SWURES11-013: การศึกษาเบื้องต้นของคุณสมบัติการสร้างกระดูกใหม่ในสิ่งมีชีวิต ด้วยการใช้โครงเลี้ยงเซลล์ไคโตซาน/กรดไดคาร์บอกซิลิก ที่ฝังเซลล์เอ็นยึดปริทันต์ของมนุษย์

PRELIMINARY STUDY OF IN VIVO BONE REGENERATION USING CHITOSAN/DICARBOXILIC ACID SCAFFOLD IMPLANTED WITH HUMAN PERIODONTAL LIGAMENT CELLS

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บทคัดย่อ

การละลายตัวของสันกระดูกขากรรไกรภายหลังการสูญเสียฟันนั้นส่งผลต่อการใส่ฟันทดแทนโดยเฉพาะการฝังรากฟันเทียม โดยการละลายตัวของสันกระดูกขากรรไกรนั้นสามารถป้องกันได้ด้วยกระบวนการคงสภาพสันกระดูกขากรรไกร ซึ่งสามารถทำได้หลายวิธี หนึ่งในวิธีการที่ได้รับความสนใจคือวิธีการทางวิศวกรรมเนื้อเยื่อโดยการใช้โครงเลี้ยงเซลล์และเซลล์ต้นกำเนิดในการกระตุ้นให้เกิดการสร้างกระดูกใหม่ขึ้นมาในบริเวณที่ต้องการ คณะผู้วิจัยได้พัฒนาโครงเลี้ยงเซลล์ไคโตซาน ที่ขึ้นรูปผ่านการละลายและเชื่อมโยงข้ามด้วยกรดคาร์บอกซิลิกชนิดมีหมู่คาร์บอกซิลสองหมู่ขึ้น แต่ยังไม่มีการศึกษาวิจัยเกี่ยวกับคุณสมบัติของวัสดุชนิดนี้ในการกระตุ้นการสร้างกระดูกใหม่ในสิ่งมีชีวิต การศึกษาวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาเปรียบเทียบคุณสมบัติการกระตุ้นการสร้างกระดูกใหม่ของโครงเลี้ยงเซลล์ไคโตซาน/กรดไดคาร์บอกซิลิก ซึ่งได้รับการฝังหรือไม่ฝังเซลล์เอ็นยึดปริทันต์ของมนุษย์ ในรอยวิการกะโหลกศีรษะหนูทดลอง

วิธีดำเนินการวิจัย: ศึกษาในสัตว์ทดลองโดยใช้รอยวิการกะโหลกศีรษะหนูเมาส์ สายพันธุ์ C57BL/6Mlac จำนวน 18 ตัว แบ่งออกเป็น 3 กลุ่ม กลุ่มละ 6 ตัว ได้แก่ กลุ่มที่ใส่โครงเลี้ยงเซลล์ไคโตซานชนิดที่ฝังเซลล์เอ็นยึดปริทันต์ กลุ่มที่ใส่โครงเลี้ยงเซลล์ไคโตซานชนิดที่ไม่ฝังเซลล์เอ็นยึดปริทันต์ และกลุ่มควบคุมที่ไม่ใส่

โครงเลี้ยงเซลล์ลงในรอยวิการกะโหลกศีรษะ และทำการวิเคราะห์เปรียบเทียบปริมาณกระดูกที่สร้างขึ้นใหม่ด้วยการถ่ายภาพรังสีไมโครคอมพิวเตดโทโมกราฟฟีในสัปดาห์ที่ 6 และสัปดาห์ที่ 12 หลังการผ่าตัด

ผลการศึกษา: พบว่ากลุ่มที่ใส่โครงเลี้ยงเซลล์ไคโตซานชนิดที่ฝังเซลล์เอ็นยึดปริทันต์ และกลุ่มที่ใส่โครงเลี้ยงเซลล์ไคโตซานชนิดที่ไม่ฝังเซลล์เอ็นยึดปริทันต์ สามารถกระตุ้นการสร้างกระดูกใหม่ได้มากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ที่ระยะเวลา 6 และ 12 สัปดาห์

สรุปผลการวิจัย: โครงเลี้ยงเซลล์ไคโตซาน/กรดไดคาร์บอกซิลิก มีคุณสมบัติที่ดีในการเป็นโครงยึดเกาะให้กับเซลล์ต้นกำเนิด และสามารถกระตุ้นการสร้างกระดูกใหม่ในรอยวิการกะโหลกศีรษะของหนูทดลอง แต่อย่างไรก็ตาม ยังคงต้องการการศึกษาวิจัยเพิ่มเติม เพื่อยืนยันถึงข้อสรุปดังกล่าว

คำสำคัญ: โครงเลี้ยงเซลล์ไคโตซาน/กรดไดคาร์บอกซิลิก เซลล์เอ็นยึดปริทันต์ การสร้างกระดูก การคงสภาพสันกระดูกขากรรไกร

Abstract

After tooth loss, a consequent atrophy of the alveolar process always takes place and adversely affect the reconstruction process especially by dental implant. Tissue engineering has emerged as a novel treatment for alveolar ridge preservation. A novel chitosan/dicarboxylic acid scaffold (CS/DA scaffold) has been developed and proven to be an excellent candidate material in bone tissue engineering. This study aimed to evaluate the effects of a CS/DA scaffold with and without seeded primary human periodontal ligament cells (hPDLCs) in mouse calvarial defect model.

Methods: In vivo bone regeneration was performed in C57BL/6Mlac mouse calvarial defects. Eighteen mice were divided into 3 groups of 6 each. Four-millimeter calvarial defects were created on both side of parietal bone and implanted with either CS/DA scaffold or CS/DA scaffold with hPDLCs. The empty bony defects were kept as control. Micro-CT scanning were used to quantify the amount of new bone form at 6 and 12 weeks after surgery.

Results: In vivo bone regeneration at 6 and 12 weeks was significantly enhanced by CS/DA scaffold alone and CS/DA scaffold implanted with hPDLCs ($P < 0.05$).

Conclusion: Our study proposes CS/DA scaffold as a novel bone regenerative material with good osteoinductive/osteoconductive properties. Further study should be conducted in more detail.

Keywords: Chitosan/dicarboxylic acid scaffold, Periodontal ligament cells, Bone regeneration, Alveolar ridge preservation

Introduction

After tooth loss, a consequent atrophy in height and width of the alveolar process always takes place. Many studies have shown that resorption of the alveolar process adversely result in the loss of functional and esthetical consequences. Insufficient vertical and horizontal volume of alveolar bone usually found at the extraction site which often impair the execution of both traditional dentures and dental implant. [1-2] Alveolar ridge preservation methods have been introduced to maintain a sufficient ridge contour in

extraction areas especially at the site with aesthetic concern. In fact, several methods have already been introduced for alveolar ridge preservation such as socket grafting with autografts, allografts, xenografts and alloplasts, however, each of which has a limitation. Some approaches are even far from ideal, the material may not successfully be replaced by bone for years. Development of reliable bone tissue engineering materials is therefore a crucial step in advancing alveolar ridge preservation techniques. [3-5]

In bone tissue engineering, the 3D porous scaffold plays an indispensable role in controlling osteoblasts function and promote new bone formation. The required property of scaffolding materials for bone tissue engineering is osteoconductive in which osteoprogenitor cells can migrate, adhere, differentiate, proliferate and finally differentiate to form new bone. [6-7] Chitosan have been introduced to use as tissue regenerative scaffolds because its provide good structure for mechanical support as well as promote cell attachment, proliferation, and differentiation. As natural-derived product, chitosan has high biocompatibility and therefore meet all criteria to be an excellence candidate for tissue engineering materials. Still, one of the major limitations of chitosan is its poor solubility in aqueous solutions. Chitosan can be characterized by its solubility in aqueous acidic solutions due to the presence of the amino group. While chitosan scaffold is considered to be an ideal polymer for making bioactive compounds, there is a potential in their toxic byproducts. The use of acid and/or chemical reagents for solvents and crosslinkers raise questions about chemical reagent contamination. [8-9]

Previously, the conventional method to prepare chitosan is to dissolve in monocarboxylic acids, acids with only one carboxyl group, such as acetic acid and formic acid. This reaction requires the subsequence utilization of crosslinking agent such as glutaraldehyde in order to form the scaffold. Besides monocarboxylic acids, there are many kinds of multi-carboxylic acids with more than one carboxyl group. Several of these acids are naturally non-toxic solvent and widely use in food and medicinal-related industries. Moreover multi-carboxylic acids not only solubilized the chitosan in water but also improved the property of the scaffold through its non-covalent cross-linking interaction with chitosan. [10-12] Recently, Valderruten et al. [13] reported the novel preparation of chitosan hydrogels using natural dicarboxylic acids as nontoxic dissolving and cross-linking agents, serving as high biocompatibility material. This method was the green method for chitosan preparation, greatly reduced contamination of the final byproduct. However, there was no studies report the effect of chitosan prepared by multi/di-carboxylic acids dissolving/cross-linking method on bone tissue regeneration properties. In the present study, we evaluated bone regeneration property of a novel chitosan scaffold prepared by utilizing dicarboxylic as dissolving and cross-linking agents implanted with or without primary human periodontal ligament cells in mouse calvarial defects by micro-computed tomography analysis.

Objectives

To evaluated bone regeneration property of chitosan/dicarboxylic acid scaffold with and without primary human periodontal ligament cells in mouse calvarial defects using Micro-CT analysis.

Methods

Isolation and culture of the primary human periodontal ligament cells

Teeth from healthy young individuals, age 18–25 year-old, were extracted as recommended by their dentist. Each subject was without systemic and oral infection. The periodontal ligament attached to the root surface of the middle one-third was scraped off with a surgical blade. The tissue explants were seeded in culture medium (10% FBS, 1% L-Gluamine, 0,5 mg/ml gentamicin and 3 mg/ml amphotericin B in DMEM, #11960, Gibco, Life Technologies Corporation, Grand Island, NY) until outgrowing cells reached confluence. The cells were incubated at 37° C humidified atmosphere with 5% CO_2. The primary human PDL cells at the 3^{rd}–8^{th} passage were used for the following experiments. The Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Thailand was approved the study to be carry out according to the protocol.

In vitro differentiation assay

In vitro differentiation to osteogenic lineage was performed as follows. hPDLCs were initially culture in growth medium. After confluence, the media will be replaced by osteogenic medium. The cells were incubated in osteogenic medium (DMEM supplemented with 10% FBS, 50mg/ml L-ascorbate-2-phosphate, 0.25mM dexamethasone, and 5 mM β-glycerophosphate) for 10 days. Mineral deposition was analyzed by staining with Alizarin red.

Preparation chitosan/dicarboxylic acid scaffold

Preparation from The Petroleum and Petrochemical College, Chulalongkorn University, Thailand. Briefly, chitosan was dissolved homogeneously in succinic acid solution (4% w/w). After stir for 10 min, the viscous solution was poured into a plastic beaker and frozen at –20C for 36 h. The frozen mass was immersed in 1N NaOH, and then washed with distilled deionized water to a pH 7 to obtain transparent chitosan/succinic acid hydrogels. Then, freeze dry the hydrogels to obtain chitosan/dicarboxylic acid scaffold and cut into cylinder shape 4x1 mm pieces.

Mouse calvaria defect model

Eighteen 8-week-old male C57BL/6Mlac mice (National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand) were used in this study. The experiment was approved by Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand.

The animal procedure used in this study modified from the protocol by Spicer *et al.* [14] and Huynh *et al*.. [15] On the operation day, 1×10^6 hPDLCs in 100 μl of growth medium were seeded onto the scaffold for 1 h prior to implantation. General anesthesia was induced by intraperitoneal injection of

pentobarbital (NEMBUTAL® Sodium Solution, Akorn, Inc., Lake Forest. IL). Surgical sites were exposed with a 1.5 cm sagittal incision. Two bilateral full-thickness bony defects (4mm in diameter) in the center of each parietal bone were created using a biopsy punch (Stiefel, GSK, NC, USA) with a normal saline rinsed.

The mouse were randomly divided into three groups of 6 each as follows: (1) the defects were filled with Chitosan scaffold, (2) the defects were filled with Chitosan scaffold with hPDL cells and (3) the defects were left empty. [Fig. 1].

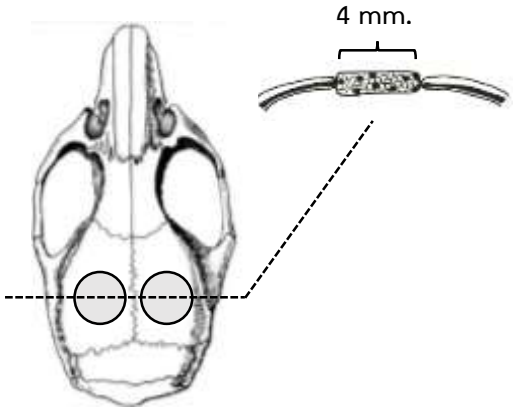

Figure 1. Anatomical location of defects and scaffold in mouse calvaria.

Micro-Computed Tomography (Micro-CT)

After being scarified at 6 weeks and 12 weeks, calvaria bones were carefully excised, cleaned, and fixed immediately with 10% buffered formalin. Bone formation in the defect were analysed using micro-CT imaging according to Bouxsein's guideline. [16] Samples were scanned using a micro-CT scanner (SCANCO Medical AG, μCT 35, Switzerland). The exposure parameters were 70 kV, 114 μA, 8 W. The mineral density and the morphology of skull cavity were evaluated. The analyses were performed using 3D analysis software from Scanco Medical (SCANCO Medical AG, Switzerland).

Statistical Analysis

For statistical analysis, One-way analysis of variance (ANOVA) were utilized for mineral density of micro-CT data. Statistical significant was consider at $P < 0.05$. All statistical analyses were performed using Statistical Package for the Social Science (SPSS) Statistics software package version 22 (IBM, New York, USA).

Results

Human periodontal ligament cells isolation and differentiation

The isolated cells after initial plating of the primary culture had typical fibroblastic morphology with spindle shape (Fig. 1A). The primary hPDLCs reached confluence after 14 days in culture. The hPDLCs were then tested for its osteogenic differentiation potential by bone nodule formation assay. After being

cultured in osteogenic medium for 10 days, the line demonstrated intense staining for calcium deposition resemble mineralization nodules were chosen for further in vivo study (Fig.1B).

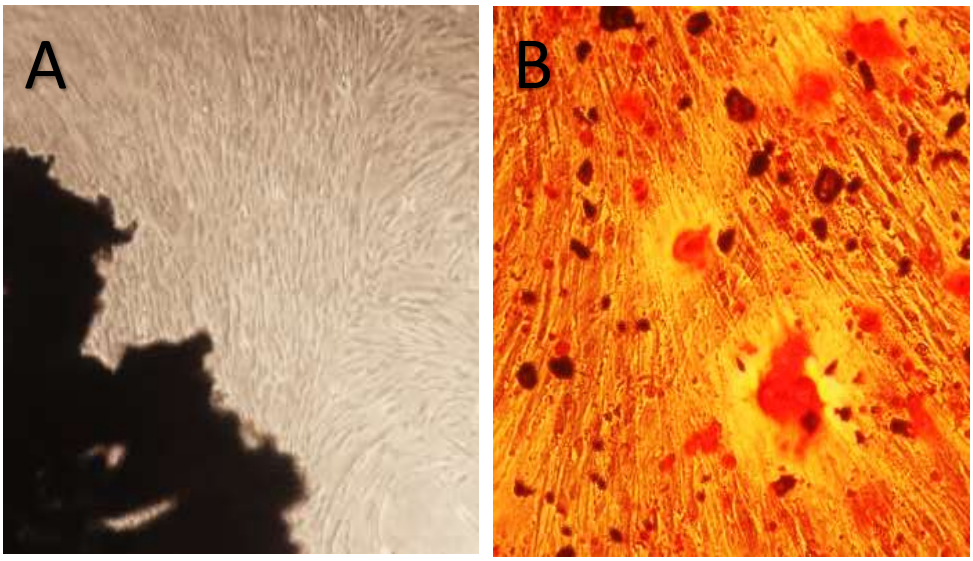

Figure 1. (A) The primary periodontal ligament cells isolated from human periodontal ligament were cultured for 14 days. (B) Mineral deposition of hPDLCs after 10 days in osteogenic medium observed by Alizarin red staining.. Magnification, x10 for A; Magnification, x40 for B.

CS/DA scaffold enhanced in vivo bone regeneration in mice calvariae

CS/DA scaffold could enhance in vivo bone regeneration in mice calvariae defect. New bone formation as assessed by Micro-CT analysis demonstrated the promoting effect apparently both at 6 and 12 weeks after implantation (Fig. 2). Quantification of bone volume/tissue volume (BV/TV) showed amount of new bone formation at 6 weeks of the CS/DA scaffold implanted with hPDLCs group was significantly greater than those of CS/DA scaffold alone or control defects ($P < 0.05$). While at 12 weeks, CS/DA scaffold implanted with hPDLCs group and CS/DA scaffold alone group was significantly higher than surgical control group ($P < 0.05$).

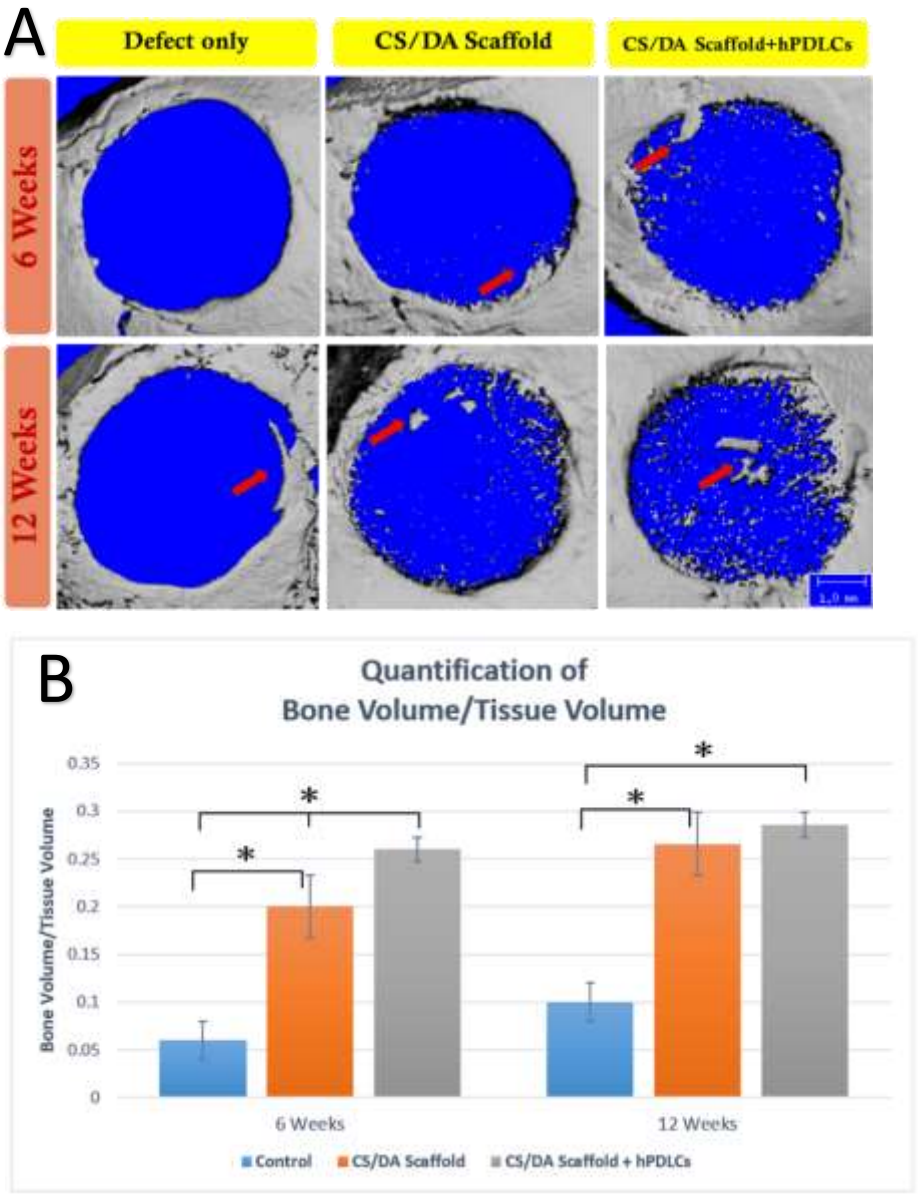

Figure 2. Effect of CS/DA scaffold on bone regeneration in mouse calvaria defects as assessed by micro-CT scanning. (A) New bone formation in mouse calvaria defect (red arrows). (B) Quantification of bone volume related to tissue volume (BV/TV). One-way ANOVA, Turkey HSD post hoc test , * p = 0.05.

Conclusions and Discussion

Regenerative medicine is a challenging science in the way to induce tissue repair in live tissue. Advance development in this field have been attempted to provide the availability of bioactive compounds for repairing damaged tissue. [17-18] Currently, significant progresses have been achieved to treat the loss of bone defect and stimulate of new bone formation with cell and scaffold based tissue engineering

strategies. Chitosan is one of the most promising biopolymers for tissue engineering and possible orthopedic applications. Chitosan scaffold has been extensively used in bone tissue engineering since it was shown to promote growth and mineral rich matrix deposition by osteoblasts in culture. Also chitosan is biocompatible, biodegradable, intrinsic antibacterial nature, and can be molded into porous structures to allows osteoconduction. [19]

Recently, Valderruten and colleagues [13] reported the novel fabrication of chitosan using dicarboxylic acids as nontoxic dissolving and cross-linking agents which greatly reduced contamination of the final toxic byproducts. In this study, we demonstrated the use of novel 3D porous CS/DA scaffold with and without seeded primary human periodontal ligament cells (hPDLCs) in mouse calvarial defects. We found that CS/DA scaffold was able to promote significant new bone formation either with or without hPDLCs. This is a first report on evaluation of osteogenic potential of CS/DA scaffold with and without seeded stem cells. Osteogenic differentiation of stem cells seeded on scaffold is key issue determining the success in new bone formation. [20] Corresponded with previous reports [21-22], hPDLCs demonstrated excellence capability to differentiate into bone forming osteoblasts as shown by intense staining for calcium deposition and formed mineralization nodules as revealed by Alizarin red staining.

The osteogenic properties of chitosan have been reported in various animal models. Pang et al. [23] presented chitosan solution promoted the synthesis of collagen type I and facilitated the differentiation of hPDLCs into osteoblast and has significant potential to accelerate new bone formation in rat calvarial defects. Nandi et al. [24] developed controlled release growth factor incorporated in chitosan scaffold and reported their ability to promote bone healing and regeneration in a rabbit model. Our data clearly demonstrated a significant increase in BV/TV in the defects of the CS/DA scaffold implanted with hPDLCs and CS/DA scaffold alone both at 6 and 12 weeks. Especially at 6 weeks, defect closure and new bone area in the CS/DA scaffold implanted with hPDLCs group were significantly greater than those in the other groups ($p < 0.05$). The μCT images also supported the enhanced bone ingrowth in CS/DA scaffolds cultured with hPDLCs. Some of the images clearly show an appearance of a mineralization nodule in the central part of the defect. However, by 12 weeks, no significant difference between scaffolds alone groups and scaffolds loaded with stem cells can be observed. hPDLCs can accelerates early onset of osteogenesis but does not affect bone formation over time. Forasmuch hPDLCs exhibit osteoprogenitor cell properties, thus the presence of more amount of osteoprogenitor cells, therefore, can stimulates early onset of bone formation. However when entering reparative phase of healing, mesenchymal stem cells derived from surrounding environment might have been recruited, proliferate, differentiate into osteogenic cells and generate bony callus formation in the defect. This might explain why we observed no difference between scaffolds alone and scaffolds loaded with hPDLCs at later time point, 12 weeks. Altogether the results of the present study demonstrated that CS/DA scaffold has significant potential to induce new bone formation in vivo.

In conclusion, this pilot study suggests that CS/DA scaffold with and without seeded primary hPDLCs were suitable for bone defect repair in a critical size mouse calvarial defect model. The novel CS/DA scaffold could be serve as carrier for stem cells to repair bone defect and this scaffold has an enormous potential to develop as materials for bone tissue engineering applications. Further experimental and clinical studies should be conducted to confirm these results.

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