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Cyst-Like Osteolytic Formations in Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) Augmented Sheep Spinal Fusion

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Cyst-Like Osteolytic Formations in

Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2)

Augmented Sheep Spinal Fusion

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in Oral Biology

by

Hsin Chuan Pan

2018

ABSTRACT OF THE THESIS


Cyst-Like Osteolytic Formations in

Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2)

Augmented Sheep Spinal Fusion



by



Hsin Chuan Pan

Master of Science in Oral Biology

University of California, Los Angeles, 2018

Professor Kang Ting, Chair

Multiple case reports using recombinant human bone morphogenetic protein-2 (rhBMP-2) have reported complications. However, the local adverse effects of rhBMP-2 application are not well documented. In addition to promoting lumbar spinal fusion through potent osteogenic effects, rhBMP-2 augmentation promotes local cyst-like osteolytic formations in sheep trabecular bones that have undergone anterior lumbar interbody fusion. Conventional computed tomography showed rhBMP-2 application groups could fuse, whereas no fusion was observed in the control group. Micro-computed tomography revealed core implant area's bone volume fraction and bone mineral density increased proportionately with rhBMP-2. Multiple cyst-like bone voids were observed in peri-implant areas using rhBMP- 2, and these sites showed significant bone mineral density decreases in relation to unaffected regions. Biomechanically, these areas decreased in

strength by 32% in comparison with noncystic areas. Histologically, rhBMP-2 affected void sites had an increased amount of fatty marrow, thinner trabecular bones, and significantly more adiponectin and cathepsin K-positive cells. Despite promoting successful fusion, rhBMP-2 use in clinical applications may result in local adverse structural alterations and compromised biomechanical changes to the bone.

The thesis of Hsin Chuan Pan is approved.

Shen Hu

Flavia Queiroz Pirih

Kang Ting, Committee Chair

University of California, Los Angeles

2018

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# ACKNOWLEDGEMENTS

I would like to thank Drs. Kang Ting, Chia Soo, Xinli Zhang and Jin Hee Kwak, my mentors, for their continual support and guidance during my MS program during orthodontic residency. Their patience, guidance, work ethic, passion, and enthusiasm in research are the driving forces behind this thesis work.

I am thankful for Mr. Matthew Dingman at the Oral Biology department for his assistance in resolving administrative difficulties.

Lastly, I want to thank all the current and past members of Ting Lab for their support and contributions to this project.

**Introduction**

Recombinant human bone morphogenetic protein-2 (rhBMP-2) has been recognized as an effective osteoinductive growth factor and has been found to induce bone regeneration in clinical trials and previous studies[1]. Its capacity for limiting donor site morbidity and enhancing arthrodesis led to the United States Food and Drug Administration (FDA) approving it for limited indication use in anterior lumbar interbody fusion (ALIF), open tibial shaft fractures, and certain oral and maxillofacial uses. Consequently, the approved rhBMP-2 product, INFUSE® Bone Graft (Medtronic, Memphis, Tennessee), is produced in various doses. Many surgeons rapidly embraced this product, outside its indicated applications, for use in posterolateral fusions (PLF), transforaminal lumbar interbody fusions (TLIF), and anterior cervical discectomy and fusions (ACDF)[2]. Its use in these procedures lacks site-specific data and evidence for appropriate dosage, safety, or efficacy, but rhBMP-2 is still used for its favorable outcomes over autogenous bone graft procedures[2].

Recently, researchers have reported adverse events associated with rhBMP-2, which range from cyst-like voids in developing bone, radiculopathy, retrograde ejaculation, and cancer[3-10]. However, since gaining FDA approval, local adverse effects of rhBMP-2 have not been evaluated. Specifically, cyst-like bone voids in relevant spinal fusion models have yet to be reported[11]. Recent advancements in imaging technology, such as high-resolution micro-computed tomography (micro-CT), permit exceptional micro-scale analysis of the bony structure to observe changes in bone structure induced by rhBMP-2, both quantitatively and qualitatively. We report that, despite its potent osteogenic effect, rhBMP-2 augmentation appears responsible for the osteolytic effects observed in local trabecular bone near the rhBMP-2 implants in sheep. The structural alterations in the affected bone, along with the aberration of biomechanical strength, are demonstrated and

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discussed for their clinical implications.

**Materials and Methods**

Animals and experimental design

The Colorado State University IULAC approved all sheep surgery protocols. Six skeletally mature Rambouillet × Columbian ewes (Three JP; LLC livestock suppliers, La Junta, CO) underwent a two-level ALIF surgery with radiolucent interbody vertebral spacers (Vertebral spacer-CR 889.915; Synthes, Monument, CO) fusion at two lumbar levels, L3/L4 and L5/L6. In total, 12 fusion sites were prepared. These levels were selected based upon a prior study's findings[12]. The radiolucent spacer was used to evaluate the longitudinal spinal fusion radiographically. The animals were divided into 3 groups; a control group receiving phosphate buffered saline (PBS) and an absorbable collagen sponge (ACS) in the spacer (n = 4), the dose-I group receiving 0.43 mg/mL (0.65 mg in total[13]) rhBMP-2 and an ACS in the spacer (n = 4), and a dose-II group receiving 1.5 mg/mL (2.25 mg in total[14]) rhBMP-2 and an ACS in the spacer (n = 4). Dose-I of rhBMP-2 was based on the manufacturer's sheep study recommendation, and dose-II of rhBMP-2 was the FDA's approved dosage in humans[13, 14]. Postoperatively, X-rays and conventional computed tomography (CT) were used to evaluate the fusions. Three months postoperatively, the sheep were euthanized and L3/L4 and L5/L6 samples were assessed by micro-CT, biomechanical, and histological analyses in a blinded fashion (Fig. 1).

Implant preparation

Before surgery, freeze-dried rhBMP-2 was reconstituted to 0.43 mg/mL and 1.5 mg/mL. 1 mL of the rhBMP-2 solution was administered drop wise onto a 2.5 cm × 5 cm type I ACS

(Helistat, Integrated Life Sciences, Plainsboro, NJ), and bound to the sponge for 30 minutes. The sponge was subsequently inserted into the cages.

Surgical procedures

Ketamine was used perioperatively as an analgesic. After anesthesia, the sheep were placed in a right lateral recumbent position. Access to L3/L4 and L5/L6 was made by a ventrolateral retroperitoneal approach, through the oblique abdominal muscles, to the plane ventral to the transverse processes. After identifying the L5/L6 disc space, an annulotomy was performed. The endplate was appropriately sized using a Midas-Rex burr. The disc space was opened with a vertebral spreader, and the cage (containing ACS with either PBS or rhBMP-2) was inserted into the disc space. After the same procedure was performed at L3/L4, each animal had 2 implantation sites. After implantation, the wound was closed and cefazolin was injected to prevent infection.

X-ray and conventional CT imaging acquisition and analysis

Anterior-posterior (AP) and lateral view X-ray images were acquired immediately after surgery and at months 1, 2, and 3 post-surgery. At 2 and 3 months post-surgery, conventional CT images were taken at a slice thickness of 1.5 mm, peak voltage 130 kVp, and current of 175 μA, which resulted in a plane resolution of 0.35 mm/pixel per image. Successful spinal fusions were defined as $\geq$ 50% of a contiguous bone bridge area within the implant on conventional CT, following the criteria from a previous report[15].

Micro-CT imaging acquisition and analysis

Three months post-surgery, the sheep were euthanized by barbiturate overdose. The L3/L4 and L5/L6 spine segments were extracted and fixed in formaldehyde. Sample images were captured with a high-resolution micro-CT (SkyScan 1176 microCT, Bruker, Kontich, Belgium). Images were obtained at tube potential of 90 kVp and 278 μA with a 1 mm copper filter set at a voxel size (resolution) of 18 μm. Three-dimensional reconstruction of the samples was obtained with NRecon software (Version 1.7.0.4, Bruker).

For comprehensive image analysis, three distinctive volumes of interest (VOIs) were generated. VOI-1; The core implant VOI, with a diameter and height of 7 mm, was centered in an anterior-posterior position within the spacer relative to the cylindrical fusion site. It consisted of various concentrations of rhBMP-2 enclosed by the spacer. VOI-2; The peri-implant VOI was taken 7 mm above and below the implant spacer, which is an area associated with increased probability of materialized cyst-like osteolytic formations. The areas above and below the implant regions were separated into individual datasets. In all samples, the VOI range was determined after measuring the relative distance of bone quality changes from the center of the spacer. The volume was segmented following the spacer's contour to better examine the direct effect around the implant. VOI-3; Cyst-like bone void VOI was generated to examine the cystic changes with greater precision. A standardized 27 mm$^3$ VOI was positioned in various osteolytic locations to analyze changes within the same sample. The bone volume fraction (bone volume/tissue volume, BV/TV), bone mineral density (BMD), and trabecular thickness (Tb.Th) were calculated at a threshold of 60 with CTAn software (Version 1.16.4.1, Bruker).

Finite element analysis (FEA)

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Biomechanical strength was evaluated using FEA. Micro-CT data were converted to DICOM images using NRecon software. Three-dimensional mesh models were subsequently generated from VOI-3, which enclosed the cystic change volume, to investigate precise mechanical changes. Model meshing was done with Mimics 3-Matics (Version 11.0, Materialise, Leuven, Belgium), and FEA analysis was accomplished with ABAQUS (Version 6.14, Dassault Systemes Simulia Corp, Warwick, RI, USA). While the inferior bone surface was encastred as a boundary condition, a compressive stress of 0.5 MPa was evenly applied to the superior surface and the equivalent tensile stress experienced by each element was calculated.

Histological analysis

For histological fixation, specimens were decalcified using 10% HCl solution, washed under running tap water, and stored in 75% ethanol. Specimens were then embedded in paraffin and cut into 5 mm coronal sections. Hematoxylin and eosin (H & E) and immunohistochemical (IHC) staining were performed as previously described[16]. Anti-cathepsin K (ab10927) and anti-adiponectin (ab62551) were used at a dilution of 1:100. The biotinylated anti-rabbit IgG secondary antibody (Dako North America, Inc, Carpinteria, CA, USA) was used at a dilution of 1:200. Photomicrographs were acquired using Olympus BX51 (×200 magnification lens, UPLanFL; Olympus, Center Valley, PA, USA) and SZX12 microscopes (×8.4 magnification lens, DF PLAPO 1.2× pf; Olympus, Center Valley, PA, USA).

Statistical analysis

The mean ± standard deviation of the data was calculated. Data were tested for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. To test for significance between two

groups, the Mann Whitney U test was employed, while the Kruskal–Wallis test with post hoc Bonferroni tests was utilized when testing for significance between more than two groups. The statistical software SPSS (Version 18.0, IBM, Armonk, New York, NY, USA) was used for all statistical analyses. Statistical significance was determined at $p < 0.05$.

**Results**

Conventional CT showed cyst-like bone void lesions in both rhBMP-2 groups

Post-operatively, implanted spacer position was confirmed with X-ray imaging (Supplemental Fig. 1). Conventional CT revealed that the control group did not demonstrate fusion in any of the sites, while both rhBMP-2 groups were completely fused in all sites (100%) ($p < 0.05$) (Fig. 2). Bone volume fraction within the spacer was calculated, and defined as a volumetric deformity when the bone volume fraction was less than 90%. The prevalence of volumetric deformities in the dose-I group was significantly higher than the other groups when imaged 2 months post-operatively ($p < 0.05$). In all evaluation periods, radiolucent lesions, resembling possible osteolytic changes around the implant, were observed by conventional CT in both rhBMP-2 groups. However, due to the conventional CT's low resolution, the extent of the cyst-like bone voids was not quantifiable. Fusion qualities are summarized in Table 1.

Micro-CT revealed quantitative volumetric deformities of newly formed bone

In axial and coronal micro-CT views, the control spines exhibited fragmented new bone growth within the implant, with new bone growth discontinuation at the center. In contrast, complete fusion of vertebrae through the center of the spacer was achieved in all dose-I and dose-II site samples (Fig. 3). The volumetric deformity was better observed using micro-CT, which

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revealed that the dose-I group had a decreased new bone diameter, $5.919 \pm 1.311$ mm, compared to the other groups ($p < 0.05$) (Table 1).

Micro-CT demonstrates dose-dependent increase of bone quality in core implant area

The dose-II core implant sites (VOI-1) exhibited significant increases in all parameters (BMD, BV/TV, Tb.Th) when compared to the other groups ($p < 0.01$) (Fig. 4A). Additionally, denser bone was formed in the implant core area of both rhBMP-2 treatment groups (Fig 3A and Fig 4A). Despite a 100% fusion rate in both rhBMP-2 groups, VOI-2 did not demonstrate the same dose-dependent trend shown in the core implant analysis. Dose-I had significantly lower BMD and Tb.Th than the control ($p < 0.05$, in both) (Fig. 4B).

Cyst-like bone void demonstrate poor bone quality and weakened mechanical strength

Cyst-like bone voids were identified and localized after cross-referencing the micro-CT datasets with conventional CT, and especially in VOI-2 (Fig. 4C). In both rhBMP-2 groups, the cyst-like bone voids were near the implant, but varied in shape and size. These formations were not observed in the control group. The dose-I group showed a cluster distribution of smaller cyst-like bone voids above and below the implant spacer, but not in the center region that directly contacted the concentrated implant site. In contrast, the cystic change in dose-II exhibited larger solitary bone voids. The bone volume fraction and BMD of VOI-3 were significantly decreased in relation to the unaffected regions in both rhBMP-2 groups (Fig. 5). Biomechanical analysis utilizing FEA further confirmed the micro-CT quantification, and all the cyst-like bone void areas exhibited significantly increased stress levels, at 32%, compared to the unaffected structures ($p < 0.05$) (Fig. 6).

<u>Cyst-like bone voids attributed to increased adipogenesis and osteoclastogenesis</u>

Histologically, trabecular bone complete bony connectivity was observed in both rhBMP-2 treatment groups, while the cartilaginous tissue band in the middle of the implant's central area was observed only in the control group. Within the vicinity of the spacer surfaces, cyst-like bone void lesions were readily identifiable in the rhBMP-2 treatment groups (Fig. 7A-C). In both rhBMP-2 treatment groups, as compared to the control group, the cyst-like bone voids exhibited a thinner trabecular bone formation with larger cavities that were filled with fatty marrow (Fig. 7D-I). Notably, a focal lesion with inflammatory cells, dilated blood vessels, and a piece of dead bone were observed in the cyst-like bone voids of the dose-I group samples (Supplemental Fig. 2A).

To further evaluate possible causes of the cyst-like bone voids in the rhBMP-2 samples, in situ expressions of representative adipogenesis protein markers, adiponectin and osteoclastogenesis cathepsin K, were detected by IHC. The adiponectin positive cells, a protein synthesized and secreted exclusively by mature adipose tissue and its progenitor cells, were abundant, with a higher adiponectin expression level in the rhBMP-2 groups than the control group (Fig. 8A-C). Interestingly, there were two types of adiponectin staining patterns that may reflect the phenotypic differences between the smaller progenitor cells and the mature fatty drop-filled adipocytes (Supplemental Fig. 2B). Like adiponectin staining, more cathespin K-positive osteoclasts were detected along the thinner trabecular bones in the rhBMP-2 groups (Fig. 8D-F). Additionally, there were more cathespin K positive cells distributed within marrow cavity in the rhBMP-2 samples. In general, this may indicate the increased number of osteoclastic cells brought about by rhBMP-2 application. By including a negative staining control through replacing specific

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antibodies of adiponectin or cathepsin K with PBS, the IHC positive results appear to be antibody specific (Supplemental Fig. 2C).

**Discussion**

Studies demonstrated that INFUSE® Bone Graft had a positive effect on new bone formation and healing existing bone by stimulating the recruitment and differentiation of bone-forming cells[17]. In clinical practice, rhBMP-2 has been used as much as 85% for non-FDA approved indications, including PLF and TLIF[18-25]. rhBMP-2's use has elicited worrisome side effects, but no guidelines for dosage have been established[26-36] (Supplemental Table 1). In our previous investigation that used a rodent model, cyst-like bone voids were observed in rhBMP-2 assisted spinal fusions (Supplemental Fig. 3). These findings necessitated the evaluation of fusion quality in larger animal models. Moreover, while several studies reported that the cyst-like bone voids were related to rhBMP-2 in both small[37] and large animal models, only conventional CT and histology have been utilized for verification[38, 39]. Although lumbar interbody fusion is the most common indication for rhBMP-2, no prior study has comprehensively evaluated its use in the lumbar interbody fusion of a large animal model. Instead, its use has been widely documented in PLF, vertebral body implantation, long bone metaphyseal defect, and radius segmental defect procedures[38-40].

High doses of rhBMP-2 in spinal fusions, compared to the small amount of rhBMP-2 found in endogenous bone (2 - 30 μg/kg), has been suggested as the main cause for the observed complications[20, 29, 41-45]. Through simulating a large animal study with comparable rhBMP-2 doses, the results suggest that the observed complications may also occur in human patients. Interestingly, the cyst-like bone voids were clearly observed in the peri-implant area (VOI-2) of both rhBMP-2

groups. These groups also had significant decreases in bone quality, but no such lesions or changes in bone quality were detected in the control group. Despite having seemingly complete fusions, the dose-I group had a lower BMD and Tb.Th in the peri-implant area in comparison to the control. In the core implant area (VOI-1), the dose-I group had a higher bone volume fraction and BMD than the control, but still exhibited an increased occurrence of volumetric deformities inside the spacer.

High resolution micro-CT was chosen as the primary tool for analyzing alternations in bone structures due to its nondestructive preservation of tissue and field of view selection flexibility.[46] Using the volumetrically reconstructed fused segment, we determined the exact location of cyst-like bone voids relative to the implant site. At the same time, through FEA, the detrimental effect of rhBMP-2 can be interpreted biomechanically, and the results alert potential users to clinical complications that could result in implant subsidence or fusion failure.

Several hypotheses were proposed for the cyst-like osteolytic formation mechanism, including osteolysis, adipocyte formation, and inflammation. Osteolysis, or bone resorption, is conceivably induced by rhBMP-2 increasing osteoclast cell activity[5, 14, 47-51] through serine/threonine receptors of bone morphogenetic protein (BMP) and the downstream molecules. In accordance with previous reports, we detected much more cathepsin K positive cells in rhBMP-2 samples than in the control. Alternatively, BMP-2 and other BMPs are well-known upregulators of adipogenesis by enhancing peroxisome proliferator-activated receptor-γ signaling[52]. Previous studies have shown that cyst-like bone voids with fatty marrow are observed with high doses of rhBMP-2 (greater than 150 mg/mL)[53]. In this study, the fatty marrow profoundly filled the cyst-like bone voids between thinner trabecular bones. Adiponectin, a representative marker for adipogenesis, was readily detectable in both adipocytes and smaller progenitor cells throughout

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the fatty marrow in the rhBMP-2 samples, in contrast to fewer positive cells in the control. Lastly, BMP's ability to induce inflammation appears essential to the in vivo osteoblastic signaling cascade[54]. In mice, BMP has been shown to cause the release of key inflammatory cytokines, including tumor necrosis factor-α, interleukin-1, and interleukin-6[8, 54-56]. However, overwhelming inflammation induced by high dose rhBMP-2 can also contribute to cyst-like bone void development[57]. Vertebral osteolysis after posterior lumbar interbody fusion may occur through the inflammatory effects of BMP[14], yet this likely did not happen in this study, as only a local inflammatory lesion was observed in the dose-I samples. Inflammatory infiltrates were not consistently observed in rhBMP-2 samples. Furthermore, studies have reported that high doses of rhBMP-2 were associated with many life-threatening complications, which can be traced and attributed to an inflammatory reaction[58].

**Conclusion & Future Direction**

Some study limitations of the present study include the lack of long-term follow-up to determine if the cystic voids persist, enlarge, or are replaced by new bone. Whether these findings are transient phenomena remains unknown. Using a larger number of animals with variable follow up periods is recommended for further studies. At the same time, additional biochemical tests could aid in validating our FEA findings.

In conclusion, although the use of rhBMP-2 effectively promotes lumbar fusion in sheep, the cystic changes to the bone brought about by rhBMP-2, especially in adjacent areas of the implants, were readily identifiable and compromised bone strength. The increased adipogenesis and osteoclastogenesis resulting from rhBMP-2 augmentation may be the primary cause of the cyst-like bone voids. Ultimately, physicians should remain aware of rhBMP-2's ability to produce

cyst-like bone void changes, even if the spine is successfully fused, and should carefully evaluate

bony structural changes proximal to the implant.

**Figures**



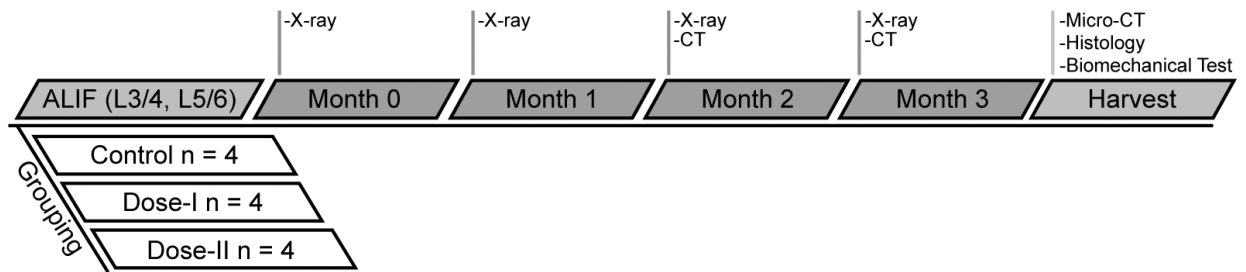Fig. 1. Experimental design. Spinal fusion (ALIF) surgery was performed on animals randomly assigned to three different groupings. The postoperative analysis included monthly X-ray scans, and conventional CT scans at months 2 and 3. At 3 months post-operation, samples were harvested and underwent high-resolution micro-CT, biomechanical, and histological analyses. ALIF: Anterior lumbar interbody fusion, CT: Computed tomography.
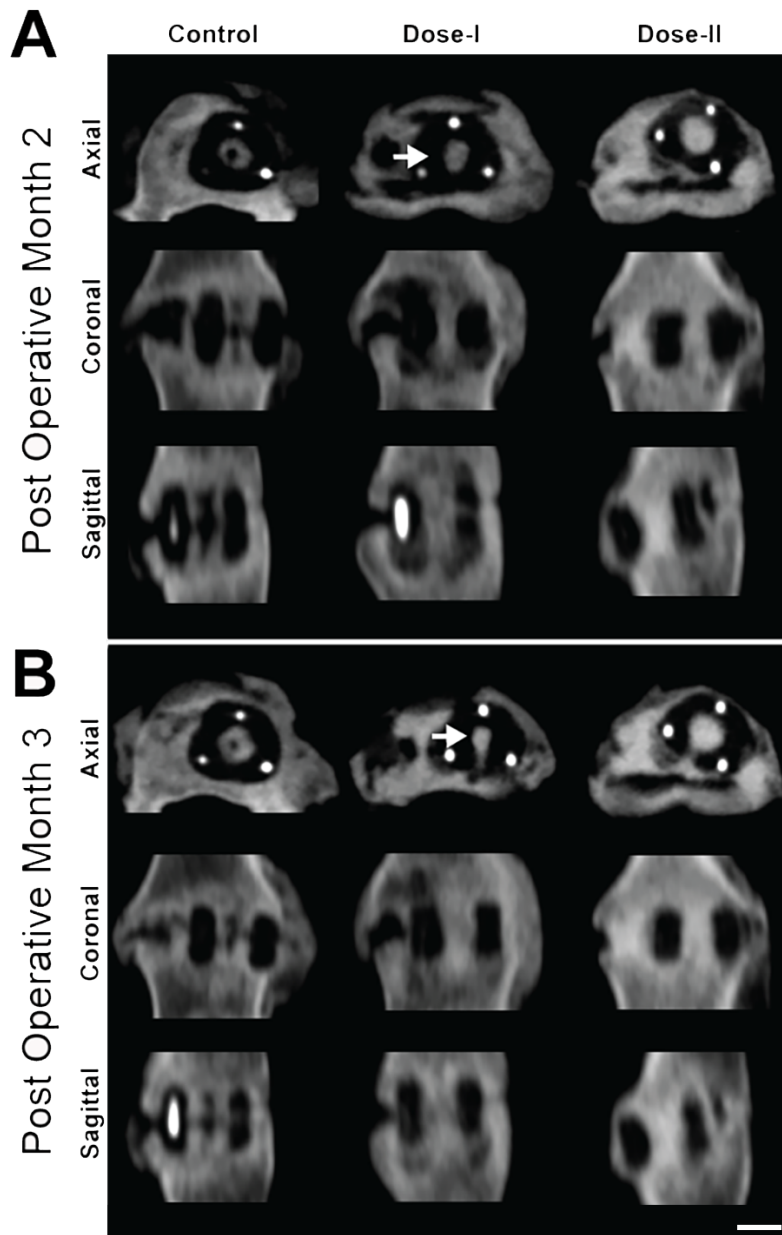
Fig. 2. Results of conventional CT. Representative conventional CT images from each group (A) 2 months and (B) 3 months after the operation. Successful fusion was observed in all rhBMP-2 groups, and no union was observed in the control group. The bone volume fraction within the spacer was calculated by measuring the ratio of new bone volume against the entire spacer volume at five vertical levels that were evenly distanced when positioned from an axial view. Volumetric deformity was defined as the bone volume fraction occupying less than 90% of the spacer. In an axial view, a volumetric deformity of the implant core (arrow) was observed in the dose-I group at both time points. CT: Computed tomography, rhBMP-2: Recombinant human bone morphogenetic protein-2. (Scale bar = 5 mm)

Fig. 3. Three-dimensional orthogonal slice representatives from micro-CT. (A) The control group presented with a clear bony gap in all samples. Both rhBMP-2 groups showed solid fusion, but the dose-I group revealed a volumetric deformity toward the center. (B) Axial view slice selection was based on the midpoint of the implant core's vertical dimension. Sagittal and coronal slices were determined from the center of the implant. CT: Computed tomography, rhBMP-2: Recombinant human bone morphogenetic protein-2. (Scale bar = 5 mm)
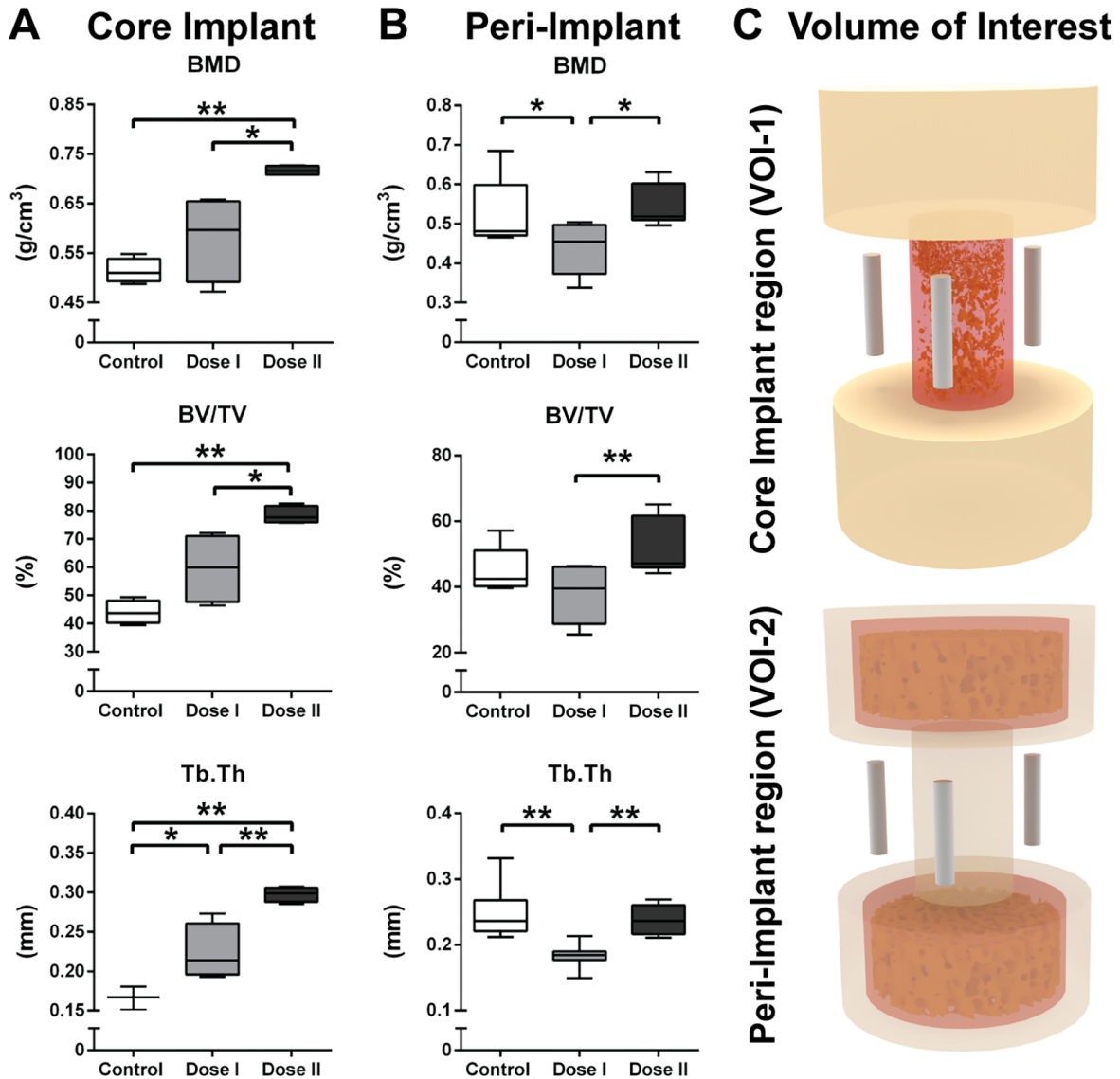
Fig. 4. Quantitative analysis of fusion mass with high-resolution micro-CT. Fusion quality was quantitatively analyzed by dividing the sample into the core and peri-implant areas. (A) As expected, the core area showed dosage dependent BMD and BV/TV increases. (B) Contrary to expected result, the peri-implant area showed that the BMD was significantly lower in the dose-I group compared to the control group. (C) Core implant VOI-1 is defined as a cylindrical volume of 7 mm in height and diameter in the center of the spacer. Peri-implant VOI-2 is defined as the volume directly above and below the implant spacer, and following the spacer contour. The volume height was 7 mm, as this was the furthest distance after comparing all samples in which cyst-like bone voids were observed. CT: Computed tomography, BMD: Bone mineral density, BV/TV: Bone volume/tissue volume, Tb.Th: Trabecular thickness, VOI: Volume of interest. $*P < 0.05$, $**P < 0.01$
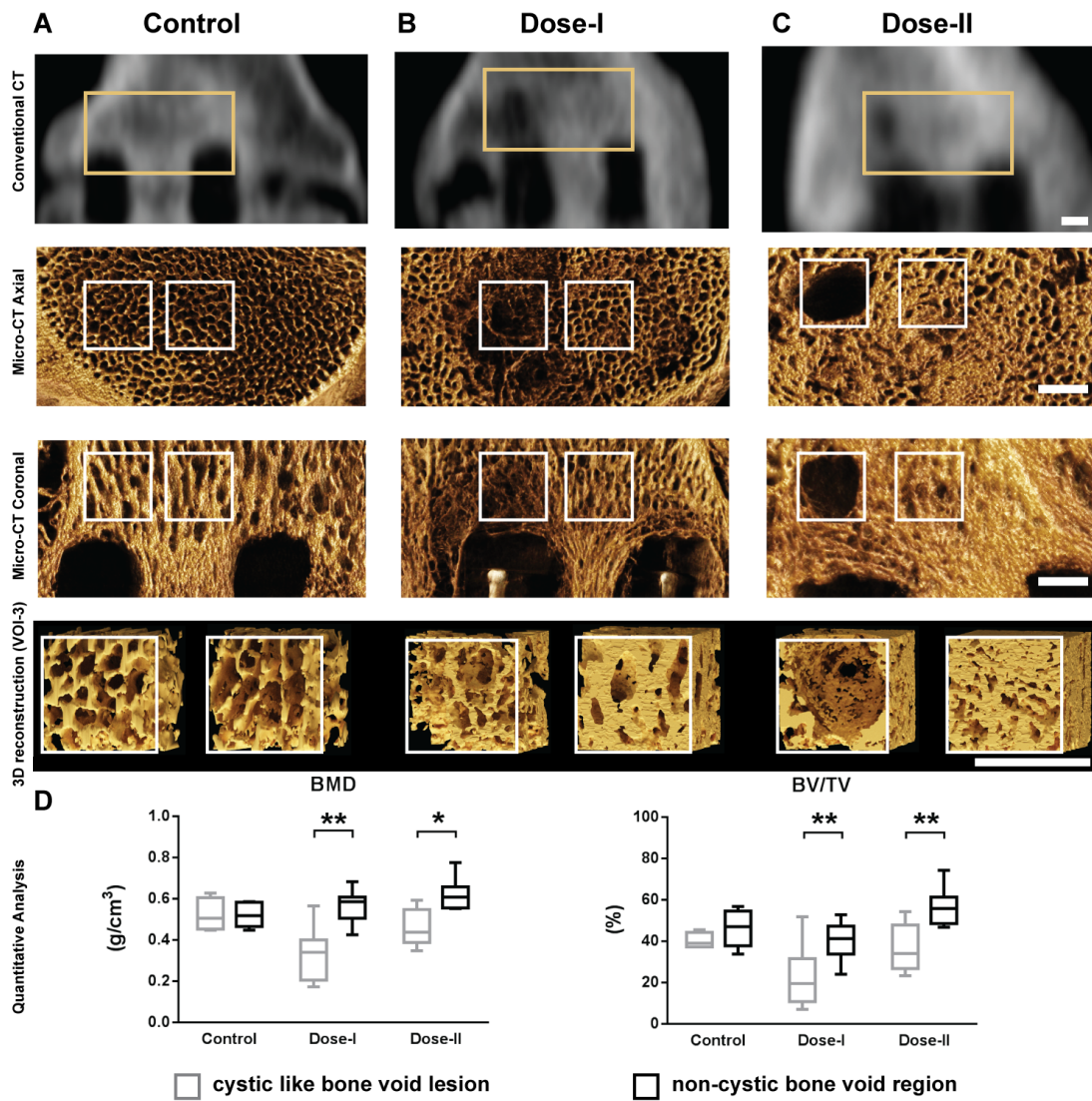
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Fig. 5. Representative images of cyst-like bone voids and quantitative analysis. From a macro to micro scale, the cyst-like bone void sites were analyzed. (A) For the control, no cystic change was found throughout the sample. (B)(C) The dose-I group showed small clustered bone cysts over an area, where as dose-II group sample could be localized as a solitary lesion. A 27 mm³ volume of interest (VOI-3) was selected after reviewing all the cyst-like bone void regions, and analyzed quantitatively to investigate the relative density/volume change. (D) The cubic volume was selected based on its relative position to the implant core, with one cubic volume directly above it and another more distant, but still adjacent to the spacer. The cyst-like bone void area showed significant bone weakness (low BMD and BV/TV) compared to the unaffected sites. Orange boxed areas are locations where subsequent micro-CT illustrations (shown below) were extracted from, with white boxed areas depicting the 27 mm³ cubic volumes used for analysis. VOI: Volume of interest, BMD: Bone mineral density, BV/TV: Bone volume/tissue volume, CT: Computed tomography. $*P < 0.05$, $**P < 0.01$ (Scale bar = 3 mm)
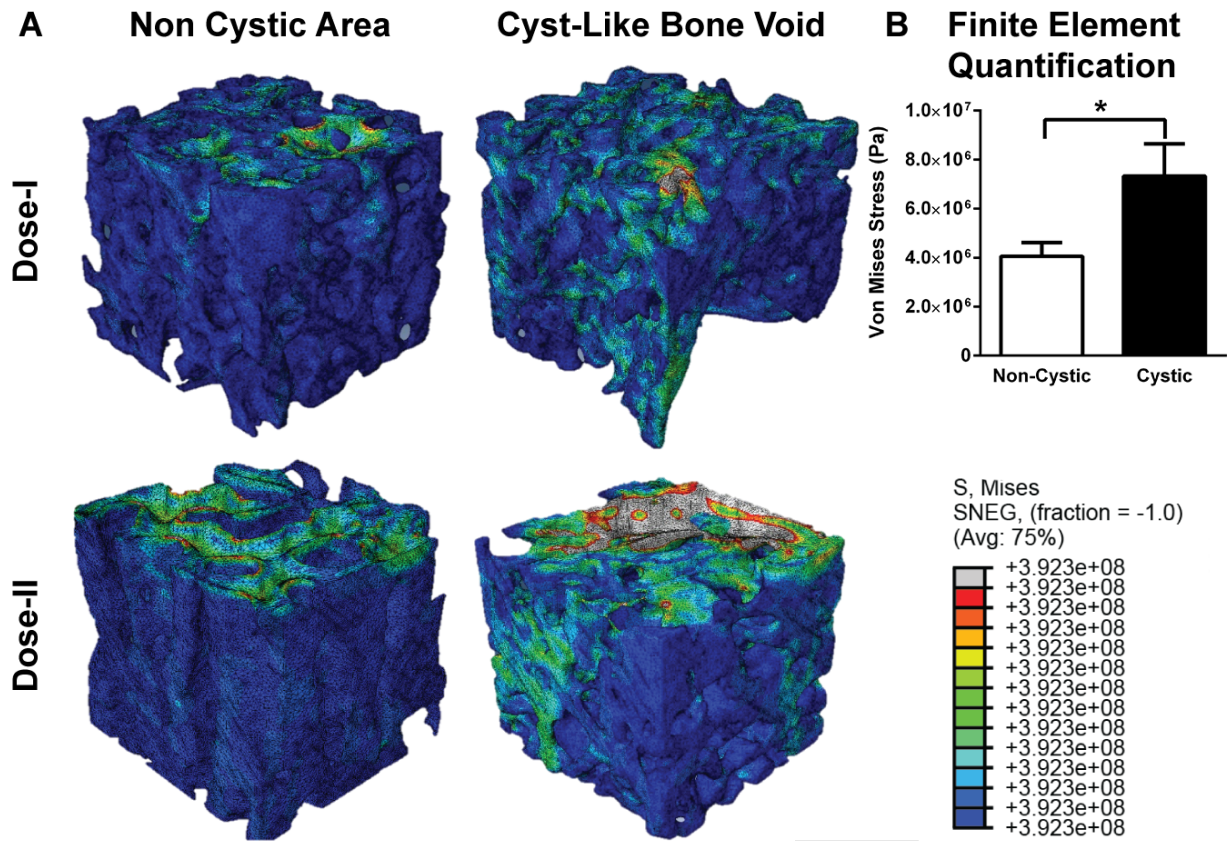
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Fig. 6. Biomechanical testing using FEA. A uniform compressive force of 0.5 MPa was applied to the superior surface of the 27 mm$^3$ cubic-shaped VOI of both non-cystic and cyst-like bone void areas. (A) Cuboidal specimens from cyst-like bone void area showed an increased von Mises stress with higher intensity of colors (grey regions indicates all values exceeding 25 MPa). (B) Quantification of cuboidal segments of each sample bone demonstrated significantly increased von Mises stress in the cyst-like bone void, which suggests deteriorated bone strength. FEA: Finite element analysis, VOI: Volume of interest. *$P < 0.05$ (Scale bar = 3 mm)
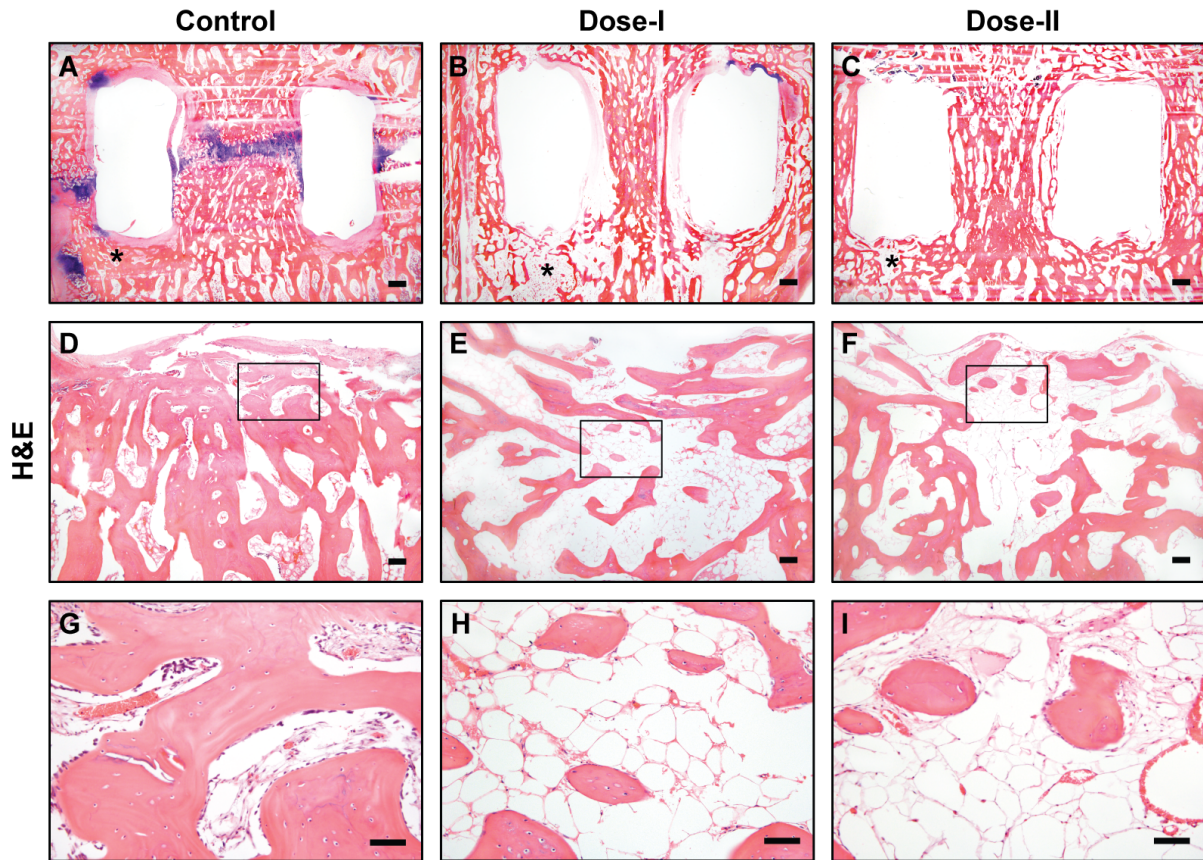
Fig. 7. Histology of cyst-like bone voids. (A-C) The cyst-like bone voids were located immediately next to rhBMP-2 implants in both treatment groups as opposed to the control (*). (D-F) The expanded spaces that had fewer and thinner trabecular bones were seen in the cyst-like bone voids, but not in the control sample; (G-I) There is a profound amount of fatty marrow filling the cyst-like bone voids in both rhBMP-2 samples. rhBMP-2: Recombinant human bone morphogenetic protein-2. Middle row figures (D-F) are magnified views from asterisk areas in the top row (A-C), at the same time boxed areas (D-F) are shown below at higher magnification (G-I). (Scale bar = 1 mm, 200 μm, 100 μm top to bottom row)
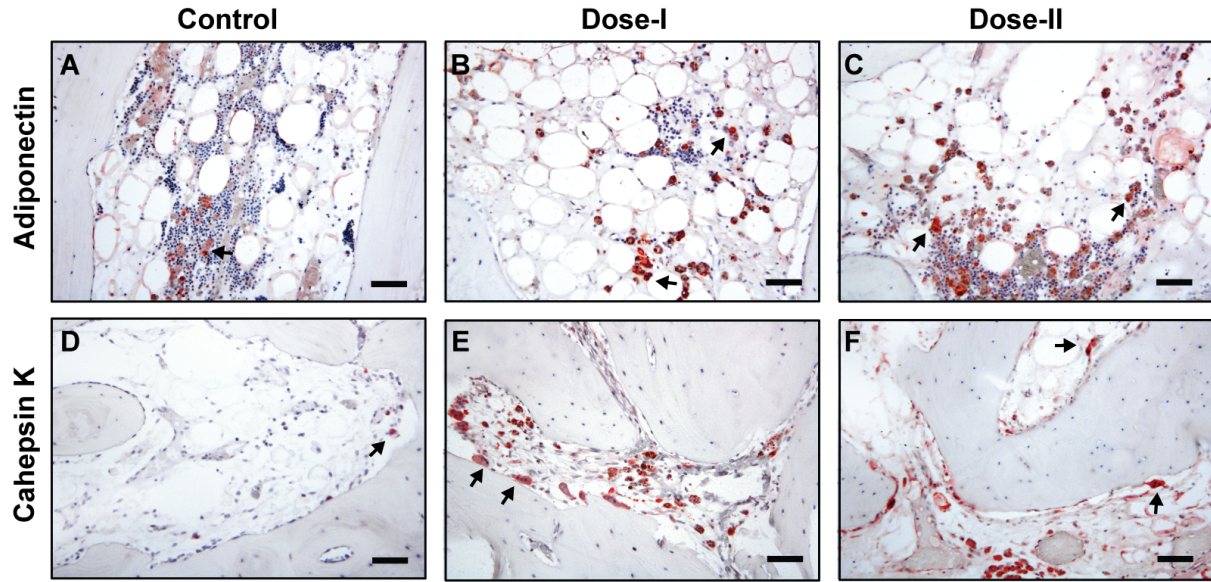
Fig 8. Immunohistochemistry of adiponectin and cathepsin K. (A-C) Many more adiponectin positive cells present in the marrow cavity were detected in the rhBMP-2 groups (arrows) than the control sample (arrow). (D-F) An increased number of cathespin K-positive osteoclasts along trabecular bones and in marrow cavity were detected in rhBMP2 groups (arrows) than in the control group (arrow). rhBMP-2: Recombinant human bone morphogenetic protein-2. (Scale bar = 100 μm)
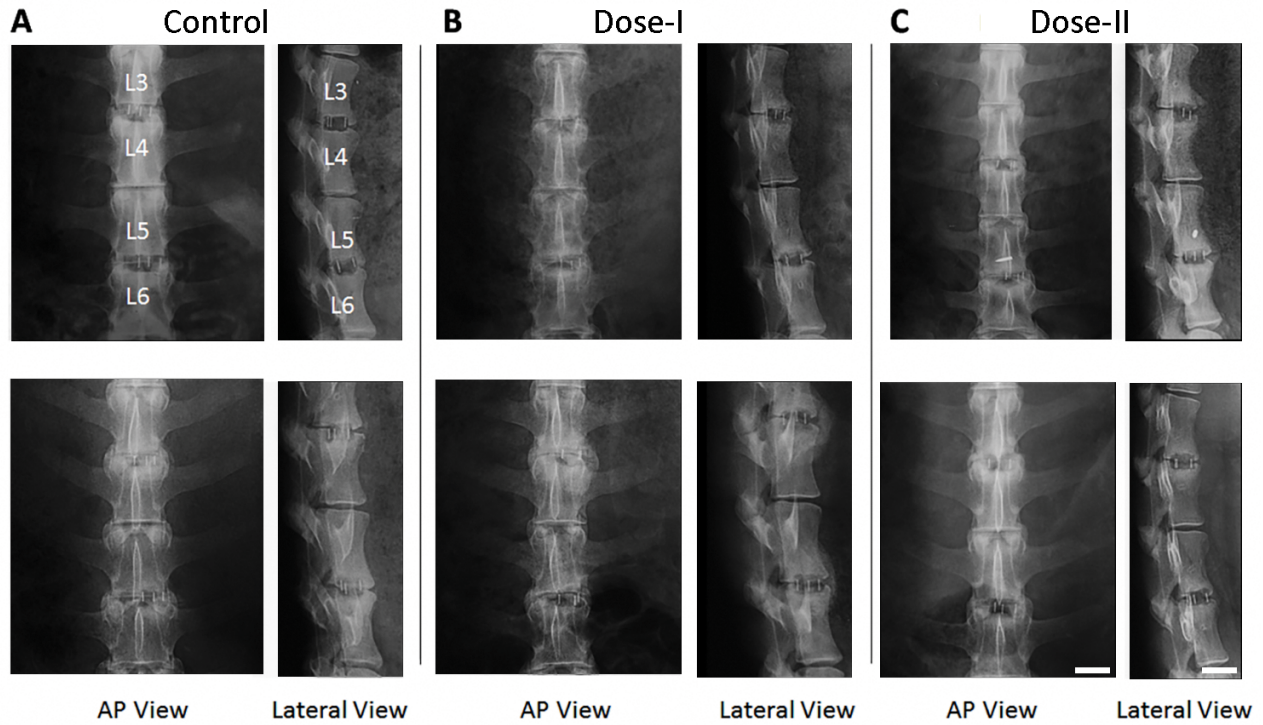
Supplementary Fig. 1. Immediate post-operative X-ray of the lumbar spine. The implant position was confirmed post-operatively with anterior-posterior and lateral X-ray radiographs. The subsequent positioning and fusion assessments took place immediately after the operation, and 1, 2, and 3 months post-operatively. Vertebral spacers are observed at both L3/L4 and L5/L6 levels of each group. (Scale bar = 3 cm)

| H&E | Adiponectin | Negative Staining Control |

**Dose-I**
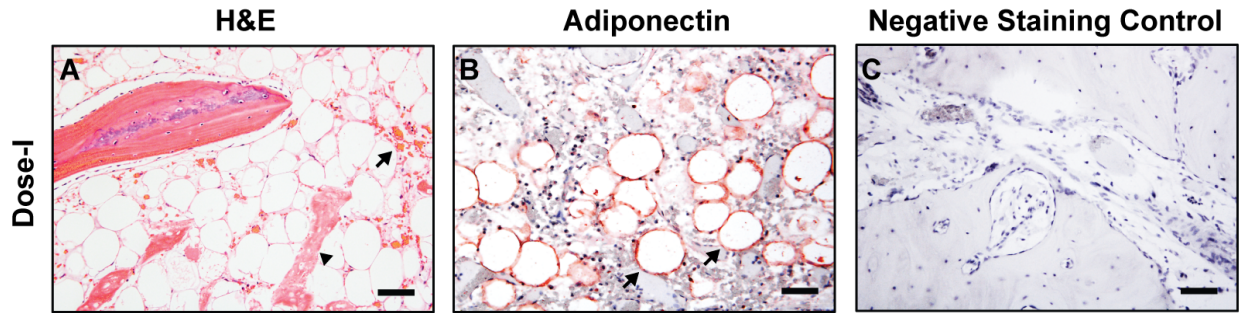
Supplementary Fig. 2. Additional histology and immunohistochemistry on voids.
(A) The inflammatory cells and a dilated blood vessel (arrow) and a piece of dead bone (arrowhead) were observed in the rhBMP-2 sample. (B) Adiponectin positive staining pattern on mature fatty drop-filled adipocytes (arrows). (C) The negative staining control created by replacing specific adiponectin or cathepsin K antibodies with PBS on a rhBMP-2 sample. PBS: Phosphate buffered saline, rhBMP-2: Recombinant human bone morphogenetic protein-2. (Scale bar = 100μm)
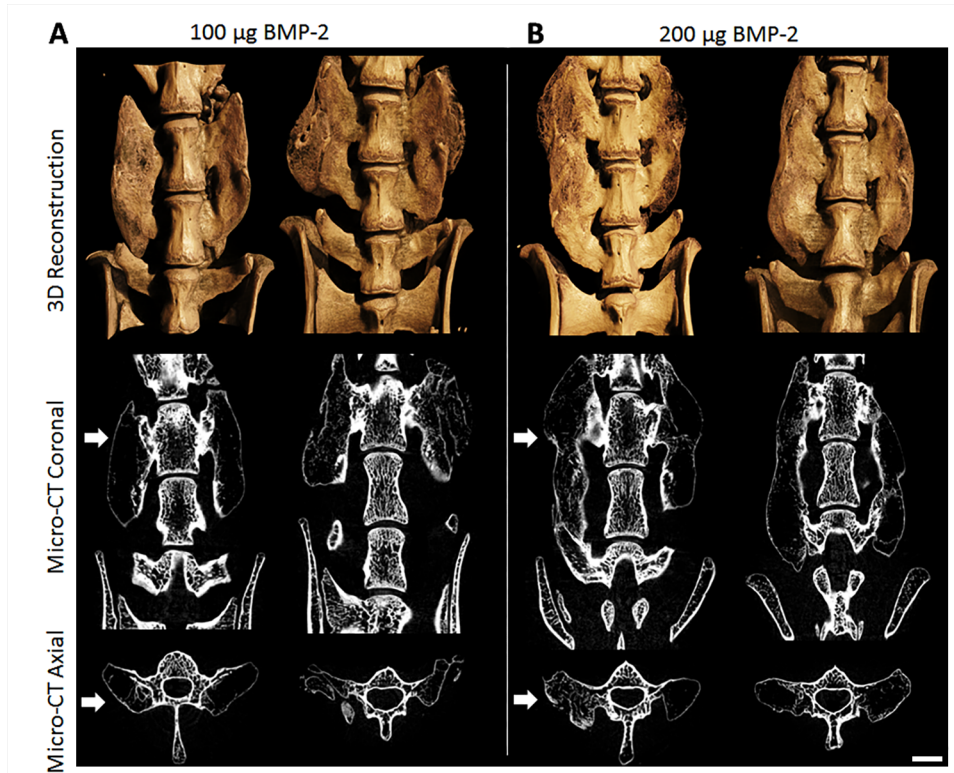
Supplementary Fig. 3. Rat posterolateral spinal fusion using rhBMP-2. As the preliminary study, posterolateral spinal fusions were performed using two different doses of rhBMP-2: (A) 100 μg treatment group and (B) 200 μg treatment group. Samples from the rats were harvested 3 weeks after surgery. While both groups had successful implant fusions, as observed in the three-dimensional reconstructed images, large volumes of cyst-like bone voids (arrow) were apparent in the orthogonal sectional images. As a result, the implant fusions were not completely solid. rhBMP-2: Recombinant human bone morphogenetic protein-2. (Scale bar = 3 mm)

Table 1. Fusion quality summary.

| | | Control (n = 4) | Dose-I (n = 4) | Dose-II (n = 4) |
|---|---|---|---|---|
| **Conventional CT** | | | | |
| Post-op[±] | Fusion (n, %) | 0 (0%) | 4 (100%)* | 4 (100%)* |
| month 2 | Volumetric deformity (n, %)[§] | 1 (25%) | 4 (100%)*[ǀ] | 0 (0%) |
| Post-op[±] | Fusion (n, %) | 1 (25%) | 4 (100%)* | 4 (100%)* |
| month 3 | Volumetric deformity (n, %) | 0 (0%) | 2 (50%) | 0 (0%) |
| **Micro-CT** | | | | |
| | Fusion (n, %) | 1 (25%) | 4 (100%)* | 4 (100%)* |
| Post-harvest | Cyst-like bone void (n, %)[¶] | 0 (0%) | 3 (75%)* | 4 (100%)* |
| | Core diameter (mm)[+] | $6.590 \pm 0.590$ | $5.919* \pm 1.311$ | $6.350 \pm 0.551$ |

*: Significant compared to control ($p < 0.05$).

[ǀ]: Significant compared to dose-II ($p < 0.05$).

[±]: Operation

[§]: Decreased bone formation within the spacer compared to the core dimension.

[¶]: Cyst-like bone void indicates number of samples containing any number of cyst-like bone void lesions.

[+]: Core diameter measurement taken at 5 vertical levels to measure circumferential changes at different levels inside the implants.

Supplementary Table 1. Summary of rhBMP-2 dose variations in spinal fusion studies

| Surgery | Lowest dose[¶] | | | Highest dose[¶] | | |
|---|---|---|---|---|---|---|
| | Dose (mg) | Fusion Rate | Complications | Dose (mg) | Fusion Rate | Complications |
| ALIF* | 3 | 100% | n/a[26] | 6 | 90.6% | Implant subsidence[27] |
| ACDF | 0.26 | 100% | n/a[28] | 2.1 | 100% | Dysphagia and cervical swelling[28] |
| | | | | 2.1 | 91.7% | Hematoma, swelling/breathing difficulties[29] |
| PCF | 1.8 | 100% | Superficial wound infection, adjacent-level degeneration[30] | 4.4 | 89.5% | Pseudoarthrosis, Wound complications[31] |
| | 1.3 | 100% | Neurologic deficit, swelling[32] | | | |
| PLF | 4.2 | 82.4% | Dural tear, cardiac problems, GI problems, UTI, neurological deficit, DVT, wound infection[33] | 42 | 96% | Superficial dehiscence, seroma, erythema, persistent discharge, wound infection, nonunion, post-op ileus, insomnia[34] |
| TLIF/ PLIF | 1.4 | 91.6% | Heterotropic ossification, perineural inflammatory cyst formation, vertebral body osteolysis with nonunion[35] | 12 | 44% | Pseudoarthrosis, dural tears, wound infections, neuroforaminal bone growth, osteolysis[36] |

Only human data utilizing rhBMP-2 without Medtronic commercial sponsorship were included except ref. 26, 34 since 2002 after US FDA approval.

* Approved indication by US FDA

¶Lowest or highest dose of rhBMP-2 for each type of surgery among the previous papers expressed by mg/level.

rhBMP-2: recombinant human bone morphogenetic protein-2, ALIF: anterior lumbar interbody fusion, ACDF: anterior cervical discectomy and fusion, PCF: posterior cervical fusion, PLF: posterolateral lumbar fusion, TLIF: transforaminal lumbar interbody fusion, PLIF: posterior lumbar interbody fusion, GI: gastrointestinal, UTI: urinary tract infection, DVT: deep vein thrombosis.

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