Guided bone regeneration using a hydrophilic membrane made of unsintered hydroxyapatite and poly(L-lactic acid) in a rat bone-defect model

Reo IKUMI, Takayuki MIYAHARA, Norio AKINO, Noriko TACHIKAWA and Shohei KASUGAI

Department of Oral Implantology and Regenerative Dental Medicine, Tokyo Medical and Dental University, Tokyo 113-8549, Japan

Corresponding author, Reo IKUMI; E-mail: reoirm@tmd.ac.jp

The effectiveness of a previously developed unsintered hydroxyapatite (uHA) and poly(L-lactic acid) (PLLA) hydrophilic membrane as a resorbable barrier for guided bone regeneration (GBR) was evaluated. Critical-size 8-mm diameter bone defects were surgically generated in the parietal bones of 24 12-week-old male Wistar rats, which were then divided into three groups in which either a uHA/PLLA or a collagen membrane or no membrane (control) was placed onto the bone defect. Following sacrifice of the animals 2 or 4 weeks after surgery, bone defects were examined using microcomputed tomography and histological analysis. Bone mineral density, bone mineral content, and relative bone growth area values 2 or 4 weeks after surgery were highest in the uHA/PLLA group. Four weeks after surgery, the relative bone growth area in the uHA/PLLA group was larger than that in the collagen group. The resorbable uHA/PLLA membrane is thus potentially effective for GBR.

Keywords: Bone-defect model, Guided bone regeneration, Biomaterials, HA/PLLA membrane, Hydrophilic

INTRODUCTION

Currently, oral rehabilitation using dental implants is considered an effective and reliable management strategy; however, its effectiveness is often limited by the lack of sufficient supporting bone. This problem can be overcome by bone augmentation through guided bone regeneration (GBR), which involves the creation of a space, with placement of a barrier membrane to preserve the blood clot, prevent soft tissue invasion, and promote bone regeneration in the created space1-3). This technique has been clinically applied and extensively reported4-7).

Membranes used for GBR include those made of nonresorbable materials, such as nitrocellulose or expanded polytetrafluoroethylene (e-PTFE); however, these membranes require surgical removal after bone augmentation8-13). Thus, resorbable GBR membranes are more desirable. Bovine or porcine collagen has been used as a material for resorbable GBR membranes7,14-19); however, although collagen is a highly biocompatible major matrix protein, the risk of infection with known or unknown diseases from the source animals cannot be completely eliminated, because of the physicochemically mild collagen extraction procedure20-22). An unfavorable immune reaction to the heterogeneous protein, although rare, is another concern regarding this material.

Poly(L-lactic acid) (PLLA) or poly(lactic-co-glycolic acid) (PLGA) is a synthetic biodegradable polymer that is also used as a material for biodegradable medical membrane23,24). It does not trigger the infections and immune responses characteristic of animal-derived biomaterials, and thus use of PLLA as a biomedical material has been widely studied in the areas of bone healing materials, tissue engineering, and drug delivery.

A biocompatible and biodegradable osteosynthesis membrane (Super-Fixsorb-MX, Takiron, Osaka, Japan), made from composites of uncalcined and unsintered hydroxyapatite (uHA) particles and PLLA, was developed in previous studies and has been clinically applied25,26). This material exhibits a mesh-like structure, an elasticity close to that of the natural cortical bone, and high strength during the period required for bone healing, making it superior to unfilled PLLA27). Furthermore, it demonstrates optimal degradation and resorption properties, osteoconductivity, and bone binding capabilities27). Interestingly, it has been reported that hydrophilization treatment of this material further enhances its properties28,29).

However, the hydrophilized material has not been compared radiologically or histologically to other materials which are clinically used. Accordingly, in the present study, we evaluated the osteogenic potential of the newly developed hydrophilized uHA/PLLA membrane in GBR using a rat bone-defect model.

MATERIALS AND METHODS

All animal experiments were performed in accordance with the guidelines laid down by the National Institute of Health (NIH; Bethesda, MD, USA) regarding the care and use of animals for experimental procedures and in accordance with local laws and regulations. All possible measures were taken to minimize the pain or discomfort of the animals. The experimental protocol for this study was approved by the Institutional Committee for Animal Care, Tokyo Medical and Dental University (0160196A).

Animals

In total, 24 male Wistar rats (12-weeks-old; weight, 280–320 g; Sankyo Labo Service, Toyama, Japan) were divided into two experimental groups and one control
group according to the membrane used for GBR. All animals were provided with water and a standard laboratory diet ad libitum.

**GBR membranes**

uHA/PLLA membranes, with a diameter of 11 mm and thickness of 0.1 mm, were prepared, with a small modification (40 wt% unsintered HA and 60 wt% PLLA), from the material used in the aforementioned biodegradable bone fixation membrane (Super Fixorb MX30, Takiron), which comprises 50 wt% unsintered HA and 50 wt% PLLA. The prepared membranes were treated with ultraviolet (UV) photofunctionalization (wavelength, 172 nm; tube wall illumination, 13 mW/cm²; period, 8 min; intensity, 6.26 J/cm²) to increase the hydrophilicity of the material. All the uHA/PLLA membranes used in this study were provided by the Takiron. The scanning electroscope images of this membrane were shown in Fig. 1. This membrane was not porous.

Collagen membranes (30×40 mm² BIOMEND®, Zimmer-Dental, Indianapolis, IN, USA) were also used in this study (Figs. 1 and 2). According to the instructions of the material, the thickness of this membrane was 0.2 mm and this membrane was porous: The pore size of this membrane was 0.004 µm. These membranes were trimmed to a circular shape with an 11-mm diameter.

**Surgical methods**

The animals were anesthetized with a combination of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg) via an intramuscular injection. The surgical field was disinfected with 10% povidone iodine solution, and 2% xylocaine/epinephrine (1:80,000) was injected along the incision line. Then, a 2-cm horizontal incision was placed between the ears. The musculature and periosteum were laterally reflected to expose the parietal bone. A diamond-coated trephine bur with a diameter of 8 mm was used in a surgical dental engine (Comfort II, Novel Biocare Japan, Tokyo, Japan) to create a critical size calvarial bone defect at the center of the parietal bone under saline irrigation (Fig. 3). The drilling speed was between 900 and 1,200 ppm. After drilling, the bone plate was peeled off from the parietal bone without causing damage to the dura mater and veins. In the experimental groups, either the uHA/PLLA...
membrane (uHA/PLLA group) or the collagen membrane (collagen group) was placed on the bone defect. In the control group, no membrane was placed. The periosteal membrane was sutured at three places using 6-0 nylon (Crownjun Kono, Chiba, Japan). Then, the skin was sutured with 4-0 nylon (ELP, Akiyama medical FMG, Fukushima, Japan).

Microcomputed tomography (μCT) analysis
At 2 weeks postoperatively, the half of the animals of the three groups were sacrificed using an overdose of isoflurane for preparing specimens for analysis. At 4 weeks postoperatively, all the remaining animals were sacrificed.

The bone defects in all three groups were analyzed using μCT (R.mCT2®, Rigaku, Tokyo, Japan) at 2 and 4 weeks after surgery. The volume of newly formed bone was measured using TRI/3DBone (RATOC System Engineering, Tokyo, Japan). In the micro CT image, the newly formed bone and the original basal bone could be easily distinguished based on the difference of radiopacity. A cylindrical region (diameter and height of 8 and 1 mm, respectively) that covered the entire thickness of the calvarial bone was selected as the region of interest (ROI).

The amount of newly formed bone was calculated by assessing the radiopaque voxels obtained from the calvarial defect using Image J (NIH) to analyze the size of the defect at 2 and 4 weeks after surgery. The bone mineral density (BMD), bone mineral content (BMC), and relative bone growth area were measured within the ROI.

Histological analysis
Both undecalcified and decalcified specimens were used for histological analysis. All samples were harvested and fixed in 10% formalin solution for 48 h. Then, half of the samples were decalcified by immersion in 10% EDTA solution, dehydrated with a graded series of ethanol, soaked serially in xylene, and embedded in paraffin. These blocks were cut into 8-μm-thick sections using a microtome (REM-710, Yamato, Saitama, Japan). Subsequently, all sections were stained with hematoxylin and eosin according to standard procedures.

The other half of the samples were dehydrated with ascending grades of alcohol and methyl methacrylate (MMA; Technovit 7200, Heraeus Kulzer, Wehrheim, Germany), embedded in MMA, and polymerized. Following polymerization, the samples were cut and ground from the center of the defects, perpendicular to the sagittal suture, using exact cutting and grinding equipment (Exact Apparatebau, Norderstedt, Germany); the final thickness was 80 μm. These specimens were fixed on microscopic slides using acrylic cement (Technovit 7210 VLC, Heraeus Kulzer). Then, one central section was prepared from each specimen, reduced to a final thickness of 20–30 μm using a microgrinding-polishing unit (Le Cube, PRESI, Brié-et-Angonnes, France), and stained with toluidine blue (Toluidine Blue O, Sigma-Aldrich, St Louis, MO, USA). Histological analysis was performed using an optical/fluorescence microscope (Biozero-8000, Keyence, Osaka, Japan), and the obtained data were analyzed using a software (BZ-Analyzer, Keyence).

Statistical analysis
All data are expressed as means±standard deviations. Multiple comparisons between groups were performed using Tukey’s honest significant difference test with one-way analysis of variance. A $p$-value of <0.05 was considered statistically significant. All statistical analyses were performed using statistical software (SPSS v. 18, IBM, Armonk, NY, USA).

RESULTS
Healing progressed uneventfully in all animals, and no postoperative complications were noted during the observation period. Three days after surgery, all animals exhibited normal behavior without any limitations.

μCT images clearly demonstrated new bone formation in the uHA/PLLA and collagen groups (Fig. 4). The BMD increased in all groups from 2 to 4 weeks after surgery, with the uHA/PLLA group exhibiting the highest value among the three groups. At 2 weeks after surgery, BMD was significantly greater in the uHA/PLLA group than in the other two groups ($p<0.05$; Fig. 5a).

The BMC also increased in all groups from 2 to 4 weeks after surgery. At 2 weeks after surgery, the uHA/PLLA group showed the highest BMC value among the three groups, with a significant difference as compared with the control group ($p<0.05$; Fig. 5b).

The relative bone growth area, calculated as the ratio of the new bone area to the original bone defect area, slightly increased in all groups from 2 to 4 weeks. At 2 and 4 weeks after surgery, the relative bone growth area was significantly larger in the uHA/PLLA group than in the control and collagen groups ($p<0.05$; Fig. 5c).
Figure 6 shows histological images of a nondecalcified section (toluidine blue staining) prepared for all groups at 2 and 4 weeks. At 2 weeks, a small amount of new bone was observed in the control group (Fig. 6a), and new bone under the membrane was more evident in the collagen and uHA/PLLA groups than in the control group (Figs. 6b, c). At 4 weeks, although new bone increased at the edge of the bone defect in the control group, there was no bone formation at the center of the defect (Fig. 6d). At 4 weeks, new bone was clearly observed at the center of the defect in the collagen group (Fig. 6e). In the uHA/PLLA group at 4 weeks, new bone was evident under the membrane, including the center of the defect, and it was more mature than the bone formed in the other two groups (Fig. 6f).

Figure 7 presents histological images of decalcified sections (hematoxylin and eosin staining) prepared for all groups at 4 weeks. In the control group, new bone was observed only in the region closed to the original bone (Figs. 7a, d). More new bone was observed in the collagen group (Figs. 7b, e), and compared with this bone, that in the uHA/PLLA group was more mature, exhibiting a layered structure (Figs. 7c, f).

While the uHA/PLLA group exhibited mature bone in the region closed to the original edge of the defect (Fig. 6i), the collagen group showed less mature and irregular bone (Fig. 6h). On the other hand, connective tissue was observed at the defect margin of the control group specimens (Fig. 6g).

**DISCUSSION**

In the present study, we evaluated the osteogenic potential of a newly developed hydrophilized uHA/PLLA membrane used for GBR in a rat bone-defect model. Currently, a collagen membrane is the most frequently used clinically. We thought that by comparing the new uHA/PLLA membrane with a commercially available collagen membrane, we could demonstrate the effectiveness of the new uHA/PLLA membrane. Thus,
we used a collagen membrane in the control group in the present study.

GBR is based on the concept of bone regeneration through the creation of a space with a barrier membrane\(^2\). During regeneration, the barrier membrane provides a sealed space that allows bone regeneration, rather than internal growth of connective tissue\(^3\). The success of GBR, which is reflected by a higher regenerative potential relative to unsupported healing conditions, depends on the properties of the barrier membrane materials, particularly their interactions with osseous and surrounding tissues\(^4\). The membrane materials should exhibit mechanical properties compatible with bone tissue\(^5\), which means that the membrane requires sufficiently strong and balanced mechanical strength to maintain the space for bone regeneration during the healing process\(^6\). Furthermore, it should be flexible to prevent damage to surrounding tissues and facilitate
surgical procedures.

In recent years, composites of HA and PLLA have been clinically used in various fields, including oral and orthopedic surgery, because of their various advantages\(^\text{22}\). Interestingly, increasing hydrophilicity by UV-irradiation significantly improves the biocompatibility of this composite material\(^\text{23,24}\).

Moroi et al. have reported that the contact angle between water and uHA/PLLA surface was changed by the UV irradiation from 76±3.5 degree to 3±3.5 degree. Furthermore, this decreased contact angle was stable for one year after UV treatment. They demonstrated enhancement of cell attachment and differentiation on uHA/PLLA surface when the surface had been UV-irradiated\(^\text{25}\). In addition, the UV-irradiated uHA/PLLA mesh stimulated bone regeneration in the rabbit nasal bone defect compared to the uHA/PLLA mesh without UV-irradiation\(^\text{26}\).

Shikinami et al.\(^\text{27}\) showed that forged composites reinforced with uHA particles and PLLA were replaced by bone tissue; exhibited bioactivity, bioreabsorbability, and high mechanical strength; and resulted in a favorable tissue response. In the present study, the uHA/PLLA membranes adhered well to the bone around the defect, without requiring fixation pins. Moreover, the membrane remained in place throughout the experimental period and maintained the space for bone regeneration.

uHA/PLLA material exhibits osteoconductivity and bone-binding capabilities\(^\text{28}\). The µCT images obtained in the present study showed that bone formation occurred irregularly in the collagen group, whereas it was uniform under the membrane and occupied more than 60% of the original defect at 2 weeks in the uHA/PLLA group. Histological analysis showed that the lamellar bone was formed from the edge of the defect in the uHA/PLLA group, whereas a lamellar structure and clear bone formation were not present in the collagen group. These results suggest that osteoblasts regularly formed new bone underneath the uHA/PLLA membrane and that enough space was maintained for bone regeneration in this group.

In the present study, BMD was analyzed as an index of the quality and strength of the newly formed bone. The uHA/PLLA group exhibited the highest BMD value at 2 weeks after surgery, which was maintained at 4 weeks after surgery. Moreover, the BMD measurements corresponded well with the findings of the histological analysis, which demonstrated that mature bone was formed early on in the calvarial defect under the uHA/PLLA membrane. Notably, such mature bone was not observed in the other groups.

The reason why uHA/PLLA membrane produced more newly-formed bone of good quality compared to the collagen membrane is not clear. Obviously, collagen is more biocompatible than PLLA. However, since uHA is highly biocompatible, it is likely that supplementing PLLA with uHA improves the biocompatibility of PLLA making this material more osteoconductive together with improving the mechanical property of this material. This speculation is supported by the new bone formation along the uHA/PLLA membrane. On the other hand, new bone formation was observed in the collagen membrane. Thus, the pattern of new bone formation between the uHA/PLLA group and the collagen group was different, which might affect the quality of new bone.

The results of the present study demonstrated the potential effectiveness of the uHA/PLLA membrane as a resorbable membrane for GBR. However, a bone fixation membrane made of the same material as the uHA/PLLA membrane used in the present study has been reported to require 3 to 5 years for complete resorption after surgery\(^\text{29}\). Therefore, in the future it is required to evaluate how the uHA/PLLA membrane degrades with time and how the membrane degradation process affects the newly-formed bone and the surrounding tissues. Furthermore, experiments in large animals mimicking the clinical situation of GBR are required.

**CONCLUSION**

The results of the present study suggest that the resorbable uHA/PLLA membrane is a potentially effective membrane for GBR compared to the collagen membrane, which is currently used clinically.

**ACKNOWLEDGMENTS**

We would like to thank N Akino and M Htet for help with the animal experiments. This work was supported by Teijin Medical Technologies and JSPS KAKENHI (Grant Number 26893073). The authors have no conflicts of interest to declare.

**CONFLICTS OF INTEREST**

The authors have no conflicts of interest to declare.

**REFERENCES**


