Effects of Low-intensity pulsed ultrasound (LIPUS) on osteogenic differentiation of murine periosteum-derived cells

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Abstract
The replacement of lost natural teeth using osseointegrated implants require adequate bone volume permitting their anchorage. The periosteum is a specialized fibrous tissue composed of fibroblast, osteoblast, and progenitor cells and optimal source for bone tissue engineering based on its accessibility and rapid proliferation in vivo and in vitro. Low-intensity pulsed ultrasound (LIPUS) has shown successful bone healing of delayed, non-unions and fracture healing in animal models and in clinical treatments. This study aimed to investigate the effects of LIPUS on the proliferation and osteogenic differentiation of murine calvarial periosteum-derived cells (PDCs). PDCs were treated with daily ultrasound stimulation with an intensity of 30 mW/cm², frequency of 3 MHz, pulse repetition frequency (PRF) of 1 kHz and exposure time of 20 min per day up to 21 days. The cell proliferation and viability were analyzed with PrestoBlue Cell Viability assay. Osteogenic differentiation was determined with alkaline phosphatase (ALP) staining, ALP activity assay, alizarin red staining, and real-time reverse transcriptase-polymerase chain reaction. The results indicated that LIPUS stimulation did not significantly affect the viability of PDCs. LIPUS significantly increase ALP activity on day 7 of culture and markedly promoted the formation of mineralized nodules on day 21. The mRNA expression of ALP, RUNX2, OSX, and OCN was significantly up-regulated by LIPUS stimulation. Thus, LIPUS stimulation increases osteogenic differentiation of PDCs and is useful for potential bone regeneration.

Keywords
Low-intensity pulsed ultrasound; LIPUS; periosteum; gelatin; osteogenic Differentiation.

1. Introduction
The use of dental implants for the rehabilitation of missing teeth has increased dramatically in the past few decades and is expected to expand further in the future. The success of dental implants largely depends on the quality and quantity of the soft and hard tissues in the recipient site. Because of its osteoconductive, osteoinductive, and osteogenic properties and absence of immunological reactions, autologous bone grafts have been considered as the “gold standard” and most effective material in bone regeneration procedures. However, they may result in
donor site morbidity and limited availability. A possible alternative to current bone graft technology is stem cells based bone tissue engineering.

The periosteum is composed of a fibrous and a cambium layer\(^1\). The fibrous layer of the periosteum contains fibroblasts in a collagen fiber matrix, together with microvascular network\(^2,3\). The cambium layer consists of fibroblasts, osteoblasts and osteochondral precursor cells. This layer is responsible for producing osteoblasts and chondroblasts for bone formation or regeneration\(^4,5\). The skull, clavicle and mandible are formed by intramembranous ossification during fetal development\(^6\). Therefore, the periosteum might be related to bone growth and regeneration\(^7,8\). MSCs can be isolated not only from bone marrow but also from other tissues such as adipose tissue, gingiva, umbilical cord blood and amniotic fluid\(^9,10\). Recently periosteum-derived cells have become a highly potential source in bone tissue engineering and regenerative medicine because PDCs can be easily and safely acquired using less-invasive methods than bone-marrow mesenchymal stem or progenitor cells\(^11\).

Low-intensity pulsed ultrasound (LIPUS) is a clinically established physiotherapeutic treatment used to accelerate the healing of bone fractures and delayed union or nonunion of bone fractures\(^12,13\). Its effectiveness has been demonstrated in numerous in vivo studies\(^14,15\) and supported by in vitro examinations using cell culture systems\(^16,17\). Several studies have demonstrated stimulatory effects of LIPUS, such as osteogenic differentiation in different types of cells\(^18-20\). To the best of our knowledge, there have been no reports on the effects of LIPUS on the osteogenic differentiation of murine calvarial periosteum-derived cells isolated by using gelatin. Therefore, the present study aims to investigate the effect of low-intensity pulsed ultrasound stimulation on the proliferation and osteogenic differentiation potential of murine calvarial periosteum-derived cells.

Materials and Methods
The Institutional Animal Care and Use Committee of Tokyo Medical and Dental University approved the protocol design and procedures (approval number A2017-343A).

1) Isolation and culture of PDCs
Ten female SLC: ICR mice (4 weeks old) (30g ± 3g) were purchased from Sankyo Laboratory services, Inc. (Tokyo, Japan). Isolation of murine calvarium was performed as previously described with modification\(^21\). (Fig. 1A). Skulls with the periosteum were collected after pentobarbitone sodium euthanasia. After trimming the skulls to 10 × 10 mm\(^2\) in size (Fig. 1B) and washing thoroughly with phosphate-buffered saline (PBS), the periosteum was collected (Fig. 1C).

Isolation of PDCs was carried out as previously described with modification\(^22\). Briefly, 600 mg of gelatin was dissolved in 6 mL of Dulbecco’s modified Eagle’s medium - high glucose (DMEM containing 4.5 g/L glucose, L-glutamine, and sodium bicarbonate, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich) and 1% (v/v) Penicillin-Streptomycin-Amphotericin B Suspension (Wako) in 60-mm culture dishes (Fig. 1D). The cells were incubated at 37 °C in a humidified incubator consisting of 95% air and 5% CO\(_2\). After 2-3 days, cells started to come out from the calvarial periosteum. The medium was changed every 72 hours until confluence was reached.
Fig. 1. Isolation of murine calvarial periosteum-derived cells (PDCs)
(A) At the base of the skull, a cut was made through the skin. An incision was made from the base of the skull to the bridge of the nose. The skin was removed. The calvaria was dissected as indicated by the dotted line and all the soft tissues were removed. (B) The skull was trimmed to 10 × 10 mm². (C) The Periosteum was peeled off using a tweezer. (D) The periosteum were placed onto the gelatin which becomes aqueous at 37°C.

2) Multipotent differentiation of periosteum-derived cells

① Osteogenic differentiation
To induce osteogenic differentiation, the third passages of PDCs were seeded in 24-well plates at an initial density of 5 × 10⁴ cells/well. They were then cultured in osteogenic medium supplemented with DMEM containing 10% FBS, 10⁻⁸ M dexamethasone, 10 mM β-glycerophosphate disodium and 50 μg/mL L-ascorbic acid up to 21 days with medium changes every 72 hours. On day 7, ALP-positive cells were stained with the ALP Stain kit (Wako). On day 21, calcium formation was detected with Alizarin Red S staining (Sigma - Aldrich).

② Adipogenic differentiation
To induce adipogenic differentiation, the third passages of PDCs were seeded in 24-well plates at an initial density of 5 × 10⁴ cells/well. They were then cultured in adipogenic medium supplemented with DMEM containing 10% FBS, 10 μg/ml insulin solution, 2.5 μM Dexamethasone solution, 0.5 mM 3-isobutyl-1-methyloxanthine solution (Adipoinducer reagent, TaKaRa) for 14 days with medium changes every 72 hours. On day 14, oil globules were detected with Oil Red O staining (Sigma - Aldrich).

③ Chondrogenic differentiation
To induce chondrogenic differentiation, the third passages of PDCs were seeded in 24-well plates at an initial density of 1 × 10⁵ cells/well. They were then cultured in chondrogenic medium (STEMPRO Chondrogenesis Differentiation Kit; Gibco) for 21 days with medium changes every 72 hours. On day 21, the presence of cartilage-specific proteoglycan core protein was
detected by Alcian Blue staining (Sigma - Aldrich).

3) **Ultrasound treatment**

Cells at the passage of 3 were seeded at $3 \times 10^5$ cells per well into 35-mm culture dishes or $5 \times 10^3$ cells per well in 96-well plates and cultured in DMEM. After 24 or 72 hours of incubation, the cells were exposed to LIPUS and/or osteogenic medium. The PDCs were divided into four groups: L+O+ (ultrasound with osteogenic medium), L+O− (ultrasound without osteogenic medium), L−O+ (no ultrasound with only osteogenic medium) and L−O− (control group; without ultrasound and osteogenic medium) Each group contained three samples ($n = 3$). The experimental schedule is shown in (Fig. 2A).

OSTEOTRON D2 (Ito Co., Ltd, Japan) was used. (Fig. 2B). A 35-mm diameter circular transducer was used, and the ultrasound intensity was nearly constant throughout its cross-sectional area. The transducer surface is perfectly compatible with a 35-mm culture dish. The culture plates were placed on the transducer with the coupling gel in between them (Fig. 2C). The cells were exposed to ultrasound irradiation as per the following: 3MHz frequency, 30 mW/cm$^2$ intensity, pulse repetition frequency of 1kHz, 1:4 pulse duty cycle (2 ms “on” and 8 ms “off”), spatial-average temporal-average (SATA). Cells are treated with LIPUS for 20 min daily. Controls are treated in the same way without switching on the machine.

4) **Cell Proliferation/ cytotoxicity**

The cells in suspension were seeded at $5 \times 10^3$ cells/well in a 96-well microtiter plate. Cell proliferation or cytotoxicity was analyzed using PrestoBlue Cell Viability assay as
mentioned in the manufacturer’s protocol. Briefly, PDCs were seeded as above. After 24 hours incubation in DMEM, the cells were treated with ultrasound stimulation for up to 10 days \((n = 6)\). Controls were prepared in the same way without switching on the machine. The culture medium was replaced every 72 hours. On days 0, 1, 3, 7 and 10, 10 μL PrestoBlue Cell Viability Reagent was added and incubated for 1 hour at 37 °C. Then measured the absorbance at 570 nm with a microplate reader. The cell density was calculated from the absorbance values using a standard curve.

5) ALP-positive cell staining
After osteogenic induction with or without LIPUS for 7 days, PDCs were stained using the ALP Stain kit (Wako). First, the cells were washed thoroughly using PBS for 2 times, then fixed with 3.7% formalin for 10 min, cells were then washed again with PBS and incubated in 1 mL ALP staining solution for 45 min at 37 °C to identify ALP-positive (blue) cells. The cells were then washed with distilled water (DW) to stop the staining reaction. Digital images were captured with Biozero BZ-8000 microscope (Keyence, Japan).

6) Alizarin red S staining
After 21 days of osteogenic induction with or without LIPUS, PDCs were washed twice with PBS and fixed with 70% ethanol for 1 hour and subsequently stained for 10 min using 1ml of 2 % Alizarin Red solution. Then, washed the cells with DW to stop the reaction and calcium nodule formation was captured using Biozero BZ-8000 microscope (Keyence, Japan). Intensities of the alizarin red staining were calibrated with image analyzing software (ImageJ 1.8.0_112; NIH) and expressed in ratios.

7) Alkaline phosphatase (ALP) activity assay
On day 7, the cells were lysed with 500 μl solution of 0.1% Triton solution following the instructions of the manufacturer. Quantitative ALP activities were analyzed by using colorimetric p-nitrophenyl phosphate (pNPP) (Lab Assay, Wako). Then measured the absorbance at a wavelength of 405nm. The average measurement of blank standard replicates was substrate from the standard and sample replicates. A standard curve was prepared by plotting the optical density of standard versus its concentration in nmol/μL. ALP activity (units/μl) was calculated using the manufacturer’s formula.

To determine protein concentration, sample were mixed with bicinchronin acid using Pierce Rapid Gold BCA Protein Assay Kit (Thermofisher). Samples were then quantified with a microplate reader measuring the absorbance at a wavelength of 480nm. The average measurement of blank standard replicates was subtracted from the standard and sample replicates. A standard curve was prepared by plotting optical density (OD) of each Bovine Serum Albumin (BSA) standard versus its concentration in μg/μL. The protein concentration of the samples was determined by the standard curve. ALP activity was normalized to the total protein concentration in each sample (units/μg protein/min). Each experiment was performed in three replicates, and data are expressed as means ± standard deviations of three replicates.

8) Real-time polymerase chain reaction (RT-PCR)
Total RNA was extracted from each sample on days 7 and 14 using the Trizol reagent (Invitrogen, Japan), and cDNA synthesis was conducted with the SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitro-
gen, Japan). RT-PCR was carried out using PowerUp™ SYBR® Green Master Mix (Invitrogen, Japan) with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Japan) with the following program: 95 °C for 5 min; 35 cycles of 95 °C for 15 s, and 60 °C for 1 min. The sequences of specific primers used in the present study are described in Table 1. The relative expression level of each gene of interest was normalized to that of the housekeeping gene GADPH and expressed relative to the values in the control group (L-O-) using the $2^{\Delta \Delta C_T}$ method.

9) Statistical analysis
Statistical analysis was performed using statistics software SPSS version 22. All data are presented as means ± standard deviations. Differences between groups were evaluated with one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test or Student’s t-test. A value of P<0.05 was considered statistically significant.

Result
1) Cell expansion and characterization of periosteum-derived cells

Table 1. Real-time PCR primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tr>
<td>ALP</td>
<td>CTTCATAAGCGGCGGCGG</td>
<td>TGCGATGGCCAGTACTAAA</td>
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<tr>
<td>RUNX2</td>
<td>TGCTATGCCCAGATTTGC</td>
<td>GAGGGGAAATGCAAATAA</td>
</tr>
<tr>
<td>OSX</td>
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<tr>
<td>GADPH</td>
<td>ACCCAGAGACTGTGGATGG</td>
<td>CACATTGGGAGTGAACG</td>
</tr>
</tbody>
</table>
Fig. 3. Periosteum-derived cells culture
(A) The first adherent PDCs appeared at day 3. (B) The isolated PDCs at the passage of 3 displayed spindle-shaped, fibroblast-like morphology attached to culture flasks under a light microscope. Scale bar represents 200 μm.

Fig. 4. Multipotent differentiation of PDCs.
(A-D) Osteogenic differentiation of PDCs. (A) Control on day 7 after ALP staining and (B) Alkaline positive (blue) cells. (C) Control on day 21 after Alizarin Red S staining and (D) mineralized nodule formation. (E) Control on day 14 after Oil Red O staining and (F) many oil droplets were observed. (G) Control on day 21 after Alcian Blue staining and (H) chondrogenic differentiation was detected.

2) Effect of LIPUS stimulation on cell Proliferation and viability
The proliferation of PDCs was measured in the presence or absence of LIPUS stimulation for up to 10 days of culture using PrestoBlue Cell Viability Reagent. There was no significant difference between groups in each timepoint. Ultrasound stimulation did not produce a significant impact on cell proliferation or viability compared with the control cells ($p > 0.05$) (Fig. 5).

3) Effect of LIPUS stimulation on ALP-positive cells and ALP activity
PDCs were cultured in osteogenic medium or DMEM culture medium. ALP-positive cells were evaluated on day 7 with or without LIPUS exposure. The results exhibited that ALP-positive
cells were more prominent in LIPUS-treated cells than in control cells (Fig. 6A). For ALP activity, there was no significant difference between L+O+ and L-O-. L+O+ showed significantly higher ALP activity compared to L+O-. ALP activity was significantly higher in L-O+ and L+O- than L-O- (p < 0.05) (Fig. 6C).

Fig. 5. Proliferation and cell viability of PDCs were analyzed by PrestoBlue Cell Viability Assay. Murine PDCs were cultured with or without LIPUS stimulation and the cells numbers were determined on days 0, 1, 3, 7 and 10 of culture (n = 6). The data are shown as mean ± standard deviation SD. No significant difference was analyzed between groups at each time point (p > 0.05).

Fig 6. Effect of LIPUS stimulation on osteogenic differentiation (A) Alkaline phosphatase staining (B) Alizarin red staining (C) Measurement of ALP activity. Quantitative ALP activity was measured at day 7 of culture. Data are expressed as mean ± SD (n = 6). (D) Measurement of mineralization. Relative intensities of alizarin red staining were measured using ImageJ image analyzing software. Data are expressed as mean ± SD (n = 3). O= osteogenic medium, L = low-intensity pulsed ultrasound. (* , P < 0.05 ).
4) Mineralized nodule formation

The result exhibited that ultrasound treatment increased the formation of mineralized nodules, as displayed more intense Alizarin Red S staining in LIPUS-treated cells than in control cells in both osteogenic medium and DMEM culture medium (Fig. 6B). In the quantitative analysis of alizarin red staining using ImageJ software, L+O+ exhibited higher intensity than L+O-. There was no significant difference between L+O+ and L-O+ groups. L-O- showed increased alizarin red staining significantly than L-O- (p < 0.05) (Fig. 6D)

5) Effect of LIPUS stimulation on osteogenesis-related gene expression

The effect of LIPUS stimulation on the expression of ALP, RUNX2, OSX, OCN was evaluated by real-time quantitative PCR. The results are presented in Fig. 7.

Fig. 7. Osteogenic gene expressions measured by RT-qPCR. The mRNA levels of ALP (A), RUNX2 (B), OSX (C) at day 7 and OCN (D) on day 14 after osteogenic induction were measured. The data are expressed as mean ± SD (n = 3). GADPH gene was used as a reference gene. (* , P < 0.05; ** , P < 0.01).

ALP mRNA expression of L+O+ seemed to be decreased than L-O+ but there was no significant difference between the two groups. L-O- showed significantly higher ALP expression than L-O- (p < 0.01). L+O- showed an increase in ALP mRNA expression level significantly higher in comparison with L-O- ( p < 0.05) (Fig. 7A). For RUNX2 expression, there were no significant differences between L+O+ and L+O-. L+O- exhibited significant RUNX2 expression than L-O- ( p < 0.05). L-O+ exhibited increased
RUNX2 expression compared with L-O- (p < 0.01) (Fig. 7B). OSX gene expression was significantly higher in L+O- than in L-O- (p < 0.05). L+O+ showed increased OSX expression than L-O- (p < 0.05). L-O+ significantly increased OSX expression than L-O- (p < 0.01). There is no significant difference in OSX gene expression in L+O+ compared to L-O+ (Fig. 7C). L+O+ showed an increase in OCN expression although there was no significant difference in comparison with L-O+. L+O+ showed significantly higher OCN expression than L+O- (p < 0.01). L-O+ showed more OCN expression significantly than L-O- (p < 0.01). L+O- showed significantly higher OCN gene expression compared with L-O- (p < 0.05) (Fig. 7D).

**Discussion**

Periosteum-derived cells became potential autologous cell source for bone tissue engineering because of their easy access and high regenerative potential. LIPUS has been widely used as an efficient therapeutic approach in bone regeneration as it can transmit mechanical energy into tissues as an acoustic pressure wave that produces biochemical reaction inside the cells. In this study LIPUS device was set with the following parameters: an intensity of 30 mW/cm² SATA, a frequency of 1.5-3 MHz, pulse repetition frequency (PRF) of 1 kHz with a duty cycle of 20%. 1 kHz PRF significantly increased calcium accretion levels. 30mW/cm² and 20 mins daily showed a positive effect on osteogenic differentiation and increased mineralization of rat bone marrow stromal cells.

LIPUS, through its nonthermal effects promote an osteoinductive response in vitro and accelerates bone regeneration in vivo. This previous study demonstrated that LIPUS can contribute to the healing of bone fractures, angiogenesis, chondrogenesis, and bone remodeling. However, the use of ultrasound in these treatments is still controversial because of adverse side effects, and the biophysical mechanisms involved in the fracture healing process need to be further studied.

In the present study, LIPUS did not significantly increase the cell density of PDCs for up to 10 days. Ultrasound stimulation did not produce a significant impact on cell viability. These results indicated that LIPUS did not cause any harm to cells and may be safe for clinical use. After 7 days of LIPUS stimulation using 30mW/cm² for 20 min daily, a significant increase in ALP activity in PDCs was observed. L+O- showed an increase in ALP staining in comparison with L-O-. L+O- group showed significantly higher ALP activity than L-O- group (p < 0.05). Ultrasound stimulation alone increased an ALP activity significantly in DMEM culture medium. ALP activity result was in accordance with the result of ALP staining. These findings show similar results with previous reports that ALP activity in osteoblasts or osteoblast-like cells increased from day 5 after transient LIPUS treatment. ALP is presented in the prophase of ossification. Increased expression of ALP in PDCs was represented as well differentiated osteoblasts. The activity of ALP in vitro is considered the biomarker of osteoblast differentiation. These results show LIPUS treatment stimulated the differentiation of PDCs into osteogenic lineage by increasing ALP activity.

Alizarin red staining in L+O+ showed higher intensity than all other groups. LIPUS stimulation together with an osteogenic medium can enhance calcium nodule formation. In quantitative analysis of alizarin red staining, L+O- showed a significant
increase in calcium nodule formation in compared with L-O- (p < 0.05). Daily LIPUS stimulation for 20 min up to 21 days significantly increased mineralized nodule formation in DMEM culture medium. It has been demonstrated that the stimulation of bone healing was a result of an increase in LIPUS-induced intracellular Ca\textsuperscript{2+} concentration\textsuperscript{33}. Daily LIPUS stimulation with 3MHz up to 14 days can enhance bone formation\textsuperscript{34}.

To confirm LIPUS-induced osteogenic differentiation of PDCs, the expression of osteogenesis-related markers such as ALP, RUNX2, OSX, OCN was evaluated by real-time qPCR. In these gene expressions, There were no significant differences between L+O+ and L+O- group. L+O- group showed a significant difference than L-O- (p < 0.05). Ultrasound stimulation increased ALP gene expression significantly in DMEM culture medium. The result is in accordance with ALP staining and ALP activity. Thus, LIPUS treatment stimulated the PDCs into osteogenic differentiation. L+O- also showed significantly higher in RUNX2, OSX and OCN gene expression than L-O- group. Osteocalcin plays a role in bone-building and bone mineralization, it is as a late-stage marker of osteogenic differentiation and bone formation process. For PDCs cultured in osteogenic medium, ultrasound treated cells did not showed significant differences with control cells. However, LIPUS stimulation in DMEM culture medium increased the osteogenesis-related genes compared with control cells. These data suggest that ultrasound stimulation enhanced the osteogenic differentiation of PDCs.

In conclusion, our findings demonstrate that PDCs can be differentiated into osteogenic, adipogenic and chondrogenic lineages and have the potential for multipotent differentiation. PDCs might be the potential stem cell source for bone tissue engineering. LIPUS stimulation increased alkaline phosphatase staining and ALP activity in PDCs, and promoted mineralized nodule formation and up-regulated the expression of ALP, RUNX2, OSX, and OCN. Based on these findings, we conclude that LIPUS may promote the osteogenic differentiation of murine calvarial periosteum-derived cells by increasing the mRNA expression of osteogenesis-related genes and the formation of mineralized nodules in vitro. Our results may provide significant evidence for clinical usage of LIPUS in bone regeneration. Since these in vitro findings could not be associated with in vivo directly, further studies are necessary to clarify potential benefits and side effects of LIPUS and its underlying mechanisms for its safe and efficacious use.

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Conflict on interests
The authors declare no competing interests.

References


33) Parvizi J, Parpura V, Greenleaf JF, Bolander ME: Calcium signaling is
required for ultrasound-stimulated
aggrecan synthesis by rat chondro-
34) Nakanishi Y, Wang P-L, Ochi M,
Nakanishi K, Matsubara H: Low-
intensity Pulsed Ultrasound Stimula-
tion Significantly Enhances the
Promotion of Bone Formation
Around Dental Implants. J Hard
Tissue Biology 20:139-146. 2011