Original

Evaluating the Bone Regeneration of nHAp/PPC Membrane in Rabbit Calvarial Defect

Chuanjian Lan^{1,2)}, Xingchen Xiang³⁾, Xi He¹⁾, Yongsheng Pan¹⁾, Duo Sun¹⁾ and Jiang Li¹⁾

¹⁾ Department of Prosthodontics, School and Hospital of Stomatology, Jilin University, Changchun, China

²⁾ Jilin Provincial Key Laboratory of Tooth Development and Bone Remodeling (School and Hospital of Stomatology, Jilin University), Changchun, China

³⁾ Department of General Dentistry, School and Hospital of Stomatology, Jilin University, Changchun, China

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Abstract: This study aims to evaluate the ability of poly(propylene carbonate) (PPC)-loaded nanohydroxyapatite (nHAp) membrane to regenerate bones. **Methods:** Six New Zealand white rabbits were randomly and equally divided into two groups. Four 6 mm diameter transosseous round defects were made at the parietal bone of each rabbit. One defect was not covered with the membrane, and the others were covered with 10% or 20% nHAp/PPC membrane or HEAL-ALL membrane. Animals of each group were sacrificed at the 4 and 12 weeks after the operation, respectively. The rabbit parietal bones were removed for radiological and histological evaluation. **Results:** Micro-computed tomography results showed that the bone regeneration of the experimental group was significantly higher than that of the control group in the absence of bone powder after 4 and 12 weeks, and the 20% nHAp/PPC group showed the most significant effect on bone regeneration. Histological analysis found that the nHAp/PPC membranes and HEAL-ALL membranes of the experimental groups prevent the growth of soft tissues during bone regeneration. In summary, the nHAp/PPC membrane showed good biocompatibility during the repair of rabbit parietal defects, and the 20% nHAp/PPC membrane is prominent.

Key words: Poly(propylene carbonate), Nanohydroxyapatite, Guided bone regeneration technique, Micro-computed tomography

Introduction

Guided bone regeneration (GBR) has been widely used in clinical practice^{1,2)} and is the most commonly used technique for restoring alveolar bone defects and treating bone defects around implants^{3,4)}. GBR uses the membrane as a barrier to prevent connective tissues and mucosal epithelial cells from growing into the defects^{5,6)}, thereby allowing underlying bones to regenerate. Currently, various GBR membranes have been developed, which can be divided into non-absorbable and absorbable membrane⁷⁾. The expanded polytetrafluoroethylene membrane is the first non-absorbable GBR membrane. Titanium alloys have been widely used as non-absorbable GBR membranes. Non-absorbable GBR membranes have good biocompatibility and space maintenance⁸⁾ and are mostly used in vertical bone defects⁹⁾. However, the disadvantages of non-absorbable membrane are prominent. Several studies have shown that non-absorbable GBR membranes are prone to result in soft tissue exposure¹⁰⁾ because of the excessive tension generated by soft tissues. Moreover, a second surgery is usually needed to remove a non-absorbable membrane completely. Meanwhile, absorbable GBR membranes, which are considered patient-friendly barrier membranes, are self-degradable, and their removal does not require secondary surgery¹¹⁾. Currently, most clinically used absorbable membranes are collagen components. However, despite their advantages, absorbable GBR membranes have poor strength and rapidly degrade. Therefore, developing a novel absorbable GBR membrane is necessary.

Poly(propylene carbonate) (PPC) is an aliphatic polyester with good

biocompatibility and biodegradability¹²⁻¹⁴⁾ and can be used as a medical material¹⁵⁾. Nanohydroxyapatite (nHAp) is the main inorganic component in bone tissues and has good biocompatibility^{16,17)}. Additionally, nHAp plays an important role in bone cell differentiation and mineralization and promotes bone growth^{18,19)}. In our previous study, nHAp and PPC were mixed into an nHAp/PPC GBR membrane by solvent casting/particulate leaching method, and the mechanical properties and cell compatibility of the resulting membrane were studied^{20,21)}. The results indicated that nHAp/PPC membrane is a biocompatible membrane^{20,21)} that promotes the proliferation and differentiation of MG63 cells²¹⁾. The nHAp/PPC membrane containing 10% and 20% nHAp by weight exhibited excellent mechanical properties. To further explore the feasibility of nHAp/PPC membrane as a guide membrane for bone regeneration, this study evaluated the effect of 10% and 20% nHAp/PPC groups on bone defect repair *in vivo*. Moreover, HEAL-ALL membrane, which is a resorbable membrane for clinical applications, is used as positive control.

Materials and Methods

Preparation of nHAp/PPC GBR and HEAL-ALL membranes

The nHAp/PPC membranes were prepared according to the solvent casting/particulate leaching method used in our previous experiments²⁰⁾. Briefly, nHAp particles were added to a chloroform solution containing PPC, and the resulting solution was mixed and poured into a glass petri dish containing NaCl particles. After the chloroform was volatilized, the remaining material was soaked in deionized water for the removal of the NaCl particles.

The prepared 10% and 20% nHAp/PPC membranes and the HEAL-ALL membranes (Yan TaiZhenghai Biotechnology Corporation,

Correspondence to: Dr. Jiang Li, Department of Prosthodontics, School and Hospital of Stomatology, Jilin University, Changchun 130021, China; Tel: +86-0431-88796018; E-mail: 444706646@qq.com

Figure 1. Surgical procedure. A: Four defects ($\phi = 6$ mm) in the calvarium of 6 rabbits. B: Random assignment of defects, control group (left down), 10% nHAp/PPC group (left top), 20% nHAp/PPC (right top), and HEAL-ALL (right down).

Table 1. Micro-CT tomography morphometric parameters of bone

Abbreviation	Parameter	Explanation	Unit
BV/TV	Bone volume fraction	Ratio of bone volume to total volume in a region of interest	%
Tb.N	Trabecular number	Average number of intersections between bone and non-bone tissues per mm	1/mm
Tb.Th	Trabecular thickness	Average thickness of the trabecular Bone	mm
Tb.Sp	Trabecular separation	Average width of the medullary between the trabeculae	mm

Shandong, China) were cut into squares of 7 mm $\times$ 7 mm size. Then, the membranes were used for surgery after sterilization.

Animal and surgical procedure

Six adult male New Zealand white rabbits (Yisi Experimental Animal Technology Co., Ltd., Changchun, China, license number: SCXK [Ji]-2016-0004) weighing 2.5–3.5 kg were randomly and equally divided into two groups. The animal experiments were approved by the ethics committee of Jilin University (No. 201825). The rabbits were fed separately in cages by the animal experiment center of basic medical college of Jilin University. The animals were fed uniformly for 1 week before the operation for them to adapt to the new environment and were fasted on water for 12 h before the operation.

The surgical procedures are shown in Fig. 1. The rabbits were weighed and administered with intramuscular anesthesia SuMianXin II (0.1 ml/kg to 0.2 ml/kg, intramuscularly, Quartermaster University of PLA, Changchun, China, Military Veterinary Institute). In each rabbit, local anesthesia was used to infiltrate the operative area (2% lidocaine, 1:100,000 epinephrine), and hair covering the skull of the rabbit was cut. The surgical area was disinfected with iodophor disinfectant, and a longitudinal incision of approximately 3 cm in size was made from the nasal bone to the occipital bone to remove the periosteum and expose the parietal bone. After washing with normal saline, a bone drill was used to make four round 6 mm bone defects that did not damage the dura. Care was exercised to avoid injury of the dura. Two defects were formed on each side of the middle cranial suture. The spacing between each adjacent bone defect was more than 2 mm (Fig. 1A). Three bone defects were covered with 10% nHAp/PPC membrane, 20% nHAp/PPC membrane, and HEAL-ALL membrane, respectively. Each membrane was fixed with titanium nails. The remaining defect was left without membrane (Fig. 1B). The flaps were then repositioned without tension

and sutured with a 4-0 suture material. Lastly, the surgical sites were sutured, and the animals received antibiotics (penicillin, 100,000 U/days) for 3 days. Each group comprising three animals was sacrificed at the 4 and 12 weeks after surgery.

Clinical observations

The animals were carefully observed and evaluated for inflammation, allergic reactions, and other complications around the surgical site 4 and 12 weeks after surgery.

Micro-computed tomography (CT) Evaluation

The parietal bone containing the healed sites were surgically harvested and immediately fixed in neutral buffered formalin (10%) for 48 h. Each parietal bone was scanned for new bone formation at 50 kV, 800 μ m, 1,304 μ m $\times$ 1,024 μ m with Micro-CT scanners (Skyscan 1076, Force, Belgium). The four 6 mm $\times$ 6 mm regions on the middle line of the skull were set as areas of interest for the three-dimensional reconstruction (region of interest). The threshold was selected according to specimen-specific thresholds²². The volume of interest (VOI) was selected corresponding to the dimensions of the defect sites, with 6-mm full-thickness cylinders for measurement of mineralized tissue volume and mineralized tissue volume was measured in VOI. N-recon software was used for three-dimensional image reconstruction. Basing on the Micro-CT images, CT-AN software (Amira, Visualization Sciences Group, Düsseldorf, Germany) analyzed the formation of new bones and trabecular bones (Table 1).

Histologic analysis

After Micro-CT analysis, each parietal bone was decalcified in a glass vial containing 30 ml of 10% ethylene diamine tetraacetic acid (EDTA) solution for 21 days. The EDTA solution was changed daily.

Figure 2. 3D reconstructed micro-CT images of defects at 4 and 12 weeks.

Then, the parietal bone was subjected to ethanol gradient dehydration, wax penetration, and embedding. The embedded wax block was placed in a -20°C refrigerator and frozen for more than 2 h. The embedded block was then placed in a room temperature environment for future use. A $5\text{ }\mu\text{m}$ -thick tissue section was cut in the middle of the bone defect. Then, the tissue section was stained with hematoxylin-eosin. The stained histologic slides were examined with a light microscope (Olympus BX41, Tokyo, Japan) equipped with a camera (Olympus DP70, Tokyo, Japan).

Statistical analysis

All data were initially analyzed to test their normality using the Shapiro-Wilk test. Independent sample t-test and one-way analysis of variance (ANOVA) (p value <0.05 was considered significant) were performed in SPSS 22 software. The data were presented as means $\pm$ standard deviation.

Results

Clinical observations

All rabbits survived the operation in good health without any abnormal conditions. No bleeding, inflammation, swelling, and other postoperative complications were observed. After 4 and 12 weeks, the skin of each group healed well, and the tissue and membranes were not exposed.

Micro-CT analysis

The three-dimensional reconstruction images of the rabbit parietal bone 4 and 12 weeks after surgery are illustrated in Fig. 2. Four weeks after surgery, the defect edges of the four defects in the absence of bone powder were visible although bone regeneration was observed. In the control group, a large number of bone defects were not repaired. In the 10% and 20% nHAp/PPC and HEAL-ALL groups, the repair ranges of the bone defects were higher than those of the control group. Meanwhile, 12 weeks after surgery, the extent of bone repair in the four groups of defects increased. The 10% and 20% nHAp/PPC groups showed higher extent of bone repair than the other groups.

The ratio of bone volume and total volume in each of the affected areas 4 and 12 weeks after surgery is displayed in Fig. 3. The BV/TV

Figure 3. The percentage of new bone (BV/TV) between the four groups. Data are presented as means. Error bars represent mean $\pm$ standard deviation, $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

values of the 10% and 20% nHAp/PPC and HEAL-ALL groups were higher than those of the control group at 4 and 12 weeks. And the BV/TV values of 10% and 20% nHAp/PPC groups were higher than that of the HEAL-ALL group, while the 20% nHAp/PPC group had the highest value. Micro-CT analysis revealed that no significant difference was observed between the volume of mineralized tissues in nHAp/PPC group and HEAL-ALL group at 4 weeks (Fig. 3). But at the 12 weeks, both 10% and 20% nHAp/PPC groups significantly promoted mineralized tissue volume when compared to control and HEAL-ALL group (Fig. 3).

The Micro-CT bone morphometry data at 4 and 12 weeks after surgery are shown in Table 2. The large values of Tb.Th and Tb.N indicate the maturity and stability of the bone structure. By contrast, if the value of Tb.Sp, which represents the degree of connectivity of the trabecular bone, is large, the arrangement of the bone structure is poor²³. It was found that the Tb.N and Tb.Th of the three groups were higher than those of the control group from Table 2 at postoperative weeks 4 and 12. By contrast, the Tb.Sp of the three experimental groups was lower than

Table 2. The micro-CT bone morphometry datas at 4 and 12 weeks after surgery

		Control	10% nHAp/PPC	20% nHAp/PPC	Heal-ALL
Tb.N	4w	0.834±0.072	0.837±0.122 ^b	0.917±0.134 ^c	0.943±0.210
	12w	1.398±0.447	1.546±0.554	1.692±0.714	1.414±0.396
Tb.Th	4w	0.097±0.004	0.105±0.015	0.116±0.010 ^{ab}	0.103±0.007 ^a
	12w	0.163±0.012	0.196±0.005 ^{ab}	0.200±0.009 ^{ab}	0.169±0.012 ^a
Tb.Sp	4w	0.981±0.006	0.856±0.010 ^a	0.800±0.071 ^a	0.766±0.106 ^a
	12w	0.812±0.103	0.595±0.086 ^a	0.652±0.144 ^a	0.659±0.080 ^a

a: $p < 0.05$ vs. control; b: $p < 0.05$ vs. Heal-ALL; c: $p < 0.05$ vs. 10% nHAp/PPC

Figure 4. Histological specimens at 4 and 12 weeks of healing (Hematoxylin and Eosin staining). A, a: staining images of control group at 4 weeks. B, b: staining images of 10% nHAp/PPC group at 4 weeks. C, c: staining images of 20% nHAp/PPC group at 4 weeks. D, d: staining images of HEAL-ALL group at 4 weeks. E, e: staining images of control group at 12 weeks. F, f: staining images of 10% nHAp/PPC group at 12 weeks. G, g: staining images of 20% nHAp/PPC group at 12 weeks. H, h: staining images of HEAL-ALL group at 12 weeks. Original magnification for A, B, C, D, E, F, G and H, $\times 4$; and original magnification for a, b, c, d, e, f, g and h, $\times 20$. (*: original bone; N: new bone; yellow arrows: bone trabeculae; yellow arrowheads: bone defect; black arrows: reversal cement line; black arrowheads: osteoblastic rimming)

that of the control group. The 20% nHAp/PPC group showed better performance than the other groups, indicating that mature and well-connected bone tissue had formed.

Histologic analysis

The images of tissue sections stained by hematoxylin-eosin are displayed in Fig. 4. In all the tissue sections, necrotic tissue and inflammation or foreign body reaction was not observed. Four weeks after surgery, a large number of loose connective tissues were found in the control group, whereas a small amount of newly formed bone (denoted as yellow arrow) was seen at the edge of the defect area. More new bone

appeared in the three experimental groups (denoted as yellow arrow). In the 10%, 20% nHAp/PPC and HEAL-ALL groups, bone-like structures (denoted as N) surrounded by osteoblasts (denoted as black arrowheads) were observed (Fig. 4). Meanwhile, 12 weeks after surgery, newly formed bone tissues were in the stage of remodeling and became more mature, and more lamellar bone formed (denoted as N). But for the control group in Fig. 4(E), we found that minimal new bone tissue could be observed. In the other three groups, the newly formed bone tissues had almost occupied the whole defect and more lamellar bone had formed and also became thicker. The 20% nHAp/PPC group had the most new bone at either 4 or 12 weeks. The results of hematoxylin-eosin staining

was consistent with that of Micro-CT.

Discussion

The ideal GBR membrane should have good mechanical strength, biocompatibility, and biodegradability. In our previous experiments, we prepared a nHAp/PPC GBR membranes²⁰, where in one side of the bio-film is smooth and prevents soft tissue penetration and the other is porous and facilitates cell adhesion consistent with previous studies by other scholars^{24,25}. Moreover, we found in our previous study that the 10% and 20% nHAp/PPC groups had good mechanical properties and biocompatibility. Thus, the two groups were selected as the experimental groups in the present experiment, which aims to evaluate bone regeneration in rabbit calvarial defects covered with our proposed biofilm through Micro-CT and histological observation. HEAL-ALL membrane is a kind of GBR membrane, which is widely used in clinical practice in China. Its main component is collagen extracted from the dermal matrix of cattle skin. The HEAL-ALL membrane is supposed to degrade completely within 6 months according to the manufacturer²⁶. The success of the GBR procedure depends on space maintenance for bone formation at a sufficient period (of at least 6 weeks)²⁷. Given that the stabilization of bone remodeling occurs approximately 3 months after surgery, bone regeneration in nHAp/PPC was observed 4 and 12 week safter surgery.

In the present study, we performed three-dimensional reconstruction and data analysis on micro-CT data. The results showed that the volumes of the newly formed bone tissues of the 10% and 20% nHAp/PPC and the HEAL-ALL groups were larger than that of the control group at 4 weeks after surgery. And the BV/TV values of 10% and 20% nHAp/PPC groups were higher than that of the HEAL-ALL group. Over time, although extent of bone formation increased in the four groups 12 weeks after surgery, the extent of bone formation in the 10% and 20% nHAp/PPC groups was higher, and the 20% nHAp/PPC group had highest extent. The results of analysis of variance showed that the differences among the groups were most statistically significant. The reason that the new bone of the 10% and 20% nHAp/PPC groups was higher than that in the HEAL-ALL group is explained as follows: Although the HEAL-ALL group has a bilayer structure composed of a compact and porous layer²⁶, it does not have Ca⁺ ions and can only maintain the bone defect space during osteogenesis; this observation is consistent with a previous study²⁶. The greater the value of Tb.T and Tb.N, the more mature and stable the bone structure. In contrast, Tb.Sp stands for the degree of connectivity of trabecular bone; if the value of Tb.Sp is bigger, the arrangement of the bone structure would be worse. The results of this experiment showed that the 10% and 20% nHAp/PPC and the HEAL-ALL groups had higher Tb.Th and Tb.N values and lower Tb.Sp values than the control group after 4 and 12 weeks of surgery. Furthermore, the 10% and 20% nHAp/PPC and the HEAL-ALL groups had higher volumes of regenerated bones covered by the membranes and the qualities of their newly formed bone tissues were better than those of the control group. This outcome may be attributed to the membranes, which resisted fibroblasts, maintained the space of the bone defects, and provided an anti-interference environment for osteogenesis.

Four and twelve weeks after surgery, no inflammation and necrotic tissues were detected in the rabbit parietal defect by histological observations. At 12 weeks, the presence of nHAp/PPC membrane was still visible to the naked eye. This phenomenon indicated that the prepared 10% and 20% nHAp/PPC films had good biocompatibility and sufficient degradation time to meet the requirements of bone regeneration. The hematoxylin-eosin stains showed that a large amount of loose connective tissue was present in the bone defect of the control group at 4 weeks and

more new bone formation in the bone defects of the 10% and 20% nHAp/PPC and the HEAL-ALL groups, showing newly formed bone marrow cavities. At 12 weeks, a small amount of new bone tissue formed in the control group, whereas the newly formed bone tissues in the 10% and 20% nHAp/PPC and the HEAL-ALL groups were numerous and thicker. The extent of bone formation in the nHAp/PPC group was obvious possibly because of Ca⁺ ions production during the degradation of the nHAp/PPC membrane. The process provided the raw material for bone formation. Furthermore, the 20% nHAp/PPC group contained many Ca⁺ ions, and thus generated a large volume of bone tissues. The results of hematoxylin-eosin staining were consistent with the results of micro-CT.

Similar to the HEAL-ALL membrane, the synthesized nHAp/PPC membrane had good biocompatibility and good effect on GBR. The micro-CT and histology analysis results showed that the 20% nHAp/PPC group had good effect on bone regeneration, and these results are consistent with our *in vitro* results²¹. However, to perform further studies to explore the mechanism of bone regeneration before the clinical applications of nHAp/PPC membrane is still necessary.

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Conflict of Interest

The authors have declared that no COI exists.

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