Original Research
Periodontics

Low-intensity pulsed ultrasound promotes tissue regeneration in rat dental follicle cells in a porous ceramic scaffold

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Abstract: The aim of this study was to investigate the effects of low-intensity pulsed ultrasound (LIPUS) on the osteogenic differentiation of dental follicle cells (DFCs) in vitro and on the regenerative effects of DFC-OsteoBoneTM complexes in vivo. DFCs were isolated and characterized. In the in vitro study, DFCs were cultured in an osteogenic medium in the presence or absence of LIPUS. The expression levels of ALP, Runx2, OSX, and COL-I mRNA were analyzed using real-time polymerase chain reaction (RT-PCR) on day 7. Alizarin red staining was performed on day 21. The state of the growth of the DFCs that were seeded on the scaffold at 3, 5, 7, and 9 days was detected by using a scanning electron microscope. In our in vivo study, 9 healthy nude mice randomly underwent subcutaneous transplantation surgery in one of three groups: group A, empty scaffold; group B, DFCs + scaffold; and group C, DFCs + scaffold + LIPUS. After 8 weeks of implantation, a histological analysis was performed by HE and Mason staining. Our results indicate that LIPUS promotes the osteogenic differentiation of DFCs by increasing the expression of the ALP, Runx2, OSX, and COL-I genes and the formation of mineralized nodules. The cells can adhere and grow on the scaffolds and grow best at 9 days. The HE and Mason staining results showed that more cells, fibrous tissue and blood vessels could be observed in the DFCs + scaffold + LIPUS group than in the other groups. LIPUS could promote the osteogenic differentiation of DFCs in vitro and promote tissue regeneration in a DFCs-scaffold complex in vivo. Further studies should be conducted to explore the underlying mechanisms of LIPUS.

Keywords: Ultrasonic Waves; Dental Sac; Osteogenesis; Regeneration.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Periodontal disease is characterized by the progressive destruction of tooth-supporting tissues, such as alveolar bone, periodontal ligaments and cementum, and the subsequent loss of teeth.[1] It affects a large population worldwide.[2,3] Conventional treatment strategies, such as oral hygiene instruction and scaling and root planing, aim to prevent the disease, halt its progress and maintain therapeutic outcomes achieved by long-

term control of dental plaque accumulation,[4] which has little effect on the regeneration of periodontal tissue.[5] In recent years, efforts have focused on regenerating lost alveolar bone through the use of autografts (cortical/cancellous bone and bone marrow), allografts (demineralized freeze-dried/ freeze-dried bone) and alloplastic materials (bioglass, polymers and hydroxyapatite).[6] However, the use of such materials for periodontal regeneration has been questioned due to issues such as the variability in safety, clinical effectiveness and stability over time of these agents.[7,8,9]

More recently, cell-based tissue engineering technology has emerged as a possible alternative to previously used treatments. Cell-based tissue engineering technology has been reported to supplement traditional restorative or surgical techniques when replacing injured or pathologically damaged tissues.[10] This therapeutic procedure has three principal components: scaffolds, seed cells and growth factors.[11] Scaffolds are temporary structures that are used to provide a three-dimensional microenvironment where cells can proliferate, differentiate and generate the desired tissue.[12] The OsteoBone™ scaffold (Yenssen Biotech, Jiangsu, China) is an inorganic ceramic material with a porous structure that has a biomimetic 3D internal geometry that is favorable to cell seeding and new bone and blood vessel growth.[13] Grafting of exogenous cells has been shown to facilitate the generation of new tissue and/or the formation of a local microenvironment that is more suitable for the stimulation of endogenous progenitors, with promising results.[14] Dental follicle cells (DFCs) are recognized as the progenitor cells of periodontal ligament cells (PDLCs), cementoblasts and osteoblasts within the dental follicle.[15,16] These cells can develop into the components that constitute different periodontal tissues, such as periodontal ligament fibers and cementum and alveolar bone, when dental tissue injury occurs.[17] Moreover, human DFCs can be isolated from impacted teeth that have been clinically discarded and can be frozen and stored for many years. Hence, DFCs have been considered ideal and promising candidate seed cells for periodontal tissue engineering. Indeed, the application of exogenous growth factors may enhance the osteogenic differentiation of cells.[18,19,20] However, the large-scale utilization of growth factors is clinically impracticable due to safety concerns.[21] Therefore, it is imperative to seek a new modality that can enhance the regenerative effect of cell-based tissue engineering.

A variety of mechanical stimuli have been actively studied to induce osteogenic differentiation, such as shock waves,[22] pulsed electromagnetic fields[23] and LIPUS (low intensity pulsed ultrasounds).[24] Of these, LIPUS (intensity ranging from 30-100 mW/cm^2) is a source of mechanical energy transmitted as acoustic pressure waves into biological tissues and subsequently evoking biochemical effects at the cellular level.[25] At present, it has been widely used for the treatment of bone fractures and nonunions in the clinic.[26] As a form of non-invasive and safe mechanical stimulation, LIPUS also shows some advantages in the treatment of periodontal disease. Ikai H et al.[27] showed that LIPUS could enhance periodontal wound healing and bone repair by affecting osteoblasts and cells in the gingival epithelium and periodontal ligament. Studies have also reported that LIPUS can promote periodontal tissue repair and alveolar bone healing of extraction sockets by accelerating the calcium salt deposition and new bone formation in animal models.[28,29,30] In addition, LIPUS is expected to prevent root resorption and accelerate its repair.[31] Based on these findings, LIPUS has been considered as a promising therapeutic tool for periodontal regeneration.

We hypothesized that LIPUS might promote the osteogenic differentiation of DFCs and enhance the regenerative effects of the DFC-scaffold complex. Therefore, we investigated the possible effect of LIPUS on the osteogenic differentiation of DFCs in vitro and on tissue regeneration in DFC-OsteoBone™ scaffolds implanted into the subcutaneous dorsa of the nude mouse.

Methodology

Experimental animals

Six- to 7-day-old Sprague-Dawley (SD) rats and nude mice were provided by the animal center of Chongqing Medical University, Chongqing, China.

All animal experiments were approved by the Ethics Committee of Chongqing Medical University.

Separation and cultivation of rat DFC Cells

The isolation and culture of DFCs were performed as previously described.[32] Dental follicles were carefully separated from the mandibular first molars of 6- to 7-day-old SD rats under a dissecting microscope. The dental follicle tissues were digested with 1 milligram per milliliter of type I collagenase (Sigma, Shangai, China) in PBS at 37°C for 30 min with frequent gentle agitation. After they were fully digested, the dental follicles and cell suspensions were centrifuged at 800 r/min for 5 min. The supernatant was discarded, and the cell pellet was resuspended in complete medium (containing 10% fetal bovine serum (FBS)) and seeded into 25 T-flasks (Corning, Shangai, China). The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. The cell culture medium was changed every 2 days. The DFCs were passaged at a 1:2 ratio until they reached approximately 70% confluence. The cells were passaged for purification.

Flow cytometric surface marker expression analysis

Flow cytometric analyses were performed to measure the expression of mesenchymal stem cell-associated surface markers. DFCs from the third passage were digested and washed twice with 1% FBS in PBS, followed by incubation with anti-rat CD34 (FITC), anti-rat CD73 (PE), anti-rat CD146 (PE), and anti-rat STRO-1 (FITC) antibodies in the dark for 20 min according to the manufacturer's protocol (BD Biosciences, USA). After washing, flow cytometry was used to analyze the stained cells.

Osteogenic, adipogenic and chondrogenic differentiation

DFCs were seeded at a density of 1×10^5 cells per well in 6-well plates. After reaching 80% confluence, the DFCs were cultured in osteogenic differentiation medium (Biowit, China), adipogenic differentiation medium (Biowit, Shenzhen, China) or chondrogenic differentiation medium (Biowit, China). The cell culture medium was changed every 2 days. After 21 days of osteogenic induction, the samples were washed gently with PBS 3 times, fixed with 4% polyoxymethylene for 20 min, and then stained at room temperature for 1 h with Alizarin Red solution (Beyotime, Shangai, China) to assess mineral deposition. After 2 weeks of adipogenic induction, the differentiated cells were fixed for 20 min and then stained at room temperature for 1 h with 0.3% Oil Red O solution (Sigma, Shangai, China) to evaluate adipogenesis. After 21 days of chondrogenic induction, the cells were fixed and stained at room temperature for 30 min with 1% Alcian blue solution (Sigma. USA) to evaluate chondrogenesis.

Exposure to LIPUS

The LIPUS exposure devices were provided by the National Engineering Research Center of Ultrasound Medicine (Chongqing Medical University, Chongqing, China). As described in our previous studies,[24,33,34] the parameters for LIPUS were as follows: an intensity of 90 mW/cm^2, a frequency of 1.5 MHz, a pulse duration of 200 μs, a repetition rate of 1.0 KHz, and a process time of 20 min/day. For the in vitro study, the induction of osteogenic differentiation in the DFCs was performed in an osteogenic medium as described above. The culture plates from the LIPUS group (LIPUS (+) group) were placed on the ultrasound transducer with a thin layer of water to maintain contact at 37°C. For the in vivo study, the mice were fixed, and the ultrasound transducer was placed into contact with the skin of the target region. To ensure favorable ultrasonic transmission, a coupling gel was used between the transducer and the skin.

Real-time polymerase chain reaction (RT-PCR)

After 7 days of treatment, the expression of osteogenesis-related genes (ALP, Runx2, OSX, and COL-I) was determined by RT-PCR. Briefly, the total RNA in the cells was isolated using a MiniBEST Universal RNA Extraction Kit (Takara, Dalian, China), and reverse transcription was performed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dailian, China) to produce complementary deoxyribonucleic acid (cDNA). The RT-PCR was

performed using a FX9600 Connect Real-Time PCR Detection System (Bio-Rad, Hercules, USA) under the following conditions: the reaction mixture was preheated for 15 min at 96°C to activate the Taq enzyme and denatured for 5 min at 96°C, which was followed by 40 cycles of 30 s at 96°C, 30 s at 57°C and 30 s at 72°C. For each reaction, a melting curve was generated to test for primer dimer formation and nonspecific priming. The sequences of the primers and probes are listed in Table. The quantification was performed with the 2-ΔΔCt method.

Osteogenic analysis

After 21 days of treatment, the formation of mineralized nodules in the DFCs was examined by Alizarin red staining. To quantify the mineralization, the cells were stained with Alizarin Red and were then destained with 10% cetylpyridinium chloride, monohydrate (Solarbio, Beijing, China). Then, the extracted stain was transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader (Perkin Elmer, Waltham, USA).

Seeding of DFCs onto the OsteoBone™ scaffold

The DFCs were collected and seeded onto the OsteoBone™ scaffold (Yenssen Biotech, Jiangsu, China) prior to the subsequent morphological analysis and subcutaneous implantation. Briefly, the DFCs were detached from the dishes and centrifuged to remove the supernatant. Then, the cells were resuspended in culture medium supplemented with FBS at a final density of 5×10^5 cells per milliliter. One milliliter of cell suspension was gently added to the Osteobone™ scaffold and wetted with culture medium every two hours for a total of three times. The cell-scaffold complexes were further cultured in complete medium in a humidified atmosphere with 5% CO_2 at 37°C.

Scanning electron microscopy (SEM)

Cell attachment and spreading on the scaffolds were visually assessed after 3, 5, 7, and 9 days. At every time point, the specimens were washed with PBS twice, fixed with 2% glutaraldehyde at 4°C for 12 h, dehydrated with a graded ethanol series, dried in a critical point dryer, coated with conductive material, and finally observed under a scanning electron microscope (SEM, Hitachi, S-3000N, Tokyo, Japan).

Histological analysis of the in vivo experiments

To evaluate the combined effect of LIPUS and the OsteoBone™ scaffold on the osteogenic differentiation of DFCs, either the scaffold alone or the cell (DFC) scaffold complex was transplanted into the subcutaneous dorsa of a nude mouse with or without LIPUS stimulation. A total of nine nude mice were randomly assigned to one of three groups: group A, empty scaffold; group B, DFCs + scaffold; group C, DFCs + scaffold + LIPUS. All

Table 1. Primer sequences used for quantitative real-time polymerase chain reaction.

Target gene	Primers (5′-3′)	Fragment size (bp)
ALP	F: 5′CACAGCTTCAGTTCCCCCTCAG 3′ R: 5′CCCCGCCATGGACTTTAGTAACC 3′	182 bp
Runx2	F: 5′CCCCCTTGCTCTCTGTTCCTTC 3′ R: 5′TTTCCCCCTCAATTTGTGTCAG 3′	146 bp
OSX	F: 5′GGGGCAATTGGTTAGGTGGTG 3′ R: 5′GGGGCAAAGTCAGACGGGTAAG 3′	122 bp
COL-1	F: 5′CCCCAAAGACACAGGAAATAATGC 3′ R: 5′CCCAGCACAGGCCCTCAAAAAC 3′	224 bp
β-actin	5′ACCCCGTGCTGCTGACCGAG 3′ 5′TCCCGGCCAGCCAGGTCCA 3′	249 bp

nude mice were placed under general anesthesia by intraperitoneal injection of 10% chloral hydrate prior to surgical procedures, followed by local anesthesia with 0.5 milliliter of 1% lidocaine with epinephrine (1:100000). In group A, the empty scaffold material was implanted into the subcutaneous dorsa of the nude mouse at the muscle surface and sutured. In group B, DFCs were seeded onto the OsteoBone™ scaffold and cultured for 9 days, and the cell scaffold complex was then implanted into the subcutaneous dorsa of the nude mice. In group C, following 9 days of culture in vitro, the cell scaffold complex was implanted into the subcutaneous dorsa of the nude mice and treated with LIPUS after wound healing. The nude mice were sacrificed 8 weeks after surgery. The specimens were sectioned and fixed with 4% polyoxymethylene at 4°C for 24 h. The transplants were decalcified with 10% EDTA at pH 8.0, dehydrated with a graded ethanol series, and then embedded in paraffin. Five microliter-thick paraffin sections were prepared for HE and Mason staining. All samples were examined under a compound microscope (Olympus, Tokyo, Japan).

Statistical analysis

The data are expressed as the mean ± standard deviation. SPSS version 19 (IBM SPSS, Armonk, USA) was used to analyze the data. The statistical analysis was performed using a two-tailed Student's t-test, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Morphology and identification of rat DFC Cells

After subculturing, the rat DFCs exhibited the typical spindle shape of mesenchymal cells (Figure 1A). The expression of markers at the DFC surface determined by flow cytometric analysis at passage 3 is presented in Figure 1B. The DFCs were positive for CD73, CD146 and STRO-1 and were negative for CD34. These data indicate that rat DFCs have characteristics of mesenchymal cells. After induction in osteogenic, adipogenic and chondrogenic media, the differentiated cells were analyzed for the formation of mineralized nodules, the accumulation of lipid clusters and the production of proteoglycan (Figure 1C). The formation of calcium mineralized nodules stained with Alizarin red solution was observed in adherent cell cultures of rat DFCs. The intercellular formation of lipid droplets, as indicated by Oil Red O staining, was clearly exhibited in rat DFCs; proteoglycan was also found in the DFCs.

Effects of LIPUS on the osteogenic differentiation of rat DFCs

The RT-PCR results showed that rat DFCs exposed to LIPUS for 7 days expressed significantly ($p < 0.05$) higher levels of ALP, Runx2, OSX, and COL-I mRNA compared with those of DFCs from the non-LIPUS treatment groups (Figure 2A). We also found that the formation of mineralized nodules significantly increased with LIPUS stimulation, resulting in nodules with observably stronger Alizarin red staining (Figure 2B, C).

Adhesion and spreading of DFCs on the material

Under the scanning electron microscope, we observed that the DFCs could adhere and grow on the surfaces of the porous scaffolds (Figure 3). After 3 days of culturing, a small number of cells was observed on the surface of the material. The number of cells increased slightly after 5 days and increased significantly after 7 days of culture. After 9 days of culture, many cells were observed to have spread on the surface and to have grown into the pore. Therefore, these results suggest that the scaffold facilitated the adhesion and spread of the DFCs onto its surface. The cell scaffold complex on day 9 of incubation was selected for in vivo implantation.

Histological analyses of the in vivo experiments.

All nude mice recovered well after surgery. The effect of LIPUS on the regeneration of the OsteoBone™-DFC complex was evaluated after 8 weeks of subcutaneous implantation in nude mice by histological analysis using HE staining and Mason staining. In group A (empty scaffolds), we did

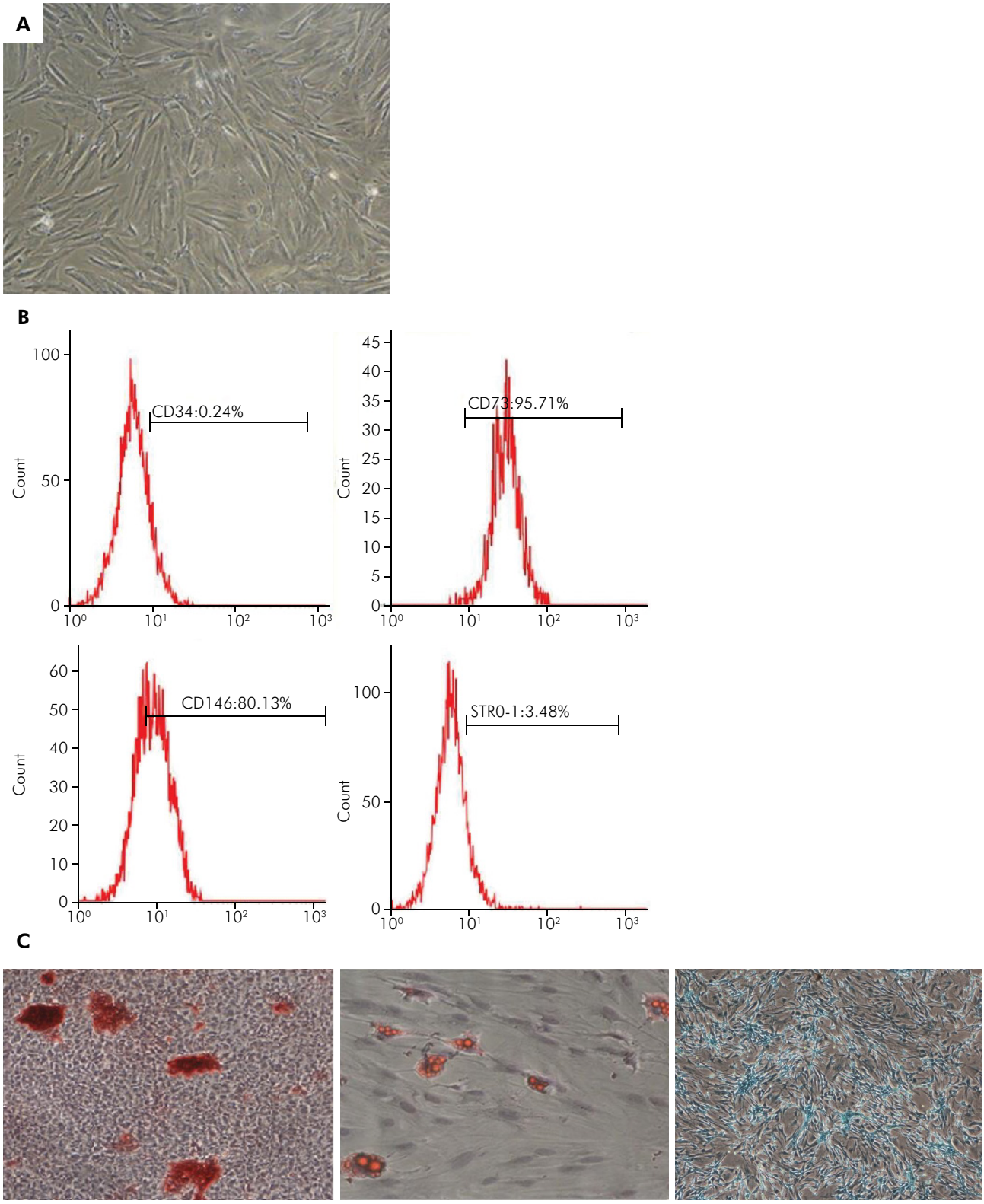

Figure 1. A: Morphological characteristics of DFCs at the third passage were observed under a light-inverted microscope. DFCs exhibit typical fibroblast-like spindle morphology. B: Flow cytometric analysis indicated that the DFCs were of mesenchymal origin (positive for CD73 and negative for CD34) and that the DFCs were stem cells (positive for CD146 and STRO-1). C: Osteogenic (Os), adipogenic (Ad) and chondrogenic (Ch) differentiation of DFCs.

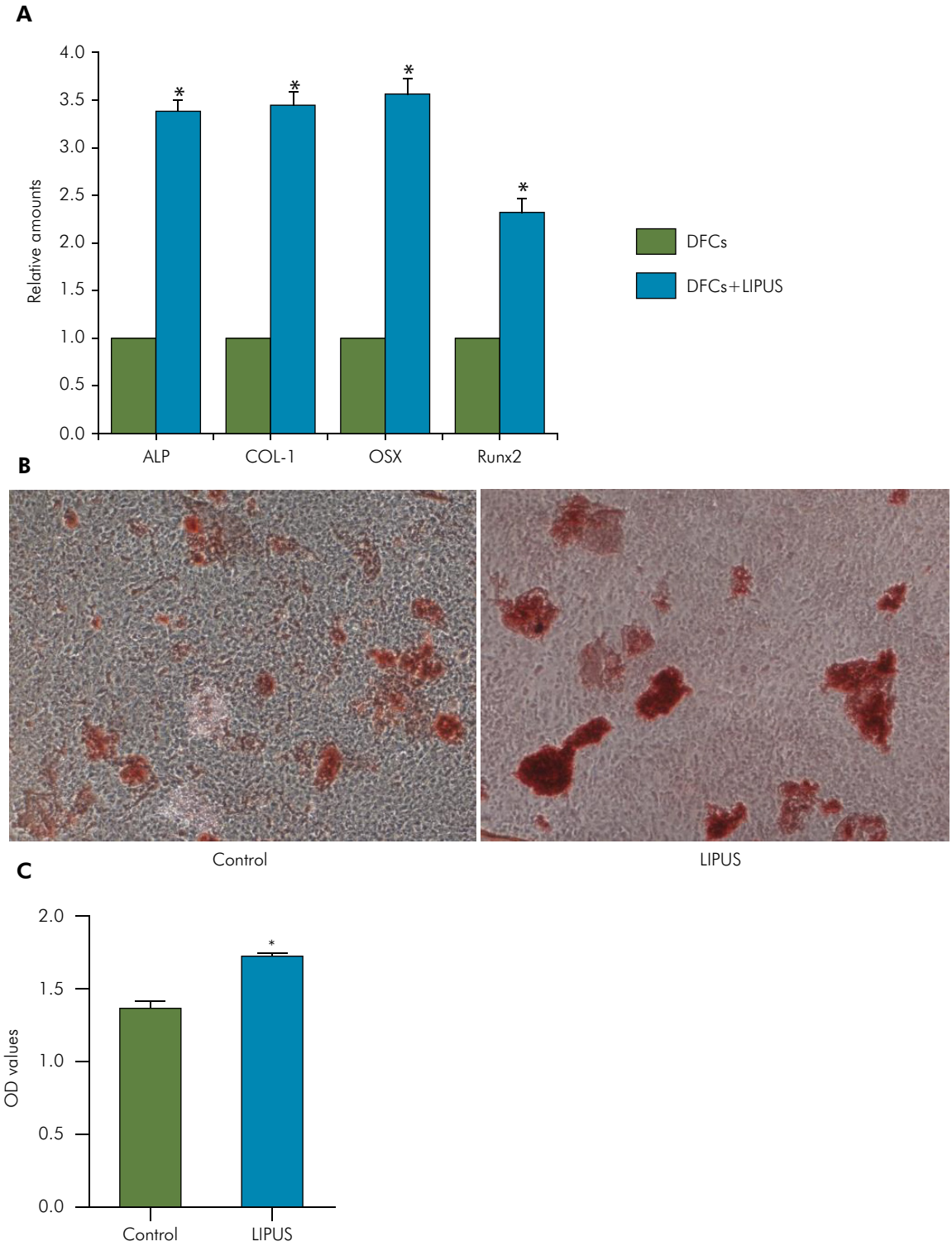

Figure 2. Effect of LIPUS stimulation on the osteogenic differentiation of DFCs. The cells were cultured in the presence or absence of LIPUS stimulation in an osteogenic medium. (−): control; (+): LIPUS stimulation. A: RT-PCR analysis of the expression levels of ALP, COL-1, OSX and Runx2 in DFC and BMSC cultures after 7 days of induced differentiation. B: The formation of mineralized nodules in DFCs was examined by staining with Alizarin red on day 21 of culture. C: The mineralization was quantified by destaining cells stained with alizarin red. $P < 0.05$ for LIPUS-treated versus control cells.

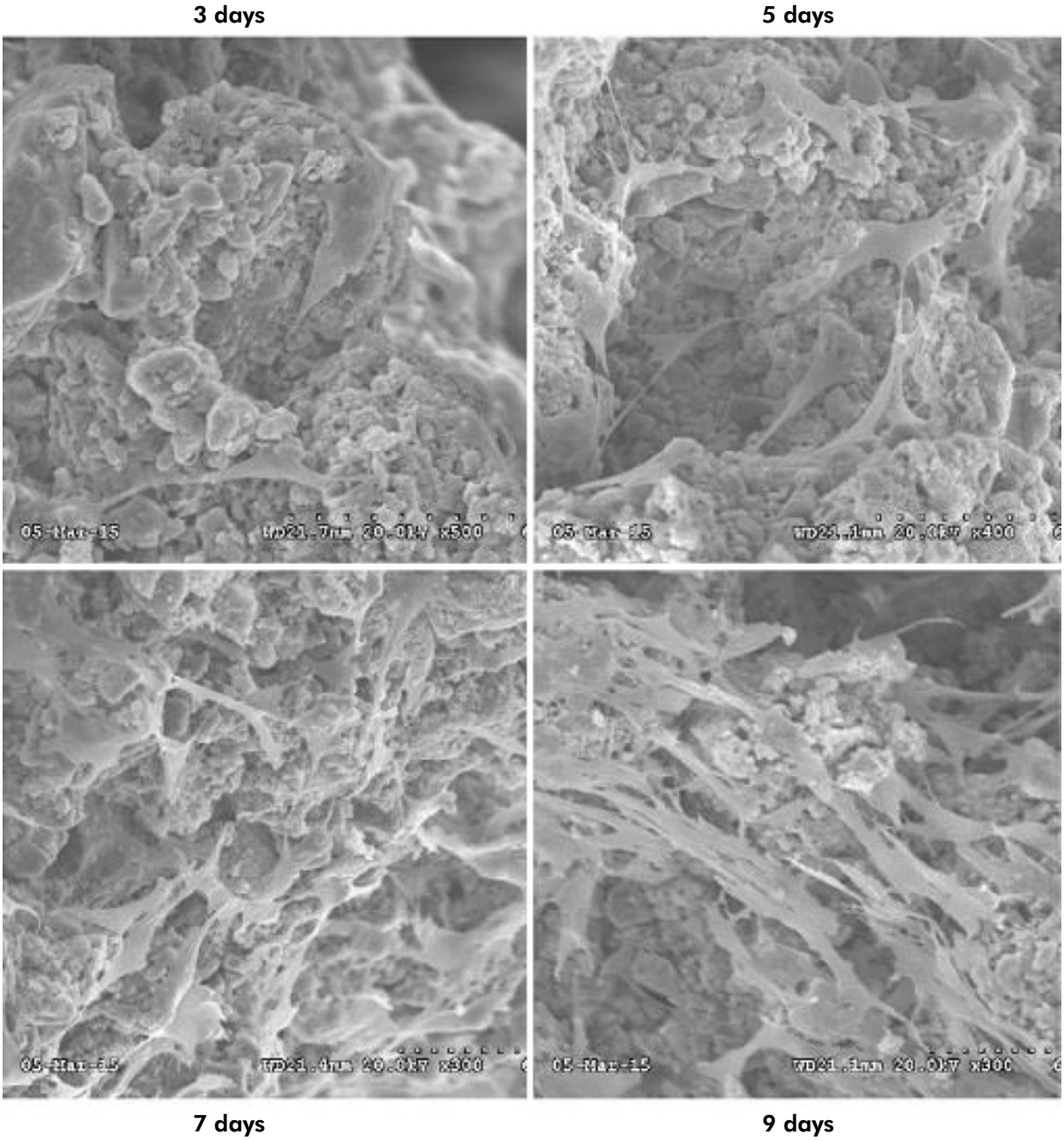

Figure 3. Scanning electron micrographs of DFCs cultured on a 3D OsteoBone™ scaffold. The DFCs were seeded on scaffolds after 3, 5, 7, and 9 days.

not observe any tissue formation or vascularization inside the implants (Figure 4A). However, the scaffolds seeded with DFCs (group B) exhibited obvious fibers and vessels that invaded the scaffold (Figure 4B). Moreover, group C also showed a significant increase in newly formed fibers and vessels (Figure 4C).

Discussion

DFCs have the capability for self-renewal and the potential to differentiate into multiple cell types, and they have been used in stem cell-based periodontal tissue engineering.[35] More importantly, DFCs are

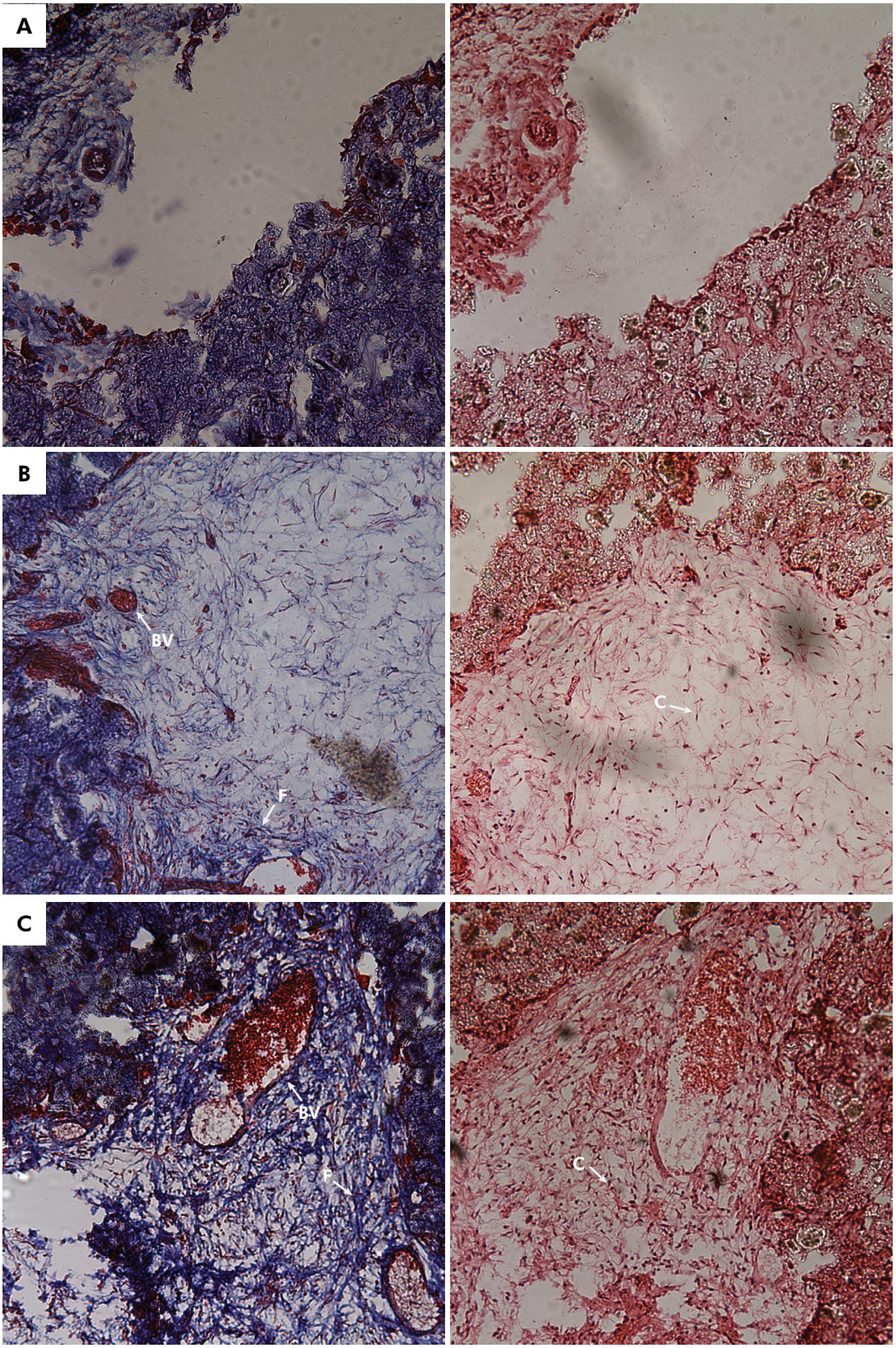

Figure 4. Histological examination of the harvested complexes at 8 weeks. A: empty scaffold; B: DFCs + scaffold; C: DFCs + scaffold + LIPUS. C, cell; F, newly formed fibrous tissue; BV, newly formed blood vessels.

one of the most accessible sources of seed cells. Therefore, we used DFCs as seed cells in our study. Simultaneously, the OsteoBone™ scaffold was used as a carrier for seed cells because it is an inorganic porous and biomimetic 3D scaffold that has a distribution of spatial pores of 100-300 μm in diameter and interporous channels of 350-500 μm in diameter. Its main components are calcium, silicon, and phosphorus without organic components, and the scaffold has been shown to exhibit better biocompatible and degradable properties.[36] Although growth factor is one of the key components of cell-based tissue engineering technology, we did not use it in our study. Instead, we used LIPUS to promote the osteogenic differentiation of DFCs and the regeneration of the DFC-scaffold complex, as LIPUS has been widely used as a type of mechanical stimulation for therapies in various medical fields, especially for the promotion of new bone formation.[37]

Based on gene expression levels, our experiments demonstrated that the expression of the ALP, Runx2, OSX and COL-I mRNAs increased significantly after 7 days of LIPUS exposure. These findings are consistent with those of previous studies showing that LIPUS promotes osteogenic differentiation by upregulating the expression of osteogenesis-related genes in PDLCs,[24] human alveolar-derived mesenchymal stem cells[38] and even adipose-derived stem cells.[39] Our findings thus provide evidence at the molecular level that explains the LIPUS-promoted osteogenesis of DFCs. Runx2 is an important transcription factor involved in the process of osteogenic differentiation because it activates osteoblast-specific genes, such as COL-I and ALP.[40] Li et al.[41] found that cyclic tensile stress promoted the osteogenic differentiation of periodontal ligament cells via activation of the ERK1/2-Elk1 MAPK pathway and the upregulation of Runx2. Previous studies by our research team found that the p38-MAPK[42] pathway and the BMP-Smad[34] pathway were involved in the process of LIPUS-induced osteogenic differentiation of periodontal ligament cells. We speculated that LIPUS probably promoted the osteogenic differentiation of DFCs via the MAPK-related and BMP-Smad pathways. However, this speculation needs to be verified, and further studies are necessary to clarify how LIPUS activates the MAPK-related and BMP-Smad pathways.

In addition, we examined the formation of mineralized nodules in the presence or absence of daily LIPUS stimulation. We found that the formation of mineralized nodules significantly increased in the presence of LIPUS stimulation, resulting in nodules with observably stronger Alizarin red staining. These results show that LIPUS stimulation may promote the formation of bone by DFCs, which is in accordance with the gene expression results.

Before implantation, we determined whether the scaffold we selected was biocompatible with DFCs. We observed that DFCs spread along the OsteoBone™ scaffold surface and grew into the pores of the scaffold after being cultured in vitro. These results suggest that OsteoBone™ has good biocompatibility and is favorable for the adhesion and growth of DFCs. Sun et al. observed similar results when seeding rabbit BMSCs on an OsteoBone™ scaffold.[13] Furthermore, the best conditions in terms of growth and adhesion on the scaffold surface were observed after the cells were cultured for 9 days. Therefore, we selected 9 days of culture as optimal for transplantation in the subsequent animal experiments.

Finally, we conducted an in vivo study to investigate whether LIPUS can enhance the regenerative effects of DFC-OsteoBone™ complexes implanted into the subcutaneous dorsa of nude mice. Histologically, we observed no new tissue formation in the empty scaffold group, which indicated that the OsteoBone™ scaffold alone was not capable of promoting tissue regeneration in a subcutaneous implant model in nude mice. In the DFCs + scaffold group, we observed more obvious newly formed tissues in the scaffold than in the empty scaffold group. This result demonstrated the indispensability of seed cells in the regenerative process. As expected, the DFCs + scaffold + LIPUS group showed the most obvious new tissue formation in the scaffold. In other words, LIPUS could further promote the regenerative effects of the OsteoBone™-DFC complex. It is worth noting that the regenerated tissues contained some cells, fibrous tissue and blood vessels without obvious newly formed osteoid, which was not in full accordance with the results of the study

that found that LIPUS may promote the osteogenic differentiation of DFCs in vitro. Therefore, further studies should be conducted to clarify the factors that influence subcutaneous heterotopic osteogenesis and to select the most optimized parameters.

Conclusion

Based on these findings, we conclude that LIPUS may promote the osteogenetic differentiation of rat DFCs by increasing the mRNA expression of osteogenesis-related genes and the formation of mineralized nodules in vitro. LIPUS may enhance the regenerative effect of a DFC-OsteoBone™ scaffold complex implanted into the subcutaneous dorsa of a nude mouse, although obvious newly formed osteoid was not observed in the histological sections. Further studies should be conducted to clarify the underlying mechanisms of LIPUS and to select the most optimized parameters to make the best use of cell-based tissue engineering.

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