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ORIGINAL ARTICLE

Enhanced Bone Regeneration by Bone Morphogenetic Protein-2 after Pretreatment with Low-Intensity Pulsed Ultrasound in Distraction Osteogenesis

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Abstract

BACKGROUND: Bone morphogenetic protein 2 (BMP-2) and low-intensity pulsed ultrasound (LIPUS) have been used to enhance bone healing in distraction osteogenesis (DO). The aim of this study was to assess the synergistic effect of BMP-2 and LIPUS on bone regeneration in DO and to determine the optimal treatment strategy for enhanced bone regeneration.

METHODS: Rat mesenchymal stromal cells were treated with various application protocols of BMP-2 and LIPUS, and cell proliferation, alkaline phosphatase activity, and osteogenesis-related marker expression were evaluated. *In vivo* experiments were performed in a rabbit DO model according to the application protocols with different timings of BMP-2 and LIPUS application.

RESULTS: Application of BMP-2 after LIPUS pretreatment (BMP-2 after LIPUS) showed greater cell proliferation than LIPUS treatment alone, and higher ALP activity than all other treatment protocols. BMP-2 after LIPUS also exhibited increased gene expression levels of *ALP*, *Cbfa1*, and *Osterix* compared with LIPUS treatment alone. *In vivo* experiments revealed no significant differences in bone healing based on the timing of LIPUS treatment in DO. The combination of BMP-2 and LIPUS resulted in increased bone volume and bone mineral density compared with BMP-2 or LIPUS. Regarding the timing of BMP-2 application, the application of BMP-2 after LIPUS pretreatment led to greater bone volume than the application of BMP-2 before LIPUS.

CONCLUSION: The results of this study suggest that the combined treatment of BMP-2 and LIPUS can lead to enhanced bone healing in DO and that effective bone healing can be achieved through the application of LIPUS before BMP-2.

Keywords Bone regeneration · Bone morphogenetic protein 2 · Low-intensity pulsed ultrasound distraction osteogenesis

1 Introduction

Distraction osteogenesis (DO) is a procedure used to obtain new bone by slowly moving the bone segment after osteotomy and rhythmically applying tensile force to the callus, which can avoid morbidities in the donor site for bone grafting and simultaneously expand overlying soft tissues. Despite the advantages of DO, its main drawback is that it requires a long treatment period for bone lengthening and subsequent bone consolidation, which increases the risk of local infection around the transcutaneous or transmucosal site for the device, causes psychological problems in patients, and increases inconvenience to the family

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[1, 2]. In addition, early removal of the device may lead to a relapse of skeletal lengthening, nonunion, and fracture [3]. To overcome these drawbacks and reduce morbidities in DO, studies have been conducted on supplementary treatment methods to enhance bone formation and maturation [4–6].

Low-intensity pulsed ultrasound (LIPUS) is a special type of high-frequency acoustic pulsed energy that has been shown to stimulate bone healing in fresh fractures or delayed union and nonunion [7]. Studies have reported that LIPUS increases bending strength, accelerates the callus formation and endochondral ossification, stimulates aggrecan gene expression, and modulates transforming growth factor-beta synthesis and calcium uptake [8–11]. In addition to fracture healing and restoration of bone defects, several investigations have applied LIPUS to DO to improve bone healing and shorten the bone maturation period [6, 12, 13]. El-Bialy et al. [12] applied LIPUS during mandibular DO in rabbits and reported a significant increase in the new bone photodensity, vibratory coherence, and mechanical stiffness at the distraction gap. In a study by Schortinghuis et al. [13], LIPUS was applied during mandibular vertical DO in patients with a severely resorbed mandible. Xie et al. [6] reported that early bone formation was enhanced when LIPUS was applied during mandibular DO in a rabbit model.

Since Urist [14] introduced and described bone morphogenetic proteins (BMPs) in 1965, BMPs, especially BMP-2, BMP-7, and BMP-9, have been extensively studied to improve bone repair and regeneration with osteogenic potential. BMP-2 is the clinically approved and most actively studied representative BMP, and has been successfully used to restore bone defects in various medical fields, including orthopedics and dentistry. In the oral and maxillofacial regions, BMP-2 has been used in bone grafting for dental implants, such as in maxillary sinus floor augmentation and guided bone regeneration, bone regeneration after various intraosseous tumors, and treatment for intractable osteomyelitis [15–17]. Studies have also shown that BMP-2 enhances bone regeneration as an adjunctive treatment for mandibular and tibial DO [18–20].

Several studies have attempted to enhance the bone regeneration effect of supplemental treatment by combining BMP-2 and LIPUS, which are known to promote bone formation when used alone [21–23]. In an ectopic implant animal model, the combination of LIPUS and BMP-2 resulted in increased bone regeneration beyond the enhancement of bone regeneration by BMP-2 only [23]. In a study using a rat model of critical-sized femoral segmental defect by Angle et al. [21], LIPUS improved bone regeneration at a low dose of rhBMP-2 and callus maturation at a relatively high dose of rhBMP-2. However, to our knowledge, no studies have been conducted on the potential and feasibility of using BMP-2 and LIPUS together to promote osteogenesis and maturation in DO. When the two treatment modalities are used together, the synergistic effect may vary depending on the application method of the two treatments; thus, a specialized strategy is required to improve the synergistic effect of the two treatments. Therefore, the purpose of this study was to evaluate the synergistic effect of the combination of BMP-2 and LIPUS on bone regeneration in DO and to determine the optimal treatment strategy for improving bone regeneration.

2 Materials and methods

2.1 Rat mesenchymal stem cell culture

Rat mesenchymal stem cells (rMSCs) were obtained from the tibial bone marrow of 4-week-old Sprague–Dawley rats. The bone marrow suspension was collected in a syringe containing 6000 U/mL heparin, mixed with an equal volume of phosphate-buffered saline (PBS) solution, and centrifuged at 2500 rpm for 10 min. After aspirating the upper PBS layer, the marrow suspension was laid over Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) at a 1:5 ratio and centrifuged at 1200 × g for 30 min. Nucleated cells concentrated at the interface were collected and washed with PBS. Adherent cells were plated at a density of 2×10^6 cells/100 mm plate and cultured in an expansion medium containing alpha-minimum essential medium (α-MEM; Welgene, Inc., Gyeongsan, Korea), 100 units/mL penicillin (Gibco, Rockville, MD, USA), 100 μg/mL streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum (Gibco) in a humidified atmosphere of 5% carbon dioxide at 37 °C. The medium was changed every 3 or 4 days. Cells were passaged when they reached 70% confluence, and the sixth-passage cells were used for *in vitro* experiments. For osteogenic studies, 1×10^4 rMSCs were seeded onto 35 mm culture dishes in osteogenic differentiation medium of α-MEM supplemented with 50 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich). The medium was changed every 3 days. The following five groups were investigated, and the detailed experimental protocols are shown in Fig. 1: 1) Control group, 2) LIPUS group, 3) BMP-2 group, 4) LIPUS after BMP-2 group, and 5) BMP-2 after LIPUS group. LIPUS was applied using an ultrasound exposure device with a frequency of 1.5 MHz and an intensity of 60 mW/cm^2 (SonoTess, HYEMIN, Seoul, Korea) for 10 min per day (Fig. 2). The culture dishes were

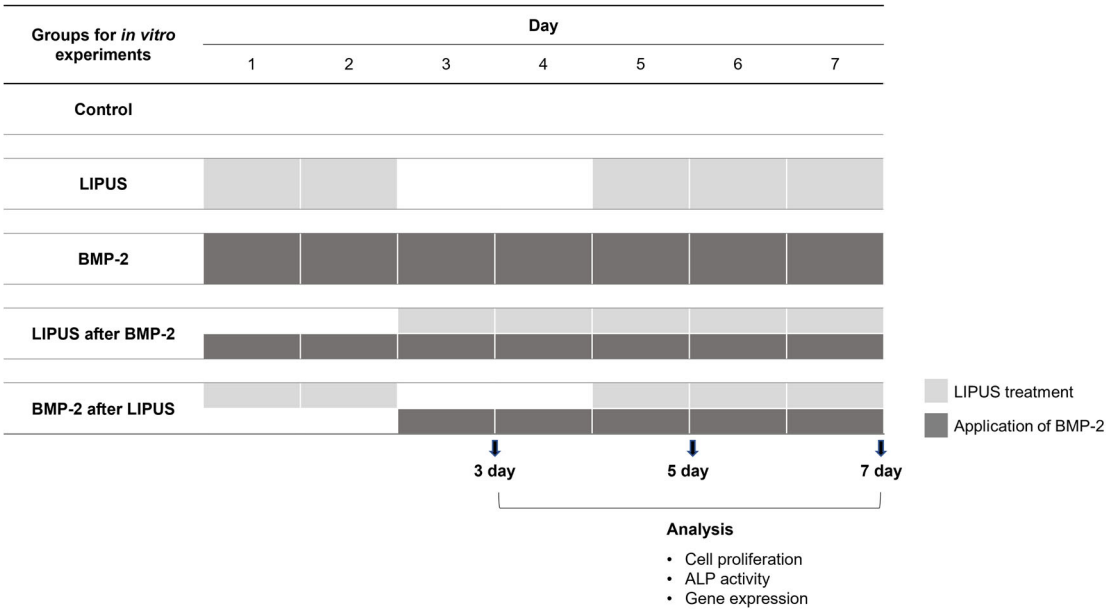

Fig. 1 Schematic representation of *in vitro* experimental design. Cell proliferation, alkaline phosphatase activity, and gene expression were analyzed on Days 3, 5, and 7 of culture in each application protocol

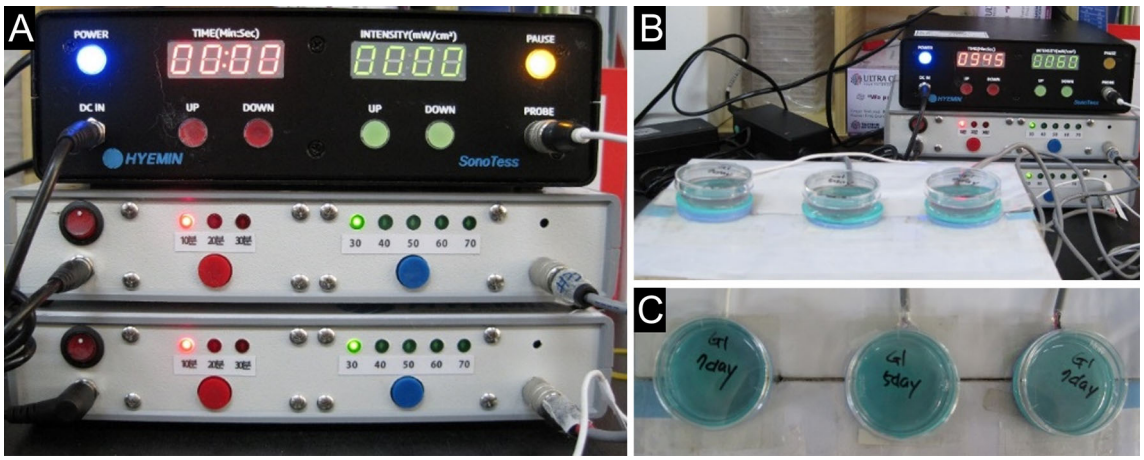

Fig. 2 Application of low-intensity pulsed ultrasound for *in vitro* experiments. **A** Ultrasound exposure device **B, C** Culture dishes were placed on the circular ultrasound transducers with a thin layer of high-viscosity gel and exposed to ultrasound with a frequency of 1.5 MHz and an intensity of 60 mW/cm^2 for 10 min per day

placed on circular ultrasound transducers (38 mm) with a thin layer of a high-viscosity gel. The groups in which LIPUS application was planned were exposed to LIPUS for 5 days. Specifically, the LIPUS and the BMP-2 after LIPUS groups were exposed to LIPUS on days 1, 2, 5, 6, and 7, and the LIPUS after BMP-2 group was exposed to LIPUS on days 3, 4, 5, 6, and 7. Recombinant human BMP-2 (Daewoong, Seoul, Korea) was administered at a concentration of 200 ng/ml and the same concentration was maintained when changing the medium. On days 3, 5, and 7 of culture, cell proliferation and alkaline phosphatase (ALP) activity were analyzed, and gene expression related to bone formation or bone resorption was investigated.

2.2 Cell proliferation assay

rMSCs were seeded onto 35 mm culture dishes at a density of 1×10^4 in osteogenic differentiation medium. While performing the experimental protocols for the groups to which the cells belonged, cell proliferation was measured after trypan blue staining on days 3, 5, and 7 of culture.

2.3 ALP activity

To determine ALP activity, the amount of ρ-nitrophenol produced using the ρ-nitrophenol phosphate substrate was measured. After 3, 5, and 7 days of culture, the cell layers

were rinsed with PBS and lysed in alkaline lysis buffer solution. Cell lysates were mixed with ρ-nitrophenol phosphate at room temperature for 30 min. After stopping the reaction by adding 0.05 N NaOH, the absorbance was measured at 405 nm and compared with a standard curve prepared with a ρ-nitrophenol standard solution. ALP activity was expressed as nanomoles of ρ-nitrophenol produced/min/mg of protein. The total protein concentration of the cell lysates was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

2.4 Real-time reverse transcription-polymerase chain reaction (RT-PCR)

After 3, 5, and 7 days of culture, the cells were harvested and analyzed for bone formation and bone resorption marker gene expression by quantitative real-time RT-PCR. Total RNA was extracted from each cell culture using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The 1 μg RNA was reverse-transcribed into cDNA by using SuperScript^{TM} Reverse Transcriptase II and oligo $(dT)_{12–18}$ primer (Invitrogen) in a 20 uL reaction volume according to the manufacturer's instructions, and RNA complementary to the cDNA was removed using *Escherichia coli* RNase H (Invitrogen). For quantitative real-time RT-PCR, the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) was used with SYBR Green PCR Master Mix (Applied Biosystems). The following PCR conditions were used: pre-denaturation at 95 °C for 10 min, amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, extension at 60 °C for 1 min) for 30 cycles, and a final dissociation cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The primers for target genes were designed using Real-Time PCR System Sequence Detection Software v1.3 (Applied Biosystems), and their sequences are shown in Table 1. Fold differences of each gene were calculated for each treatment group by using normalized C_T values of the housekeeping gene β-actin according to the manufacturer's instructions of Applied Biosystems.

2.5 Experimental animals

All animal experiments were performed in accordance with the "Recommendations for Handling of Laboratory Animals for Biomedical Research" compiled by the Committee on Safety and Ethical Handling Regulation for Laboratory Experiments of the School of Dentistry at Seoul National University. Fourteen adult male New Zealand rabbits (weight 3.0–3.5 kg) were used in this study. The rabbits were kept individually in cages for 1 week. All operations were performed under general anesthesia using ketamine (20 mg/kg, Yuhan, Seoul, Korea) and xylazine (Rompun, 10 mg/kg, Bayer, Leverkusen, Germany). After disinfection of the skin overlying the mandible with 10% povidone-iodine (Betadine; Purdue Pharma, Stamford, CN, USA), a skin incision was made along the inferior border of the mandible, and the mandibular body was exposed through periosteal elevation. Vertical osteotomy for DO was performed on the mandibular body between the first premolar and mental foramen by using a fissure bur with sterile saline irrigation. The mandibular distractor was adapted to the lateral surface of the mandible perpendicular to the vertical osteotomy line (Fig. 3). After sufficient sterile saline irrigation at the surgical site, closure of the surgical wound was performed using layer-by-layer sutures. The same surgical procedure as that for DO was performed on the contralateral side of the mandible. After surgery, antibiotics (cefazolin; 55 mg/kg; ChonKunDang Pharm, Seoul, Korea) were administered intramuscularly to all rabbits for 3 days.

2.6 Distraction procedure

After a latency period of 7 days, all rabbits underwent mandibular lengthening on both sides at a rate of 1.5 mm/day for 5 days. On day 30 of the consolidation phase, all animals were sacrificed, and further analysis was performed to evaluate bone regeneration. In this study, 28 surgical sites in 14 rabbits were divided into six groups, and four or five surgical sites were assigned to each group (Table 2 and Fig. 4).

Table 1 Primer sequences used for real-time reverse transcription-polymerase chain reaction

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
ALP	ATG TCT GGA ACC GCA CTG AAC	TTC TTT GTC AGG ATC CGG AGG
Cbfa1	ACC ATG GTG GAG ATC ATC GC	GCC ATG ACG GTA ACC ACG G
Osterix	AGC TCT TCT GAC TGC CTG CCT AGT	TTG GGC TTA TAG ACA TCT TGG GGT
VEGF	TTT CTC CGC TCT GAA CAA GGC	TGC AGA TCA TGC GGA TCA AAC
OPG	GGA GAG TGA GGC AGG CTA TTT GA	CTA CAA ATG GGA TAA GGC TCG TG
RANKL	ACT CTG GAG AGC GAA GAC ACA GAA	ATC AGG TTA TGC GAA CTT GGG ATT
β-actin	CCT GAG GAG CAC CCT GTG CTG CT	CAA CAC AGC CTG GAT GGC TAC GT

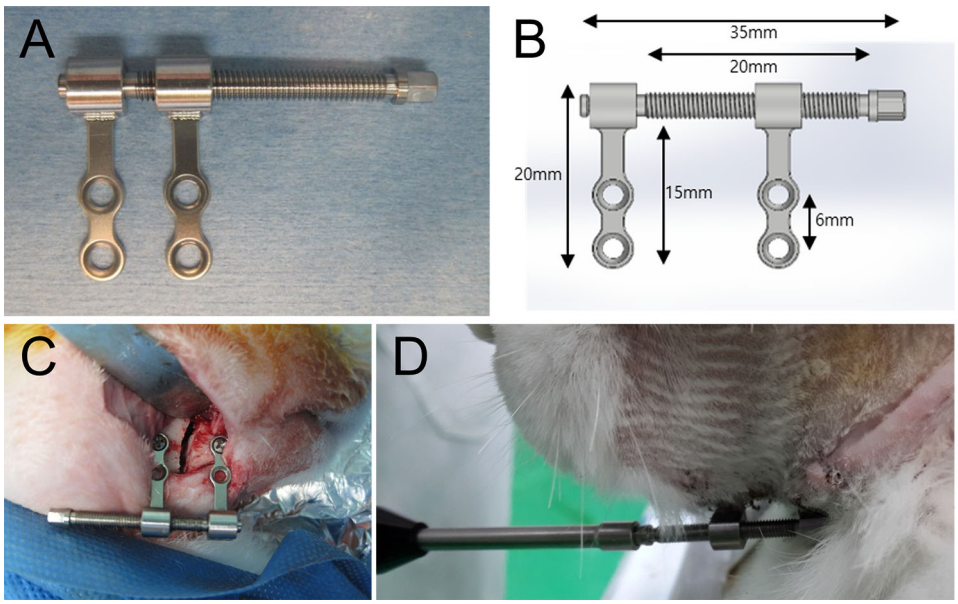

Fig. 3 Mandibular distraction osteogenesis in a rabbit. **A, B** Distractor used in this study **C** Fixation of the distractor for a mandibular distraction osteogenesis **D** Lengthening of the mandible using the distractor

Group 1 (control group): DO without application of LIPUS or BMP-2.

Group 2 (BMP-2 group): BMP-2 application 2 weeks after the distraction phase.

Group 3 (LIPUS after distraction group): application of LIPUS after the distraction phase.

Group 4 (LIPUS during distraction group): application of LIPUS during the distraction and consolidation phases.

Group 5 (BMP-2 before LIPUS group) application of BMP-2 before LIPUS application.

Group 6 (BMP-2 after LIPUS group): application of BMP-2 after LIPUS application.

The animal experiment used a spit-mouth design; thus, groups 1 and 2, groups 3 and 4, and groups 5 and 6 were conducted on the same subject. Groups 1, 3, and 4 were compared to evaluate the effect of LIPUS application timing on bone regeneration. The synergistic effect of BMP-2 and LIPUS on bone regeneration was assessed by comparing groups 1, 2, 3, and 6. The effect of BMP-2 application timing on bone regeneration in the combined BMP-2 and LIPUS treatment for DO was evaluated through comparisons among groups 1, 5, and 6. For each animal, 20 ug of BMP-2 was injected directly into the distraction gap. LIPUS with a frequency of 1.5 MHz and 60 mW/cm^2 was applied on the distraction gap for 20 min per day, 5 days per week, for 4 weeks of the consolidation phase (Fig. 5). For group 4, LIPUS was additionally applied for 3 days during the distraction phase before consolidation.

2.7 Micro-CT evaluation

All the animals were sacrificed on day 30 of the consolidation phase. Mandibular specimens with a length of 2 cm containing a central distraction gap were harvested and fixed in 10% formalin for 1 week. For a quantitative evaluation of bone regeneration, a micro-CT scan was performed on the obtained specimens by using a SkyScan 1172 microfocus X-ray system (Brunker microCT, Kontich, Belgium). This system is equipped with a microfocus X-ray tube with a focal spot of 2 μm, producing a cone beam detected by a 12-bit cooled X-ray camera CCD (charge coupled device) fiber-optically coupled to a 0.5 mm scintillator. The resulting images were 2000 × 1048 pixel square images with an aluminum filter used to produce the optimized images. A second-order polynomial correction of the algorithm was used to reduce the beam-hardening effect for all the specimens. Reconstruction of the CT scan data was performed using NRecon reconstruction software (Brunker microCT, Kontich, Belgium), and subsequent analysis was conducted using CTAn software (Brunker microCT, Kontich,

Table 2 Assignment of surgical site for each group

Right mandible	Left mandible	Number of animals
Group 1: Control	Group 2: BMP-2	5
Group 3: LIPUS after distraction	Group 4: LIPUS during distraction	4
Group 5: BMP-2 before LIPUS	Group 6: BMP-2 after LIPUS	5

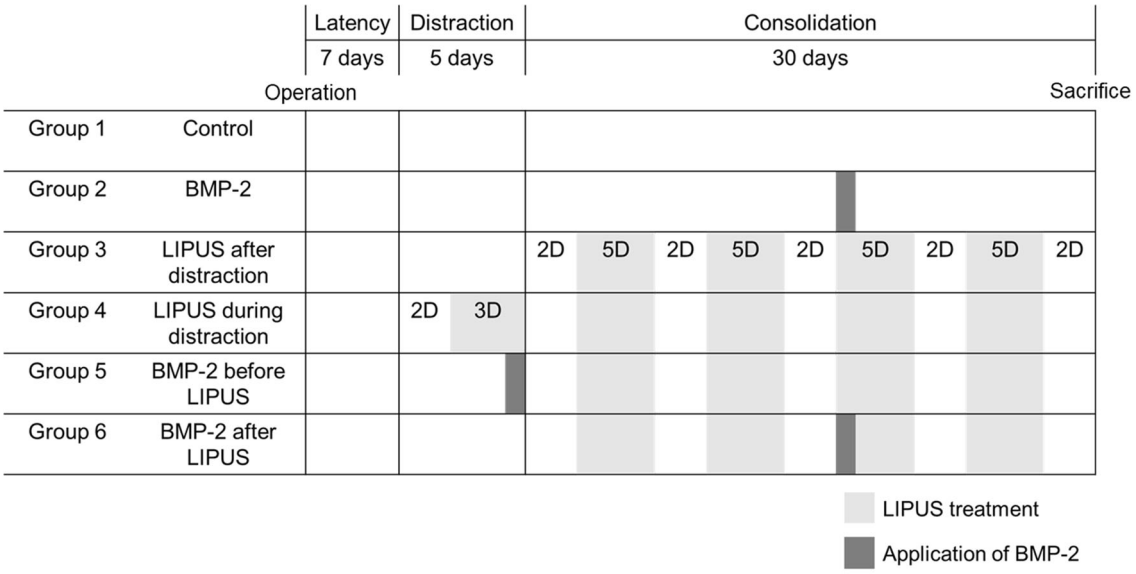

Fig. 4 Schematic representation of *in vivo* experimental design. D, days

Fig. 5 Application of the low-intensity pulsed ultrasound with a frequency of 1.5 MHz and an intensity of 60 mW/cm^2 for 20 min. A distractor screwdriver is connected to indicate the direction of the distractor

Belgium). For measurements of the newly formed bone, a rectangular area of the central region in the distracted callus was defined as the region of interest (ROI) in the two-dimensional images. The ossification area in the defined ROI was reconstructed in 3D using the threshold value presented in grayscale units. The following morphological parameters were analyzed using a CT-analyzer in direct 3D based on a surface-rendered volume model according to the manufacturer's instructions: bone volume (BV), bone volume to total volume ratio (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th). For the measurement of bone mineral density (BMD), attenuation data for the ROIs were converted into Hounsfield units and expressed as BMD values by using phantom scans (SkyScan). BMD values were expressed in grams per cubic centimeter of calcium hydroxyapatite (CaHA) in distilled water.

2.8 Histological and immunohistochemical evaluation

After micro-CT, the mandibular specimens, including the distraction gap, were cut in half parallel to the buccal surface of the mandible. The specimens were decalcified by incubation in a 7% EDTA solution (pH 7.0) for 10 days, during which time the solution was changed every 2 days. The specimens were then dehydrated in 70% ethanol and embedded in paraffin. After cleaning the decalcified paraffin sections with xylene for 10 min, the specimens were cut to 4 μm thickness without any trimming and stained with Masson's trichrome (MT). Digital images of the stained sections were acquired using an Axioskop microscope (BX51; Olympus Corporation, Tokyo, Japan).

2.9 Statistical analysis

Statistical analysis was performed using SPSS software (version 25.0, IBM SPSS, Chicago, IL, USA). The normal distribution of the data was determined using Kolmogorov–Smirnov tests. Data between the control and experimental groups were compared using one-way analysis of variance with Bonferroni post hoc multiple comparison tests. The level of significance was set at $p < 0.05$.

3 Results

3.1 Proliferation and osteogenic differentiation of rMSCs

In the assessment of cell proliferation on the days 3,5, and 7 of culture, the BMP-2 after LIPUS group showed significantly greater cell proliferation than the LIPUS group on day 7 of culture ($p = 0.019$), and no significant differences were observed among the other groups (Fig. 6). The effect of LIPUS and BMP-2 on osteoblast differentiation of rMSCs was evaluated by assessing ALP activity at 3, 5, and 7 days after culturing in the osteogenic culture medium. On day 5 of culture, the LIPUS after BMP-2 group exhibited significantly higher ALP activity than the control ($p = 0.001$) and LIPUS groups ($p = 0.005$), whereas the BMP-2 group and BMP-2 after LIPUS group showed no significant differences in ALP activity compared with the control and LIPUS groups. On day 7 of culture, the BMP-2 and LIPUS after BMP-2 groups showed significantly higher ALP activities than the control and LIPUS groups with equivalent statistical significance (vs. control group, $p < 0.01$; vs. LIPUS group, $p < 0.001$). For the BMP-2 after LIPUS group, ALP activity was significantly higher than that in all other groups (vs. control and LIPUS groups, $p < 0.001$; vs. BMP-2 and LIPUS after BMP-2 groups, $p < 0.01$), showing the best results in osteoblast differentiation of rMSCs.

3.2 Gene expression according to different BMP-2 and LIPUS application protocols

The expression of bone formation markers, including *ALP*, *Cbfa1*, *Osterix*, and *VEGF*, and bone resorption markers, including *OPG* and *RANKL,* was examined on days 3, 5, and 7 of culture under different BMP-2 and LIPUS protocols. The expression of *ALP* was significantly upregulated on day 5 of culture in the LIPUS after BMP-2 group and BMP-2 after LIPUS group compared with the control and LIPUS groups (LIPUS after BMP-2 vs. control, $p = 0.001$; LIPUS after BMP-2 vs. LIPUS, $p = 0.001$; BMP-2 after LIPUS vs. control, $p < 0.001$; BMP-2 after LIPUS vs. LIPUS, $p < 0.001$); and on day 7 of culture, only the BMP-2 after LIPUS group showed significantly increased gene expression of *ALP* compared with the control and LIPUS groups (vs. control group, $p < 0.05$; vs. LIPUS group, $p < 0.001$). For *Cbfa1*, the BMP-2 group showed significantly increased gene expression compared with the control group on day 5 of culture ($p < 0.05$), whereas on day 7 of culture, unlike the expression level of *ALP*, the LIPUS after BMP-2 group showed the most increased gene expression, namely, where it showed higher significance in comparisons with the control ($p < 0.001$) and LIPUS ($p < 0.001$) groups and lower significance in comparison with the BMP-2 group ($p < 0.05$). *Osterix* showed significantly different levels of gene expression by group from day 3 of culture. The BMP-2 and LIPUS after BMP-2 groups showed significantly increased gene expression compared with the control (vs. BMP-2, $p < 0.05$; vs. LIPUS after BMP-2, $p < 0.05$), LIPUS (vs. BMP-2, $p < 0.01$; vs. LIPUS after BMP-2, $p < 0.01$), and BMP-2 after LIPUS groups (vs. BMP-2, $p < 0.01$; vs. LIPUS after BMP-2, $p < 0.01$). On day 5, the BMP-2, LIPUS after BMP-2, and BMP-2 after LIPUS groups showed significantly increased gene expression compared to the control and the LIPUS groups ($p < 0.001$). The BMP-2 after LIPUS group exhibited significantly increased gene expression compared with the BMP-2 group ($p < 0.05$); however, no significant differences in gene expression were observed in the comparison of the BMP-2 after LIPUS and LIPUS after BMP-2 groups. On day 7 of culture, the BMP-2, LIPUS after BMP-2, and BMP-2 after LIPUS groups showed significantly increased gene expression compared with the control and LIPUS groups with equivalent significance (vs. BMP-2, $p < 0.001$; vs. LIPUS after BMP-2, $p < 0.05$; vs. BMP-2 after LIPUS, $p < 0.001$), although no significant differences were observed among these three groups that showed increased gene expression. For *VEGF*, gene expression was significantly upregulated in the LIPUS after BMP-2 and BMP-2 after LIPUS groups compared with the control (vs. LIPUS after BMP-2, $p < 0.05$; vs. BMP-2 after LIPUS, $p < 0.01$) and LIPUS (vs. LIPUS after BMP-2, $p < 0.05$; vs. BMP-2 after LIPUS, $p < 0.01$) groups on day 5 of culture. On day 7 of culture, the expression in the LIPUS after BMP-2 and BMP-2 after LIPUS groups decreased, and only the BMP-2 after LIPUS group showed a significantly increased gene expression compared with the control group ($p < 0.01$).

Regarding bone resorption markers, the LIPUS group showed significantly increased gene expression of *OPG* on day 5 of culture compared with all other groups (vs. control, LIPUS after BMP-2, and BMP-2 after LIPUS groups, $p < 0.01$; vs. BMP-2 group, $p < 0.001$). On day 7, the gene expression of *OPG* decreased in the LIPUS group and increased in the BMP-2 group, and only the BMP-2 group showed significantly higher gene expression than the control group ($p < 0.05$). The expression level of *RANKL* showed a similar tendency on days 5 and 7 of the culture, where three groups with BMP-2 exhibited significantly increased gene expression compared with the control and LIPUS groups (for both control and LIPUS groups; vs. BMP-2 group, $p < 0.001$ on day 5 and $p < 0.05$ on day 7; vs. LIPUS after BMP-2 group, $p < 0.001$ on day 5 and $p < 0.01$ on day 7; vs. BMP-2 after LIPUS group, $p < 0.01$ on days 5 and 7). With regard to the *RANKL*/*OPG* ratio, the

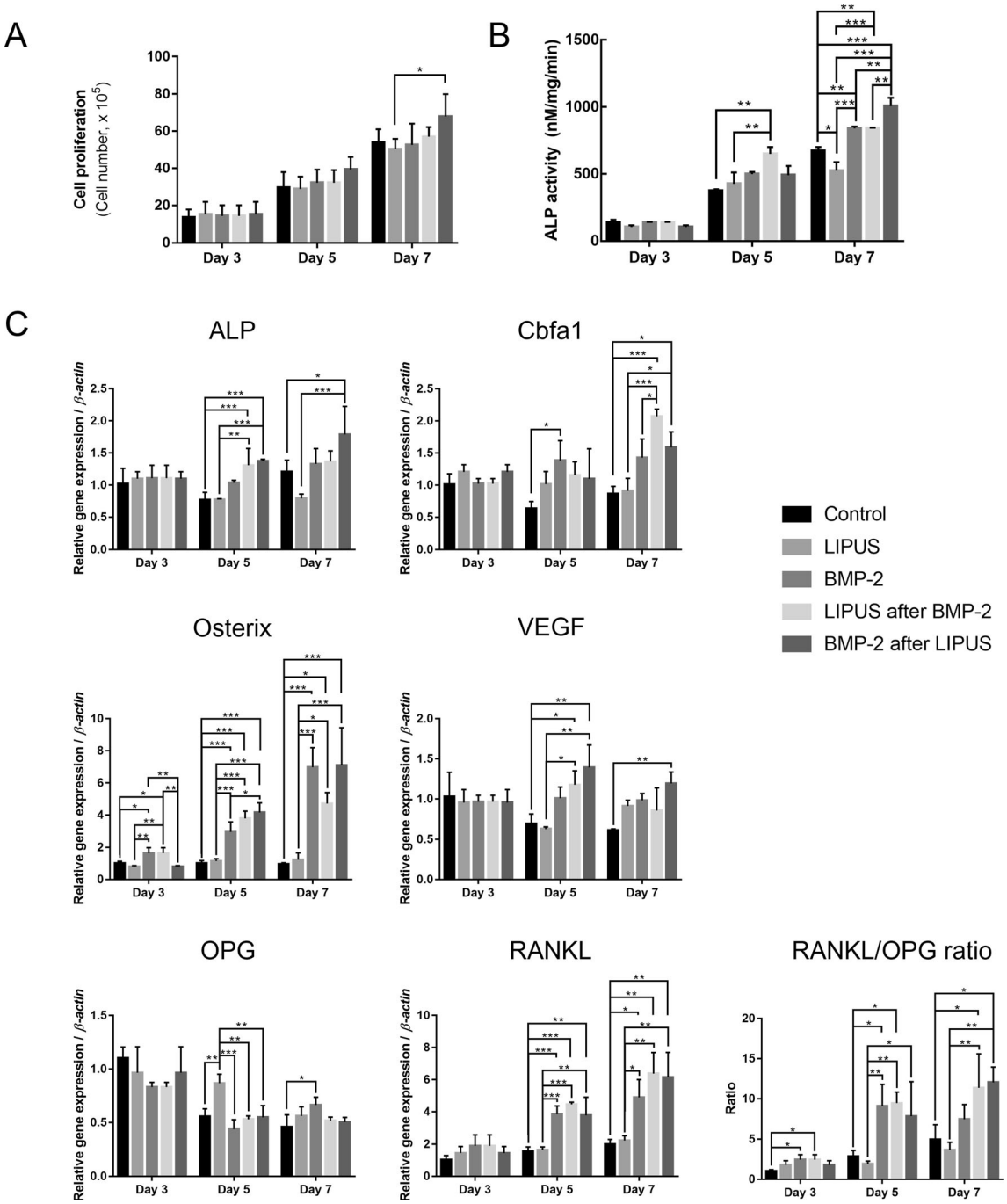

Fig. 6 Results of cell proliferation, ALP activity, and gene expression level of osteogenesis-related marker. **A** Cell proliferation, **B** ALP activity, **C** Gene expression level of osteogenesis-related marker. Significant difference between the groups is indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

BMP-2 and LIPUS after BMP-2 groups showed a significant increase on the day 5 with relatively high significance compared with the LIPUS group ($p < 0.01$), and with a low significance compared with the control group ($p < 0.05$), whereas the BMP-2 after LIPUS group showed a significant increase with a low significance in comparison with the LIPUS group ($p < 0.05$). On day 7, the BMP-2 after LIPUS and LIPUS after BMP-2 groups exhibited a significantly increased RANKL/OPG ratio, showing a relatively high significance in the comparison with the LIPUS group ($p < 0.01$), and a low significance in the comparison with the control group ($p < 0.05$).

3.3 *In vivo* experiment

3.3.1 Effect of timing of LIPUS application

The results of micro-CT analysis are presented in Table 3. To assess the effect of the timing of LIPUS application on new bone formation, groups 1 (control), 3 (LIPUS after distraction), and 4 (LIPUS during distraction) were compared (Fig. 7). Compared with the control group, the BV for groups 3 and 4 increased by 14.4% and 21.4%, respectively, but no significant differences were observed among the three groups. BV/TV and Tb.Th showed a similar tendency to bone volume, in which the values were highest in group 4 and lowest in group 1; however, no significant differences were observed among the three groups. Tb.N, Tb.Sp, and BMD also showed no statistically significant differences among the three groups. In the histological examination of MT-stained sections and comparison of cross-sectional views of three-dimensionally reconstructed mandibles, groups 3 and 4 exhibited relatively greater new bone formation in the distraction gap than group 1; however, there was little difference between groups 3 and 4 (Fig. 8).

3.3.2 Determination of synergistic effect of LIPUS and BMP-2

By comparing groups 1 (control), 2 (BMP-2), 3 (LIPUS after distraction), and 6 (BMP-2 after LIPUS), we verified whether the combination of BMP-2 and LIPUS increased new bone formation more than the single use of each treatment (Fig. 9). BV increased by 13.8% in group 2, 14.4% in group 3, and 44.4% in group 6 compared with group 1, and group 6 showed significantly higher BV than groups 1, 2, and 3 (vs. group 1, $p < 0.01$; vs. group 2, $p < 0.05$; vs. group 3, $p < 0.05$). In the evaluation of BV/TV and BMD, group 6 exhibited significantly higher BV/TV and BMD values than groups 1 and 2 (vs. group 1, $p < 0.01$; vs. group 2, $p < 0.01$). However, no significant differences were observed among the four groups in Tb.Th, Tb.N, and Tb.Sp. The histological examination and cross-sectional view of the three-dimensionally reconstructed mandible also revealed that the combination of LIPUS and BMP-2 resulted in the greatest bone formation among the four groups.

3.3.3 Effect of timing of BMP-2 application

The synergistic effect of BMP-2 and LIPUS was confirmed by micro-CT analysis and histological examination. To determine the optimal application time of BMP-2 for the maximum synergistic effect, group 5, in which BMP-2 was applied before LIPUS, and group 6, in which BMP-2 was applied after LIPUS, were compared (Fig. 10). Both groups were compared with the control group (group 1). The BV and BV/TV in the distraction gap were significantly greater in group 6 than in group 5 (BV, $p < 0.001$; BV/TV, $p < 0.001$). Compared with group 1, groups 5 and 6 exhibited significantly increased BV by 20.3% ($p < 0.01$) and 44.4% ($p < 0.001$), respectively. BV/TV showed a similar tendency to BV, where BV/TV was significantly higher in groups 5 (32.7%) and 6 (39.1%) than in group 1 (27.2%; vs. group 5, $p < 0.01$; vs. group 6, $p < 0.001$). In

Table 3 Morphometric measurements in the micro-CT analysis

Group	BV (mm^3)	BV/TV (%)	Tb.Th (mm)	Tb.N (1/mm)	Tb.Sp (mm)	BMD (g/cm^3)
Group 1	49.36 ± 4.13	27.23 ± 2.28	0.29 ± 0.06	0.98 ± 0.20	0.92 ± 0.13	1.36 ± 0.01
Group 2	56.18 ± 7.52	28.36 ± 4.29	0.29 ± 0.07	0.99 ± 0.25	0.93 ± 0.21	1.36 ± 0.02
Group 3	56.47 ± 11.83	31.21 ± 6.63	0.31 ± 0.08	1.08 ± 0.46	0.96 ± 0.08	1.38 ± 0.03
Group 4	59.93 ± 3.53	33.06 ± 1.95	0.39 ± 0.07	0.96 ± 0.27	0.96 ± 0.11	1.37 ± 0.01
Group 5	59.36 ± 2.97	32.75 ± 1.64	0.25 ± 0.02	1.30 ± 0.16	0.85 ± 0.08	1.39 ± 0.01
Group 6	71.27 ± 2.78	39.11 ± 1.78	0.33 ± 0.04	1.27 ± 0.19	0.87 ± 0.06	1.42 ± 0.03

BV bone volume, *TV* total volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular separation, *BMD* bone mineral density; group 1, control group; group 2, BMP-2 group; group 3, LIPUS after distraction group; group 4, LIPUS during distraction group; group 5, BMP-2 before LIPUS group; group 6, BMP-2 after LIPUS group

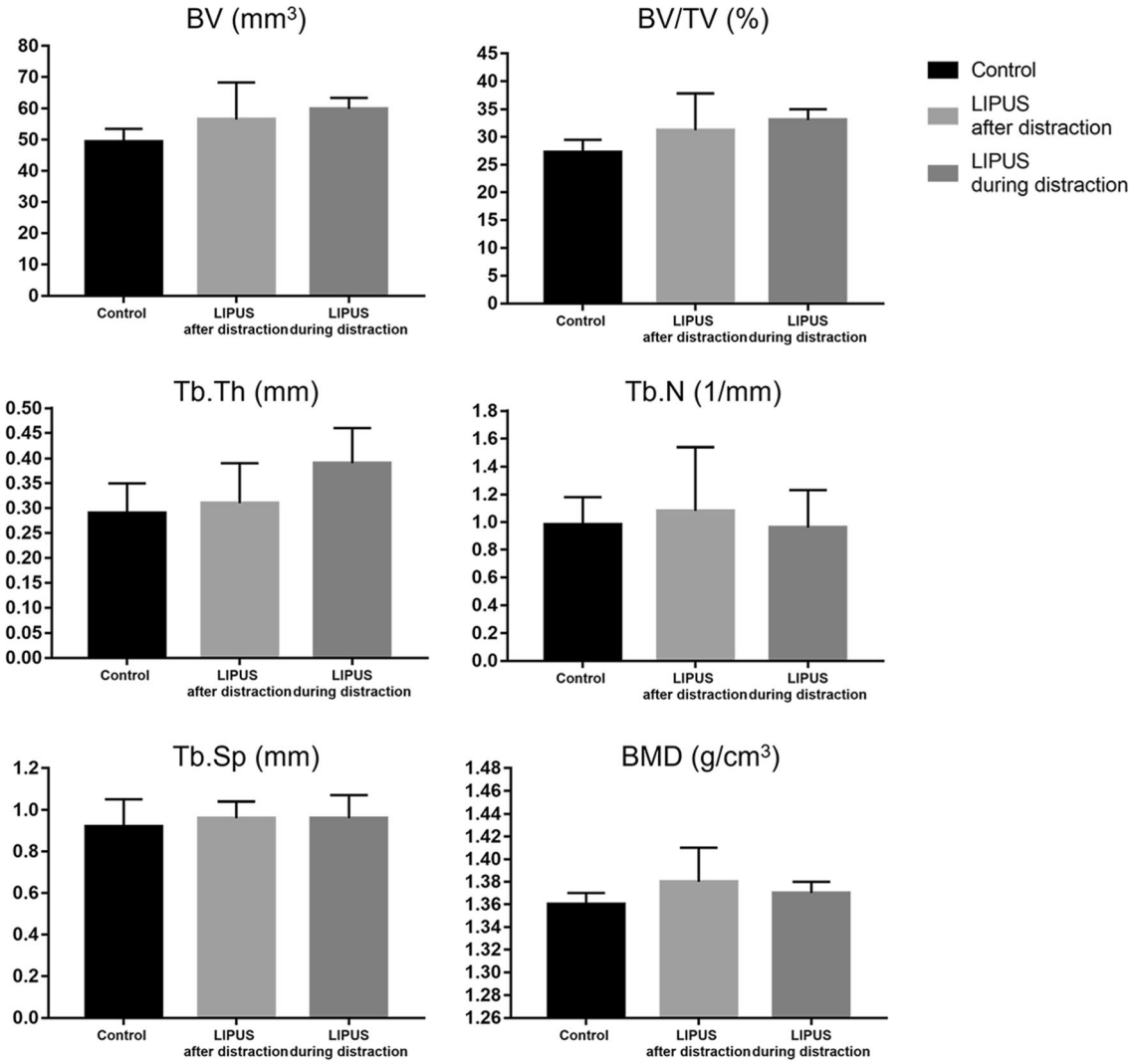

Fig. 7 Effect of timing of LIPUS application on bone regeneration. The results of micro-CT analysis were compared among the control, LIPUS after distraction, and LIPUS during distraction groups. No statistically significant differences were observed among the three groups. *BV* bone volume, *TV* tissue volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number; *Tb.Sp* trabecular separation, *BMD* bone mineral density

terms of BMD, Group 6 showed significantly higher BMD than group 1 ($p < 0.01$), however, no significant difference was observed between groups 5 and 6. For Tb.Th, Tb.N, and Tb.Sp, no significant differences were observed depending on the timing of BMP-2 application when a combination of BMP-2 and LIPUS was provided. The histological examination and cross-sectional view of the three-dimensionally reconstructed mandible exhibited results consistent with the results of micro-CT analysis, where the new bone formation was greatest in group 6, followed by group 5, and group 1.

4 Discussion

In this study, the synergistic effect of BMP-2 and LIPUS on bone regeneration in DO was evaluated in *in vitro* and *in vivo* experiments, and the optimal treatment strategy for bone regeneration was determined. The results revealed that BMP-2 enhanced the osteogenic differentiation of hMSCs, as expected, and the most enhanced osteogenic differentiation was observed when BMP-2 was injected after LIPUS application. In addition, we found that applying BMP-2 after LIPUS application had a synergistic effect on hMSC cell proliferation compared with LIPUS alone. In *in vivo* experiments using a rabbit DO model, the combination of BMP-2 and LIPUS led to a significantly increased new bone formation in the distraction gap

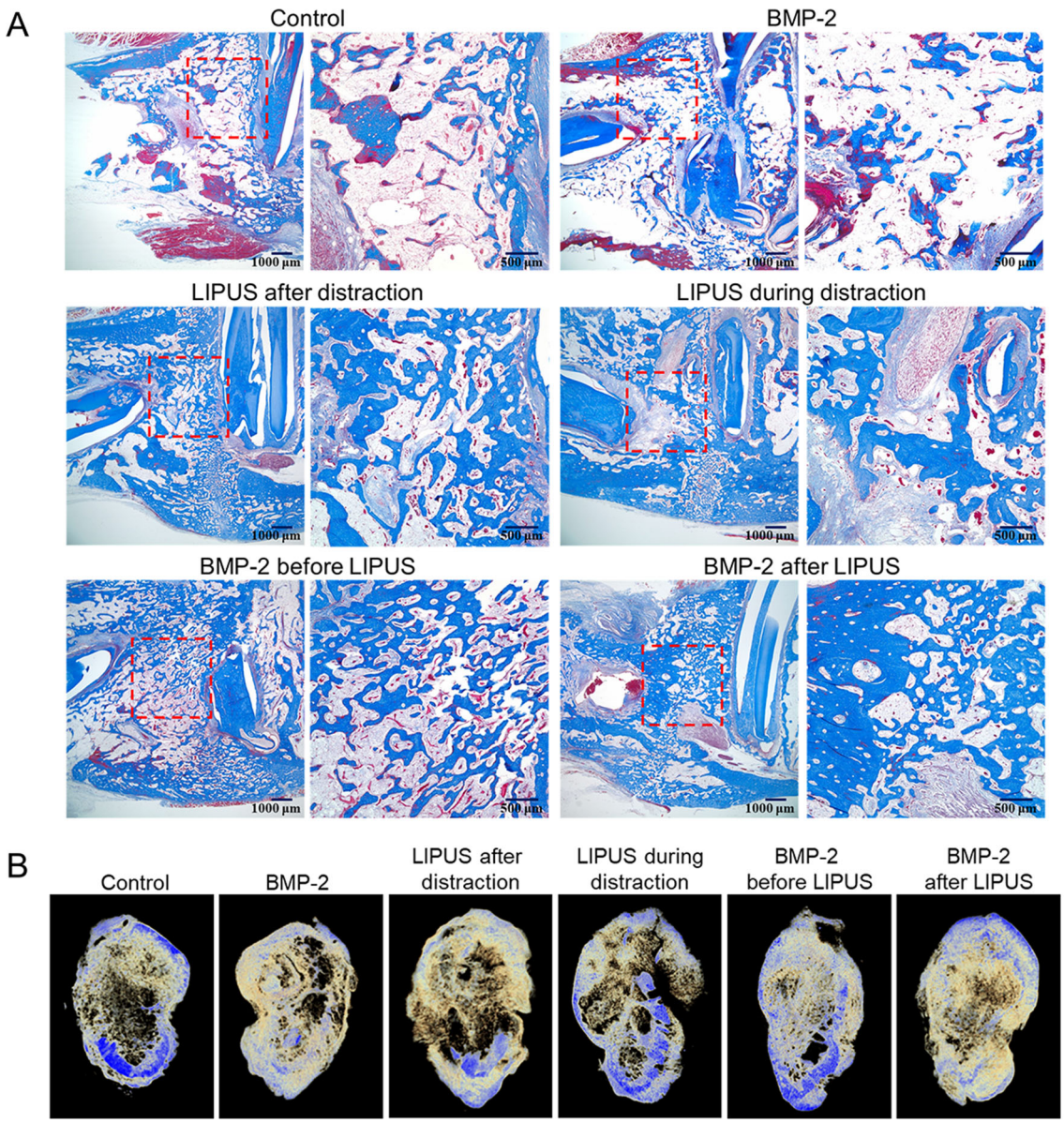

Fig. 8 **A**, **B** Histological image and cross-sectional view of three-dimensionally reconstructed mandible **B** at the distraction gap. The square region (red) of the histological image is magnified and presented on the right for each group

compared with the single treatment modality, where the most effective bone regeneration occurred when BMP-2 was injected after LIPUS application.

DO consists of a three-stage treatment process after osteotomy: latency, distraction, and consolidation phases. The effect of LIPUS on bone formation or maturation may differ by the phase of application of LIPUS [24]. In the experimental design in the literature, the timing of LIPUS application during DO was distinct in each study. In early studies, LIPUS was applied daily during the consolidation phase, starting after the distraction phase was completed, and it accelerated the maturation of the newly formed bone [25, 26]. By contrast, El-Bialy et al. [12] provided LIPUS for mandibular distraction in a rabbit model during and for 4 weeks after the distraction phase and reported that LIPUS significantly enhanced new bone photodensity, vibratory coherence, and mechanical stiffness at the distraction site. In a clinical study on mandibular vertical distraction by Schortinghuis et al. [13], ultrasound was applied for 20 min daily from the first day of distraction and continued during the consolidation period; however, ultrasound treatment did not appear to enhance bone formation after vertical DO in the severely resorbed mandible. To determine the optimum timing of applying LIPUS for improved bone regeneration, Sakurakichi et al. [24] applied LIPUS for 7 days during the latency, distraction, or

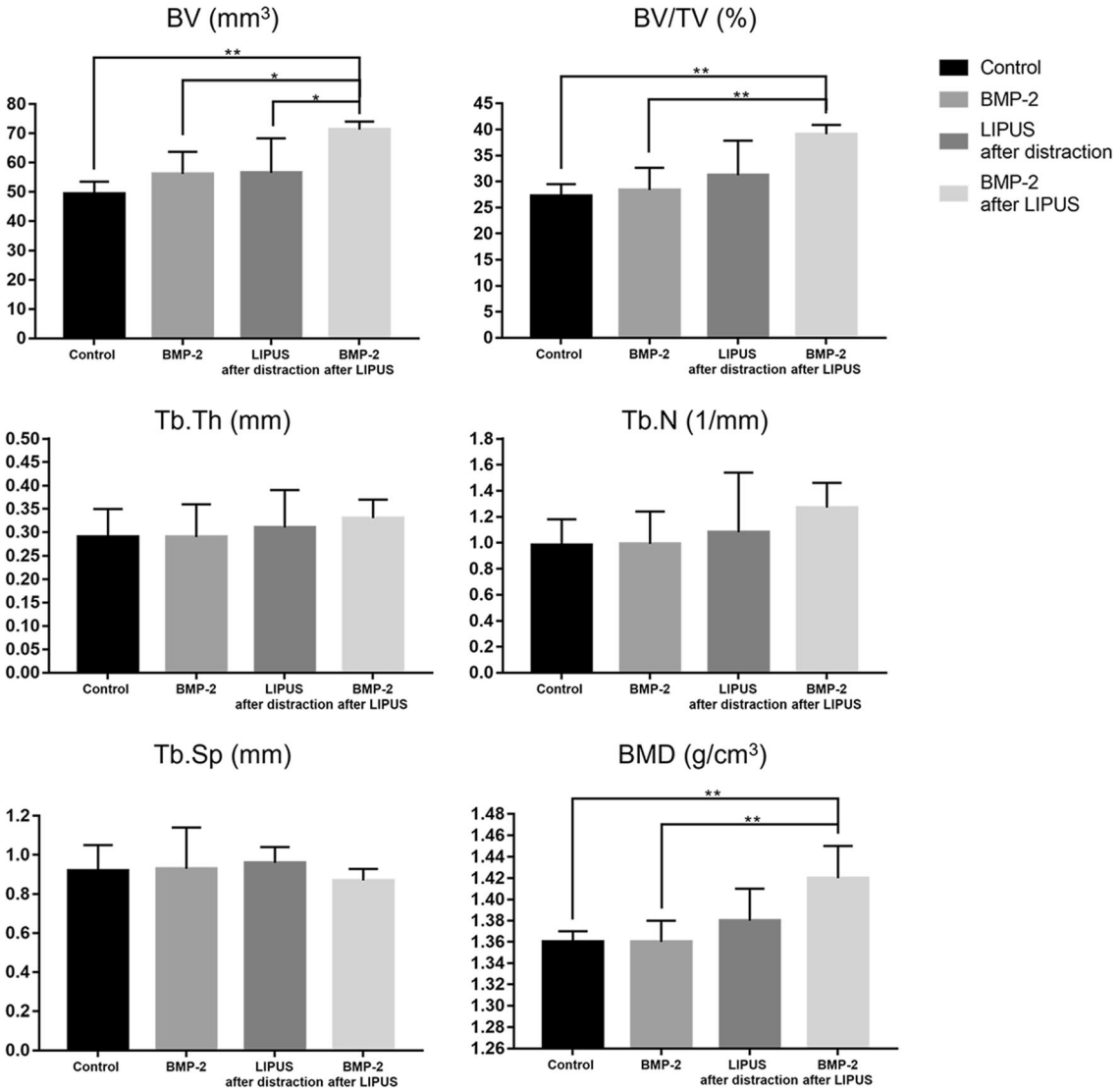

Fig. 9 Determination of synergistic effect of BMP-2 and LIPUS on bone regeneration. Results of micro-CT analysis were compared among the control, BMP-2, LIPUS after distraction, and BMP-2 after LIPUS groups. Significant difference between the groups is indicated by $*p < 0.05$ and $**p < 0.01$. *BV* bone volume, *TV* tissue volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular separation, *BMD* bone mineral density

consolidation periods of tibial DO in rabbits and compared bone formation according to the timing of LIPUS application. In that study, BMD and mechanical strength were the greatest when LIPUS was applied during the distraction period, and endochondral bone formation occurred earlier when LIPUS was applied during the distraction or consolidation period than when LIPUS was applied during the latency period or when LIPUS was not applied. In this study, the group that received LIPUS from the distraction phase to the consolidation phase was compared with the group that received LIPUS only during the consolidation phase. The two groups showed comparable BV, BMD, and other histomorphometric findings in the distraction gap. These results are inconsistent with those of study by Sakurakichi et al. [24]. First, in this study, the period of applying LIPUS during the distraction phase was 3 days of the 5-day distraction phase, which may have been a relatively short period for LIPUS to exert its effect. In addition, because the micro-CT analysis and histologic analysis were conducted after 4 weeks of consolidation, even if the early bone regeneration or maturation was improved by applying LIPUS during the distraction period, the amount of bone formation and the degree of maturation in the group in which LIPUS was applied only for the consolidation phase may have reached a level comparable to those in the group in which LIPUS was applied from the distraction period at the analysis period after 4 weeks of consolidation. This explanation is supported by the results of another study [6],

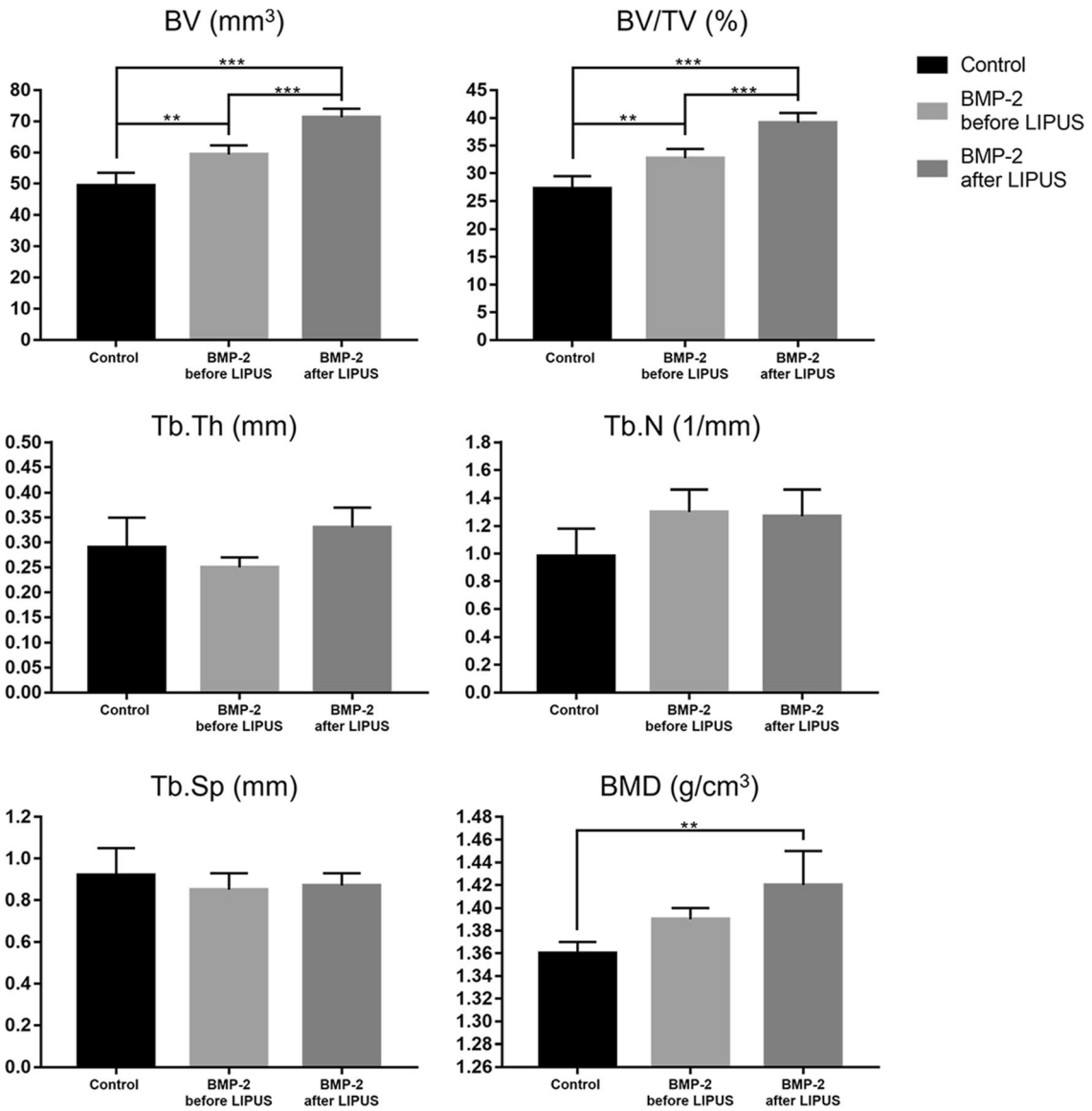

Fig. 10 Effect of timing of BMP-2 application on bone regeneration in the combination of BMP-2 and LIPUS. Results of micro-CT analysis were compared among the control, BMP-2 before LIPUS, and BMP-2 after LIPUS groups. Significant difference between the groups is indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$. *BV* bone volume, *TV* tissue volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular separation, *BMD* bone mineral density

in which the group with LIPUS from the first day of distraction showed higher radiopacity, microhardness, and bone formation than the group without LIPUS until 2 weeks of consolidation, although no significant differences were observed in radiopacity, microhardness, and bone formation at 4 weeks of the consolidation phase.

In this study, BMP-2 and LIPUS, which are known to promote bone regeneration when used alone, were combined to enhance bone regeneration in DO. The combination of BMP-2 and LIPUS to enhance bone growth and healing was first conducted by Sant'Anna et al. [22]. In an *in vitro* study evaluating the expression of several genes involved in osteogenesis, including *Cbfa-1*, *Runx2*, *IGF-receptor*, *Alk-3*, *ALP*, *osteopontin*, *TGF-β1*, and *BMP-7*, the combination of BMP-2 and LIPUS did not show clear synergistic effects on bone marrow stromal cells after 7 days of culture. Another study also reported that no significant differences were observed in the mRNA expression of *Runx2* and *ALP* among the LIPUS, BMP-2, and LIPUS/BMP-2 treatments [27]. Consistent with the literature, the gene expression of *ALP* in this study showed no significant differences among LIPUS, BMP-2, and LIPUS after BMP-2 treatments. Unlike the combination strategy in which BMP-2 was applied before or simultaneously with LIPUS in literature, in this study, gene expression was additionally evaluated in cells in which

LIPUS was applied first, followed by the application of BMP-2. The BMP-2 after LIPUS group resulted in a significantly increased gene expression of *ALP* compared with the LIPUS group. Regarding the *Cbfa1*, the LIPUS after BMP-2 group upregulated gene expression remarkably compared with the LIPUS and BMP-2 groups, and the BMP-2 after LIPUS group also exhibited markedly increased gene expression compared with the LIPUS group. However, with respect to other bone formation and resorption markers analyzed in this study, such as *ALP*, *Osterix*, *VEGF*, *OPG*, and *RANKL*, the three groups treated with BMP-2 (BMP-2, LIPUS after BMP-2, and BMP-2 after LIPUS groups) showed similar gene expression levels. In the assessment of ALP activity, the BMP-2, LIPUS after BMP-2, and BMP-2 after LIPUS groups showed markedly increased ALP activity than LIPUS group, and among these three groups, the BMP-2 after LIPUS group showed the highest ALP activity. In addition to the enhancement of cell differentiation, the application of BMP-2 after LIPUS increased the proliferative activity of rMSCs compared with LIPUS treatment alone in this study, which is consistent with the results in the literature [28]. In the study by Lou et al. [28], the transfer of the *BMP-2* gene into mesenchymal stem cells increased cell proliferation as well as differentiation into osteoblasts. In the application of pulsed electromagnetic field (PEMF), another type of stimulation to promote bone regeneration, the combination of PEMF and BMP-2 showed an additive effect on cell proliferation compared with PEMF alone [29].

Although the synergistic effect of the combination of LIPUS and BMP-2 has not been clearly demonstrated in *in vitro* studies in the literature, several *in vivo* studies have shown the synergistic effect of these two stimuli on bone regeneration. In an animal study by Wijdicks et al. [23], who reported little indication of the synergistic effect of BMP-2 and LIPUS in their prior *in vitro* study, micro-CT findings, including BV and BMD, and histological results revealed that LIPUS enhanced rhBMP-2 induced ectopic bone formation. The combination of BMP-2 and LIPUS also led to enhanced bone formation beyond the extent of bone formation through BMP-2 in a rat model with a critical-sized femoral defect [21]. Recently, BMP-9 was reported to have a synergistic effect on bone formation when used together with LIPUS in a rat calvarial defect model [30]. Although several investigations have assessed the potential bone formation capacity of combined BMP-2 and LIPUS in bone defect models, such as the tibia or mandible, according to our review of the literature, the synergistic effects of BMP-2 and LIPUS on bone formation in DO have not been reported. In this study, the group that received combined BMP-2 and LIPUS exhibited significantly greater bone formation than the BMP-2 and LIPUS groups, and higher BV/TV and BMD than the BMP-2 group at the distraction gap after 30 days of consolidation after mandibular distraction.

When applying BMP-2 to enhance bone healing in the treatment of DO with LIPUS, BMP-2 can be injected at different time points based on the phases of DO or the relative application timing of LIPUS, and the differences in the application timing may affect bone regeneration. In terms of the phases of DO, BMP-2 has been applied at the start of distraction or at the time of the consolidation period in the literature, and bone regeneration with the use of BMP-2 was improved compared with bone regeneration of the control group, regardless of the timing of BMP-2 application [18, 31]. In this study, BMP-2 was applied during the consolidation period after distraction was completed for all experimental groups in which BMP-2 injection was planned. Regarding the combination strategy of BMP-2 and LIPUS, previous studies have applied LIPUS after implanting an absorbable collagen sponge soaked with BMP into the defect or subcutaneously, and it has been reported that LIPUS enhances the bone formation induced by BMP [21, 23, 30]. In this study, to assess the degree of bone healing according to the injection timing of BMP-2 in the combined BMP-2 and LIPUS treatment, BMP-2 was injected before or after LIPUS treatment, and the BMP-2 after LIPUS group that received LIPUS for 2 weeks, BMP-2 injection, and LIPUS for 2 weeks sequentially during the consolidation period induced more bone formation than the group with BMP-2 injection before LIPUS application. Although comparing the results of this study with those in the literature is difficult because this study is the first study on the application timing of BMP-2 in combined BMP-2 and LIPUS, the results of the *in vivo* experiments in this study are consistent with the results of the *in vitro* experiments, in which the cells treated with BMP-2 after LIPUS exhibited the greatest osteogenic differentiation in the analysis of ALP activity. In addition, only the cells treated with BMP-2 after LIPUS showed markedly increased cell proliferation compared with the cells with LIPUS. These results suggest that the combination treatment of BMP-2 and LIPUS can promote bone formation in DO and that applying BMP-2 after LIPUS can lead to improved bone formation in terms of the application strategy.

There have been concerns about the production of antibodies as an immunologic response to BMP-2, which may have a potential effect on the safety and efficacy of BMP-2 [32–35]. In a segmental tibial defect of a sheep, anti-BMP antibody was significantly increased at 3 and 6 weeks after repair of the defect using a composite implant consisting of the BMP and type VI collagen, and impaired mechanical properties of tibia repair was observed at 16 weeks after implantation [34]. In contrast,

in the study using a sheep skull defect model, the serum level of anti-BMP antibody remarkably increased at 3 weeks after implantation of type VI collagen mixed with BMP, however, it returned to basic level at 6 weeks after implantation [35]. With respect to the effect of the production of anti-BMP antibody on bone healing, no significant systemic inhibitory effect on normal bone healing with low risk of immunological response have been reported [32, 33]. In this study, BMP-2 was applied through direct injection into distraction gap, unlike the application methods of previous studies. After injection of BMP-2, an antibody may be produced as a response to BMP-2, and it can inhibit the function of endogenous BMP-2. Therefore, further evaluation of the production of the antibody after direct injection of BMP-2 and the effect of the antibody on bone healing is necessary to assess the long-term safety of the combination of BMP-2 and LIPUS treatment.

In conclusion, we evaluated the synergistic effect of BMP-2 and LIPUS combination therapy on bone regeneration in DO and conducted a study to determine an effective combination strategy. The results of this study suggest that the combined treatment of BMP-2 and LIPUS can lead to enhanced bone healing in DO and that effective bone healing can be achieved through the application of LIPUS before BMP-2.

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Declarations

Conflict of interest The authors have no financial conflicts of interest.

Ethical statement The animal studies were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC no. SNU-141120-2).

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