Sequential Fluorescent Labeling Observation of Maxillary Sinus Augmentation by a Tissue-engineered Bone Complex in Canine Model

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Abstract

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Aim To evaluate the effects of maxillary sinus floor elevation by a tissue-engineered bone complex of β -tricalcium phosphate (β -TCP) and autologous osteoblasts in dogs.

Methodology Autologous osteoblasts from adult Beagle dogs were cultured *in vitro*. They were further combined with β -TCP to construct the tissue-engineered bone complex. 12 cases of maxillary sinus floor elevation surgery were made bilaterally in 6 animals and randomly repaired with the following 3 groups of materials: Group A (osteoblasts/ β -TCP); Group B (β -TCP); Group C (autogenous bone) ($n=4$ per group). A polychrome sequential fluorescent labeling was performed post-operatively and the animals were sacrificed 24 weeks after operation for

histological observation.

Results Our results showed that autologous osteoblasts were successfully expanded and the osteoblastic phenotypes were confirmed by ALP and Alizarin red staining. The cells could attach and proliferate well on the surface of the β -TCP scaffold. The fluorescent and histological observation showed that the tissue-engineered bone complex had an earlier mineralization and more bone formation inside the scaffold than β -TCP alone or even autologous bone. It had also maximally maintained the elevated sinus height than both control groups.

Conclusion Porous β -TCP has served as a good scaffold for autologous osteoblasts seeding. The tissue-engineered bone complex with β -TCP and autologous osteoblasts might be a better alternative to autologous bone for the clinical edentulous maxillary sinus augmentation.

Keywords maxillary sinus augmentation, tissue engineering, β -tricalcium phosphate, osteoblasts, fluorescent labeling

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Introduction

As the bony height from the alveolar crest to the sinus floor is usually not adequate enough for dental implantation due to sinus pneumatization or alveolar resorption, the lack of initial stability at edentulous sites would usually lead to the failure of osseointegrated implants (Tiwana *et al.*, 2006; Browaeys *et al.*, 2007). As a consequence, the augmentation of the deficient osseous ridge has become an integral part of therapeutic procedures in pre-prosthetic, pre-implantology surgery (Woj-

towicz *et al.*, 2007).

Among the various techniques used to regain the height of resorbed maxilla, maxillary sinus floor elevation is regarded as an effective way to restore the upper jaw (Jensen *et al.*, 1998). The use of autogenous bone grafts in sinus augmentation has been considered as “gold standard” since its introduction (Boyne and James, 1980) because of their excellent survival for loaded implants and the degree of functionality they can afford. However, when harvesting autologous bone, side effects including donor-side morbidity, infection, pain and

blood loss were reported (Ueda *et al.*, 2001). Allografts and xenografts are susceptible to immunoreaction and carry the disease transmission risks. Some synthetic materials, on the contrary, have limited potential for osteoconduction, as well as osteoinduction (Sun *et al.*, 2008).

Due to the disadvantage of the above methods, other alternative therapies need to be explored for maxillary sinus augmentation. Recent technological advances have allowed dentists to explore regeneration of bone by using novel tissue engineering techniques (Hollinger *et al.*, 2000) where biomaterials and cells are among the key elements for the principles (Langer and Vacanti, 1993). Though there have been few animal studies or clinical case reports applied in maxillary sinus augmentation with this method, these studies only gave some preliminary evaluation of the effects (Schimming and Schmelzeisen, 2004; Beaumont *et al.*, 2008). In this study, we have used porous β -TCP granules for the first time to combine with autologous osteoblasts to augment the maxillary sinus floor in a canine model, and a sequential fluorescent labeling observation was taken to evaluate the outcome used by this tissue-engineered bone complex.

Methods and materials

Animals

A total of 6 adult beagle dogs in healthy condition, 18 months of age 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 Peoples Hospital affiliated to Shanghai JiaoTong University, School of Medicine.

Cell culture

Under general anesthesia with 5% sodium pentobarbital (0.5 mL/kg), a 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, and placed immediately into phosphate buffered saline (PBS) containing of 100 units/mL penicillin and 100 mg/mL

streptomycin. After washing thoroughly by phosphate buffered saline (PBS) to remove blood components, the samples were cut into small fragments and cultured in 100 mm dishes (Corning, USA) with standard DMEM : F12 (1 : 1) (Gibco BRL, USA) culture medium containing 10% (*V/V*) fetal bovine serum (FBS; Hyclone, USA), 100 units/mL penicillin and 100 mg/mL streptomycin. Explants were incubated under 95% humidity, 5% CO₂, 37°C which were subsequently fed continuously by replacing the medium every 3–4 days until cell density reached 70%–80% confluence. Then cells were detached with 0.25% trypsin/EDTA, subcultured at a density of 1×10^5 cells/cm² in 100 mm dishes. The cells at passage 2–3 were used in our study.

Alkaline phosphatase staining and alizarin red S calcium nodules staining

After culturing for another 14 days, the expanded cells were measured by alkaline phosphatase (ALP) staining and alizarin red S staining. Briefly, the cells were fixed for 10 minutes at 4°C and incubated with a mixture of naphthol AS-MX phosphate, N,N-dimethylformamide and fast blue BB salt (ALP kit, Hongqiao, China)(Jiang *et al.*, 2006). The calcium nodules staining method consisted of the cells being cultured with 10 mg/L alizarin red S (Sigma Aldrich, USA) for 5–7 days before observed using a fluorescent microscope (Leica DM 1RB, Germany).

Preparation of Osteoblasts/ β -TCP construct

β -TCP granules (Shanghai Bio-Lu Biomaterials Co., Ltd., China; diameter: 1.5–2.5 mm;) were sterilized by ⁶⁰Co irradiation before use. 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 combined with the β -TCP granules till a final saturation. After being incubated for additional 4 hours to allow cell's initial attachment, implantation surgeries was performed *in vivo*.

In a parallel experiment, 3 mm×3 mm×3 mm cuboids were prepared and seeded with osteoblasts at an identical cell density. 1 day after, the

constructs were fixed in 2% glutaric dialdehyde for 2 hours, cut into two halves, and then subjected for scanning electron microscopy examination (Philips SEM XL-30, Netherlands).

Surgical procedure

Under general anesthesia through intramuscular injection of ketamine (10 mg/kg) all dogs were prepared by extraction of maxillary third premolar and first molar bilaterally 8 weeks before the maxillary sinus augmentation surgery, and gingival was closed after the operation. 12 alveolar augmentation surgeries in 6 animals were made bilaterally and randomly repaired with the following 3 groups of grafts: Group A consisted of tissue-engineered osteoblasts/ β -TCP complex ($n=4$); Group B consisted of β -TCP alone ($n=4$); as a positive control, Group C consisted of autogenous bone obtained from iliac bone ($n=4$).

Based on the technique described by Kent and Block (Kent and Block, 1989), bilateral maxillary sinus elevation surgeries were carried out identically. The edentulous region was opened by crestal incision. The mucoperiosteal flap was reflected on the buccal cortical plate, extending from the first maxillary premolar to the second maxillary molar. A bone window of 1.5 cm $\times$ 2 cm was marked using round burs. After removal of the bone, the sinus membrane was elevated from the maxillary sinus floor, taking care to avoid perforation of the antral membrane. After creating the extrasinusoidal space, and around 1.2 mL tissue-engineered bone substitutes or autologous bone were inserted to elevate the sinus to reach an average height of 10 mm to the floor (Figure 1). Finally, the mucoperiosteal flap was repositioned and sutured.

Figure 1 Maxillary sinus augmentation surgery

For the autogenous bone graft, an incision of 5 cm was made and a corticocancellous bone block was harvested from the anterior iliac crest. The periosteum and skin flap were replaced and sutured. The corticocancellous bone block was ground with a bone mill (Leibinger, Germany) and the bone graft was stored in a physiologic saline solution before it was grafted to the maxillary sinus.

Sequential fluorescent labeling

A polychrome sequential fluorescent labeling method was carried out to label the mineralized tissue and assess the time course of new bone formation and mineralization. At 4, 12, 20 weeks after the operation, the animals were intraperitoneally administered with 25 mg/kg hydrochloride tetracycline (TE) (Sigma Aldrich, USA), 20 mg/kg calcein (CA) (Sigma Aldrich, USA), and 30 mg/kg alizarin red S (AL) (Sigma Aldrich, USA) respectively.

Sample preparation, the height as well as histological and fluorescent analysis

The dogs were sacrificed at 24 weeks after surgery. The augmented maxillary sinus was fixed in 10% buffered formalin (pH 7.4). One half cut along the perpendicular plane was dehydrated in ascending concentrations of alcohol from 75% to 100%, and finally embedded in polymethylmethacrylate (PMMA). The specimens were cut in 150 μ m thick sections using a microtome (Leica, Germany), and were subsequently ground and polished to a final thickness of about 40 μ m.

The measurements of elevated height were performed on gross cross section. It was defined as the distance between the bottom and the top of augmented maxillary sinus on undecalcified specimens, and the mean value of four specimen was used to calculate the height of each group.

Undecalcified sections were observed for fluorescent labeling under confocal laser scanning microscope (CLSM) (Leica TCS Sp2 AOBS, Germany), excitation/emission wavelengths for each of the fluorescence was used as 405 nm/580 nm (TE), 488 nm/517 nm (CA), 543 nm/617 nm (AL), respectively. Then the sections were stained with

Figure 2 Cell culture and osteoblastic phenotype tests

(A): Cells were found growing around bone fragments after initial incubation (100×). (B): Cells proliferated quickly afterwards to reach a confluence (100×). (C): Alkaline phosphatase-positive staining area (50×). (D): Mineralized calcium nodules shown by alizarin red S staining (100×).

van Gieson's picro fuchsin for histologic observation.

Statistical analysis

Statistically significant differences ($P < 0.05$) between the various groups were measured using ANOVA and SNK post hoc. All statistical analysis was carried out using a SAS 6.12 statistical software package (Cary, USA).

Results

Cell culture and osteoblastic phenotype tests

Cells were found growing around bone fragments 5–9 days after initial incubation (Figure 2A), and proliferated quickly afterwards to reach a confluence after approximately another 4–7 days (Figure 2B) for subculture. Around 14 days after seeding for expanded cells, ALP-positive staining (Figure 2C) and mineralized calcium nodules

alizarin red S staining (Figure 2D) were observed in cultured cells, demonstrating that those cultured cells maintained the osteoblastic phenotype after sufficient *in vitro* expanding.

Adhesion and spreading of osteoblasts on the material

Scanning electron microscope was used to determine the pores of β -TCP with a dimension of 450 μm and the interconnection pores diameter of (150 ± 50) μm (Figure 3A). 1 day after the osteoblasts were combined with the material, cells attached to and spread on the surface of the scaffold *in vitro* (Figure 3B). The results suggested that the material was a suitable biomaterial as it facilitated osteoblasts initial adhesion and spreading onto its surface.

The height of augmented maxillary sinus and histological analysis

To evaluate the effects of augmented maxillary

Figure 3 Scanning electron microscopic evaluation of the scaffold microstructure, and osteoblasts attaching to and spreading on the surface of the scaffold

(A): Scanning electron microscopic evaluation of the scaffold microstructure (50 $\times$). (B): 1 day after the osteoblasts were combined with β -TCP, they could be seen attaching and spreading on the surface of the scaffold (600 $\times$).

Figure 4 The height of augmented maxillary sinus analysis

There is significant difference between Group A and Group B or Group C ($P < 0.05$).

(*indicates significant differences $P < 0.05$).

sinus, the height was measured. As Figure 4 showed, the height of tissue-engineered maxillary sinus augmentation remained with (6.55 $\pm$ 1.21) mm at 24 weeks, by comparison, the augmented height decreased significantly to only (4.51 $\pm$ 0.69) mm for group B (β -TCP control), which is comparable to the autogenous bone group (4.26 $\pm$ 1.03) mm ($P > 0.05$). Both Group B and Group C were significant lower than group A ($P < 0.05$). Under light microscopy (Leica DM 2500, Germany), the specimens in group A demonstrated a significant amount of new bone formation even at center portion of the block, which is obviously higher than that of group B or group C (Figure 5A, 5B, 5C).

Fluorochrome microscopy

The deposition of mineralized bone matrix was

observed in Group A, Group B and Group C for different time points as demonstrated by tetracycline (yellow), calcein (green) and alizarin red S (red). At week 4, fluorescent-labeling areas in Group A were larger than those in Group B or Group C, as evidenced by the fluorescence of tetracycline (yellow). There was no significant difference among the three Groups detected by calcein (green) fluorescent labeling at week 12, however, 20 weeks later, the mineralization of new bone was present more frequently in Group C than in Group A or Group B according to alizarin red S labeling. These data suggested that seeding osteoblasts contributed to the enhanced mineralized area at an earlier stage for the tissue-engineered group. While in Group C, the absorption of autogenous ilium bone was probably predominant at earlier stage, since obvious new bone regeneration and mineralization could be seen till at a later stage of week 20 (Figure 6A, 6B, 6C).

Discussion

Sinus augmentation has become a standard procedure to increase bony height in the posterior maxilla. Though several procedures and materials for augmenting bone height have been reported (Browaeys *et al.*, 2007; Knabe *et al.*, 2008; Yamada *et al.*, 2008), there was still a lack of objective scientific data regarding the beneficial effects about using tissue-engineered bone complex for augmentation of maxillary sinus. In this study, we

Figure 5 The photomicrograph of bone formation inside augmented maxillary sinus for different groups (40×)

Figure 6 New bone formation and mineralization were determined by fluorescent observation the mineralization level for different groups

Determined by TE, CA and AL fluorescent observation the mineralization level at 4, 12, and 20 weeks after operation for different groups (scale bar 100 μ m).

demonstrated that tissue-engineered bone with autologous osteoblasts and a biodegradable β -TCP scaffold achieved an earlier bone formation and mineralization and maximally maintained the elevated space of the maxillary sinus when compared with β -TCP scaffold alone or even autogenous bone, which may facilitate to ensure the placement of dental implants with sufficient length and satisfy initial stability.

Autologous osteoblasts derived from periosteum or bone, has been successfully used previously to regenerate bones in animal experiments, or in clinic (Rai *et al.*, 2004; Strietzel, 2006). They are easy to harvest, and compared with mixed properties of bone marrow stromal cells, they are relatively pure, and have inherent bone formation capability (Hu *et al.*, 2003; Lee *et al.*, 2006). We have been able to isolate the osteoblasts from a small sheet of cortical bone in mandible through an intraoral surgical procedure. We were able to obtain enough cells in 3 passages with definite osteoblastic phenotypes, which was sufficient for constructs to fill a void volume of as large as 3 cm³. Such a surgical biopsy is minimally invasive, and convenient for oral surgeons.

The β -TCP we selected has good biocompatibility and osteo-conductive capacity. Compared with other bone substitutes (e.g. collagen scaffolds), β -TCP is characterized by its precisely defined physical and chemo-crystalline properties, high level of purity and uniformity of chemical composition, so that its biological reactions can be predicted reliably (Horch *et al.*, 2006). It can be fabricated into high porosity scaffolds with good interconnectivity, which will ensure intercellular communication among osteogenic cells rested in lacunae. The macro-porosity of the material will facilitate cells adhesion and growth, and facilitate bony ingrowth and especially vascularization (Dong *et al.*, 2002). It has been used in oral and maxillofacial surgery such as sinus floor augmentation and repair of deficient alveolar bone at immediate implant (Zerbo *et al.*, 2001; Ormianer *et al.*, 2006). However, its potential as a tissue-engineered scaffold is not well established for maxillary sinus augmentation.

A polychrome fluorescent labeling observation in the current animal study showed that new bone formation and mineralization in Group A (osteoblasts/ β -TCP) was more than Group B (β -TCP) or

Group C (autologous bone) at 4 weeks according to the area of TE labeling (yellow). There were no significant difference were detected among three groups at 12 weeks according to CA labeling (green). While 20 weeks after operation, AL labeling (red) was more obviously observed in Group C. The data indicated that, in Group A, seeding osteoblasts with osteoconductive β -TCP, could promote new bone formation and mineralization inside the elevated space at a much earlier stage, while for β -TCP scaffold control, the osteoconductive β -TCP alone can be used to achieve only a delayed and less effective mineralization in augmented maxillary sinus. Unexpectedly, for autogenous bone, resorption of grafts seemed to be the dominant phenomena with only few new bone deposition and mineralization in maxillary sinus being observed at an earlier period, which increased till at a later stage of 20 weeks.

Coincided with the mineralization observation, the histological observation of new bone formation at 24 weeks after operation showed that new bone area inside the tissue-engineered bone was much larger than those of the control groups. At this point in time, the augmented sinus height analysis suggested that the tissue-engineered bone could maximally maintained the augmented space. For group B, β -TCP alone achieved a much less elevated space than group A, indicating that, without osteoblasts, β -TCP granule may be resorbed more rapidly. Interestingly, the height of the autograft also reduced significantly at 24 weeks postoperation. Similar trend on autologous bony resorption were reported in other studies (Watanabe et al., 1999; Wada et al., 2001). One explanation might be that cancellous bone in the autogenous bone graft could not withstand sinus pressures for long periods of time, and as such, start to lose their density and height during the first several weeks (Jensen et al., 1998). Some published clinical data showed that the absorption rate using autogenous bone in sinus augmentation was 47% 6–7 months after surgery (Johansson et al., 2001). In fact, the resorption of autogenous bone graft has not only been reported in a sinus lifting model but has also been described in studies that explored ridge augmentation and bone defect's restoration (Roccuzzo et al., 2007). By comparison, the tissue-engineered bone complex in the current

study might have withstood the sinus air pressure and maintained the highest elevated space to achieve an increased effect than scaffold alone or autologous bone.

Conclusion

In conclusion, porous β -TCP had served as a good scaffold for autologous osteoblasts seeding, and tissue engineered bone complex with β -TCP and autologous osteoblasts might be a superior alternative to autologous bone for the clinical edentulous maxillary sinus augmentation.

Acknowledgements

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