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Safety and Efficacy of a New Moldable and Compression Resistant Matrix Carrier with Recombinant Human Bone Morphogenetic Protein-2 in the Rabbit Bone Defect Model

Jae-Min AHN[1], Seung-Jae HYUN[2], Ki-Jeong KIM[2]

[1]Soonchunhyang University Cheonan Hospital, Department of Neurosurgery, Cheonan, Korea
[2]Spine Center, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Department of Neurosurgery, Seongnam, South Korea

Corresponding author: Seung-Jae HYUN ✉ hyunsj@snu.ac.kr

ABSTRACT

AIM: Recombinant human bone morphogenetic protein-2 (rhBMP-2) was developed in the 1990s. We developed a new moldable and compression-resistant matrix (CRM) carrier. We conducted an animal experimental study to evaluate the safety and efficacy of the CRM carrier for rhBMP-2 in osteogenesis.

MATERIAL and METHODS: New moldable CRM carrier, and with rhBMP-2 (new CRM carrier with rhBMP-2) were prepared as the experimental groups. Pre-existing synthetic bone graft material was prepared as a control graft group. A total of 24 rabbits were included in the study. Defects were made and grafts were performed, and radiographic and histopathologic findings were evaluated to assess fusion.

RESULTS: In the computed tomographic scan, new bone formation was superior in 16.0%, 39.3%, 64.7%, and 81.1% of the total defect volume at 4, 8, 12, and 16 weeks in the new CRM carrier with rhBMP-2 group. In the new CRM carrier group, new bone formation was observed in 10.6%, 26.3%, 53.1%, and 71.4%, respectively. In the control graft group, new bone formation was observed in 10.1%, 26.6%, 53.4%, and 72.1%, respectively. On histopathologic evaluation, new CRM carrier with rhBMP-2 group showed better new bone formation compared with those of other groups.

CONCLUSION: The new moldable CRM carrier and the CRM carrier with rhBMP-2 showed preclinical safety and efficacy in new bone formation. In particular, the CRM carrier with rhBMP-2 was considered to be an effective bone graft material for bone fusion.

KEYWORDS: rhBMP-2, Compression resistant matrix, Pseudarthrosis, Hydroxyapatite, Tricalcium phosphate

INTRODUCTION

Spinal fusion is widely used to reconstruct the spine and restore spinal stability after surgery for degenerative spinal disease, spinal deformity correction, and trauma (4,6,36,42). Although it has been used for a long time, fusion failure and pseudarthrosis have been identified as major complications, and there is an ongoing debate regarding the selection of the ideal graft material for bone fusion. Moreover, many studies have reported fusion failure in 35% of cases due to pseudarthrosis, which leads to significant morbidity and increased healthcare costs (13,18,20,43).

The iliac bone is the most common source of autologous bone graft. Moreover, it has been considered the gold standard fusion material because it best provides both cortical bone and cancellous bone for grafting and there are no chances of biological rejection due to its autologous nature. However, graft harvesting from the iliac crest is often associated with significant complications, such as nerve injury, sacroiliac joint injury, chronic pain and infection at the donor site, and pelvic instability (7,15,35,42). Therefore, research is underway to identify suitable substitutes, such as synthetic bone grafts, calcium phosphatases, ceramics, demineralized bone matrix, and bone morphogenetic proteins (BMPs) (10,16,21,45).

Jae-Min AHN : 0000-0002-3427-8807 Seung-Jae HYUN : 0000-0003-2937-5300 Ki-Jeong KIM : 0000-0001-8547-8545

In 2002, the FDA approved the use of recombinant human bone morphogenetic protein-2 (rhBMP-2); since then, it has been widely used for spinal fusion (23,37). Although rhBMP-2 is highly efficient in improving osteoblast formation and activating osteogenesis and bone healing, it has some adverse effects, such as heterotopic ossification near critical vital structures, diffusion to the surrounding areas, and osteoclastic reaction due to rapid release of rhBMP-2 that causes bone resorption (21,29,34,39,41).

To minimize these adverse effects and maximize the effect of fusion activation, several carriers of rhBMP-2, such as collagen sponge, degradable synthetic polymers, hydroxyapatite (HA), and tricalcium phosphate (TCP), ceramics have been introduced; however, the use of these materials is still controversial (25,31).

Herein, we developed a new moldable and compression-resistant matrix (CRM) carrier to address the complications associated with the use of pre-existing carriers for rhBMP-2 before testing its efficacy in humans. This study aimed to elucidate the safety and efficacy of the new moldable CRM carrier and rhBMP-2 in a rabbit bone defect model.

This study was conducted from January 2018 to October 2018 and was approved by the Institutional Animal Care and Use Committee (Approval No. DGMIF-17120401-00, 2017-12-04).

Twenty-four skeletally mature (2.5–3.5 kg, all males) New Zealand white rabbits were prepared, and two defects were induced in the occipital bone for each animal. A total of 48 skull bone defect models were divided into four groups. The first group was designed as a defect-only negative control group. The second group was subjected to the application of the new moldable CRM carrier (collagen/β-TCP composite material) with rhBMP-2 application (*E. coli*-derived rhBMP-2, 1 mg per 1 g of the new CRM carrier). The third group was subjected to the application of the new CRM carrier alone (Figure 1). The second and third groups formed the experimental group. The fourth group, the control graft group, was subjected to the application of a licensed synthetic bone graft product, OSTEON II (GENOSS Co., Ltd. Suwon-Si, Gyeonggi-do, Republic of Korea) comprising collagen/HA and β-TCP at a ratio of 30:70). Twelve bone defect models were used in each group.

Bone defects of 8.0 mm width were induced on both sides of the rabbit occipital bone in each group, which has been reported to be a reliable model for evaluating subcritical defects (27,28,30,32,38). Then, at 4, 8, 12, and 16 weeks, six experimental animals (three per group) were euthanized for histological and radiological evaluation at each time point (Figure 2).

All procedures were performed under intramuscular anesthesia using Rompun (5 mg/kg) and Zoletil (15 mg/kg). To prevent perioperative infection, subcutaneous gentamycin (5 mg/kg) and Metacam (0.2 mg/kg) were used. After shaving the sur-

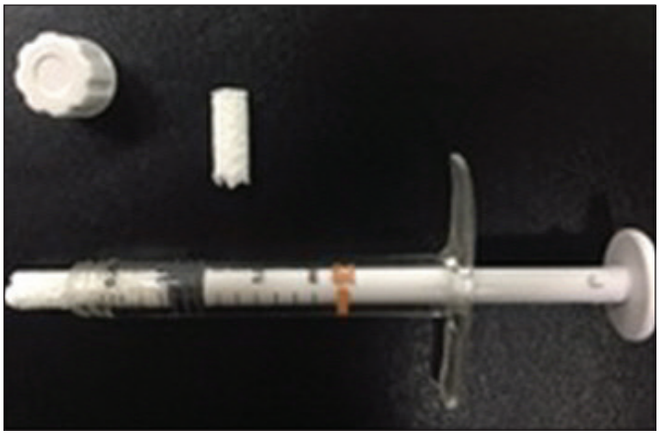

Figure 1: New moldable CRM carrier.
CRM: Compression resistant matrix.

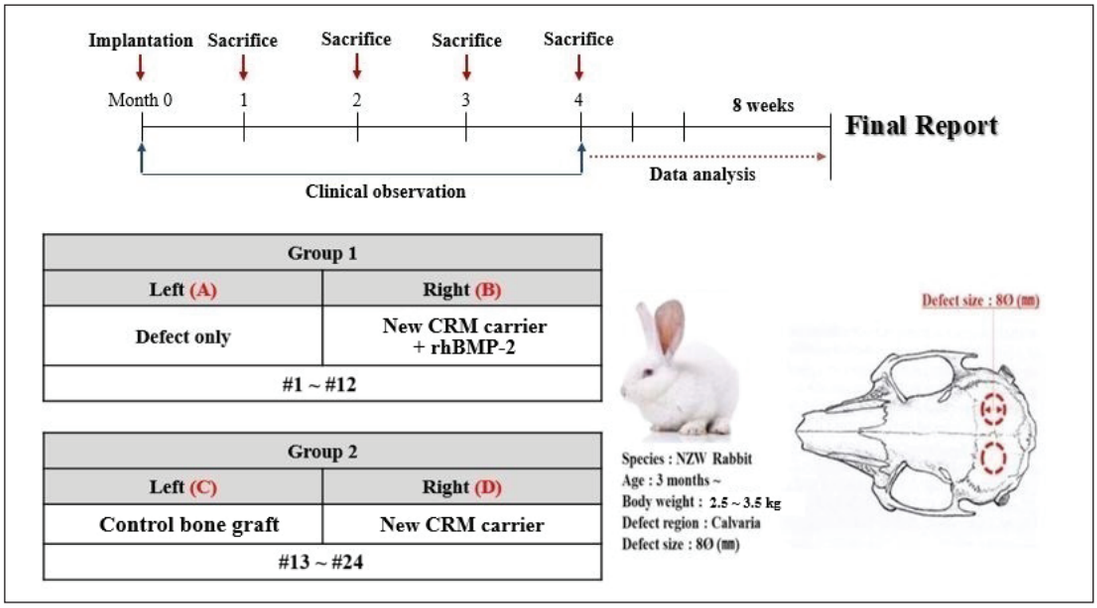

Figure 2: Graft assessment scheme of rabbit bone defect model.
CRM: Compression resistant matrix,
rhBMP-2: Recombinant human bone morphogenetic protein-2.

gical site, the skin was prepared with alcohol and povidone, and draped in a sterile fashion. A midline scalp incision was performed and the occipital bone was exposed after cutting off the periosteum. A Trephine bur was used to induce defects measuring 8.0 mm in width on both left and right sides (two per animal) of the occipital bone at least 3 mm apart to prevent the experimental materials from spilling to the other side. After confirming the defect size, the experimental materials were applied to each defect site. After implantation, a collagen shield was applied between the periosteum and graft material to prevent their interaction, and the periosteum was sutured so that the applied material was fixed well onto the defect site. After suturing the wound, the surgical site was treated with povidone, and antibiotics were administered for 3 days (Figure 3).

All animals used in the experiment were euthanized at 4, 8, 12, and 16 weeks after experimental matrix application. Inflammation and other abnormalities around the surgical site were observed before bone tissue harvest. A reciprocal saw was used to harvest the bone defect site, including normal bone tissue, while preserving the bone graft.

A micro-computed tomography (CT) scan (Quantum FX micro-CT, PerkinElmer, USA) was used to confirm the distribution of the implant and the degree of bone formation at the defect site. The conditions were as follows: voltage value, 90 kVp; current, 180 μA; field of view area, 24 mm; scan time: 4.5 min, region of interest, 60; and threshold value, 4,000–8,000. Micro-CT was performed at 4, 8, 12, and 16 weeks. To quantitatively evaluate newly formed bone, a region of interest with a diameter of 6 mm was concentrically positioned over the defect site (17). The Hounsfield unit (HU) of the newly formed bone was measured to qualitatively evaluate the newly formed bone. The HU threshold was set to ~225 HU for soft tissue, 148–661 HU for cancellous bone, and 662–1988 HU for cortical bone (5).

The collected tissues were divided at the center of the defect site into two halves for two different staining, and each side was fixed in 10% formalin solution. The samples were demineralized in 10% EDTA solution for 4 weeks. The demineralized tissues were trimmed, dehydrated in alcohol using a tissue pretreatment machine, and pretreated for paraffin infiltration. The tissue blocks were fixed with paraffin and cut into 5-μm sections. After hematoxylin–eosin and Masson's trichrome staining, the bone regeneration effect was evaluated by optical microscopy (Nikon Eclipse Ti, Japan).

Student's t-test was performed to compare body weight between the groups. Bone fusion properties were compared among the four groups using one-way analysis of variance

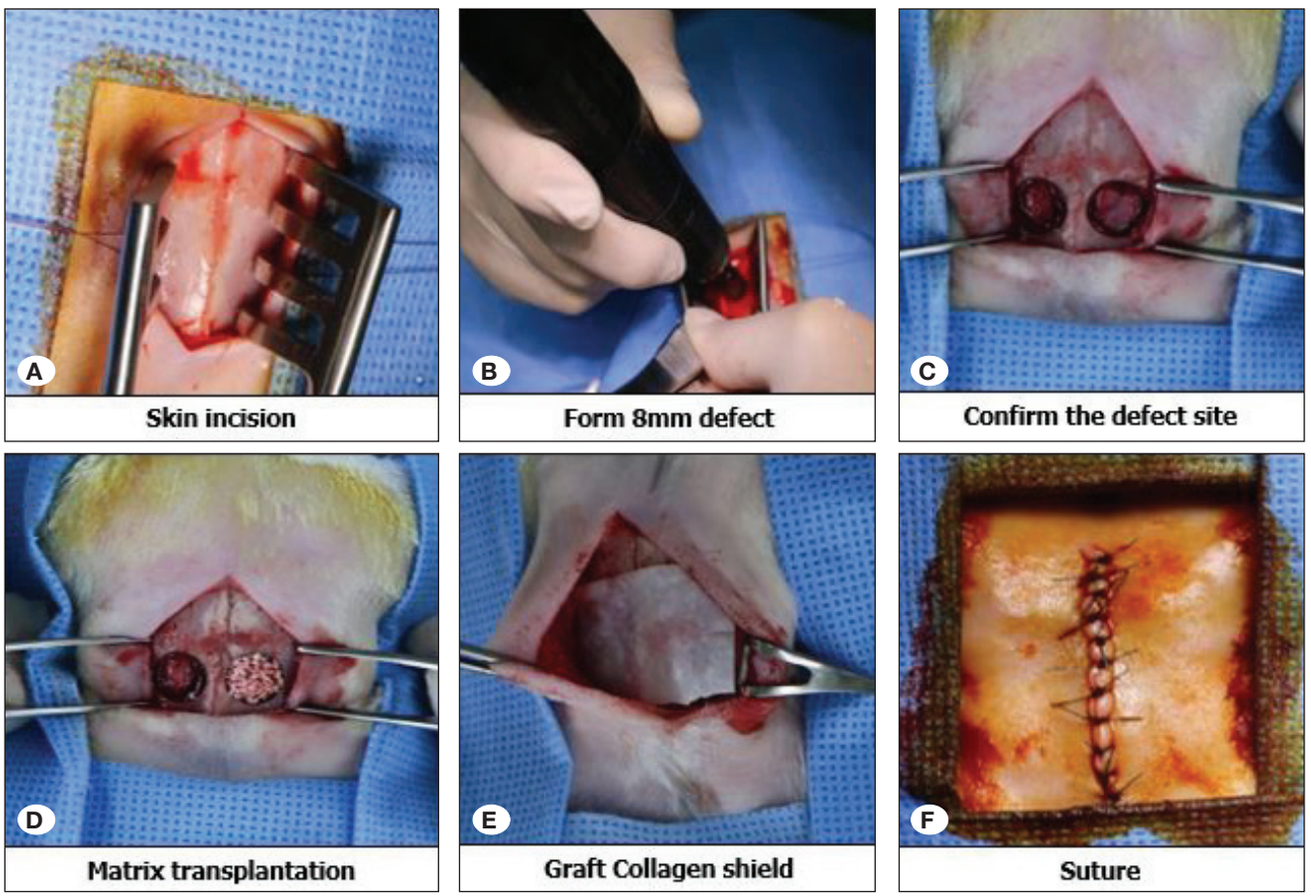

Figure 3: Surgical procedure. **A)** Midline scalp incision was done. **B,C)** 8 mm width defect was made on the both side of occipital bone. **D)** Experimental material transplantation. **E)** Collagen shield graft between the periosteum and graft material.

test and Tukey's post-test. All statistical analyses were performed using SPSS software version 25.0 (IBM Corp., USA), and statistical significance was set at a p-value of <0.05.

RESULTS

No abnormal signs or symptoms, such as inflammation and necrosis, were observed around the surgical site. Appearance, feed intake, and water intake were normal, and no specific symptoms were observed in any of the experimental groups. After operation, body weight was measured weekly for 16 weeks, and no significant difference was observed between the groups (p>0.05). Weight loss of over 10% did not occur during the experimental period in any of the animals (Figure 4).

Micro-CT results revealed that bone formation throughout the entire experimental period in the new CRM carrier, new CRM carrier with rhBMP-2, and pre-existing synthetic graft material-treated control graft groups was superior to that in the negative control group (Figure 5).

In the new CRM carrier with rhBMP-2 group, new bone formation was superior in 16.0%, 39.3%, 64.7%, and 81.1% of the total defect volume at 4, 8, 12, and 16 weeks, respectively. In the new CRM carrier group, new bone formation was observed in 10.6%, 26.3%, 53.1%, and 71.4% of the total defect volume at 4, 8, 12, and 16 weeks, respectively. In the control graft group, new bone formation was observed in 10.1%, 26.6%, 53.4%, and 72.1% of the total defect volume at 4, 8, 12, and 16 weeks, respectively (Table I). The new moldable CRM carrier with rhBMP-2 group demonstrated significantly higher bone formation throughout the entire experimental period (p<0.05).

At 4 weeks, the HU measurement showed 554.95 ± 35.96 HU in the new CRM carrier with rhBMP-2 group, 434.57 ± 14.01 HU in the new CRM carrier group, and 365.80 ± 13.03 HU in the control graft group. At 8 weeks, HU measurements were as follows: the new CRM carrier with rhBMP-2 group, 670.57 ± 10.42 HU; the new CRM carrier group, 551.23 ± 21.26 HU; and the control graft group, 544.95 ± 26.78 HU. At 12 weeks, the HU measurements were as follows: the new CRM carrier with rhBMP-2 group, 1285.46 ± 24.77 HU; the new CRM carrier group, 1018.90 ± 41.36 HU; and the control graft group, 1008.47 ± 19.10 HU. At 16 weeks, the HU measurements were as follows: new CRM carrier with rhBMP-2 group, 1568.66 ± 36.96 HU; new CRM carrier group, 1331.70 ± 55.04 HU; and control graft group, 1319.03 ± 54.93 HU (Table II). In the new CRM carrier with rhBMP-2 group, bone formation was substantially accelerated from the 4th week to the 16th week compared with that in the new CRM carrier and the control graft groups (Figure 6).

In the negative control group, connective tissue filled the bone defect and no new bone formation was observed. New bone formation at the margins of the defect area was more prominent in the new CRM carrier with rhBMP-2 group than in the new CRM carrier or control graft group.

In the negative control group, marginal new bone formation was observed at the site of bone defects; however, the degree of bone formation was lesser than that in the experimental and control groups. The highest level of new bone formation was

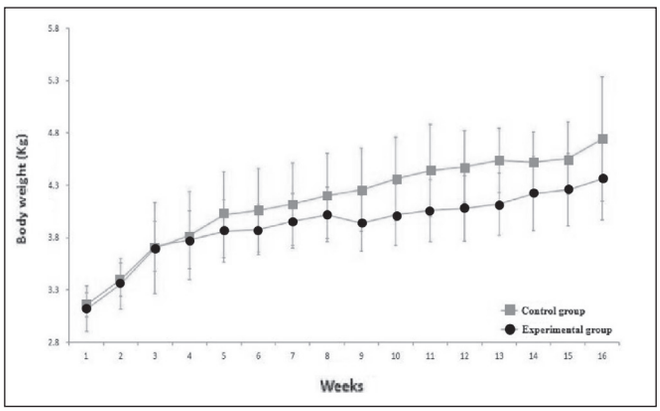

Figure 4: Weight change of experimental animals at each weeks.

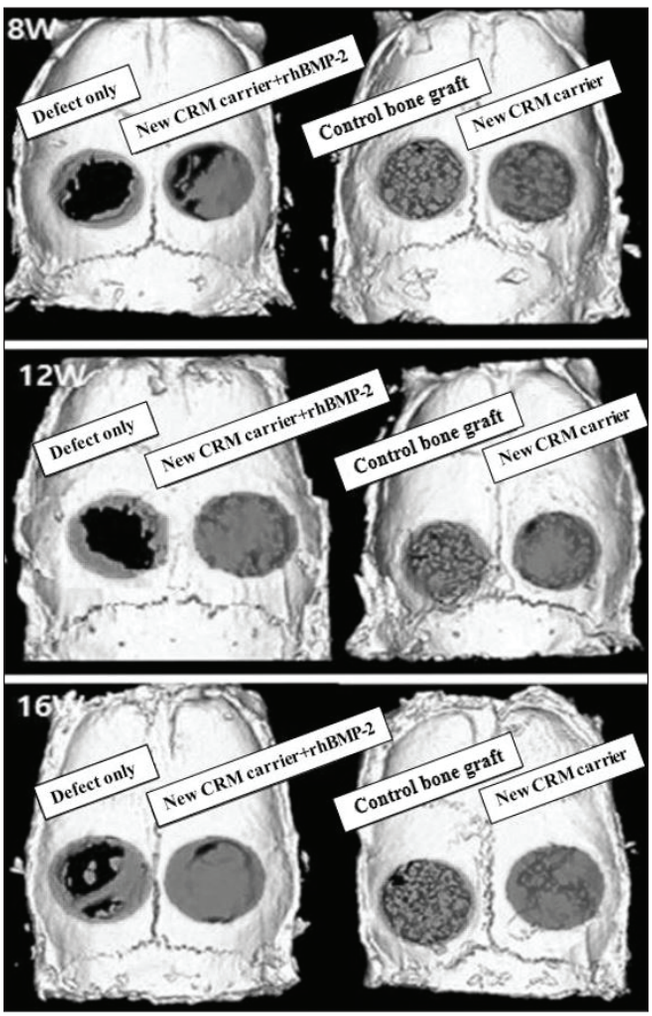

Figure 5: 3-dimensional reconstruction image using micro-computed tomographic scan.
CRM: Compression-resistant matrix, **rhBMP-2:** Recombinant human bone morphogenetic protein-2.

observed at the edge and center in the new CRM carrier with rhBMP-2 group. However, in the new CRM carrier and control graft groups, new bone formation was observed only in the lower periosteum, and both groups showed similar degrees of new bone formation (Figure 7A).

In the negative control group, thin new bone formation was observed in the lower periosteum. In the new CRM carrier with rhBMP-2 group, the bone graft material was partially degraded, and the rate of new bone formation was superior to that in the other groups. The new CRM carrier and control graft

Table I: Comparison of new bone formation volume with each materials

			4 week	8 week	12 week	16 week
Bone defect volume (mm^3)			**100.3**			
Group 1	Defect only	Bone volume (%)	3.2	17.6	19.8	35.3
			p<0.001	p<0.001	p<0.001	p<0.001
	New CRM carrier+rhBMP-2	Bone volume (%)	16.0	39.3	64.7	81.1
Group 2	New CRM carrier	Bone volume (%)	10.6	26.3	53.1	71.4
			p<0.001	p<0.001	p<0.001	p=0.014
	Control bone graft	Bone volume (%)	10.1	26.6	53.4	72.1
			p=0.001	p<0.001	p<0.001	p=0.022

Each p-value was obtained by comparison with new CRM carrier+rhBMP-2 group. Three defects per group were analyzed at each time point.
CRM: *Compression resistant matrix,* ***rhBMP-2:*** *Recombinant human bone morphogenetic protein-2.*

Table II: Comparison of Hounsfield unit value with each materials

		4 week	8 week	12 week	16 week
Group 1	Defect only	165.6 ± 8.2	245.0 ± 9.5	784.5 ± 11.6	993.1 ± 36.9
		p<0.001	p<0.001	p<0.001	p<0.001
	New CRM carrier +rhBMP-2	554.9 ± 35.9	670.5 ± 10.4	1285.4 ± 24.7	1568.6 ± 36.9
Group 2	New CRM carrier	434.5 ± 14.0	551.2 ± 21.2	1018.9 ± 41.3	1331.7 ± 55.0
		p<0.001	p<0.001	p<0.001	p=0.001
	Control bone graft	365.8 ± 13.0	544.9 ± 26.7	1008.4 ± 19.1	1319.0 ± 54.9
		p<0.001	p<0.001	p<0.001	p=0.001

Each p-value was obtained by comparison with new CRM carrier+rhBMP-2 group. Three defects per group were analyzed at each time point.
CRM: *Compression resistant matrix,* ***rhBMP-2:*** *Recombinant human bone morphogenetic protein-2.*

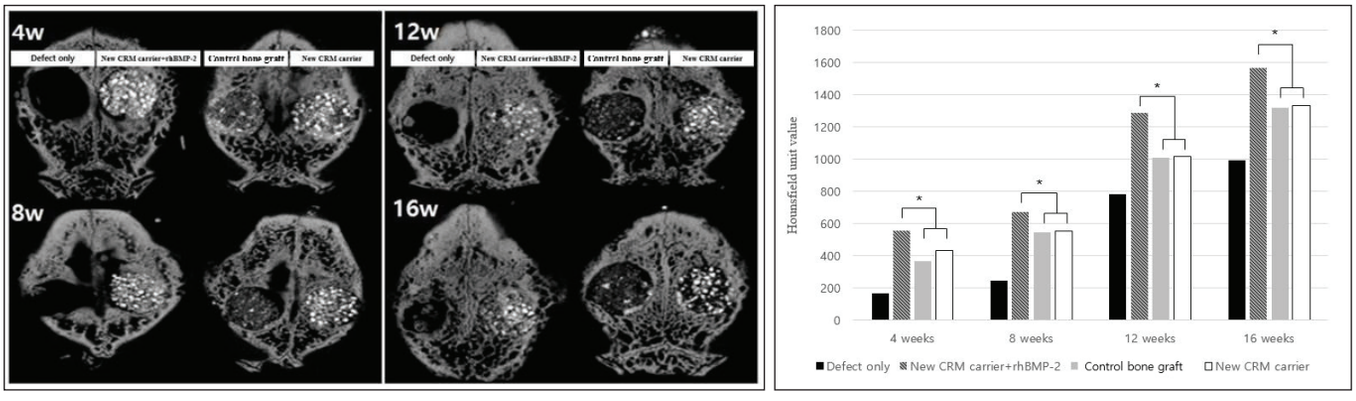

Figure 6: Hounsfield unit values with micro-computed tomographic scan and comparison by group.
CRM: compression-resistant matrix, **rhBMP-2:** recombinant human bone morphogenetic protein-2. *statistically significant (p<0.05)

groups showed similar degrees of new bone formation (Figure 7B).

There was some new bone formation in the negative control group; however, the extent was much lesser than that in the experimental and control groups. New bone formation from the existing bone and shrinkage of the bone defect site were confirmed in the negative control group. In the new CRM carrier with rhBMP-2 and the new CRM carrier groups, dense new bone formation was observed throughout the bone defect site. However, in the control graft group, new bone formation was mainly observed around the edge of the bone defect (Figure 7C).

DISCUSSION

Spinal fusion is widely used to restore spinal stability. Although autologous iliac bone grafting is superior to that with other synthetic graft materials in terms of fusion and rejection rates and is considered the gold standard, its drawbacks are not negligible. The suboptimal fusion rate using autologous iliac bone is reported to be less than 90% (13,33). Furthermore, fusion failure, such as pseudarthrosis, can occur in approximately 45% of uninstrumented cases (1,24).

Recent studies have reported good results in terms of successful fusion rates with rhBMP-2 in comparison with autologous bone grafts (42). BMPs are proteins that pertain to transforming growth factor-beta and can induce new bone formation. BMPs exert their function by triggering osteoblast differentiation, direct bone matrix production, and angiogenesis. BMP-2 is one of the most effective BMPs in terms of osteoinductivity (22,42).

As the importance of BMPs increases, the importance of the carrier has also been highlighted. Several carriers have been developed and investigated, including collagen sponges, biodegradable synthetic polymers, and calcium phosphate ceramics, such as HA, TCP, and biphasic calcium phosphate (BCP), a mixture of the above two materials (12,26,31).

An ideal carrier should match the rates of new bone formation and resorption. If the carrier is reabsorbed before proper osteodeposition, bone formation will be insufficient, and adverse effects, such as pseudarthrosis and fusion failure, may occur. If resorption is too slow, bone formation may be inhibited (31).

Several studies have shown that collagen sponge can be a satisfactory carrier for rhBMP-2 in animal models (1,14). However, the vulnerability of collagen sponge to compression by

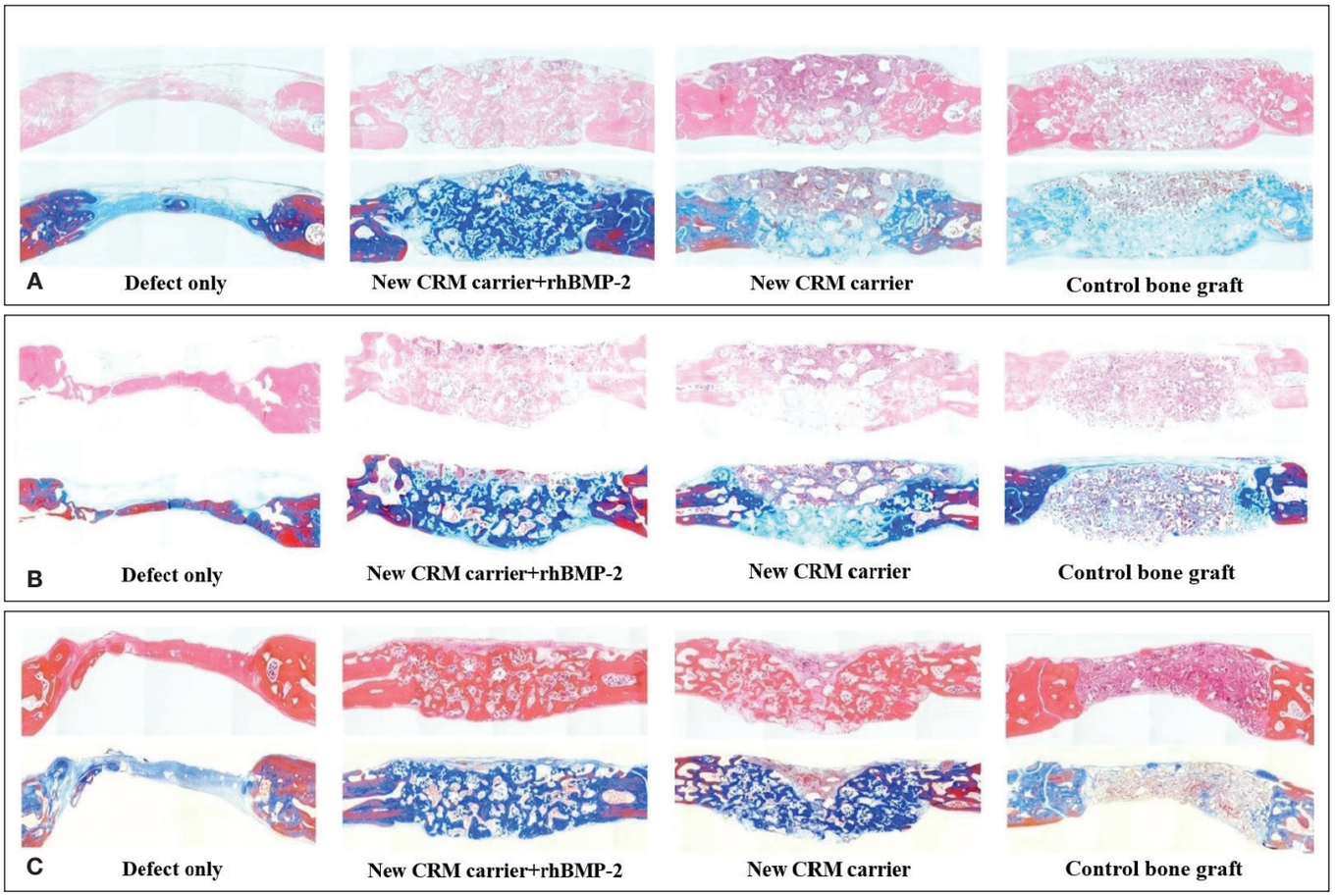

Figure 7: A) Histological examination (Week 8). **B)** Histological examination (Week 12). **C)** Histological examination (Week 16). Hematoxylin-eosin staining (top), Masson's trichrome staining (bottom) (X4).
CRM: Compression resistant matrix, **rhBMP-2:** Recombinant human bone morphogenetic protein-2.

the paraspinal muscle and mechanical compressive force during implantation is a huge limitation, and higher doses of rhBMP-2 are required for successful bone fusion. Furthermore, mechanical diffusion by compressing the vulnerable carrier containing rhBMP-2 may cause considerably harmful effects on the surrounding tissues, such as unexpected heterotopic ossification, osteolysis, massive tissue inflammation, and painful seroma formation. This compressibility problem requires the consideration of an alternate carrier (10,25).

Resistance to compression is an important characteristic of ceramics that can overcome the above mentioned drawbacks. As an alternative to spinal fusion materials, BCP has advantages such as good biocompatibility, osteoconductive capabilities, and biodegradability (19). Moreover, BCP can change the ratio of HA to TCP, altering the mechanical strength, degree, and speed of resorption (25). However, despite the numerous advantages and variety depending on the composition ratio, the use of ceramics also has limitations. Depending on the HA to TCP ratio, a higher HA content can increase the radiopacity, making it difficult to determine whether early bone formation occurs. As HA is the most stable calcium phosphate ceramic, its degree of biodegradability is very low. Conversely, if the proportion of TCP is increased, the reabsorption rate increases; therefore, there is still a debate regarding the appropriate adjustment of the ratio (11). Moreover, there is a serious disadvantage that any carrier developed so far can easily spread throughout the body, resulting in heterotopic ossification of nearby critical neural structures, soft tissue swelling, seroma formation, or systemic absorption.

A new CRM carrier and new CRM carrier with rhBMP-2 having moldable and compression resistance characteristics were developed to overcome these limitations. We conducted a rabbit bone defect model experiment to confirm the performance of the newly developed CRM using radiographic and histological evaluations. No local adverse reactions, such as inflammation, seroma, and necrosis were observed around the surgical site during the period of test material application, and no abnormal symptoms were noted.

Micro-CT evaluation revealed that new bone formation was more active in the new CRM carrier with rhBMP-2 and the new CRM carrier and control graft groups than in the negative control group (bone defect only group).

Micro-CT and dual-energy X-ray absorptiometry have been widely used for bone fusion assessment. Several studies have reported that the amount of bone formation and the quality of the newly formed bone can be estimated by measuring HU using CT, whereas only the quantity of bone formation can be evaluated using dual-energy X-ray absorptiometry (2,3,8,9,40,44). When bone mineral densitometry was analyzed using HU, the bone mineral densitometry of the new CRM carrier with rhBMP-2 group was 1568.66 ± 36.96 HU, that of the new CRM carrier group was 1331.70 ± 55.04 HU, and that of the control graft group was 1319.03 ± 54.93 HU at 16 weeks. The new CRM carrier with rhBMP-2 group was superior to the other materials not only in terms of bone formation but also in terms of bone quality.

When the degree of new bone formation and the extent of degradation of the product were confirmed by histopathological evaluation, degradation of the material was observed at 8 weeks of the implantation period, and new bone formation in the new CRM carrier with rhBMP-2 group was superior to that in the other groups. At 16 weeks, the newly formed bone was dense, and the degree of degradation of the implant was not significantly increased compared with that at 8 and 12 weeks.

In the new CRM carrier group, degradation of the product was observed at 12 weeks, and the graft material significantly decreased at 16 weeks, indicating further degradation. Furthermore, new bone formation increased with the transplantation period starting from the 8th week, and dense new bone formation was observed at the entire bone defect site at 16 weeks. However, in the control graft group, although the degree of new bone formation increased over time, new bone formation was observed mainly at the edge of the bone defect site, and degradation was observed from the 8th week.

Histopathological evaluation revealed that unlike in the new CRM carrier and control graft groups, in the new CRM carrier with rhBMP-2 group, there was a small amount of new bone growth from the existing bone at the edge of the bone defect site from the 4th week. At 16 weeks, osteogenesis observed in the new CRM carrier with rhBMP-2 group was substantially denser than that in the other groups. In the control graft group, osteogenesis was observed at the margins due to the rapid degradation rate of the graft material compared with the regeneration of the new bone.

In 2009, Toth et al. reported that hyperconcentrating rhBMP-2 could lead to concentration-dependent osteoclastic resorption of the peri-implant bone (39). In the present study, osteoclastic activity was not found in the histopathologic analysis nor was there peri-implant soft tissue swelling or heterotopic ossification.

These results confirmed the preclinical safety and efficacy of the new CRM carrier and the new CRM carrier with rhBMP-2. In particular, the new CRM carrier with rhBMP-2 was superior to other graft materials in terms of new bone formation and is considered a suitable material for bone grafting.

This study has several limitations. We confirmed the effect of the experimental matrix on bone formation; however, we did not confirm its advantages, such as compression resistance and absence of diffusion to surrounding tissues. Although heterotopic ossification due to peripheral diffusion of the experimental matrix was not observed, its absence can be attributed to the simple shape of the bone defect. The rabbit bone defect model used in this study had a simple cylindrical bone defect compared with the complex structure of the spine. Therefore, there is a limitation in confirming the compression resistance and the effect of preventing mechanical diffusion into the surroundings, which are advantages of the present invention. Second, the rabbit skull defect model was an 8-mm cylindrical, relatively small model; therefore, the exact amount of carrier and rhBMP-2 contained therein could not be measured. Though significant bone regeneration effect could be confirmed, rhBMP-2 at 1 mg per 1 g of the new CRM carrier is a relatively small dose, which could explain the absence of

complications related to rhBMP-2. Third, in the present study design, it was not possible to compare the bone regeneration effect of licensed products containing rhBMP-2, as the purpose of this study was to confirm the safety and efficacy of the newly developed CRM. Therefore, the experimental group included animals with and without rhBMP-2, and it was confirmed that there were no adverse events or complications. The absence of rhBMP-2 in licensed products is because the efficacy of rhBMP-2 has already been demonstrated in many clinical trials.

As the safety and efficacy of the experimental matrix have been proven previously, its merits can be confirmed through other animal experiments and human clinical trials.

CONCLUSION

In the experimental group, the moldable CRM carrier with rhBMP-2 was a safe carrier that prevented adverse effects, such as systemic diffusion of rhBMP-2, seroma formation, and heterotopic ossification. Through this experimental study, we confirmed the preclinical efficacy of the new CRM carrier and new CRM carrier with rhBMP-2.

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AUTHORSHIP CONTRIBUTION

Study conception and design: SJH

Data collection: SJH

Analysis and interpretation of results: JMA, SJH

Draft manuscript preparation: JMA, SJH

Critical revision of the article: KJK

Other (study supervision, fundings, materials, etc...): JMA, SJH, KJK

All authors (JMA, SJH, KJK) reviewed the results and approved the final version of the manuscript.

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