Enhancement of Cardiomyogenesis in Stem Cells by Low Intensity Pulsed Ultrasound

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Abstract. Low intensity pulsed ultrasound (LIPUS) has been shown to enhance bone and cartilage regeneration from stem cells. Gene expression of angiotensin II type 1 (AT1) receptor can be increased in LIPUS-treated osteoblasts. The AT1 receptor is a known mechanoreceptor in cardiomyocytes. It suggests that LIPUS may enhance cardiomyogenesis via mechanotransduction by increasing AT1 expression. Murine embryonic stem cells (ESCs) were treated daily by 10-min 1MHz LIPUS at spatial-average temporal-peak acoustic intensities of 30 mW/cm² and 300 mW/cm² in both continuous and pulsed wave (20% duty cycle) for 10 days. Polymerase chain reaction (PCR), immunocytochemistry, and beating rate were used to evaluate the cardiac viability quantitatively. After the treatment of LIPUS, beating rate of contractile areas and cardiac gene expression, such as α- and β-myosin heavy chain, were improved. Furthermore, no deleterious effects to the development of cardiac proteins were observed. All results suggest that LIPUS stimulation has the capacity of enhancing cardiomyogenesis from embryonic stem cells. With the benefit and the ease in incorporating LIPUS into various culture platforms, LIPUS has the potential to produce cardiomyocytes for clinical use in the future.

INTRODUCTION

Low intensity pulsed ultrasound (LIPUS) is a medical technology, using ultrasound pulses at 1-2 MHz with a pulse width much longer than that of diagnostic one (ms vs. µs) at a low intensity (< 2 W/cm²). It is becoming popular in the rehabilitation and has been approved by the Food and Drug Administration (FDA) of USA for use in orthopedics, such as promoting bone-fracture healing, treating orthodontically induced root resorption, regrow missing teeth, enhancing mandibular growth in children with hemifacial microsomia, promoting healing in various soft tissues such as cartilage, inter vertebral disc, and improving muscle healing after laceration injury ¹.

Although many differentiation studies investigating LIPUS effects were mostly on osteogenesis and chondrogenesis, a study showed that LIPUS was also able to control myoblast differentiation toward the matured myosin lineage ². Under daily stimulation with 10 mins of 30 mW/cm² LIPUS for 6 days, myoblasts showed the presence of tropomyosin expression, which in contrast was absent in the control cells. Here it can be deduced that LIPUS provided mechanical cues that were responsible for inducing differentiation towards the myocyte lineage. Moreover, in a study on osteoblasts, there was an increased gene expression of angiotensin II type 1 (AT1) receptor in LIPUS-treated osteoblasts ³. The AT1 receptor is a known mechanoreceptor in cardiomyocytes. Mechanical stimulation has been shown to improve cardiomyocyte differentiation from cell sources like neonatal cardiomyocytes, MSCs and ESCs ⁴,⁶. These studies suggest that LIPUS may have a great potential in enhancing cardiomyocytes differentiation and maturation.

We hypothesized that mechanical cues from LIUS could enhance cardiomyogenesis via mechanotransduction. In this study, effects of LIPUS on cardiomyocyte differentiation of murine embryonic stem cells (ESCs) were
investigated. The cells were subjected to a daily treatment of LIPUS for 10 min at two different intensities (30 an 300 mW/cm²) and two different wave generation modes (continuous and pulsed wave). Promising results showed that LIPUS can enhance cardiomyogenesis in stem cells.

**METHODS**

Murine ESCs (E14TG2a, ATCC, VA, USA) were uniformly distributed in 0.1% porcine-gelatin (Sigma, MO, USA) coated flasks under ESC maintenance medium. The maintenance medium comprises of high-glucose Dulbecco Modified Eagle's Medium (DMEM) (Gibco, Life Technologies, NY, USA), 10% gold-standard fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), 0.1 mM 2-mercaptoethanol (Sigma, MO, USA), 2 mM L-glutamine (Gibco, Life Technologies, NY, USA), 1x penicillin-streptomycin (PAA Laboratories) and 1000 U/mL leukemic inhibitory factor (LIF) (Chemicon, Millipore, MA, USA). Medium was changed daily and cells passage every 2-3 days. Murine ESCs were primed toward the mesodermal lineage by culturing on gelatin-coated flasks in HepG2-CM for 3 days (Hwang et al. 2006; Lake et al. 2000). Next, the formation of embryoid bodies (EB) was initiated. Murine ESCs were gently detached from the gelatin-coated flasks using 0.25%-EDTA trypsin (Gibco, Life Technologies, NY, USA) and small clumps of mESCs were re-plated onto bacteriological grade petri dishes containing EB medium, which consists of high-glucose Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Life Technologies, NY, USA), 20% gold-standard FBS, 2 mM L-glutamine, 1x penicillin-streptomycin and 450 μM 1-thioglycerol (Sigma, MO, USA). The EBs were left to grow for 5 days with medium being changed every two days. To induce cardiomyocyte differentiation, suspended EBs were plated onto gelatin-coated 6-well plates, and 0.25 μM Cardiogenol C (Sigma, MO, USA) was added into the EB medium as the cardiomyogenic-inducing agent. This differentiation medium was used for the rest of the culture till Day 21 and changed every two days.

On Day 11, the plated EBs were subjected to daily treatments of LIPUS for 10 mins. The LIPUS probe working frequency of 1 MHz and active area of 10 cm² (Model X, Rich-Mar, TN, USA) was placed underneath cell-seeded plates and various treatment conditions were tested: (1) 30 mW/cm² LIPUS at a pulse repetition frequency (PRF) of 1 Hz and duty cycle (DC) of 20% (30pul), (2) 30 mW/cm² continuous LIPUS (30cont), (3) 300 mW/cm² LIPUS at PRF of 1 Hz and DC of 20% (300pul), and (4) 300 mW/cm² continuous LIPUS (300cont).

**RESULTS**

After 3, 7 and 10 days of LIPUS treatment (Day 14, 18 and 21 of culture, respectively), cardiac gene expressions were compared between treated groups and the control on Day 14 (Fig. 1). After only 3 days of LIPUS treatment (Day 14), the upregulation of Brachyury T, a marker for mesodermal commitment of ESCs, was found to be higher in all LIUS treatment conditions as compared to the control group (Fig. 1a). This showed that LIPUS could produce cues that induced mesodermal lineage commitment, and would have further beneficial effects for cardiomyogenesis. Under the markers for late cardiomyocyte differentiation (α-MHC, β-MHC, cTNT), the upregulation under 30pul and 300cont LIUS treatments were more pronounced than the other two treatment conditions. On Day 18, cells treated with 300cont LIUS showed significant upregulation of the two isoforms of the cardiac myosin heavy chains (α-MHC and β-MHC) compared to the controls. Meanwhile, cells treated by 30pul LIUS did not show any significant increase in cardiac gene expressions on Day 18, but the expressions for cTNT and α-MHC were significantly increased on Day 21. It showed that the 30pul LIUS condition could also enhance cardiomyogenesis of ESCs but the observed effects were slower than those with 300cont LIUS. Since the significant increased gene expression definitely occurs no matter of LIUS intensity, the enhancements of 30cont and 300pul might be missed between Day 18 and 21.
FIGURE 1. Gene expression fold changes in cardiac markers (a) Brachyury T, (b) α-MHC, (c) β-MHC, and (d) cTNT during the treating period of LIPUS. Bracket: significant difference between sample and its control. *: significant difference between sample and its intensity counterpart. ^: significant difference between sample and its PRF counterpart. p < 0.05.

On Day 18 and 21, beating colonies found in differentiated mESCs were quantitatively evaluated by measuring the beating rate. Figure 2 summarizes the beating rate of cells corresponding to the trends we observed in the upregulation of cardiac genes. On day 18, beating rate of cells under 300cont LIPUS treatment was significantly increased while all the other experimental groups showed no significant difference. On Day 21, 30pul and 300pul LIUS treatments had also produced higher beating rate than that of the controls. These briefly showed that LIPUS could generally enhance ESC-differentiated cardiomyocytes’ contractile function. This result agrees with the one of gene expression.

FIGURE 2. Beating rate of contracting areas in differentiating cells under various LIPUS treatments. Same statistical legends as Fig. 1. p < 0.05.

We also evaluated whether LIPUS would cause any deleterious effects to the development of structural and cardiac proteins on cell surface. Surface expressions of structural proteins and cardiac markers in mESC differentiated cells were evaluated via immunostaining for sacromeric α-actinin (ACTN) and cardiac troponin I (cTNI) on Day 21 (Fig. 3). Figure 3 (a), (d), and (g) shows the nuclei stained images. Sacromeric α-actinin is a
binding protein that attaches actin filament to dense striations in cardiomyocytes and is represented by the green fluorescence in Fig. 3 (b), (e) and (h). Cardiac troponin, a regulatory protein involved in calcium signalling for the contractile motion of cardiomyocytes, is represented by the red fluorescence in Fig. 3 (c), (f) and (i). The expression levels of both proteins in all samples were intense, extensive and fairly uniform in comparison to their respective nuclei stained images. It implies that LIPUS caused no deleterious effects to the development of cardiac proteins.

FIGURE 3. Immunostaining micrographs of control, 30pul and 300cont on day 21. Scale bar represents 50 μm.

DISCUSSION

LIPUS has produced a temporal increase in Brachyury-T expression, which signified that the sonication could increase mesodermal commitment of differentiating ESCs. In addition, comparisons of late cardiac gene expressions (α-MHC, β-MHC and cTNT) and beating rate showed that 300 mW/cm² continuous LIUS and 30 mW/cm² pulsed LIPUS could enhance cardiomyogenesis. A possible reason is a form of mechanical stimulation and the subsequent biophysical effect induced by the LIPUS, such as acoustic radiation force, microstreaming and shock wave generating by bubble cavitation. Though LIPUS can be utilised for enhancing cardiomyogenesis, different parameters of LIPUS, e.g. intensity and duty cycle, will influence its impact. For instance, we observed that 30 mW/cm² pulsed LIPUS required a longer period of LIPUS treatment than 300 mW/cm² continuous LIUS to produce enhanced cardiomyogenesis. 300 mW/cm² continuous LIUS produced a higher US pressure in the medium and thus could have created higher mechanical stress and a stronger mechanotransduction effect on the ESCs. Therefore, the time required for enhanced cardiomyocyte differentiation was shorter than the 30 mW/cm² LIPUS. This implies that optimal LIPUS conditions will vary for different applications in tissue engineering and regenerative medicine.

After the treatment of LIPUS, beating rate of contractile areas and cardiac gene expression were improved. LIPUS stimulation has the capacity of enhancing cardiomyogenesis from embryonic stem cells. Furthermore, LIPUS does no harm to the development of cardiac proteins. With the benefit and the ease in incorporating into various culture platforms, LIPUS has the potential to produce cardiomyocyte for clinical use in the future.

REFERENCES

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