

Application of a Novel Resorbable Membrane in the Treatment of Calvarial Defects in Rats

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Abstract

Diplen-Gam (DG) is a novel absorbable guided bone regeneration (GBR) membrane. This study was designed to evaluate the capacity of bone repair of DG compared with that of Bio-Gide (BG). Critical size defects were created in both sides of the calvarium of 36 Sprague–Dawley rats. Defects were assigned to six groups and each group was subjected to one of the following treatments: (A1) unfilled defects, (A2) Bio-Oss (BO) grafts, (B1) DG membrane, (B2) BG membrane, (C1) DG membrane + BO grafts and (C2) BG membrane + BO grafts. The animals were killed at 2, 4, 8 and 12 weeks after the operation. The defects and surrounding tissues were examined by gross observation and X-ray examination. The paraffin sections were subjected to HE (hematoxylin and eosin) staining and IHC (immunohistochemistry) for bone morphogenetic protein-2 (BMP-2). The X-rays showed that, at 12 weeks, the DG and BG group exhibited more new bone formation than CSD blank group did; the BG group exhibited more new bone formation than the DG group did ($t = 5.240$, $P = 0.035$), the BG + BO group showed no significant differences in bone formation compared with the DG + BO group ($t = 1.246$, $P = 0.339$). By IHC staining, BMP-2-positive results could be seen inside the DG membrane, on the surface of the new bone, and inside the new bone. It can be suggested that BG membrane achieved better effects in guided bone regeneration compared with DG membrane. No significant differences were found between the two membranes in their bone healing ability when they are used with BO. Therefore, DG membrane shows clinical effectiveness, but should be used in combination with bone substitute.

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Keywords

Guided bone regeneration, resorbable membrane, bone substitute, critical size defects, Diplen-Gam membrane

1. Introduction

Guided tissue regeneration (GTR), introduced by Nyman *et al.* [1], is a technique that uses a barrier membrane, which allows the repopulation of periodontal ligament-derived cells onto the dental root surface. Dahlin *et al.* [2] originally ap-

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plied this technique to bone regeneration in a bone loss area to establish the concept of osteopromotion. Based on Dahlin's technique, Buser *et al.* [3] proposed the term guided bone regeneration (GBR), which aims to promote bone augmentation by a barrier membrane. This technique has been applied in clinical dentistry to various cases including dental implant therapy with an insufficient volume of bone in recipient site. The most popular nonabsorbable membrane currently utilized for GBR is an expanded polytetrafluoroethylene (e-PTFE) membrane, which is a biologically inert material and can be safely applied clinically [4, 5]. Despite the many advantages of this membrane, however, it must be removed by a secondary operation that is required to repair dehiscence because of its natural unresorbability. The most popular absorbable membrane for GBR is Bio-Gide (BG). Bio-Gide membrane is a natural biomaterial of a collagenous bioresorbable membrane, composed of porcine type-I and type-III collagen fibers without any organic components and/or chemicals. Although Bio-Gide membrane in combination with Bio-Oss (BO) appears to obtain the same results as the grafting material in combination with an e-PTFE membrane [6], Bio-Gide is too expensive to be used widely in the clinic.

Diplen-Gam (DG) is a novel absorbable membrane, which consists of both a hydrophilic layer and a hydrophobic layer. The hydrophilic layer of the film includes a biocompatible structure containing hydroxyapatite and calcium phosphate. Meanwhile, the hydrophilic layer can be tightly fixed on the surface of the bone in the field of defects and exists for several weeks. This adhesive character allows it to be used without additional fixing elements [7, 8].

The present study was designed to obtain radiographic and histological data to guide the clinical application of DG membrane, and study its bone healing ability when used in combination with bone substitute (Bio-Oss) to effect bone regeneration. To this end, we chose radiographic and histological techniques to study bone regeneration effect in rat calvarias bone defects, compare the healing capability of DG membrane with that of BG membrane when applied alone or with BO.

2. Materials and Methods

All animal experiments in this study were conducted under the Peking University Guidelines for Animal Experimentation.

2.1. Animals

Male Sprague–Dawley rats ($n = 36$, 10 weeks old, weighing 260–280 g) were used. This weight corresponds to an average age of 10 weeks, which is considered adult. They were obtained from Peking University Experimental Animal Center and kept under a standard light-dark schedule and standard relative humidity. Stock diet and tap water were available.

2.2. Experimental Procedures

The rats were anaesthetized by an intraperitoneal injection of 8% chloralhydrate (400 mg/100 g body weight), the surgical area was shaved and the skin was washed



Figure 1. Critical size defect (5 mm diameter) created in the calvarium. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

with a mixture of iodine and 70% ethanol before surgical draping. Local anesthesia using 2% lidocaine with 1/100 000 epinephrine was performed to control bleeding and to provide additional local anesthesia. Surgical sites were exposed with a sagittal incision through the skin and the periosteum at the midline of the calvarias using sterile surgical technique. A stainless trephine bur (5 mm outer diameter), operated at low speed with sterilized physiological saline cooling, was used to make standardized transosseous defects 5 mm in diameter on both parietal bones, 1.5–2 mm lateral to the sagittal suture (Fig. 1).

The 36 rats were assigned into six groups as follows. In the BO and blank control groups (12 rats), BO was placed in the left bone defect and the right one was used as control. In the DG and BG groups (12 rats), DG was placed over the left bone defect and BG was placed over the right bone defect. In the DG–BO and BG–BO groups (12 rats), both of the defects were filled with BO and then covered with DG (left defect) and BG (right defect) membranes. After the placement, the periosteum and subcutaneous tissues were replaced and the surgical wounds closed with sutures. Three rats from each experimental group were killed at 2, 4, 8 and 12 weeks after surgery.

2.3. Quantitative Radiographic Analysis

The calvarias were radiographed by means of a microradiograph unit (Soredex Miniray) under standardized conditions: 70 kV, 7 mA, 0.06 s exposure time, 20 cm film–radiation beam distance. Radiographic density of the digital radiographs within the defects was measured using Image-Pro plus 6.0 (Media Cybernetics). A gray scale was used with 0 equaling black (or absolute radiolucency) and 255 equaling white (or absolute radiopacity). All data are expressed as mean \pm SD [9]. A paired *t*-test was used to analyze the difference between groups.

2.4. Histological Study

After anesthesia with diethyl ether and an injection of pentobarbital, the rats were fixed by perfusion with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4).

The skulls were quickly dissected and immersed in the same fixative overnight. The specimens were rinsed in 0.1 M cacodylate buffer and decalcified with 10% EDTA for 4 weeks at 4°C. The skulls were then divided along the sagittal suture, dehydrated in a series of increasing concentrations of ethanol, and embedded in paraffin. Sagittal sections, 5.0 µm thick at the middle part of the defect, were selected from serial sections and stained with hematoxylin and eosin. Some sections were used for immunohistochemical studies.

2.5. *Histochemical Studies*

Specimens were used to demonstrate the distribution of bone morphogenetic protein 2 (BMP-2), a marker for osteoblasts and osteoblast-like cells, by immunohistochemistry. The sections were treated with 3% H₂O₂ in methanol for 20 min to inhibit endogenous peroxides, followed by preincubation for 30 min with phosphate-buffered saline (PBS) containing goat serum albumin. Then the specimens were incubated at 4°C overnight, with antiserum rabbit anti-rat BMP-2 (Abcam) at a dilution of 1:200 in PBS, followed by incubation for 30 min with anti-rabbit IgG at adulation of 1:100 in PBS. Immunoreactivity was visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3-diaminobenzidine and 0.002% H₂O₂. All incubations were done at room temperature. When a control solution of PBS containing no antiserum rabbit anti-rat BMP-2 was used, no non-specific reaction in tissues was found. The sections used for histochemical studies were counterstained with hematoxylin.

3. Results

3.1. *Gross Finding*

After surgery all animals recovered well and the expected increase in weight during the postsurgical period was then recorded. Neither side-effects, such as paralysis, convulsions, respiratory distress, or signs of pain, nor any clinical signs of wound infections were observed. Skin tissues were in fair condition and scars were not proliferative.

3.2. *X-Ray Radiographs*

X-ray radiographs showed that the defects of control group had not been completely repaired with new bone in week 12. The BO group showed apparent displacement of the Bio-Oss grafts. In the DG, BG, DG-BO and BG-BO groups, the defects were almost completely repaired with new bone in week 12 (Figs 2–4).

3.3. *Quantitative Radiographanalysis*

As the BO group showed apparent displacement of the Bio-Oss grafts, and had little new bone formation, we excluded this group from data analysis. In the DG, BG, DG-BO and BG-BO groups, bone repair proceeded gradually and were almost complete at postoperative week 12. The BG group showed significantly more

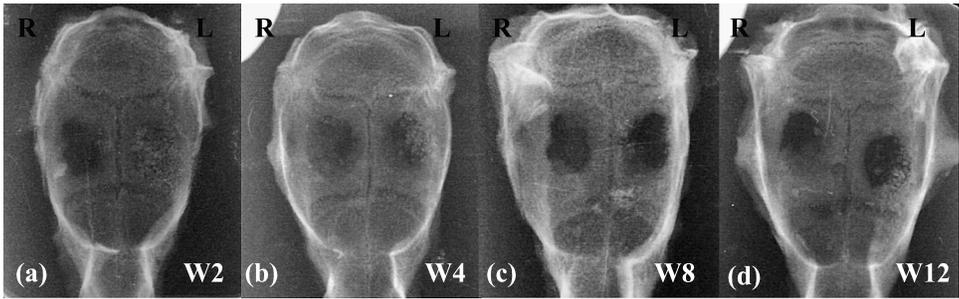


Figure 2. Radiographs of bone repair in defects in week 2 (a), 4 (b), 8 (c) and 12 (d). The left side is the BO group (BO was placed in the left bone defect), apparent displacement of the Bio-Oss grafts, little new bone formation. The right side is the control group, the defect was not completely repaired with new bone in week 12.

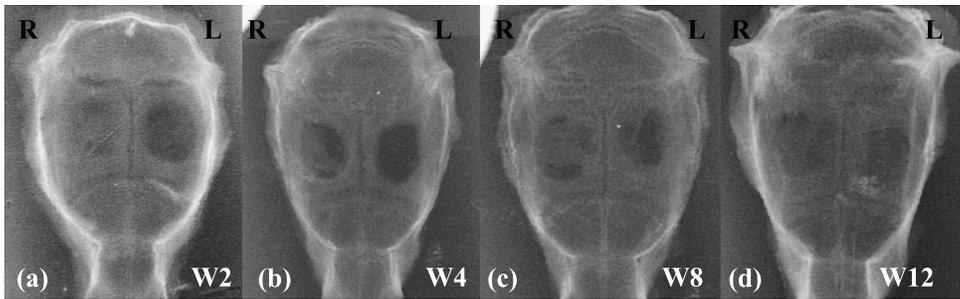


Figure 3. Radiographs of bone repair in defects in week 2 (a), 4 (b), 8 (c) and 12 (d). The left side is the DG group (bone defects covered by DG). The right side is the BG group (bone defects covered by BG), in the BG group there is a little more new bone formation compared with the DG group in week 12.

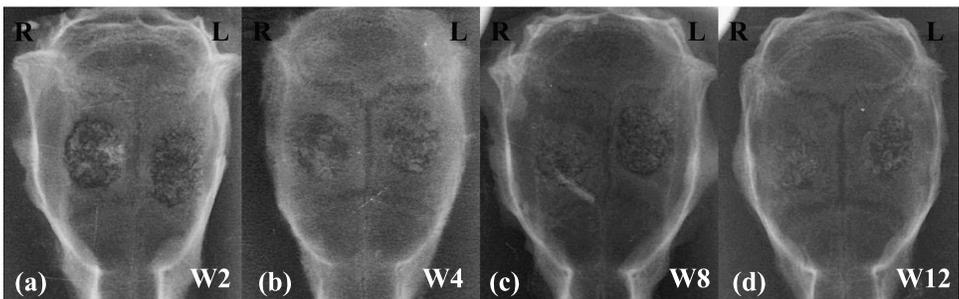


Figure 4. Radiographs of bone repair in defects in week 2 (a), 4 (b), 8 (c) and 12 (d). The left side is the DG-BO group (bone defects filled with BO and then covered by DG), the right side is the BG-BO group (bone defects filled with BO and then covered by BG).

Table 1.

Quantitative radiographic analysis for the each group (gray value)

Group	(-)	DG	BG	DG + BO	BG + BO
2w	90 ± 5.4	91 ± 5.8	103 ± 3.6	111 ± 2.7	113 ± 3.3
4w	104 ± 0.5	108 ± 4.6	115 ± 3.7	117 ± 0.4	120 ± 1.5
8w	105 ± 1.2	114 ± 3.7	118 ± 1.7	121 ± 5.2	125 ± 4.0
12w	107 ± 3.9	116 ± 3.3	124 ± 4.6	124 ± 3.6	128 ± 2.7

Table 2.Results of data analysis (paired *t*-test)

	2w	4w	8w	12w
BG group vs DG group	<i>t</i> = 4.178 <i>P</i> = 0.053	<i>t</i> = 1.210 <i>P</i> = 0.350	<i>t</i> = 4.628 <i>P</i> = 0.044*	<i>t</i> = 5.240 <i>P</i> = 0.035*
BG-BO group vs DG-BO group	<i>t</i> = 4.409 <i>P</i> = 0.048*	<i>t</i> = 3.160 <i>P</i> = 0.087	<i>t</i> = 2.308 <i>P</i> = 0.147	<i>t</i> = 1.246 <i>P</i> = 0.339

* *P* < 0.05.

bone formation than the DG group in week 8 (*t* = 4.628, *P* = 0.044) and 12 (*t* = 5.240, *P* = 0.035). However, The BG-BO group only showed significantly more bone formation than the DG-BO group in week 2 (*t* = 4.409, *P* = 0.048), and no significant difference was found between the BG-BO and the DG-BO group in any of the following weeks (Tables 1 and 2).

3.4. Histology

3.4.1. Control Group

At postoperative week 2, new bone was formed in the defect from the outer and inner surface of the parietal bone and made up the projection. The bone formation was more obvious on the inner surface than on the outer surface. There was also some new bone formation in the defect. At postoperative week 12, even though more new bone was observed, the defect was not completely repaired yet, and filled with both fibrous connective tissue and new bone. The repaired bone was lined with bone lining cells (Fig. 5).

3.4.2. BO Group

At postoperative week 2, the defect was filled with Bio-Oss grafts and no obvious new bone formation was observed. The grafts were surrounded by fibrous connective tissue. At postoperative week 12, no Bio-Oss grafts were left in the defect. The defect was filled with fibrous connective tissue (Fig. 6).

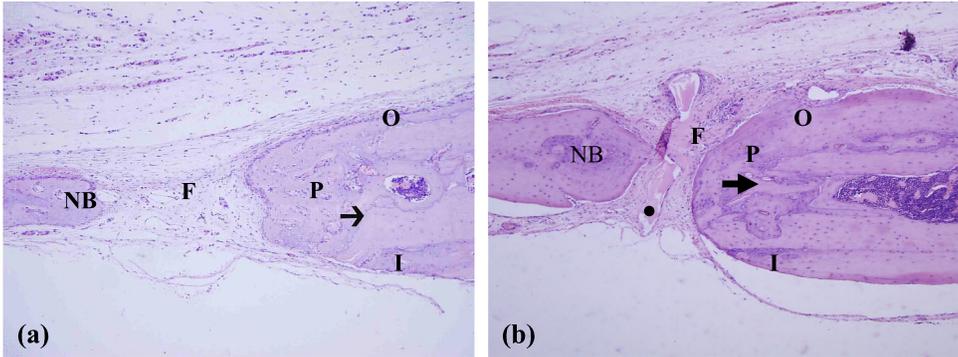


Figure 5. Histology of the blank control group. Bone is formed in the defect from the outer (O) and inner (I) surfaces of the parietal bone in week 2 (a) and makes up the projection (P). The bone formation is more noticeable on the inner surface. There is also some new bone (NB) formation in the defect (a). The defect is not completely filled with bone and the unrepaired defect is filled with fibrous connective tissue (F) and some Bio-Oss graft (●) shifting from the BO group side in week 12 (b). Projection (P) was new bone that formed in the defect from the outer and inner surface of the parietal bone. NB was new bone that formed in the defect. They are both 'new bone', formed in different locations. The arrows point at the edge of the original defect, $\times 100$. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

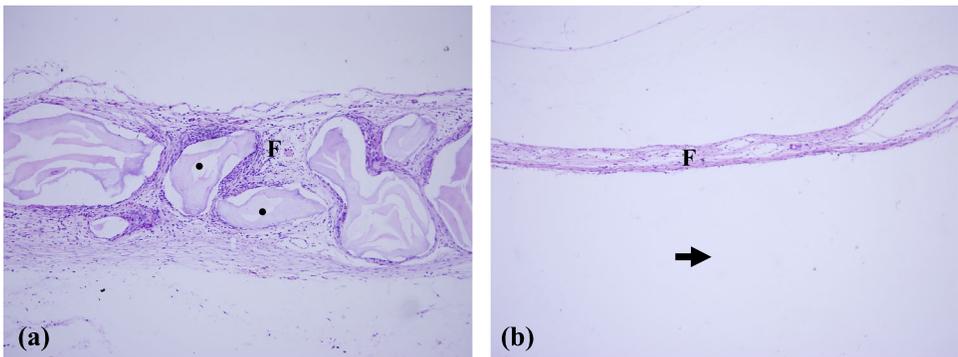


Figure 6. Histology of the BO group. The defect is filled with Bio-Oss grafts (●) and the grafts are surrounded by fibrous connective tissue (F) in week 2 (a). At postoperative week 12, there were no more Bio-Oss grafts left in the defect. The defect was filled with fibrous connective tissue (b), $\times 100$. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

3.4.3. DG Group

At postoperative week 2, the defect was filled with newly formed bone. A bony layer lining the inner surface of the dura mater of the parietal bone had formed. At postoperative week 12, more new bone had formed and extended upward. New bone fully occupied the space of the defect (Fig. 7).

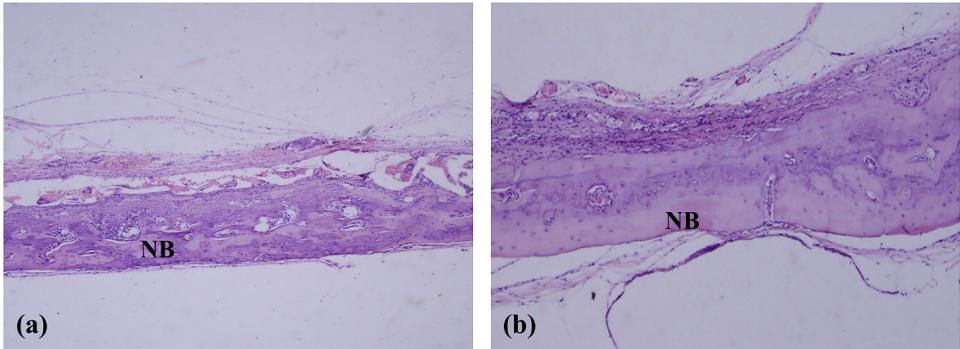


Figure 7. Histology of the DG group. Newly formed bone (NB) had filled in the defect in week 2. A bony layer lining the inner surface of the dura mater of the parietal bone had formed (a). More new bone had formed and had extended upward in week 12 (b), $\times 40$. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

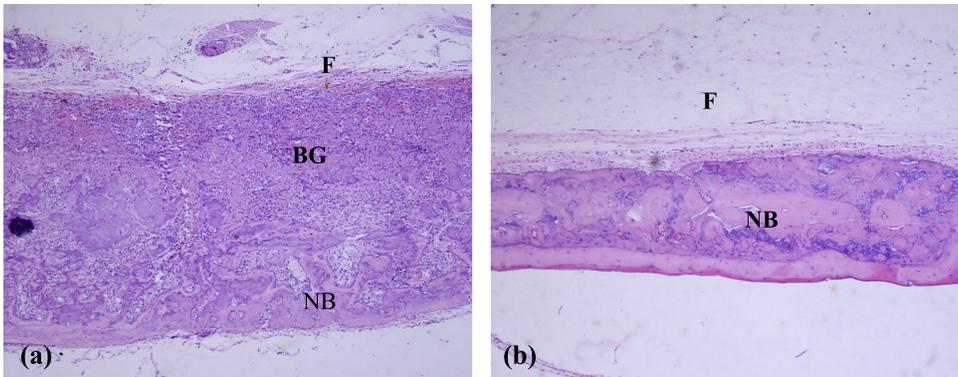


Figure 8. Histology of the BG group. A bony layer lining the inner surface of the dura mater of the parietal new bone (NB) had formed. There was no obvious degradation of BG membrane and the fibrous connective tissue (F) was blocked on the outer surface (a). Most of the BG membrane degraded and the residual part was surrounded by the new bone in week 12. New bone had fully occupied the space of the defect (b), $\times 40$. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

3.4.4. BG Group

At postoperative week 2, a bony layer lining the inner surface of the dura mater of the parietal bone had formed. There was no obvious degradation of BG membrane and the fibrous connective tissue was blocked on the outer surface. At postoperative week 12, most of the BG membrane had degraded and the residual part was surrounded by the new bone. New bone fully occupied the space of the defect (Fig. 8).

3.4.5. DG–BO Group

At postoperative week 2, the defect was filled with BO grafts and covered by DG membrane. The fibrous connective tissue (F) was blocked on the outer surface. At

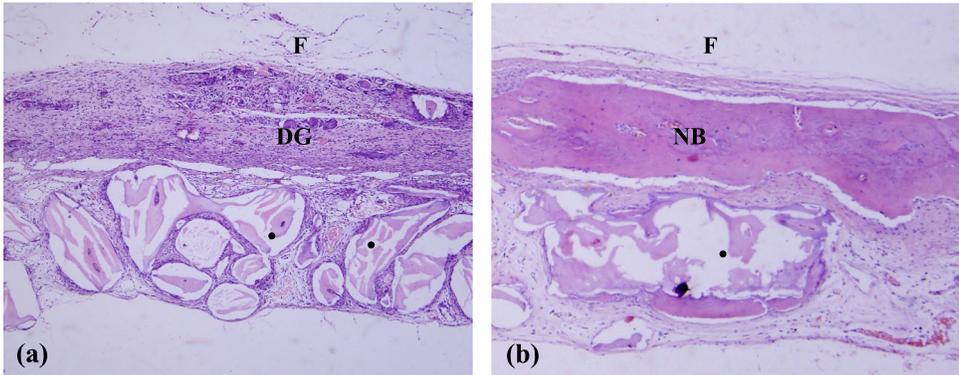


Figure 9. Histology of the DG–BO group. The defect was filled with BO grafts and covered by DG membrane in week 2. The fibrous connective tissue (F) was blocked on the outer surface (a). Most of the DG membrane degraded and the new bone fully occupied the space of the defect in week 12 (b), $\times 40$. This figure is published in colour in the online edition of this journal, which can be accessed via <http://www.brill.nl/jbs>

postoperative week 12, most of the DG membrane had degraded and the new bone had fully occupied the space of the defect. The tissue around the BO grafts is a blood clot in the organization process, including the vascular tissue, fibrous tissue and newly formed bone tissue (Fig. 9).

3.4.6. BG–BO Group

At postoperative week 2, the defect was filled with BO grafts and covered by BG membrane. There was no obvious degradation of BG membrane and the fibrous connective tissue (F) was blocked on the outer surface. At postoperative week 12, most of the BG membrane had degraded and the residual part was surrounded by the new bone. New bone fully occupied the space of the defect. The tissue around BO grafts is a blood clot in the organization process, including the vascular tissue, fibrous tissue and newly formed bone tissue (Fig. 10).

3.5. Immunohistochemistry

By immunohistochemistry staining, the distribution of osteoblasts, osteoblast-like cells and bone matrix was clearly demonstrated. BMP-2-positive results could be seen inside the DG membrane, on the surface of the new bone and inside the new bone (Fig. 11).

4. Discussion

Nowadays, the most popular membranes utilized for GBR are Bio-Gide and e-PTFE [4–6]. Bio-Gide (BG) is limited in clinical application due to its high price. e-PTFE must be fixed with additional elements in the surgical site and removed in a secondary operation because of its natural property. Diplon-Gam (DG) is a novel absorbable membrane, needs no secondary operation, and can be tightly fixed on

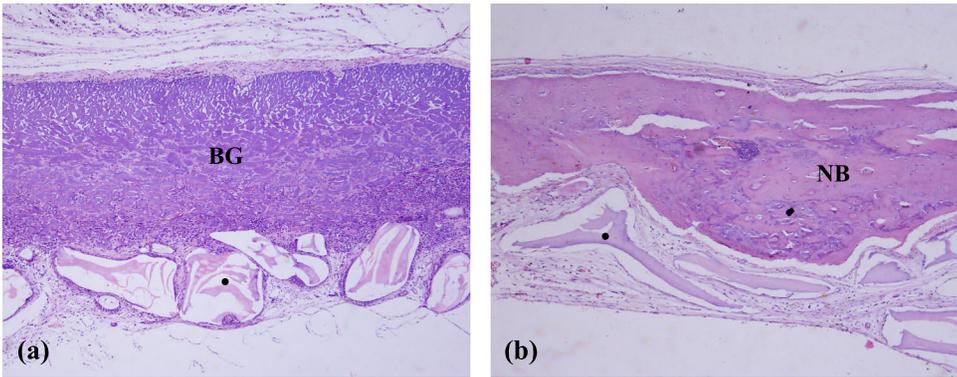


Figure 10. Histology of the BG-BO group. The defect was filled with BO grafts and covered by BG membrane in week 2. The fibrous connective tissue (F) was blocked on the outer surface (a). The new bone fully occupied the space of the defect in week 12 (b), $\times 40$. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

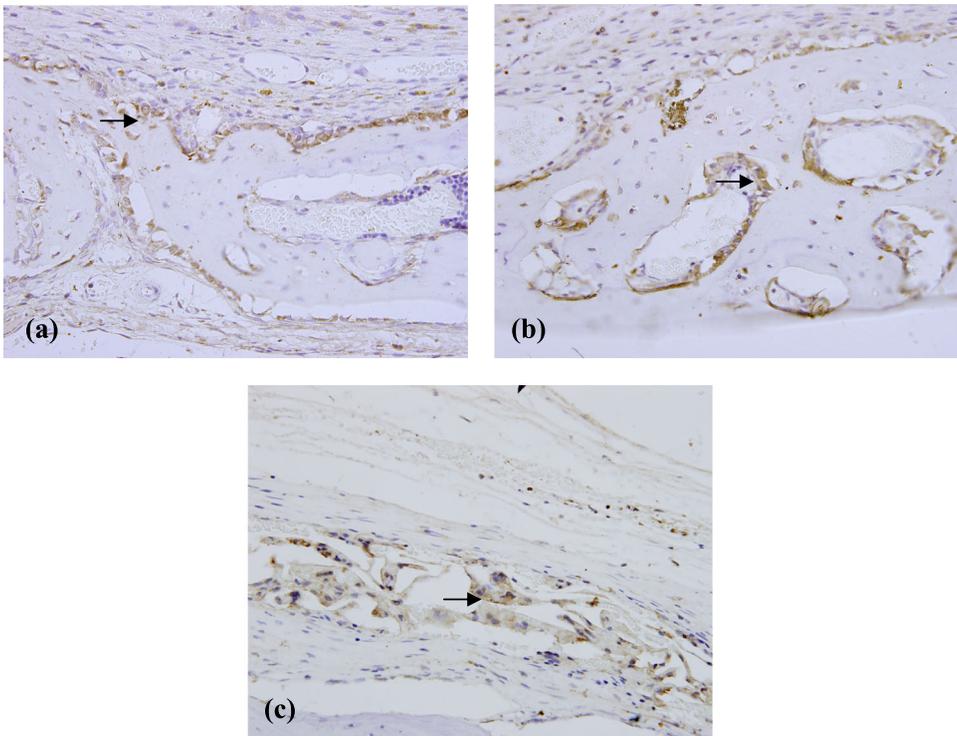


Figure 11. Immunohistochemistry staining. Brown granules, indicating BMP-2-positive results, could be seen (black arrow). (a) Brown granules on the surface of the new bone. (b) Brown granules inside of the new bone. (c) Brown granules inside of the DG membrane, all $\times 200$. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

the surface of the bone in the field of defects without additional fixing elements. In this research, we have investigated the bone healing capacity of DG when compared with that of BG, in order to provide evidence for clinical application of this biomaterial.

DG consists of both a hydrophilic layer and a hydrophobic layer. The biocompatible structure in the hydrophilic layer contains hydroxyapatite and calcium phosphate, which are the necessary elements for bone regeneration. The hydrophilic layer can be tightly fixed on the surface of the bone and stays for several weeks. This adhesive character allows it to be used without additional fixing elements in the animal experiment. The condition in the bone defect under the DG membrane can be observed during the operation because of its transparent character. The DG membrane is easy to trim to the size and shape of the operating region.

Critical size defects in cranium, mandible, femur and other portions of the rat skull are often used as experimental models to test bone repair materials [10–12]. A calvaria defect model has many similarities to defects in maxillofacial region. Calvarias develop from a membrane precursor and, thus, morphologically and embryological resemble the membrane bones of the face. Anatomically, the calvaria consist of two cortical plates with regions of intervening cancellor's bone which is similar to the mandible [13]. We choose a rat cranium model in our study. Defects of 5 mm [14] and 8 mm [9, 15] in diameter are the most commonly used in the rat cranium defect model. A critical size defect (CSD) is defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal [16]. With a 8 mm CSD in the rat cranium, the sagittal suture will be involved in the defect, which cannot simulate the jawbone exactly. What is more, the experimental defect and the control defect will not be established in one rat with a 8 mm CSD. Therefore, 5 mm CSD models were chosen in our study. In our study, the blank control group was found not to heal well at week 8 and 12 due to the interference of the fiber connective tissue. This behaved in accordance with the definition of the CSD. Therefore, it also proved that the 5 mm CSD model is reliable.

It has been suggested that the bone repair of the CSD model is more noticeable on the surface of the bony edge around the original defect. The results from our study suggested that bone repair proceeds from the periosteum on both sides of the parietal bone and surface of the bony edge around the original defect. Bone formation was more noticeable from the periosteum but not the edge around the original defect. Honma *et al.* [17] found similar results. The periosteum may have been affected less by the surgery than the edge around the original defect. However, how the repaired bone is connected to the original bony edge surface remains unknown.

Nowadays, many studies use type-I collagen, osteocalcin and osteopontin antibodies as markers for osteoblasts [10, 18, 19]. In our study, we wanted to use multiple ways, such as gross finding, radiographs quantitative radiograph analysis and histology methods, to evaluate the capacity of bone repair of DG compared with that of BG. As a bone growth factor, BMP-2 emerges at the early process

of osteogenesis [20]. We wanted to know the active osteogenesis period through BMP-2 detecting. Therefore, we detected the new bone formation by BMP-2 staining, combined with other methods.

In our study, when the BG–BO group was compared to the BG group, and the DG–BO group to the DG group, the quantitative data of the BG–BO group and DG–BO group are larger, because of the opacity of BO, but we could not detect the difference in sections, maybe the difference of new bone quantity could not yet be seen by the naked eye. We did not compare the BG–BO group to the BG group, and the DG–BO group to the DG group, by quantitative radiograph analysis, but compared the BG group to the DG group and the BG–BO group to the DG–BO group. The BG–BO and DG–BO groups were in the same rat, left and right. All of the main experimental process were carried out by one laboratory person; thus, the quantity of BO between the two groups was equal. Finally, we used the difference of gray value dates between DG–BO and BG–BO groups to do a paired *t*-test, whereas the BG group also could be compared to the DG group.

The results showed that the bone formation in the BO group is even less than in the control group. This could be explained by the fact that the stability of the bone formation space was affected by the replacement of the BO grafts. It also suggests that the membrane used to provide a containing and stable bone formation space is an essential prerequisite for bone healing.

The X-ray result was a reflection of the accumulated bone formation in the defect. At postoperative week 12, The BG group showed significantly more bone formation than the DG group ($t = 5.240$, $P = 0.035$). However, no significant differences in bone formation were found between the BG–BO group and the DG–BO group at postoperative week 12 ($t = 1.246$, $P = 0.339$). This suggested that better effects in guided bone regeneration can be achieved with the BG membrane than with the DG membrane. When used in combination with BO, no significant differences in bone formation were found between the two membranes. One of the most difficult complications is the membrane collapse resulting from external mechanical forces and soft tissue pressure, which might result in a reduced volume of bone regeneration [3, 4, 21]. The lower bone formation capability of the DG membrane may be due to its weaker mechanical strength. When the membrane was used in combination with BO grafts, the BO grafts may compensate for the mechanical strength of the DG membrane. Also, the BO grafts have good osteoconduction and osteoinduction in bone formation. Therefore, the DG membrane achieved the same result in GBR as the BG membrane.

In conclusion, the BG membrane showed better results in GBR than the DG membrane. When used in combination with BO, both membranes showed satisfying results in bone formation. Therefore, the DG membrane is clinical applicable, but should be used in combination with bone substitutes.

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