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Combined Use of Low Intensity Pulsed Ultrasound and rhBMP-2 to Enhance Bone Formation in a Rat Model of Critical-Size Defect

Siddhesh R. Angle^{1,3,*}, Kotaro Sena^{1,**}, Dale R. Sumner^{1,2}, Walter W. Virkus², and Amarjit S. Viridi^{1,2,+}

¹Department of Anatomy and Cell Biology, Rush Medical College, Rush University Medical Center, Chicago, IL 60612

²Department of Orthopedic Surgery, Rush Medical College, Rush University Medical Center, Chicago, IL 60612

³Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607

Abstract

Background—Bone repair is regulated by biological factors and the local mechanical environment. We hypothesize that the combined use of low intensity pulsed ultrasound (LIPUS) and recombinant human bone morphogenetic protein-2 (rhBMP-2) will synergistically or additively enhance bone regeneration in a model simulating the more difficult scenarios in orthopedic traumatology.

Methods—Femoral defects in rats were replaced with absorbable collagen sponges carrying rhBMP-2 (0, 1.2, 6 or 12µg; n=30). Each group was divided equally to receive daily treatment of either LIPUS or sham stimulation. At 4 weeks new bone formation was assessed using quantitative (radiography and microcomputed tomography), qualitative (histology) and functional (biomechanical) endpoints.

Results—LIPUS with 1.2µg of rhBMP-2 significantly improved the radiographic healing as compared to its sham control starting as early as 2 weeks. Quantitatively, the use of LIPUS with 6µg of rhBMP-2 significantly increased the bone volume. However, using LIPUS with 12µg of rhBMP-2 indicated a reduction in callus size, without compromising the bone volume, which was also observable histologically, showing organized lamellar bone and repopulated marrow in the original defect region. Histologically, 1.2µg of rhBMP-2 alone showed the presence of uncalcified

⁺Corresponding author: Amarjit S. Viridi, Ph.D. Anatomy and Cell Biology, 600 S. Paulina Street, Rm 507, AcFac, Chicago, Illinois 60612, Tel: (312) 942 5143, Fax: (312) 942 5744, amarjit_viridi@rush.edu.

^{*}**Present address:** Institute for Complex Engineered Systems, Carnegie Mellon University, 5000 Forbes Avenue, 2214 Hamburg Hall, Pittsburgh, PA 15213

^{**}**Present address:** Department of Periodontology, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima, 890-8544, JAPAN

Siddhesh R. Angle, Ph.D., Anatomy and Cell Biology, 600 S. Paulina Street, Rm 507, AcFac, Chicago, Illinois 60612

Kotaro Sena, D.D.S., Ph.D., Anatomy and Cell Biology, 600 S. Paulina Street, Rm 507, AcFac, Chicago, Illinois 60612

D. Rick Sumner, Ph.D., Anatomy and Cell Biology, 600 S. Paulina Street, Rm 507, AcFac, Chicago, Illinois 60612

Walter W. Virkus, M.D., Orthopedic Surgery, 1611 W. Harrison Street, Chicago, Illinois 60612

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cartilage in the defect which was reduced with LIPUS treatment. Biomechanically, LIPUS treatment significantly increased the peak torsion and stiffness in the 6µg and 12µg rhBMP-2 group.

Conclusion—LIPUS enhances rhBMP-2 induced bone formation at lower doses (1.2µg and 6µg) and callus maturation at 12µg dose delivered on ACS for bone repair in a rat critical-sized femoral segmental defect.

Keywords

rhBMP-2; Low Intensity Pulsed Ultrasound; Critical size defect; trauma; bone regeneration

Introduction

Traumatic injuries, non-unions or orthopedic interventions such as bone tumor resection may result in impaired healing in large defects. While the conventional method of autologous bone grafting has potential complications at the donor and/or host site¹⁻³, the alternative of using allografts can increase complications such as immunorejection and transmission of pathogens^{4,5}. This situation is creating a huge burden on fracture care and limits the options for surgeons to repair large defects.

Tissue engineering utilizes the innate potentials of cells to regenerate and repair defective tissues. Mechanism of bone repair is complex and regulated by multiple factors, including ECM scaffolds, angiogenic and osteogenic growth factors, and the osteogenic potential of cells. Because of the relative ease of use, local delivery of growth factors for bone repair is now becoming a common clinical approach^{6,7}. Many growth factors play important roles in the cascade of fracture healing and have shown satisfactory results in healing fractures⁸⁻¹². Furthermore, the mechanical environment at the repair site also plays a very important role in bone healing by altering the bio-physical signals¹³. Low-intensity pulsed ultrasound (LIPUS) stimulation is a clinically established, widely used and FDA approved intervention for accelerating bone growth during healing of fractures, non-unions and other osseous defects¹⁴⁻²⁰. LIPUS has been shown to increase the rate of fracture repair at all stages of the healing process, and is most effective when applied during all stages of the repair²¹.

Previous studies have reported on combining growth factors or engineering cues that influence cell behavior to enhance bone regeneration²²⁻²⁴. Like most other stimuli, LIPUS and rhBMP-2 are also known to exert their action on cells via intracellular signaling. They may work through analogous yet distinct mechanism, raising the possibility that their combined use might be more effective than the use of either one alone. However there is no direct *in vivo* evidence on their combined interactions on bone formation. Nonetheless, Sant'Anna et al, have shown the combination to cause a temporal variation in the expression of osteogenic genes indicating the difference in signal transduction pathways of the stimulus in bone marrow stromal cells²⁵. Wijdicks et al, have reported LIPUS to significantly increase the bone volume in the 1µg and 5µg rhBMP-2 treated implants in an ectopic model²⁶.

We have reported that in the rat critical-sized defect rhBMP-2 dose response curve exhibited a bi-phasic mode, with the calculated optimum dose of $12\mu\text{g}$ ²⁷. In the current study, we hypothesize that the combined use of LIPUS and rhBMP-2 will synergistically or additively enhance bone regeneration in a model system simulating the more difficult scenarios in orthopedic traumatology. We quantitatively, qualitatively and functionally assessed the outcome of combining LIPUS with optimum ($12\mu\text{g}$) and sub-optimum ($10\%=1.2\mu\text{g}$ and $50\%=6\mu\text{g}$) dose of rhBMP-2.

Materials and Methods

Study Design and Surgical Model

A 5-mm mid-diaphyseal segment of bone was removed from the left femur of male Sprague-Dawley rats (400–425g) and replaced with an absorbable collagen sponge (ACS) soaked with either buffer ($0\mu\text{g}$) or $1.2\mu\text{g}$, $6\mu\text{g}$, or $12\mu\text{g}$ rhBMP-2 ($n=30$ per main group)^{26,27}. Each group was further divided equally to receive daily treatment of either LIPUS or sham stimulation ($n=15$ for each group studied). 12 additional animals were used as intact controls. All animals were sacrificed at 4 weeks and denuded femurs stored at -20°C .

Low-intensity pulsed ultrasound treatment

LIPUS was administered daily for 20 min for four weeks using a EXOGEN™ Device ($30\text{mW}/\text{cm}^2_{\text{SATA}}$, 1.5 MHz, 20% duty cycle, 1kHz repetition rate) (Smith & Nephew Inc., Memphis, TN, USA) under 1–3% isoflurane anesthesia. Sham animals were handled the same as LIPUS animals, except the power to the LIPUS generator was not turned on.

Radiographs

Medio-lateral radiographs at 30 kVp for 10s (Faxitron MX-20, Lincolnshire, IL) were obtained each week to examine the progression of bone healing. In addition, orthogonal radiographs of harvested femora were obtained at sacrifice. Radiographs were scored for callus formation, quality of union, and bone remodeling by 2 independent blinded observers according to a published scoring system²⁸.

Microcomputed Tomography

A 10mm mid-shaft region of the bones spanning the defect was scanned in saline using $30\mu\text{m}$ isotropic voxels at 55kVp, $145\mu\text{A}$, and 300ms integration time ($\mu\text{CT}40$, Scanco Medical, Wayne, PA). The manufacturer's software was then used to filter noise ($\text{Sigma}=0.8$, $\text{Support}=1$) and segment the data for the newly formed bone at a threshold of 1432 Hounsfield Units in the created defect. The bone volume (BV, mm^3), total tissue volume (TV, mm^3), percentage of bone volume over tissue volume (BV/TV) and bone mineral content (BMC, mg of HA) were calculated. A 100 slice thick mid-shaft region of the intact bones was scanned and evaluated for BV/TV using identical methods as above.

Mechanical torsion tests

All torsion tests were performed using a custom designed linear to torsion test fixture (Test Resources, Shakopee, MN) along with a uniaxial testing frame (MTS Insight 5, Eden

Prairie, MN). Femurs were thawed to room temperature and embedded distally and proximally into acrylic resin, exposing 9 mm of the diaphysis. The femurs were subjected to a slow load in external rotation on the distal end at 3°/sec until failure^{29,30}. The torsion stiffness (N-m/deg), ultimate torsion strength (UTS, N-m) and maximum degree of deformation (deg) was calculated from the displacement curve.

Histology

For undecalcified histology, the specimens (n=2, per group) were embedded in polymethylmethacrylate (PMMA), longitudinally sectioned (100µm) and stained with basic fuchsin-toluidine blue³¹. For decalcified histology, one representative sample from each group was decalcified and embedded in paraffin. Serial sections (6µm) were stained with Hematoxylin-Eosin, Fast Green-Safranin-O or Tartarate Resistant Acid Phosphatase (TRAP)-Fast Green-Hematoxylin.

Statistical analyses

All data were expressed as mean $\pm$ standard error of the mean (SEM). One sample was excluded from the (12µg rhBMP-2+LIPUS) group due to misalignment of the fixator. The normality of the data was examined so that the appropriate parametric or nonparametric tests can be used. For the morphological endpoints from µCT, radiographic score and the mechanical endpoints, we used multivariate ANOVA (with dose and group as the main factors, with the dose-by-group interaction also examined) because they have relatively large sample sizes and were normally distributed. The effect of LIPUS on its respective sham control was analyzed using Student's t-test. Data analysis and plotting used commercial programs (SPSS, version 14.0; Microsoft Excel version 2007; GraphPad Prism 5.04).

Results

Mean body weight at the time of surgery was 430.10 g (SEM = 2.39) and on day of sacrifice was 495.94 g (SEM = 3.33) with no significant differences in weight gain between groups.

Radiographic Evaluation

The *in vivo* radiograph showed a significant increase in healing with the use of LIPUS with 1.2µg of rhBMP-2 at 2 weeks (Fig. 1) compared to sham control. There was also a significant increase in the healing observed with 6µg rhBMP-2+LIPUS and 12µg rhBMP-2+LIPUS from 2 weeks onwards as compared to all other groups.

The *ex vivo* radiographs showed that all doses of rhBMP-2 resulted in increased healing as compared to the control (Fig. 2). While control and LIPUS only defects continued to exhibit non-unions, all rhBMP-2 treated defects showed a progressive bridging with varied levels of callus formation (Fig. 2). The contact radiograph showed a near significant increase in radiographic healing with the use of LIPUS and 1.2µg rhBMP-2 compared to 1.2µg rhBMP-2 and sham at 4 weeks. There was also a significant increase in the healing with 6µg rhBMP-2, 6µg rhBMP-2+LIPUS (6.70 $\pm$ 0.94), 12µg rhBMP-2 and 12µg rhBMP-2+LIPUS as compared to all other groups. Defects with higher doses of rhBMP-2 (6µg and 12µg) showed

increased bridging in the gap with some levels of remodeling observed with the use of LIPUS as evident in Fig. 2.

Microcomputed Tomography (μ CT)

There was a significant increase in BV when LIPUS was used with 6 μ g rhBMP-2 compared to 6 μ g rhBMP-2 sham control at 4 weeks (Table I). The use of LIPUS alone (0 μ g rhBMP-2) did not show significant increase in the BV when compared to its respective sham control. However, 6 μ g $\pm$ LIPUS and 12 μ g $\pm$ LIPUS exhibited a significant increase in the BV compared to sham control, LIPUS control and 1.2 μ g $\pm$ LIPUS. A synergistic increase was observed with the use of LIPUS with the 6 μ g rhBMP-2 as compared to LIPUS control. This BV with 6 μ g rhBMP-2 with LIPUS was comparable to the BV with 12 μ g of rhBMP-2 sham. At 4 weeks the BMC showed similar trend to the BV (Table I).

The 3D reconstruction showed a progressive increase in bridging with the use of rhBMP-2 while the control groups showed minimal or no bridging. The newly formed bone was not only evident in the defect but also on the periosteal and endocortical surfaces of the native bone (Fig. 3). However, there appeared to be a decrease in the callus volume with the use of LIPUS with 1.2 μ g and 12 μ g rhBMP-2. This was apparent when we evaluated TV in the defect region (Table I). Thus, to estimate the correct amount of bone formation in the defect region and account for the potential remodeling occurring in the LIPUS groups, we evaluated the BV/TV for all the samples which is the ratio of mineralized tissue to the total callus tissue. A significant increase was observed in the 1.2 μ g rhBMP-2 when LIPUS was added (Table I). A similar significant increase was also observed in the optimum dose of 12 μ g rhBMP-2 when LIPUS was added.

Biomechanical Test

Daily LIPUS treatment for 4 weeks significantly increased the peak torsion in the 6 μ g rhBMP-2 group (Fig. 4a). With the LIPUS treatment there was also a significant increase in the 12 μ g rhBMP-2 group (Fig. 4a). Torsion force with 6 μ g+LIPUS was not different from the 12 μ g sham group. The peak torsion with LIPUS and 12 μ g rhBMP-2 was closest to that of intact bone. LIPUS treatment significantly decreased the deformation angle (Fig. 4b) and increased the stiffness in the 6 μ g rhBMP-2 group (Fig. 4c). LIPUS treatment also significantly increased the stiffness without changing the deformation angle in the 12 μ g rhBMP-2 group. Peak torsion, deformation angle and stiffness of defects treated with 6 μ g rhBMP-2 and LIPUS treatment had comparable values to the defects treated with 12 μ g rhBMP-2 alone, with no statistical changes observed in either (Fig. 4a, 4b and 4c).

Histology

Histology revealed changes in soft tissue (callus, cartilage, marrow, ACS) or provided information on bone structure. Due to space constraint, hematoxylin and eosin, and TRAP data is discussed but not shown.

At the end of 4 weeks there was a prominent callus formed in all groups with varied levels of tissue mineralization and architecture depending upon the rhBMP-2 dose and/or LIPUS treatment. The control groups showed mineralized bone only at the ends of the defect (Fig.

5a & Fig. 5e) surrounded by TRAP positive osteoclasts in the intra-medullary space. Some of the endosteal surfaces in the LIPUS control were lined with osteoblasts. There was no or minimal activity seen in the actual defect region. With the use of 1.2µg of rhBMP-2 there was mineralized bone at the ends (Fig. 5b & Fig. 5f) of the defect with new trabecular bone islands in the defect region surrounded with active TRAP positive osteoclasts and the presence of some uncalcified cartilage (Fig. 6b). However the cartilage levels appeared to have diminished with the treatment of LIPUS with 1.2µg of rhBMP-2 (Fig. 6f) and replaced by bone instead. Treatment with LIPUS also showed the presence of newly forming and mature blood vessels in the defect region.

With the use of 6µg rhBMP-2 the defect region mainly consisted of trabecular bone (Fig. 5c) with some degree of cartilage (Fig. 6c). The treatment of LIPUS with 6µgrhBMP-2 qualitatively appeared to provide a better union and cortical remodeling when compared to its respective sham control (Fig. 5g). The defect consisted of mostly trabecular bone (Fig. 6g) with higher numbers of TRAP positive osteoclasts. The hard callus on the periphery qualitatively appeared to be undergoing active bone turnover with active osteoblasts and osteoclasts present on most surfaces.

The optimum dose of rhBMP-2 (12µg) showed full bridging with a hard callus undergoing cortical remodeling. The defect region adjacent to the native cortex showed trabecular bone which was not fully resorbed (Fig. 5d). Nevertheless, the hard callus was well integrated with the existing native cortex (Fig. 6d). Active osteoblasts were noted on most of the peripheral surfaces with some osteoclasts present actively resorbing the bone surfaces. The LIPUS treatment along with the optimum dose of 12µg rhBMP-2 in addition brought about cortical remodeling based on the lamellar bone organization (Fig. 6h). The defect region showed faint traces of resorbed trabecular bone with a distinct marrow cavity with repopulated marrow in the void spaces (Fig. 6h).

Discussion

rhBMP-2 when delivered exogenously can accentuate the healing process by initiating chemotaxis, proliferation and mitosis of mesenchymal cells, inducing mesenchymal commitment to osteoblast or chondrocyte differentiation³². 12µg of rhBMP-2 was expected to show the maximum volume of bone regenerated in the defect, maximum bridging and highest functionality mechanically²⁷. However it was suspected to be capable of completely masking the effect of a subtle stimulus like LIPUS. Hence in our study we decided to assess the outcome of the use of LIPUS in combination with the optimum dose, 12µg; and two sub-optimum doses 1.2µg (10%) and 6µg (50%) of rhBMP-2.

We are aware, at 4 weeks, an empty (ACS only) defect would develop into a critical-sized non-union model²⁷. The use of LIPUS alone in an empty defect did not significantly increase the healing. This is consistent with a previous report with large (5mm) mandibular defect in the rat³³ and is not surprising as LIPUS is not osteoinductive³⁴ but is able to increase osteogenic differentiation³⁵. rhBMP-2 to begin with, even in small doses, can chemotactically recruit mesenchymal stem cells and osteoblasts in the defect region. In addition, the periosteal cells at the defect site are a ready source of osteo-chondroprogenitor

cells which could be stimulated by LIPUS. With just 1.2µg of rhBMP-2 there was a significant change in bone volume, with LIPUS increasing this. Radiographically, the effect was evident from 2 weeks onwards. It is known that LIPUS stimulates cartilage proteoglycan (chondroitin sulfate biglycan) through induction of aggrecan gene expression *in vitro*³⁶ and in the early stages of fracture healing³⁷. A significant increase in the BV/TV shows the ability of LIPUS to accelerate endochondral bone formation which is evident from the observed newly formed bone at 2 weeks and the capillary invasion. Similar observations in the stimulation of endochondral ossification by LIPUS has been presented in distraction osteogenesis studies^{38,39}. However the lack of union in either groups hamper the ability to show any difference in any functional biomechanical test. When 6µg of rhBMP-2 was used, the volume, quality and functional strength of the newly formed bone was significantly higher than control and 1.2µg irrespective of the use of LIPUS. The absence of cartilage with the use of LIPUS with 6µg rhBMP-2 suggests that the defect has gone through the endochondral phase completely. Although not apparent radiographically, quantitative assessment with µCT, showed LIPUS to have significantly increased the BV, and torsional strength of the regenerate tissue. Statistical analyses reveal it to be functionally as strong as using 12µg of rhBMP-2 alone.

As expected, 12µg proved to increase the bone formation to the greatest extent. With the use of LIPUS along with the 12µg of rhBMP-2 there was no change in the BV or BMC, but a decrease was seen in the volume of the callus. This suggests that LIPUS was able to accelerate the callus maturation with the optimum dose of rhBMP-2. As a result there was a significant increase in the BV/TV. These findings are not only supported by the histological findings, where a remodeled hard callus was seen, but also by the significant increase seen in the mechanical strength of the bone. Previous studies have shown the ability of LIPUS to promote remodeling or callus maturation in an osteotomy⁴⁰, segmental transport⁴¹, and distraction osteogenesis^{39,42–44}.

LIPUS is able to enhance healing in critical-sized femoral segmental defect by possibly stimulating two exclusive mechanisms of healing. In the first mechanism, as seen with sub-optimum levels, LIPUS is able to enhance the bone formation induced by the rhBMP-2 to result in higher bone volumes and increase the bridging of the defect. One could say, in this mechanism the fractured bone is brought back to its “form” by increasing the activity of the pertinent cells; osteoblast, fibroblast or chondrocytes in response to the “function” created by LIPUS. This could be a direct effect of LIPUS on the osteogenic activity of the stromal cell population^{35,45} or an indirect effect by enhancing blood flow around the fracture site⁴⁶. On the other hand, in an optimized healing system, the activity of the cells is already at its peak. The bone takes its form at a faster pace where LIPUS is able to stimulate callus maturation and remodeling. This could be a direct effect of LIPUS on osteoclast-like cells which have invaded the regenerated bone²¹ or the indirect effect of enhancing blood flow too. LIPUS has been shown to accelerate clinical fracture healing of delayed unions of the fibula by increasing osteoid thickness, mineral apposition rate, and bone volume, indicating increased osteoblast activity, at the front of new bony callus formation⁴⁷. However, the increased vascularity at the fracture site is crucial in providing a normoxic environment suitable for intramembranous bone formation⁴⁸. It not only brings in with it multiple growth

factors to the healing site but also multiple cell types which are required at a complex site like that of fracture healing.

In conclusion, LIPUS enhances rhBMP-2 induced bone formation at suboptimum doses and callus maturation at optimum dose delivered on ACS for bone repair in a rat critical-sized femoral segmental defect. The optimum dose of rhBMP-2 is not yet defined for clinical use, but LIPUS could potentially enhance bone healing induced by a clinically safe dose of rhBMP-2. Moreover, having had no proven side effects, LIPUS can be used with lower doses of rhBMP-2 to achieve equally strong regenerated bone.

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Fig. 1.

Average radiographic score of the weekly *in vivo* radiographs of the defect undergone combined treatment of rhBMP-2 and LIPUS. All data in the Fig. represented as mean±SEM.

*P<0.05 compared to 1.2µg Sham #P<0.05 compared to 0µg±LIPUS and 1.2µg±LIPUS

Fig. 2.

Representative (defect with the median value) contact radiographs of defect undergone combined treatment of rhBMP-2 and LIPUS. All radiographs are shown in the mediolateral views. Average radiographic scores after 4 weeks of ACS implantation with rhBMP-2 and daily LIPUS or sham treatment is shown in the graph. All data represented as mean±SEM. P=0.051 compared to 1.2µg Sham

Fig. 3.

Three dimensional reconstructions of the microCT slices. Representative (defect with the median value) microCT images of the segmental defect with varying rhBMP-2 doses loaded on ACS with or without LIPUS after four weeks of treatment (Blue: newly formed bone in the 5mm critical sized defect. Yellow: native cortical bone with periosteal reaction).

Fig. 4. Graphical representation of the biomechanical properties; peak torsion (a), torsion deformation (b) and stiffness (c), of the regenerated tissue after 4 weeks of ACS implantation with rhBMP-2 and daily LIPUS or sham treatment. All torque (N-mm), deformation angle (degree) and stiffness (N-mm/degree) data represented as mean $\pm$ SEM. *P<0.05 and **P<0.01 compared to respective sham control.

Fig. 5.
Representative histological tissue mineralization with basic fuchsin stained sections of the defect after 4 weeks of rhBMP2 and daily sham (a–d) or LIPUS (e–h) treatment. Bar = 2mm.

Fig. 6. Representative histological tissue mineralization with Safranin O and fast green stained sections of the defect after 4 weeks of rhBMP2 and daily sham (a–d) or LIPUS (e–h) treatment. Bar = 500µm.

Microcomputed tomography values after 4 weeks of ACS implantation with rhBMP-2 and daily LIPUS or sham treatment. All data represented as mean (SEM).

Table 1

rhBMP-2 LIPUS	0µg		1.2µg		6µg		12µg		Intact	
	-	+	-	+	-	+	-	+	-	+
BV	6.54 (1.17)	7.35 (1.21)	35.16 ^d (5.68)	44.30 ^d (5.46)	79.94 ^{d,e} (3.51)	92.00 ^{d,d,e} (5.69)	95.54 ^{d,e} (5.89)	94.68 ^{d,e} (5.23)	-	-
TV	74.15 (6.27)	78.42 (6.96)	93.82 (5.34)	90.42 (6.33)	136.43 (5.93)	155.93 (6.36)	195.16 (14.79)	159.12 ^a (9.63)	-	-
%BV/TV	9.22 (1.68)	9.90 (1.60)	35.66 (5.17)	48.81 ^a (4.70)	58.87 (1.45)	58.81 (2.35)	50.58 (2.66)	60.71 ^b (2.79)	66.83 (1.93)	66.83 (1.93)
BMC	3.25 (0.56)	3.81 (0.65)	18.93 ^d (3.04)	25.23 ^d (3.29)	44.64 ^{d,e} (1.99)	51.28 ^{c,d,e} (3.36)	53.73 ^{d,e} (2.94)	55.93 ^{d,e} (2.66)	-	-

^a P<0.05 versus respective sham,

^b P<0.01 versus respective sham,

^c P=0.05 versus respective sham,

^d P<0.01 versus 0µg rhBMP-2± LIPUS,

^e P<0.05 versus 1.2µg rhBMP-2± LIPUS N=15 in all groups, except 12µg rhBMP-2+ LIPUS (N=14) and Intact (N=12)