



The Potential Application of Pulsed Ultrasound on Bone Defect Repair via Developmental Engineering: An In Vitro Study

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Abstract: Repairing bone defect by recapitulation of endochondral bone formation, known as developmental engineering, has been a promising approach in bone tissue engineering. The critical issue in this area is how to effectively construct the hypertrophic cartilaginous template in vitro and enhance in vivo endochondral ossification process after implantation. Pulsed ultrasound stimulation has been widely used in the clinic for accelerating bone healing in fractures and nonunions. The aim of this study was to investigate whether ultrasound (US) could accelerate in vitro chondrogenesis and the hypertrophic process in certain microenvironments. Rat bone marrow mesenchymal stem cells were chondrogenic or hypertrophic differentiated in a three-dimensional pellet culture system with different media, and treated with different

intensities of US. US exposure promoted chondrogenic differentiation of stem cells and inhibited their transition into the hypertrophic stage in a chondrogenic-friendly microenvironment. US significantly advanced hypertrophic differentiation of bone marrow stem cell pellets in hypertrophic medium after chondrogenesis. Our data indicated that pulsed US promoted in vitro chondrogenic and hypertrophic differentiation of stem cell pellets in specific culture conditions. The present study proves the potential application of US in the in vitro stage of “developmental engineering” for bone development and repair. **Key Words:** Ultrasound—Bone repair—Developmental engineering—Cartilage—Mesenchymal stem cells.

Endochondral ossification is an important pathway for bone development during embryonic development. The concept of repairing bone defects by recapitulation of endochondral bone formation has been proposed as a promising approach in bone tissue engineering, known as “developmental engineering” (1). Briefly, mesenchymal stem cells (MSCs) are induced to form hypertrophic cartilaginous constructs as templates in vitro. The templates are then implanted into the bone defect. The defect is repaired through endochondral ossification (2,3).

The critical issue in this area is how to effectively construct the hypertrophic cartilaginous template in vitro and enhance its in vivo endochondral ossification after implantation (4,5).

Pulsed ultrasound (US), a clinically established and widely used mechanical stimulation method, has been an effective and noninvasive tool to repair disorders in bone and cartilage for a long time (6–9). Pulsed US functions as an advanced form of bioreactor that incorporates cavitation effect and acoustic streaming of extracellular fluid (10,11). Therapeutic US with frequencies between 0.5 and 1.5 MHz is known to facilitate fracture healing, bone growth, and cartilage repair (12–15). Studies on bone fracture repair have shown that US could stimulate osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs), increase cell proliferation and alkaline phosphatase activity of osteoblasts, and enhance mineralization in bone for-

doi:10.1111/aor.12578

Received March 2015; revised May 2015.

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mation and endochondral ossification in fracture site (16,17). US promoted the expression of chondrogenic markers of stem cells, enhanced cartilage matrix formation, and accelerated chondrocyte differentiation in chondrogenesis-related studies (18,19). US demonstrated effective stimulation effect on osteogenesis, chondrogenesis, and endochondral ossification during different biological process (20–22).

Based on previous clinical observations and scientific studies, the present article hypothesizes that the stimulation effect of US mainly depends on the biochemical condition of the local microenvironment. US could promote chondrogenic and hypertrophic differentiation in specific culture medium in vitro. Thus, US can be used in developmental engineering for the in vitro stage of bone defect repair.

BMSCs are an attractive cell source for tissue engineering approaches (23). In the present study, rat BMSC pellets were cultured in vitro in chondrogenic medium for 4 weeks, or in chondrogenic medium for 3 weeks followed by 1 week in hypertrophic medium to examine the effect of US of various intensities in different culture conditions and differentiation stages. We demonstrated that US significantly promoted chondrogenic differentiation and maintained chondrocyte phenotype in a chondrogenic-friendly microenvironment, while it switched to stimulate hypertrophic differentiation in a hypertrophic-friendly microenvironment. US can be used as an accelerator during the in vitro stage of repairing bone defects via developmental engineering.

MATERIALS AND METHODS

Cell culture

All animal procedures were reviewed and approved by the Sichuan University Animal Care and Use Committee. MSCs were isolated from bone marrow of 2-week-old male Sprague-Dawley rats (Experimental Animal Center of Sichuan University, Chengdu, China). Briefly, both femora and tibiae were removed and soft tissues were detached. Metaphysis from both ends were resected and bone marrow cells were collected by flushing the diaphysis with 2 mL/bone of Eagle's alpha minimum essential medium (α -MEM; Gibco, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), and 1% penicillin/streptomycin. Cells were resuspended in 5 mL of complete medium, plated in a 75-cm² glass tissue culture flask, and cultured in a humidified atmosphere containing 5% CO₂ at 37°C. After 2 days, the culture medium and nonadherent cells were removed. The medium was changed two or three

times a week. As the culture reached almost complete confluence, cells were subcultured and passage 3 cells were used for subsequent experiments.

Flow cytometry analysis of rat BMSC phenotype

Third passage BMSCs processed as single-cell suspensions at 5×10^6 /mL were prepared in 10% FBS in phosphate-buffered saline (PBS). Fluorescence-labeled fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD29 and CD44, and allophycocyanin (APC)-conjugated anti-mouse CD34 and CD45 were used (all purchased from Abcam, Cambridge, MA, USA). Tubes with CD29 and CD34, and tubes with CD44 and CD45 were incubated in the dark on ice for 60 min, respectively. Cells were then washed twice in PBS (containing 10% FBS). Finally, cells were mixed with PBS and kept cold before analysis by flow cytometry. Nonspecific staining was assessed using relevant isotype controls. Analysis was performed using a fluorescence-activated cell sorter (FACS Calibur; BD Biosciences, San Jose, CA, USA) and data analysis was performed using WinMDI2.8 software (The Scripps Institute, West Lafayette, IN, USA). For each sample, a region for live cells was defined, according to the forward scatter (FSC) and side scatter (SSC) signals, which excluded aggregated cells from the analysis.

Chondrogenic and hypertrophic induction of rat BMSC pellets culture

Passage 3 cells were dissociated, centrifuged, and resuspended to a concentration of 5×10^5 cells/mL in chondrogenic medium (RASMIX-90042, Cyagen Biosciences, Guangzhou, China) containing 100 μ L/L dexamethasone, 3 mL/L ascorbate, 10 mL/L insulin-transferrin-selenium (ITS), 1 mL/L sodium pyruvate, 1 mL/L proline, and 10 mL/L transforming growth factor- β 3 (TGF- β 3). Cells were then centrifuged at $150 \times g$ for 5 min and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h, cells were aggregated into pellets, and cell pellets were carefully transferred to 6-well plates, 8 pellets per well with 2 mL chondrogenic medium. The medium was changed every 2–3 days, and cells were cultured for 4 weeks. In order to induce a hypertrophic phenotype, BMSC pellets were cultured for an additional week in hypertrophic medium lacking TGF- β 3, but supplemented with 50 nM thyroxine (T1775, Sigma-Aldrich, St. Louis, MO, USA) after 3 weeks of chondrogenic induction (4,24). The hypertrophic medium also contains 7.0×10^{-3} M



FIG. 1. US device for the application of pulsed US in vitro. The US device consists of a control box and an array of two transducers, each 30 mm in diameter specifically designed for a 6-well culture plate. Each channel could be individually tuned to give an output of 100, 150, and 200 mW/cm². The culture plates were all placed in the incubator (37°C, 5% CO₂, 95% humidity).

β -glycerophosphate, 10⁻⁸ M dexamethasone, and 2.5×10^{-4} M ascorbic acid (all from RASMX-90021, Cyagen Biosciences).

US stimulation

The US device (working frequency at 1 MHz, pulse repetition frequency at 1 kHz, duty ratio of 20%) was applied for 15 min per day for 4 weeks (17–22). It consisted of an array of two transducers, each 30 mm in diameter, specifically designed for a 6-well culture plate (Fig. 1). Culture dishes were placed on the US transducer array with a thin layer of coupling gel. One culture plate was used for each treatment group. Controls (0 mW/cm²) were handled in the same way using separate culture plates. All US treatments were performed with the culture plates in the tissue culture incubator (37°C, 5% CO₂, 95% humidity).

The experimental setup is shown in Table 1. Chondrogenically differentiated rat BMSC pellets were divided into three different groups based on the intensity: group US1, 100 mW/cm²; group US2, 150 mW/cm²; and group US3, 200 mW/cm². Group USh was cultured in chondrogenic medium for 3 weeks followed by 1 week in a hypertrophic medium with US stimulation of 150 mW/cm² in the whole duration. Group US1 pellets displayed the strongest biological effect among groups US1, US2, and US3 in the first part of our study. Therefore 150 mW/cm² intensity was adopted in Group USh. Group C was the nega-

tive control consisting of uninduced pellets. Group N was the chondrogenic positive control consisting of pellets that were chondrogenic induced for 4 weeks with no US stimulation. Group Nh was the hypertrophic positive control consisting of pellets that were cultured in hypertrophic medium for 1 week without US stimulation, after 3 weeks of chondrogenic induction.

Immunohistochemical and histochemical staining

The cell pellets collected in each groups were fixed in 4% paraformaldehyde for 48 h, rinsed with distilled water, dehydrated in a graded series of ethanols, embedded in paraffin, and cut into 5- μ m thick sections that were collected on slides. The sections were subjected to hematoxylin and eosin (H&E) and safranin O-fast green staining. Immunohistochemical staining of collagen type I, collagen type II, collagen type X, and bone sialoprotein (BSP) was conducted using specific primary antibodies as follows: collagen type I (08A001277; MP Biomedicals, Solon, OH, USA), collagen type II (08A001280; MP Biomedicals), collagen type X (ab7046; AbCam), BSP (A4232.1/A4232.2; Immundiagnostik, Bensheim, Germany), as well as biotinylated secondary antibodies (Dako, Carpinteria, CA, USA). The immunobinding was detected using the appropriate avidin-biotin complex (ABC PK-4000; Vector Laboratories, Peterborough, UK). Image-Pro Plus 6.0 software (Media Cybernet-

TABLE 1. Experimental groups, the intensity of the US stimulus, and the culture conditions in each of seven experimental groups of rat BMSC pellets

Group	Procedure
Group C	No induction + No US stimulation
Group N	CI for 4 weeks + No US stimulation
Group Nh	CI for 3 weeks + HI for 1 week + No US stimulation
Group US1	CI for 4 weeks + 100 mW/cm ² US for 4 weeks
Group US2	CI for 4 weeks + 150 mW/cm ² US for 4 weeks
Group US3	CI for 4 weeks + 200 mW/cm ² US for 4 weeks
Group USh	CI for 3 weeks + HI for 1 week + 150 mW/cm ² US for 4 weeks

CI, chondrogenic induction; HI, hypertrophic induction. Groups C, N, and Nh were control groups.

ics, Silver Spring, MD, USA) was used for semi-quantitative analysis of immunohistochemical images using the following scores: area, integrated optical density (IOD), and mean optical density ($\text{MOD} = \text{IOD SUM} / \text{area SUM}$).

Real-time quantitative polymerase chain reaction (PCR) analysis

Twelve pellets per condition after 4-week culture were homogenized in 1 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA). Equal amounts of RNA samples were reverse-transcribed using the SYBR PrimeScrip RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's protocol. Each real-time PCR was run in triplicate in the ABI PRISM 7300 Fast Real-time System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (TaKaRa). The sense and antisense sequences of primers were as follows: Col2a1 (forward, 5'-GAAGCACATCTG GTTTGGAG-3'; reverse, 5'-TTGGGGTTGAGG GTTTTACA-3'), aggrecan (forward, 5'-TAGAG AAGAAGAGGGGTTAGG-3'; reverse, 5'-AG CAGTAGGAGCCAGGGTTAT-3'), Sox9 (forward, 5'-TGCTCGGAACTGTCTGGAAACT-3'; reverse, 5'-GAGGAGGAGGGAGGGAAAACA-

3'), Col10a1 (forward, 5'-GATGCCTCTTGTCAGT GCTAAC-3'; reverse, 5'-GATCTTGGGTCAT AGTGCTGCTG-3'), Col1a (forward, 5'-GGC AAGACAGTCATCGAATACA-3'; reverse, 5'-GATGGAGGGAGTTTACACGAAG-3'), Runx2 (forward, 5'-GAAATGCCTCTGCTGTTATGAA-3'; reverse, 5'-AAAGTGAAACTCTTGCCCTCGTC -3'), Osterix (forward, 5'-AAGTTCACCTGTCTG CTCTGCTC-3'; reverse, 5'-GGCTGATTGGCTTC TTCTTCC-3'), TGF- β 3 (forward, 5'-AGGTTTT CCGTTTCAATGTGTC-3'; reverse, 5'-TTGGCTA TGTGTTTCATCAGGTC-3'), and β -actin (forward, 5'-ACGGTCAGGTCATCACTATCG-3'; reverse, 5'-GGCATAGAGGTCTTTACGGATG-3'). The PCR amplification reaction was carried out for 40 cycles by denaturing at 95°C for 5 s, and annealing at 60°C for 30 s, followed by melting curve analysis. β -actin was used for normalization. Value of the negative control group (group C) was designated as 1.

Statistical analysis

We performed three or more independent sets of experiments, and each experiment was run at least three times. Data are provided as mean \pm standard deviations (SDs). A one-way analysis of variance

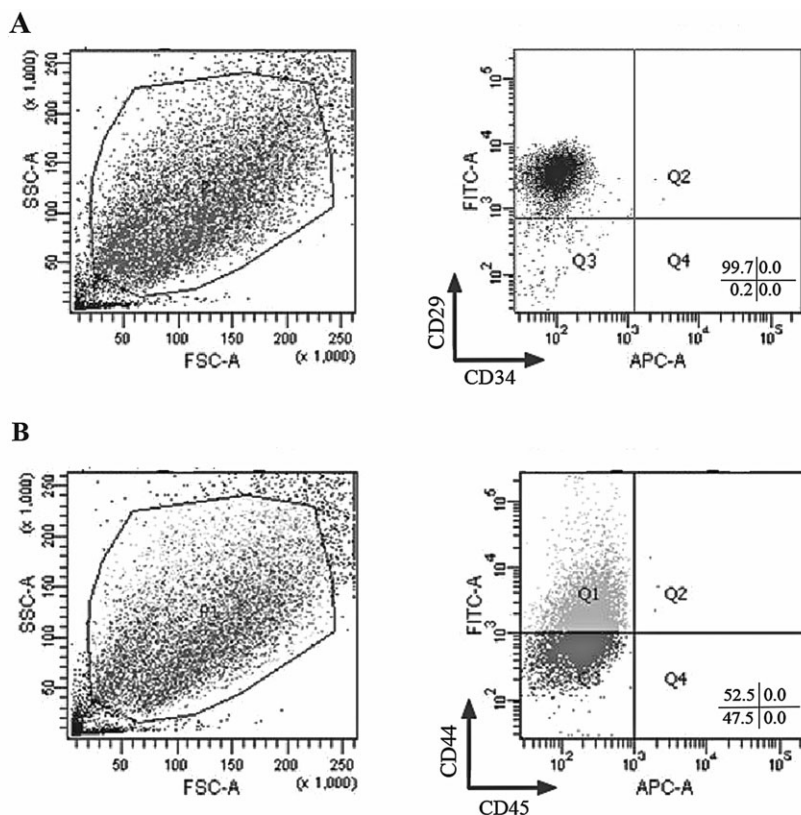


FIG. 2. Flow cytometry analysis of cell surface markers on passage 3 BMSCs. BMSCs were stained for FITC-conjugated anti-mouse CD29 and APC-conjugated anti-mouse CD34 (A) and FITC-conjugated anti-mouse CD34 and APC-conjugated anti-mouse CD44 (B). Left panels: dot plots showing size distribution of all events (cells and debris). Right panels: gates were drawn from isotype control stainings. Percentage of cells in each quadrant is indicated. Plots show data from one experiment out of three.

(ANOVA) was used to evaluate the significant differences among group means. When ANOVA indicated a significant difference between the means, the differences were evaluated using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

BMSC characterization

A representative flow histogram is shown in Fig. 2. BMSCs were positive for CD29 (+) ($99.7 \pm 2.07\%$), CD44 (+) ($52.5 \pm 3.48\%$), and negative for CD34 (–) ($0 \pm 0.12\%$), CD45 (–) ($0 \pm 0.04\%$) (Fig. 2).

US stimulation promoted chondrogenic-related gene and cartilage extracellular matrix expression in chondrogenic medium

Safranin O staining results demonstrated that US exposure groups displayed a more compact pellet structure and higher safranin O staining level compared with group N (Fig. 3, right panel). Immunohistochemical staining revealed that US upregulated expression of collagen type II in comparison with group N. The positive staining was expressed in the extracellular matrix and heightened in confined spots within the pellets. Furthermore, group US2 (150 mW/cm^2) and group US3 (200 mW/cm^2) displayed higher collagen type II expression than group US1 (100 mW/cm^2) (Fig. 4).

Real-time RT-PCR showed that mRNA expression of collagen type II and aggrecan was upregulated in US groups over group N. And the stimulation effect of 150 and 200 mW/cm^2 was stronger than that of 100 mW/cm^2 . In addition, 150 mW/cm^2 US significantly upregulated the gene expression of TGF- $\beta 3$. Interestingly, a significant decrease of Sox9 mRNA expression was detected in US groups compared with group N (Fig. 5).

US inhibited expression of hypertrophic and osteogenic markers in chondrogenic medium

US significantly decreased collagen type I protein expression in US groups compared with group N. The positive staining area was confined predominantly to the outer rim of the pellets and the staining intensity decreased with increasing intensity of US. BSP expression was rarely detected in US groups, whereas it was weakly expressed in group N (Fig. 4). The expression of type X collagen gene (Col10a1), a marker for chondrocyte hypertrophic differentiation, was significantly lower in US groups compared with group N. The mRNA expression of Runx2 was downregulated in US groups.

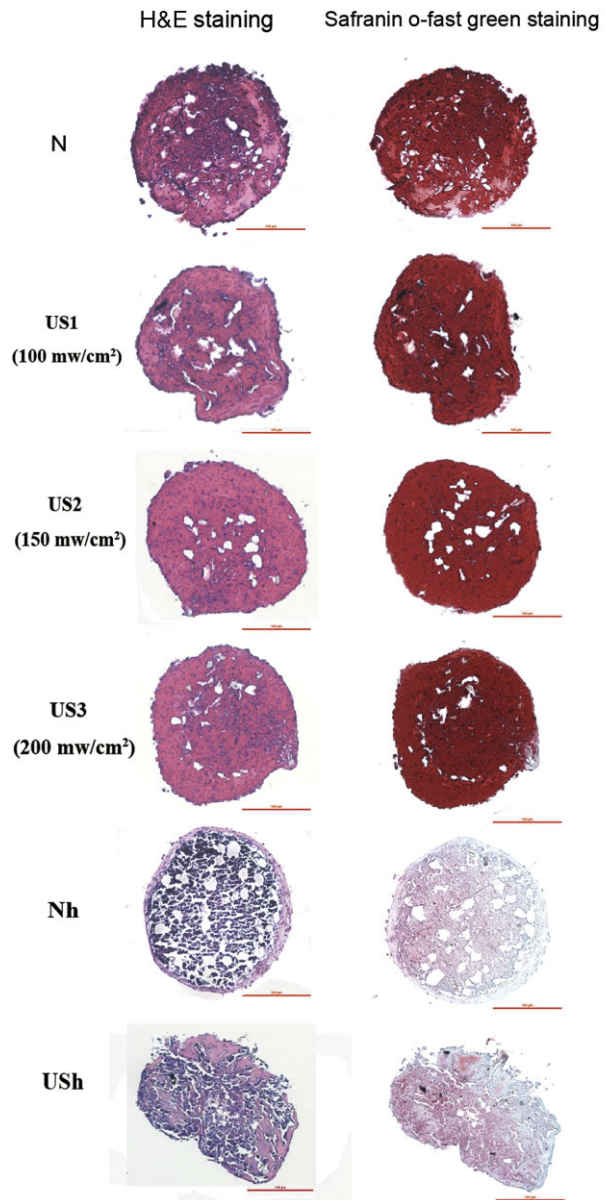


FIG. 3. Safranin red O-fast green staining of rat BMSC pellets. US1, US2, US3: chondrogenic groups exposed to US (US1, 100 mW/cm^2 ; US2, 150 mW/cm^2 ; US3, 200 mW/cm^2) for 15 min/day for 4 weeks. Group N: chondrogenic positive control consisting of pellets that were chondrogenic induced for 4 weeks with no US stimulation. Group USH: cultured in hypertrophic medium for 1 week with 100 mW/cm^2 US, after 3 weeks of chondrogenic induction. Group Nh: hypertrophic positive control consisting of pellets that were cultured in hypertrophic medium for 1 week without US stimulation, after 3 weeks of chondrogenic induction. Scale bar = $100 \mu\text{m}$.

Stimulation of 100 and 150 mW/cm^2 also inhibited Osterix (Osx) mRNA expression significantly (Fig. 5).

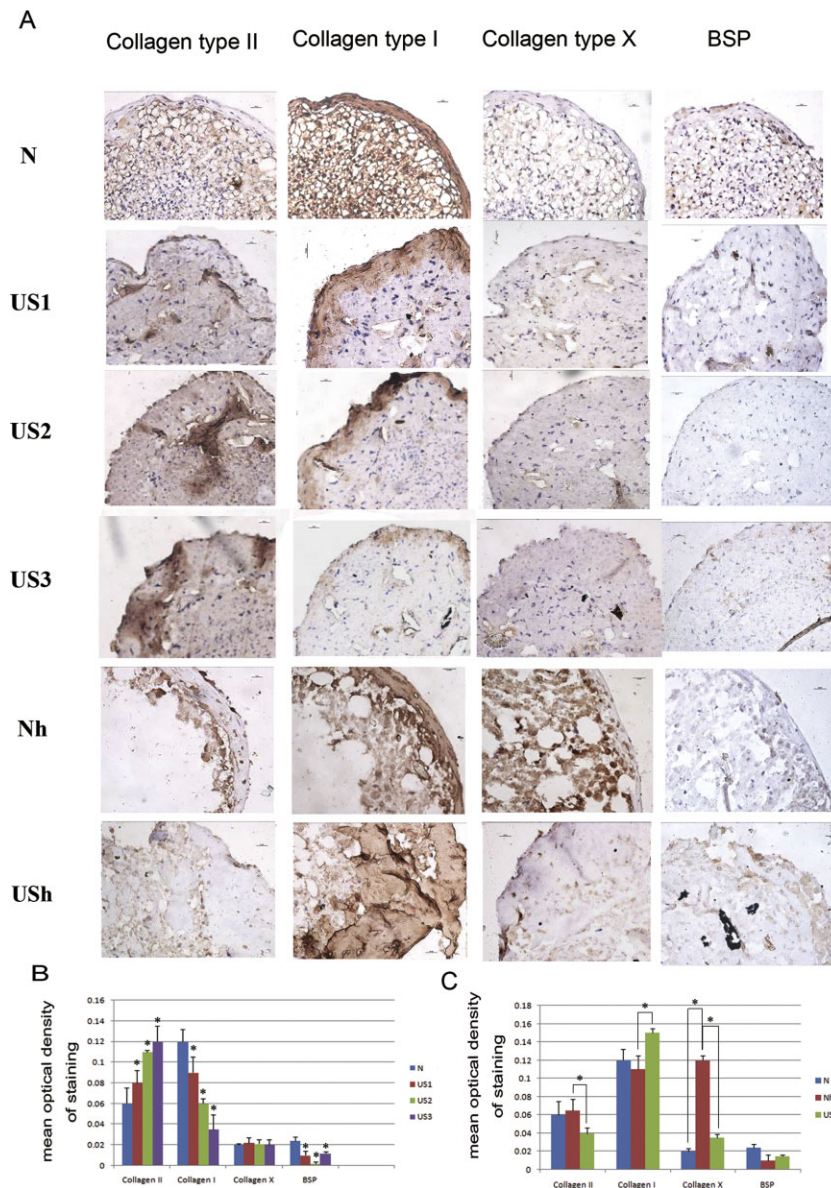


FIG. 4. Effect of US treatments on chondrogenic- and osteogenic-related protein expression in rat BMSC pellets. (A) Immunohistochemistry results of collagen II, collagen I, collagen X, and BSP in BMSC pellets with US stimulation. Scale bar = 10 μ m. (B) Mean optical density of collagen II, collagen I, collagen X, and BSP in immunohistochemical staining of US-stimulated chondrogenic groups. * $P < 0.05$ when compared with group N. (C) Mean optical density of collagen II, collagen I, collagen X, and BSP in immunohistochemical staining of US-stimulated hypertrophic group. * $P < 0.05$ when compared with group Nh.

US stimulation promoted hypertrophic differentiation of rat BMSC in the hypertrophic induction medium

By H&E staining, the hypertrophic-induced pellets (group Nh) displayed a basophilic matrix and marked necrosis centrally with a ring of peripheral cells. In group USh, the hypertrophic pellets stimulated with 150 mW/cm² showed a scattered eosinophilic matrix without obvious peripheral cells (Fig. 3, left panel). Safranin O staining in both group USh and group Nh was almost negative (Fig. 3, right panel). US stimulation reduced collagen type II and collagen type X protein expression in group USh compared with group Nh. Collagen type I protein expression in group USh was significantly higher

than group Nh. And unlike the “shell”-like distribution of the positive staining in group Nh, group USh displayed diffused deposition of collagen type I, which accumulated mainly in the external zone of the pellets (Fig. 4). Consistent with the immunohistochemistry observations, US increased gene expression of collagen type I and Osterix, and decreased mRNA expression of Sox9 and Col10a1 in group USh significantly compared with group Nh (Fig. 6).

DISCUSSION

Evidence has shown that bioreactors that impart multifactorial mechanical stimulation, such as US,

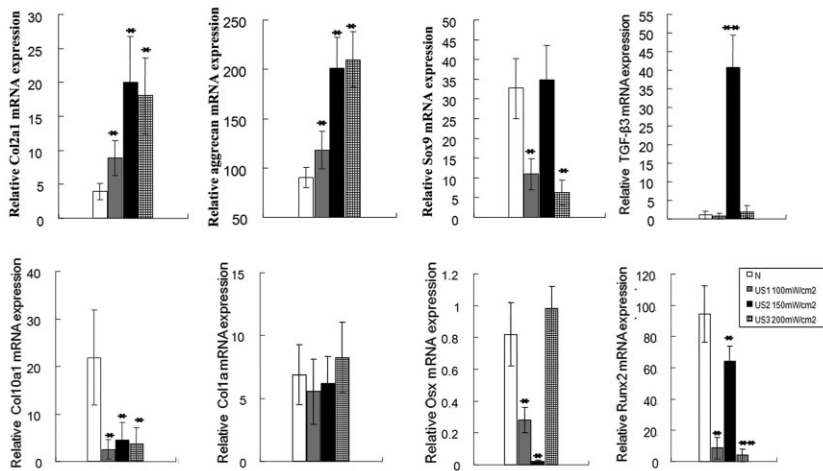


FIG. 5. Effect of US stimulation on chondrogenic and osteogenic gene expression in chondrogenic rat BMSC pellets. Quantitative real-time RT-PCR analysis of chondrogenic- and osteogenic-related gene expression levels using total RNA isolated from US-stimulated chondrogenic pellets. * $P < 0.05$ when compared with group N (Y-axis: $2^{-\Delta\Delta Ct}$).

further enhance the chondrogenic and osteogenic response of MSCs to mechanical loading (11). In the present study, US was applied and supposed to stimulate the process of in vitro chondrogenic and hypertrophic differentiation in certain medium. US applied in this study was generated by a custom-designed machine. Several important parameters of the device could be modulated within certain range, including the duration time, pulse repetition frequency, duty ratio, and the US intensity.

Chondrogenic, hypertrophic, and osteogenic-related markers were evaluated to detect the different differentiation trend in the present study. Briefly, collagen type II, aggrecan, and TGF- β 3 are known as related to cartilaginous differentiation and formation of new cartilage (25). Runx2 is a master regulatory transcription factor for osteogenesis

(26,27). Col10a1 is the only known hypertrophic chondrocyte-specific molecular marker and a direct transcriptional target of Runx2 during chondrogenesis (28). Osterix is located downstream of Runx2, which controls osteoblast lineage commitment and subsequent bone formation. BSP has been reviewed as a valuable marker for osteogenic differentiation and de novo mineralization in bone formation (29).

In the US groups, US significantly enhanced the protein and gene expression of collagen type II and aggrecan. TGF- β 3 gene expression was significantly upregulated by 150 mW/cm² US. US inhibited the protein expression of collagen type I and BSP, as well as the gene expression of collagen type X. These results indicated that US significantly promoted chondrogenic differentiation and the synthesis of cartilaginous extracellular matrix. US inhibited the

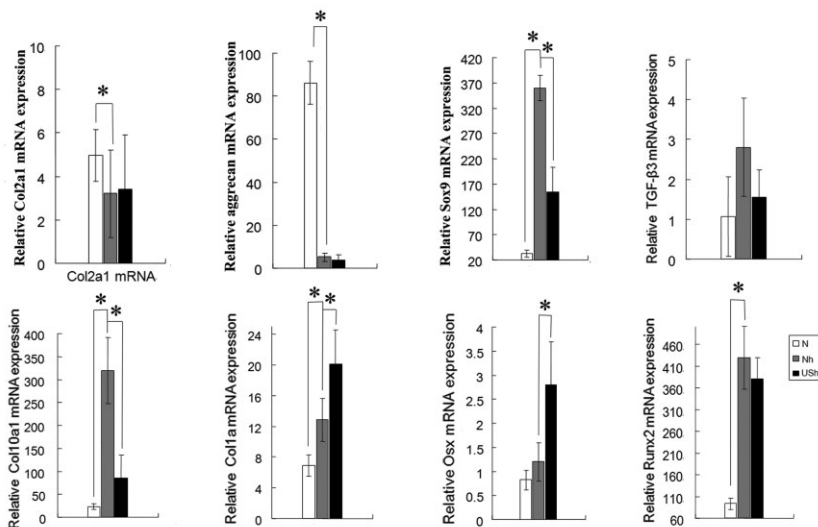


FIG. 6. Effect of US stimulation on chondrogenic and osteogenic gene expression in hypertrophic-differentiated rat BMSC pellets. Quantitative real-time RT-PCR analysis of chondrogenic- and osteogenic-related gene expression levels using total RNA isolated from US-stimulated hypertrophic pellets. * $P < 0.05$ when compared with group Nh (Y-axis: $2^{-\Delta\Delta Ct}$).

hypertrophic differentiation in the chondrogenic medium. US plays a role in chondrogenesis and the maintenance of cartilage phenotype in chondrogenic-friendly microenvironment.

Interestingly, a decreased Sox9 gene expression was detected in chondrogenic BMSC pellets imposed to US in US groups. Sox9 is a key regulator for early stages of chondrogenesis, chondrocyte proliferation, and expression of cartilage matrix genes (30,31). As the Sox9-dependent chondrocyte-specific proliferation gene, Col2a1 expression was upregulated by US stimulation. It is supposed that US may accelerate the in vitro chondrogenesis process in chondrogenic-friendly microenvironment. The downregulation of Sox9 gene expression was possibly due to the fact that stem cell pellets have moved into the late stage of chondrogenic differentiation after 4-week in vitro culture.

When rat BMSC pellets were switched to hypertrophic medium after 3-week chondrogenic induction, expression of cartilage-related collagen type II protein and Sox9 gene was down-regulated in group USh. US inhibited the expression of chondrogenic and cartilaginous related markers. As to hypertrophic-related factors, a decrease of hypertrophy-related collagen type X protein and gene expression was detected in group USh. Researchers revealed that the expression level of collagen X is increased when the immature chondrocytes differentiate into hypertrophic chondrocytes, and decreased when the hypertrophic chondrocytes differentiate into osteoblasts (28,32). US stimulation significantly upregulated collagen type I, Runx2, and Osterix expression in hypertrophic-induced stem cell pellets in the present study. The decrease of collagen type X could be explained as US enhanced hypertrophic differentiation and promoted hypertrophic BMSC to differentiate into osteoblasts. Thus, in this study, US was demonstrated to promote hypertrophic differentiation of BMSC pellets in hypertrophic-friendly culture medium after chondrogenic induction.

CONCLUSIONS

The present study showed that ultrasound has played a positive regulation role in chondrogenic and hypertrophic differentiation of rat bone marrow mesenchymal stem cell pellets in certain culture microcircumstances. US can be applied to the in vitro stage of developmental engineering for bone defect repair. In future experiments based on this work, the effect of US on the later in vivo stage of developmental bone engineering after implantation of intermediate hypertrophic cartilaginous template should be

studied. In conclusion, US could accelerate in vitro chondrogenic and hypertrophic processes and work as a potential strategy for bone formation and repair in the concept of “developmental engineering.”

Acknowledgments: This study was supported by grants from the National Natural Science Foundation of China (No. 30900287 and 81030034) and Sichuan Provincial Sci. & Tech. Department, PR China (No. 2013SZ0057).

Author contributions: Jue W., N. T., Q. X., L. Z., Y. L., J. L., Jun W., Z. Z., and L. T. designed research; Jue W., N. T., Q. X., L. Z., Y. L., and J. L. performed research; Jue W., N. T., Q. X., Jun W., Z. Z., and L. T. analyzed data; and Jue W., N. T., Q. X., J. L., Jun W., Z. Z., and L. T. wrote and revised the article.

Conflict of Interest: The authors declare no conflict of interest.

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