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Vertical alveolar ridge augmentation with β-tricalcium phosphate and autologous osteoblasts in canine mandible

Shaoyi Wang [a], Zhiyuan Zhang [a], Jun Zhao [a], Xiuli Zhang [b], Xiaojuan Sun [c], Lunguo Xia [a], Qing Chang [d], Dongxia Ye [a], Xinquan Jiang [b,*]

[a] Department of Oral and Maxillofacial Surgery, Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai 200011, China
[b] Oral Bioengineering Lab, Shanghai Research Institute of Stomatology, Ninth People's Hospital Affiliated to Shanghai Jiao Tong University, School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai 200011, China
[c] Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Ningxia Medical University, Ningxia 750004, China
[d] Wuxi Fourth People's Hospital, Wuxi, Jiangsu 214000, China

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ABSTRACT

A tissue-engineered bone has become a viable alternative to autologous bone for bone augmentation in atrophy alveolar ridge. The aim of the present study was to evaluate porous β-tricalcium phosphate (β-TCP) combined with autologous osteoblasts to augment edentulous alveolar ridge in a canine model. Autologous osteoblasts were expanded and combined with β-TCP scaffold to fabricate a tissue-engineered bone. 12 bilateral alveolar ridge augmentation surgeries were carried out in 6 beagle dogs with the following 3 groups: β-TCP/osteoblasts, β-TCP alone and autogenous iliac bone control ($n = 4$ per group). Sequential fluorescent labeling and radiographs were used to compare new bone formation and mineralization in each group. 24 weeks later, animals were sacrificed and non-decalcified and decalcified sections were evaluated histologically and histomorphometrically. Results indicated that the tissue-engineered bone dramatically enhanced new bone formation and mineralization, increase the new bone area, and maintain the height and thickness of the augmented alveolar ridge when compared with β-TCP alone group. More importantly, the tissue-engineered bone achieved an elevated bone height and thickness comparable to that of autogenous iliac bone graft. This study demonstrated the potential of porous β-TCP as a substrate for autogenous osteoblasts in bone tissue engineering for alveolar ridge augmentation.

1. Introduction

Dealing with a resorbed edentulous mandible or maxilla remains a major challenge in modern dentistry [1,2]. A deficient alveolar ridge fails to provide sufficient support and retention for dentures. That will not only compromise the soft tissue support and the lower anterior facial height, but also preclude dental implants placement [3,4], which may dramatically reduce the quality of life for patients. Alveolar augmentation of the deficient osseous ridge have become an integral part of therapeutic procedures in pre-prosthetic, pre-implantology surgery [5].

Several techniques have been suggested to reconstruct or enlarge a deficient alveolar ridge, including autologous bone grafts [6–11], distraction osteogenesis [12], and guided bone regeneration (GBR) [13]. Autologous bone grafts remains the gold standard for regeneration of alveolar bone [14,15]; however, they require extensive harvest of healthy tissue from a distant location. They are also limited by the unpredictability of remodeling with reports showing the complication rate ranging from 33% to >70% using the mandibular symphysis grafts [2,16,17]. Distraction osteogenesis, which was previously employed for lengthening of bones and now applied to increase alveolar crest, has a reported complication rate of ~70%, including basal bone and transport segment fractures, fixation screw loss, non-union, premature consolidation, and lingual positioning of transport segment [18]. In addition, it is technically demanding and often unacceptable for patients who cannot tolerate intraoral distraction devices, Finally, the guided-bone regeneration, using membranes to create a space for the development of new bone and subsequent implant placement, may face an unexpected exposure of the barrier membrane, which may

* Corresponding author. Tel.: +86 21 6313 5412; fax: +86 21 6313 6856.
E-mail address: xinquanj@yahoo.com.cn (X. Jiang).

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cause secondary infections and consequently failure of bone regeneration [19].

Due to the disadvantage of these methods, other alternative therapies need to be explored for alveolar bone augmentation. Recent technological advances have allowed researchers to explore regeneration of bone by using novel tissue engineering techniques [20], where design, construction, modification and growth of living tissue are the goal. Biomaterial and cells are among the most important key elements for tissue engineering [21]. So far, there has been a case report which used the principles of tissue engineering to augment alveolar ridge. In Strietzel et al.'s report, a tissue-engineered bone obtained by autogenous periosteum cells and polymer fleece was used for lateral alveolar augmentation [22]. However, this study only provided preliminary data. Though it demonstrated certain success, there was no sequential analysis conducted, and the mechanism of bone regeneration was largely unknown. Thus comprehensive pre-clinical investigations are necessary before extensive clinical applications with the techniques.

In this study, we used a porous β-TCP block combined with autologous osteoblasts to explore its effect on vertical alveolar ridge augmentation in a canine model. Autogenous bone or β-TCP alone served as controls.

2. Materials and methods

2.1. Animals

A total of 6 adult beagle dogs in healthy condition, aged 18–24 months old with an average weight of 12.5 kg were used in this study. The experimental protocol was approved by the Animal Care and Experiment Committee of Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine.

2.2. Cell culture

Under general anesthesia with 5% sodium pentobarbital (0.5 ml/kg), a 3 mm × 3 mm bony sheet biopsy was obtained from the lateral cortex of the mandibular body in the apical region of the second molar area by an intraoral buccal approach. The explants were placed immediately into a solution of phosphate buffered saline (PBS) containing of 100 units/ml penicillin and 100 units/ml streptomycin. After washing thoroughly by PBS to remove blood components, the explants were cut into small fragments and cultured in 100 mm dishes (Falcon, Franklin Lakes, NJ) with DMEM:F12 (1:1) culture medium containing 10% (v/v) FBS (Hyclone), 100 units/ml penicillin and 100 units/ml streptomycin. Explants were incubated at 37 °C under 95% humidity with 5% CO_2. Explants were subsequently maintained by replacing the medium every 3–4 days until cell density reached confluence. Then cells were detached with 0.25% trypsin/EDTA, subcultured at a density of 1×10^5 cells/cm^2 in 100 mm dishes. Cells at passage 2–3 were used for the following experiments.

2.3. Phenotypes characterization of cultured cells

The osteoblastic phenotypes of these expanded cells were confirmed by following tests.

2.3.1. Reverse transcription-polymerase chain reaction (RT-PCR)

The transcription of osteoblastic marker of Collagen I, Osteopontin (OPN) and Osteocalcin (OCN) was detected by RT-PCR. Briefly, the total cell RNA was prepared by using TRIzol Plus RNA purification kit (Invitrogen) according to manufacturer's instructions. 1.0 μg total RNA was used as template for the synthesis of cDNA with OligodT and AMV reverse transcriptase (TaKaRa, Japan). The following PCR amplification reaction utilized Taq polymerase and specific primers. All primer sequences were designed using Primer Premier 5.0 Software, synthesized commercially (Shenneng Co. Ltd. Shanghai, China) and the specific primers sets are outlined in Table 1.

2.3.2. Osteocalcin immunohistochemistry

To further evaluate osteoblastic differentiation level of expanded cells in vitro, immunostaining with OCN antibodies was carried out. The cells were seeded and grown on cover glass for 4 days and sections were subsequently fixed with 4% paraformaldehyde. Endogenous peroxidase was blocked with H_2O_2 for 30 min at room temperature. Non-specific binding was blocked with goat normal serum for 30 min at room temperature. Then the sections were incubated with mouse monoclonal antibodies against OCN (Abcam, Cambridge, UK) overnight at 4 °C in a moist chamber at 1:200 concentration and washed three times with PBS (PH 7.3). Goat biotinylated anti-mouse secondary antibody (Boster Co. Ltd, China) was incubated for 30 min at room temperature and washed three times with PBS (pH 7.3). Streptavidin biotin complex (Boster Co. Ltd, China) was incubated with the cells for 20 min at room temperature and the cells were washed five times with PBS (pH 7.3). Staining was performed by DAB substrate (Boster Co. Ltd., China). The sections were counterstained with hematoxylin, dehydrated through a series of alcohols and xylene, and then mounted with mounting medium. Positive cells stained brown, negative cells only showed blue/green nuclei but no brown cytoplasm.

2.3.3. Alkaline phosphatase and Von Kossa staining

After culturing for another 14 days, alkaline phosphatase (ALP) activity of the cells were stained by fixing the cells for 10 min at 4 °C and incubating the cells with a mixture of naphthol AS–MX phosphate and fast blue BB salt (ALP kit, Hongqiao, Shanghai, China) [23]. The Von Kossa staining was performed by fixing the cells in 70% ethanol and staining with 5% silver nitrate, and 5% Na_2SO_3 [24].

2.4. Preparation of osteoblasts/β-TCP construct

β-TCP scaffolds (Shanghai Bio-Lu Biomaterials Co. Ltd., Shanghai, China) were molded into cuboids (20 mm × 6 mm × 6 mm) and were sterilized by ^{60}Co irradiation before use. The scaffolds had volume porosity of 70% with average pore diameter of 450 ± 50 μm. For cell seeding, osteoblasts were detached from culture dishes, centrifuged to remove supernatant, and then resuspended in the culture media without FBS at a density of 2×10^7 cells/ml. Cells in suspension were slowly added to the β-TCP cuboids till a final saturation. After incubation for an additional 4 h to allow cell attachment, the scaffolds were used as described in Section 2.5.

In a parallel experiment, 3 mm × 3 mm × 3 mm cuboids were prepared and seeded with osteoblasts at an identical cell density. Four and 24 h later, the constructs were fixed in 2% Glutaric dialdehyde for 2 h, cut into two halves, and then subjected for scanning electron microscopy examination (Philips SEM XL-30, Amsterdam, Netherlands).

2.5. Surgical procedure

All mandibles were prepared by extraction of bilateral premolar as well as molar teeth 8 weeks before the alveolar augmentation surgeries. Under general anesthesia, 12 alveolar augmentation surgeries in 6 animals were made bilaterally and randomly divided into 3 groups: Group A consisted of tissue-engineered osteoblasts/β-TCP complex ($n = 4$), Group B consisted of β-TCP alone ($n = 4$), and Group C consisted of autogenous bone obtained from iliac bone ($n = 4$) as a positive control. For the autogenous bone grafts, an incision of 5 cm was made on top of the anterior iliac crest and a corticocancellus bone blocks of the same size (20 mm × 6 mm × 6 mm) were harvested from the anterior iliac crest. During the operation, full thickness mucoperiosteal flaps were elevated to expose the underlying alveolar crests bone. All soft tissue remnants were carefully removed from the alveolar ridge and different groups of grafts were put into place to augment the

Table 1
Primers for RT-PCR.

Gene	Primer sequence	Product Size (bp)	Denaturation	Annealing	Elongation	PCR cycles
Collagen type 1	5′GAGCGGAGAATACTGGATTGA3′ 5′CGGGAGGTCTTGGTGGTT3′	498	95 °C for 30 s	53 °C for 30 s	72 °C for 30 s	30
Osteopontin	5′CACTGACATTCCAGCAAC3′ 5′CTTCCATACTCGCACTTT3′	188	95 °C for 20 s	53 °C for 30 s	72 °C for 30 s	30
Osteocalcin	5′TCACAGACCCAGACAGAACCG3′ 5′AGCCCAGAGTCCAGGTAGCG3′	207	95 °C for 20 s	53 °C for 30 s	72 °C for 30 s	30
β-actin	5′CCTGTGGC ATCCACGA AACT3′ 5′GAAGCCATTTGCGGTGGACGA3′	307	95 °C for 20 s	53 °C for 30 s	72 °C for 30 s	30