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Low-intensity Pulsed Ultrasound Promotes Osteogenic Differentiation of Reamer-Irrigator-Aspirator Graft-Derived Cells *In Vitro*

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Abstract

Recently, reamer-irrigator-aspirator (RIA) systems have been increasingly used to harvest autologous bone grafts. RIA graft materials contain bone marrow, which provides a viable source to derive large numbers of mesenchymal stem cells. Low-intensity pulsed ultrasound (LIPUS) significantly accelerates the differentiation of stem cells derived from bone marrow. This *in vitro* study investigated the effect of LIPUS on the osteogenic activity and differentiation of RIA graft-derived cells. A small amount of RIA graft was obtained from seven patients. After culturing the cells derived from RIA grafts, they were divided into two groups: the LIPUS and control groups. LIPUS was applied once daily for 20 min (1.5 MHz; pulse duration, 200 μ s; pulse repetition rate, 1 kHz; spatial average-temporal average intensity, 30 mW/cm²). Significantly higher alkaline phosphatase activity (113.4% and 130.1% on days 7 and 14), expression of osteoblast-related genes (ALP, Runx2), and mineralization (135.2% on day 21) of the RIA graft-derived cells was observed in the LIPUS group than in the control group. However, it did not affect the cell proliferation of RIA graft-derived cells. This study indicates that LIPUS may enhance the healing process of nonunion and critical bone defect treated by autologous bone grafting using the RIA system.

Keywords: Low-intensity pulsed ultrasound; Reamer-irrigator-aspirator; Osteogenic differentiation; Fracture healing

Introduction

Treatment of nonunions and critical bone defects remains challenging. In treating these cases, autologous bone grafting, where osteogenic cells, osteoconductive bone matrix, and osteoinductive growth factors are contained, is often required. Although autologous bone grafting obtained from the iliac crest is considered to be standard (Miclau and Sen 2007), various complications such as donor site pain, potential infection, bone resection-related fracture, blood loss, and limited bone availability are noted (Younger and Chapman 1989; Goulet et al. 1997; Cox et al. 2011; Kanakaris et al. 2011).

In recent years, the reamer-irrigator-aspirator (RIA) system (DePuy Synthes, Westchester, Pennsylvania, USA) has been increasingly used as an alternative system to harvest autologous bone grafts (Schmidmaier et al. 2006; Belthur et al. 2008). RIA is composed of a reamer head, tube assembly, drive shaft, and closed graft filter bottle. The drive shaft is hollow and contained in a plastic tube, allowing for continuous irrigation and aspiration of the femoral canal contents during reaming (Giannoudis et al. 2009). A graft filter bottle is placed between the reamer and the suction canister to trap particulate matter like the bone marrow contents and bony fragments. Although the RIA system was originally developed to reduce the incidence of fat embolism and thermal necrosis by aspiration and irrigation of the canal during reaming of the long bone (Higgins et al. 2007; Dimitriou et al. 2011), the ability to harvest a large amount of autologous bone in this process has been attracting attention. Cortical perforation and fracture have been reported as complications of the RIA system; however, the total complication rate is reported to be lower than that of iliac crest bone grafting (Dimitriou et al. 2011; Niikura et al. 2020).

The RIA graft contains bone and bone marrow, which is a viable source to derive large numbers of mesenchymal stem cells (MSCs) (Kuehlfluck et al. 2015; Toosi et al. 2019). Several reports have revealed that the MSCs derived from the RIA graft exhibited a similar or even better osteogenic potential than those

derived from the iliac crest bone marrow in *in vitro* studies (Sagi et al. 2012; van der Bel and Blokhuis 2014), although it was also reported that there is no difference in bone union rate and time to union (Dawson et al. 2014). There remains the problem of nonunion and delayed union even in cases of bone grafting using RIA.

On the other hand, it is well known that low-intensity pulsed ultrasound (LIPUS) accelerates the healing of fractures and nonunions by physical stimulation to the fracture and nonunion site. Although the actual determinant of bone healing by LIPUS is not fully understood, there are several sources that explain possible mechanisms. LIPUS waves cause nanomotion at the fracture site, and mechanical signals are translated to biochemical signals through integrin mechano-receptors into the cells (Harrison et al. 2016). The cells stimulate the production of cyclooxygenase 2 (COX-2), which accelerates bone remodeling, resulting in accelerated fracture healing. Several reports have shown that LIPUS significantly promotes the differentiation of bone marrow MSCs (Sant'Anna et al. 2005; Sena et al. 2005). Moreover, LIPUS is known as a noninvasive therapy for fractures and nonunions and is effective in clinical studies (Busse et al. 2002). It would be beneficial for patients if LIPUS treatment helps fracture healing in bone grafting cases using RIA. To our knowledge, there are no reports that LIPUS stimulation was performed on RIA graft *in vitro* or fracture site using RIA grafting *in vivo*.

We hypothesized that LIPUS treatment would promote the osteogenic activity and differentiation of cells contained in the RIA graft. This study aimed to evaluate the effect of LIPUS on the osteogenic activity and differentiation of the RIA graft-derived cells *in vitro*.

Materials and Methods

Patients

Seven patients who underwent autologous bone grafting between July 2018 and January 2020

using RIA systems to treat nonunion, malunion, or osteomyelitis were included in this study (Table 1). All seven patients were males, with a mean age of 49.4 years (range: 40–60 years). RIA was used in the femur in all cases. The Institutional Review Board of Kobe University Hospital approved this study, and written informed consent was obtained from all patients (Project ID 180067).

Isolation and Culture of RIA Graft-Derived Cells

A small part of the RIA graft was obtained for this study. The RIA graft was usually sherbet-like and amorphous, and the average amount of graft was 0.75g (0.66–0.80g). Samples were minced into small pieces with growth medium, which was α -modified minimum essential medium (Sigma-Aldrich, St Louis, MO) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco, Grand Island, NY), and antibiotics, on 10 cm culture dishes. The cultures were incubated at 37 °C under 5% humidified carbon dioxide. Seven days after initiating the culture, the growth medium was changed and exchanged twice weekly. Approximately 2 weeks later, the adherent cells were harvested with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (Wako, Osaka, Japan) and passaged into 150 cm² culture flasks with a density of approximately 4×10³ cm⁻² for further expansion. Cells at passage two or three were used in the following assays. RIA graft-derived cells were seeded into 6-well plates at 3.3×10⁴ cells per well. They were incubated in the growth medium at 37 °C under 5% humidified carbon dioxide for 4 days; then, it was changed to osteogenic medium. The osteogenic medium was the growth medium supplemented with 10 mol/L β -glycerophosphate (Sigma-Aldrich) and 50 μ g/mL ascorbic acid (Wako, Tokyo, Japan). The volume of the growth and osteogenic media in each well was 2 mL. The cells were divided into two groups: the LIPUS and control groups.

LIPUS Treatment

We used a LIPUS exposure device (Teijin Pharma Ltd, Tokyo, Japan) that was adapted to the 6-

well graft cell culture plate as in previous studies (Ito et al. 2000; Hasegawa et al. 2009; Lee et al. 2013; Koga et al. 2013). Photos and schematics are shown in Figure 1. LIPUS was generated by an array of six lead zirconate titanate transducers (diameter, 2.5 cm) fixed on the frame to ensure correct positioning of the array beneath each well of a 6-well culture plate. The culture plate was placed 5 mm above the array (Iwabuchi et al. 2005). To initiate LIPUS stimulation, the LIPUS device was immersed in a 37 °C water bath maintained in a CO₂ incubator. The culture plate was placed in a 10 mm water layer over the ultrasound transducer. This device was set at 1.5 MHz; 200 µs burst width sine wave, with a repeating pulse at 1 kHz; and a spatial and temporal average intensity of 30 mW/cm² (Heckman et al. 1994). Above the transducer, there is water, bottom surface of the plate, cells, medium, and air through which the ultrasound waves transmit. When ultrasound waves pass through different materials, they are separated into transmitted waves and reflected waves. Reflectance is defined by the acoustic impedance of each material:

$$R = |Z_2 - Z_1| / (Z_1 + Z_2)$$

where R is the reflectance, Z is the acoustic impedance of each material. Acoustic impedance value of each material is shown in Table 2 (Sabri et al. 2013; Ohtani and Ogawa. 2016). Since the acoustic impedance values of water and polystyrene, which comprise the material of the plate, are not so different, assumably, about 22% of the ultrasound waves are reflected in the layer between water and polystyrene while the rest of them are transmitted that further irradiate the cells. Most of the ultrasound waves beyond the cell layer are reflected by the layers of the medium and air due to the difference of impedance between them, which can cause multiple reflection and affect the cells. In addition, the thickness of each material relative to the wavelength can also affect the degree of reflected waves because of wave interference. As multiple factors could influence the ultrasound waves, we actually measured ultrasound waves from the device by a hydrophone (NH-2000; Precision Acoustics Ltd, Dorset, UK) and an ultrasound power meter (UPM-DT-

1PA; Ohmic Instruments Co, Easton, MD). We confirmed that the proper intensity of LIPUS was correctly transferred through the water layer to the bottom of each well using the hydrophone and ultrasound power meter. All six wells in the plate were stimulated simultaneously, and there was no interference of LIPUS among the wells. The measurement results on the culture plate are shown in Table 3. The waveform setup was equivalent to the wave conditions of a Sonic Accelerated Fracture Healing System (Teijin Pharma Ltd). To exclude the effect of reflected waves by the layers of the medium and air on the target cell plane, a method using a silicon absorption chamber has been reported (Iwabuchi et al. 2005); however, we did not use the silicon chamber due to the problem of contamination in this study. LIPUS was applied through the bottom of the culture plates for 20 min daily at 37 °C in the LIPUS group only.

Cell Proliferation

A total of 3.3×10^4 RIA graft-derived cells per well was seeded into a 6-well plate in a stationary culture system in growth medium for 4 days. The LIPUS stimulation described above was conducted for 2, 4, and 7 days. RIA graft-derived cells were detached using 0.05% trypsin-0.02% ethylenediaminetetraacetic acid. The number of RIA graft-derived cells was counted twice using a hemacytometer, and the mean was calculated. The cell viability was determined to be >99% using the Trypan Blue dye (Gibco) exclusion technique. Results were presented as the relative percentages of the total number of viable cells compared with the control group on each timepoint.

Alkaline Phosphatase Activity Assay

On days 7 and 14, the cellular alkaline phosphatase (ALP) activity was determined by the ALP activity assay. We washed the cell layer from each well twice with phosphate-buffered saline, sonicated with a Microson Ultrasonic Cell Disruptor XL2000 (Misonix, Farmingdale, NY) and stored at -20 °C until assay. ALP activity was assayed as the release of *p*-nitrophenol from *p*-nitrophenylphosphate, pH 9.8; the *p*-

nitrophenol release was monitored by optical density at 405 nm using the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Corp, San Jose, CA). The value of ALP activity was expressed as the relative percentage compared with the control group on day 7.

Real-time Polymerase Chain Reaction

On days 0, 3, 7, 10, and 14, total RNA was extracted from each culture with the RNeasy Mini kit (Qiagen, Valencia, CA). The total RNA was reverse transcribed into single-strand complementary DNA using a high-capacity complementary DNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reactions were performed in duplicate on the complementary DNA with an ABI Prism 7700 sequence detection system (Applied Biosystems) and SYBR Green reagent (Applied Biosystems) according to the recommended protocols. The primer sequences used in the experiments were as follows (Table 4). The expression levels of each gene were normalized by those of glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene, and expressed relative to the control culture levels on day 0. Gene expression was quantified using $\Delta\Delta CT$ methods (Applied Biosystems) (Livak and Schmittgen 2001).

Mineralization

Mineralization assays were performed to evaluate calcification of the extracellular matrix during osteogenic differentiation. On day 21, the cells were fixed for 1 h with 95% ethanol at 20 °C, and the plate was stained with 1% Alizarin Red S (Hartman Leddin, Philadelphia, PA). The mineralized extracellular matrix was stained red. Alizarin Red S staining was released from the cell matrix by incubation in 10% ethylpyridinium chloride for 15 min, and the amount of released dye was quantified by spectrophotometry at 562 nm (Lee et al. 2006). The intensity of Alizarin Red S staining in the sample was expressed as relative intensity levels compared with the control group on day 21.

Statistical Analysis

Each measurement was made in duplicate and averaged. Seven independent repetitions were used to determine the group means and statistical comparisons ($n = 7$). Statistical comparisons at each measurement time between the groups were made using the Mann-Whitney U test. A p -value of ≤ 0.05 was considered statistically significant. All data were shown as the mean \pm standard error (SE).

Results

Cell Proliferation

The obtained cells were fibroblast-like, spindle, and adhesive (Figure 2A). Cell proliferation in the LIPUS group compared to the control group on each timepoint was $106.2\% \pm 6.4\%$, $107.6\% \pm 10.1\%$, and $103.7\% \pm 11.4\%$ on days 2, 4, and 7, respectively. There was no significant difference in cell counts between the LIPUS and control groups on days 2, 4, and 7 ($p > 0.05$; Figure 2B).

Alkaline Phosphatase Activity Assay

The ALP activities of the RIA graft-derived cells in the LIPUS group on days 7 and 14 and in the control group on day 14 compared to that in the control group on day 7 was $113.4\% \pm 5.8\%$, $156.2\% \pm 9.9\%$, and $120.1 \pm 7.8\%$, respectively. The ALP activities in the LIPUS group were significantly higher than those in the control group on each timepoint by 13.4% and 30.1% on days 7 and 14, respectively ($p < 0.05$; Figure 3).

Osteogenic Gene Expression

The expression of five osteoblast-related genes is shown in Figure 4. In the LIPUS group, the expression level of ALP mRNA was significantly upregulated compared with the control group on days 3, 7, 10, and 14. The expression level of Runx2 mRNA in the LIPUS group was significantly higher than the control group on days 10 and 14. At any time point, there was no significant difference in the expression levels of OSX, OC, and ATF4 mRNA between both groups.

Mineralization Assay

Alizarin Red S staining of the LIPUS and control groups at day 21 is shown in Figure 5. The intensity of Alizarin Red S staining in the LIPUS group was $135.2\% \pm 5.9\%$ compared with that of the control group on day 21 and significantly higher than the control group.

Discussion

Previous studies investigating the effect of LIPUS on various cells *in vitro* have been reported (Kokubu et al. 1999; Ito et al. 2000; Saito et al. 2004; Sant'Anna et al. 2005; Sena et al. 2005; Gleizal et al. 2006). However, to the best of our knowledge, this is the first study to demonstrate that the biological effect of LIPUS on the cells was derived from the RIA graft. This study confirms that LIPUS can promote the osteogenic activity and differentiation of the RIA graft-derived cells *in vitro*.

Materials harvested by femoral reaming with the RIA system contained MSCs (Kuehlfluck et al. 2015). Previous studies have revealed qualitative and quantitative differences between bone graft obtained from the medullary canal with the RIA system and iliac crest of the same patient (Sagi et al. 2012). The RIA graft contained more MSCs and higher levels of the expression of bone morphogenic proteins (BMPs) and vascular endothelial growth factor receptors than the iliac crest bone graft. In the porcine model, the expression level of ALP and matrix mineralization by RIA graft-derived cells were significantly higher than those by the iliac crest bone graft cells (van der Bel and Blokhuis 2014). In the comparative study of MSCs from RIA and iliac crest bone grafts, bone formation was significantly higher in the MSCs from RIA grafts than those from iliac crest bone grafts, with or without stimulation with BMP-7 (Hoellig et al. 2017).

Several studies have shown that LIPUS promotes differentiation of a variety of cells including MSCs. We previously reported that LIPUS increased the osteogenic activity of human fracture hematoma-derived cells (Hasegawa et al. 2009). LIPUS upregulated the SDF-1/CXCR4 pathway, which is an essential

factor in bone fracture healing, in MSCs (Wei et al. 2014).

In the present study, LIPUS treatment of the RIA graft-derived cells resulted in higher ALP activity on days 7 and 14 compared with the control group. Furthermore, the ALP mRNA level on days 3, 7, 10, and 14 were significantly increased compared with the control, which is consistent with the above results. Additionally, the Runx2 mRNA level on days 10 and 14 were significantly upregulated by LIPUS treatment. Finally, LIPUS treatment dramatically accelerated the extracellular matrix mineralization of RIA graft-derived cells on day 21.

The gene expression levels of ALP and Runx2, osteogenic markers of early stage (Ducy et al. 1997), were significantly upregulated by LIPUS, while the gene expression levels of OSX, OC, and ATF4, osteogenic markers of late stage (Malaval et al. 1994; Nakashima et al. 2002; Yang X et al. 2004), did not exhibit significant change during the time course. These results were supposedly related to the phase of osteogenesis that we investigated in this study.

There was no significant difference in proliferation of the RIA graft-derived cells between the LIPUS and control groups. This indicates that LIPUS does not affect cell proliferation. Several reports indicated that LIPUS mainly affects differentiation rather than proliferation in human MSCs (Noriega et al. 2007), human fracture hematoma-derived cells (Hasegawa et al. 2009), rat femoral fracture cells (Jingushi et al. 2007), and rat fracture callus cells (Gebauer et al. 2002). In contrast, others indicated that LIPUS affects the proliferation of osteoblasts in rat calvaria (Sun et al. 2001), in CD-1 mice (Gleizal et al. 2006), and in human periosteal cells (Leung et al. 2004). Although there were various reports on the effect of LIPUS on cell proliferation, our results suggested that LIPUS affected the differentiation of RIA graft-derived cells rather than their proliferation.

The Masquelet technique, which was reported for the first time by a French surgeon, is a relatively

new two-staged surgical procedure for critical bone defect consisting of temporary cement spacer placement and following bone grafting (Masquelet et al. 2000). We reported on LIPUS treatment for bone defect patients with Masquelet technique using RIA and observed acceptable clinical results. Based on the result of this study, it is possible that LIPUS treatment promotes osteogenic differentiation and calcification of RIA graft-derived cells, resulting in adequate clinical results.

This study had a few limitations. Firstly, ultrasound waves stimulated the cells within the near field in this study. The diffraction pattern of the ultrasound waves produced by the LIPUS transducer has two characteristic zones: the near field and the far field. It is recommended to place the *in vitro* cell culture within the far field where the ultrasound pressure wave and temporal variation of intensity are more uniform (Padilla et al. 2014). However, placement within the near field more closely matches the clinical situation because the majority of human bones receive the ultrasound within the near field in clinical use of LIPUS treatment (Harrison et al. 2016). Most previously reported *in vitro* and *in vivo* experiments were performed in the near field (Azuma et al. 2001; Freeman et al. 2009; Hasegawa et al. 2009; Lee et al. 2013). Secondly, there are reflected waves that occur between water and dish, or between medium and air. We think that reflected waves may affect the cells, however, it is difficult to calculate their effects. Instead, we made measurements of acoustic field using a hydrophone and recognized that they would not have a significant effect. Finally, our study only evaluated the response to the direct effect of LIPUS treatment on RIA graft-derived cells *in vitro* and could not simulate the ultrasound exposure to bone and cells *in vivo* completely. Since the LIPUS setting in this study are the same in clinical use and measurement results using hydrophone are near the LIPUS setting value, the effect of promoting osteogenic differentiation at the bone grafting site using RIA can be expected; however, further experiments *in vivo* are needed to investigate the effect of LIPUS treatment on autologous bone grafting using RIA in the living body.

The results of this study provide significant clinical implications. In the previous randomized controlled trial, patient outcomes were compared between bone grafting using RIA and iliac crest bone graft for treating nonunion or segmental bone defect (Dawson et al. 2014). The average union rate and time to union of RIA patient were 82.1% and 25.8 weeks and equivalent to iliac crest bone graft. Another study has reported that the union rate in treating nonunion or bone defect cases was similar between two groups, while the time to union, operative time, and length of hospital stay were shorter and complication rate was lower in the RIA group (Le Baron et al. 2019). On the basis of our findings, it is suggested that adding LIPUS to nonunion or bone defect treatment with bone grafting using RIA may improve the union rate and time to union.

Conclusions

In this *in vitro* study, we demonstrated the significant effect of LIPUS on the osteogenic activity and differentiation of RIA graft-derived cells for the first time. Significantly higher alkaline phosphatase activity, expression of osteoblast-related genes, and mineralization of the RIA graft-derived cells was observed in the LIPUS group than in the control group; however, LIPUS did not affect the cell proliferation of RIA graft-derived cells. These results suggested that LIPUS affected the differentiation of RIA graft-derived cells rather than their proliferation. This study indicates that LIPUS may be an additional tool to enhance the healing process of nonunion and critical bone defect treated by autologous bone grafting using RIA system.

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261 **Conflict of Interest Statement**

262 We have no conflicts of interest directly relevant to the content of this article.

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381

Figure Legends

Fig. 1A. LIPUS exposure device *in vitro*. Left images show an array of ultrasound transducer and right images show 6-well culture plate on the array.

Fig. 1B. A schematic of LIPUS device. Experimental setup with the key parameters are shown.

Fig. 2A. RIA graft-derived cells. They are fibroblast-like, spindle, and adhesive.

Fig. 2B. Proliferation of RIA graft-derived cells relative to day 0 level on days 2, 4, and 7 (NS, not significant; $*p \leq 0.05$).

Fig. 3. Relative ALP activity of RIA graft-derived cells on days 7 and 14. The ALP activity in each sample was expressed relative to the level in the control group on day 7 ($*p \leq 0.05$).

Fig. 4. Quantitative real-time polymerase chain reaction analysis of RIA graft-derived cells. The mRNA expression levels of ALP (A), Runx2 (B), OSX (C), OC (D), and ATF4 (E) were evaluated and normalized by the mRNA expression level of glyceraldehyde-3-phosphate dehydrogenase. The mRNA levels are expressed relative to those for the controls on day 0 (value set at 1 for each marker, $*p \leq 0.05$).

Fig. 5. Alizarin Red S staining of the LIPUS and control groups at day 21. Photographs show that the control cells (A) had less Alizarin Red S staining than the LIPUS-treated cells (B). The intensity of Alizarin Red S staining in the LIPUS group was expressed relative to the level in the control group (C; $*p \leq 0.05$).

404 Table 1. Clinical data of the seven patients

Patient	Gender	Age	Disease	Comorbidity	Smoking (cigarettes/day, years)	Other factors affecting osteogenic activity
1	M	55	Non-infected nonunion/Tibia	Hypertension	20, 35	-
2	M	51	Chronic osteomyelitis/Femur	-	30, 5	-
3	M	42	Acute osteomyelitis/Tibia	-	-	-
4	M	54	Acute osteomyelitis/Femur	Alcoholic liver disease	20, 35	-
5	M	40	Non-infected nonunion/Tibia	-	10, 20	-
6	M	60	Infected nonunion/Tibia	-	20, 20	-
7	M	44	Infected nonunion/Tibia	-	20, 25	-

405

407 Table 2. Acoustic impedance value of each material

408

Material	Acoustic impedance Z, kg/(m²s)
Air	428.6
Water	1.50×10^6
Polystyrene	2.37×10^6
Silicone	1.50×10^6

409

410 Table 3. Quantitative data on acoustic output measurement

Parameter	Manufacturer's Characteristics	Measured Characteristics
I_{SATA} , mW/cm ²	30.0	28.0
Frequency, MHz	1.5	1.5
Pulse repetition rate, kHz	1	1
Pulse duration, μ S	200	200

411 I_{SATA} : spatial-average temporal-average intensity

412 Table 4. Details of the primers used for amplification

Gene	Primer sequences (5' to 3') (forward/reverse)
ALP	CTCGTTGACACCTGGAAGAGCTTCAAACCG GGTCCGTCACGTTGTTTCCTGTTTCAGC
Runx2	ATGCTTCATTTCGCTCACAAC CCAAAAGAAGTTTTGCTGACATGG
OSX	CGGGACTCAACAACCTCT CCATAGGGGTGTGTCAT
OC	CATGAGAGCCCTCACA AGAGCGACACCCTAGAC
ATF4	CTGACCACGTTGGATGACAC GGGCTCATAACAGATGCCTCT
GAPDH	CGTCTTCACCACCATGGAGA CGGCCATCACGCCACAGTTT

413 *ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; OSX, osterix; OC, osteocalcin;

414 ATF4, activating transcription factor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

415



Fig. 1A

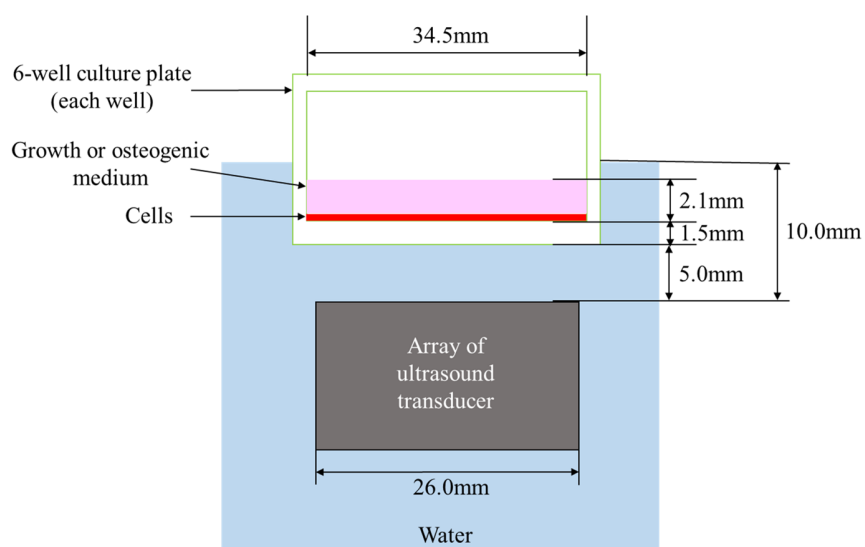


Fig. 1B

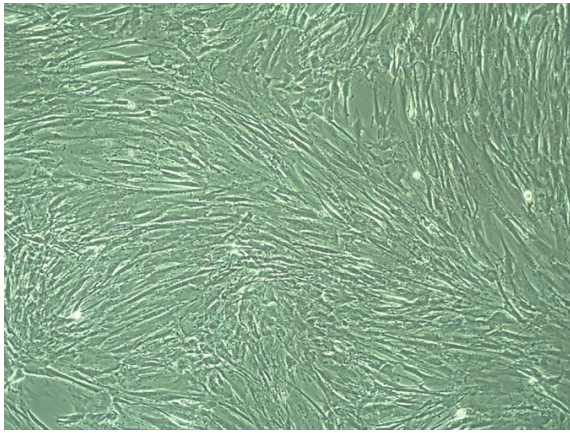


Fig. 2A

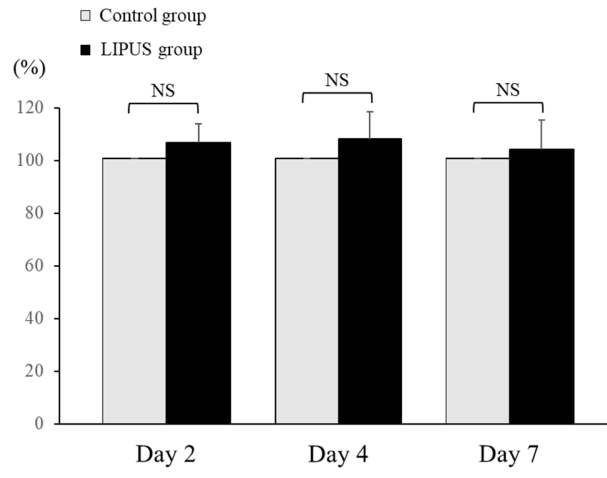


Fig. 2B

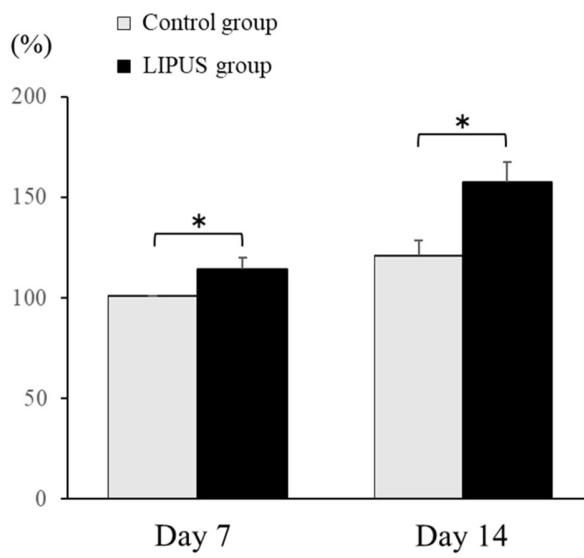


Fig. 3

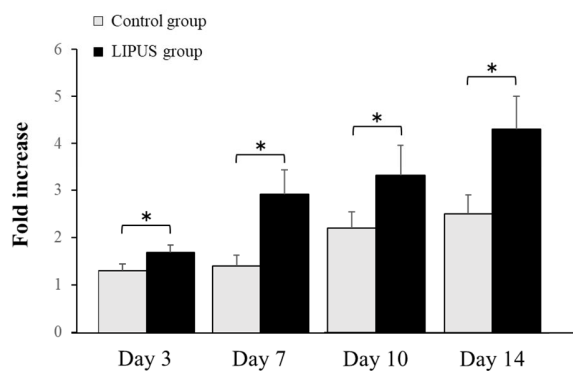


Fig. 4A (ALP)

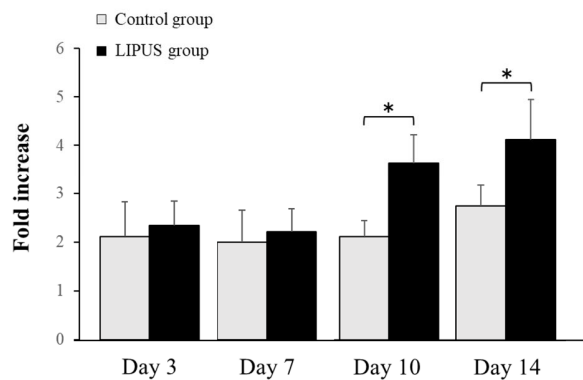


Fig. 4B (Runx2)

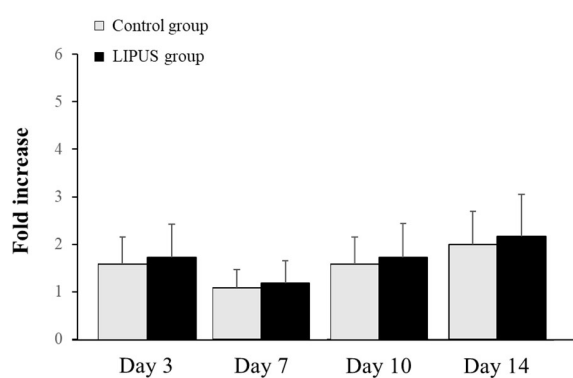


Fig. 4C (OSX)

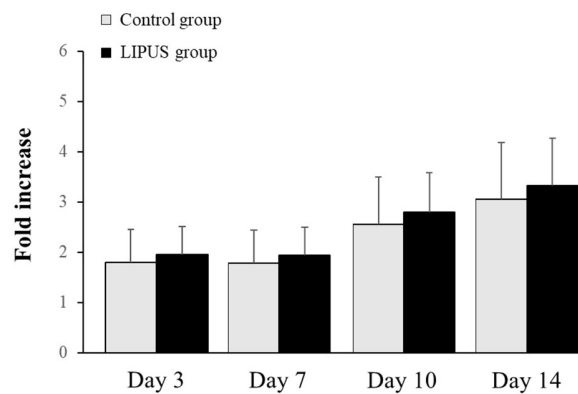


Fig. 4D (OC)

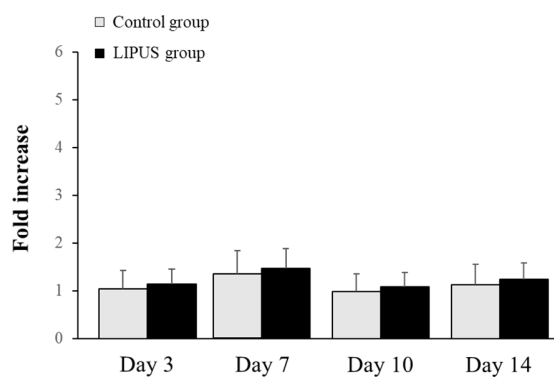
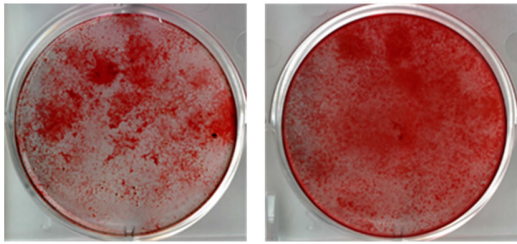


Fig. 4E (ATF4)



Control group

LIPUS group

Fig. 5A

Fig. 5B

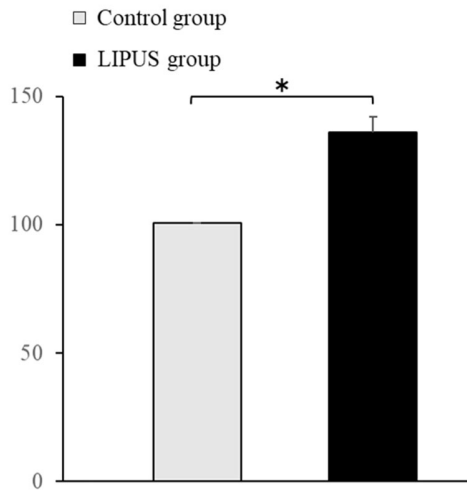


Fig. 5C