Paracrine effect of the bone morphogeneticprotein-2 at the experimental site on healing of the adjacent control site: a study in the rabbit calvarial defect model

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Purpose: The aim of this study was to assess the possible paracrine effect of bone morphogeneticprotein-2 (BMP-2) at the experimental site on the adjacent control site for validating a rabbit calvarial defect model as a means of verifying the effect of BMP-2.
Methods: Sixteen rabbits were divided into two groups (n = 8 in each) according to whether or not BMP-2 would be used. Two circular defects (8 mm in diameter) were created side by side, 2 mm apart, in the calvarium of all of the rabbits. In each animal, one of the defects was grafted with either BMP-2-loaded carrier or carrier material alone. The control defects adjacent to these grafted defects, designated CB (the nongrafted defect adjacent BMP-2-loaded carrier-grafted defect) and CC (the nongrafted defect adjacent to carrier only-grafted defect), respectively, were the focus of this study, and were filled only with a blood clot in all of the animals. Histologic observation and histomorphometric analysis were performed at 2 and 8 weeks (n = 4 animals per point in time) after surgery.
Results: There was no noteworthy difference in the healing pattern, and no statistically significant differences in histomorphometric parameters such as the defect closure, new bone area, or total augmented area between the CC and CB groups.
Conclusions: The results of this study suggest that rabbit calvarial defects separated by a distance of 2 mm are suitable for evaluating the effects of BMP-2 and the control defect can be regarded not to be affected by BMP-2 applied defect.

Keywords: Animal experimentation, Bone morphogenetic protein 2, Bone regeneration, Research design.

INTRODUCTION

Numerous treatments have been developed to augment atrophic alveolar ridges, and their clinical efficacy has been demonstrated in both clinical and preclinical studies. However, defects with less osteogenic potential, such as vertical deficiencies, have been found to exhibit variable regenerative outcomes. Therefore, predictable measures for enhancing osteogenesis with a bone substitute have been sought. Among many efforts, the introduction of growth factors such as recombinant bone morphogeneticprotein (BMP), vascular endothelial growth factor, and fibroblast growth factor have certainly raised the prospect of predictable bone regeneration [1-4], and recombinant human BMP-2 (rhBMP-2) has been highlighted as a particularly promising candidate [5].

The rabbit calvarial model is one of the better-established models for evaluating the efficacies of bone substitutes. Several circular defects can be made on the rabbit calvarium,

with the sizes of the defects being determined according to the characteristics of the biomaterials being used, the healing period, and more importantly, the aim of individual studies [6-8]. For example, circular 8-mm defects were created for evaluating a newly developed bone-graft material, with the treatment modality and the number of defects created differing between studies [1,6,7]. Rabbit calvaria have also been used for evaluating growth factors such as rhBMP-2 [2,9]. In order to accurately determine the effect of BMP-2, the experimental site grafted with carrier-material-loaded BMP-2 should have no or only a minimal effect on the adjacent, control site. Researchers ordinarily establish a negative control group, often a positive control group grafted with carrier only, and a group grafted with a well-documented carrier material loaded the growth factor to be tested. Moreover, individual differences among the experimental animals, even within the same species, must be taken into consideration. Therefore, all experimental groups are logically required to be tested on the same animal simultaneously. In this sense, where several defects are created on the rabbit calvarium, each defect is bound to be adjacent to another [2]. This has raised concerns as to whether the BMP-2 grafted site has a paracrine effect on the adjacent, control defect, thus confounding the findings. If there is such a paracrine effect, the influence of the tested growth factor—and especially a low dose thereof—would be underestimated, thus attenuating any measured statistically significant differences between findings at the control and experimental sites. However, not much is currently known about the possible effect of BMP-2 in an experimental site on the adjacent, control site.

The aim of this study was therefore to determine whether grafting of rhBMP-2 into an experimental site has a paracrine effect on the adjacent control site, thus validating the rabbit calvarial model as a means of verifying the effect of growth factors.

MATERIALS AND METHODS

Animals

Sixteen male New Zealand white rabbits (age, 9–20 months; body weight, 3–3.5 kg) were used in this study. The animals were housed in divided cages under standard laboratory conditions and fed a standard diet. The selection of experimental animals, their management, and the surgical protocol followed routines approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea.

Materials

BMP-2 was chosen as the growth factor because it is the most frequently used growth factor in the dental field. The BMP-2 used in this study was provided by Dentium (Seoul, Korea). Lyophilized BMP-2 was vortexed with distilled water, yielding a concentration of 0.05 mg/mL. Biphasic calcium phosphate (BCP) was used as the carrier material (OsteonII, Genoss, Suwon, Korea). OsteonII is composed of hydroxyapatite and beta-tricalciumphosphate at a ratio of 30:70, and has a particle diameter of 0.5–1.0 mm. OsteonII was loaded with growth factor by soaking it in 0.1 mL of BMP-2 for 5 minutes. The loading time was followed by previous studies [10,11].

Table 1. Group assignment.

	Treatment for the defect	Treatment for the adjacent defect
CC	Blood clot only	BCP only
CB	Blood clot only	BCP + BMP-2

CC: control defect adjacent to a defect grafted with biphasic calcium phosphate (BCP) carrier only, CB: control defect adjacent to a defect grafted with biphasic calcium phosphate carrier loaded with bone morphogenetic protein-2 (BMP-2).

Study design

Two circular defects (8 mm in diameter) were made side by side, 2 mm apart on each rabbit calvarium. The rabbits were divided into two groups: BMP-2+carrier and carrier only (n = 8 animals in each group). Block randomization was performed for random allocation. The surgeon had not been informed of the allocation until the defects were created. In each animal, one of the defects was designated a control defect, and was filled with a blood clot only. The experimental sites were filled with either BCP carrier alone (BCP animals) or with BMP-2-loaded BCP carrier (BCP+BMP-2 animals). The control defects in each group were the subject of the present study, and were labeled as follows (Table 1):

- CC group: in the BCP (carrier)-only animals, the defect located adjacent to the BCP-grafted site.
- CB group: in the BCP+BMP-2 animals, the defect located adjacent to the site grafted with BMP-2-loaded BCP.

Surgical protocol

The rabbits were anesthetized using an intramuscular injection of 4:1 solution of ketamine hydrochloride (Ketalar, Yuhan, Seoul, Korea) and xylazine (Rompun, Bayer Korea, Seoul, Korea). The surgical site was shaved, then disinfected with povidone iodine, and then infiltration anesthesia was induced by injection with 2% lidocaine (lidocaineHCl, Huons, Seoul, Korea). An incision was made in the sagittal plane, and a full-thickness flap was elevated. Two circular defects with a diameter of 8 mm were made with a trephine drill; the distance between the defects was set to 2 mm (Fig. 1). The assigned grafting material (i.e., BCP only or BCP+BMP-2) was grafted into one of the defects, and a blood clot was placed into each of the CC and CB defects. Barrier membrane was not used in this study because the membrane protection could make the favorable circumstance for bone formation like guided bone regeneration in each control defect. The flap was repositioned and sutured layer by layer using 4-0 glyconate absorbable monofilament (Monosyn, B. Braun Aesculap AG & Co KG, Tuttlingen, Germany). Postoperative antibiotics were administered by daily intramuscular injection of gentamicin (5 mg/kg body weight) for 1 week postoperatively. The stitches were removed 1 week after the operation. The animals in each group were sacrificed at either 2 weeks (n = 4

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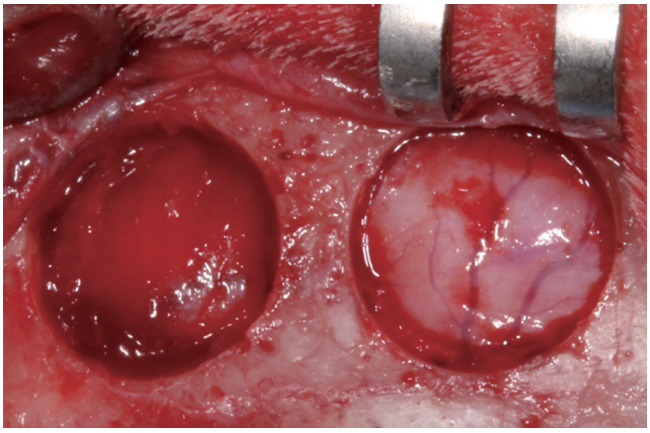

Figure 1. Clinical photograph of the experiment. Two 8-mm diameter defects were made, 2 mm apart, in the calvaria of 16 rabbits.

per group) or 8 weeks (n = 4 per group) postoperatively by an overdose of anesthetic.

Histologic processing

Blocks that included the adjacent tissues were harvested. The blocks were fixed in 10% buffered formalin for 10 days, decalcified in 5% formic acid for 14 days, and then embedded in paraffin. Serial sections were cut at 5 μm through the center of the defects. The two center-mostsections were selected from each block and stained with hematoxylin and eosin.

Evaluation methods

Clinical observations

The animals were carefully observed for any adverse signs such as inflammation, allergic reactions, and other complications throughout the postoperative healing periods.

Histologic observations

The specimens were examined using a binocular microscope (DM LB, Leica Microsystems, Wetzlar, Germany) equipped with a camera (DC300F, Leica Microsystems) by a single observer (J.W.L.) who was blinded to the experimental conditions.

Histomorphometric analysis

Histomorphometric data regarding the following parameters were obtained with an automated image-analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA; Fig. 2): (1) the percentage of defect closure (DF, %)—the percentage of new bone in relation to the original defect length; (2) the total augmented area (TA, mm^2)—the sum of all tissues between the defect margins, including new bone, connective tissue, and vessels; and (3) new bone area (NB, mm^2)—the area of new bone between the defect margins.

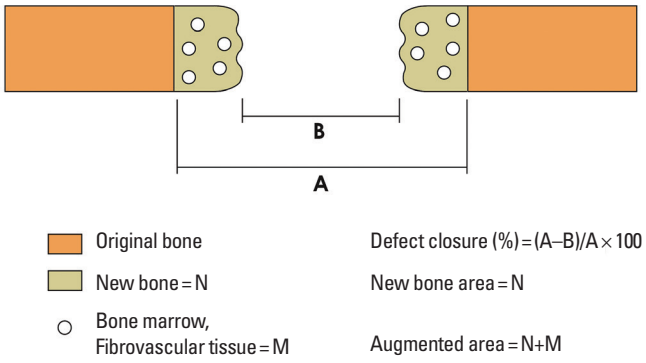

Figure 2. Schematic diagram of a calvarial defect showing the parameters that were analyzed histomorphometrically. The calculations for various measurements are shown.

Statistical analysis

Independent-samples *t*-tests were conducted using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). The data are presented as mean ± standard deviation values, and the cutoff for statistical significance was set at $P < 0.05$.

RESULTS

Clinical findings

Healing was uneventful during the postoperative period, and no sign of adverse events was observed.

Histologic findings

The histologic features were generally similar in the two groups at both healing times. Most of the defects in both groups were largely filled with fibrous connective tissue at 2 weeks postoperatively, and were concave in shape. A minimal amount of wedge-shaped immature new bone was formed at the defect margins (Fig. 3); the amount of new bone was increased at 8 weeks postoperatively. A demarcating line was observed in the newly formed bone. Bone islets were observed in some samples. The defect closure in the 8-week group was greater than in the 2-week group (Fig. 4).

Histomorphometric analysis

A summary of the histomorphometric analysis is given in Table 2. DF, NB, and TA all increased with healing time, with differences over time reaching statistical significance ($P < 0.05$). However, there were no significant differences for each variable between the BCP- and BMP-2+BCP-grafted defects and the CC and CB defects, respectively, at either healing time.

DISCUSSION

This study evaluated the possible paracrine effect of BMP-2 grafted into an experimental site on the adjacent control site in a rabbit calvarial model. This model has frequently been used be-

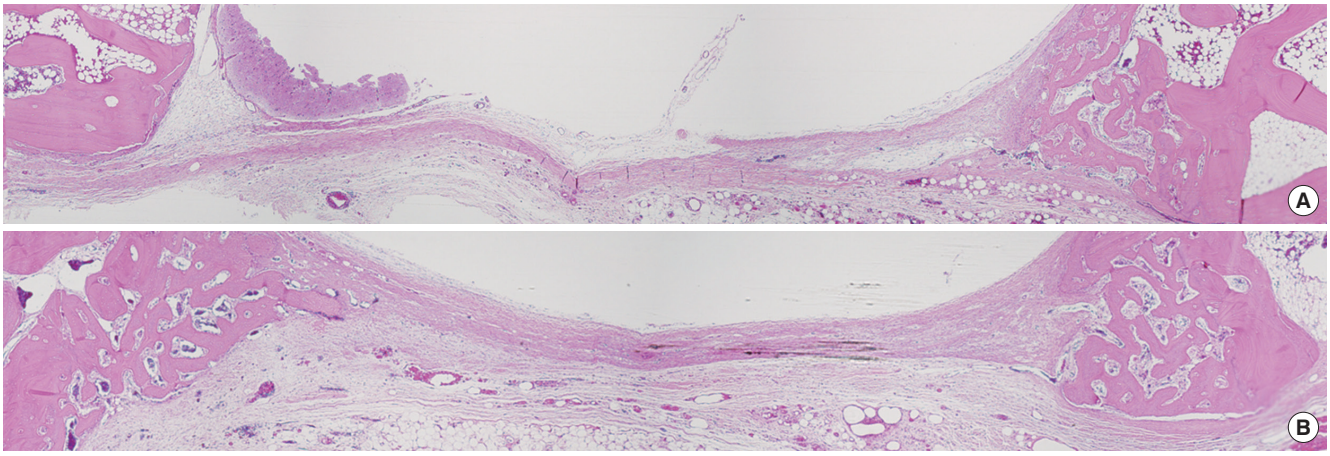

Figure 3. Representative photomicrographs obtained at 2 weeks postoperatively: the CC group (A) and the CB group (B) (A, B: H&E, ×40). CC: control defect adjacent to a defect grafted with biphasic calcium phosphate carrier only, CB: control defect adjacent to a defect grafted with biphasic calcium phosphate carrier loaded with bone morphogenetic protein-2.

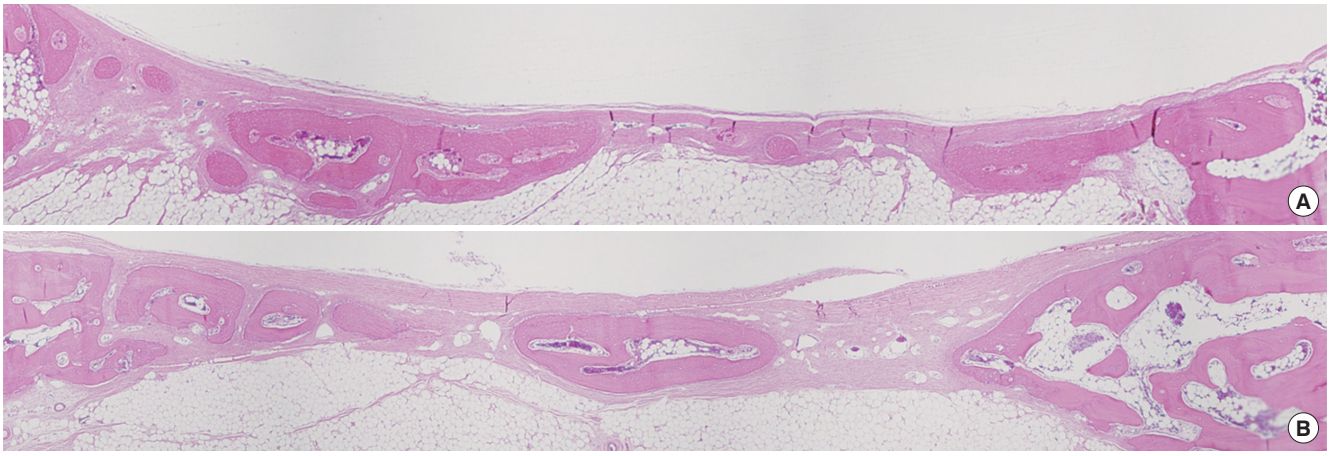

Figure 4. Representative photomicrographs obtained at 8 weeks postoperatively: the CC group (A) and the CB group (B) (A, B: H&E, ×40). CC: control defect adjacent to a defect grafted with biphasic calcium phosphate carrier only, CB: control defect adjacent to a defect grafted with biphasic calcium phosphate carrier loaded with bone morphogenetic protein-2.

Table 2. Histomorphometric measurements.

	DF (%)		NB (mm^2)		TA (mm^2)	
	2 Weeks	8 Weeks	2 Weeks	8 Weeks	2 Weeks	8 Weeks
CC	24.8±5.24	66.03±3.35*	0.62±0.25	3.26±0.14*	1.06±0.32	3.58±0.27*
CB	22.6±1.78	59.28±1.43*	0.67±0.19	3.13±0.28*	1.25±0.28	3.75±0.43*

Values are presented as mean±standard deviation.
DF: defect closure, NB: new bone area, TA: total augmented area.
*Statistically significant difference compared to the 2-week group ($P<0.05$).

cause it is relatively easily handled, the bone healing rate is good, and the marrow space is sufficient for stimulating new bone formation [12,13]. Critical-size defects (CSDs) must be created to evaluate biomaterials. A CSD is defined as the smallest defect that would not heal spontaneously without intervention for a certain period of healing [14], or which results in less than 10% bone healing during the remaining life span of the experimental animal [15]. The size of CSDs in the rabbit is reportedly up to 17 mm×17 mm [16]; however, many studies have adopted circular 8-, 6-, and even 5-mm defects [2,7,17,18]. Circular 8-mm diameter defects were chosen for the present study. Although an 8-mm defect is smaller than some other CSDs, this size of defect has been sug-

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gested to be appropriate for evaluating the early phase of bone healing [8], and it has been shown that 8-mm defects do not fully heal within 8 weeks [8]. The proportions of defect closure and new bone were approximately 65% and 39%, respectively. Therefore, the bone-regenerative potential can be evaluated after a healing period of 8 weeks.

The advent of the growth factor era has led to some side effects when they are used in certain procedures, such as when BMP-2 is used in bone regeneration. After spinal fusion surgery using BMP-2, ectopic bone formation was observed in a remote site that was not related to the surgery [19,20]. Accordingly, it is logical to suspect that a grafted growth factor could also affect the area adjacent to the surgical site. Moreover, in experimental research using growth factors, it is possible that, for example, BMP-2 grafted into experimental sites could leak into other sites, such as neighboring negative or positive control sites. This is a particularly important issue given that researchers often establish experimental and control sites in the same bony structures. If, as is often the case, four 8-mm circular defects are created in the rabbit calvarium, the distance between each defect margin would typically be 2–4 mm, and possibly smaller with inexperienced surgeons.

We set the distance between each defect margin at 2 mm in the present study. Histologic and histomorphometric evaluations after 2 and 8 weeks did not reveal any differences in either the healing pattern or the characteristics of each variable. Sohn et al. [8] studied various sizes of circular defect in the rabbit calvarium. At 2 weeks, the authors reported almost 1.9 mm^2 of NB and a DF of 41%, which is higher than the present study; however, this does not indicate that the present study was inappropriate. Rather, the difference might stem from the healing potentials of the individual animals. At 8 weeks, the NB and DF measured in this study (CC group: 3.26 mm^2 and 66.03%, respectively; CB group: 3.13 mm^2 and 59.28%, respectively) were similar to those reported by Sohn et al. [8] (3.2 mm^2 and 65%, respectively).

In the present study, the BCP carrier material was soaked in 0.05 mg/mL BMP-2 solution for use in the BCP+BMP-2 experimental animals. At 2 and 8 weeks, the defect grafted with the carrier-loaded BMP-2 demonstrated statistically significant new bone formation compared to the defect grafted with the carrier only (data not shown). These results indicate that the dose of BMP-2 used in the present study was effective, and support the minimal influence of BMP-2 on the adjacent control (CB) defect.

Within the limitations of the present study, BMP-2 application to experimental defects did not have a significant influence on the healing of the adjacent control defects in a rabbit calvarial model. Therefore, it can be concluded that rabbit calvarial defects that are separated by more than 2 mm are suitable for evaluating the effects of BMP-2. Future studies should assess this issue with other kinds of growth factor, lower doses of BMP-2, and extended healing periods.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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