

LIPUS Actuates and Increases Cell-to-Cell Communication and Connexin-43 Expression in Bone Marrow Stromal Cells

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INTRODUCTION:

The medical application of low-intensity pulsed ultrasound (LIPUS) is an established therapy for fracture repair. Despite its pronounced effects during fracture repair, the underlying mechanisms of action of LIPUS remain unclear.

Cell-to-cell communication is critical for bone development and remodeling. In the bone marrow, nonhematopoietic-bone marrow stromal cells (BMSC) provide an important microenvironment for regulations of differentiation of both hematopoietic and skeletal cells. The function of the stromal cell is based on cell-cell communication and secretion of soluble factors. Morphological studies have shown that gap junctions are channels that span the transmembrane space which exists between stromal cells and bone cells. The gap junctions mediate the intracellular exchange of regulatory ions and small molecules that allow metabolic cooperation between adjacent cells which control cell differentiation and growth. These junction complexes are formed by proteins of the connexin family. The major gap junction protein connexin43 (Cx43) has been identified in stromal cells and bone cells. Gap junctions have been suggested to be central to the transmission of biophysical stimuli^{1,2} and mechanical stimulation has been shown to increase production of connexins.³ We hypothesized that LIPUS renders its effect on fracture repair by actuating cell-to-cell communication as well as Cx43 in BMSC.

METHODS:

Tissue culture: Rat BMSC were obtained from adult male Sprague-Dawley rats using standard procedures.⁴ Cells were incubated in DMEM containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37 °C.

LIPUS treatment:⁵ LIPUS exposure (operating frequency=1.5 MHz, intensity=30 mW/cm², 20% duty cycle) for 20 min was applied by a Sonic Accelerated Fracture–Healing System device, consisting of an array of 6 transducers specifically designed for a 12-well culture plate. Ultrasound transducers were placed under 6 wells using a coupling gel. Sham controls were handled in the same way using separate culture plates, but the ultrasound generator was not switched on.

Preloading (“parachute”) assay:⁶ Donor BMSCs were preloaded with calcein-AM, trypsinized to become single cell suspension and a small number of cells were added (“parachuted”) to the unstained-monolayer acceptor cells. After single LIPUS or sham treatment, calcein fluorescence was monitored by epifluorescence microscopy and the dye coupling was assessed by counting the number of cells acquiring dye per parachuted donor cell.

Real-time quantitative PCR for Cx43: Total RNA was isolated and reverse transcribed into cDNA and used as template for PCR.

Quantitative gene expression analyses were carried out using real-time PCR by means of the SYBR® Green I and the Smart Cycler® system as described previously.⁵ Data were normalized to 18S rRNA.

Modification of LIPUS-induced cell signaling by gap junction inhibitor: To block gap junction channels, 18β-glycyrrhetic acid was applied to BMSC 4h before the LIPUS treatment. For intracellular signaling, cells were harvested 30 min after a single LIPUS treatment and analyzed for activation of ERK 1/2 and p38 MAPK pathways by Western blots.

Statistical analysis: Data are presented as mean ± S.D. Significance was defined as probability values less than 0.05 with Mann-Whitney test.

RESULTS:

Effect of LIPUS on calcein dye coupling:

After a single treatment, cells that underwent LIPUS treatment had a higher number of coupled cells compared to sham control. (Fig. 1)

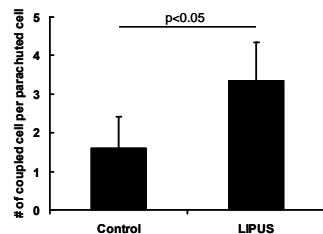


Fig.1 Effect of LIPUS on dye coupling.

Effect of LIPUS on Cx43 gene:

Cx43 showed a trend of elevated expression of mRNA in LIPUS treated cells over sham controls at 3 hr after the ultrasound exposure (Fig. 2). LIPUS treatment demonstrated a 2-fold higher expression level compared to control.

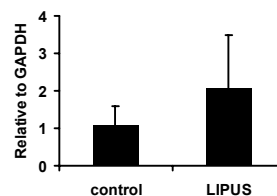


Fig. 2 Effect of LIPUS on Cx43 gene expression.

Effect of gap junction inhibitor on phosphorylation of cell signaling via LIPUS:

LIPUS showed increased levels of phosphorylated ERK 1/2 and p38 as compared to sham (Fig.3). Addition of 18β-glycyrrhetic acid abolished the increased levels of phosphorylated ERK 1/2 and p38 by LIPUS.

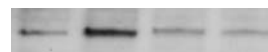


Fig. 3 Effect of LIPUS on phosphorylation of p38.

DISCUSSION:

We have clearly demonstrated that LIPUS stimulates cell-to-cell communication and increases the expression of Cx43 gene. Moreover, we have shown that cell-to-cell communication, i.e. gap junction, is a critical factor for LIPUS to affect BMSC through cell signaling. Recent studies have demonstrated the importance of Cx43 in bone development and remodeling. The skeleton of Cx43 null newborn mice reveals defects in intramembranous and endochondral ossification, that leads to skull abnormalities, brittle, misshapen ribs, and delayed mineralization, primarily of intramembranous bones.⁷ Osteoblasts isolated from calvaria of Cx43 deficient mice exhibit a reduced osteogenic differentiation, mineralization, and decreased expression of bone matrix proteins.⁷ Moreover, Cx43 mutations have been linked to the human disease oculodentodigital dysplasia (ODDD), an autosomal dominant disorder characterized by limb malformations and craniofacial abnormalities.⁸ Taken together with our finding, anabolic effect of LIPUS on fracture healing might be regulated by modulating cell-to-cell communication as well as Cx43. Indeed, previous reports indicate the role of gap junctional intercellular communication on bone-cell response to biophysical signals, such as electromagnetic fields or fluid flow.²

These results emphasize the importance of gap junctions on LIPUS stimulation in BMSC, which may also explain the underlying mechanism of action of LIPUS in fracture healing.

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