Development of \(\beta\)-tricalcium Phosphate/Collagen Sponge Composite for Bone Regeneration

Tomonori MATSUNO\(^1\), Tatsuo NAKAMURA\(^2\), Koh-ichi KUREMOTO\(^3\), Syunsuke NOTAZAWA\(^4\), Taka NAKAHARA\(^5\), Yoshiya HASHIMOTO\(^6\), Tazuko SATOH\(^7\) and Yasuhiro SHIMIZU\(^2\)

\(^1\)Department of Oral & Maxillofacial Surgery, The Nippon Dental University School of Dentistry at Tokyo, 1-9-20, Fujimi, Chiyoda, Tokyo, 102-8159, Japan
\(^2\)Department of Bioartificial Organs, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo, Kyoto, 606-8507, Japan
\(^3\)Department of Removable Prosthodontics and Occlusion, Osaka Dental University, 1-9-20, Fujimi, Chiyoda, Tokyo, 102-8159, Japan
\(^4\)Department of Material Chemistry, Kyoto University, Yoshida-Honmachi, Sakyo, Kyoto, 606-8501, Japan
\(^5\)Department of Removable Prosthodontics and Occlusion, Osaka Dental University, 1-9-20, Fujimi, Chiyoda, Tokyo, 102-8159, Japan
\(^6\)Department of Biomaterials, Osaka Dental University, 8-1, Kuzuahanazono-cho, Hirakata, Osaka, 573-1121, Japan

Received August 30, 2005/Accepted December 16, 2005

Synthetic biomaterials have been developed and used for bone grafting. Here, we developed a biodegradable sponge composite for bone tissue engineering by combining \(\beta\)-tricalcium phosphate (\(\beta\)-TCP) and collagen. In addition, we sought to determine the optimal \(\beta\)-TCP granules/collagen ratio by evaluating and bone formation in vivo. Porous \(\beta\)-TCP granules were mixed with atelocollagen hydrochloride solution at various ratios — 0.02, 0.05, 0.1, and 0.2 g/mL. The resultant mixtures were freeze-dried and subjected to dehydrothermal treatment in vacuo. The final composites obtained were designated \(\beta\)-TCP/collagen sponge composites (\(\beta\)-TCP/CS). Through compression testing, it was found that the stress values for \(\beta\)-TCP/CS (0.2 g/mL) were higher than those of the other three composites over the whole strain range. Histological evaluation at four weeks after implantation revealed that the collagen sponge had degraded and newly formed bone was present on the surface of the \(\beta\)-TCP granules. At 12 weeks, the \(\beta\)-TCP granules were completely degraded and remodeling of the lamellar bone was observed.

Key words: \(\beta\)-tricalcium phosphate/collagen sponge composite, Biodegradable scaffold, Bone tissue engineering

INTRODUCTION

In oral and maxillofacial surgery, bone defects occur due to infections, trauma, tumors, and cysts, as well as clefts of the alveolar bone and palate. Bone resorption caused by periodontal diseases often results in tooth loss. Although autologous bone grafts have been transplanted into such bone defects or resorption sites, these autografts have inherent donor site limitations, including insufficient bone volume, donor site morbidity and discomfort\(^{1,2}\). Therefore, synthetic biomaterials have been developed for bone grafting, and synthetic calcium phosphate ceramics — such as hydroxyapatite (HA: Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\)) and \(\beta\)-tricalcium phosphate (\(\beta\)-TCP: \(\beta\)-Ca\(_3\)(PO\(_4\))\(_2\)) — are commonly used as bone graft substitutes\(^{3-6}\).

HA and \(\beta\)-TCP implants are both well known to possess high tissue compatibility, and that new bone can form directly on these calcium phosphates. Moreover, the regular and uniform surface morphology of HA and \(\beta\)-TCP is a key factor that promotes cell proliferation and differentiation\(^7\). However, since \(\beta\)-TCP biodegrades more rapidly than HA ceramics and since HA usually shows minimal resorption\(^8\), \(\beta\)-TCP is considered to be a more suitable scaffold candidate for bone tissue than HA.

Collagen has been conventionally applied in the clinic in various forms, such as gels or sponges, since it shows good biocompatibility and absorbability. Cross-linked collagen sponges are considered to be a useful scaffold matrix for several tissues, such as the esophagus, trachea, peripheral nerves, and periodontal tissues\(^{13-19}\). In addition, dehydrothermal collagen sponges have been reported to be effective carriers for the controlled release of growth factors\(^{19}\). Type I collagen is not only a major component of the bone matrix and useful as a carrier of osteoblasts\(^{20}\), but that osteoblast cells had also well invaded collagen sponge with porous hydroxyapatite frame\(^{21}\). In the light of these reported benefits, we prepared collagen sponges of \(\beta\)-TCP/CS by a dehydrothermal cross-linking method to bond both the collagen fibrils and \(\beta\)-TCP granules.

In this study, we developed \(\beta\)-TCP/CS for the regeneration of alveolar bone defects. To determine the optimal ratio of \(\beta\)-TCP granules to collagen solution of \(\beta\)-TCP/CS for this purpose, we evaluated its mechanical properties using a compression test and...
its effects on bone regeneration after implantation into canine tibial bone defects.

MATERIALS AND METHODS

Preparation of \( \beta \)-TCP/CS composites

The collagen used was extracted from porcine skin by enzymatic treatment with pepsin (Nippon Meat Packers, Osaka, Japan). During this process, the telopeptides of collagen, which are considered to have antigenicity, were removed. Through this atelocollagen preparation, types I (70-80\%) and III (20-30\%) collagen were obtained. \( \beta \)-TCP granules (OSferion Type G1, Olympus Optical Co., Tokyo, Japan) were used, which were prepared according to a mecanochemical method. After autoclaving, \( \beta \)-TCP granules of 0.5-1.5 mm diameter were subjected to the following procedure.

The atelocollagen (2.5 g) was dissolved in 0.1 mol/L HCl aqueous solution (16 mL) at pH 3 to give a final concentration of 1.0 wt\%. The collagen solution was homogenized at 8000 rpm on ice for 15 minutes using a homogenizer (Nissei AM-3, Nihonseiki Kaisha, Tokyo, Japan). Next, \( \beta \)-TCP granules were mixed with the homogenized collagen solution at ratios of 0.02, 0.05, 0.1, and 0.2 g/mL (\( \beta \)-TCP granules/collagen solution). Each resultant \( \beta \)-TCP/collagen mixture was poured into two plastic molds. For the mechanical test, the sample mold was 10.0 × 10.0 × 10.0 mm\(^3\), while that for the animal test was of 5 mm diameter and depth. Immediately after pouring into plastic molds, all samples were frozen to −80°C and freeze-dried for 24 hours. The freeze-dried \( \beta \)-TCP/collagen composites resembled sponge-like structures, and were subsequently cross-linked in vacuo at 140°C for 24 hours. Microstructures of the resultant scaffolds were then observed using an SEM (S-450, Hitachi Ltd., Tokyo, Japan). Collagen sponges without \( \beta \)-TCP granules were used as controls.

Mechanical testing

A universal testing machine (RTM-500, Orientec Co., Tokyo, Japan) was used for the mechanical testing. Two parallel plates of 10 cm diameter (upper plate) and 15 cm diameter (lower plate) were used for the compression tests. Cross-head speed was set to 10 mm/min. Samples were compressed in the radial direction (Fig. 1), and the force and displacement were monitored. Measurements were taken in a dry condition for the various \( \beta \)-TCP/CS composites (0.02, 0.05, 0.1, and 0.2 g/mL) as well as for the control collagen sponges without \( \beta \)-TCP granules. The same compression test was also conducted after the same samples were immersed in a saline solution (Otsuka Normal Saline, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) at 20°C for 20 minutes. Each compression test was performed using five samples (n = 5).

In vivo implantation

A total of eight adult (2-5 years old) beagle dogs weighing 4-12 kg were used. Each animal was anesthetized with an intramuscular injection of atropine sulfate (Atropine Sulfate Injection, Tanabe Seiyaku Co. Ltd., Osaka, Japan; 0.5 mg/mL) followed by ketamine (Ketalan®, Sanka Co. Ltd., Tokyo, Japan; 10 mg/kg) and xylazine (Rompun® Inj. Solution 2%, Bayer Health Care, Leverkuse, Germany; 3 mg/kg). To reduce the risk of postoperative infections, each dog was administered an antibiotic (Vicilln®, Meiji Seika Kaisha Ltd., Tokyo Japan; 50 mg/kg) immediately after operation. As for all surgical procedures, they were carried out under additional local anesthesia (2% Xylocain, Fujisawa Pharmaceutical Co., Osaka, Japan).

An initial incision was made in the skin covering an appropriate part of the tibia, and the skin and subcutaneous tissue were separated from the periosteum. A second incision was made in the periosteum of the tibia, and the periosteum was elevated and carefully dissected from the underlying tibia. After exposure of the tibia, five bone defects with diameter and depth of 5 mm were prepared at 5-mm intervals using a trephine bur at a low rotational speed under continuous saline cooling. Subsequently, each defect was implanted with one of the four \( \beta \)-TCP/CS composites (0.02, 0.05, 0.1, and 0.2 g/mL) or a control collagen sponge. The periosteum was then sutured over the implant with 5-0 Vicryl® (Johnson and Johnson, New Jersey, USA) and the skin closed using 3-0 nylon sutures.

All animals were sacrificed with an overdose of intravenously administered pentobarbital sodium at four (n=3) or 12 (n=3) weeks after surgery. Two dogs were excepted from this experiment because of local infection. Tissue blocks were removed and fixed in 10% (v/v) buffered formalin. After decalcification and paraffin embedding, sections were cut at the
center of each defect in a uniaxial plane and subjected to hematoxylin and eosin (H&E) staining for histological examination. All procedures were performed in accordance with the Guidelines for Animal Experiments of Kyoto University (1989).

Histometric evaluation
Histometric evaluation was carried out for the areas of new bone formation in the specimens at 12 weeks after surgery. Computer-assisted histomorphometric measurements of newly formed bone were obtained using Image pro® Plus (Media Cybernetics®, Maryland, USA), and the area of newly formed bone (expressed in percentage) was calculated according to the following formula:

Area of newly formed bone = Newly formed bone area/Cortical bone defect area × 100%

Statistical analysis
The mean and standard deviation of each parameter were obtained for each group. Statistical analysis was performed using one-way factorial ANOVA and Scheffe’s F test by means of “SPSS 13.0” computer software (SPSS Inc., Troy, NY, USA).

RESULTS

β-TCP/CS composites
Fig. 2 shows the appearance of a β-TCP/CS composite (0.2 g/mL). All the composites were elastic and could be easily cut with scissors or a sharp knife. Therefore, β-TCP/CS can be formed into various shapes and fitted into bone defects. The microstructure of a β-TCP/CS composite (0.2 g/mL) is shown in Fig. 3. The composites were composed of β-TCP granules (ranging from 0.5 to 1.5 mm in diameter) and a three-dimensional porous structure (with an anastomosing network).

Compression test
As shown in Fig. 4A, the stress (σ) values for β-TCP/CS (0.02, 0.05, and 0.1 g/mL) were almost the same in the low strain region (ε < ~20%) in the dry condition, but differed markedly in the 30% strain region. However, the σ value for β-TCP/CS (0.2 g/mL) was significantly greater than those of the other three composites over the whole strain range. In the wet condition (Fig. 4B), the σ values (ε > ~40%) for β-TCP/CS (0.02, 0.05, and 0.1 g/mL) decreased by more than 80-90% of the dry condition, whereas that of β-TCP/CS (0.2 g/mL) decreased by about 20%. In both conditions, the σ value for the control sponges was almost 0.

Table 1 shows the Young’s moduli of the β-TCP/CS composites and control sponges. The Young’s moduli of β-TCP/CS (0.2 g/mL) were significantly higher than those of β-TCP/CS (0.1, 0.05, and 0.02 g/mL) in both dry and wet conditions.

Histological evaluation and histometric analysis
Fig. 5 shows the histological sections of canine tibial bone defects implanted with β-TCP/CS (0.2 g/mL) or control collagen sponge at four and 12 weeks after implantation. For all the β-TCP/CS composites, bone repair was observed in the tibial defects at four weeks (Fig. 5A). The newly formed, woven bone was directly deposited on the surface of β-TCP granules (Fig. 5B). Further, the newly formed bone was interconnected and included many osteocytes in an irregular pattern. The composites were regularly lined by large numbers of cuboidal osteoblasts — indicating active bone formation, and many capillary vessels...
were observed within the bone defects. This bone formation was initiated from the surface of the β-TCP granules, and not from the edge or bottom of the bone defect. In the bone defects implanted with a control collagen sponge without β-TCP granules, the collagen sponge was completely degraded by four weeks (Fig. 5C). In these samples, the top of the bone defect had been invaded by connective tissue, although trabecular bone was observed in the center of the defect.

At 12 weeks after implantation, the β-TCP granules showed further degradation and continuous cortical bone formation covered the top of the bone defect (Fig. 5D). In the central part of the bone defect, resorption of the newly formed bone was observed around the β-TCP granules. Furthermore, bone marrow tissue and fatty marrow were observed in this area. These findings indicated that bone formation and maturation had progressed, as well as subsequent bone remodeling. Over time after implantation, the β-TCP granules continuously degraded without inflammation. Within the evaluation period in this study, the β-TCP granules were almost completely degraded. In the control group, the bone defect surface became hollow and was filled with soft connective tissue, whereby no continuity of cortical bone was observed (Fig. 5E).

Results of the histometric analysis are shown in Fig. 6. The percentage of newly formed bone area in the cortical bone defect (i.e., new bone area in %) ± S.D. was measured for each composite or control. Amount of new bone formation in the cortical bone area increased with increasing content of the β-TCP granules. The new bone area (%) for β-TCP (0.2 g/mL) was significantly higher than those of the other three composites and the controls.

**DISCUSSION**

β-TCP ceramics show strong osteoconductivity and high biocompatibility due to two advantageous characteristics: their chemical composition is similar to that of natural bone and that they degrade in vivo. Furthermore, β-TCP is available in various forms, such as porous blocks, granules, and powder. For these reasons, β-TCP has been widely applied to bone defects as a scaffold for bone tissue engineering. Atelocollagen molecules do not contain telopeptides and show extremely low antigenicity when adopted as a basic material for medical use. Type I collagen is a major component of the bone matrix and is useful as a carrier of osteoblasts. Indeed, it has been reported that osteoblast cells had well invaded collagen sponge with porous hydroxyapatite frames. Moreover, extracellular matrix molecules – such as laminin and fibronectin – can be adsorbed onto the collagen scaffold to aid cell

---

**Table 1** Young’s moduli of β-TCP/CS composites

<table>
<thead>
<tr>
<th>β-TCP/collagen (g/ml)</th>
<th>Dry (kPa)</th>
<th>Wet (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.4 ± 0.49</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>0.02</td>
<td>18 ± 2.38</td>
<td>1.2 ± 0.21</td>
</tr>
<tr>
<td>0.05</td>
<td>47 ± 3.82</td>
<td>1.6 ± 0.27</td>
</tr>
<tr>
<td>0.1</td>
<td>62 ± 3.41</td>
<td>1.8 ± 0.30</td>
</tr>
<tr>
<td>0.2</td>
<td>1400 ± 62.6</td>
<td>340 ± 37.4</td>
</tr>
</tbody>
</table>

---

Fig. 4A Compression testing of β-TCP/CS composites in dry condition.

Fig. 4B Compression testing of β-TCP/CS composites in wet condition.

---
migration and proliferation\textsuperscript{24}.

Ishaug \textit{et al.}\textsuperscript{27} suggested that scaffold materials used for bone formation should meet the following criteria. First, the scaffold material must allow osteoblast attachment, since these anchorage-dependent cells require a supportive matrix to survive. Second, the scaffold must provide an appropriate environment for osteoblast proliferation and function. Third, the scaffold should allow ingrowth of vascular tissue to ensure the survival of transplanted cells. Fourth, the scaffold material should be biodegradable and its degradation products should be easily metabolized and excreted. Finally, the scaffold material should be able to be processed into irregular three-dimensional shapes. The results of the present study indicated that $\beta$-TCP/CS satisfied all the

Fig. 5 Histological section of canine tibial defects (H&E staining).

A: $\beta$-TCP/CS (0.2 g/mL) at 4 weeks post-implantation.
B: Higher magnification of A, where asterisk: $\beta$-TCP granules; arrow: newly formed bone.
C: Collagen sponge (control) at 4 weeks post-implantation.
D: $\beta$-TCP/CS (0.2 g/mL) at 12 weeks post-implantation.
E: Collagen sponge (control) at 12 weeks post-implantation.

(A, C, D, and E: 2.5 mm scale bar; B: 1.0 mm scale bar)
above-mentioned tissue engineering scaffold criteria for bone regeneration.

Biomaterials for bone grafting need to possess sufficient mechanical strength to sustain the shape until they become replaced by newly formed bone. However, a material that is too stiff poses a problem since it is difficult to adapt the shape that is to be fitted into a bone defect. The β-TCP/CS composite can be easily cut with scissors or a sharp knife, and can therefore be easily molded for use for various tissue disorders, such as periodontal bone defects, cyst cavities, maxillary sinus floor augmentation, and alveolar bone augmentation.

Through compression testing, it was shown that the stress (σ) values of β-TCP/CS (0.2 g/mL) were significantly greater than those of β-TCP/CS (0.1, 0.05, and 0.02 g/mL) over the whole strain range. Furthermore, the stress-strain curves of β-TCP/CS (0.2 g/mL) differed from those of the other three composites in both dry and wet conditions. In particular, the initial Young’s moduli of β-TCP/CS (0.2 g/mL) differed significantly from those of the other three composites. These results suggested that the optimal mixing concentration of β-TCP granules and the atelocollagen solution was 0.2 g/mL.

With regard to the biodegradability of β-TCP/CS, the control collagen sponge scaffold was completely degraded at four weeks after implantation. Although this collagen sponge scaffold was replaced not only by soft connective tissue but also newly formed bone, the new bone did not show the formation of a typical bone network. In contrast, the β-TCP/CS scaffolds were replaced by a network of newly formed bone. Furthermore, the remaining β-TCP granules in the defect were in direct contact with the newly formed bone. Osteogenic progenitor cells migrated from the adjacent bone edge or b-
tom, adhered to the surface of the β-TCP granules, and subsequently formed new bone. These results suggested that with an adequate three-dimensional porous structure consisting of β-TCP granules and a collagen sponge, it served to provide an appropriate spatial arrangement for the osteogenetic cells as well as facilitate vascular invasion. Slight degradation of the β-TCP granules was observed at four weeks, while almost all the granules had degraded by 12 weeks. Continuous, thick lamellar structural bone was formed at the top of the defect, while newly formed bone at the center of the defect was resorbed and replaced by bone marrow tissue at 12 weeks after operation. These observations indicated that the β-TCP/CS composites supported bone regeneration, including lamellar and trabecular bone formation and remodeling.

Strategies for engineering bone tissue have been developed using three components: a scaffold, growth factors, and stem cells. By incorporating transforming growth factor-β1 into a dehydrothermally cross-linked collagen sponge, the former was released in a biologically active form as a result of sponge biodegradation, resulting in enhanced bone repair of skull defects. Yamanouchi et al. suggested that dexamethasone-treated human osteoblasts within collagen sponge possessed differentiated osteoblastic functions and were able to form bone tissue in vitro. In this connection, β-TCP may act as a scaffold for cultured bone derived from bone marrow and thereby promote the regeneration of bone tissue. Based on the results of this study, it may be possible to expand the potential applications of β-TCP/CS.

In conclusion, this study demonstrated that composites created using β-TCP granules and collagen sponges possessed adequate mechanical strength and were sufficiently adaptable for treating bone defects. Furthermore, the biodegradable β-TCP/CS was replaced by newly formed bone without any adverse responses. Therefore, this newly developed type of sponge composite may be useful for bone tissue engineering.

ACKNOWLEDGEMENTS

This work was supported in part by a grant-in-aid from the Osaka Dental University Joint Research Funds (No. B 03-01).

REFERENCES

β–TCP/COLLAGEN SPONGE FOR BONE REGENERATION