Original Article
Effect of low-intensity pulsed ultrasound on the biological behaviors of bone marrow mesenchymal stem cells on titanium with different surface topographies

Yanxin An[1,2,3]*, Yan Song[4]*, Zhaoling Wang[4]*, Jing Wang[4], Gaoyi Wu[4], Guoxiong Zhu[4], Lei Chen[1,2]

[1]Department of Orthodontics, School of Stomatology, Shandong University, Jinan, Shandong Province, People's Republic of China; [2]Shandong Provincial Key Laboratory of Oral Tissue Regeneration, School of Stomatology, Shandong University, Wenhua Xi Road No. 44-1, Jinan 250012, Shandong Province, People's Republic of China; [3]Department of General Surgery, Jinan Military General Hospital, Jinan 250031, Shandong Province, People's Republic of China; [4]Department of Stomatology, Jinan Military General Hospital, Jinan 250031, Shandong Province, People's Republic of China. *Equal contributors.

Abstract: The use of low-intensity pulsed ultrasound (LIPUS) is a promising approach to promote osteogenesis. However, few studies have reported the influence of this technique on the osseointegration of endosseous implants, especially regarding different implant topographies. We focused on how the initial interaction between cells and the titanium surface is enhanced by LIPUS and the potential regulatory mechanisms. The bone marrow mesenchymal stem cells (BMSCs) of rats were cultured on two types of titanium surfaces (polished surface, Flat and large grain blast acid etched, SLA) under LIPUS stimulation or control conditions. The cell proliferation on the implant surfaces was significantly promoted by LIPUS, which stimulated the increase in the number of microfilaments, pseudopodia formed and extracellular matrix mineralization nodules compared with those in the control group. The expression of osteogenesis-related genes, including OPN, OCN, BMP-2, ALP, Runx2 and Col-1, were up-regulated on all the surfaces by LIPUS stimulation. Our findings suggest that LIPUS enhances osteoblast differentiation from BMSCs on titanium surfaces. The use of LIPUS might be a potential adjuvant treatment to improve the osseointegration process.

Keywords: Low-intensity pulsed ultrasound, bone marrow mesenchymal stem cells, osseointegration, implant topography

Introduction

Implant dentures can effectively restore the oral function in patients with the absence of dentition. With the development of technology, the achievement ratio of implant dentures was greatly improved; however, due to deficiency or low-quality of bone mass, the failure of implantation still occurs [1]. Recently, many studies have shown that rapid and successful osseointegration is a critical factor necessary for implant fixation [2, 3]. Implant surface modifications, drug delivery systems and biophysical stimulation have been considered to achieve better osseointegration [4, 5]. Because of a non-invasive and locally applied strategy, bio-

physical stimulation is the research topic of interest to enhance bone regeneration around implants among these methods.

Low-intensity pulsed ultrasound (LIPUS) is a type of high-frequency sound wave that could be transmitted in vivo through mechanical vibration [6-8]. It has been demonstrated to have beneficial therapeutic effects on a variety of bone- and cartilage-related disorders [8-11]. Recently, many animal and clinical studies have shown that the frequency of 0.5-1.5 MHz, intensity of 30-200 mW/m^2 ultrasound intervention will promote bone healing, deposition and growth [12]. Although considerable research on the osteogenic effects of LIPUS has

been reported, few studies have evaluated the osteogenic effects of LIPUS on titanium implant surfaces or the underlying mechanism.

It is known to all that bone marrow mesenchymal stem cells (BMSCs) had the potentiality of multi-directional differentiation. To explore whether the LIPUS can promote BMSC differentiation toward osteogenesis, primary BMSC culture under no stimulation (Control) and LIPUS stimulation were evaluated on implant surfaces in this work. Two representative implant surface topographies, SLA and Flat, were used as substrates. The proliferation and differentiation of BMSCs, observation of the microfilaments, pseudopodia formed, the number of mineralization nodules with or without LIPUS stimulation were investigated. Furthermore, to define the initial molecular mechanism associated with BMSC differentiation activated by LIPUS, the expression profile of alkaline phosphatase (ALP), osteocalcin (OCN), type 1 collagen (COL-1), bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor 2 (Runx2) were also investigated.

Materials and methods

Cell culture

The animal procedures were approved by the Ethics Committee of the Jinan Military General Hospital. Briefly, BMSCs were obtained by digesting the marrow cavity of Wistar rats that weighed between 70 and 80 g [13, 14]. The complete BMSCs were cultured in low-glucose DMEM supplemented with 10% bovine calf serum (BCS, Gibco) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; Sigma). Cells were grown in a humidified atmosphere with 5% CO_2 and 95% air at 37°C. Cells from passage 3 were used.

Specimen preparation

Pure titanium was cut into 10 mm × 10 mm × 1.0 mm and 20 mm × 20 mm × 1.0 mm samples through machining. Next, the specimens were polished using 200 #, 400 #, 600 #, 800 #, 1000 #, 1200 # and 1500 # grit of silicon carbide sandpaper. After polishing and cleaning ultrasonically, the substrates were dried in hot air. To prepare sand-blasted SLA surfaces, the substrates were alumina-blasted with large-grit particles (an average grit size of 250

μm) and then acid-etched using a hot solution of HCl/H_2SO_4 following the proprietary process of Institute Straumann AG [15]. The morphologies of the two surfaces were examined by field-emission scanning electron microscopy (FE-SEM; JEOL JSM-4800, Hitachi Corporation, Japan). All specimens were sterilized by ultraviolet irradiation for 30 min before use.
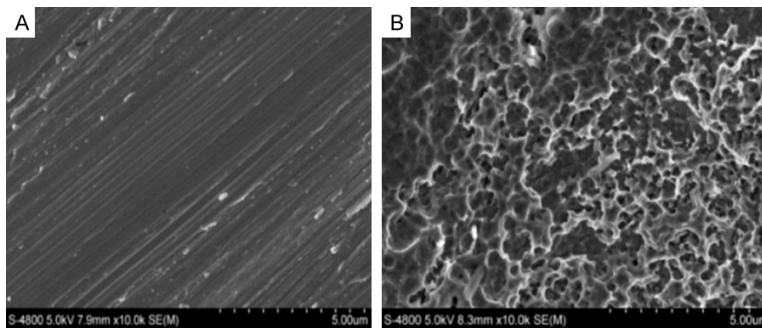
Cell proliferation

A 1-ml aliquot of the cell suspension was seeded onto each specimen at a density of 2×10^4 cells/ml and then was cultured in DMEM with 10% BCS. After culturing for 1, 3 and 5 days, the level of cell proliferation was assessed using the CCK-8 Detection Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The specimens were then incubated with CCK-8 solution at 37°C for 3 h. The optical density (OD) was measured at 450 nm using a spectrophotometer (Bio-Tek). The LIPUS parameters were as follows: intensity: 100 mW/cm^2, frequency: 1.0 MHz, duty ratio: 10%, time: 10 min. In the control group, the 24-well cell culture plate was also placed in another solenoid without stimulation.
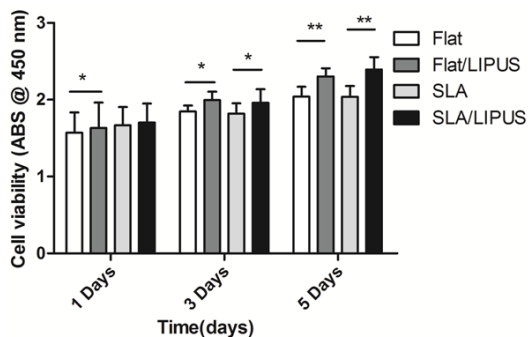
Cell morphology

FE-SEM: The titanium specimens were divided into four groups-, Flat group, Flat/LIPUS group, SLA group, SLA/LIPUS group. The suspension of BMSCs was seeded onto each specimen at a density of 1×10^4 cells/ml. After culturing in DMEM with 10% BCS for 24 h, the samples were fixed with glutaraldehyde solution for 12 h at 4°C, rinsed with PBS three times and dehydrated in a series of acetonitrile washes (50%, 70%, 80%, 90% and 100%). All samples were dried to the critical point, coated with gold and examined using SEM (Hitachi JSM-4800).

CLSM: Confocal laser scanning microscopy (CLSM; Olympus FV 1000) was used to examine the cell morphology and cytoskeletal arrangement of the BMSCs seeded onto the titanium surfaces. After culturing for 24 h, the specimens were stimulated by LIPUS (intensity: 100 mW/cm^2, frequency: 1.0 MHz, duty ratio: 10%, time: 10 min). Cells were fixed in glutaraldehyde solution for 20 min and then were permeabilized with 0.1% Triton X-100. The cells were blocked with VCL (1:100, red fluorescence; Abcam) overnight at 4°C in the dark. Next, the

Figure 1. SEM images at 10,000 × showing the microscale topography. A. Flat. B. SLA.

Figure 2. BMSC proliferation after incubation for 1, 3 and 5 days (*P<0.05, **P<0.01).

ously described, and then the cells were fixed with 4% paraformaldehyde for 20 min. ALP staining was performed using BCIP/NBT solution or 0.1% Alizarin red solution according to the manufacturer's instructions, followed by observation under a stereoscopic microscope.

Western blot analysis

After culturing for 14 days and 21 days, the protein samples were solubilized in RIPA (Beyotime) containing protease inhibitors (Roche, Switzerland). The concentration of the protein was determined using the BCA kit, and western blot analysis was performed as previously described [16]. Primary antibodies against OPN (1:500, Abcam), Runx2 (1:1000, Abcam), BMP-2 (1:1000, CST) were purchased from Abcam or CST. An antibody against β-actin (Sigma) was used as an internal control.
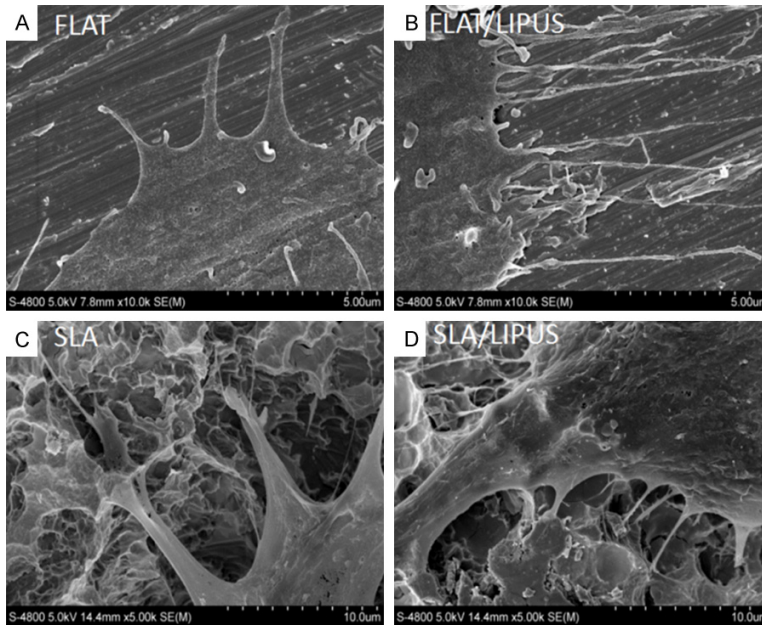
Osteogenesis-related gene expression

The expression levels of osteogenesis-related genes were evaluated using real-time PCR. The cells were seeded at $2 × 10^4$ cells per well. After 14 days' and 21 days' incubation, total RNA was isolated using Trizol reagent (TaKaRa), and RNA was reverse transcribed to cDNA using the PrimeScript RT reagent kit (TaKaRa RR037A). Osteogenesis-related genes, including OCN, OPN, BMP-2, ALP, COL-1 and Runx2, were analyzed by Applied Biosystems 7500 using SYBR Premix Ex Taq IITAKARA (RR820A). The data were analyzed using the iQ 5 optical system software, version 2.0 (Bio-Rad). The relative expression level of each gene of interest was normalized to that of the housekeeping gene GAPDH.

Statistical analysis

The data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by an SNK post hoc test and paired t test was used to determine the level of significance. p<0.05 was considered significant, and p<0.01 was considered highly significant.

cells were blocked with FITC (1:100, green fluorescence; Sigma) for 1 h. DAPI (1:100, blue fluorescence; Sigma) was used to stain the nuclei for 5 min. The cells were analyzed using CLSM.

Alkaline phosphatase activity assay

Cells were seeded at the density of $2 × 10^4$/ml on titanium specimens and were cultured for 3 d and 7 d, followed by centrifugation at 1000 r/min for 10 min. The reagent was added according to the specification of the alkaline phosphatase assay kit, and a microplate reader measured the OD value at 520 nm. ALP activity was calculated according to the following formula: Activity of alkaline phosphatase (Kim/100 ml) = (OD - blank OD/standard OD - blank OD) × standard phenol concentration (0.02 mg/ml) × 100 ml × samples before dilution.

Alkaline phosphatase and Alizarin red staining

Cells were seeded at the density $2 × 10^4$/ml on titanium specimens and were cultured for 14 d. Ultrasound treatment was performed as previ-

Figure 3. SEM images of BMSC adhesion on two surfaces after 48 h. A. Flat/Control. B. Flat/LIPUS. C. SLA/Control. D. SLA/LIPUS. The images were taken at high magnification (5000 ×) to display the detail of cells adhesion on Flat and Micro surfaces.

and spread completely after LIPUS stimulation.

CLSM: The confocal microscopy images show that BMSCs that were stained with vinculin and F-actin to label the cytoskeleton (**Figure 4**). LIPUS stimulation increased the expression of vinculin on all two surfaces, and the expression in the Flat/LIPUS group was increased significantly. More microfilaments were observed in the SLA/LIPUS group. By contrast, most of the cells in the control group did not exhibit a well-developed cytoskeleton. Additionally, there was no obviously difference between the two surface topographies in affecting cytoskeletal morphology.

Results

SEM characterization

The surface morphologies of samples were examined by SEM (**Figure 1**). Residual parallel grooves were observed on the flat samples (**Figure 1A**), and SLA samples exhibited micropitted morphology. SLA treatment caused the surface to become irregular and induced the formation of micropits (**Figure 1B**).

Cell proliferation

According to the results of screening, LIPUS significantly increased cell proliferation on both Flat flat and SLA surfaces (**Figure 2**). On day 1, no significant difference was found between the LIPUS and Control groups regarding the SLA specimens. However, the cell viabilities on all surfaces in the LIPUS-stimulated group were obviously higher than those for the non-stimulated group on days 3 and 5.

Cell morphology

SEM: The SEM images revealed that the BMSCs generated more pseudopodia on the titanium surfaces under LIUPS stimulation (**Figure 3**). Most of the BMSCs on the Flat and SLA specimens exhibited a spindle fibroblast-like shape

ALP activity

As shown in the quantitative experiment in **Figure 5A**, LIPUS stimulation significantly increased the level of ALP activity only on the SLA surface after 3 days of culture (P<0.01); however, after 7 days of culture, the level of ALP activity was increased on both the flat and SLA surfaces (P<0.05). The results of ALP staining showed that, compared with the control group, LIPUS stimulation led to slightly higher ALP activity on all two surfaces after 2 weeks of osteogenic induction (**Figure 5B**).

ECM mineralization

ECM mineralization was assayed by Alizarin red staining (**Figure 6**). The applied LIPUS led to more mineralized nodules on both Flat and SLA surfaces. In the control group, no obvious difference in matrix mineralization was observed between the Flat and SLA surfaces.

Osteogenesis-related gene expression

The expression levels of osteogenesis-related genes, including OPN, OCN, BMP-2, ALP, Col-1, and Runx2, are presented in **Figure 7**. Generally, LIPUS stimulation up-regulated the mRNA levels of all osteogenesis-related genes. The expression of OPN was up-regulated slightly after
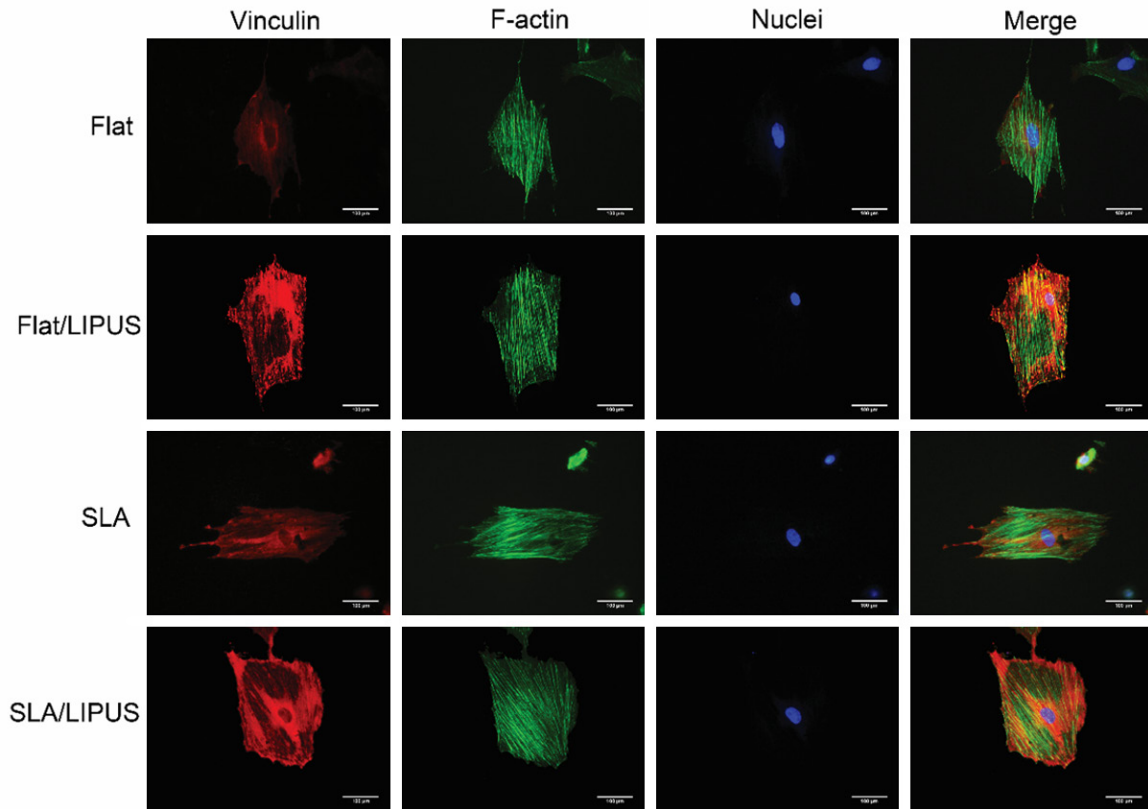
Figure 4. Representative CLSM images of cells stained with red fluorescence to show vinculin, with DAPI to show the nuclei (blue), with F-action to show the actin filaments (green) and with VCL to show the vinculin (red).

LIPUS stimulation at day 14 (P<0.05) but was significantly up-regulated at day 21 (P<0.01, **Figure 7A**). Col1 was most responsive to the applied LIPUS, with maximum increases in expression of 5-fold (Flat/LIPUS) at day 14, and 7-fold (Flat/LIPUS) at day 21, over the corresponding controls in the control group (**Figure 7E**). The expression of OCN was up-regulated by 3-fold (SLA/LIPUS) after LIPUS stimulation at day 14 (**Figure 7B**). BMP-2 was also up-regulated by LIPUS, with increases of 1.8-fold (SLA/LIPUS) at day 14 (**Figure 7C**); however, there was no difference between day 14 and day 21. The expression of ALP was up-regulated in both Flat and SLA group after LIPUS stimulation at day 14 but was decreased after day 21 (**Figure 7D**). Runx2 was also up-regulated by LIPUS in both Flat and SLA groups (**Figure 7F**).

*Osteogenesis-related protein expression*

The expression levels of osteogenesis-related proteins, including OPN, BMP-2 and Runx2, are presented in **Figure 8**. Similar to the gene expression levels, LIPUS stimulation up-regulated the protein levels of all samples. The expression of OPN was up-regulated slightly after LIPUS stimulation at day 14 but was significantly increased at day 21. BMP2 was most responsive to the applied LIPUS, with maximum increases in expression at day 21 over the corresponding controls in the control group. Runx2 was also slightly up-regulated by LIPUS at day 14; however, its expression showed no difference between day 14 and day 21.

Discussion

As a safe and non-invasive physical technique, LIPUS has been used in the delayed healing of bone fracture by the FDA [17]. LIPUS has been shown to exert strong effects on bone repair processes through many mechanisms, including stimulating calcification, increasing the blood supply, inhibiting the resorptive phase and increasing the activity of osteoblasts [18-20]. Recently, several studies have demonstrated the positive effects of LIPUS on implants; however, most of them were observation studies in animals [21, 22]. Few studies have re-
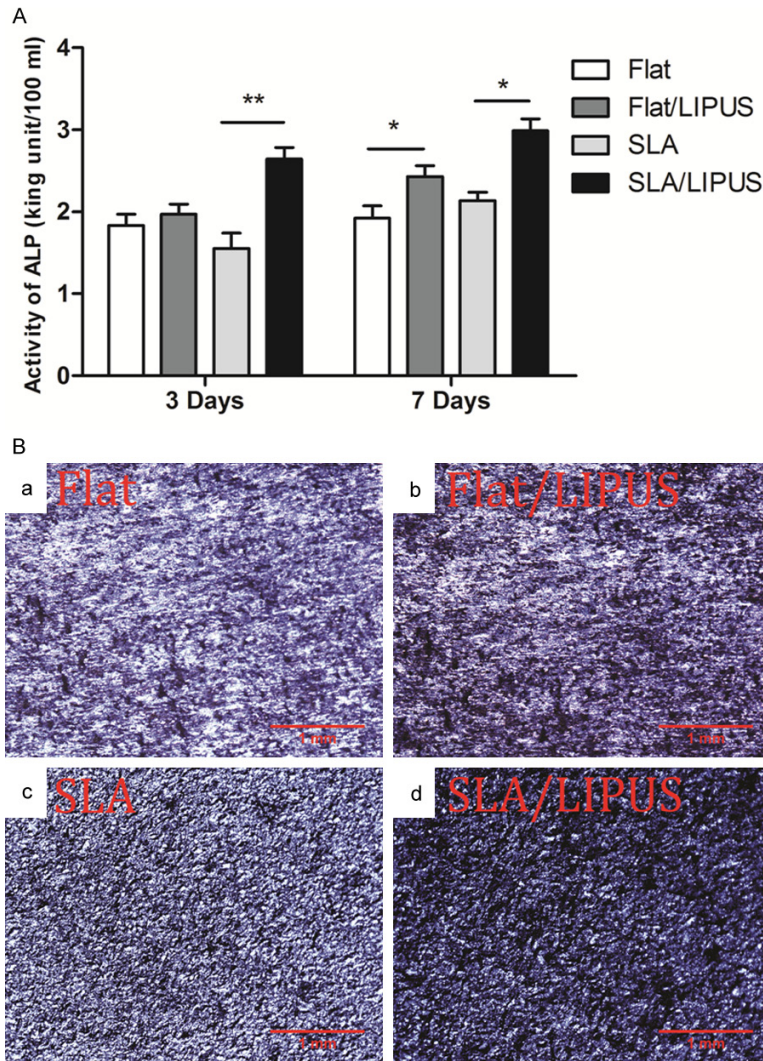
Figure 5. A. ALP activity in different groups cultured for 3 or 7 days. *P<0.05, **P<0.01. B. Optical images of ALP-stained osteoblasts after 2 weeks of incubation.
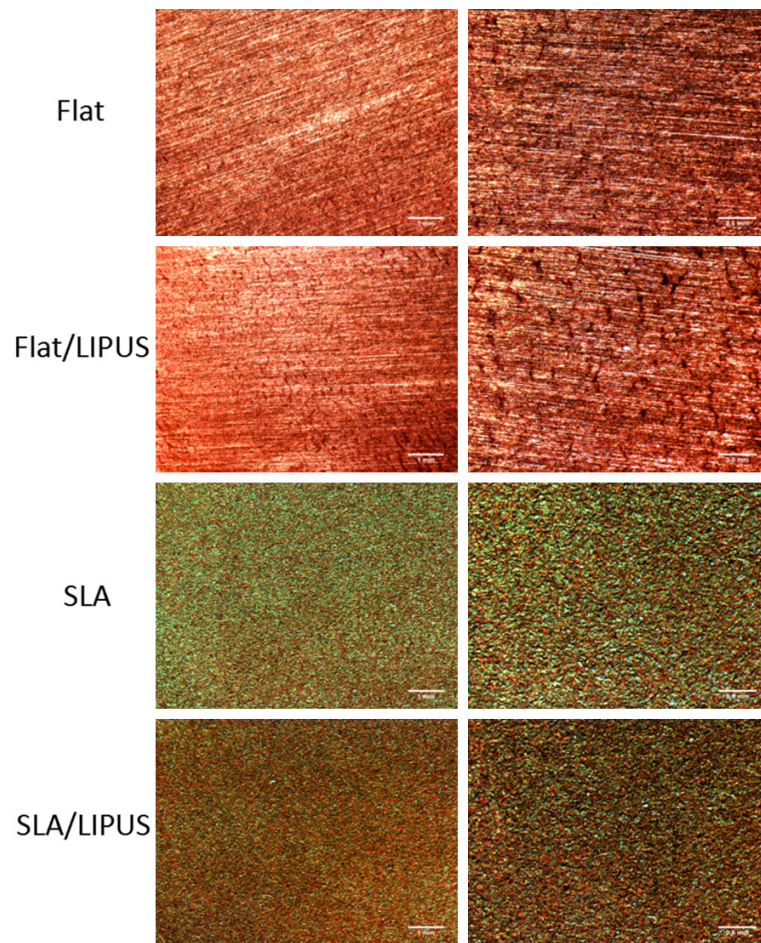
and to understand whether this physical stimulation could make the titanium implant more active for osseointegration in the dental and orthopedic fields.

First, BMSCs are separated from rats, and their character was identified, indicating they were obtained successfully. Second, according to our previous experience [15], two topographies of implants were prepared to use for subsequent detection. Additionally, the optimal parameters of LIPUS stimulation were screened through cell proliferation experiment: intensity: 100 mW/cm^2, frequency: 1.0 MHz, duty ratio: 10%, and time: 10 min. Our results demonstrate that, compared with the control group, LIPUS stimulation promoted BMSC activity in all of the tested surfaces (Flat and SLA). The microscopic results indicated that the cells exhibited dramatic microstructures and orientations after LIPUS stimulation. The SEM results showed that increased filipodia were attached to the surfaces in the LIPUS stimulation group. These results were consistent with those of previous studies [26]. However, this stimulation did not affect the pseudopodia form of BMSCs. In the CLSM experiment, more intracellular microfilaments were observed. By contrast, most cells in the control group did not exhibit well-developed cytoskeletons. Moreover, increased expression of VCL by CSLM also proved that LIPUS promoted cell adhesion and extension in both implants on implant topographies in this study.

ported the effects of LIPUS on the functions of osteoblasts when cultured on titanium implants, especially on Flat or SLA surfaces.

BMSCs play an important role in the progress of bone remodeling. Because of the potential of multi-directional differentiation, BMSCs are regarded as the very important cell in bone healing and regeneration [23]. Several studies have shown that LIPUS could up-regulate the expression of bio-markers of ossification and matrix protein in BMSCs, further increasing the number of osteoblasts [24, 25]. However, BMSCs are selected as the target cells, and the aim of this study was to evaluate the effects of LIPUS on the functions of BMSCs growing on implant surfaces with different topographies

ALP is presented in the prophase of ossification. Increased expression of ALP in BMSCs is represented as well differentiated osteoblasts. Thus, the activity of ALP in vitro is considered the bio-marker of osteoblast differentiation [27, 28]. Our study showed that LIPUS stimulation significantly promotes ALP activity, especially

Figure 6. Analysis of ECM mineralization after 3 weeks of incubation. Images taken at low magnification (Bar = 1 mm) and high magnification (Bar = 0.5 mm).

Regarding the expression of genes associated with osteogenesis, the present study showed that OCN, OPN, ALP, BMP-2, Col-1, and Runx2 were up-regulated in cells on the implant surfaces after LIPUS stimulation. As non-collagen proteins, OCN and OPN were the markers of mature osteoblasts. OPN plays an important role in cell adhesion and mineralization [32, 33]. We found that the expression levels of OCN and OPN were significantly increased by LIPUS stimulation, especially at day 21. The same results were also found by western blotting. It has been established that BMPs affect many stages of endochondral bone formation and mediate the recruitment of cells to repair sites through chemotaxis [34, 35]. Col-1 is another important marker of osteoblast differentiation. The up-regulation of Col-1 transcription might account for the increased ECM formation by LIPUS. BMP2 is a very important member of the TGF-β superfamily, which regulates Runx2 by activating the BMP/Smad pathway [36, 37]. Runx2 is essential for the differentiation of osteoblasts from mesenchymal precursors and the formation of mineralized bone [38, 39]. Taking the above into consideration, we speculated that the BMP2 signaling pathway may play an important role in promoting osteoblast functions under LIPUS stimulation. However, to verify the speculation, more work should be performed to elucidate the process between LIPUS stimulation and conformational changes in biomolecules.

It is well known that implant surface morphology affects the attachment, proliferation and differentiation of osteoblasts and osteogenesis-related gene expression [40]. Our results showed that there was no significant difference between Flat and SLA surfaces, but the SLA surface exhibited greater cell proliferation, ECM
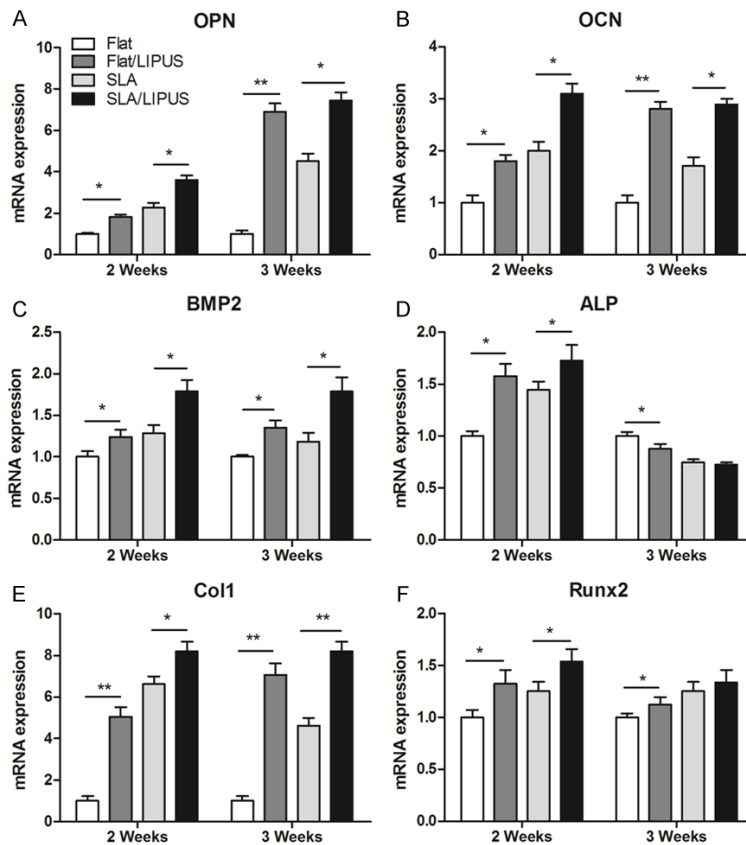
on day 14. However, the expression of ALP is reduced on day 21. Due to the expression of hydroxyapatite, osteocalcin and other genes in the bone mineralization, the activity of ALP was reduced. Additionally, the results of Alizarin red staining showed that more mineralization nodules were observed in the LIPUS stimulation group on day 21, corresponding to the activity of ALP. These results demonstrate that LIPUS can promote the differentiation of BMSCs toward osteoblasts, a finding that was with that in other studies [29-31]. The ALP activity did not differ significantly on the Flat and SLA surfaces between the LIPUS and control groups, but LIPUS stimulation significantly promoted the formation of ECM nodules on the SLA surface. This result is consistent with the findings of previous studies using cell culture plates. Thus, the consensus is that LIPUS exerts a reproducible osteogenic effect.

Figure 7. Relative expression levels of (A) OPN, (B) OCN, (C) BMP2, (D) ALP, (E) Col1 and (F) Runx2. *P<0.05, **P<0.01.
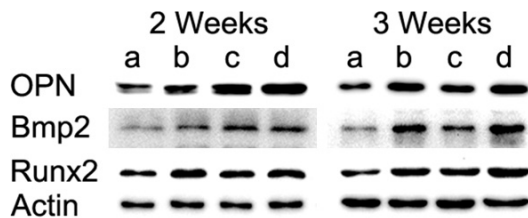
Figure 8. Expression of OPN, BMP2 and Runx2 after osteogenic induction for 2 and 3 weeks.

mineralization and COL-1 gene expression than the Flat surface. The cause may be that the micro-structure increased the surface area of SLA. This result is also consistent with the findings of previous studies [41].

Although we attempted to control other factors that would affect the experiment, some limitations must be considered when interpreting our results. For example, we analyzed ECM mineralization only at day 21. That observation time point is not sufficient to draw the time variation

curve of ECM mineralization. Importantly, LIPUS only induces physiological effects within certain parameter windows, but the mechanism remains unclear. Further studies will be required to elucidate the interaction between surface topography and LIPUS stimulation and how the mechanical irritation translates into biochemical signals.

Conclusions

LIPUS can have beneficial effects on the functions of BMSCs on implant surfaces with different topographies (Flat and SLA). This stimulation significantly increased cell proliferation and filipodia on the implant surfaces, resulting in the formation of more intracellular microfilaments. LIPUS can accelerate BMSC proliferation and differentiation on implant surfaces by up-regulating the expression of relevant genes. The results of the study suggest that LIPUS could be used as a potential adjuvant treatment to improve the osseointegration of titanium implants.

Disclosure of conflict of interest

None.

Address correspondence to: Lei Chen, Department of Orthodontics, College of Stomatology, Shandong University, Number 44, Wen Hua Xi Lu, Jinan 250-012, Shandong Province, People's Republic of China. Tel: +86 15168856191; Fax: +86531 829-50154; E-mail: sdcl8006@163.com

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