Ovariectomy-Induced Osteoporosis Does Not Impact Fusion Rates in a Recombinant Human Bone Morphogenetic Protein-2–Dependent Rat Posterolateral Arthrodesis Model

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Abstract

Study Design  Randomized, controlled animal study.
Objective  Recombinant human bone morphogenetic protein-2 (rhBMP-2) is frequently utilized as a bone graft substitute in spinal fusions to overcome the difficult healing environment in patients with osteoporosis. However, the effects of estrogen deficiency and poor bone quality on rhBMP-2 efficacy are unknown. This study sought to determine whether rhBMP-2-induced healing is affected by estrogen deficiency and poor bone quality in a stringent osteoporotic posterolateral spinal fusion model.
Methods  Aged female Sprague-Dawley rats underwent an ovariectomy (OVX group) or a sham procedure, and the OVX animals were fed a low-calcium, low-phytoestrogen diet. After 12 weeks, the animals underwent a posterolateral spinal fusion with 1 μg rhBMP-2 on an absorbable collagen sponge. Representative animals were sacrificed at 1 week postoperative for alkaline phosphatase (ALP) and osteocalcin serum analyses. The remaining animals underwent radiographs 2 and 4 weeks after surgery and were subsequently euthanized for fusion analysis by manual palpation, micro–computed tomography (CT) imaging, and histologic analysis.
Results  The ALP and osteocalcin levels were similar between the control and OVX groups. Manual palpation revealed no significant differences in the fusion scores between the control (1.42 ± 0.50) and OVX groups (1.83 ± 0.36; p = 0.07). Fusion rates were 100% in both groups. Micro-CT imaging revealed no significant difference in the quantity of new bone formation, and histologic analysis demonstrated bridging bone across the transverse processes in fused animals from both groups.

Keywords

► spinal fusion
► rhBMP-2
► osteoporosis
► rats
► pseudarthrosis
**Introduction**

Osteoporosis, a metabolic disease characterized by reduced bone mineral density and deterioration of bony microarchitecture, affects over 12 million people in the United States.\(^1\) Resulting in the compromise of load-bearing capacity of bones, the disease contributes to over 2 million fractures each year, including 547,000 involving the spine.\(^2\) These vertebral fractures account for over $1 billion in health care costs in the United States annually. With an aging population, the burden of osteoporosis on the health care system is expected to worsen, as the number of fractures is projected to increase by 50% by 2025.\(^3\)

Patients with osteoporosis are not only at increased risk of fractures; poor bone healing in these individuals often compounds the difficulty associated with orthopedic procedures. Spinal fusion, a surgical procedure utilized to increase the stability of adjacent vertebral bodies, is performed in over 490,000 individuals in the United States annually.\(^4\) Despite advances in surgical technique, pseudarthrosis after spinal surgery occurs in 30% of patients with osteoporosis,\(^5\) with the associated estrogen deficiency remaining a strong risk factor for instrumentation failure and subsequent nonunion.\(^6\)\(^–\)\(^9\)

Although autologous iliac crest bone grafting has been the historical gold standard to achieve successful fusion, complications including persistent donor site pain have encouraged the development and use of bone graft substitutes including growth factors such as recombinant human bone morphogenetic proteins (rhBMPs).\(^10\)\(^–\)\(^11\) Utilization of these proteins in spinal fusion has increased dramatically since 2001, with use in over 30% of primary posterolateral fusions and nearly half of all posterior lumbar interbody fusions.\(^12\)

Despite the advances in bone graft substitutes, little is known of the effects of estrogen deficiency and metabolic bone disease on the osteoinductive capacity of these growth factors. Utilizing a well-established osteoporotic animal model (ovariectomized rats), Moazzaz et al studied the effect of rhBMP-7 in posterolateral transverse process spinal fusion and concluded that the growth factor was unable to overcome the inhibitory effect of estrogen deficiency on spinal fusion.\(^13\)

Meanwhile, Lu et al employed the same animal model and determined that rhBMP-7 delivered on a composite carrier with a threefold higher dose was able to overcome an estrogen-deficient state and successfully achieve fusion.\(^14\)

Despite the prevalence of rhBMP-2 in spinal fusion procedures, our understanding of the effects of estrogen deficiency and bone quality on healing with the use of this growth factor is limited. Recent controversy surrounding the use of supraphysiologic doses of rhBMP-2 in this setting has caused many surgeons to reduce the dose they use in fusion procedures. As such, understanding the potential complications and limitations of rhBMP-2 use in an osteoporotic animal model would not only provide surgeons with valuable insight into the clinical value of the product for use in osteoporotic individuals, but could also provide an indication of whether dosing should be adjusted. To this end, we employed an osteoporotic posterolateral spinal fusion model to determine whether established estrogen deficiency and decreased bone mineral density impact fusion rates elicited by rhBMP-2.

**Materials and Methods**

**Ovariectomy Procedure**

Institutional Animal Care and Use Committee approval was obtained prior to all animal procedures. Twenty-four female Sprague-Dawley rats aged to 24 weeks underwent either an ovariectomy (OVX group) or a sham procedure (\(\rightarrow\) Fig. 1). Animals were maintained under continuous anesthesia with an inhalational isoflurane anesthetic delivery system and were monitored by an assistant for cardiac and respiratory anomalies throughout the procedure. Each animal in the OVX group (n = 12) underwent a bilateral ovariectomy using a previously described surgical technique,\(^14\) and each animal in the control group (n = 12) underwent a sham procedure. In the investigational group, a 5-mm incision was made through the skin, fat, and muscle at the angle formed by the lowest rib and the spinal column. The uterine horn was tied with a 3–0 Monocryl absorbable suture (Ethicon, Inc., Somerville, NJ, United States), and the ovary and associated fat pad were resected. After wound closure, the procedure was repeated on the contralateral side. Warmed lactated Ringer’s solution (5 mL) was then administered intraperitoneally for fluid replacement. The rats were maintained on a heating pad and checked every 15 minutes until they fully recovered. Analgescs were administered for up to 3 days as needed. The animals in the OVX group were placed on a low-calcium (0.2%), low-phytoestrogen diet after the surgery, and the control rats continued to receive a standard diet.

**Spinal Fusion Procedure**

Twelve weeks after ovariectomy or sham procedure, each animal underwent a spinal fusion procedure under continuous anesthesia with the same anesthetic delivery system. Using our previously described surgical technique,\(^15\)\(^–\)\(^16\) two separate fascial incisions were made 4 mm from the midline exposing the L4 and L5 transverse processes. rhBMP-2 (1 µg) on an absorbable collagen sponge (ACS) measuring 2 x 2 x 16 mm was implanted bilaterally between the L4 and L5 transverse processes after decortication with an oscillating burr. This dose was chosen based on previous studies that

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**Conclusions** This study demonstrates that estrogen deficiency and compromised bone quality do not negatively influence spinal fusion when utilizing rhBMP-2, and the osteoinductive capacity of the growth factor is not functionally reduced under osteoporotic conditions in the rat. Although osteoporosis is a risk factor for pseudarthrosis/nonunion, rhBMP-2-induced healing was not inhibited in osteoporotic rats.
found that 1 µg rhBMP-2/ACS elicits fusion at a rate ranging from 40 to 100%, indicating that this dose approaches but does not exceed the threshold dose required for successful fusion in this model.\textsuperscript{17,18} After graft implantation, the fascial incisions were closed with a 3-0 Monocryl absorbable running suture. The same postoperative care including fluid replacement and analgesia as after the OVX or sham procedure was provided. Although all animals recovered from surgery without incident, one animal from the control group was found deceased 5 days postoperatively.

**Alkaline Phosphatase and Osteocalcin Activity Assays**

One week after the spinal fusion procedure, four animals from the OVX group and three animals from the control group underwent serum cytokine analysis obtained via cardiac puncture before the animals were euthanized. Whole blood was transferred to microfuge tubes and allowed to settle for 10 minutes at room temperature. Samples were then centrifuged at 10,000g for 10 minutes, and serum supernatants were then removed and stored at -80°C until analysis. The alkaline phosphatase (ALP) activity was quantitated using a colorimetric assay according to the manufacturer's instructions (Anaspec, Inc., Fremont, CA, United States). Absorbance was read at 405 nm on a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA, United States) at the Simpson Querrey Institute for BioNanotechnology at Northwestern University. The U.S. Army Research Office, the U.S. Army Medical Research and Materiel Command, and Northwestern University provided funding to develop this facility.

Osteocalcin activity was quantitated according to the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA, United States). Diluted (1:20) serum (25 µl) was mixed with 100 µl of osteocalcin antiserum and incubated at 37°C for 3.5 hours. The plates were then washed five times with phosphate-buffered saline and incubated with 100 µl of donkey anti-goat immunoglobulin G peroxidase for 1 hour. The plates were again washed three times with phosphate-buffered saline and then incubated with 100 µl of 3,3',5,5'-tetramethyl benzidine and hydrogen peroxide for 30 minutes. The reaction was terminated with a stop solution and absorbance was read at 450 nm. Serial dilutions were utilized to generate the standard curves to determine protein concentration.

**Assessment of Bone Mineral Density with Radiographs**

All eight animals in each treatment group underwent in vivo plain anteroposterior radiographs with an APR-VET Console (Sedecal USA, Inc., Buffalo Grove, IL, United States) at both 2

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**Fig. 1** Study design. Animals were divided into two groups of 12 animals. Animals in both groups were aged to 24 weeks and underwent either a sham procedure (control group) or ovariectomy (OVX group). Prior to undergoing the spine fusion procedure, the control group received a regular diet and the OVX group received a low-calcium, low-phytoestrogen diet for 12 weeks. One week after the spine fusion procedure, four animals from the OVX group and three animals from the control group underwent serum cytokine analysis. The other eight animals in each group were euthanized at 4 weeks postoperatively for further evaluation with wet uterine weights, radiographs, manual palpation, micro-computed tomography (microCT), and histology.
and 4 weeks following the spinal fusion procedure. As in our previously published work,\textsuperscript{15} these times were chosen to obtain radiographs at the halfway point (2 weeks) and the time of euthanasia (4 weeks). Settings including voltage, current, exposure time, and distance were kept constant for all radiographs to standardize brightness and contrast. Radiopacity analysis was performed on the radiographs taken at both 2 and 4 weeks with ImageJ software (National Institutes of Health, Bethesda, MD, United States). Rectangular regions of interest (ROIs) were standardized to size and location and drawn to include the L3 vertebral body but not the transverse processes. The mean gray values within these ROIs were calculated using voxel-based content calculations with ImageJ software. The L3 vertebral body was chosen because it was not manipulated during the spinal fusion procedure yet remained in close proximity to the surgical site.

**Assessment of Reduced Estrogen State with Wet Uterine Weights**

At 4 weeks, animals were sacrificed and uteri were harvested for further evaluation. Each uterus was weighed immediately after harvest to obtain a wet uterine weight as a marker of successful OVX.

**Determination of Fusion**

The lumbar spines were harvested for further evaluation at 4 weeks, immediately after harvesting uteri. All spines were manually palpated for evidence of successful fusion by three independent, blinded observers using a previously published scoring system,\textsuperscript{15–17} in which 0 indicates no fusion, 1 indicates fusion unilaterally with evidence of bridging bone, and 2 indicates fusion bilaterally with bridging bone. The fusion scores for each specimen were averaged. Any spine with an average score greater than or equal to 1 (unilateral fusion) was considered to be successfully fused. Previous studies have demonstrated the interobserver variability with this protocol to be very low.\textsuperscript{16,17}

**Micro–Computed Tomography Analysis**

Four specimens per group underwent three-dimensional micro–computed tomography (CT) analysis to compare the amount of new bone formed between the L4–L5 transverse processes, using a Scanco MicroCT-40 system (Scanco Medical AG, Brüttisellen, Switzerland). In a modification of our previously described protocol,\textsuperscript{15} two spines were scanned simultaneously, with their axes parallel to the rotation axis of the scanner. The X-ray tube was operated at 70 kVp and 114 µA, with an integration time of 200 milliseconds per projection. Micro-CT scans were performed with 37-µm isotropic volume elements (voxels), and the 540 contiguous slice data sets encompassed the L4 and L5 transverse processes.

The amount of newly formed bone between the L4 and L5 transverse processes was quantified for each specimen using ImageJ software analysis tools. To accomplish this, the two posterolateral intertransverse fusion beds of each spine were included in the manually defined ROI for each slice. Voxels absorbing more than a predefined threshold were identified as bone and summed using the Scanco software (Scanco Medical AG, Brüttisellen, Switzerland) to give the total bone volume. A 100 mg/cm\textsuperscript{3} threshold was identified by examining several specimens and selecting the value best reproducing the structure of newly formed bone, as seen in the grayscale reconstructions. The host bone volume in the L4 and L5 transverse processes was quantified in four control animals outside of the study groups and averaged (266 ± 32 mm\textsuperscript{3}). The volume of new bone formed was calculated by subtracting the mean host bone from the total bone volume measured in each animal.

**Histologic Analysis**

Representative spines from each group underwent histologic analysis, which was performed utilizing a previously described protocol at the Northwestern University Mouse Histology and Phenotyping Laboratory Core Facility. This facility is supported by a Cancer Center Support Grant (NCI CA060553)\textsuperscript{15,16} After detachment of surrounding soft tissue, spine specimens were fixed in 10% neutral-buffered formalin, decalcified in HCl/ethylenediaminetetraacetic acid, and embedded in paraffin. Serial sagittal 4-mm cuts were made along the L4 and L5 transverse processes. The sections were subsequently stained with hematoxylin and eosin. The selection criteria for histologic sections to be analyzed include sections that (1) were perpendicular to the transverse process, (2) demonstrated cancellous bone within the transverse processes (not too lateral), (3) demonstrated interposing soft tissue between transverse processes at other vertebral levels (not too medial), and (4) had minimal artifact in histologic and cytological preparation.

**Statistical Analysis**

Statistical analysis was performed using SPSS Statistics v. 21.0 (SPSS Inc., Chicago, Illinois, United States). Uterine wet weights, mean gray value, fusion scores, ALP activity, and osteocalcin activity were compared between the control and OVX groups with two-tailed Student \textit{t} tests for normally distributed data. The fusion rates between the groups were compared utilizing a Fisher exact test. Statistical significance was accepted with a \textit{p} value less than 0.05.

**Results**

The mean uterine wet weight at sacrifice of the control group was 556.3 ± 168.2 mg (standard deviation), and the mean weight of the OVX group was significantly lower, 78.9 ± 15.9 mg (\textit{p} < 0.001). The lowest uterine wet weight in the control group was 374.0 mg, and the highest uterine wet weight in the OVX group was 101.3 mg.

Radiographic image analyses qualitatively identified unilateral or bilateral bridging bone at the L4–L5 transverse processes in the animals in both groups (\textit{\textbullet} Fig. 3a and 3b). When comparing the mean gray value of the L3 vertebral body in the radiographs taken 2 weeks after surgery, the mean of the control group (97.8 ± 8.4 units) was significantly greater than the mean of the OVX group (82.8 ± 7.6 units; \textit{\textbullet} Fig. 3c; \textit{p} = 0.002). In the radiographs taken 4 weeks after surgery, the mean of the control group (88.4 ± 6.5 units)
was significantly greater than the mean of the OVX group (79.7 ± 7.3 units; Fig. 3d; p = 0.02).

Manual palpation of the harvested spines revealed no significant differences in the fusion score (p = 0.07) or fusion rate (p = 1.00) between the two groups. The mean manual palpation fusion score of the control group was 1.42 ± 0.50 and that of the OVX group was 1.83 ± 0.36 (Fig. 4a). Based on manual palpation scores, all animals in the control and OVX groups were considered fused at 4 weeks, and accordingly, the fusion rates of both groups were 100 ± 0% (Fig. 4b).

Micro-CT analysis demonstrated that the mean new bone formation in the OVX group (1312.7 ± 382.9 mm³) was lower than that of the control group (1646.9 ± 621.2 mm³) at 4 weeks (Fig. 5). However, this difference was not statistically different (p = 0.39).

The serum ALP activity levels were similar between the two groups at 1 week postoperatively. The mean ALP activity level for the control group was 5.22 ± 2.24 ng/mL and for the OVX group was 2.31 ± 0.89 ng/mL (Fig. 6a, p = 0.08). The serum osteocalcin levels were also similar between the two groups. The mean osteocalcin level for the control group was 14.6 ± 2.23 ng/mL and for the OVX group was 13.73 ± 1.12 ng/mL (Fig. 6b, p = 0.35).

The histologic analysis demonstrated fusion mass with evidence of collagenous tissue and woven bone between the transverse processes of L4 and L5 in the spine specimens from both groups (Fig. 7). Mineralization was present throughout each fusion mass.

**Discussion**

Advancing age leads to an increase in bone resorption by osteoclasts at the endosteal surface relative to new bone formation by osteoblasts in the region called a basic multicellular unit, which results in net bone loss. In women, menopause accelerates the loss of bone because the fall in circulating estrogen levels results in an increase in the number of basic multicellular units. Accordingly, the increase in bone remodeling after menopause leads to trabecular thinning and increased intracortical porosity. This decrease in bone mineral density contributes to a weaker bone–metal interface in orthopedic procedures including spinal fusion.

In addition, decreased bone quality in patients with osteoporosis negatively impacts the tissue’s structural competence, and accordingly, may complicate bone healing itself.

An animal model for osteoporosis utilizing ovariectomized rats has previously been established. In this study, we utilized this model with stringent criteria, which included the ovariectomy surgical procedure, rats aged to 24 weeks prior to ovariectomy then to 36 weeks prior to posterolateral fusion, and a low-calcium, low-phytoestrogen diet. The highly significant decrease in the uterine weight between the two groups demonstrated the successful implementation of the ovariectomy procedure in all animals in the OVX group and an estrogen-deficient state in those animals. Moreover, the
significant difference in the radiographic mean gray values of the L3 vertebral body indicated a significant reduction in the bone mineral density of animals in the OVX group compared with those in the control group. The histologic analyses in previous studies utilizing the OVX-induced osteoporosis have also demonstrated a decrease in bone mineral density as well as an increase in the number of osteoclasts in this model.12,22

Autologous iliac crest bone graft is the historical gold standard for spinal fusions in humans. Although its success is dependent on bone quality, which is negatively impacted by osteoporosis, successful fusions have been achieved with iliac crest bone graft in osteoporotic individuals.23–25 However, neither autograft nor absorbable collagen sponge alone leads to a reliable fusion in the rodent posterolateral model,26–28 Accordingly, reliable healing in this model is dependent on the use of bone graft substitutes, such as rhBMP-2/ACS. Park et al recently showed that when combined with iliac crest autograft and a collagen scaffold, 90 µg rhBMP-2 elicited fusion in 6/6 rats in a similar osteoporotic model.26 However, this rhBMP-2 dose exceeds that required to achieve successful fusion in this model by 9- to 30-fold.15–18,28,30 Furthermore, the 90 µg of rhBMP-2 was applied only to one side of the spine, whereas dosing for this model is typically reported on a per animal basis, with the total dose divided between both L4–L5 fusion beds. We hypothesized in this study that if the rhBMP-2 dose sufficiently exceeds the threshold required for successful fusion in this model, any impaired fusion capacity in an osteoporotic model might be masked by the exceedingly high dose of the growth factor.

Historically, 3 to 10 µg rhBMP-2/ACS has proven sufficient to achieve a 100% fusion rate in this model.15–18 Therefore, we chose a dose expected to elicit fusion at a rate approaching 100% (i.e., a near-therapeutic dose). Previous studies have indicated that that 1 µg rhBMP-2/ACS represents a dose expected to achieve fusion at a rate of 40 to 100%.17,18 Therefore, with this experimental approach, if the osteoporotic condition were to in fact lower the efficacy of rhBMP-2, that effect could be observed experimentally. To the contrary,
we found in this study that even at the low dose of 1 µg rhBMP-2/ACS, not only was successful fusion achieved in all animals in both groups, but the average fusion scores between the two groups were also not significantly different. We concluded that rhBMP-2-dependent healing in this animal model was not affected by the decreased circulating estrogen levels and decreased pre-existing bone quality in the OVX group. Further studies are needed to determine if the effect of estrogen deficiency and bone quality impacts the efficacy of other bone graft substitutes.

rhBMP-2 may have the potential to overcome the difficult bony microenvironment in the setting of osteoporosis by several different mechanisms. Turgeman et al showed that systemic intraperitoneal injections of rhBMP-2 in osteopenic mice resulted in the increase of proliferation of bone marrow–derived mesenchymal stem cells, the increase in their osteogenic activity, and the decrease in apoptosis of these cells.\(^{31}\) In an ovine model of osteoporosis, Wu et al demonstrated that local administration of rhBMP-2 in vertebrae resulted in increased bone mineral density, greater biomechanical strength, and improved microarchitecture, including greater trabecular thickness and less trabecular separation.\(^{32}\) Accordingly, these effects of rhBMP-2 may be enough to improve the poor healing microenvironment of osteoporosis in spinal fusion to render the healing with rhBMP-2 independent of the bone quality. In our study, the results revealed no statistically significant difference in new bone formation as determined by quantitative micro-CT imaging analysis in the OVX group compared with the control group. Moreover, our results suggest that it may not be necessary for surgeons to use a higher rhBMP-2 dose (more INFUSE kits, for example) to achieve fusion in patients with osteoporosis. This finding is significant in light of recent concerns over the use of supraphysiologic doses of rhBMP-2 in humans.\(^{32-36}\)

In this study, we found that the ALP activity 1 week after surgery in the OVX group was less than that of the control group, with a trend toward significance (\(p = 0.08\)). Atalay et al demonstrated that the serum ALP activity, a measure of bone turnover, was elevated in postmenopausal women in comparison with premenopausal women.\(^ {37}\) In the osteoporotic rodent model, ALP levels have been shown to be elevated in OVX animals compared with controls.\(^ {38,39}\) In this study, there was a trend toward significance of lower ALP levels in the OVX group. However, we measured the ALP levels 1 week after spinal fusion surgery, and this essential period of bone healing may not reflect the same metabolic state as other studies that measured the ALP activity in this model. Instead, a decreased ALP value during this period may reflect decreased bone formation in the OVX group in comparison with the control group. In addition, we found no difference in serum osteocalcin levels 1 week after surgery. Atalay et al demonstrated that serum osteocalcin, a noncollagenous protein produced and released by osteoblasts, was elevated in postmenopausal women in comparison with premenopausal women.\(^ {37}\) In vitro studies have demonstrated that osteocalcin is also released during bone resorption, suggesting that it should be considered a marker for bone turnover as well.\(^ {40}\) However, osteocalcin-deficient rats were found to have increased bone formation without impaired bone formation.\(^ {41}\) Further studies on the changing profile of the ALP and osteocalcin levels after spinal fusion and orthopedic procedures are needed to fully understand its diagnostic utility.

There are several limitations to this study. First, the findings in the osteoporotic rodent posterolateral spinal fusion

![Fig. 6](image_url)

**Fig. 6** Serum (a) alkaline phosphatase (ALP) and (b) osteocalcin levels in the control and ovariectomy (OVX) groups measured 1 week postoperatively.

![Fig. 7](image_url)

**Fig. 7** Hematoxylin and eosin stain of sagittal cross sections of the transverse processes at L4 and L5 of animals from (a) the control and (b) ovariectomy groups. Qualitative analysis demonstrates solid fusion masses between L4 and L5 transverse processes in both groups with no substantial differences.
model are specific to this animal, and its applicability to clinical practice is unproven. In addition to the different anatomy of the spine compared with humans, bone healing in rats occurs at a faster rate due to their higher metabolic rate. Studies in higher phylogenetic species that better mimic human anatomy and allow similar surgical techniques as in clinical practice may provide further evidence on the effect of estrogen deficiency on spinal fusion with rhBMP-2. In addition to comparison of quantitative bone formation, the biomechanical strength of the fusion mass, which was not evaluated in this study, may provide more information on the quality of fusion between the two groups. We felt that a global difference in bone quality between the groups would have confounded any potential differences seen in the data. Finally, we assessed the effect of estrogen deficiency on only a single bone graft substitute and did not vary the dose of rhBMP-2 used in the study. Accordingly, further evaluation of different bone graft substitutes at varying doses in an osteoporotic animal model may provide more information on the effects of estrogen deficiency in spinal fusion.

In conclusion, rhBMP-2-dependent healing in a rodent posterolateral spinal fusion model was not dependent on the circulating estrogen levels and bone quality. This study suggests that estrogen deficiency does not negatively influence spinal fusion when utilizing rhBMP-2.

Disclosures
Jason H. Ghodasra, none
Michael S. Nickoli, none
Sohaib Z. Hashmi, none
John T. Nelson, none
Marco Mendoza, none
Joseph D. Nicolas, none
Sharath S. Bellary, none
Kevin Sonn, none
Amruta Ashitekar, none
Christian J. Park, none
Jacob Babu, none
Chawon Yun, none
Anjan Ghosh, none
Abhishek Kannan, none
Stuart R. Stock, none
Wellington K. Hsu, Consultant: Stryker, Globus, AONA; Synthes, Medtronic, Pioneer, Bioventus, Lifenet; Speaker’s bureau: AONA; Editor: Journal of Spinal Disorders and Techniques; Board membership: American Academy of Orthopaedic Surgery, Cervical Spine Research Society, Lumbar Spine Research Society, North American Spine Society; Research support: Medtronic
Erin L. Hsu, none

Acknowledgment
This study was supported by the Department of Orthopaedic Surgery at the Northwestern University Feinberg School of Medicine.

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