Use of a gelatin hydrogel membrane containing β-tricalcium phosphate for guided bone regeneration enhances rapid bone formation

Kanako NORITAKE1,2, Shinji KURODA1, Myat NYAN1, Yuji ATSUZAWA3, Motohiro UO4, Keiichi OHYA5 and Shohei KASUGAI6

1 Section of Oral Implantology and Regenerative Dental Medicine, Department of Masticatory Function Rehabilitation, Tokyo Medical and Dental University, Tokyo 113-8549, Japan
2 Oral Diagnosis and General Dentistry, University Hospital of Dentistry, Tokyo Medical and Dental University, Tokyo 113-8549, Japan
3 Nippi Research Institute of Biomatrix, Tokyo 120-8601, Japan
4 Section of Advanced Biomaterials, Tokyo Medical and Dental University, Tokyo 113-8549, Japan
5 Pharmacology, Tokyo Medical and Dental University, Tokyo 113-8549, Japan

Corresponding author, Kanako NORITAKE; E-mail: noritake.irm@tmd.ac.jp

INTRODUCTION

Oral rehabilitation with dental implants is effective, predictable, and can provide functional and esthetically pleasing treatment outcomes. However, this modality is difficult when the bone at the implant installation site is insufficient. Autologous bone graft is regarded as the gold standard for bone augmentation; however, it increases the burden on patients, and induces donor site inflammation1. Therefore, an effective method without this complication is desirable for both patients and clinicians. Guided bone regeneration (GBR) is a clinical technique developed by Dahlin et al. that regenerates bone at sites where the existing bone is insufficient2-4.

This technique is based on the concept that application of a membrane creates a protected space which facilitates the proliferation of angiogenic and osteogenic cells from the basal bone without interference from proliferating fibroblasts in the surrounding soft tissue5.

To develop an ideal bioresorbable membrane for GBR, we chose gelatin because it has been used as a carrier of recombinant proteins, such as fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs)6-9, and lacks antigenicity10.

However, a membrane prepared from gelatin alone cannot function as a GBR membrane due to its mechanical weakness. To overcome this issue, β-tricalcium phosphate (β-TCP) powder was mixed with the gelatin solution. β-TCP has often been used as a bone substitute, because it is osteoconductive and biodegradable11. These characteristics are also expected to contribute a better outcome of this novel membrane as a GBR membrane.

Although the combination of gelatin and β-tricalcium phosphate has already been applied as a biomaterial8,9, an ideal, thin, mechanically strong, safe, and effective membrane for GBR has not yet been developed. Since a GBR membrane is covered by the periosteum and mucosal tissue, it should be as thin as possible, and still maintain sufficient mechanical integrity to reduce complications, such as flap dehiscence and subsequent infection12.

We used a commercially available, medical-grade, low-endotoxin gelatin and β-TCP powder to prepare gelatin hydrogel membranes containing β-TCP (G-TCP) by thermal cross-linking. In order to plan optimization of the membrane fabrication, we carried out preliminary experiments to compare several types of the density of the aqueous gelatin, the ratio of β-TCP, crosslinking condition evaluating by in vitro and in vivo studies before starting this study (date not shown). The purpose of the present study was to evaluate effects of this novel G-TCP membrane on rat bone marrow cell proliferation and differentiation in culture and bone regeneration of parietal bone defects in rats.

Keywords: β-Tricalcium phosphate, Gelatin hydrogel, Guided bone regeneration, Dental implant, Bone defect
MATERIALS AND METHODS

The animal experiments in this study were performed in accordance with the Guidelines for Animal Experimentation of Tokyo Medical and Dental University, Tokyo, Japan. The Animal Welfare Committee of Tokyo Medical and Dental University approved the animal protocols used in this study.

Membrane preparation

A gelatin with an isoelectric point of 5.1 was kindly supplied by Nippi, Inc. (MediGelatin®, HMG-BP, Tokyo, Japan). This material was prepared from porcine skin through an alkaline pre-treatment. β-TCP granules and glycerol were purchased from Wako Pure Chemical Industries, Ltd. (Apatite β-TCP, triclinic, code:018-14912, and Glycerol, code:075-00616, Osaka, Japan).

The aqueous gelatin (5 wt%) was prepared with mixing gelation powders and water at 60°C. After gelatin powders were totally dissolved in the water, glycerol (10 wt%) was added to the gelatin solution to improve the membrane’s suppleness. Then, ground β-TCP powders (0.1 mg/10 mL, ranging from 0.5–1.5 μm in diameter) were added to the solution. This mixture was poured into an 10 cm×10 cm×1 cm dish with 1.5 mm height and dried in air. The membrane was cut into 6 mm×13 mm rectangles for the in vivo experiment and into 14-mm diameter disks for the in vitro study. Next, these membranes were soaked in deionized water, freeze-dried, and cross-linked by heating at 150°C for 6 h to fabricate the β-TCP-containing gelatin hydrogel membrane (G-TCP). The membrane has different characteristics of its front and back because the β-TCP particles precipitate at the bottom of the plate during the freeze-dried step, we used the back surface as an experimental surface for following experiments. Subsequently, the G-TCP membranes were sterilized in an electron accelerator (15 kGy, 11 s, Dynamitron®, Ion Beam Application, Inc., Louvain-La-Neuve, Belgium). The thickness of the membranes was measured with a digital micrometer. To prepare samples for scanning electron microscopy (SEM) (S-4500, Hitachi Ltd., Hitachinaka, Japan), a set of the G-TCP membranes was sputter coated with Pt, and the surface and cross-sectional morphology were observed (accelerated voltage: 15 kV, magnification: 400×). A commercially available collagen (Col) membrane (Koken Tissue Guide, Koken, Tokyo, Japan) was also processed in the same manner.

Mechanical testing

To characterize the mechanical properties of the G-TCP and the Col membranes, a modified tensile test was employed. The prepared membrane sheets described above were hydrated, papered, and stuck on the rim of the longitudinal end of 9-mm diameter plastic tubes to fully cover the bore of each tube. The center of each membrane was then pushed perpendicularly with the rounded end of a 4-mm diameter cylinder, and then the points of failure, the points at which the membranes tore, were evaluated (Micro Material Testing Machine, MMT-250N, Shimadzu Corp., Kyoto, Japan).

Bone marrow cell culture

After they were killed, 4-week-old male Wistar rats were used as sources of BMSCs, which were obtained from the femoral medulla and were prepared as follows. Both the proximal and distal ends of the femurs were resected from the epiphyses, and the marrow was aspirated with 10 mL of alpha minimum essential medium (αMEM) (MEMs, Life Technologies, Carlsbad, CA, USA) expelled from a syringe through a 21-gauge needle. The released cells were collected in a 175-mL flask (BD Biosciences, Bedford, MA, USA) containing 20 mL of αMEM supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) and 1% antibiotics (100 U/mL penicillin, Life Technologies, Carlsbad, CA, USA; 0.25 mg/mL amphotericin B, Sigma-Aldrich, St. Louis, MO, USA). To remove non-adherent cells, the medium was replaced with fresh medium after 24 h of culture. Subconfluent cells in the culture were harvested by treatment with 0.25% trypsin/0.05 mM EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) and were subcultured in the same medium. During the culture period, the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂, the medium was replaced every 2 or 3 days, and the cells were detached and reseeded into fresh flasks when they reached confluence.

At passage 4, the cells were seeded at an initial density of 1×10⁵ cells onto 4 G-TCP and 4 Col membranes placed in the wells of 24-well tissue culture plates after moistening both membranes in 500 μL of FBS for 24 h. Each well received 1 mL of osteogenic medium comprising αMEM supplemented with 10% FBS, 50 ng/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 1 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), and 10⁻⁸ M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA)²⁰. Finally, cells were cultured on the membranes for the following analyses.

Cell proliferation assay

A Cell Count Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to quantitatively evaluate cell proliferation. Although metabolic assays cannot be used to directly quantify the cells, the amount of formazan dye generated by intracellular dehydrogenase correlates with the number of living cells¹⁰. After the BMSCs were cultured on the membranes for 4 h or 1, 3, 7, or 14 days, the membranes were carefully transferred into a new 24-well plate. The cells on the membranes were then incubated in 500 μL of osteogenic medium with 50 μL of CCK-8 reagent for 2 h at 37°C in 5% CO₂ to form water-insoluble formazan. The medium (550 μL total volume) was collected, and 400 μL of it was evenly divided into 4 wells of a 96-well plate to generate technical replicates of each sample. The optical density (OD) of the solution was measured at 450 nm using a micro-plate reader (Wallace 1420 ARVOx multi-label counter, PerkinElmer Co., Ltd., Tokyo, Japan), and the OD was converted to a cell number using a standard curve. The averages OD of the 4 wells containing the
same samples were compared between the 2 groups at the different culture periods.

**Alkaline phosphatase staining**
Another set of membranes cultured with cells for 7 days were washed twice with phosphate buffered saline, fixed with 10% neutral buffered formalin, and washed with deionized water. Cells expressing alkaline phosphatase (ALP) were stained with 0.6 mg/mL Fast Blue RR Salt and 0.1 mg/mL naphthol AS-MX phosphate alkaline (Sigma-Aldrich, St. Louis, MO, USA). The positive-stained cells on the membranes were observed under an optical microscope (BZ-8000; Keyence, Osaka, Japan).

**Surgical protocol**
Twenty-seven adult (18-week-old) Wistar rats were used for the in vivo experiment. Under anesthesia induced with a combination of Ketamine (40 mg/kg) and Xylazine (5 mg/kg), a mucoperiosteal flap of the parietal bone was raised, and 2 symmetrical full-thickness bone defects (5 mm in diameter) were created with a bone trephine bur under saline irrigation. The animals were divided into 3 groups: in 1 group of 9 animals, the defects were covered by G-TCP membranes (the experiment surface faced to the bone defect), in the second group of 9 animals, the defects were covered by Col membranes and in the third group of 9 animals (the negative controls), and the defects were left uncovered. In the control group, the defects were empty, without any membrane application. The flap was then repositioned and sutured.

**Radiographic analyses**
Three of the nine animals in each group were sacrificed at 2, 4, and 8 weeks after surgery, and were subjected to X-ray and histological analyses. After dissecting the skin, the defect sites were removed along with their surrounding bone and soft tissues, and the samples were fixed in 10% neutralized formalin for 1 week. Next, X-ray imaging was performed with a micro computed tomography (μCT) scanner (InspeXio, Shimadzu Science East Corporation, Tokyo, Japan) at a voxel size of 70 μm/pixel. Tri/3D-Bon software (RATOC System Engineering Co. Ltd, Tokyo, Japan) was used to make 3D reconstructions from the obtained scans. From the entire 3D data, a region of interest (ROI), a 5-mm diameter cylinder of sufficient height to cover the entire thickness of the calvarial bone, was used for precise analysis of the volume of regenerated bone. Masking was used to binarize the new bone in the ROI by using an intensity threshold, and then the volume was measured15).

**Histological sectioning**
The specimens were subsequently decalcified in 10% EDTA for 4 weeks. Then, an incision was made exactly through the middle of the bone defect to ensure that the microtome sections were made in the ROI, and then the samples were dehydrated in a graded series of ethanol. The samples were then embedded in paraffin, and 5-μm-thick coronal sections were prepared, stained with hematoxylin-eosin, and observed under an optical microscope (BZ-8000, Keyence, Osaka, Japan).

**Statistics**
The thickness of the membranes, the mechanical testing data, and the results of the cell proliferation assay were analyzed by Student’s t-tests. The volume of the newly formed bone was analyzed by one-way ANOVA with Tukey’s post hoc multiple comparison tests. p-Values less 0.05 were considered statistically significant.

**RESULTS**
The thickness of the G-TCP membranes was 0.12±0.03 mm, and that of the Col membranes was 0.08±0.001 mm, and these values were not statistically different. The images in Fig. 1 show the microstructure of the membranes as observed by SEM. The topography of the G-TCP membrane is characterized by a rough and wavy surface lacking large pores, the β-TCP particles were evenly distributed in the membrane; however, there was a tendency that the β-TCP particles accumulated more at lower part of the membrane than the upper part, whereas that of the Col membrane is characterized by a relatively smooth, net-like surface. The mechanical properties of the G-TCP membranes resulted in minimal perpendicular force tore the membrane. Although the force required to tear the G-TCP membranes was approximately 2.5 times larger than that required to tear the Col membranes, the scores were not statistically different (Fig. 2). The results of the CCK-8 assay are shown in Fig. 3. There were no significant differences between the groups during the observation period, and
the number of the cells on both membranes increased in a time-dependent manner until day 14. As shown in Fig. 4, ALP-positive cells were observed on both membranes as early as day 7.

All rats recovered well after the surgery. No macroscopic infection of the wounds was noted, and the soft tissue was observed to heal uneventfully, without prominent clinical signs, such as inflammation or swelling. The swelling and scabbing at the incised area abated in all groups by 2 weeks.

The volume of newly formed bone in the defects is shown in Fig. 5. An increase in bone volume was observed in all 3 groups over time. There was no significant difference in bone volume at 2 weeks among the groups. At 4 weeks, the bone volume in the G-TCP group was significantly greater than in the Col group. At 8 weeks, the volume in both the G-TCP and Col groups were significantly greater than that in the control group.

Histological images of the calvarial defects at 2 weeks are shown in Fig. 6. Although there was no significant difference in the μCT analysis of the bone volume in the defects among the groups at 2 weeks, bone bridges had formed in half of the defects in the G-TCP group as early as 2 weeks, whereas bone bridges were not observed in the other 2 groups at 2 weeks. In addition, the newly formed bone was observed not only under the membrane but also between the periosteal flap and the membrane, i.e., on the membrane, in the G-TCP and Col membrane groups.

Initial membrane absorption was only observed in the G-TCP group, whereas total absorption was observed in the Col group at 4 weeks (Fig. 7). Maturation of the
newly formed bone was prominent in the G-TCP and Col groups at 4 weeks compared with that at 2 weeks.

In the G-TCP group, new bone regenerated underneath the G-TCP membrane, whereas newly formed bone evenly and gradually replaced the collagen membrane in the Col group. The thickness of the newly formed bone in both membrane groups was greater than that of the control group.

The maturity of the newly formed bone in the defects of the G-TCP and Col groups was identical to that of the surrounding host bone at 8 weeks (Fig. 8). Membrane absorption was evident in both the G-TCP and Col groups at 8 weeks.

**DISCUSSION**

The objective of this study was to examine the effects of our novel G-TCP membrane on rat bone marrow cell proliferation and differentiation and the effects of this membrane on bone regeneration in rat parietal bone defects. Although the combination of gelatin and β-tricalcium phosphate has already been used as a biomaterial⁸,⁹, some issues, such as material safety and its properties remain to be solved. The G-TCP membranes were developed with the concept to apply not for big bone defects such as the critical-sized defects, but for smaller bone defects. Usually non-resorbable membranes such as titanium mesh are used for a big defect because it needs more mechanical strength and space keeping ability. On the other hand, most resorbable membranes such as collagen membranes are used for not so big defect clinically. In this sense, the bone defect size for in vivo experiment was selected smaller defect than the
critical-size defect.

In regards to material safety, glutaraldehyde (GA) is commonly used to strengthen the material in cross-linking processes. However, GA is extremely cytotoxic and elicits inflammatory cell responses. Although it is usually carefully rinsed away with water after the completion of cross-linking, residual GA in a material is usually carefully rinsed away with water after the completion of cross-linking, residual GA in a material might enhance cell toxicity.

In addition, ethylene oxide gas (EOG) is commonly used to sterilize materials, including heat-sensitive medical devices, and the amount of EOG remaining in a device partly depends on the type and size of the polymeric material. Therefore, to avoid any potential problems, we changed our cross-linking method to heating, and used an electron accelerator for sterilization in our fabrication process.

The thickness and mechanical properties of a membrane that is used as a GBR membrane are important. A GBR membrane is covered by periosteum and mucosal tissue, and clinical complications, such as dehiscence followed by infection, sometimes occur. Therefore, a GBR membrane that is a thin membrane with the appropriate mechanical strength is ideal. In the present study, the thickness of the G-TCP membrane was as thin as that of a commercially available collagen membrane. Moreover, the mechanical strength of the G-TCP membranes was approximately 2.5-times that of the Col membranes. This thin and strong G-TCP membrane might allow for easy handling by clinicians during surgery and could reduce the risk of dehiscence, infection, and necrosis of the mucosa after the surgery; complications that negatively affect the outcome of GBR.

Overcoming these potential material issues, our G-TCP membrane, which is thin, strong, and safe, seems to be ideal for GBR. However, the effectiveness of G-TCP as a GBR membrane should be assessed.

The microstructure of the G-TCP membrane is characterized by a rough surface lacking large pores, which prevents cell migration into the membrane, the β-TCP particles gathered one of the surface of the membrane, whereas the smooth, net-like property of the Col membrane allows cells to migrate inside. The cells on the G-TCP membrane proliferated time dependently. The cells on the G-TCP membrane as well as expressing ALP as early as day 7 that means the cells have an ability to differentiate into osteoblastic cells cultured on the G-TCP membrane. Although the cell cannot migrate inside the G-TCP not like the Col membrane, the G-TCP membrane has hydrophilic features and the osteoconductivity of the β-TCP particles that may support efficient cell growth and accelerated the ALP activity of the cells similar to the commercially available collagen membranes.

The present in vitro studies demonstrated that this novel G-TCP membrane is suitable for use as a GBR membrane. Furthermore, the G-TCP membrane allowed for rapid formation of new bone as determined by the in vivo studies. In the present animal experiments, bone bridge formation was observed at the surgery site of the G-TCP group 2 weeks after surgery. Although the bone matrix of the G-TCP group at 2 weeks in the histology looks more well-formed than the other groups, there were no significant different of bone volume at 2 weeks by μCT analysis. Because the intensity threshold to binarize for μCT analysis was set to devide the bone defect into mature bone and the other tissue. The degree of the calcification of the bone matrix at 2 weeks may be less-matured to be selected as a ROI by μCT analysis. At 4 weeks, significantly more bone volume was observed in the G-TCP group than in the Col group. This difference can be also observed in the histology. In addition, osteogenesis was observed to occur primarily along the G-TCP membrane surface, whereas it occurred primarily inside the Col membrane. This difference may affect bone quality during the early phase of bone formation.

Although there were no significant differences between the volume of bone in defects treated with the G-TCP and Col membranes at 8 weeks, both membranes performed well as GBR membranes, as the volume of bone formed in these defects was significantly higher than formed in the non-treated control defects.

The present results indicate that G-TCP might accelerate bone healing due to the membrane modifications reported here. Although the osteoconductivity of the β-TCP particles may contribute this, the mechanism underlying the accelerated bone healing in the bone defect induced by the G-TCP membrane is not yet known. We will investigate this mechanism in future studies. In addition, the efficacy of G-TCP as a GBR membrane for jawbone defects should also be investigated.

In conclusion, the G-TCP membrane generated in this study has suitable membrane properties for use as a GBR membrane, and it allows for more rapid bone formation than Col membranes.

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