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# Manipulation of anabolic and catabolic responses with bone morphogenetic protein and zoledronic acid in a rat spinal fusion model

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ABSTRACT

Bone fusion involves a complex set of regulated signaling pathways that control the formation of new bone matrix and the resorption of damaged bone matrix at the surgical site. It has been reported that systemically administering a single dose of zoledronic acid (ZA) at the optimal time increases the strength of the bone morphogenetic protein (BMP)–mediated callus. In the present study, we aimed to investigate the effect of BMP-2 and ZA in a rat spinal model. Sixty-seven rats were divided into 6 groups: group I (n = 11) animals were implanted with a carrier alone, group II (n = 12) animals were implanted with a carrier and a subcutaneous injection of ZA was administered 2 weeks after surgery, group III (n = 12) animals were implanted with a carrier containing 1 μg of rhBMP-2, group IV (n = 12) animals were implanted with a carrier containing 1 μg of rhBMP-2 and a subcutaneous injection of ZA was administered 2 weeks after surgery, group V (n = 10) animals were implanted with a carrier containing 3 μg of rhBMP-2, and group VI (n = 10) animals were implanted with a carrier containing 3 μg of rhBMP-2 and a subcutaneous injection of ZA was administered 2 weeks after surgery. The rats were euthanized after 6 weeks, and their spines were explanted and assessed by manual palpation, radiography, high-resolution micro-computerized tomography (micro-CT), and histologic analysis. The fusion rates in group VI (60%) were considerably higher than those in the groups I (0%), II (0%), III (12.5%), IV (20.8%), and V (35%), ($P < 0.05$). Additionally, the radiographic scores of group VI were higher than those in the other groups, ($P < 0.05$). In micro-CT analysis, the tissue and bone volumes of the callus were significantly higher in group VI than those in the other groups, ($P < 0.05$). The trabecular number was significantly higher and the trabecular spacing was significantly lower in group VI than those in the other groups, ($P < 0.05$). The combination of rhBMP-2 and ZA administered systemically as a single dose at the optimal time was efficacious in our rat spinal fusion model. Our results suggest that this combination facilitates spinal fusion and has potential clinical application.



## Introduction

Spinal arthrodesis is a fundamental treatment option for spinal pathologies and one of the most common spinal procedures, with more than 200,000 surgeries performed in the United States each year [1]. This procedure is the gold standard for treatment of degenerative and traumatic spine diseases associated with severe neck or back pain, and sometimes, neurologic problems. In this procedure, bone grafts are used to restore mechanical stability to the affected spinal segment by providing bridging bone between vertebrae. Because successful bone fusion between unstable spinal segments leads to pain relief and neurologic recovery, the efficacy of this procedure has gained wide acceptance, and the number of these types of surgery has increased annually with the increase in the aged population [2–5].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily [6], and are powerful osteoinductive molecules. An in vitro study has shown that BMPs work by stimulating pluripotent mesenchymal cells to differentiate into osteoblasts, thereby producing a bone matrix. BMPs also are considered to promote new osteoclast formation since they stimulate the production of receptor activator of nuclear factor kappa-B ligand (RANKL) osteoblasts and help ensure mature osteoclast survival; therefore, BMPs participate in bone matrix resorption [7,8].

The osteoinductive effects of recombinant human BMPs (rhBMPs) for spinal fusion have been shown in animal models and clinical trials [9–13]. Although BMPs are approved for clinical use, clinical trial results have shown that high doses are required to induce adequate bone fusion because of the following reasons: (1) solubility of the molecules, (2) easy diffusion of the molecules away from the fusion site, and (3) in vivo inactivation [14]. In addition, BMPs are expensive; therefore, their usefulness may be limited by their expense. As a result, a number of strategies are being developed to provide a safer, less expensive, and more efficacious spinal fusion using rhBMP.

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Bisphosphonates mediate complex effects on bone: they primarily show anticatabolic effects. Circulating bisphosphonates bind to bone mineral. When the bone is resorbed by osteoclasts, the osteoclasts undergo apoptosis [15,16]. Bisphosphonates have improved the clinical outcomes of osteoporosis, Paget's disease, and metastatic bone disease [17–19]. Zoledronic acid (ZA), the most potent bisphosphonate, can be administered as a single systemic dose. In a rabbit spinal fusion model, systemic ZA administration increased fusion mass size, bone mineral content, and fusion rate [20]. On the other hand, long-term bisphosphonate administration leads to decreased bone formation, which is thought to result from uncoupling of the balance between osteoclastic and osteoblastic activity [21]. Following the use of bisphosphonates in millions of patients in clinical practice, some unexpected possible adverse effects have been reported, including osteonecrosis of the jaw, and atypical femur fractures [22].

Although many factors influence bone fusion modification, the ultimate result is determined principally by the balance between anabolic osteoblast and catabolic osteoclast responses. We previously reported that the synergic effect of rhBMP-2 and ZA administered systemically as a single dose at the optimal time was efficacious for fracture repair, and significantly enhanced bone fusion in a rat femoral fracture model [23]. However, the mechanism of these effects in spinal bone fusion remains unclear. We also previously tested the osteogenic activity of rhBMP-2 in a rodent spinal fusion model, and tried to provide a safer, less expensive, and more efficacious bone fusion using rhBMP-2 [24]. The purpose of the present study was to elucidate the synergic effect of rhBMP-2 and ZA administered as a single systemic dose for spinal fusion and to examine its feasibility for clinical application by using a rat spinal fusion model.

## Materials and methods

### *Preparation of matrices*

MedGEL (MedGEL, Kyoto, Japan) is a biodegradable gelatin hydrogel scaffolding for cellular attachment [25]. The MedGEL was cut using a scalpel into 5 mm × 10-mm strips. To prepare MedGEL incorporating rhBMP-2 (Peprotech, Rocky Hill, NJ), 100 μL of phosphate-buffered saline solution (PBS, pH 7.5) containing 1 μg or 3 μg of rhBMP-2 was dropped onto MedGEL and left overnight at 4 °C on an Eppendorf tube prior to implantation. Similarly, 100 μL of rhBMP-2-free PBS was dropped onto MedGEL to obtain the rhBMP-2-free empty MedGEL.

### *Study groups*

Sixty-seven male Sprague–Dawley rats (16–18 weeks old; CLEA Japan, Inc., Tokyo, Japan) were divided into 6 groups: group I (n = 11) animals were implanted with MedGEL alone, group II (n = 12) animals were implanted with MedGEL and a subcutaneous injection of ZA was administered 2 weeks after surgery, group III (n = 12) animals were implanted with MedGEL containing 1 μg rhBMP-2, group IV (n = 12) animals were implanted with MedGEL containing 1 μg of rhBMP-2 and a subcutaneous injection of ZA was administered 2 weeks after surgery, group V (n = 10) animals were implanted with MedGEL containing 3 μg of rhBMP-2, and group VI (n = 10) animals were implanted with MedGEL containing 3 μg of rhBMP-2 and a subcutaneous injection of ZA was administered 2 weeks after surgery. ZA was prepared in sterile saline from commercial 4-mg vials (Novartis Pharma KK, Tokyo, Japan) and administered as a single subcutaneous injection of 0.1 mg/kg. The optimal time for administering systemic ZA is 2 weeks after surgery, as shown in a previous critical defect rat model [26]. Animals that were not scheduled to receive ZA were administered with control injections of saline.

### *Surgical technique for constructing L4–L5 posterolateral spinal fusion model*

Approval was obtained from Oita University's Animal Research Committee prior to animal experimentation. A posterior midline incision was made on the skin. Next, 2 separate paramedian incisions were made at 3 mm from midline in the lumbar fascia, and the transverse processes were exposed. The transverse processes of the L4 and L5 were decorticated using a low-speed burr. Subsequently, MedGEL with or without rhBMP-2 was implanted on each side. The fascial and skin incisions were closed with a 3–0 absorbable suture. Immediately following surgery and on subsequent days, the rodents received analgesics (buprenorphine subcutaneously and paracetamol). The rodents were housed in separate cages and fed food and water ad libitum, and their conditions were monitored on a daily basis. The rats were humanly euthanized 6 weeks post operatively.

### *Manual assessment of fusion*

Six weeks after implantation, explanted spines were manually tested for intersegmental motion by 3 blinded independent observers. The explanted lumbar spine was palpated gently, and lateral side-bending motion at the L4–L5 level was compared with the motion at the adjacent levels above (L3–L4) and below (L5–L6). The absence of motion was considered as successful fusion. Any motion detected between the transverse processes was considered a failure of fusion. The spine was designated as "not fused" if any of the 3 observers graded it as not fused. The spines were scored as either fused or not fused on both the right and left sides. The fusion rate then was calculated.

### *Radiographic analysis*

The explanted spines obtained at the 6-week time point were radiographed using a Softex radiograph apparatus (Softex CSM-2; Softex, Tokyo, Japan) employing an HS Fuji Softex film (Fuji Film, Tokyo, Japan) at 45 cm with 30 kV and 15 mA for 20 s. Fusion between the L4 and L5 transverse processes in each rat was recorded as a percentage of the total area between the L4 and L5 that was filled with new bone. Three blinded independent observers scored the bone formation in each rat on a 5-point scale: 0 = no bone formation; 1 = bone filling less than 25% of the area; 2 = bone filling 25–50% of the area; 3 = bone filling 50–75% of the area; and 4 = bone filling 75–100% of the area. The spines were scored on both the right and left sides.

### *Micro-CT analysis*

Next, the spines were scanned by micro-CT using SkyScan1172 (Bruker microCT, Kontich, Belgium) with voxel size of 20 μm. The data were collected at 100 kV and 100 μA, and reconstructed using the cone-beam algorithm. Each spine was set on the object stage and sample scanning was performed over 180° rotation with an exposure time of 105 ms. A cylindrical volume of interest with a diameter of 20 mm and a height of 27 mm was selected, which displayed the microstructure of the rat vertebra as comprising cortical and cancellous bone. Data analysis was performed using a CT Analyzer software (Bruker microCT). By using this software, the area from the top of the L4 transverse processes to the bottom of the L5 transverse processes, including the vertebrae, was analyzed. The spines were analyzed on both the right and left sides. In the 3-dimensional (3D) analysis, tissue volume (TV), bone volume (BV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular spacing (Tb. Sp), and bone volume fraction (BV/TV, %) were measured.

### *Histologic analysis*

Six weeks after implantation, the spines were dissected, and the specimens were fixed in 40% ethanol, decalcified using standard 10% decalcifying solution HCl (Cal-Ex; Fischer Scientific, Fairlawn, NJ), washed with running tap water, and then transferred to 75% ethanol. Serial sagittal sections near the transverse processes were cut carefully

at the level of the transverse process on both the right and left sides. The specimens were embedded in wax for sectioning. Sagittal sections (5 μm) were cut from the paraffin blocks using a microtome (LS-113; DAIWA-KOKI, Saitama, Japan). The sections were stained with hematoxylin and eosin for basic morphology. Three blinded independent observers scored histologic bone formation. Histologic fusion was defined as bony trabeculae bridging from one transverse process to the next. Fusion masses were assessed and the extent of new bone formation was scored using the following scoring criteria: 1 = fibrocartilage tissue filling less than 25% of the gap area; 2 = fibrocartilage tissue filling 25–50% of the gap area; 3 = fibrocartilage and bone tissue filling 75–99% of the gap area); 4 = bridged with bone tissue, but with the fusion masses composed of thin trabecular bone; and 5 = completely bridged with abundant mature bone tissue. The spines were scored on both the right and left sides.

### *Statistical methods*

The computer program Statistical Package for the Social Sciences (SPSS) (V13; IBM Corporation, Armonk, NY) was used for statistical analysis. Analysis of variance was used for statistical analysis. $P$ values $< 0.05$ were considered significant. A kappa statistic was calculated as a measure of interobserver reliability of the 3 independent blinded observers. The kappa statistic corrects the observed agreement for possible chance agreement among observers. Agreement was rated as follows: poor, $\kappa =$ 0–0.2; fair, $\kappa = 0.21$–0.4; moderate, $\kappa = 0.41$–0.60; substantial, $\kappa =$ 0.61–0.8; and excellent, $\kappa > 0.81$. A value of 1 indicated absolute agreement, whereas a value of 0 indicated agreement no better than chance.

## Results

No abnormal behavior was noted in the 67 operated rats. None of the rats showed any neurologic deficits before or after the surgical procedure, or at sacrifice.

### *Manual palpation*

Table 1 shows the proportion of subjects in each group judged as "fused" by the 3 independent evaluators; consistent agreement was noted among the evaluators ($\kappa = 0.892$). At 6 weeks, 12 segments (the spines were analyzed on both the right and left sides) in group VI (n = 10, segments = 20) were assessed as fused (fusion rate, 60%), while 7 segments in group V (n = 10, segments = 20) exhibited fusion (fusion rate, 35%). Five segments in group IV (n = 12, segments = 24) were assessed as fused (fusion rate, 20.8%), while 3 segments in group III (n = 12, segments = 24) exhibited fusion (fusion rate, 12.5%). None of the spines in group I (n = 11, segments = 22) or group II (n = 12, segments = 24) were fused (fusion rate, 0%). The 1-μg and 3-μg rhBMP-2 groups with systemic ZA injection (groups IV and VI) had higher fusion rates than those in the groups with no injection (groups III and V). There was no significant difference between the manual assessment scores of groups III and IV, whereas significantly higher fusion rates were observed in group VI than those in the groups I, II, III, IV, and V, ($P < 0.05$).

### *Radiographic analysis*

Radiographs of the femurs were obtained at 6 weeks. Consistent agreement ($\kappa = 0.816$) was noted among the 3 independent observers who graded the radiographs. The spines were scored on both the right and left sides. The average evaluation scores for each group are shown in Table 2, and the representative anteroposterior (AP) radiographs for each group at 6 weeks are shown in Fig. 1. Groups V and VI showed evidence of bone formation between the L4 and L5 transverse processes, and bony bridging was detected. It was difficult to detect bony bridging in groups III and IV. Groups I and II showed no evidence of bone formation. There were no differences between the 1-μg rhBMP-2 groups that did (group IV) and did not receive (group III) systemic ZA injection; their radiographic scores were also similar. On the other hand, the 3-μg rhBMP-2 groups that received systemic ZA injection (group VI) had significantly higher radiographic scores than that in the group without the injection (group V), ($P < 0.05$). The scores of group II were low and similar to those of group I.

### *Micro-CT analysis*

Three-dimensional AP images for each group are shown in Fig. 2. The gaps between the transverse processes were clearly observed in groups I and II, while both the gap and mineralized callus bridging were detected in groups III and IV. The gaps were nearly invisible in group V, though the mineralized callus bridging was deemed insufficient. There was a greater amount of bony callus and evidence of bone fusion in group VI. Computer analysis of the micro-CT images revealed the volume of new bone and the quality of the spinal fusion area. Average micro-CT data based on the histomorphometry of each group are shown in Tables 3.1 and 3.2. Quantitatively, the TV and BV were higher in group VI than those in the groups I, II, III, IV, and V, ($P < 0.05$). The Tb. N was higher and the Tb. Sp was lower in group VI than those in the groups I, II, III, IV, and V, ($P < 0.05$ for both).

### *Histologic analysis*

Histologic analysis of group I showed a paucity of new bone formation and no evidence of fusion. (Fig. 3A, B). These images clearly show muscle between the transverse processes for both specimens. There was occasional evidence of new bone formation either originating from the decorticated transverse process or from normal remodeling, but this new bone formation did not bridge the gap between the two transverse processes and was not considered as fusion. Group II showed

**Table 1**
Assessment of spinal fusion via manual palpation.

| Treatment group | | No. assessed manually for fusion | No. assessed as fused | Fusion rate (%) |
|---|---|---|---|---|
| Group I | MedGEL alone | 22 | 0 | 0 |
| Group II | MedGEL + ZA | 24 | 0 | 0 |
| Group III | MedGEL + 1 μg rhBMP-2 | 24 | 3 | 12.5 |
| Group IV | MedGEL + 1 μg rhBMP-2 + ZA | 24 | 5 | 20.8 |
| Group V | MedGEL + 3 μg rhBMP-2 | 20 | 7 | 35.0 |
| Group VI | MedGEL + 3 μg rhBMP-2 + ZA | 20 | 12 | 60.0* |

The spines were assessed on both right and left side.
ZA: Zoledronic acid.
The group VI had higher fusion rates than those in the groups I, II, III, IV, V with significant differences ($P < 0.05$).
* $P < 0.05$.

**Table 2**
Radiographic scores at 6 weeks.

| Treatment group | | No. studied radiographically | Score at 6 weeks |
|---|---|---|---|
| Group I | MedGEL alone | 22 | 0.14 |
| Group II | MedGEL + ZA | 24 | 0.21 |
| Group III | MedGEL + 1 μg rhBMP-2 | 24 | 1.04 |
| Group IV | MedGEL + 1 μg rhBMP-2 + ZA | 24 | 1.42 |
| Group V | MedGEL + 3 μg rhBMP-2 | 20 | 2.25 |
| Group VI | MedGEL + 3 μg rhBMP-2 + ZA | 20 | 3.65* |

The spines were scored on both right and left side.
ZA: Zoledronic acid.
The group VI had higher radiographic scores than those in the groups I, II, III, IV, V with significant differences ($P < 0.05$).
* $P < 0.05$.

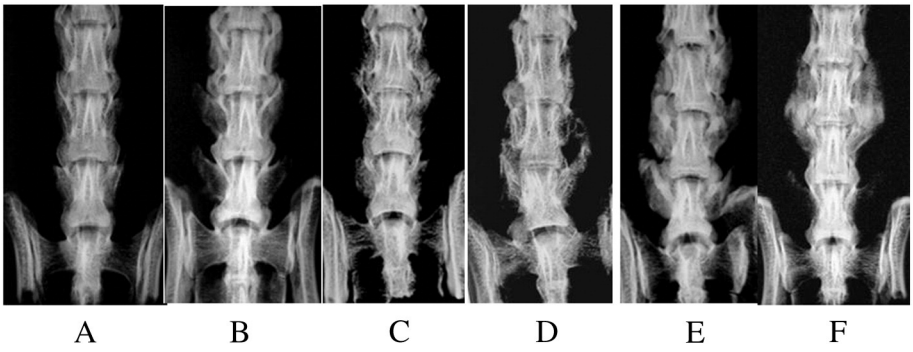


**Fig. 1.** Radiographs of the rat spines obtained 6 weeks after surgery. (A) group I (MedGEL alone). (B) group II (MedGEL and subcutaneous injection of zoledronic acid [ZA] 2 weeks after surgery). (C) group III (MedGEL containing 1-μg recombinant human bone morphogenetic protein [rhBMP]-2). (D) group IV (MedGEL containing 1-μg rhBMP-2 and administration of subcutaneous injection of ZA 2 weeks after surgery). (E) group V (MedGEL containing 3-μg rhBMP-2). (F) group VI (MedGEL containing 3-μg rhBMP-2 and administration of subcutaneous injection of ZA 2 weeks after surgery). Groups V and VI show evidence of bone formation between the L4 and L5 transverse processes, and bony bridging is detected. It is difficult to detect bony bridging in groups III and IV. Groups I and II show no evidence of bone formation.

the formation of individual trabeculare in the transverse processes, which were much thicker in size and much less narrow but there was fibrosis tissue and muscle fiber between the transverse processes and no evidence of bone fusion (Fig. 3C, D). Analysis of group III showed distribution of cartilaginous tissue and no woven bone between the transverse processes (Fig. 3E, F). Group IV showed that maturity of new bone was much more delayed and fibrocartilage tissue and immature bone was shown between the transverse processes (Fig. 3G, H). Analysis of group V showed mature bone formation and woven bone in the spinal fusion area. However, there was still distribution of immature bone between the transverse processes (Fig. 3I, J). Histologic analysis of group VI showed abundant bone bridging in the transverse processes. Mature bone formation appeared and displayed osteoid tissue connecting to form trabeculae, which were surrounded by well-developed bone marrow cavities (Fig. 3K, L). Histologic fusion scores are shown in Table 4. The spines were scored on both the right and left sides. With regard to the 1-μg rhBMP-2 groups, there were no differences between the groups that did (group IV) and did not receive (group III) systemic ZA injection; their histologic scores were also similar. In contrast, the 3-μg rhBMP-2 group that received systemic ZA injection (group VI) had significantly higher histologic scores than that in the group that did not receive the injection (group V), ($P < 0.05$). Consistent agreement ($\kappa = 0.924$) was noted among the 3 independent observers. Fig. 4A, B, C, D, E, F showed the osteoclast in groups I, II, III, IV, V, VI, respectively

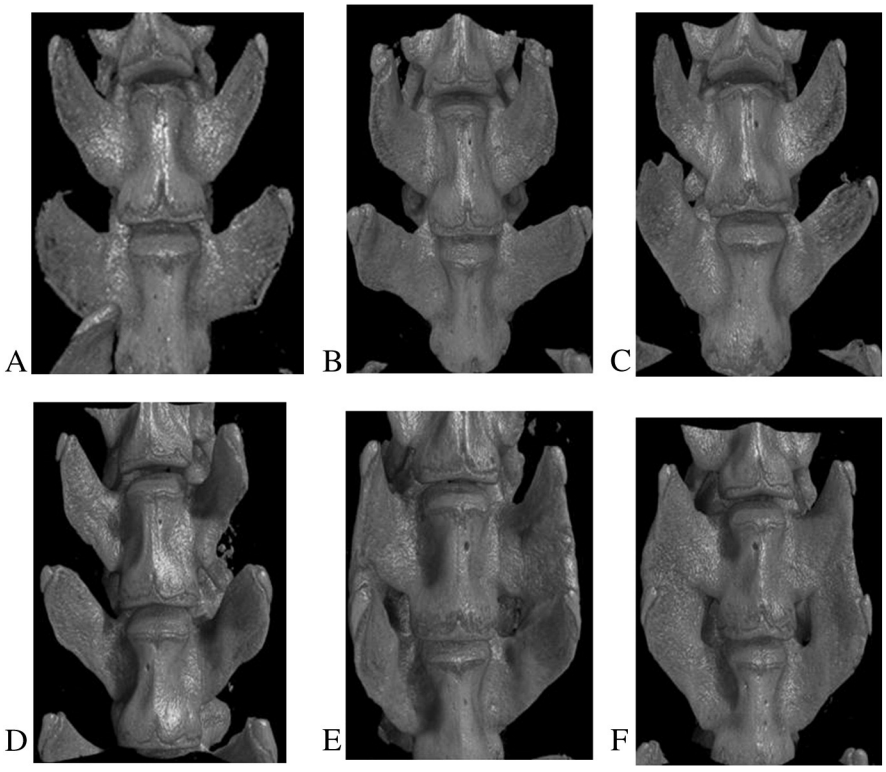


**Fig. 2.** Three-dimensional micro-computed tomography images of the rat spines obtained 6 weeks after surgery. (A) group I (MedGEL alone). (B) group II (MedGEL and administration of subcutaneous injection of zoledronic acid [ZA] 2 weeks after surgery). (C) group III (MedGEL containing 1-μg recombinant human bone morphogenetic protein [rhBMP]-2). (D) group IV (MedGEL containing 1-μg rhBMP-2 and administration of subcutaneous injection of ZA 2 weeks after surgery). (E) group V (MedGEL containing 3-μg rhBMP-2). (F) group VI (MedGEL containing 3-μg rhBMP-2 and administration of subcutaneous injection of ZA 2 weeks after surgery). The gaps between the transverse processes were clearly observed in groups I and II, while both the gap and mineralized callus bridging were detected in groups III and IV. The gaps were nearly invisible in group V, though the mineralized callus bridging was deemed insufficient. There was a greater amount of bony callus and evidence of bone fusion in group VI.

**Table 3.1**
Micro-CT based histomorphometry of spine at 6 weeks.

| Treatment group | No. studied Micro-CT | TV ($mm^3$) | BV ($mm^3$) | BV/TV (%) |
|---|---|---|---|---|
| Group I MedGEl alone | 6 | 535.43 ± 29.67 | 261.39 ± 9.13 | 48.93 ± 1.23 |
| Group II MedGEl + ZA | 6 | 564.59 ± 34.71 | 307.41 ± 21.09 | 54.45 ± 1.69 |
| Group III MedGEl + 1 μg BMP-2 | 6 | 531.80 ± 54.01 | 248.10 ± 17.36 | 46.94 ± 4.09 |
| Group IV MedGEl + 1 μg BMP-2 + ZA | 6 | 521.99 ± 47.69 | 279.86 ± 30.33 | 53.56 ± 1.92 |
| Group V MedGEl + 3 μg BMP-2 | 6 | 620.97 ± 59.65 | 281.72 ± 22.57 | 45.85 ± 5.33 |
| Group VI MedGEl + 3 μg BMP-2 + ZA | 6 | 687.55 ± 57.07[a] | 383.80 ± 37.23[a] | 55.78 ± 2.56 |

ZA: Zoledronic acid.
TV: Tissue volume, BV: Bone volume, BV/TV: Bone volume fraction.
[a] Significant vs. groups I, II, III, IV, V $P < 0.05$.

**Table 3.2**
Micro-CT based histomorphometry of spinal fusion at 6 weeks.

| Treatment group | No. studied Micro-CT | Tb. Th (um) | Tb. N (1/mm) | Tb. Sp (um) |
|---|---|---|---|---|
| Group I MedGEl alone | 6 | 0.26 ± 0.01 | 1.91 ± 0.06 | 0.49 ± 0.04 |
| Group II MedGEl + ZA | 6 | 0.28 ± 0.02 | 1.95 ± 0.08 | 0.40 ± 0.06 |
| Group III MedGEl + 1 μg BMP-2 | 6 | 0.25 ± 0.02 | 1.87 ± 0.06 | 0.47 ± 0.01 |
| Group IV MedGEl + 1 μg BMP-2 + ZA | 6 | 0.27 ± 0.02 | 2.02 ± 0.15 | 0.38 ± 0.06 |
| Group V MedGEl + 3 μg BMP-2 | 6 | 0.24 ± 0.02 | 1.91 ± 0.16 | 0.47 ± 0.09 |
| Group VI MedGEl + 3 μg BMP-2 + ZA | 6 | 0.26 ± 0.02 | 2.11 ± 0.12[a] | 0.36 ± 0.03[a] |

ZA: Zoledronic acid.
Tb. Th: Trabecular thickness, Tb. N: Trabecular number, Tb. Sp: Trabecular spacing.
[a] Significant vs. groups I, II, III, IV, V $P < 0.05$.

and osteoclasts were attached to bone surfaces and seemed to be actively attempting to resorb bone. There were no significant differences between the groups in focusing on the forms of osteoclasts.

## Discussion

In the present study, we tested the effect of rhBMP-2 and ZA administered systemically as a single dose for spinal fusion. Posterolateral lumbar fusion in rats has been well established as an acceptable model for measuring bone growth and manual palpation is the most sensitive and specific method for assessing spinal fusion [24]. Although a collagen sponge has been used as a carrier for rhBMP-2 in the clinical field [12,13], a collagen sponge begins to disintegrate after implantation.

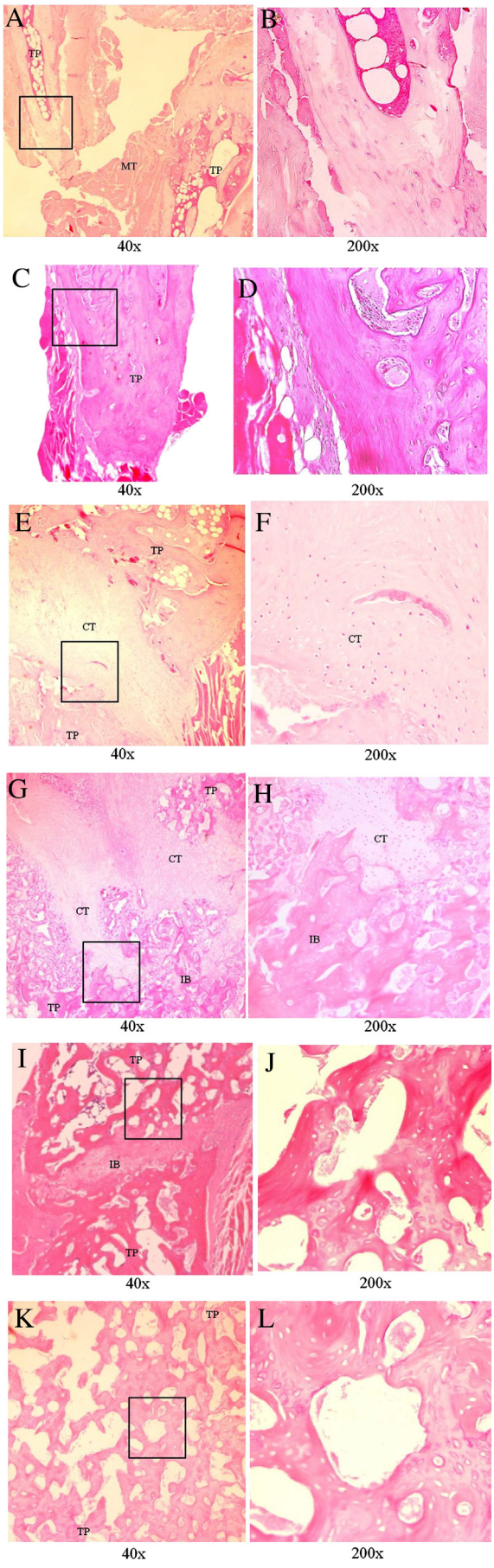


**Fig. 3.** Sagittal histologic cross section of the L4–L5 transverse processes of rat spines obtained 6 weeks after surgery. (A) group I (magnification × 40), (B) group I (magnification × 200): There was a paucity of new bone formation and no evidence of fusion. TP: transverse processes, MT: muscle tissue. (C) group II (magnification × 40), (D) group II (magnification × 200): No fusion and trabecular bone in the transverse processes is much thicker. TP: transverse processes. (E) group III (magnification × 40), (F) group III (magnification × 200): Distribution of cartilaginous tissues were shown. TP: transverse processes, CT: cartilaginous tissue. (G) group IV (magnification × 40), (H) group IV (magnification × 200): The maturity of new bone was much more delayed and distribution of cartilaginous tissues was shown. TP: transverse processes, CT: cartilaginous tissue, IM: immature bone. (I) group V (magnification × 40), (J) group V (magnification × 200): Both immature bone and mature bone formation in the spinal fusion area were shown. TP: transverse processes, IM: immature bone. (K) group VI (magnification × 40), (L) group VI (magnification × 200): Abundant bone bridging in the transverse processes is shown. The carrier has disappeared, and mature bone formation appears and displays osteoid tissue connecting to form trabeculae, which are surrounded by well-developed bone marrow cavities. TP: transverse processes.

**Table 4**
Histologic fusion score at 6 weeks.

| Treatment group | No. studied histologically | Score at 6 weeks |
|---|---|---|
| Group I MedGEl alone | 22 | 0.08 |
| Group II MedGEl + ZA | 24 | 0.25 |
| Group III MedGEl + 1 μg BMP-2 | 24 | 1.50 |
| Group IV MedGEl + 1 μg BMP-2 + ZA | 24 | 2.00 |
| Group V MedGEl + 3 μg BMP-2 | 20 | 2.92 |
| Group VI MedGEl + 3 μg BMP-2 + ZA | 20 | 4.33* |

The spines were scored on both right and left side.
ZA: Zoledronic acid.
The group IV had higher histologic scores than those in the groups I, II, III, IV, V with significant differences ($P < 0.05$).
* $P < 0.05$.

MedGEL has shown more controlled release of rhBMP-2 gradually over 4 weeks, and allows a longer local retention of rhBMP-2 at the implantation site, compared with a collagen sponge [25].

According to our previous data [24], 10 μg of rhBMP-2 was sufficient for bone fusion. Therefore, we expected that 1 μg and 3 μg of rhBMP-2 would not be sufficient to achieve 100% bone fusion in a rat spinal fusion model, and that these amounts would be suitable to investigate the combination effect of rhBMP-2 and ZA. ZA is a potent bisphosphonate and several studies have reported the effect of bisphosphonate therapy in spinal fusion. Lehman and colleagues concluded that daily administration of alendronate sodium delays bone fusion in a rabbit model [27]. On the other hand, Takahata and colleagues reported that alendronate inhibited endochondral ossification but induced the growth of a larger and denser fusion mass by strongly suppressing osteoclastic activity [28]. Bransford and colleagues investigated the effect of ZA in a rabbit model and reported an increased fusion mass size and bone mineral content [21]. On the other hand, BMPs have been reported as powerful anabolic molecules that increase bone remolding and resorption. When initial union occurs, BMPs are ideal agents because they may promote rapid remolding of abundant callus. Accordingly, we expected that rhBMP-2 would increase osteoblastic activity at the initial phase, that ZA (administered at the optimal time) would suppress bone resorption for spinal fusion, and that the combination effect of these agents would occur. In fact, we showed that this combination was efficacious for fracture repair and significantly enhanced bone fusion [22]. Little and colleagues reported that callus size and strength can be increased with the combination of BMP and ZA to synergistically modulate both anabolic and catabolic responses in a rat femoral critical defect model [29]. In the present study, the fusion rate and the radiographic scores in rhBMP-2 and ZA were considerably higher than that in rhBMP-2 without ZA. Thus, the combination effect of rhBMP-2 and ZA administered systemically as a single dose at the optimal time was shown to be efficacious in a rat spinal fusion model, and it significantly enhanced bone fusion at 6 weeks after surgery.

The reconstructed micro-CT images clearly and objectively showed the thickness and quality of the bony structures consistent with the histologic results. Additionally, the use of micro-CT allows researchers to precisely evaluate data. The 3D micro-CT images of group VI showed solid bone fusion and images of groups I and II had clear gaps between the transverse processes. Calculations using computer software revealed various parameters of bone in the target area. The TV and BV parameters in group VI were significantly higher than those in the other groups. Consistent with the micro-CT data results, ZA increases callus size and causes retention of an intricate trabecular network. Studies have reported that ZA increases callus bone mineral content volume with reduced inhibition of primary callus remolding [30–32]. These previous studies support our results, though the combination effect of rhBMP-2 was not reported.

Although BMP therapy is promising for spinal fusion in a clinical application, there is a concern that BMPs also may induce bone resorption in situations where cellular recruitment conditions favor osteoclasts over osteoblasts. Laursen and colleagues [33] reported on thoracolumbar burst fractures treated with transpedicular BMP transplantation. Excessive bone resorption was noted, and in all cases, there was loss of correction with regard to anterior and middle column height and sagittal balance at the last follow-up. McClellan and colleagues [34] retrospectively investigated cases of transforaminal lumbar interbody fusion with BMP. They observed a high rate of bone resorption defects, and assumed that the osseous remodeling potential of rhBMP-2 may lead to bone resorption within the vertebral body. Pradhan and colleagues [35] reported that the nonunion rate among patients who received femoral ring allografts with rhBMP-2 was higher than that in patients who received femoral ring allografts with the iliac bone. They concluded that these findings were because of the aggressive resorptive phase of allograft incorporation that occurs before the osteoinduction phase. These cases indicate that mistimed

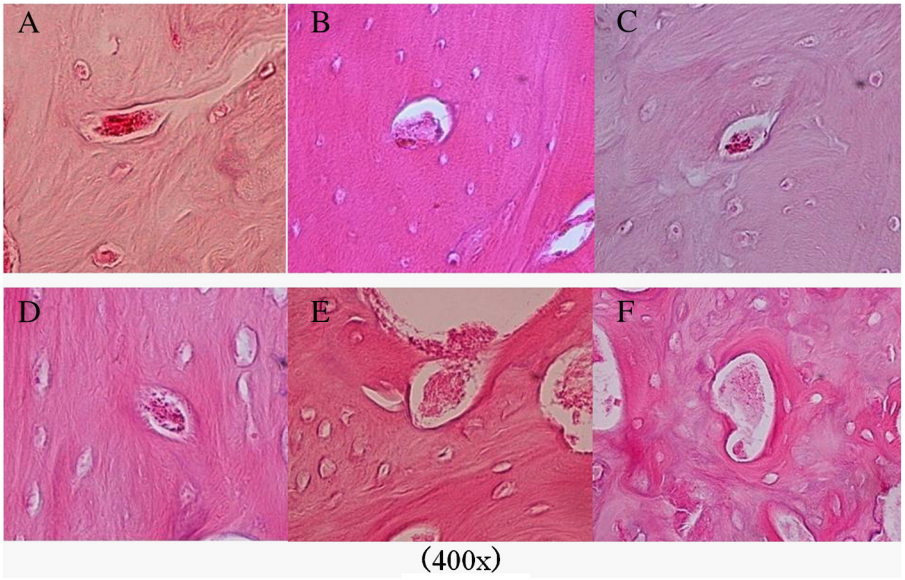


**Fig. 4.** Histological sections of osteoclasts (magnification × 400). (A) group I, (B) group II, (C) group III, (D) group IV, (E) group V, (F) group VI. There were no significant differences between the groups in focusing on the forms of osteoclasts.

catabolism occurs and that resolution of this adverse effect should be established. In clinical settings, this adverse effect is crucial and bisphosphonate administration may possibly prevent it. In a preclinical study, it was reported that BMP-2 caused initial resorption to occur when placed in the metaphysis in a nonhuman primate core defect model in which bisphosphonates were used successfully in preventing the unwanted catabolic phase induced by BMP-2 [36].

The present study was able to show that rhBMP-2 at the surgical site and administration of a single systemic dose of ZA 2 weeks after surgery are efficient for increasing callus volume and reducing osteoclastic stimulation in spinal fusion. However, in the present study, we evaluated the results at six weeks postoperatively and we have not researched it for a long time. Furthermore, it is well known that control and induction of bone volume in larger animals are more difficult, and that high bone-forming ability is required for adequate spinal fusion in humans. In addition, the long-term effects of this combination of agents remain unknown. In the present study, we have decided the doses for zoledronic acid and rhBMP-2 in reference to the previous paper. The proper doses and an increase of fusion rate are not clear and the mechanism was not showed enough in the present study. To validate efficacy and safety of this method, further large-animal and human studies are required before clinical use.

In conclusion, the present study showed the combination effect of rhBMP-2 and ZA administered systemically as a single dose in a rat spinal fusion model. The use of ZA is expected to decrease the required dose of rhBMP-2 in a clinical setting, and prevent the unwanted rhBMP-2-induced catabolic phase. However, the efficacy and safety of this combination in large animals and humans are unclear and need to be examined through further studies before clinical application. Despite these limitations, the combination effect of rhBMP-2 and ZA makes it a possible attractive therapy for spinal fusion.

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