarin Red S staining on day 7th

a. Gingival fibroblasts;

b. MG-63;

c. DPSC;

d. SHED.

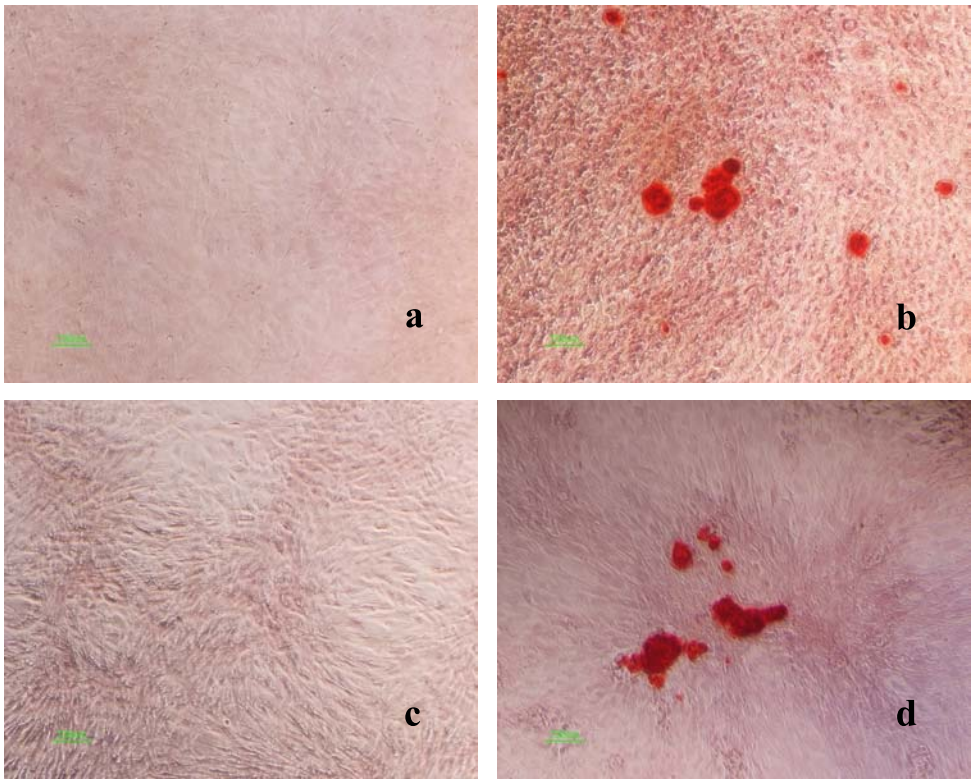


Figure 3 Mineralized nodule formation detected by Alizarin Red S staining on day 14th

a. Gingival fibroblasts;

b. MG-63;

c. DPSC;

d. SHED.

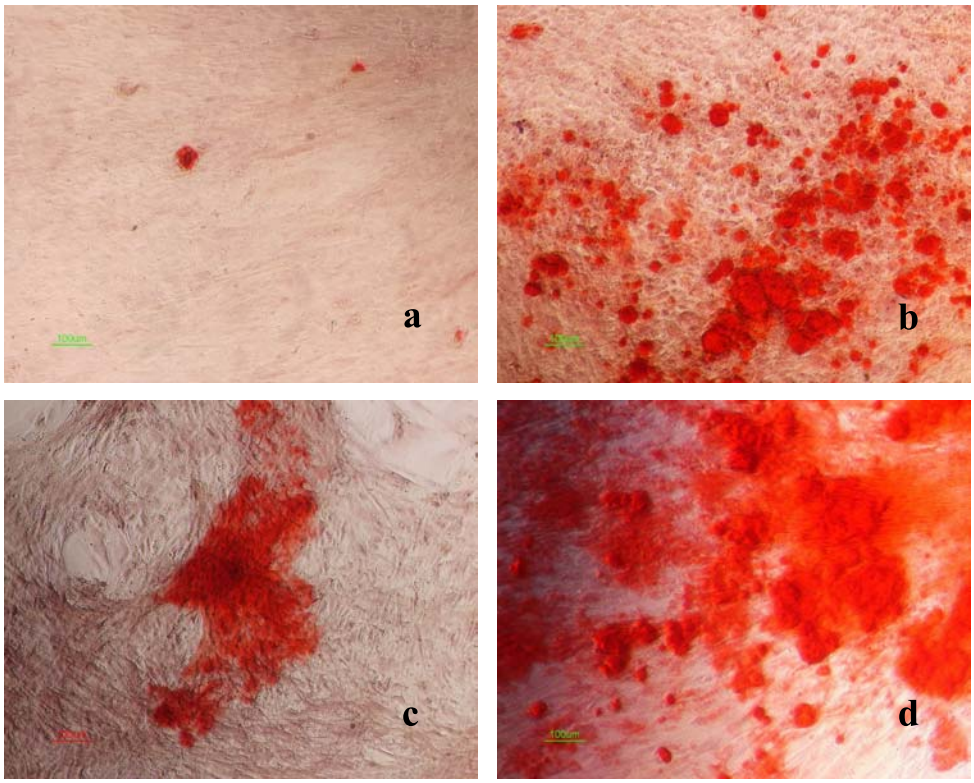


Figure 4 Mineralized nodule formation detected by Alizarin Red S staining on day 21st.

a. Gingival fibroblasts;

b. MG-63;

c. DPSC;

d. SHED.

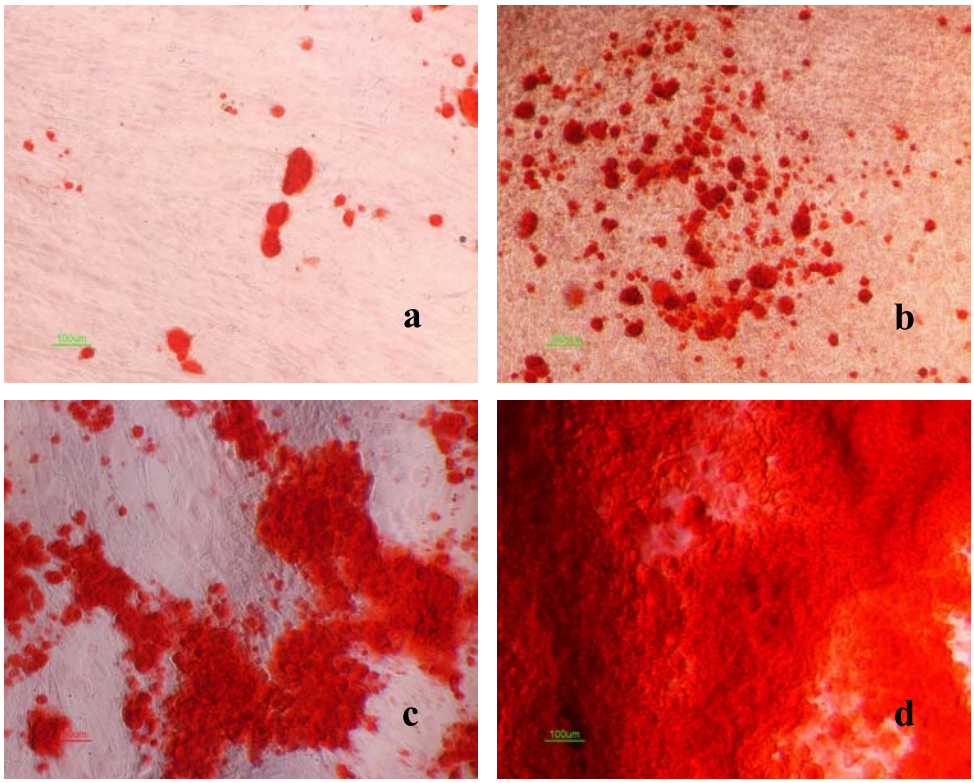


Figure 5 Mineralized nodule formation detected by Alizarin Red S staining on day 28th.

a. Gingival fibroblasts;

b. MG-63;

c. DPSC;

d. SHED.

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, $n = 9$).

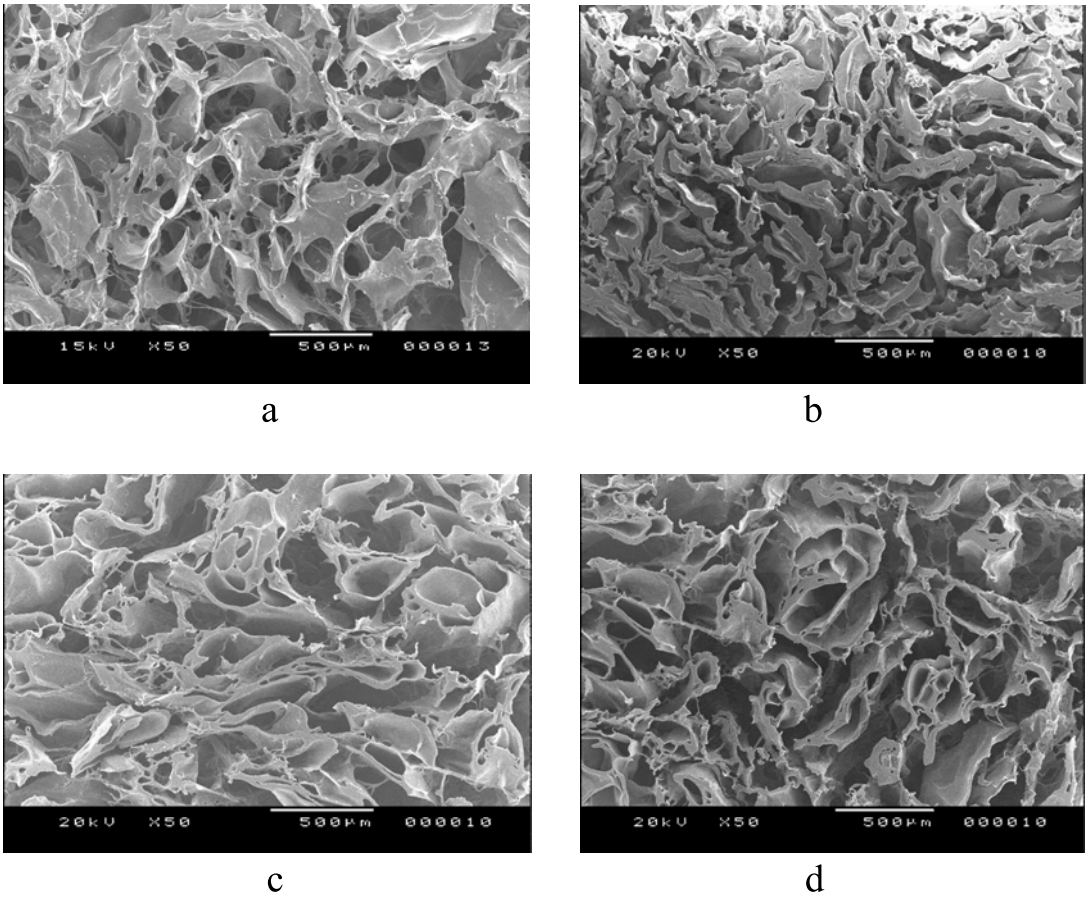


Figure 6 SEM micrographs showed the microstructures of chitosan scaffold:

a. Cross section of 2% chitosan scaffold

b. Longitudinal section of 2% chitosan scaffold

c. Cross section of 3% chitosan scaffold

d. Longitudinal section of 3% chitosan scaffold

3.4 Cytocompatibility

3.4.1 Cytotoxicity test

An indirect contact test was performed to evaluate the cytotoxicity of porous chitosan scaffold. After 24, 48 and 72h, the reaction of cells to the extraction media was evaluated microscopically. No obvious floating cells and changes in cellular morphology were observed in both treatment and control groups. Figure 7 and table 8 demonstrate MTT assay used to determine cell viability after 72h cells exposed to the extraction media 2% and 3% (extracted from 2% and 3% chitosan scaffold).

DPSC viability between extraction media of the 2% group and the 3% group were not significant different, but they were significantly higher than cell viability of the normal media control group.

For SHED and MG-63, cell viabilities of the 2% groups were higher than those of the 3% and the control groups.

The viabilities of gingival fibroblast among control, 2% and 3% groups were not significantly different.

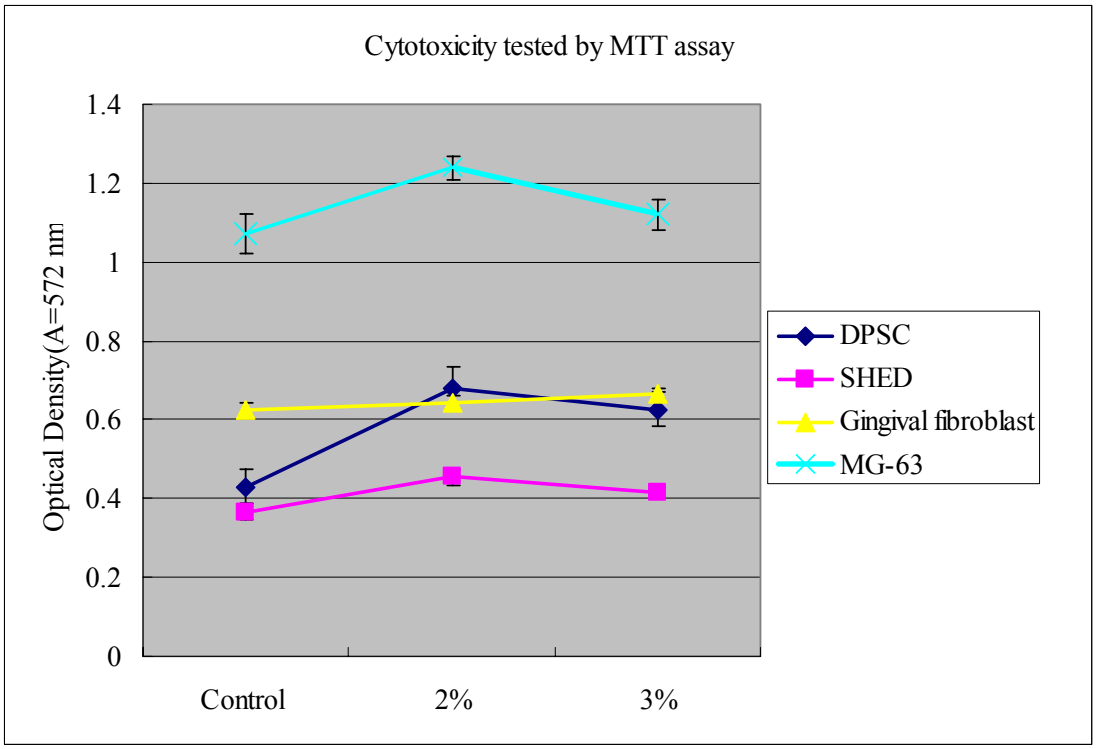


Figure 7 Cytotoxicity test by MTT assay:

Optical density (*A*=572 nm) was expressed as a measure of cell viability after exposure to the extraction media 2% and 3% 72h, and the cells cultured with normal media were set as control, *n*=6. Errors bars represent means ± *SD*.

Table 8 Cytotoxicity test by MTT assay:

Means ± *SD* of optical density in different cell group analyzed by One-way ANOVA, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.01)

	Control	2% chitosan	3% chitosan
DPSC	0.430±0.044	0.680±0.055*	0.627±0.043*
SHED	0.366±0.021	0.454±0.022*	0.417±0.016
Gingival fibroblasts	0.623±0.020	0.644±0.019	0.667±0.013
MG-63	1.070±0.050	1.239±0.029*	1.120±0.040

3.4.2 Morphology of cells seeded in the scaffolds

SEM was used to observe cellular morphology and cell attachment on the chitosan scaffold in *vitro*. Cells could attach to the bottom (Fig 8c) and wall (Fig. 8d) of each pore. They spared on the wall, and the pseudopods could be seen clearly (Fig. 8a). When the cells were confluent, they could cross the border of the pores (Fig. 8b) and connect together.

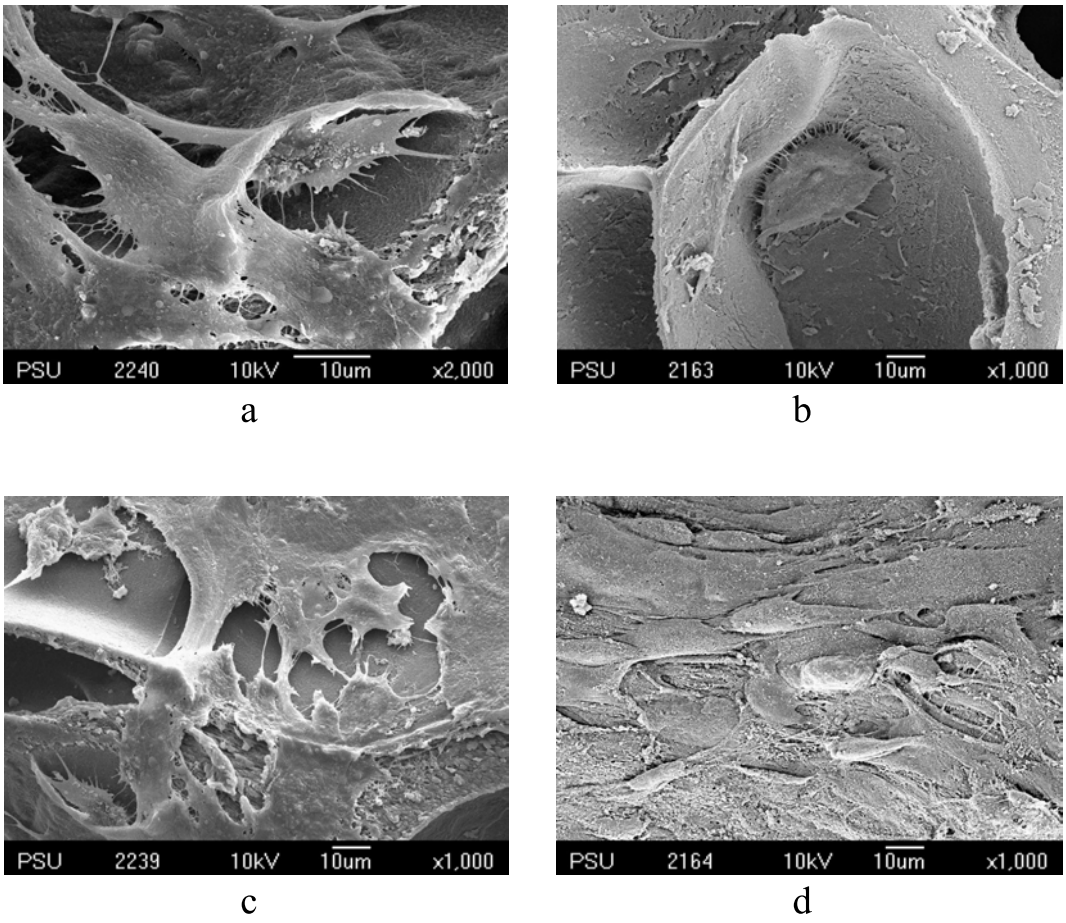


Figure 8 Scanning electron micrographs of cells cultured on chitosan scaffold for 16 days.

a. Gingival fibroblasts on the scaffold

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

3.4.3 Cell viability in scaffolds

In order to determine the viability of DPSC, SHED, gingival fibroblasts and MG-63 in the chitosan scaffold, the cells were cultured in scaffolds and the WST-1assay was performed on day 8th, 15th and 21st. The cells were cultured on plates were used as controls (Table 9).

For DPSC, the optical density (OD) of the cells within the 2% and 3% scaffolds were significantly higher than those in the control groups on day 8th, 15th and 21st. In contrast, the OD of the control group did not increase much from day 15th to day 21st. On day 21st, 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. 9a).

SHED showed similar results as DPSC except that between day 15th and day 21st, the OD of the 3% group had a decreasing trend (Fig. 9b).

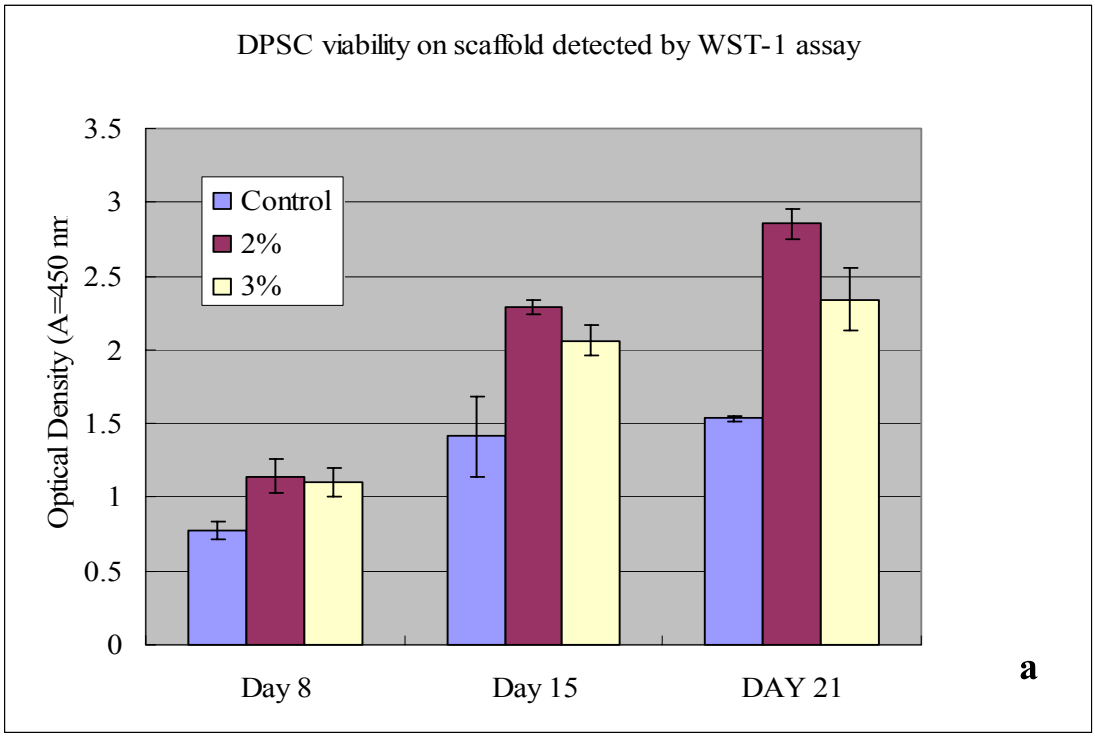
For gingival fibroblasts, the OD of the test group was higher than that of the control group since day 8th of this study, and the OD of the 2% scaffold group was higher than the 3% group started from day 15th. From day 15th to day 21st, the OD of control groups were almost the same, whereas the OD of the 3% scaffold group decreased (Fig. 9c). Compare to others, gingival fibroblasts demonstrated better compatible to 2% than 3% scaffold at all period of experiment except on day 8th when the difference was not significant.

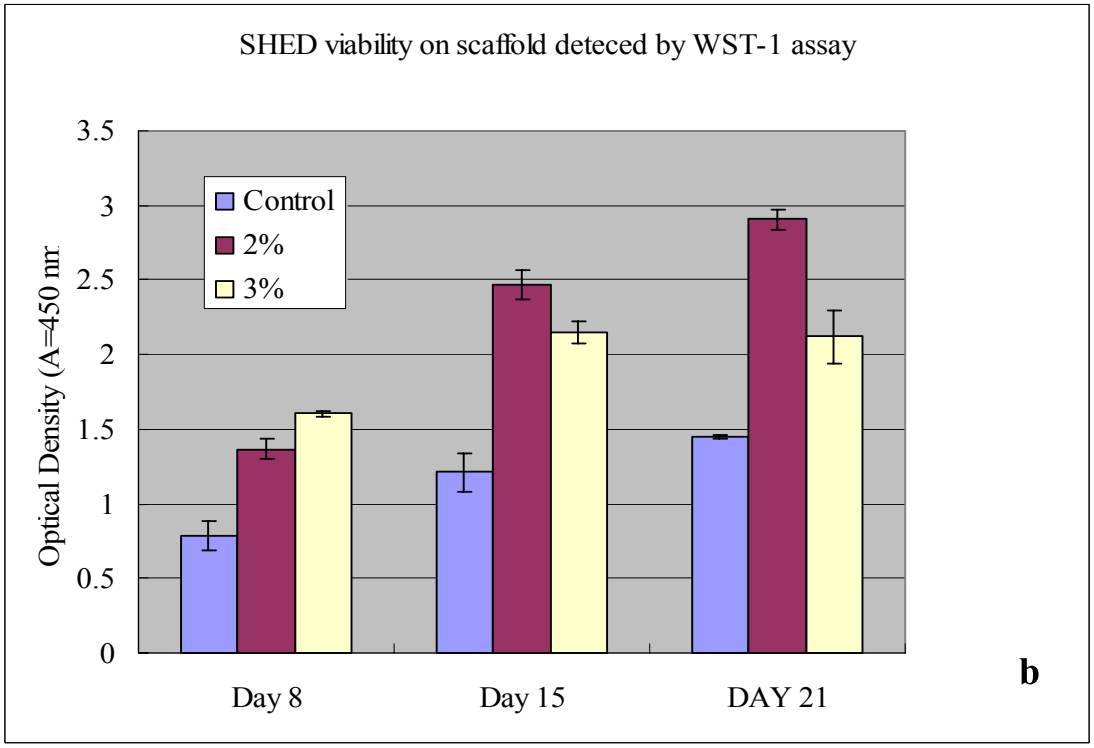
For MG-63, the OD of the test group was higher than that of control group, but there was no significant difference between the 2% and the 3% scaffold groups at all time points (Fig. 9d).

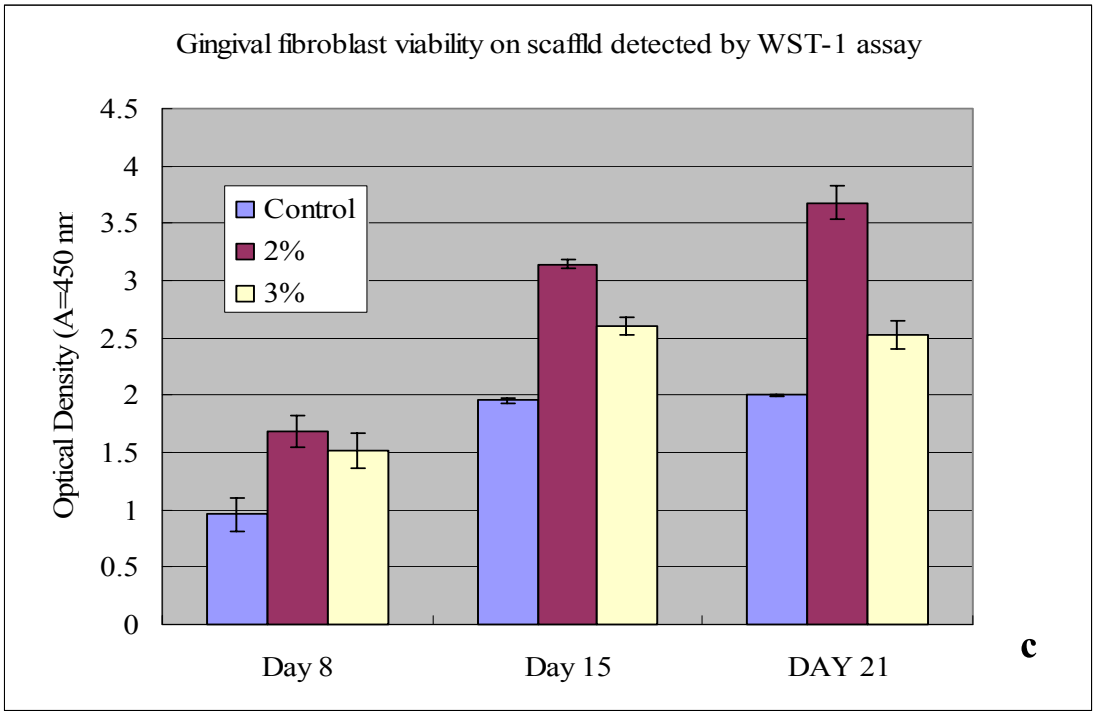
Table 9 Cell viability in scaffolds detected by WST-1 assay:

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)

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*


DPSC viability on scaffold detected by WST-1 assay
Optical Density (A=450 nm
3.5
3
2.5
2
1.5
1
0.5
0
Control
2%
3%
Day 8
Day 15
DAY 21
a


SHED viability on scaffold deteced by WST-1 assay
Optical Density (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



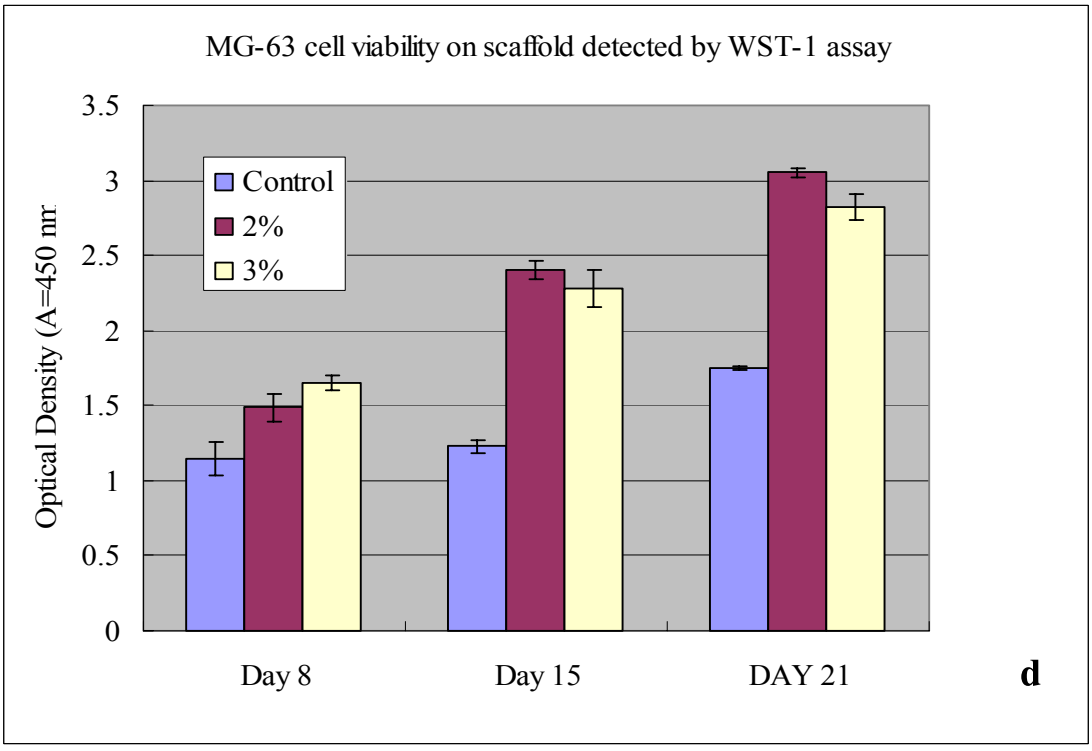


Figure 9 Cell viability in scaffolds detected by WST-1 assay Optical density (*A*=450 nm) was expressed as a measure of cell (a. DPSC; b SHED; c Gingival fibroblasts; d MG-63 cells) viability in scaffold on Day 8, 15and 21, *n*=6. Errors bars represent means ± *SD*.

CHAPTER 4

DISCUSSIONS

A functional but challenging approach for dental pulp therapy is the using of tissue engineering techniques[38]. Those techniques include the application of a scaffold on an open pulp[9, 10]. In order to develop a potential biomaterial for dental pulp regeneration and reconstitution of a complete dentin-pulp-complex, the understanding of the cell proliferation, differentiation processes as well as the characteristics of scaffold is crucial.

Two type of stem cells isolated from dental pulp tissues were reported, DPSC[12] and SHED[13]. SHED and DPSC have distinct characteristics *in vivo*[13]. Unlike DPSC, SHED is unable to generate the dentin-pulp-complex[13]. In contrast, SHED can induce the new bone formation[13]. The cell surface antigen STRO-1 is considered as an early marker of different mesenchymal stem cell populations and the marker of preosteogenic[93, 41]. DPSC and SHED, in the total cell populations derived from permanent and primary teeth, contain approximately 5-6% and 9% STRO-1 positive cell population respectively[13, 41, 94]. These maybe indicate that the primary teeth contain more progenitor cells than permanent teeth. Corresponding to previous studies[13, 95] the data in this study showed that proliferation of SHED are significantly higher than that of DPSC (Fig. 1, Table 6 and 7).

Both of DPSC and SHED have ability to differentiate into odontoblasts[12, 13] 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[43]. The cultural condition of mineralized nodule formation was established by Tsukamato *et al*[96]. In order to determine the differentiation ability of DPSC and SHED, the mineralized nodule formation were observed using Alizarin Red S staining. The osteosarcoma cell line, osteoblast-like cell MG-63[97] which can form the mineralized nodules under osteogenic condition were set as positive control, and the gingival fibroblasts were set as the negative control. The results of this study show that the nodules appeared from day 14th in test group of SHED and MG-63 (Fig. 3), and the nodule formation of DPSC can be found at 21 days (Fig. 4). On day 21st and day 28th, the nodules of SHED groups were more than in DPSC group (Fig. 4, 5). Those results suggest that the nodule formation of SHED may be earlier than that of DPSC. In contrast, although the nodules

formation of DPSC appeared late and were not as many as that of SHED, they increased very fast after their appearance. 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[12, 43, 94, 98]. One study shows mineralization of the rat dental pulp cell begins on day 11th [99]. There are many factors effect on the time of mineralized nodule appearance, such as isolation method used, seeding density and donor age[38]. 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[9]. This study suggests that SHED maybe possess stronger mineralization ability than DPSC do under the same isolation method, seeding density and culture condition. The assumption that SHED is likely to be more immature than DPSC might be one of the reasons for these results. Interestingly, the nodule formation of MG-63 was increase markedly from day 14th to day 21st. On day 28th, the circumstance of nodule formation of MG-63 cell test group was not change compare to itself at day 21st. These suggest that the mineralized ability of MG-63 may have a trend to decrease after day 21st.

In order to obtain a functional approach to restore dental structure, dental pulp stem cells play an important role to organize a net work of inducing the connective tissue to regenerate new tissue. As a carrier, scaffolds could support cell adhesion, proliferation and differentiation. The selection of an appropriate scaffold material is important. Chitosan possess a great deal of properties that make them suitable for application in biomedical field[72]. Moreover, the procedure of chitosan scaffold preparation is easy to handle and the materials are economic.

The 2% and 3% chitosan scaffolds of our study were fabricated by the undergraduate students for their junior project (Figure 10). The fabrication of chitosan scaffolds were modified from several studies. In brief, the methods contained centrifugation, free-drying, rehydration stabilization step[100-104]. They also studied the swelling and degradation ability of those chitosan.

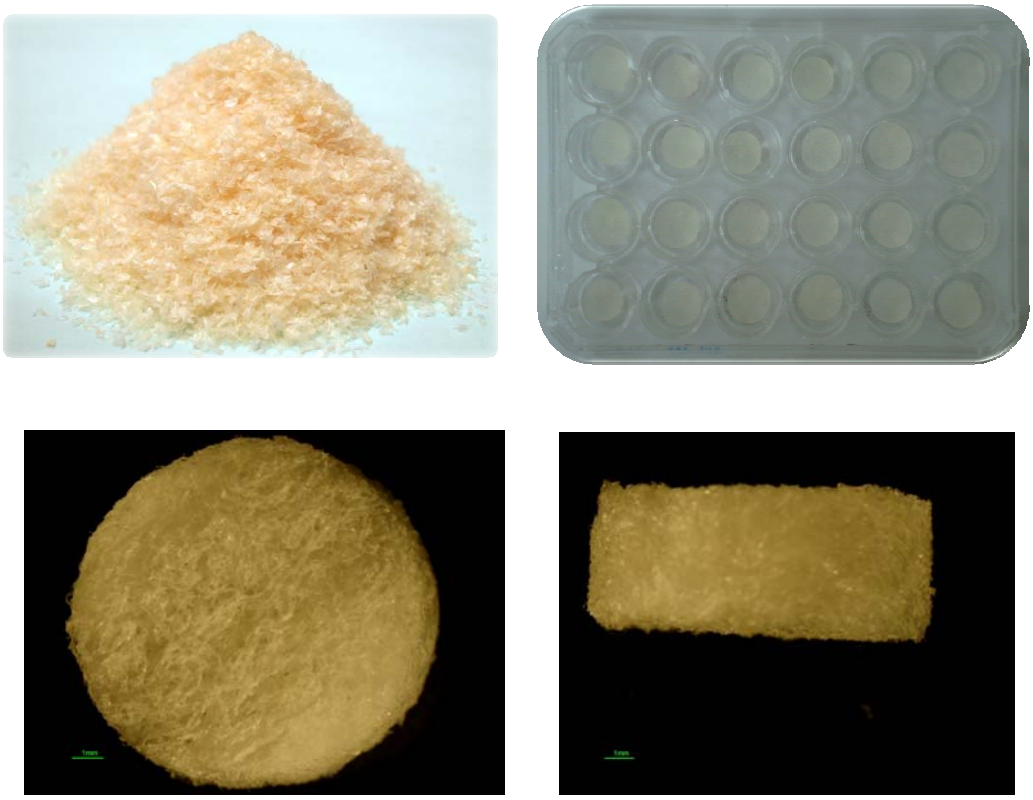


Figure 10 Morphology of chitosan from powder to scaffolds

The swelling study was designed to investigate the ability of water uptake[105] and the dimension changes of chitosan scaffolds. Stimulated body fluid (SBF) was prepared to mimic environment of the body serum. After dissolving appropriate quantities of the precursor chemicals in deionized water, the ion concentrations nearly equal to those of the inorganic constituents of human blood plasma (Table 10)[106]. Water uptake (swelling ratio) at 5 minutes (Fig. 11) and dimension changes (Fig. 12 and 13) of chitosan scaffolds within SBF were tested by using appropriate equation. The results show that the cross-sectional and longitudinal sizes of the scaffolds only significantly changed in the first 5 minutes, and the scaffolds maintained their dimension after that.

The degradation study was performed using lysozyme. Chitosan is mainly degraded by lysozyme[107]. The experimental procedures were modified from the previous study [84, 108] at the intervals of 7th, 14th and 21st [101]. Their results (Fig. 14, Tab. 11) showed that comparing to the weight loss of scaffold on day 7th, the weight loss of those scaffolds significantly increased on day 14th. Similarly, the weight loss of scaffolds on day 21st were significantly more

than that on day 14th ($P < 0.05$, analyzed by one-way ANOVA, multiple comparisons, n = 9).

Table 10 Ion concentrations (mM) of SBF and human blood plasma

Ion	Simulate Body Fluid	Blood plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	148.8	103.0
HCO^{3-}	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

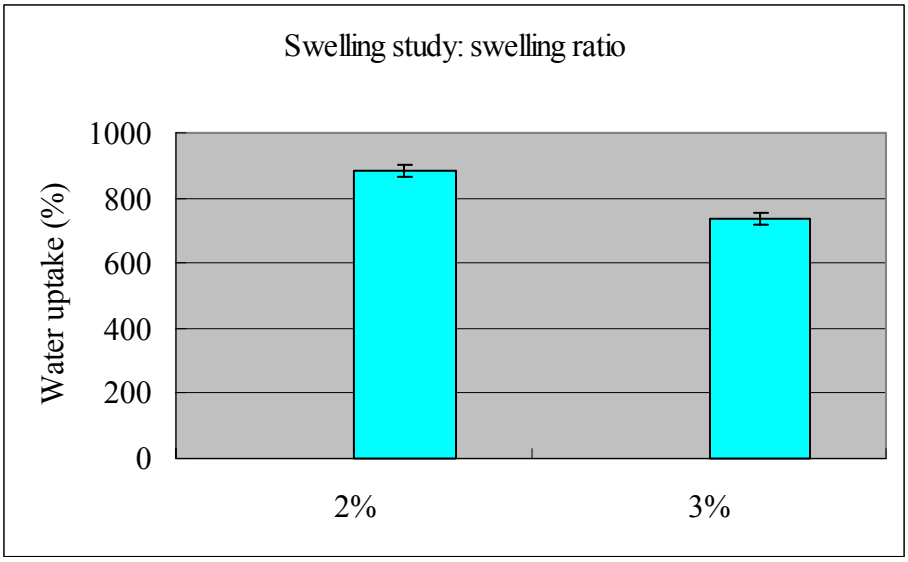


Figure 11 Swelling ratios (738.47 ± 18.27) of 3% chitosan scaffolds were significantly lower than that (883.89 ± 20.92) of 2% chitosan scaffolds ($P < 0.05$, analyzed by Mann-Whitney U test, n = 9).

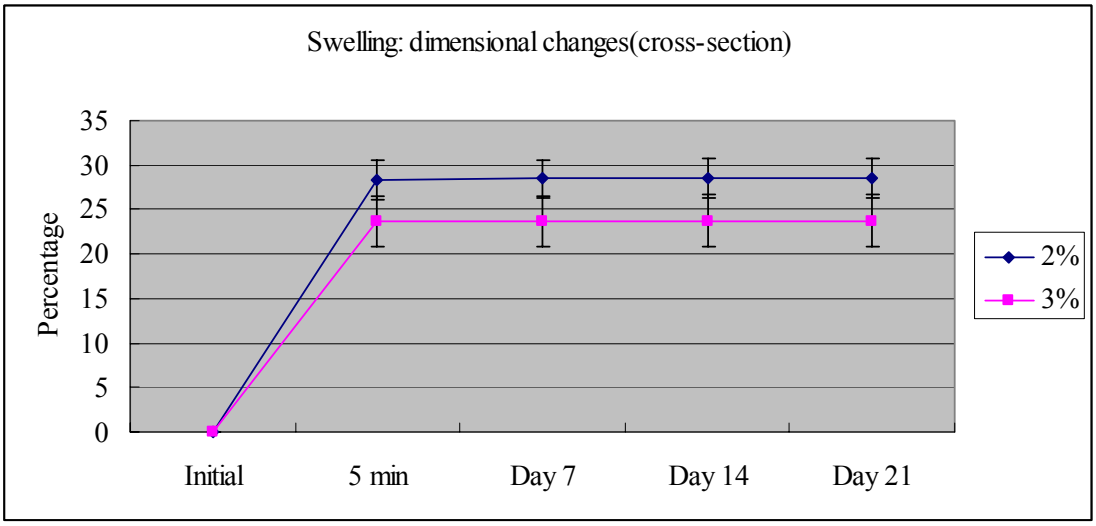


Figure 12 Dimensional changes of 2% and 3% chitosan scaffolds in cross-section

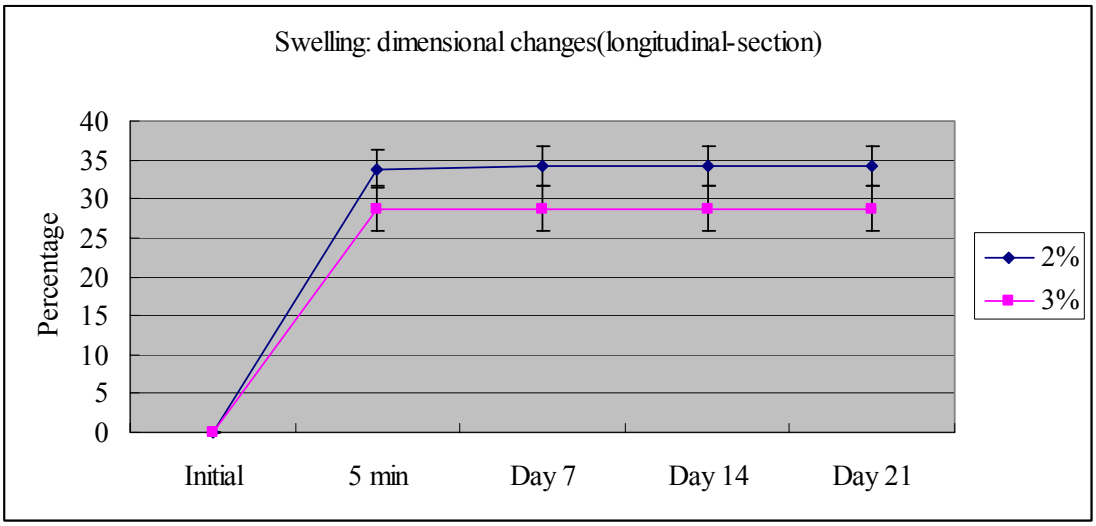


Figure 13 Dimensional changes of 2% and 3% chitosan scaffolds in longitudinal section

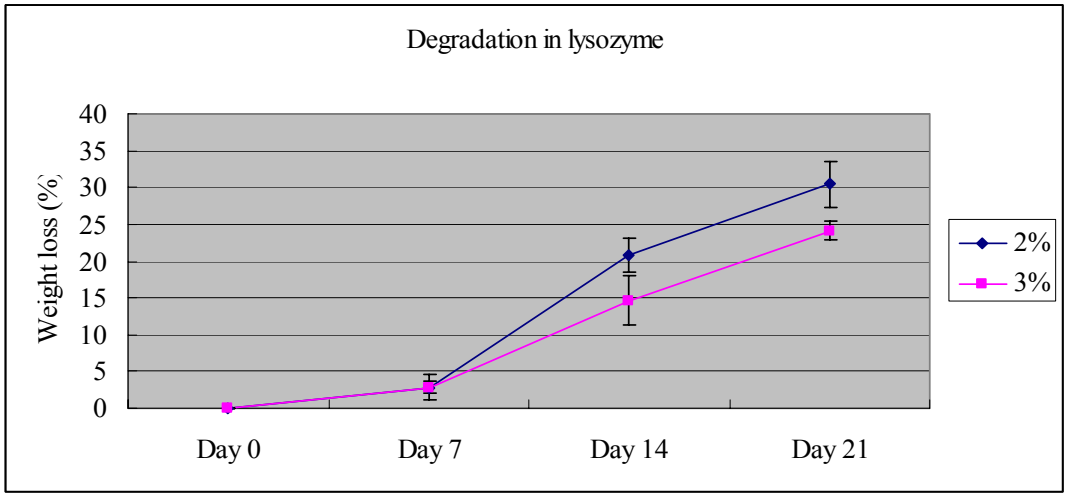


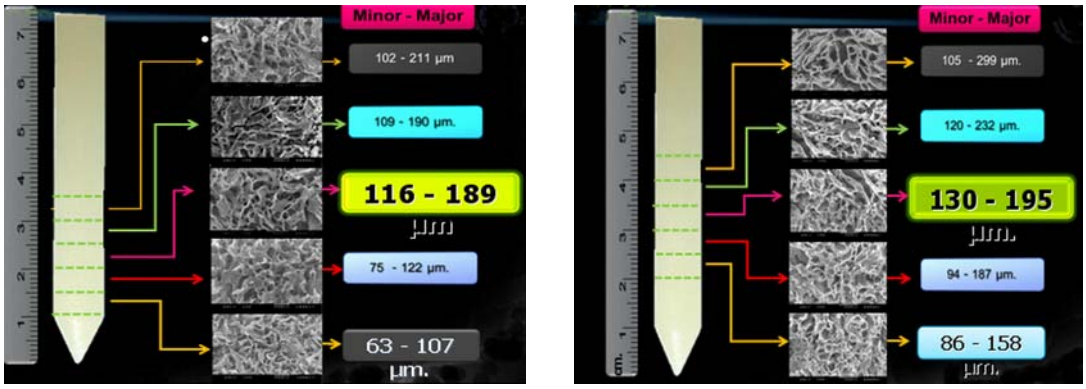
Figure 14 *In vitro* degradation of chitosan scaffolds, Errors bars represent means ± *SD*. Mann-Whitney U test

Table 11 *In vitro* degradation of chitosan scaffolds (Between different time point, $P < 0.05$, analyzed by Mann-Whitney U test, n = 9)

	Day 7	Day 14	Day 21
2%	2.88±1.75	20.86±2.32	30.42±3.20
3%	2.85±0.79	14.67±3.44	24.15±1.23

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[101]. 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[104] 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[104]. In the pilot study, we measured the pore size in different position of the 15 ml tubes after centrifugal step (Fig. 15). Researches of Arpornmaeklong *et al* and Itala *et al* showed that scaffolds with pore size less than 100 μm (50– 100 μm) allowed a favorable arrangement of cells[109, 110]. However a larger pore size promotes cell proliferation by facilitating revascularization

and transporting of oxygen as well as nutrients[111]. Pores of size 300–400 μm are optimal for in-vivo bone regeneration as they allow migration of blood vessels and promote faster migration of cells into the porous structure[112, 113]. Therefore, for different purpose of tissue regeneration, the selection of pore size is necessary. Respectively, the specimens of 2 to 2.5 cm and 3 to 3.5 cm from 2% and 3% chitosan scaffolds were chose for the further study (Fig. 15). The pore sizes of those specimens were calculated from cross-sectional SEM images (Fig. 6). According to these images, the pore sizes of 2% and 3% chitosan scaffolds were 188.71 ± 51.90 μm and 195.30 ± 67.21 μm, respectively. It can be found from the SEM images that the cross-sectional pore sizes between 2% and 3% chitosan scaffolds are similar, whereas the pore sizes of longitudinal section of 2% scaffolds are bigger than that of 3% chitosan scaffolds. Kose *et al* suggest that the average pore size should be at least three times (>100 μm) than the size of cells so that a single cell could establish contact with others [114]. 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[115]. Another study suggested that porous scaffold microstructure with minimal pore size ranging from 100 to 150 μm was usually required to allow tissue ingrowth[116]. The mean size range of osteoblasts is 10-30 μm[109]. Similarly, those cell sizes of our study were measured using cell counter (Countess, Invitrogen) in the pilot study. 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. Next, we examined the swelling ratio and strength of those chitosan scaffolds. It was demonstrated that there was no relationship between swelling ratio and DD (deacetylation degree)[101]. The results in Fig. 11 show that the chitosan scaffolds of our study possess a high swelling ratio which 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[105, 117, 118]. On the other hand, all of the chitosan scaffolds swelled and could maintain their structure without changing after their swelling (Fig. 12 and 13). The swelling promote attachment and growth of cells on the three-dimensional matrix by increasing pore size and the internal surface area[118]. These results are possibly due to the higher swelling ratio could maintain three-dimensional structure of the scaffolds[117, 118]. In addition, our results agree with the previous study[101] that swelling ratios of 3% chitosan scaffolds were significantly lower than that of 2% chitosan scaffolds since the pore sizes were relatively smaller and pore interconnectivities were lower.



a **b**

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

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

The degradation rate of the scaffolds should be compatible to the proliferation and differentiation rate of the migrated cells. An optimal degradation time is when the cells begin to differentiate. 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[72]. Chitosan is mainly degraded by lysozyme[107] which commonly exists in various human body fluids and tissues[114]. DD, distribution of acety1 groups and Mw (molecular weight) are responsible for the degradation rate of the chitosan[72]. In our study, the chitosan scaffolds significantly degraded in lysozyme solution during 7 to 14 days (Fig. 14). According to our cell proliferation and differentiation study *in vitro*, both of DPSC and SHED grew fast and differentiated after reaching confluence, and mineralized time of SHED is earlier than that of DPSC. This suggests that those scaffolds could support the proliferation of those cells, but in term of degradation rate those scaffolds are more suitable for the differentiation rate of SHED.

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. On the other hand, Mw of the chitosan did not influence toxicity[73]. DD of the chitosan in our study is 85%, which is assumed to be non-toxic. The cytotoxicity of the preparing chitosan scaffold specimens was evaluated according to ISO/EN 109935 guidelines[90] and modifying by previous studies[87, 89], that include MEM extraction, short term of indirect contact test (72h), observation of cell morphology and

floating cell under light microscope and MTT assay. The results 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. Moreover, cells of the test groups proliferated more than those of the control groups (Fig. 7 and Table 8). During the MEM extraction procedure, some chitosan oligomers maybe released. Those oligomers have been demonstrated to have biological activities[120]. *In vitro*, highly deacetylated chitosan stimulate fibroblast proliferation than less deacetylated chitosan[120]. Our results agree with those studies. Extraction media of the test group which maybe contains some chitosan oligomers. Those oligomers could stimulate the growth of cells. Similarly, those oligomers affect proliferation of SHED and DPSC positively. The results of our study indicate that those specimens are not only non-toxic but also promote growth of DPSC and SHED. On the other hand, 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 fibroblast proliferation indirectly[121], possibly through forming polyelectrolyte complexes with serum components such as heparin[121], or potentiating growth factors such as platelet derived growth factor (PDGF)[122]. 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. Therefore, DPSC maintained higher viability toward extraction media test groups due to the stimulation via chitosan oligomers. 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.

On the conventional plastic dishes, DPSC show a typical fibroblastic, spindle-shape to polygonal morphology[43, 94] and SHED present a spherical shape[13]. In the scaffolds, Figure 8 exhibits that the cells remained their normal morphology. Cells spread on the wall of the scaffolds, 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[123]. Those scaffolds might be suitable for DPSC and SHED to adhesion and proliferate.

The cytocompatibility of chitosan has been proved *in vitro* with myocardial, endothelial and epithellial cells, fibroblast, hepatocytes, condrocytes, keratinocytes[124] and periodontal ligament cells[125]. 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[126]. Cells could bind tightly within chitosan via electrostatic interaction. 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[72]. 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 the 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. 9 and Table 9). These possible reasons might be due to the initial seeding density, the size of culture plates and the opinions from Peng and Zhou: 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 contact inhibition[125]. 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 Fig. 9 and Table 9, 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 14th. On the other hand, the densities of SHED and DPSC in 2% scaffolds higher than those of 3% scaffolds could be found on day 21st. 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[124]. 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 stem cells, gingival fibroblasts and MG-63 are distinct. Those results suggest that 2% chitosan scaffolds are more suitable for cell growth.

The present study demonstrated that SHED possessed higher proliferation and earlier mineralization ability than DPSC. These results indicated that SHED was possibly immature than DPSC. 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. Those questions might be expanded by further studies. For chitosan scaffolds characteristics in this study, although those scaffolds without cell represent suitable swelling and degradation abilities in mimic body fluid and lysozyme solution respectively, the swelling and degradation toward those scaffolds with cell *in vitro* and *in vivo* are still required the further study. Present study showed that chitosan oligomers could simulate the DPSC and SHED growth in short term, but the long term effect of those is unknown. Moreover, this study suggested that culture condition of DPSC and SHED are possibly somewhat different. Further investigations are needed to prove this assumption. In addition, the further study should include the differentiation ability after cell seeding in scaffolds *in vitro*, the long-term animal trail, clinical trial, and whether chitosan scaffold could bring its antimicrobial ability into dental pulp therapy.

CHAPTER 5

CONCLUSIONS

The results of the study possibly indicated that

1. Proliferation of SHED is significantly higher than that of DPSC
2. Mineralization of SHED is possibly earlier than that of DPSC. However, although the nodules formation of DPSC appeared late and were not as many as that of SHED, they increased very fast after their appearance.
3. The fabricated chitosan scaffolds for this study are not only non-toxic but also could promote growth of DPSC and SHED.
4. Those chitosan scaffolds are suitable for DPSC and SHED to adhesion and proliferate.
5. The concentration of chitosan might affect the cell growth. 2% chitosan scaffolds are more suitable than 3% chitosan scaffolds for cell growth of SHED and DPSC. However, the response of this affection is different in each cell type.

There were some methodological limitations in the present study that should be considered when interpreting the results. Some of the results need the further investigation such as the result indicated that the culture condition of DPSC and SHED are possibly somewhat different. Moreover, the further study should include the differentiation ability after cell seeding in scaffolds *in vitro*, the long-term animal trail, and the clinical trail.

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APPENDIX

APPENDIX

Solutions

1. Antibiotics 20%

Penicillin	1,000,000 units/5 ml
Streptomycin	1 gm/5 ml
Kanamycin	1gm/5 ml
Mycostatin	5 ml

2. Alizarin Red solution

Alizarin Red powder:	0.5 g
PBS:	100 ml

Adjusted the pH to 4.1-4.3

3. Dexamethasone 500 µM (200 ng/µl) stock solution, 5 ml

Dexamethasone 500 µM	1 mg
Absolute ethanol	5 ml

4. Glutaraldehyde 2.5%

Glutaraldehyde 25%	1 ml
PB buffer 0.2 M	9 ml

5. MTT solution 5 mg/ml

MTT powder	0.5 g
Double distill water	100 ml

Filtrated the MTT solution

6. PBS

NaCL	8 g
KCL	0.2 g
Na_2HPO_4	1.44 g
KH2PO4	0.24 g

Continuous stirring for complete dissolving

Adjust volume to 1,000 ml using deionized water and adjust pH to 7.4

7. PB buffer 0.2 M

$NaH_2PO_{4:}$ 2.4 g dissolved in 100 ml deionized water $\rightarrow$ Solution A

$Na_2HPO_{4:}$ 2.84 g dissolved in 100 ml deionized water → Solution B

23 ml of Solution A + 77 ml of Solution B

Adjusted the pH to 7.2

8. PB buffer 0.2 M

   50 ml of PB buffer 0.2 M + 50 ml of deionized water

9. Trypsin/EDTA 1%

PBS	9 ml
Trypsin/EDTA 10x	1 ml

Equipments and instruments

1. Autoclave Hiclave ™, Hirayama Manufacturing Corp., Japan
2. Automatic pipetter, Portable Pipet-aid XP, Drummond Scientific Company, USA
3. Biotrak ™ II microplate reader, Amersham Biosciences, Biochrom Ltd., UK
4. Countess ™ automated cell count, , Invitrogen Corp., USA
5. Digital camera, NIKON DXM 1200, Japan
6. Hot air oven, Mammert, Germany
7. Liquid nitrogen tank, Taylor-Wharton, USA
8. Microflow laminar downflow workstation, Bioquell Lab, UK
9. Microscope, Inverted Nikon TS 100E, Japan
10. pH meter, Precisa Instruments AG, Switzerland
11. Popetter 1000-microlitre, Pipetman Gilson, France
12. Popetter 200-microlitre, Pipetman Gilson, France
13. Popetter 100-microlitre, Pipetman Gilson, France
14. Popetter 20-microlitre, Pipetman Gilson, France
15. Refrigerated centrifugator, Kendro Laboratory products, Germany
16. Scanning electron microscope, JSM-5800LV, JEOL Ltd, Japan
17. Series II water jacketed CO_2 incubator, Thermoforma, USA
18. Sputter Coater, SPI-Module ™, SPI supplies, USA
19. Vortex mixer, Vortex Genie 2, Scientific Industries Inc., USA
20. Water bath, Mammert, Germany

21. Weight meter, Adventurer ™ Electric Balance Ohaus, Ohaus Corp., USA

Disposable materials

1. 24-well plate, Nunc ™, Denmark
2. 48-well plate, Nunc ™, Denmark
3. 96-well plate, Nunc ™, Denmark
4. 100 ml culture plates, Nunc ™, Denmark
5. 70 µl cell strainer, Becton Dickinson, USA
6. Cryotube 1.8 ml, Nunc ™, Denmark
7. Centrifuge tube 15 ml, Neptune™, Switzerland
8. Centrifuge tube 50 ml, Neptune™, Switzerland
9. T-25 tissue cultural flask, TPP, Switzerland
10. T-75 tissue cultural flask, TPP, Switzerland
11. Microcentrifuge tube 1.5 ml, Treff AG, Switzerland
12. Micropipette tip 1000 µl, Treff Lab, Switzerland
13. Micropipette tip 200 µl, Treff Lab, Switzerland
14. Parafilm "M" Laboratory film, Pechiney plastic packaging, USA

Chemical agents

1. Absolute ethanol, Merck, Thailand
2. Alizarin Red S, Nacalai Tesque Inc., Japan
3. β-glycerophosphate, Sigma USA
4. Collagenase type I, Sigma, USA
5. Dexamethasone, Sigma, USA
6. Dispase, Sigma, USA
7. Dimethyl sulfoxide (DMSO), Amresco, USA
8. Dulbecco's Modified Eagel Media (D-MEM), Gibco, USA
9. Fetal bovine serum (FBS), Biochrom AG, Germany
10. Glutaraldehyde 25%, Electron Microscopy Sciences, USA
11. Hexamethyldisilazane (HMDS), Sigma, USA
12. L-ascorbic acid 2-phosphate, Sigma, USA

13. L-glutamine, Gibco, USA
14. MTT kit, Sigma, USA
15. Trypsin/EDTA solution, Biochrom AG, Germany
16. WST-1 assay, Roche, Germany

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List of Publication and Proceeding

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