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Low-intensity pulsed ultrasound promotes endothelial cell-mediated osteogenesis in a conditioned medium coculture system with osteoblasts

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Abstract

Angiogenesis plays an important role during bone regeneration. Low-intensity pulsed ultrasound (LIPUS) has been proven to accelerate the process of bone fracture healing. However, the mechanism of the effect of LIPUS on bone regeneration is still unclear. In the present study, we used human umbilical vein endothelial cell (HUVEC) and human osteosarcoma cell (MG-63) to investigate the effect of LIPUS stimulation in an endothelial cell–osteoblast coculture system. At the same time, we used transwell and in vitro angiogenesis assay to observe how LIPUS affects endothelial cells. The results demonstrated that LIPUS could significantly increase the migratory ability and promote tube formation in angiogenesis of HUVECs. Furthermore, LIPUS could significantly elevate the expression of osteogenesis-related genes on osteoblasts such as Runt-related transcription factor 2, alkaline phosphatase, Osteorix, and Cyclin-D1, indicating the pro-osteogenesis effect of LIPUS in our coculture system. In conclusion, endothelial cell is involved in LIPUS-accelerated bone regeneration, the positive effect of LIPUS may be transferred via endothelial cells surrounding fracture healing site.

Abbreviations: ALP = alkaline phosphatase, ANG = angiopoietin, HUVEC = human umbilical vein endothelial cell, LIPUS = low-intensity pulsed ultrasound, Runx2 = Runt-related transcription factor 2, VEGF = vascular endothelial growth factor.

Keywords: angiogenesis, endothelial cells, low-intensity pulsed ultrasound, osteoblasts, osteogenesis

1. Introduction

During bone fracture healing, the coupling of osteogenesis and angiogenesis plays a pivotal role in maintaining bone metabolism balance. The process of vascularization occurs during different stages of bone development and regeneration. Newly formed vessels deliver nutrients, oxygen, osteoblast progenitor cells, and other essential regulatory factors to support bone formation. Osteoblasts secret angiogenic factors, such as vascular endothelial growth factor (VEGF) and angiopoietin (ANG), that further promote vascular formation.[1,2] During bone regeneration, angiogenesis precedes osteogenesis.[3] Despite the significant regenerative capacity of bone, approximately 5% of the patients experience delayed union or nonunion after a fracture.[4] A sufficient blood supply is indispensable for successful bone fracture healing, and the study has demonstrated that the nonunion rates of fractures with large vascular injuries are much higher than global nonunion rates (46% vs 10%).[5] In addition, animal experiments have shown that inhibition of angiogenesis after a fracture results in fibrous scar tissue formation, resembling that seen when nonunion occurs in humans.[6]

Low-intensity pulsed ultrasound (LIPUS) has been approved as a noninvasive treatment for fresh fractures by the US Food and Drug Administration. The study has shown that LIPUS increases bone volume and bone callus formation, and is effective for treating delayed union and nonunion.[7–10] Several previous studies have investigated the effects of LIPUS using monoculture systems of endothelial cells and osteoblasts. However, these culture systems are limited in that the interaction between endothelial cells and osteoblasts cannot be investigated. Specifically, the process of angiogenesis and osteogenesis cannot be evaluated in a monoculture cell system at the same time. To overcome this limitation, coculture systems have been developed to investigate the interaction of paracrine signaling and cell-to-cell communication.[11]

Studies have documented the positive effect of LIPUS on bone metabolism and regeneration, and that sufficient blood flow in the fracture area leads to greater angiogenesis and osteogenesis.[12] However, few studies have investigated the link between

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LIPUS and angiogenesis during bone repair. Our previous study demonstrated that enhanced microvessel proliferation was accompanied with accelerated spinal fusion with LIPUS stimulation. However, mechanism of the effect of LIPUS on angiogenesis and osteogenesis is still unknown.

The purposes of the present study were to determine if the media of endothelial cells subjected to LIPUS influenced osteoblast proliferation and differentiation, and determine the role of the media in osteoblast differentiation.

2. Methods

2.1. Cell culture

Human osteosarcoma cells MG-63, which act like osteoblasts, and human umbilical vein endothelial cells (HUVECs) were cultured in high glucose DMEM (Gibco, CA) supplemented with 10% FBS (Gibco), 100 μg/mL streptomycin, and 100 μg/mL penicillin (Biolight, China) in a humidified incubator at 37°C containing 5% CO_2. Media was changed every 2 days, and cells were resuspended and seeded at a density of $5–7 \times 10^4$/mL in 6-well plates.

2.2. LIPUS stimulation

An Exogen 2000$^+$ (Bioventus Inc., Piscataway, NJ) LIPUS system was used in this study. The parameters used for stimulation were as follows: 1.5-MHz pulsed frequency, 200-μs pulse duration, 1.0-kHz repetition rate, and $30 \pm 5.0\,mW/cm^2$ spatial average and temporal average incident intensity. The LIPUS transducer head was centered underneath the cell culture plates, and coupling gel was carefully smeared to ensure contact between the transducer head and the plates. HUVECs in LIPUS group were treated for 20 and 40 minutes, separately. HUVECs in control group were treated with sham LIPUS stimulation for the same durations. All reagents used were analytical grade, and devices were sterilized before use. The experimental procedures were approved by the Laboratory Ethics Center of the Second Military Medical University.

2.3. Preparation of conditioned medium

HUVECs were seeded into 6-well plates at the density of 5×10^4 cells/mL. Cells were cultured for 24 hours before LIPUS treatment. Then, HUVECs were treated with LIPUS for 20 and 40 minutes as described above. After LIPUS treatment, HUVECs from each group (sham, 20 minutes, and 40 minutes of LIPUS treatment) were cultured for another 24 hours before the medium was collected into centrifuge tubes. The collected culture medium was centrifuged immediately at 200 *g* in 4°C for 10 minutes. The supernatant was extracted and mixed with equal volume of fresh culture media.

MG-63 cells in 6-well plates were cultured with 2 mL of the above mixed conditioned medium for 24 hours before total RNA was extracted.

2.4. RNA extraction and qRT-PCR analyses

Trizol reagent (Invitrogen, CA) was used according to the manufacturer's protocol to extract MG-63 total RNA. Briefly, trizol reagent (500 μL) was added into each well and incubated at room temperature for 5 minutes. Then, 100 μL chloroform (BioLight, Shanghai, China) was added into the mixture. After 5 minutes' incubation at room temperature, samples were centrifuged at 12,000 *g* for 15 minutes at 4°C. After washed with 75% ethanol (BioLight, Shanghai, China) and air-dried in room temperature for 5 minutes, the pellet was resuspended with 20 μL nuclease-free water and total RNA was stored at −80°C for further study.

The SYBR Green PCR Master Mix (TOYOBO, Japan) was used according to the manufacturer's protocol to perform qRT-PCR, and detect gene expression. Primers were designed according to PubMed Primer-Blast data (Supplemental data, http://links.lww.com/MD/B919). ABI PRISM 7700 system (Applied Biosystems, CA) was used to perform qRT-PCR. Same thermal cycle conditions were applied for all primers (60 seconds, 95°C for hot start followed by 40 cycles of 15 s/95°C; 15 s/60°C, and 45 s/72°C). C_T values were normalized with geometric mean of the endogenous control gene β-actin.

2.5. Transwell assay

For the transwell assays, 200 μl aliquots of 2×10^3 HUVECs/mL in serum-free medium were plated onto the transwell chambers of 8 μm pore, 6.5 mm polycarbonate transwell filters (Corning, NY). Then, 500 μl of DMEM medium containing 10% FBS was added to the bottom of the transwell filters. Cells were cultured for 12 hours before treatment. Cells were treated with LIPUS or/and VEGF, and cultured to migrate through the filters for 24 hours. Cells on the upper surface were wiped off with cotton swabs. Cells attached to the underside of the polycarbonate membrane were fixed in 4% paraformaldehyde for 20 minutes, and stained with 1% crystal violet for 20 minutes. The numbers of stained cells on the lower surface of the filters were quantified using a microscope. Five random fields were counted for every transwell filter.

2.6. In vitro angiogenesis assay

The tube formation capacity of HUVECs on Matrigel was measured according to the Matrigel matrix protocol. Matrigel matrix (Corning, NY) was plated into precooled 96-well chambers (100 μL/well), and solidified at 37°C in 5% CO_2 for 30 minutes. Then, 200 μL aliquots of 8 x 10^4 HUVECs/mL were resuspended in culture medium and plated onto the Matrigel matrix in the chambers. The HUVECs were incubated at 37°C for 1 hour, and then treated with LIPUS (sham, 20 and 40 minutes). After LIPUS treatment, the HUVECs were cultured for another 8 x0200A;hours to form a tube. Angiogenesis was observed with an inverted phase contrast microscope, and analyzed using NIH Image software Image J.

2.7. Statistical analysis

Data were analyzed using SPSS version 22.0. The results of the transwell assay and in vitro angiogenesis assay of different groups were analyzed by Student *t* test. A *P* value of $<.05$ was considered to be statistically significant.

3. Results

3.1. LIPUS upregulated osteogenesis-related genes expression in a coculture system

The expression of osteogenesis-related genes in MG-63 cells was determined by qRT-PCR. The results showed that the expressions of Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and Osterix in MG-63 cells cultured with

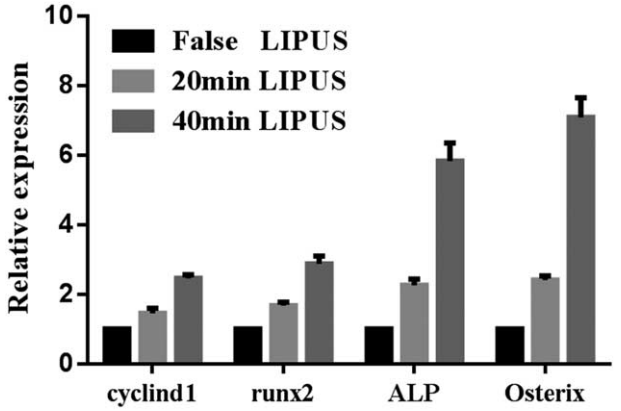

Figure 1. The expression of osteogenesis-related genes was significantly upregulated. After culture with conditioned medium, the relative expressions of differentiation markers Runx2, ALP, and Osterix in MG-63 cells were upregulated. Gene expression was detected by qRT-PCR. The expression of proliferation marker cyclin-D1 was also upregulated. Expression of these genes after 40 minutes of LIPUS treatment was significantly higher than after 20 minutes of LIPUS treatment.

conditioned medium (collected from LIPUS-treated HUVECs) were upregulated compared with the sham LIPUS group. In addition, the expressions of osteogenesis-related genes in the 40 minutes LIPUS group were higher than in the 20 minutes LIPUS group (Fig. 1). Furthermore, to understand the effect of LIPUS on angiogenesis and osteogenesis, we determined the expression of cyclin-D1. As expected, LIPUS significantly upregulated cyclin-D1 expression.

3.2. LIPUS and VEGF promote the migratory potential of HUVECs

To evaluate the effects of LIPUS and VEGF on HUVEC migration, we measured the biological function of LIPUS by transwell cell migration assays. In control group, there were approximately 275.6 ± 28.4 cells per field. Although in LIPUS and VEGF group, there were 466.8 ± 43.4 and 535.4 ± 60.8 cells per field separately. Moreover, in the group with LIPUS and VEGF, 771.6 ± 74.7 cells per field were observed. These observations indicated that LIPUS and VEGF promoted the HUVEC cells migration, and the combination of LIPUS and VEGF might have synergistic effect on HUVEC migration (Fig. 2).

3.3. LIPUS promotes tube formation of HUVECs in vitro

The tube formation assay was performed to investigate the effect of LIPUS on HUVEC angiogenesis potential. Forty minutes of LIPUS treatment had a pronounced influence on tube formation (8.5 ± 1.1 mm per field), and the total tube length was significantly higher than that sham (6.4 ± 0.5 mm per field) and 20-minute LIPUS (6.7 ± 0.9) treatment groups (Fig. 3).

4. Discussion

The results of this study support the involvement of endothelial cells in the response of bone to LIPUS stimulation, which is known to accelerate bone regeneration. We demonstrated an interaction between endothelial cells and osteoblasts with LIPUS

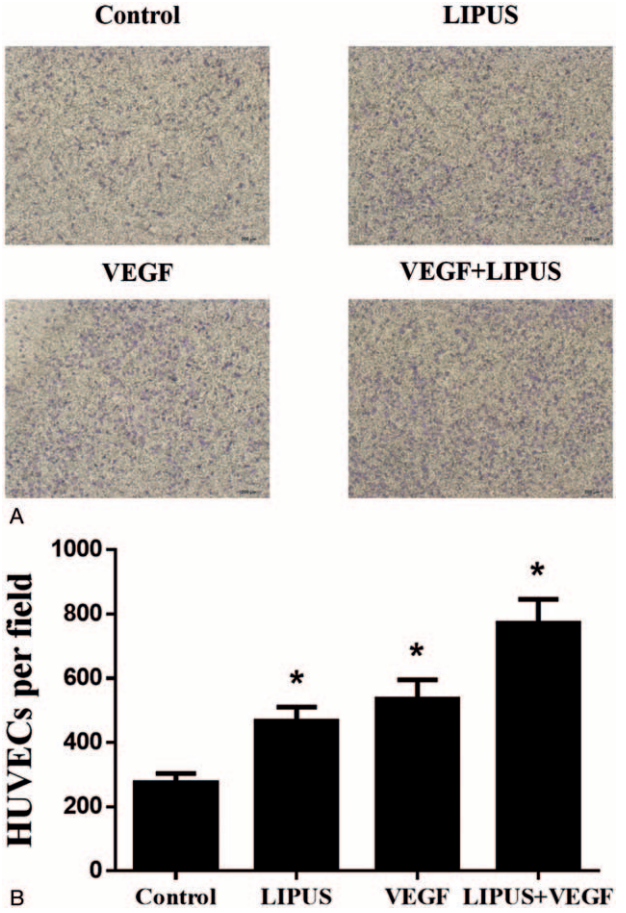

Figure 2. LIPUS and VEGF promote HUVECs migration. Cell migration ability was evaluated by transwell migration assay. LIPUS significantly increased the migratory ability of HUVECs. The addition of VEGF increased the effect of LIPUS on HUVECs. $^{*}P < .05$.

stimulation. The studies have reported that integrins, cadherins, and actins can sense mechanical force by changes to the cytoskeleton,[13,14] which then regulates the spatial organization of different intracellular signals.

Animal studies have shown that blood vessel formation surrounding implants increases 24 hours to 1 month after implantation, which was confirmed by our previous study.[15] Microstress has been proved to improve angiogenesis during bone fracture healing in the first 4 weeks. Yet, no significant difference of blood vessel formation was found with different mechanical forces after week 4 and week 8.[16] This phenomenon is generally explained by the presumption that more microvessels are rapidly formed during bone formation, and the density of microvessels decreases after the bone formation phase is stable. Therefore, understanding the bilateral regulation between angiogenesis and osteogenesis is of great importance for understanding the mechanism of LIPUS-stimulated bone regeneration.

LIPUS imparts a micromechanical force as a mechanical wave that is transmitted into biological signals with nearly no thermal effects.[17] In this study, we investigated the interaction between endothelial cells and osteoblasts, which play primary roles in angiogenesis and osteogenesis, under LIPUS stimulation. We found that LIPUS significantly promoted vessel tube formation. The effect of ultrasound on blood vessels, that blood vessels absorb ultrasound energy and this initiates effects on blood cells,

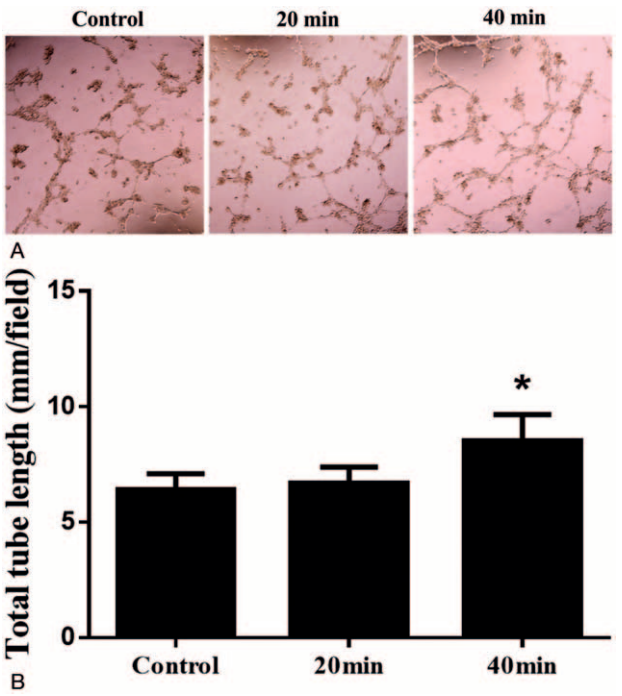

Figure 3. Tube formation assay for angiogenesis. Few tubes were formed in the sham LIPUS group and 20-minute LIPUS group, whereas in the 40-minute LIPUS group tubes were obviously more abundant. The total tube length in the 60-minute LIPUS group was significantly greater than in the 40-minute LIPUS group. $^{*}P < .05$.

was proven by Alekseev et al.[18] Hanawa et al[19] found that LIPUS improved regional blood flow and increased VEGF expression, strongly suggesting the beneficial effect of LIPUS on angiogenesis. Alternatively, different researches have believed that endothelial cells can sense dynamic mechanical strain, and release ATP in response to mechanical stimulation.[20,21] In addition, VEGFR2, a receptor of VEGF, is reported to be a mechanosignaling cascade initiator after it senses mechanical forces. The study has proven that LIPUS affects endothelial cells, and the response of endothelial cells may initiate a cascade amplification effect downstream that impacts osteogenesis.

ALP, which is closely related to calcium phosphate and its deposition in bone, is an early marker that appears before the onset of bone mineralization.[22,23] Many studies have demonstrated the osteogenic effect of LIPUS in vivo and in vitro. Runx2, Osterix, and ALP, as osteogenic differentiation markers, were found to be significantly increased after LIPUS treatment in human mandibular fracture hematoma-derived cells.[24] A study by Southwood et al. showed that the concentration of ALP was decreased in nonunion fractures, indicating the predictive ability of ALP during bone regeneration with an accuracy of 96%.[25] Runx2 acts as a nucleic acid scaffold, and regulates other factors relating to skeletal gene expression. Sugawara et al. found that Runx2 is constitutive in human bone development. In addition, Runx2 is overexpressed in osteosarcoma cells, whereas its expression is limited in less differentiated cells, such as chondrogenic tumors.[26] Osterix acts downstream of Runx2 during bone formation, as it has been shown to be absent in Runx2-null mice.[27]

During the process of bone regeneration, microvessels secrete many pro-osteogenesis molecules such as oxygen, and calcium and phosphate ions. Bo et al. found that the expression of osteocalcin and ALP mRNA was significantly elevated with the presence of HUVECs in MG-63 cell cultures, indicating the positive effect of endothelial cells on osteoblast differentiation.[28] In the present study, we investigated the expression of these factors, which are pivotal for bone regeneration. In our coculture system, LIPUS treatment of endothelial cells significantly increased the expression of the proliferation marker Cyclin-D1 and the differentiation markers Osterix, Runx2, and ALP in osteoblasts, indicating that existence of endothelial cell-promoted proliferation and differentiation of osteoblasts. These results demonstrated the involvement and pro-osteogenesis effect of endothelial cells on osteoblasts in the coculture system under LIPUS stimulation.

There are some limitations of the present study. Our study only focused on the effect of LIPUS in an endothelial cell–osteoblast coculture system. Further study should compare these results with an osteoblast monoculture system to determine if there is a synergistic effect of endothelial cells and LIPUS stimulation. Future study should also investigate the relationship between LIPUS, angiogenesis, and osteogenesis in an animal model, which takes the in vivo physical reaction into consideration.

References

[1] Shi B, Andrukhov O, Berner S, et al. The angiogenic behaviors of human umbilical vein endothelial cells (HUVEC) in co-culture with osteoblast-like cells (MG-63) on different titanium surfaces. Dent Mater 2014;30:839–47.
[2] Kanczler JM1, Oreffo RO. Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater 2008;15:100–14.
[3] Stegen S, van Gastel N, Carmeliet G. Bringing new life to damaged bone: the importance of angiogenesis in bone repair and regeneration. Bone 2015;70:19–27.
[4] Zura R, Braid-Forbes MJ, Jeray K, et al. Bone fracture nonunion rate decreases with increasing age: a prospective inception cohort study. Bone 2017;95:26–32.
[5] Dickson KF, Katzman S, Paiement G. The importance of the blood supply in the healing of tibial fractures. Contemp Orthop 1995;30: 489–93.
[6] Hausman MR, Schaffler MB, Majeska RJ. Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone 2001;29:560–4.
[7] Zura R, Della Rocca GJ, Mehta S, et al. Treatment of chronic (>1 year) fracture nonunion: heal rate in a cohort of 767 patients treated with low-intensity pulsed ultrasound (LIPUS). Injury 2015;46:2036–41.
[8] Zura R, Mehta S, Della Rocca GJ, et al. A cohort study of 4,190 patients treated with low-intensity pulsed ultrasound (LIPUS): findings in the elderly versus all patients. BMC Musculoskelet Disord 2015;16:45.
[9] Koga T, Lee SY, Niikura T, et al. Effect of low-intensity pulsed ultrasound on bone morphogenetic protein 7-induced osteogenic differentiation of human nonunion tissue-derived cells in vitro. J Ultrasound Med 2013;32:915–22.
[10] Jingushi S, Mizuno K, Matsushita T, et al. Low-intensity pulsed ultrasound treatment for postoperative delayed union or nonunion of long bone fractures. J Orthop Sci 2007;12:35–41.
[11] Goers L, Freemont P, Polizzi KM. Co-culture systems and technologies: taking synthetic biology to the next level. J R Soc Interface 2014;11: pii: 20140065.
[12] Ramasamy SK, Kusumbe AP, Schiller M, et al. Blood flow controls bone vascular function and osteogenesis. Nat Commun 2016;7:13601.
[13] Mui KL, Chen CS, Assoian RK. The mechanical regulation of integrin-cadherin crosstalk organizes cells, signaling and forces. J Cell Sci 2016; 129:1093–100.
[14] Bernal A, Pérez LM, De Lucas B, et al. Low-intensity pulsed ultrasound improves the functional properties of cardiac mesoangioblasts. Stem Cell Rev 2015;11:852–65.
[15] Xu X, Wang F, Yang Y, et al. LIPUS promotes spinal fusion coupling proliferation of type H microvessels in bone. Sci Rep 2016;6:20116.
[16] Scarano A, Perrotti V, Artese L, et al. Blood vessels are concentrated within the implant surface concavities: a histologic study in rabbit tibia. Odontology 2014;102:259–66.

[17] Tanaka E, Kuroda S, Horiuchi S, et al. Low-intensity pulsed ultrasound in dentofacial tissue engineering. Ann Biomed Eng 2015; 43:871–86.
[18] Alekseev SI, Ziskin MC. Enhanced absorption of millimeter wave energy in murine subcutaneous blood vessels. Bioelectromagnetics 2011;32: 423–33.
[19] Hanawa K, Ito K, Aizawa K, et al. Low-intensity pulsed ultrasound induces angiogenesis and ameliorates left ventricular dysfunction in a porcine model of chronic myocardial ischemia. PLoS One 2014;9: e104863.
[20] Liu J, Agarwal S. Mechanical signals activate vascular endothelial growth factor receptor-2 to upregulate endothelial cell proliferation during inflammation. J Immunol 2010;185:1215–21.
[21] Joseph EK, Green PG, Bogen O, et al. Vascular endothelial cells mediate mechanical stimulation-induced enhancement of endothelin hyperalgesia via activation of P2X2/3 receptors on nociceptors. J Neurosci 2013;33: 2849–59.
[22] Bosshardt DD. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? J Dent Res 2005;84:390–406.
[23] Unsworth J, Kaneez S, Harris S, et al. Pulsed low intensity ultrasound enhances mineralisation in preosteoblast cells. Ultrasound Med Biol 2007;33:1468–74.
[24] Imai Y, Hasegawa T, Takeda D, et al. The osteogenic activity of human mandibular fracture haematoma-derived cells is stimulated by low-intensity pulsed ultrasound in vitro. Int J Oral Maxillofac Surg 2013;43: 367–72.
[25] Southwood LL, Frisbie DD, Kawcak CE, et al. Evaluation of serum biochemical markers of bone metabolism for early diagnosis of nonunion and infected nonunion fractures in rabbits. Am J Vet Res 2003;64:727–35.
[26] Sugawara M, Kato N, Tsuchiya T, et al. RUNX2 expression in developing human bones and various bone tumors. Pathol Int 2011;61: 565–71.
[27] Baek JE, Choi JY, Kim JE. Skeletal analysis and differential gene expression in Runx2/Osterix double heterozygous embryos. Biochem Biophys Res Commun 2014;451:442–8.
[28] Bo Y, Yan L, Gang Z, et al. Effect of calcitonin gene-related peptide on osteoblast differentiation in an osteoblast and endothelial cell co-culture system. Cell Biol Int 2012;36:909–15.