การศึกษาการสร้างกระดูกโดยเซลล์ต้นกำเนิดจากฟันน้ำนมมนุษย์ ในโครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์ในสัตว์ทดลอง

นางสาวสุภัทรา ศิลานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IN VIVO STUDY OF BONE FORMATION BY STEM CELL FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) IN POLYCAPROLACTONE/HYDROXYAPATITE SCAFFOLDS.

Miss Supattra Silanon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Oral and Maxillofacial Surgery

Department of Surgery
Faculty of Dentistry
Chulalongkorn University

Academic Year 2011

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Thesis Title	IN VIVO STUDY OF BONE FORMATION BY STEM CELL FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) IN POLYCAPROLACTONE/HYDROXYAPATITE SCAFFOLDS
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สุภัทรา ศิลานนท์ : การศึกษาการสร้างกระดูกโดยเซลล์ต้นกำเนิดจากฟันน้ำนมมนุษย์ในโครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์ในสัตว์ทดลอง (IN VIVO STUDY OF BONE FORMATION BY STEM CELL FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) IN POLYCAPROLACTONE/HYDROXYAPATITE SCAFFOLDS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ทพ.ดร. อาทิพันธุ์ พิมพ์ขาวขำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, 64 หน้า.

วัตถุประสงค์ ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินการเกิดเนื้อเยื่อกระดูกของเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงฟันน้ำนมที่เลี้ยงบนโครงร่างพอลีคาโปรแลคโตน/ไฮดรอกซีอะปาไทต์ เมื่อฝังในหนูแรทจำนวน 6 ตัว

วัสดุและวิธีการ เซลล์ต้นกำเนิดที่เตรียมได้จากเนื้อเยื่อโพรงฟันน้ำนม เป็นเซลล์ที่มีคุณสมบัติหลายประการของเซลล์ต้นกำเนิด มีความสามารถในการเจริญเติบโตอย่างรวดเร็ว รวมทั้งสามารถแปรสภาพไปเป็นเซลล์ได้หลายชนิด เช่น เซลล์สร้างเนื้อฟัน เซลล์ไขมัน เซลล์ประสาท และเซลล์กระดูก หลังจากการตรวจสอบการแสดงออกของโปรตีนที่บ่งชี้ความเป็นเซลล์ต้นกำเนิดชนิดมีเซนไคม์ รวมทั้งความสามารถในการแปรสภาพเป็นเซลล์สร้างเนื้อเยื่อแข็งในจานเลี้ยงเซลล์แล้ว ในกลุ่มทดลองเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงฟันน้ำนมจะถูกหว่านลงในโครงร่างพอลีคาโปรแลคโตน/ไฮดรอกซีอะปาไทต์ และนำไปฝังในรอยแผลขนาดประมาณ 4 มิลลิเมตรบริเวณกระโหลกศีรษะของหนูแรท โดยในกลุ่มควบคุมจะทำการฝังเฉพาะโครงร่างที่ไม่มีเซลล์ต้นกำเนิด หลังจากฝังไป 8 สัปดาห์ เนื้อเยื่อจะถูกแยกออกมาผ่านกระบวนการตรึง การกำจัดเนื้อเยื่ออนินทรีย์ ฝังลงในพาราฟิน และตัดเป็นแผ่นเนื้อเยื่อทำการย้อมสีแมสซอนไตรโครม

ผลการศึกษา ผลการวิเคราะห์จากแผ่นเนื้อเยื่อ พบว่าระดับของเนื้อเยื่อคล้ายกระดูกและกระดูกชนิด woven จะเกิดขึ้นมากกว่าในโครงร่างที่มีเซลล์ต้นกำเนิด เมื่อเปรียบเทียบกับกลุ่มควบคุม

สรุป ผลการทดลองแสดงถึงความสามารถที่น่าจะเป็นไปได้ของเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงฟันน้ำนมในการนำไปใช้ในงานวิศวกรรมเนื้อเยื่อกระดูก อย่างไรก็ตามยังคงต้องมีการศึกษาเพิ่มเติมถึงความปลอดภัยในการใช้งานระยะยาวต่อไป

ภาควิชา ศัลยศาสตร์	ลายมือชื่อนิสิต
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5176139732 : MAJOR ORAL AND MAXILLOFACIAL SURGERY

KEYWORDS : Hydroxyapatite (HAp), Osteogenic differentiation, Polycaprolactone (PCL), Stem cell from human exfoliated deciduous teeth (SHED).

SUPATTRA SILANON: IN VIVO STUDY OF BONE FORMATION BY STEM CELL FROM HUMAN EXFOLIATED DICIDUOUS TEETH (SHED) IN POLYCAPROLACTONE/HYDROXYAPATITE SCAFFOLDS. ADVISOR : ASST. PROF. ATIPHAN PIMKHAOKHAM, Ph.D., CO-ADVISOR : PROF. PRASIT PAVASANT, Ph.D., 64 pp.

Objective The aim of this study was to examine the ability of SHED seeded in polycaprolactone/hydroxyapatite (PCL/HAp) scaffolds to differentiate into osteogenic cell in 6 Wistar Rat.

Materials and Methods Stem cell from human exfoliated deciduous teeth (SHED) has been documented to possess several stem cell characteristics including a highly proliferative rate and the capability to differentiate into several cell types such as odontoblasts, adipocytes, neural cells, and osteoblasts. The expression of mesenchymal stem cells as well as the ability of osteogenic differentiation of SHED in vitro was examined. SHED cells were seeded on the PCL/HAp scaffold for 30 minutes and transplant into 4 mm. diameter defects in calvarial of Wistar Rat. In control, the scaffolds without cells were transplant into the defect. After 8 weeks, the calvarials were removed, fixed with formaldehyde, decalcified with EDTA and processed for paraffin section. The sections were stained with Masson's Trichrome.

Result The results indicated the increased in osteoid formation and woven bone structures in the scaffold with SHED compared to the control using scaffold alone.

Conclusion SHED is a useful source of stem cells. The results suggest the potential to use these cells for bone tissue engineering. However, further studies are necessary to investigate the safety of the protocol in longer period of treatment.

Department : Oral and Maxillofacial Surgery .... Student's Signature ....

Field of Study : Oral and Maxillofacial Surgery .... Advisor's Signature ....

Academic Year : 2011 .... Co-advisor's Signature ....

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CHAPTER I
INTRODUCTION

Background

There is a great demand for regeneration of orofacial defects caused by trauma, tumor, genetic malformation, and periodontal diseases. Autologous bone grafting has been the gold standard because of its obvious advantages in osteogenic capacity, osteoconduction, mechanical properties, and the lack of adverse immunological response. On the other hand, autologous bone grafting techniques have some limitations, such as the requirement of additional surgery for harvesting, the availability of grafts of sufficient size and shape, and the risk of donor site morbidity, which may include fracture, long lasting pain, nerve damage, and infection. Allografts and xenografts are also used, but it can be associated with the transmission of disease and tendency to elicit an immune response.

This major clinical requirement has stimulated interest in developing new therapies that involve bone regeneration and has led to the hope that bone tissue engineering may provide alternative solutions providing "living" constructs that posses the potential to integrate with the surrounding native tissue. [1] Due to obtain quality and quantity of bone graft without subsequence problems such as disease transmission risk and secondary surgical intervention, the field of tissue engineering has developed. Tissue engineering is done by combining scaffolds, cells and suitable biochemical factors to improve or replace biological function. For bone tissue engineering, new strategies have been developed to improve the osteogenic potential of stem cells, which is combined with appropriate signaling molecule, in three-dimensional scaffolds for desired tissue.

The new source of stem cells is dental pulp stem cell which could be obtained from exfoliated deciduous teeth. Stem cells from human exfoliated deciduous teeth are derived from a readily accessible tissue source, human deciduous teeth that are expendable and routinely exfoliated in childhood with little or no morbidity to the patient. In fact, orofacial and long bones originate from neural crest cells and the mesoderm,

respectively. [2,3] Orofacial bone is not an optimal donor source, due to the limited size. Therefore, stem cells from human exfoliated deciduous teeth, originating from neural crest cells and expressing many neural cell markers, could be a practical resource for repair of orofacial bone defects. Stem cells from human exfoliated deciduous teeth possess the mesenchymal charateristics and have been demonstrated that they can differentiate into osteoblast, odontoblast adipocytes and neurons both in vitro and in vivo. Moreover, there are several reports showing that mesenchymal stem cells obtain the immunosuppressive properties and has showing recently that SHED could be used in xenograft application. However, the role of dental pulp stem cells in tissue engineering still requires more investigations.

A variety of porous scaffold polymers (naturally derived or synthetic matrices) have shown promising potential for bone tissue engineering applications. Natural polymers such as collagen are of a major interest because they are biodegradable and biocompatible, allowing cells to adhere proliferate and differentiate without the problems of inflammatory reactions and cytotoxicity which are often associated with synthetic polymers. However, natural polymers are structurally complex compare to synthetic polymers. Therefore, technical manipulation is complicated and more elaborate causing variation in processing of consistent scaffold matrices. Additionally, these polymers lack the mechanical properties, which are required to withstand forces, which for example exist in the bone environment. Synthetic polymer matrices, polylactic acid (PLA) polyglycolic acid (PGA), polycaprolactone (PCL) which are free of potential contamination, can be readily engineered. However, one of the major problems with these types of scaffolds is the lack of osteoconductivity compared to the current gold standard of allograft. Researchers have tried to overcome these problems by combining ceramics such as hydroxyapatite (HA) and calcium phosphate with the synthetic polymers. Recently, by using a solvent casting and particulate leaching technique, we have fabricated the novel PCL/HAp scaffold with 400-500 μm porosity. [4] This novel fabricated scaffold has been shown that it can support growth and differentiation of

bone cells both in vitro and in vivo. However, the scaffold's ability in supporting osteogenic differentiation of dental pulp stem cells is still not elucidated.

From all above reasons, the aim of this study is to examine the ability of SHED seeded in PCL/HAp scaffold to differentiate into osteogenic cell and to determine the immunomodulatory properties of SHED when used as a xenograft. The result of the study will provide knowledge that could be applied for dental treatment. Furthermore, the study will elucidate the possible of PCL/HAp scaffold as a material of choice for the replacement of defected bone tissues.

Research Objective

1. To examine the ability of SHED seeded in PCL/HAp scaffold to differentiate into osteoblasts in vivo.
2. To determine the immunomodulatory properties of SHED when using as a xenograft.

Research hypothesis

Ho_1: PCL/HAp scaffold can support the osteogenic differentiation in vivo.

HA_1: PCL/HAp scaffold cannot support the osteogenic differentiation in vivo

Ho_2: SHED embedded PCL/Hap scaffold can form bone in Wistar rat.

HA_2: SHED embedded PCL/Hap scaffold cannot form bone in Wistar rat

Expected Benefit

The data will be shown that SHED and the developed three dimensional PCL/HAp scaffold may be useful to induce bone regeneration. And we may use tissue engineering approach by SHED, PCL/HAp scaffold to replacing damaged or diseased bone tissue in the future.

CHAPTER II
REVIEW OF BASIC KNOWLEDGES AND LITERATURES

REVIEW OF BASIC KNOWLEDGES

Desired features of a bone tissue engineering

Bone is a high dynamic tissue which comprises mineralized extracellular matrix embedded with bone cells, blood vessels, and nerves. Bone has three major cell types that are bone-specific: osteocytes, osteoblasts, and osteoclasts. Osteocytes, mature cells inside bone lacunae, communicate with other osteocytes through long cellular processes, sense mechanical stress in bone, and send signals for bone remodeling as a result of mechanical stress. The responding cells are osteoblasts, cells specialized to secrete the unique collagen-rich extracellular matrix in bone that enables mineralization. Finally, osteoclasts are macrophage-like cells that degrade the bone structure through a combination of localized acidification (removes the minerals) and protease secretion (breaks down matrix). Osteoclasts tunnel through bone and are usually followed close behind by osteoblasts. Bone is in a constant state of remodeling in healthy individuals. [5]

Bone formation can take place via a direct (intramembranous) or indirect (endochondral) process. Intramembranous ossification occurs during embryonic development of the cranial vault bones when there is a direct ossification of the mesenchymal tissues. Primitive mesenchymal cells are transformed into osteoprogenitor cells and mature osteoblasts leading to formation of bone with all of its histological characteristics.[6] This process occurs typically in the calvarial bones, mandible, and the clavicle. In contrast, the epiphysial growth plate in the appendicular skeleton is characterized by the intracartilagenous bone formation. In this process primitive mesenchymal cells differentiate in a two-step process into mature bone. In the first step, mesenchymal cells transform into chondroblasts, form collagen and other elements of bone matrix, become ossified, and lead to mature bone. In embryonic phase, a formation of bone through intramembranous compared with intracartilaginous

process depends on the anatomical site. As stated earlier, cranial structures and mandible undergo an intramembranous growth process whereas the appendicular skeleton forms through an intracartilagenous process. [7]

To consider the desired features of potential tissue-engineering materials, it is important to understand two concepts in bone regeneration for tissue-engineering constructs: osteoconduction and osteoinduction.

Osteoconduction provides a framework to support a bone formation which will allow capillaries and osteopregenetor cells to grow inside during bone grafting. [8] A 3-dimension framework can guide a repair for bone healing. Many studies find a variety of material which can be used as a bone graft substitute such as the tubular absorbable polymers used in long bone defects. The polymers are believed to maintain an osteogenic-rich medullary environment within the defect. Therefore, they allow direct bone growth onto the polymer skeleton. [9]

In contrast, osteoinduction recruits the pluripotential cells from a non-osseous environment to differentiate into chondrocytes and osteoblasts during bone formation. An osteoinductive material repairs bone in location that normally not heals if left untreated; this property is different from osteoconductive material which repairs bone in location that heals. [10] It is mediated by graft-derived factors such as bone morphogenetic proteins and other growth factors.

Functions of bone grafts include osteogenesis and mechanical support. Osteogenesis is a formation of bone which is derived from grafted cell or host cell. It also provides a structural support for host bone as a primary function and a result of remodeling. [9]

Types of grafting are classified by origins of materials which can be autogenous, allogenous, xenogenous bone grafts or synthetic materials. An autologous bone graft is a transplantation of tissue or organ within the same subject from one site to another. The disadvantages of this method are inadequate supply, donor-site morbidity and the procedure prolongs the time of surgery. [8]

An allogenous bone graft is transplanted within the same species. It carries a risk of disease transmission as well as immune rejection. [11.12] To avoid immune reaction, xenogenous bone graft which is removed all of the organic components is used instead. This type of bone graft is derived from a donor of a different species such as a bovine bone. The remaining inorganic substances still provide a natural architectural matrix. This method still has a problem of quantity and cost. [13]

Synthetic composite materials produced from metals, alloys, ceramics and polymers are used to avoid the problem of quantify and cost. Metals and ceramics are used to replace hard tissue, while polymers are used in soft tissue applications. Composites are widely used for both applications. The details of each material are shown in figure 1.

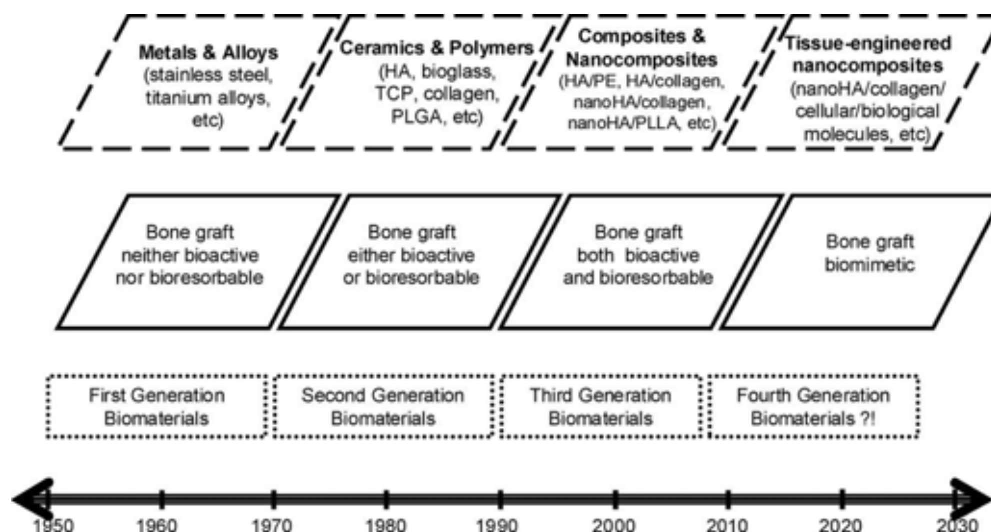


Figure 1. The evolution of biomaterials in bone grafting.

(Ref: Murugan, R. and Ramakrishna, S. Development of nanocomposites for bone grafting. Composites Science and Technology 65 (2005): 2385-2406.)

These materials used in fabrication of tissue-engineered bone have some significant advantages such as providing structural integrity within the body and eventually be able to break down at intended period.

Needs for bone grafts materials lead to the synthesis of many kinds of materials with different properties. These materials can be divided into 4 generations based on the nature of the materials, and relation between the grafts and the host tissues (Figure 1). The first generation of bone grafting biomaterials have remarkable mechanical properties but they are neither bioresorbable nor bioactive. More important, this kind of bone grafts has limited lifetime (usually less than 10-15 years) and need to be extracted and replaced surgically. Representative biomaterials from the first generation are: iron, cobalt, chromium, titan, steel and their alloys. [14, 15]

The second generation bone grafting biomaterials are at least bioresorbable or bioactive, and they do not have to be replaced in time. The representatives are calcium phosphates (especially hydroxyapatite and tricalcium phosphate), bioglasses[16], alumina[17]; zirconia[18] and the polymers such as poly ε-caprolactone[19], and polyurethanes. [20, 21]

The third generation of bone grafting biomaterials are both bioresorbable and bioactive. These biomaterials have superior properties. In addition, these biomaterials present higher specific properties than the first two generations. The materials are resorbed and new bone is formed in place of the bone graft shortly after implantaion. The properties of these nanocomposite materials are strongly influenced by composition and morphology. Thus many researchers look for materials which are similar to natural bones in composition, mineralogy, and morphology. The representative biomaterials of the third generation are (nano)hydroxyapatite/collagen[22], (nano)hydroxyapatite/collagen/hyaluronic acid [23], hydroxyapatite/poly-L-lactic acid [24], and hydroxyapatite/chitosan. [25]

Finally, the fourth generation of bone grafting biomaterials is similar to the third generation but improved by the presence of bony cells, growth factors, bone morphogenetic proteins etc.

Tissue engineering

Tissue engineering technologies involve the successful interaction between three components: (1) the scaffold that holds the cells together to create the tissue physical form, (2) the cells that create the tissue and, (3) the biological signaling molecules (such as growth factors) that direct the cells to express the desired tissue phenotype. [26]

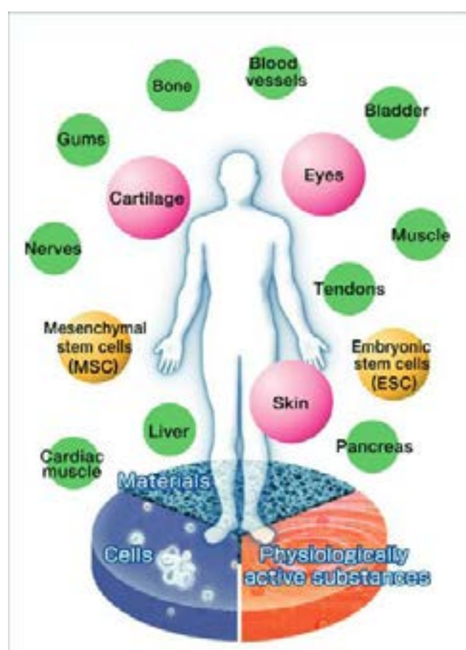


Figure 2. Component of tissue engineering

(Ref: http//www.jpte.co.jp/English/technologies/index.html)

Scaffold design

The tissue engineering scaffolds serve the following functions: (1) to encourage cell-material interactions i.e. cell attachment, differentiation and proliferation, eventually leading to the deposition of extracellular matrix, (2) to permit the transport of nutrients, wastes and biological signaling factors to allow for cell survival, (3) to biodegrade at a controllable rate which approximates the rate of natural tissue regeneration, and (4) to provoke minimal immune and/or inflammatory response in vivo. For proliferating and

maintaining their differentiation functions, the cells use a 3D scaffold that acts as a template, allowing cells to produce and deposit extracellular matrix (ECM) that would not be possible in 2D environments. [26]

Properties of scaffolds for tissue engineering

1. **Biocompatibility**

Biocompatibility is an ability of scaffold to perform in a specific application without eliciting a harmful immune or inflammatory reaction [26]. Tissue response after a polymeric scaffold implant occurs in three stages. Stage 1 happens during the first 1 to 2 weeks after implantation and is characterized by acute and chronic inflammatory responses. Acute inflammation can be sudden and last for minutes or days depending on the extent of the injury. Chronic inflammation results from long-term presence of inflammatory stimuli. It is confined to the implantation site. In general, the stage 1 response is independent of the degradation rate of the polymer. When the numbers of monocytes and macrophages increase, stage 2 will begin. In stage 2, fibrous encapsulation of the foreign material is initiated. Unlike stage 1, the length of this stage depends on the rate of biodegradation of the scaffold. Fibrous encapsulation continues in stage 3. Length of this stage depends on the degradation rate of the polymer. With slowly degrading polymers, this stage can be response lasts from weeks to months. In contrast, with rapidly degrading polymers, this stage can be as short as 1 to 2 weeks. [27]

In bone tissue engineering, the immune response is a major concern because degradation products cause failure in many orthopedic implants. Therefore, at the stage of a biomaterial design, one must consider both the initial properties of the scaffold and its degradation products and their effect on the host. Materials intended for implantation should minimize intensity and duration of response.

2. **Biodegradability**

Scaffold should degrade with a controllable degradation rate, (approximating the rate of natural tissue regeneration), as well as with controllable degradation

products. The breakdown products should be non-toxic and easily transported away from the body via metabolic pathways or renal filtration system as it degrades. [28]

There are two types of degradation: surface and bulk. [29] Surface degradation of scaffold has a character as a gradual decrease in the dimensions of the scaffold with no change in its mechanical attributes. [30] Surface degradation is look like soap dissolution. The material degrades at the surface by a constant rate. This cause a construct to thin out, yet bulk integrity and structure are maintained. When the material degrades, the size of the construct decrease, but the density remains unchanged. This character allows polymer to maintain mechanical integrity, a property critical for bone tissue engineering.

Bulk degradation is characterized by loss of material throughout the scaffold's volume during degradation. Thus, mechanical strength decreases during bulk degradation and is dependent on the degradation rate. [30]

3. Mechanical properties

The mechanical properties of the scaffold should be designed to meet the specific requirements of the tissue to be regenerated at the defect site. Furthermore, at the time of implantation, the scaffold should have sufficient mechanical integrity to allow for handling by the clinician, to be able to withstand the mechanical forces imposed on it during the implantation procedure and survive under physical conditions.

For proper tissue regeneration without significant deformation, a scaffold should provide a mechanical modulus of 10 to 1500 MPa for hard tissue and 0.4 to 350 MPa for soft tissue. [31]

4. Scaffold architecture

It is well recognized that the pore size of scaffolds plays an important role for cell binding, migration, ingrowth and tissue ingrowth and regeneration. Generally, it was reported that the large pore size or porosity of the scaffold can allow effective nutrient supply, gas diffusion and metabolic waste removal but lead to low cell attachment and intracellular signaling.

The minimum pore size required to regenerate mineralized bone is generally considered to be about 100 μm based on the early study of Hulbert et al. 1970, where calcium aluminate cylindrical pellets with 46% porosity were implanted in canine femorals. Large pores (100-150 and 150-200 μm) showed substantial bone ingrowth. Smaller pores (75-100 μm) resulted in ingrowth of unmineralized osteoid tissue. Smaller pores (10-44 and 44-74 μm) were penetrated only by fibrous tissue. However, subsequent studies have shown better osteogenesis for implants with pores>300 μm. [(32)]

Porosity is defined as "the percentage of void space in a solid" and it is a morphological property independent of the material. [(33)] Pores are necessary for bone tissue formation because they allow migration of endothelial cells: to promote osteoblasts or progenitors cells; to promote vascularization of mesenchymal cells; or facilitate osteoblast proliferation and differentiation (i.e. osteogenesis). In addition, a porous surface improves mechanical stability and interlocking at the critical interface between the implant biomaterial and the surrounding natural bone.

The necessity for porosity in bone regeneration has been show by Kuboki et al., 1998 [(34)] using a rat ectopic model and solid and porous particles of hydroxyapatite for BMP-2 delivery: no new bone formed on the solid particles, while in the porous scaffolds direct osteogenesis occurred.

An increase in the void volume results in a reduction in mechanical strength and stiffness of the scaffold, which can be critical for regeneration in load-bearing bones. Pore size can be increased in the extent which maintains mechanical requirements. It depends on a number of factors including the nature of the biomaterial and the processing conditions used in the fabrication of 3D scaffolds. [(35)]

5. **Manufacturing technology**

Surprisingly, the success of tissue engineering therapy greatly depends on the microstructure and the morphology of the porous structure, which is governed by the fabrication method. The regeneration of specific tissues aided by synthetic materials mostly depends on the porosity (void volume fraction), pore size, pore shape, pore distribution (interconnection between the pore), architecture (overall shape of the

object), as well as to the mechanical properties of the scaffolds. In general, a high porosity and high interconnectivity between the pores are necessary to allow cell growth and flow transport of nutrients and metabolic waste. [36] Therefore, several different techniques have been introduced to fabricate the porous conventional biodegradable polymer matrices. The most common techniques are listed as followed.

Solvent Casting & Particulate Leaching (SCPL): The approach is used in this experiment which allows preparation of porous structures with regular porosity, but with a limited thickness. First polymer is dissolved into a suitable organic solvent (e.g. polylactic acid could be dissolved into dichloromethane, polycaprolactone in chloroform) then the solution is cast into a mold filled with porogen particles. Such porogen can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. After the polymer solution has been cast. The solvent is allowed to fully evaporate, and then a composite structure in a mold is immersed in a bath of a liquid suitable for dissolving the porogen: water in case of sodium chloride, saccharose and gelatin or an aliphatic solvent like hexane for paraffin. Once the porogen has been fully dissolve. A porous structure is obtained. Sizes of the porogen particles affect size of the scaffold pores. The polymer to porogen ratio is directly correlated to amount of porosity of final structure. Various bioceramic fillers can be incorporated into porous polymer matrices to improve their mechanical properties or to bring bioactivity to the material. [37] Other than the small thickness range that can be obtained, another drawback of SCPL lies in its use of organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold.

Gas foaming: A technique using gas as a porogen has been developed. First disc shaped structures made of the desired polymer are prepared by means of compression molding using a heated mold. The discs are then placed in a chamber where are exposed to high pressure CO_2 for several days. The pressure inside the chamber is gradually restored to atmospheric levels. During this procedure the pores are formed by the carbon dioxide molecules that abandon the polymer, resulting in a sponge like structure. The main problems of such a technique are caused by the

excessive heat used during compression molding which prohibits the incorporation of any temperature labile material into the polymer matrix. Another concern is relating to the fact that the pores of material fabricated by this technique do not form an interconnected structure.

Emulsification/Freeze-drying: This technique uses temperature change to create a porous structure. [38] Synthetic polymer is dissolved into a suitable solvent such as PLGA are dissolved in cold solvents, glacial acetic acid, or benzene, and water is added to create an emulsion. [38-40] Before the two phases separate, the emulsion is quick-frozen (immersion into liquid nitrogen) and the resulting ice crystals are sublimed by the freeze-drying technique. This leaves a highly connected porous matrix. With this technique, pore size can be controlled by altering the freezing rate; in general, faster freezing leads to smaller pores. [39] However, it is difficult to control pore structure and porosity is often irregular. However, freeze-drying by itself is still a commonly employed technique for the fabrication of scaffolds. In particular it is used to prepare collagen sponges: collagen is dissolved into acidic solutions of acetic acid or hydrochloric acid that are cast into a mold, frozen with liquid nitrogen then lyophilized.

Phase separation: this technique is use to isolate components of a heterogenous mixture. In this process the polymer is first dissolved in a solvent such as molten phenol, naphthalene, or dioxane. The polymer solvent solution is cooled, causing liquid-liquid or solid-liquid phase separation, with the polymer in a separate phase from the solvent. The solvent is then evaporated, leading to the formation of a porous polymer structure. One considerable advantage of this technique is that biomolecules can be incorporated into the scaffold without exposure to harsh chemical or thermal conditions. [31]

CAD/CAM Technologies: Since most of the above described approaches are limited when it comes to the control of porosity and pore size, computer assisted design and manufacturing techniques have been introduced to fabricate tissue engineering materials. [41] First a three-dimensional structure is designed using CAD software, and then the scaffold is realized by using ink-jet printing of polymer powders or through Fused Deposition Modeling of a melting polymer.

Polycaprolactone/hydroxyapatite scaffold

Poly (e-caprolactone) (PCL) is aliphatic polyester with semi-crystalline properties. PCL has repeating unit of one ester group and five methylene groups. It has a low melting temperature of 58 to 63ºC and glass transition temperature of -60ºC. PCL is formed through ring-opening polymerization of the cyclic monomer ε-caprolactone. Catalysts sush as stannous octate are used to catalyse the polymerization (Figure 3). The degradation of PCL occurs by bulk or surface hydrolysis of these ester linkages, resulting in byproduct of caproic acid. [30]

Due to its low cost, sustained biodegradability, availability at low molecular weight, more stable in ambient condition, and readily available in large quantities [42], PLC is very attractive.

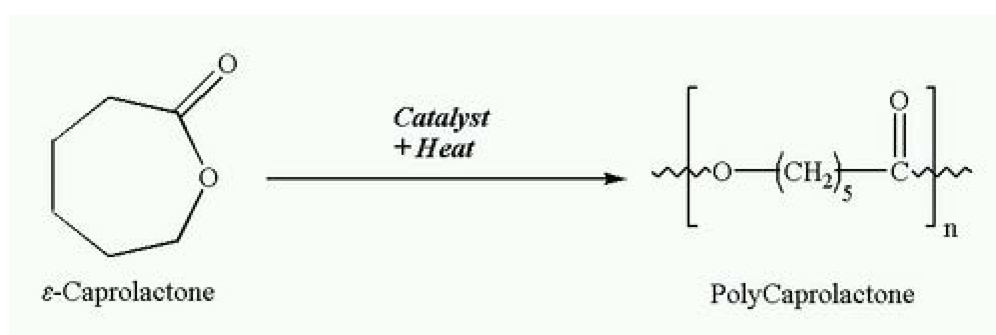


Figure 3. Ring opening polymerization of ε-caprolactone to polycaprolactone

(Ref: http://www.Synthetic Biodegradable as Medical Devices (MPB archive, Mar 98).html)

PCL is regarded as a soft and hard-tissue compatible material for applications including resorbable suture, drug delivery systems and recently bone graft substitutes. PCL has received Food and Drug Administration (FDA) approval for bone and cartilage repair. [43] Interesting property of PCL is the kinetics of biodegradation, which is considerably slower than other aliphatic polyesters. [42] For bone scaffolds this can provide good implant bonding and maintaining biological and mechanical integrity. [44, 45]

The degradation of PCL can be split into two phase:

The first stage involves a decrease in molecular weight without mass loss and deformation. Afterwards the materials gradually lost strength and broke into pieces. In the second stage, low molecular weight PCL pieces metabolized by unknown process which mainly involves intracellular phagocytosis [46, 47] and ultimately excreted from the body through urine and feces. The material did not cumulate in the any body organs. [48]

Hydroxyapatite

Hydroxyapatite($Ca_{10}(PO_4)_6(OH)_2$) is the main mineral component of bone and because of its high bioactivity and biocompatibility is commonly used as filler in polymer-based bone substitutes. [49] Scaffold materials made of hydroxyapatite have been found to have a relatively slow rate of degradation and are intrinsically brittle [50] and insufficient toughness. [51]

Due to some of the problems associated with using scaffolds synthesized from a single phase biomaterial (for example: poor mechanical properties and biocompatibility of natural and synthetic polymers respectively, and poor degradability of bioceramics), then researchers have developed composite scaffolds by comprising two or more phases to combine the advantageous properties of each phase.

The addition of hydroxyapatite (HAp) particles to PCL, which would improve the mechanical properties, especially increasing its stiffness [51] and promote osteoconductivity [52], offers a way to provide a material more generally suitable as a scaffold for hard tissue replacement. PCL/HAp composite materials often show an excellent balance between strength and toughness and usually show improved characteristics when compared to their separate components. Moreover, the basic resorption products of HA or TCP would buffer the acidic resorption by-product of the aliphatic polyester and may help to avoid the formation of an unfavorable environment for the cells due to a decrease pH. [53] Therefore, a possible way to improve mechanical properties is to reinforce PCL scaffold with rigid HAp particles, which would also improve osteoconductivity of the polymer. [54-56] In addition to this, suitable porosity achievement becomes crucial for the success of these materials as scaffolds for

orthopedic applications. A preliminary study on HAp included in PCL scaffolds with a continuous cell line has demonstrated that the presence of HAp on PCL substrates enhanced the osteoblast function and growth. [52]

Stem cell

Stem cells are defined as clonogenic, self-renewing progenitor cells that can generate one or more specialized cell types. [57] Stem cells are classified into two main types, based on their origins: embryonic stem cells (ES cells) and postnatal or adult stem cells (AS cells). Embryonic stem cells are stem cells derived from inner cell mass of an early, pre-implantation stage embryo known as a blastocyst. Adult stem cells are thought to migrate to the area of injury and differentiate into specific cell types to facilitate repair of the damaged tissues. [58, 59]

Stem cells can also be classified according to their ability to differentiate as totipotent, pluripotent, or multipotent cells. Totipotent stem cells can be implanted in the uterus of the living animal and give rise to a complete organism. Pluripotent stem cells are those that can give rise to every cell of an organism except its extraembryonic tissues, such as the placenta. This limitation restricts pluripotent stem cells from developing into a complete organism. Embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells) are pluripotent stem cells. Multipotent stem cells are adult stem cells that only generate specific lineages of cells. [58, 59]

Due to ethical issues associated with the use of embryonic stem cells, recent studies have focused on stem cells derived from adult tissues. It is thought that adult stem cells have a more restricted differentiation potential compared to embryonic stem cells. Nevertheless adult stem cells still fulfill the basic characteristics of stem cells, which are the abilities to self-renew, generate large number of progeny and differentiate into multiple mature cell types.

Mesenchymal stem cells (MSC) are adult multipotent stem cells that have two unique properties. The first one is their capacity of self renewal beyond Hayflictk's limit; this property is shared by embryonic stem cells. The second is the ability of

mesenchymal stem cells to differentiate into mesenchymal and non-mesenchymal mature cell lines including fat, bone, cartilage [60] and neural cells. [61]

Recent finding that mesenchymal stem cells, which are postnatal stem cells, can be easily isolated from various tissues and subsequently expanded render them a promising tool for regenerative medicine. Previously, the only way to obtain postnatal stem cells was from tissues such as bone marrow, neuron tissue, skin, retina, skeletal muscle and the gastrointestinal tract. [62-65] The mesenchymal stem cells are thought to possess great therapeutic potential for repairing damaged tissues caused by disease, aging or trauma.

To date, several types of adult stem cells have been isolated from teeth, including dental pulp stem cells (DPSCs) [66], stem cells from human exfoliated deciduous teeth (SHEDs) [67], periodontal ligament stem cells (PDCLs) [68], dental follicle progenitor stem cell (DFPCs) [69], and stem cells from apical papilla (SCAPs). [70]

Dental pulp stem cells

Recently, a team of researchers has identified a population of putative post-natal stem cells in human dental pulp or dental pulp stem cells (DPSCs). [66] Human DPSCs were isolated from dental pulp of adult teeth; there were populations of clonogenic cells with high proliferative capacity. These cells were successfully isolated by emzymatic digestion of pulp tissue after separating the crown from the roots. [66]

Dental pulp stem cells are multipotent cells that proliferate extensively (maintained for at least 25 passages), can be cryopreserved, possess immunosuppressive properties, and express markers such as CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146 and STRO-1, but do not express CD14, CD24, CD34, CD45, CD19 and HLA-DR. [71-73]

A number of reports showed that dental pulp stem cells (DPSCs) express bone marker such as bone sialoprotein, alkaline phosphatase, type I collagen, and osteocalcin. [74-76] Under an appropriate condition, these cells can be induced to express

markers of osteoblasts, chondroblasts, adipocytes and neurons, indicating the differentiation ability of DPSCs.

The plasticity of DPSCs has been verified through in vitro and in vivo studies. DPSCs have the ability to differentiate into odontoblast-like cells, osteoblasts, adipocytes, neural cells, cardiomyocytes, myocytes and chondrocytes in vitro. (77-80) DPSCs can form mineralized nodules with a dentine-like structure under osteoinductive conditions in vitro and reparative dentine-like tissue on surface of human dentine in vivo. DPSCs transplanted with the carrier hydroxyapatite/tricalcium phosphate (HA/TCP) produce a dentine- like structure lined with human osteoblast-like cells and surround by pulp-like interstitial tissue in vivo. (66, 81) Thus, DPSCs have the capacity to differentiate into osteoblasts in vivo and produced a bone-like tissue. (79)

Stem cells from human exfoliated deciduous teeth

Miura et al in 2003 reported the potential to obtain stem cells from human deciduous teeth. As DPSCs, these multipotent cells were derived from dental pulp explants by digestion of dental pulp tissue and had immunosuppressive properties. (67)

SHEDs have a higher proliferation rate than bone marrow mesenchymal stem cells (BMMSCs) and DPSCs and express Oct4, CD13, CD29, CD44, CD73, CD90, CD105, CD146 and CD166, but do not express CD14, CD34, or CD45. (72,82)

Stem cells from human exfoliated deciduous teeth (SHED) are capable of differentiating into a variety of cell types including neural cells, osteobalsts, odontoblasts, chondrocytes, adipocytes, and myocytes. (67, 83, 84)

From the study of Miura et al, 2003 (67) reported that SHED were capable of generating small amount of dentin and organizing significant amount of bones in vivo. However, SHED was unable to regenerate a complete dentin-pulp-like complex as DPSC. (66)

In addition, after transplantation subcutaneously on the dorsum of immunocompromised mice, SHEDs from ectopic dentine-like tissue, but are unable to regenerate the dentine/pulp-like complex. These results suggest that SHEDs can

differentiate into odontoblasts in vivo. Thus, the mineralized tissue generated by SHED in the pulp space of the tooth-slice scaffolds had morphological features of dentine, including the presence of dentinal tubules and predentine, which distinguishes it from osteoid tissue. [85]

Dental Pulp Stem Cells and Bone Regeneration

Dental pulp stem cells show differentiation profiles similar to osteoprogenitor cells during bone differentiation. [86] This event makes them very interesting as a model to study osteogenesis [87] and the relationship with scaffolds. [88]

In 2005, Laino et al. isolated a selected subpopulation of DPSC called Stromal Bone Producing Dental Pulp Stem Cells (SBP-DPSCs). SBP-DPSCs, when undergoing differentiation into pre-osteoblasts, deposit an extracellular matrix that becomes a calcified woven bone tissue called LAB (Living Autologous Bone). In addition, it has been shown that there has been no differences regarding expansion rate, number of calcified nodules obtained per well of cells selected from young (up to 29 years) and old (30-45 years) subjects. Furthermore, this study showed that LAB transplantation into immunocompromised rats, in all cases, formed a lamellar bone with osteocytes within bone and osteocytes surrounding trabeculae. [89]

Stem cells from human exfoliated deciduous teeth are also capable of repairing critical-size defects in immunocompromised mice; however, the bone regenerated by these cells lacks haematopoietic marrow elements. [67]

It has been documented that dental pulp cells obtained from exfoliated deciduous teeth possess the characteristic of mesenchymal stem cells. SHED were found to express the cell surface molecules STRO-1 and CD146 [67], two early mesenchymal stem cell markers previously found to present in bone marrow stromal stem cells (BMSSCs) and dental pulp stem cells (DPSCs).

In addition, from the study of Seo et al, 2008, [90] it provided the evidence that SHED did not only induce recipient cells to differentiate into osteogenic cells to form bone as reported previously by Miura et al, 2003, but also actively contribute to bone

formation. These results suggest that SHED were capable of forming and inducing bone formation in vivo.

Furthermore, from the study of Yamada et al. 2011, it showed a potential of using stem cells, autologous MSCs, DPSCs, and allologous deciduous tooth stem cells (DTSCs) in large-animal model (canine mandible defect). The results showed that no significant difference in newly formed bone between MSCs, DPSCs and DTSCs group when using platelet rich plasma as a carrier. [91]

Immunomodulatory of Dental Pulp Stem Cells

MSCs have been reported to have a potent immunosuppressive effect that inhibits T cells, B cells, and NK cells and dendritic cell function and be usefull for the treatment of mesenchymal disorders. In a previous study, significant immune modulatory function of MSC has been demonstrated in hematopoietic stem cell transplantation. They strongly inhibit alloantigen-induced dendritic cell differentiation, down-regulate alloantigeninduced lymphocyte expansion, decrease alloantigen-specific cytotoxic capacity mediated by either cytotoxic T lymphocytes or NK cells. Interestingly, a more effective suppressive activity on mixed lymphocyte culture-induced T-cell activation was observed when MSC were heterologous rather than autologous. It was suggested that due to these properties, MSC can be used to prevent immune complications related to both hematopoietic stem cells and solid organ transplantation and it was proposed that MSC are "universal" suppressors of immune reactivity. Moreover, it was shown that regulatory CD4+ or CD8+ lymphocytes were generated in co-cultures of peripheral blood mononuclear cells with MSC. This strongly indicates that these regulatory cells may amplify the reported MSC-mediated immunosuppressive effect. [92-94]

Recently, Human Immature Dental Pulp Stem Cells (hIDPSC), which are constituted by a homogeneous population positive for MSC markers, were shown to contribute for the reconstruction of large cranial defects produced in non immunosuppressed rats after their transplantation onto collagen membrane, without

presenting any graft rejection. [95] Furthermore, populations of dental pulp MSC (DP-MSC) similar to those of hIDPSC, with immunosuppressive activity were described. [96] Analysis of their proliferation activity demonstrated that it was significantly higher in DP-MSC, when compared to those from bone marrow. Similarly to bone marrow MSC, these cells inhibited the proliferation of phytohemagglutinin stimulated T-cells, presenting an even stronger effect than in BM-MSC. [96, 97] Other authors reported that DPSC can survive and engraft in ischemic environments in nonimmunosuppressed rats with acute myocardial infarction. [98]

Transplantation of hIDPSC (human Immature Dental Pulp Stem Cell) in golden retriever muscular dystrophy (GRMD) dogs reported by Kerkis et al. 2008 did not show any immune reaction. [99] This result could be explained by the observation that the immunosuppressive activity of human DPSC according to the previous publication was significantly higher, when compared to those from bone marrow. [96] More recently, similar results were reported with different populations of stem cells isolated from dental pulp. [95, 96, 98]

A study of Yamaza et al. 2010 examined the immunomodulatory properties of SHED in comparison to BMMSCs. Their results showed that SHED had significant superior immunomodulatory effects on inhibiting T helper 17 (Th17) cells when compared to BMMSCs in vitro. In addition, SHED transplantation, as seen in BMMSCs transplantation, is capable therapeutic effect on SLE murine model. [100]

From the study of Yamada et al. 2011, they used autologous MSCs, DOSCs, and allologous deciduous stem cells (DTSCs) to transplant in canine animal models. The results showed that DTSCs group were found to present good bone formation and no clinical symptoms of DTSCs rejection were observed in the parent recipient mandible. This successful bone regeneration by allologous DTSCs indicated that DTSCs do not cause allogenic graft rejection. [91]

SHED are not only derived from a very accessible tissue resource but also capable of providing enough cells for potential clinical application. Thus, exfoliated teeth may be an unexpected unique resource for stem-cell therapies including autologous

stem-cell transplantation and tissue engineering. [67] SHED are derived from a readily accessible source, human deciduous teeth that are expandable and routinely exfoliated in childhood with little or no morbidity to the patient. [90]

SHED are an excellent cell population that maintain a higher proliferation rate than bone marrow mesenchymal stem cells and have the capacity to provide sufficient numbers of cell for clinical therapies. [67] The novel discovery of SHED-mediated bone formation provided a promising stem cell resource for orofacial bone regeneration. [90]

CHAPTER III
MATERIALS AND METHODS

Cells isolation and characterization

1. Isolation and culture of stem cells

Dental pulp was obtained from normal exfoliated human deciduous teeth of 6-13 year-old children under local anesthesia with informed consent. The protocol was approved by The Ethical Committee, Chulalongkorn University. The pulp tissue was collected and digested in Dulbecco's modified Eagle's medium (DMEM, GIBBCO BRL, USA) containing 4 mg/ml collagenase type I (Worthington Biochemicals Corp., Freehold, NJ, USA) and 4 mg/ml dispase (Roche diagnostic/boehringer Mannheim Corp., Indianapolis, IN, USA) for 30 min at 37ºC. After digestion, the explants were plated on the 35-mm culture dish. After cells were migrated out of the explants and reach confluence (Figure 4) on the culture dish (Costar, Cambridge, MA, USA), cells were subcultured with 1:3 ratio. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBBCO BRL,USA), containing 10% FBS (GIBBCO BRL,USA), 2mM L-glutamine,100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B (GIBBCO, Grand Island, New York, USA), at 37°C in a humidified atmosphere of 95% air, 5% CO_2. The cell passages from 3 to 6 were used in this study.

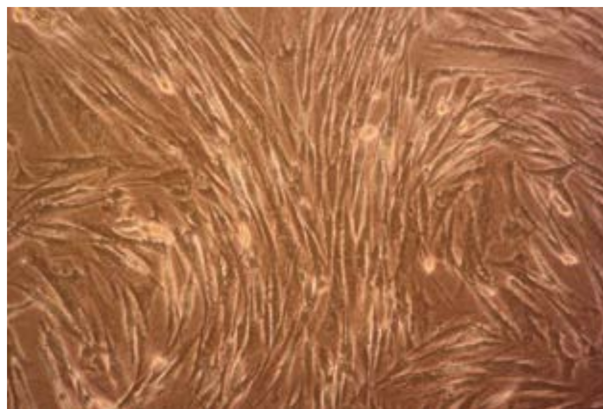


Figure 4. Stem cells from human exfoliated deciduous teeth (SHED) at confluent stage in culture plate.

2. Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Deciduous dental pulp stem cells were seeded in a six-well polystyrene tissue culture plate at a density of 200,000 cells/well. Total cellular RNA was extracted with TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. One μg of each RNA sample was converted to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Fitchburg, WI, USA) for 1.5 h at 42°C. Subsequently, a polymerase-chain reaction (PCR) was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers were CD44 forward: 5´ GCA AGT TTT GGT GGC ACG CA 3´ reverse: 5´ CAA TCT TCT TCA GGT GGA GC 3´, CD73 forward: 5´ ATT GCA AAG TGG TTC AAA GTC A 3´ reverse: 5´ AGA CTT GGC CAG TAA AAT AGG G 3´, CD105 forward: 5´ CAT CAC CTT TGG TGC CTT CC 3´ reverse : 5´ CTA TGC CAT GCT GCT GGT GGA 3´, Nanog forward: 5' TCT CTC CTC TTC CTT CCT CCA 3´ reverse: 5´ GGA AGA GTA GAG GCT GGG GT 3´, Rex-1 forward: 5´ AGA ATT CGC TTG AGT ATT CTG A 3´ reverse: 5´ GGC TTT CAG GTT ATT TGA CTG A 3´, Oct4 forward: 5´ GCA ACC TGG AGA ATT TGT TCC T 3´ reverse: 5´ AGA CCC AGC AGC CTC AAA ATC 3´, Nestin forward: 5´ CTG CGG GCT ACT GAA AAG TT 3´ reverse: 5´ AGG CTG AGG GAC ATC TTG AG 3´ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5´ ACT TTG TCA AGC TCA TTT CC 3´ reverse: 5´ TGC AGC GAA CTT TAT TGA TG 3´. Semi-quantitative PCR was performed using Tag polymerase (Tag DNA Polymerase, Invitrogen, Sao Paulo, Brazil) with a reaction volume of 25 μl containing 25 pmol of primers and 1 μl of RT product.

The PCR was performed in the DNA thermal cycler (Biometra GmbH, Goettingen, Germany). The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide (EtBr; Bio-Rad, San Diego, CA, USA) fluorostaining. All bands were recorded with photography.

3. Alizarin Red-S Staining

To examine the capability of calcified nodule, cells were seeded at a density 50,000 cells/well on culture plate. After cells reach confluence, the medium was changed into osteogenic medium [medium containing 50 µg/ml ascorbic acid (Sigma, St. Loius, MO, USA) and 5mM β-glycerophosphate (Sigma, St. Loius, MO, USA)] as experimental group. For control culture, cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBBCO BRL,USA), containing 10% FBS (GIBBCO BRL,USA), 2mM L-glutamine,100 units/ml penicillin, 100 µg/ml streptomycin, and 5 µg/ml amphotericin B (GIBBCO, Grand Island, New York, USA). Both groups were incubated further for another 15 days, with a change of the medium every other day. Calcium deposition was investigated by Alizarin Red-S staining (Sigma, St. Loius, MO, USA). Cells were fixed with cold methanol for 10 minutes, washed with deionized water and immersed in 1% Alizarin Red-S solution (Sigma, St. Loius, MO, USA) (1:100 (v/v) (in 0.4 ml. ammonium hydroxide/40 ml. water mixture (pH=4.2)) for 3 minutes. After that, removed Alizarin Red-S solution, washed with deionized water 2-3 times, waited to dry completely and recorded the staining result with photography.

In vitro biological behavior of SHED on PCL/HAp scaffold

1. Preparation of PCL/HAp scaffolds.

Porous PCL/HAp scaffolds were prepared using solvent casting and particulate leaching technique as previously described. [2] First, polycaprolactone (PCL; Aldrich, USA, Mw = 80,000 g/mol^{-1}) were dissolving in chloroform (Labscan; Asia,Thailand) at room temperature. HAp was added to the PCL-chloroform solution to yield the final 40 concentration wt% of PCL. The mixture was stirred vigorously to make a good dispersion of HAp particles within the PCL solution. Sucrose (Fluka Chemika, Switzerland) range between 400-500µm in diameter, obtained by sieving, was added to the PCL/HAp solution. The mixture was poured into a mould and allowed to dry for 24 hours. The scaffold was immersed in distilled water for a period of 2-3 days under room temperature, during which time the water was changed approximately every 4 hours for

sucrose leaching. The porous scaffold was dried in a vacuum for 48 hours to remove traces of water.

To increase hydrophilicity, scaffold was immersed in 1.0 M NaOH solution at 37°C for 6 hours. The alkaline treated scaffold was then washed thoroughly with distilled water and dried in vacuum oven for 48 hours (Figure 5). For experiment, scaffold was cut into either 1x2x3 mm^3 rectangular shape or circular shape with diameter 15 mm. (1 mm. of thickness).

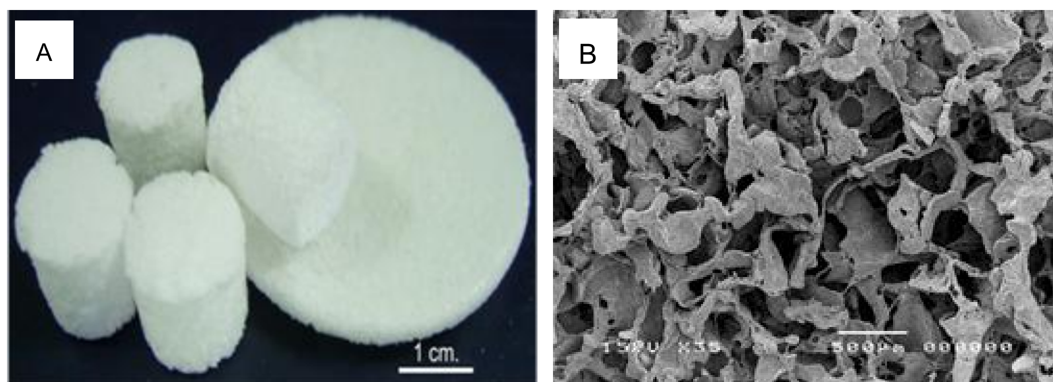


Figure 5. (A) Porous PCL/HAp scaffolds were prepared by using solvent casting and particulate leaching technique.

(B) Micrograph from Scanning Electron Microscope shows the morphological the porous scaffold (scale bar = 500 µm and magnification = 35x).

2. Cell proliferation

Cell proliferation was determined by staining with 4’, 6-diamidino-2’-phenylindole, dihydrochloride (DAPI) to locate the nucleus of the cell and counted under microscope. In brief, cells were seed into the scaffold at a density of 50,000 cells/scaffold (1x2x3 mm^3. rectangular shape) and cultured for 24, 48 and 72 hours. The medium was removed and the scaffolds were washed with sterile phosphate buffer saline (PBS). The staining was performed by adding DAPI working solution to cover the scaffold for 15 min at room temperature. Finally, DAPI solution was discarded and the

scaffolds were washed extensively with sterile PBS and mounted with ProLong™ gold antifade reagent (Invitrogen, Brazil). The staining was observed fluorescent microscope (ApoTome.2, Carl Zeiss, Germany) and recorded the staining result with photography (magnification = 20X). Then, five photographs of each group were selected by randomization and counted for positive nucleus staining.

3. Cell attachment by SEM analysis

For cell attachment analysis, cells were seeded into porous PCL/HAp scaffolds at a density 200,000 cells/scaffold (circular shape with diameter 15 mm. and 1 mm. in thickness) for 30 minutes, 1, 4, 24, 48 hours. Scaffolds were processed for SEM analysis. Briefly, scaffolds were immersed in 3% buffered glutaraldehyde for 30 minutes, Wash 2 times in buffer to remove all traces of glutaraldehyde. Dehydrate tissue through graded ethanols: 30%, 50%, 70%, 90% and 100%. Adding hexamethyldixylaxane (HMDS) 500 μl/well and leave until all of the HMDS is evaporated. The scaffolds were coated with gold using JEOL JFC-1100E sputtering device and examined with JSM-5410LV SEM (JEOL, Japan).

4. Alizarin Red-S Staining of SHED on PCL/HAp scaffold and Calcium Quantification.

To examine the capability of calcified nodule of Stem cells from human exfoliated deciduous teeth (SHED) on PCL/HAp scaffold, cells were seeded at a density 50,000 cells/well on culture plate and PCL/HAp scaffold as experimental group. After cells reach confluence, the medium was changed into osteogenic medium [medium containing 50 μg/ml ascorbic acid (Sigma, St. Loius, MO, USA) and 5mM β-glycerophosphate (Sigma, St. Loius, MO, USA)] and incubated for 15 days, with a change of the medium every other day. In another group, Primary human bone cells were used as control group. Primary human bone cells used in this experiment were obtained form alveoloplasty, torus palatinus or torus mandibularis removal for prosthodontic reasons. All patients gave informed consent. The protocol has been approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. All

bone chips were washed extensively in sterile phosphate buffer saline (PBS, pH 7.4), cut into smaller pieces and digested with 0.25% trypsin-EDTA to remove residual adipose and hematopoietic tissues. The bone chips were harvested in 35 mm culture dish (Nunc, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA), containing 15% fetal bovine serum (FBS; ICP biologicals, New Zealand), 2mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL amphotericin B (GIBCO, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After human primary bone cells reached the confluence, cells were sub-cultured at a 1:3 ratio.

Cells were stained for alkaline phosphatase (ALP) activity, using an ALP staining kit (TAKARA Bio, Japan) to confirm the primary characteristic of the bone cells. The cell passages from 3 to 6 were used in this study. Primary human bone cells were seeded at a density 50,000 cells/well on culture plate and PCL/HAp scaffold. Cells were incubated for other 15 days, with a change of the medium every other day. Calcium deposition was investigated by Alizarin Red-S staining (Sigma, St. Loius, MO, USA). Cells were fixed with cold methanol for 10 minutes, washed with deionized water and immersed in 1% Alizarin Red-S solution (Sigma, St. Loius, MO, USA) (1:100 (v/v) (in 0.4 ml. ammonium hydroxide/40 ml. water mixture (pH=4.2)) for 3 minutes. After that, removed Alizarin Red-S solution, washed with deionized water 2-3 times, waited to dry completely and recorded the staining result with photography. The amount of calcium was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma, USA) in 10 mM sodium phosphate at room temperature for 15 minutes and spectrophotometrically read at 570 nm.

Animal study

1. Rat calvarial defects and histomorphometric analysis

Cells were seeded on PCL/HAp scaffold at a density 1,000,000 cells/scaffold in 12-well plate for 30 minutes before implantation. The implantation sites were prepared in calvaria of 8-week-old-male (Wistar Rat) rats (National Laboratory Animal Centre, Mahidol University). The protocol was approved by the Animal Care and Use Ethical

Committee, Faculty of Medicine, Chulalongkorn University. Two circular calvarial defects (4 mm. in diameter) were created by trephine bur under general anesthesia with Avertin by intraperitoneal injection (200-250mg/kg). The wound was closed with a 4-0 nylon suture. A total of 6 rats were used and 12 calvarial defects were generated. The defects were implanted with PCL/HAp scaffold and stem cell from human exfoliated deciduous teeth seeded on PCL/HAp scaffold by randomization. At 8 weeks, rats were sacrificed and the calvarial bone was fixed with 4% formalin (24 hours at 4 degree celceous). The specimens were processed for paraffin embedding and sectioned (10 μm in thickness). The sections were then stained with either Masson'sTrichrome for morphological analysis. The specimens were scanned by a visual slide microscope (Mirax desk, Carl Zeiss, Germany). Histomorphometric measurements of each section were done by Image Pro Plus analysis program.

2. Immunohistochemistry

To locate the position of SHED in the section, the sections were blocked with 15% horse serum and incubated with primary anti-human CD44 antibody (1:100-1:250 dilution in15% horse serum) for 1 hour. The samples were subsequently incubated with biotinylated secondary antibody for 45 minutes followed by streptavidin conjugated with FITC for another 40 minutes. The sections were counter-stained with 4', 6-diamidino-2'-phenylindole, dihydrochloride (DAPI) for15 minutes to locate the nucleus. Sections were mounted with ProLong™gold antifade reagent (Invitrogen, Brazil), examined under fluorescent microscope and recorded the staining result with photography.

Statistical Analysis

The descriptive analysis was used in description of the SHED proliferation on PCL/HAp scaffold at the culture period 24, 48 and 72 hours. All values were expressed as mean ± standard deviation. For the experiment of in vitro calcification and in vivo bone formation, Statistical analysis of Independent t test or Mann-Whitney test was used to detect statistically significant differences between the different groups depend on the

test of normality by One-sample Kolmogorov-Smirnov test. The probability level $P < .05$ was considered as the level of statistical significant.

CHAPTER IV

RESULTS

Stem cells

1. Characterization of SHED

The mRNA expression of stem cell markers were analyzed by RT-PCR as shown in Figure 6. The results revealed that SHED expressed both the mesenchymal stem cell markers such as CD44, CD73 and CD105, these cells also expressed embryonic stem cells markers such as Nanog, Rex-1 and Oct-4. SHED also expressed Nestin, a neuronal stem cell marker. The results suggested the potential of deciduous dental pulp stem cells to differentiate into several cell types including neurons.

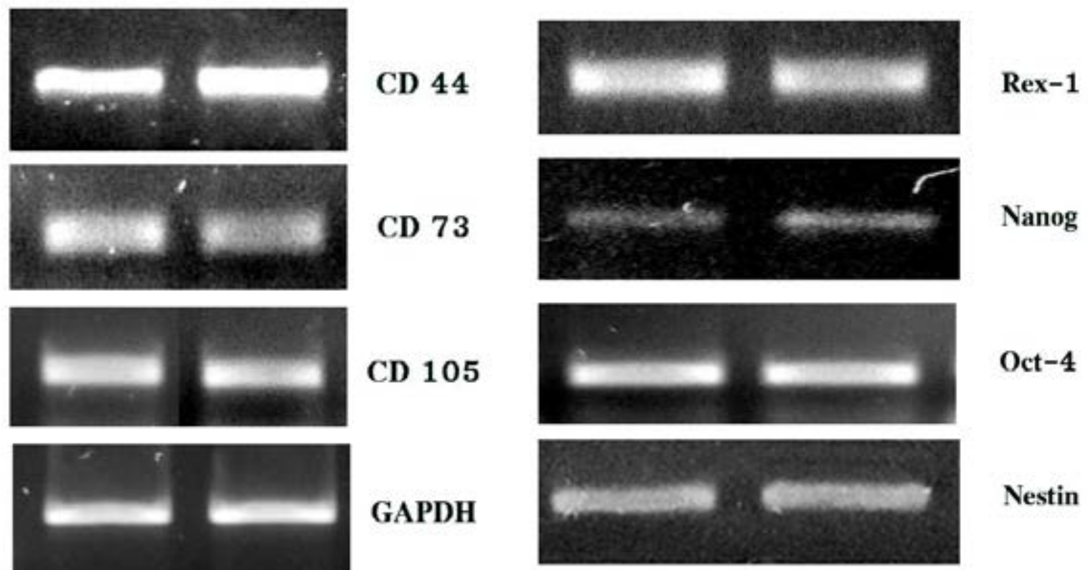


Figure 6. RT-PCR analysis showed the expression of stem cell markers in cells obtained from human exfoliated deciduous teeth (SHED).

2. In vitro calcification

Deciduous dental pulp stem cells were cultured on culture plate for 15 days and the in vitro calcium deposition was examined by Alizarin Red-S staining (Figure 7). The results revealed that the formation of mineralized nodules was found in experimental group which the medium was changed into osteogenic medium (Figure 7B). The formation of mineralized nodules was not found in control group which Dulbecco's modified Eagle's medium (DMEM) was used as medium (Figure 7A).

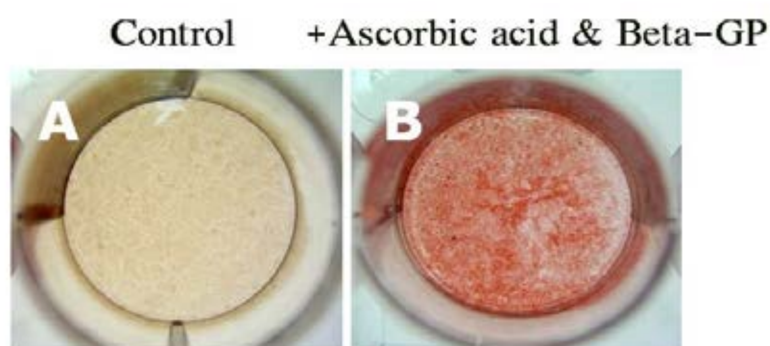


Figure 7. Photographic images illustrating Alizarin red-S staining for mineralization assessment of cellular constructs after deciduous dental pulp stem cells had been cultured on culture plate for 15 days.

In vitro biological behavior of SHED on PCL/HAp scaffold

1. Cell proliferation, cell attachment and SEM analysis

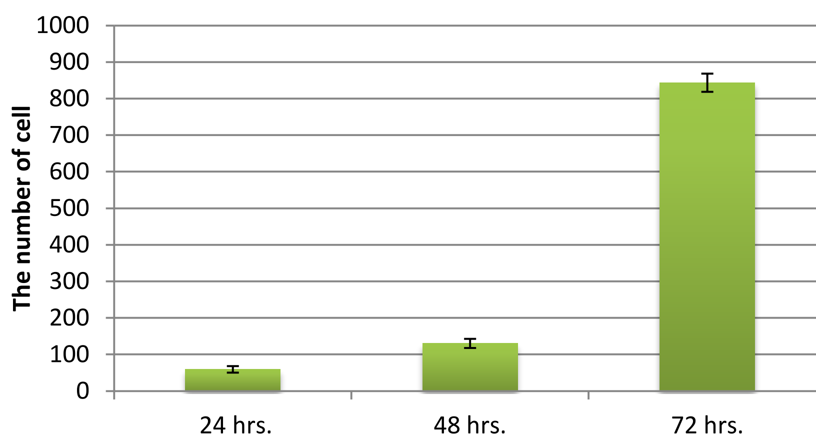


Figure 8. The number of cells grew on PCL/HAp scaffold as determined by using 4', 6-diamidino-2'-phenylindole, dihydrochloride (DAPI) assay.

The results from Figure 8 showed that SHED could survive and grow on PCL/HAp scaffold. The number of cells increased when cells were grown from 24 to 72 hours. In addition, SEM pictures revealed that cells could attach on scaffold surface within 30 minutes and fully spread after 24 hours (Figure 9).

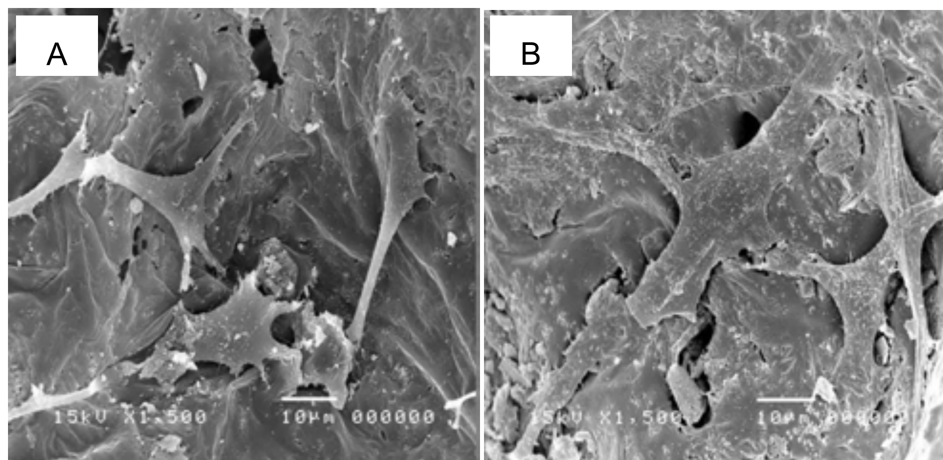


Figure 9. Micrographs from Scanning Electron Microscope showed cells culture on PCL/HAp for (A) 24 hours and (B) 48 hours (magnification=1500x and scale bar = 10 μm).

2. In vitro calcification

SHED was cultured on culture plate and PCL/HAp scaffold as experimental group. Primary human bone cells were cultured on culture plates and PCL/HAp scaffolds as control group. For 15 days in osteogenic medium, the calcium deposition in vitro was examined by Alizarin Red-S staining. The positive stained nodules are in orange-red color. The result indicated that SHED could induce calcium deposition on culture plates and PCL/HAp scaffolds similar to the ability of primary human bone cells (Figure 10). The amount of calcium deposition from Figure 10A and B was quantitated by eluting the color with cetylpyridinium chloride and measured the optical density with spectrophotometer as shown in Figure 10C. The results revealed that no significant different of the formation of mineralized nodules between primary human bone cells and SHED cultured on culture plates and PCL/HAp scaffolds.

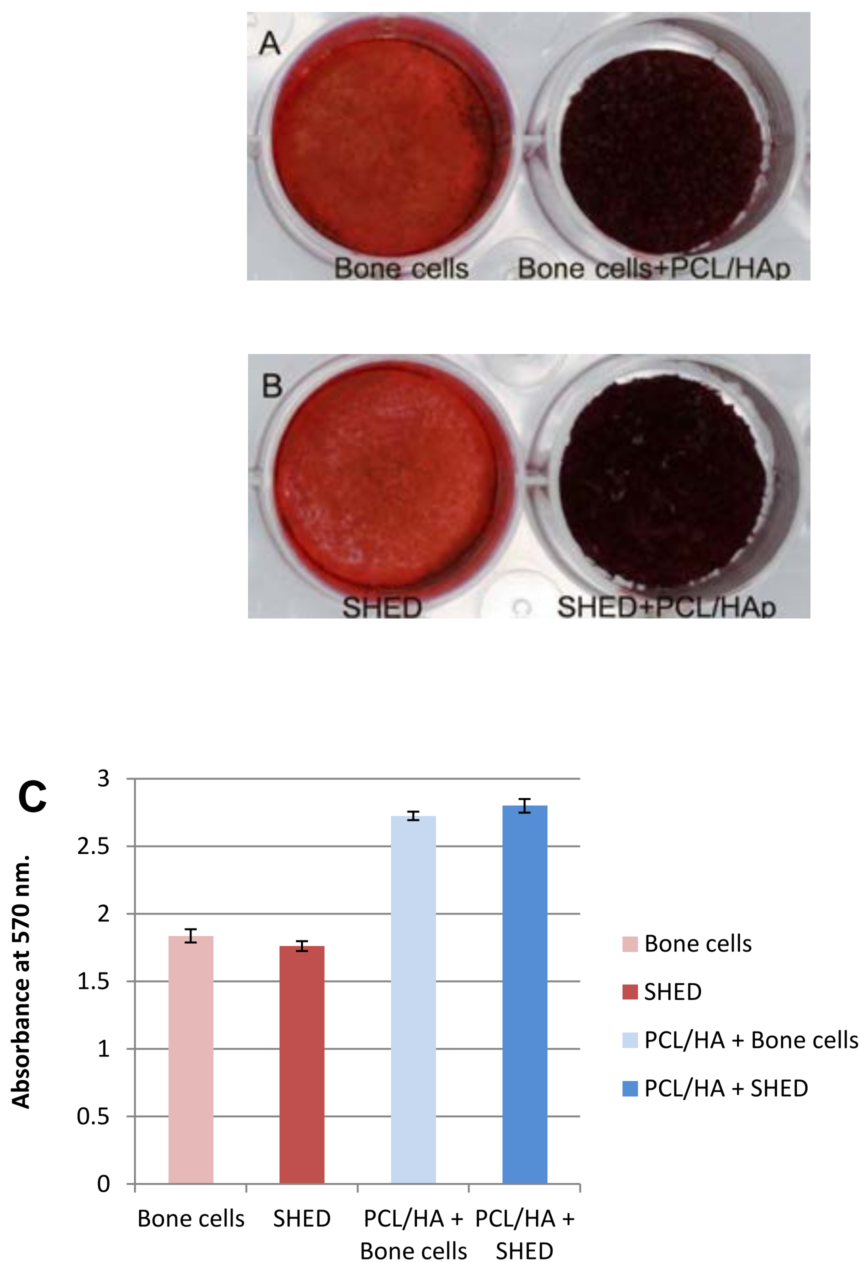


Figure 10. (A) and (B) Photographic images illustrating the Alizarin red-S staining for mineralization assessment of cellular constructs after primary bone cells (A) and Stem Cell from Human Exfoliated Deciduous teeth (B) had been cultured on culture plate and PCL/HAp scaffolds for 15 days.

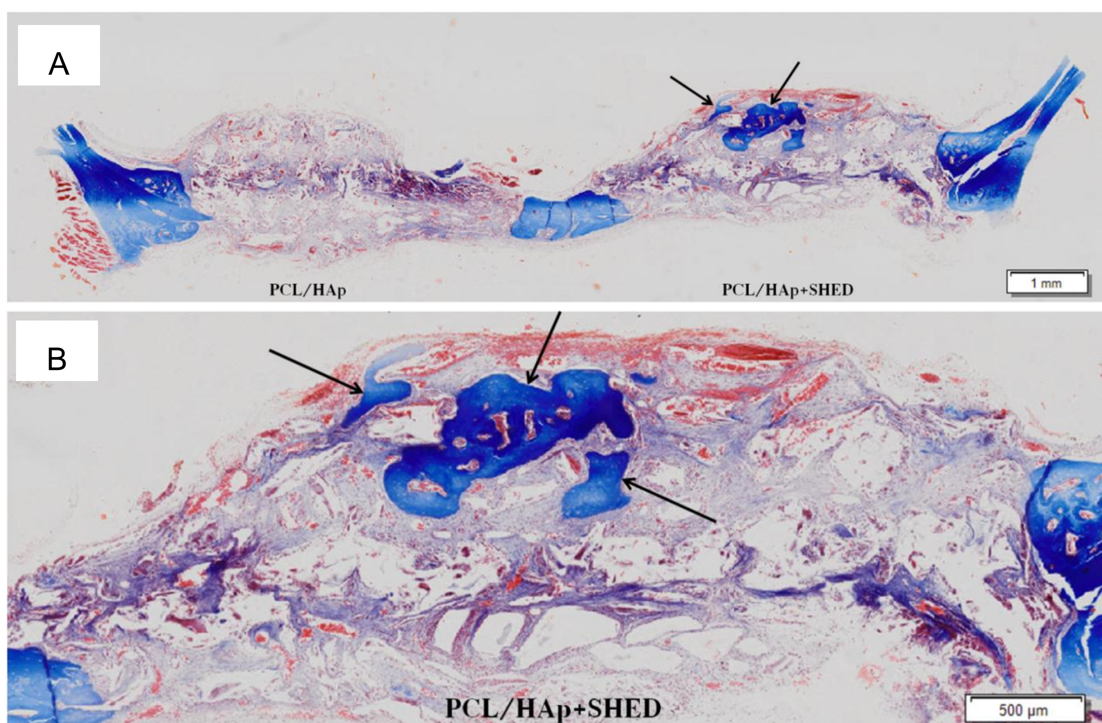
(C) Quantification of calcium deposition by elution with cetylpyridinium chloride and spectrophotometically read at 570 nm.

Animal study

1. In vivo bone formation by implantation of PCL/HAp scaffold containing SHED

PCL/HAp scaffold containing SHED was transplant into the 4 mm. diameter defect in calvarias of rats. The sections of calvaria after 8 weeks of implantation showed the bone-like formation in the calvarial defect.

As shown in Figure 11, bone formation had been detected in the defect contained PCL/HAp scaffold containing SHED (right) compared to granulation in the scaffold alone (left). The calvarial defect of the rat with scaffold alone was filled with a thin loose connective tissue with minimal mineralization. In the defect implanted with scaffold containing SHED, the scaffold was still intact with some new bone formation inside the scaffold. Numerous of vessels were found with in both scaffolds suggesting that PCL/HAp scaffold support the vascularization. Higher magnification of the section showed the osteoid formation along the edge of newly generated bone area, especially in PCL/HAp scaffold containing SHED. Histomorphometric analysis indicated an increase in the amount of new bone formation in all of PCL/HAp scaffold containing SHED implants as compared to all of PCL/HAp scaffold alone (Figure 11C).



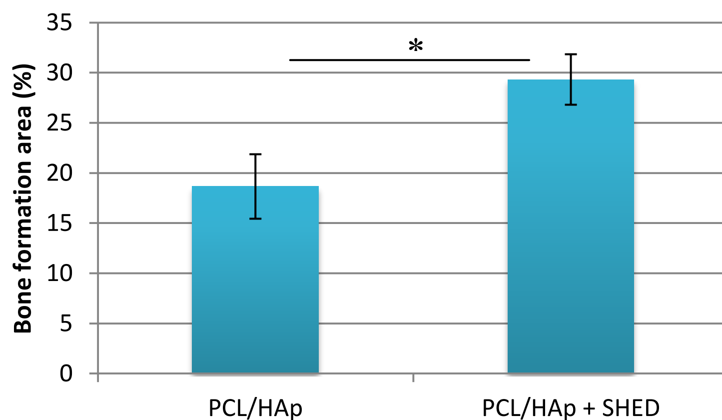


Figure 11. (A) Histological evaluation of the calvaria at 8 weeks after implantation. The sections were stained with Masson's Trichrome staining. The image showed the section of stem cells from human exfoliated deciduous teeth (SHED) seeded into PCL/HAp scaffold (right) compared to implantation of PCL/HAp scaffold (left; Control).

(B) Higher magnification of stem cells from human exfoliated deciduous teeth (SHED) seeded into PCL/HAp scaffold.

(C) Histomorphometric analysis from histological images using Image Pro Plus analysis. The graph showed the percentage of the bone formation compares between PCL/HAp scaffold alone and PCL/HAp scaffold containing SHED. * Significance at $p < 0.05$.

2. Immunohistochemistry

To further explore whether SHED participated in new bone formation in rat, the sections were immunostained with anti-human CD44. As shown in Figure 12, the positive cells for human CD44 were detected in the section.

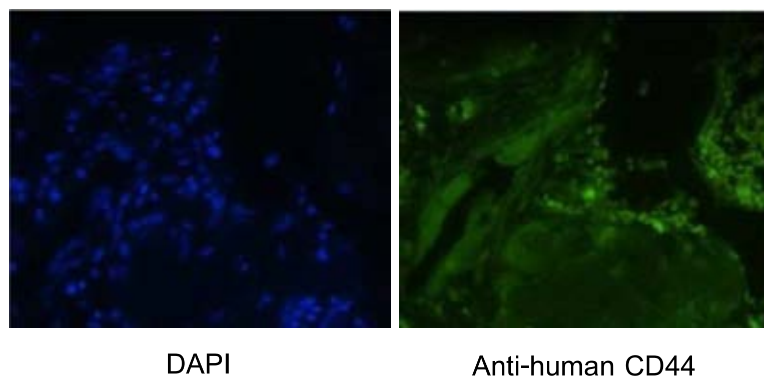


Figure 12. Immunohistochemical staining showed the presence of Anti-human CD44 labeled cells within the scaffold in transplant area (right). The same section was counter-stained with DAPI (left).

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

The results from this study demonstrate the potential of SHED in osteogenic differentiation both in vitro and in vivo experiments. The studies showed that SHED, when cultured with PCL/HAp scaffold could form in vitro calcification and participate as a xenograft that support new bone formation in rat model, suggesting the potential of SHED to be used in bone tissue engineering.

SHED possesses the mesenchymal stem cell characteristics. Many study showed that SHED express MSCs markers including STRO-1, CD146 [67, 100], CD44 [66], CD73, CD105 and CD166. [100] In this study, the expression of CD44, CD 73 and CD105, three of the reported mesenchymal stem cell markers had been detected. In addition, SHED also expressed nestin, a neural crest cell marker, as well as Rex-1, Oct-4 and Nanog, the embryonic stem cells markers. [101] These characteristic supports that SHED might be differed from other mesenchymal stem cells corresponded with their high rate of proliferation and a versatile in differentiation ability. [67]

In our previous study, we have described the novel PCL/HAp scaffold [4], fabricated by solvent casting and particular leaching technique, for bone tissue engineering. These scaffolds could enhance the osseogenesis when implanted into the mice calvarial model. In this study, the results revealed that PCL/HAp scaffold could also support growth and osteogenic differentiation of SHED in vitro. Moreover, the results further indicated the potential of our novel PCL/HAp scaffold on supporting the osteogenic differentiation of SHED in vivo.

PCL/HAp scaffold can support growth and differentiation of SHED in vitro with regard to cell attachment, proliferation and in vitro calcification. From the results of this study, SHED could survive and grow on PCL/HAp scaffold. The numbers of cells increased when cells were grown from 24 to 72 hours. Furthermore, SEM pictures

showed that cells could attach on scaffold surface within 30 minutes and fully spread after 24 hours with normal morphology.

The osteogenic potential of SHED was demonstrated based on in vitro calcification. Differentiation into osteoblasts could be demonstrated by staining with Alizarin red or von Kossa staining. In this study, the results revealed the capacity of SHED to form Alizarin red S positive condensed nodules with high calcium content like primary bone cells. Thus, this observation confirms the previous finding that DPSCs are capable of differentiation to mineralized tissue in vitro. (102)

The calvarial defect model was used to demonstrate the osteogenic potential of SHED in in vivo bone formation. Study in immunocompromised mice support that SHED in combination PCL/HAp had a better rate of bone formation when compared with PCL/HAp alone (data was shown in appendix). The immunocompromised mice model benefit the demonstration of human cell ability as xenograft application, since these mice has a defect in T cell and do not has a graft rejection potential. However, there are several reports showing that mesenchymal stem cells obtain the immunosuppressive properties (12-15) and has been shown recently that SHED could be used in xenograft application.

To test this hypothesis, we implanted the SHED in PCL/HAp scaffold in rat calvarial model. The rat calvarial defect showed a normal wound healing process. There was no pus exudates detected around the surgical area. Eight weeks after implantation, bone formation within the scaffold was observed. The histological analysis revealed the better bone formation in the implant that contained SHED compared to the implant using PCL/HAp scaffold alone. Many capillaries were also found inside and around scaffold indicated that good vascularization which is known as a one of the key phenomenon in bone development. The vascularization is important for the transportation of oxygen, nutrients and soluble factors. Furthermore, it also provides the route for the cell to migrate during bone formation. In addition, a few giant cells were found inside scaffold and around new bone formation which normally found in implanted area. No tissue reaction was observed obviously. Most importantly, immunohistochemistry

analysis revealed the positive staining of human CD44 around new bone indicated that SHED can participate in new bone formation in rat calvarial defect. This result corresponds to a study which was previously reported that mesenchymal stem cell has immunosuppressive function. [12-15]

Conclusion

PCL/HAp scaffold can support growth and differentiation of SHED in vitro with regard to cell attachment, proliferation and in vitro calcification. In addition, PCL/HAp scaffold can support SHED in bone regeneration. SHED was participated in partial bone formation of calvarial defect in immunocompromised mice. This result indicated the ability of SHED seeded in PCL/HAp scaffold to differentiate into osteogenic cell. Moreover, when we transfer calvarial defect model of immunocompromised mice to Wistar rat, SHED was also participated in partial bone formation after 8 weeks of implantation. In morphology observation, no tissue response was observed obviously. In preliminary study, immunohistochemical analysis indicated cells which are positive to human antigen around new bone. This result suggests the possibility to use SHED as a xenograft in bone tissue engineering. However, in detail of cellular and molecular response, further studies are necessary to investigate including the safety of the protocol in longer period of treatment.

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Appendix

In vivo bone formation by implantation of PCL/HAp scaffold containing SHED

Animal are routinely used as preclinical screening tool to access the feasibility of viability of implants before proceeding to human clinical trials. Immunocompromised mice have no thymus and T cells. Because of the lack of T cells, immunocompromised mice cannot reject any transplanted cells or tissues. Therefore, they have been usually used as xenograft model.

In this study, cells were seeded on PCL/HAp scaffold at a density 1,000,000 cells/scaffold in 12-well plate for 30 minutes before implantation. The implantation sites were prepared in calvaria of 8-week-old-male athymic mice (National Laboratory Animal Centre, Mahidol University). The protocol was approved by the Animal Care and Use Ethical Committee, Faculty of Medicine, Chulalongkorn University. The circular calvarial defects (2 mm. in diameter) were created by trephine bur under general anesthesia with Avertin by intraperitoneal injection (200-250mg/kg). The wound was closed with a 4-0 nylon suture. Total 6 mice were used and 6 calvarial defects were generated. The defects were implanted with PCL/HAp scaffold and stem cell from human exfoliated deciduous teeth seeded on PCL/HAp scaffold by randomization. At the 8th week, the mice were sacrificed and the calvarial bone was fixed with 4% formalin. The specimens were processed for paraffin embedding and sectioned (10 μm in thickness). The sections were then stained with either Masson'sTrichrome for morphological analysis.

Results

PCL/HAp scaffold containing SHED was transplant into the 2-mm diameter defect in calvarias of immonocompromised mice. The results from Figure 1 showed the bone-like formation in the calvarial defect after transplant .

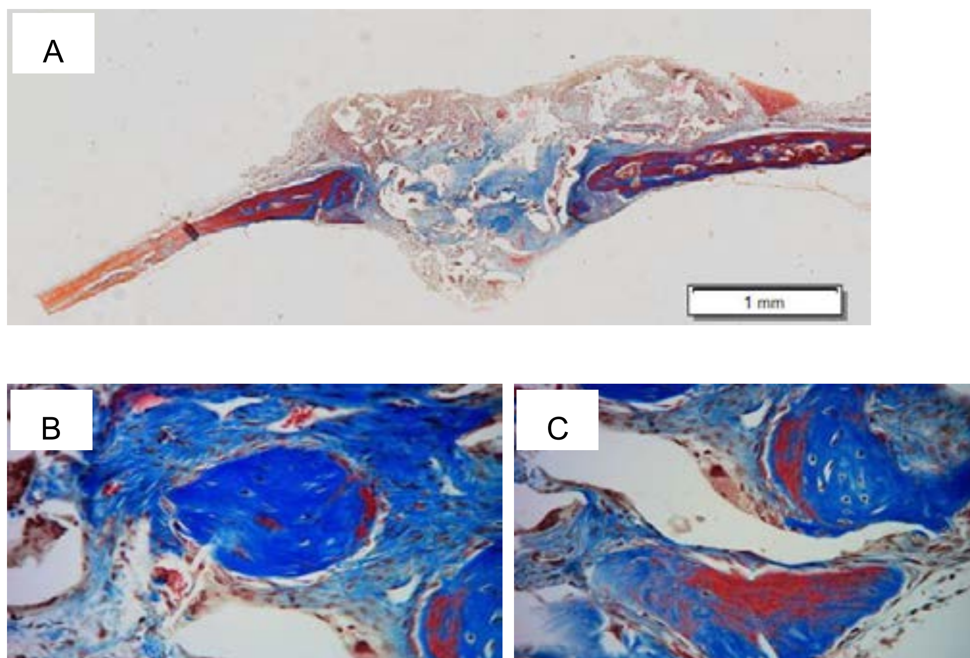

Figure 1. (A) Histological evaluation of the calvaria at the 8th week after implantation. The sections were stained with Masson's Trichrome staining. The image showed the section of stem cells from human exfoliated deciduous teeth (SHED) seeded into PCL/HAp scaffold in immnocompromised mice.

(B),(C) Higher magnification (magnification = 40X) of stem cells from human exfoliated deciduous teeth (SHED) seeded into PCL/HAp scaffold.

Limitation

Due to the very small size of calvarial in immnocompromised mice, it was difficult to create 2 defects in their calvaria. Therefore, the experiment and control groups were done in two different animals.

Tissue processing protocol

	Reagent	Time
1.	70% alcohol	1 hour
2.	95% alcohol	1 hour
3.	95% alcohol	1 hour
4.	Absolute alcohol (100%)	1 hour
5.	Absolute alcohol (100%)	1 hour
6.	Xylene	10 minutes
7.	Xylene	10 minutes
8.	Xylene	10 minutes
9.	Paraplast (Wax)	1 hour
10.	Paraplast (Wax)	1 hour

Masson's Trichrome staining

Dewaxing

1. Xylene 5 minutes
2. Xylene 5 minutes

Hydration

3. Absolute Ethanol 5 minutes
4. Absolute Ethanol 5 minutes
5. 95% Ethanol 3 minutes
6. 95% Ethanol 3 minutes
7. 70% Ethanol 3 minutes
8. Di-H2O 3 minutes

Staining

9. Filtered Weigert's haematoxylin for 10 minutes, wash in running tap water
10. Biebrich Scarlet for 5 minutes, wash in running tap water
11. Phosphomolybdic acid for 10 minutes
12. Aniline blue for 5 minutes, wash in running tap water
13. Acetic acid for 5 minutes, rinse in Di-H2O

Dehydration

14. 70% Ethanol 3 minutes
15. 95% Ethanol 3 minutes
16. 95% Ethanol 3 minutes
17. Absolute Ethanol 5 minutes
18. Absolute Ethanol 5 minutes

Clearing

19. Xylene 5 minutes
20. Xylene 5 minutes
21. Mount and cover with cover slips

Statistical analysis

1. Cell proliferation
   a. Data show descriptive analysis of SHED proliferation on PCL/HAp scaffold at the culture period 24, 48 and 72 hours.

Descriptive Statistics

GROUP		N	Mean	Std. Deviation	Minimum	Maximum
24 hours	NUMBER	5	58.8000	8.92749	51.00	72.00
48 hours	NUMBER	5	129.8000	12.51799	113.00	147.00
72 hours	NUMBER	5	843.0000	24.86966	817.00	878.00

2. In vitro calcification
   a. Data show descriptive analysis of Quantification of calcium deposition by elution with cetylpyridinium chloride and spectrophotometically read at 570 nm.

Descriptive Statistics

GROUP		N	Mean	Std. Deviation	Minimum	Maximum
Bone cells	ALIZARIN	3	1.87033	.064081	1.798	1.920
SHED	ALIZARIN	3	1.76133	.036460	1.722	1.794

b. The test of normality was showed.

One-Sample Kolmogorov-Smirnov Test

GROUP			ALIZARIN
Bone cells	N		3
	Normal Parameters[a,b]	Mean	1.87033
		Std. Deviation	.064081
	Most Extreme Differences	Absolute	.305
		Positive	.219
		Negative	-.305
	Kolmogorov-Smirnov Z		.528
	Asymp. Sig. (2-tailed)		.943
SHED	N		3
	Normal Parameters[a,b]	Mean	1.76133
		Std. Deviation	.036460
	Most Extreme Differences	Absolute	.239
		Positive	.193
		Negative	-.239
	Kolmogorov-Smirnov Z		.414
	Asymp. Sig. (2-tailed)		.995

a. Test distribution is Normal.

b. Calculated from data.

c. Independent t test was done for analyze Quantification of calcium deposition by elution with cetylpyridinium chloride and spectrophotometically read at 570 nm.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ALIZARIN	Equal variances assumed	1.585	.277	2.561	4	.063	.10900	.042566	-.0092	.22718
	Equal variances not assumed			2.561	3.172	.079	.10900	.042566	-.0224	.24040

3. In vivo bone formation

a. Data show descriptive analysis of histomorphometric analysis from histological images using Image Pro Plus analysis.

Descriptive Statistics

GROUP		N	Mean	Std. Deviation	Minimum	Maximum
PCL/HAp	BONE	3	18.67	3.215	15	21
PCL/HAp + SHED	BONE	3	29.33	2.517	27	32

b. The test of normality was showed.

One-Sample Kolmogorov-Smirnov Test

GROUP			BONE
PCL/HAp	N		3
	Normal Parameters[a,b]	Mean	18.67
		Std. Deviation	3.215
	Most Extreme Differences	Absolute	.328
		Positive	.234
		Negative	-.328
	Kolmogorov-Smirnov Z		.567
	Asymp. Sig. (2-tailed)		.904
PCL/HAp + SHED	N		3
	Normal Parameters[a,b]	Mean	29.33
		Std. Deviation	2.517
	Most Extreme Differences	Absolute	.219
		Positive	.219
		Negative	-.189
	Kolmogorov-Smirnov Z		.380
	Asymp. Sig. (2-tailed)		.999

a. Test distribution is Normal.

b. Calculated from data.

c. Independent t test was done for analyze percentage of the bone formation area.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
BONE	Equal variances assumed	.450	.539	-4.525	4	.011	-10.67	2.357	-17.211	-4.123
	Equal variances not assumed			-4.525	3.782	.012	-10.67	2.357	-17.362	-3.971

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