Published in final edited form as:

Ultrasound Med Biol. 2013 May ; 39(5): 804–812. doi:10.1016/j.ultrasmedbio.2012.11.016.

Reversal of the Detrimental Effects of Simulated Microgravity on Human Osteoblasts by Modified Low Intensity Pulsed Ultrasound

Sardar M. Zia Uddin, Michael Hadjiargyrou, Jiqi Cheng, Shu Zhang, Minyi Hu, and Yi-Xian Qin*

Orthopaedic Bioengineering Research Lab, Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281

Abstract

Microgravity (MG) is known to induce bone loss in astronauts during long duration space mission due to lack of sufficient mechanical stimulation under microgravity. It has been demonstrated that mechanical signals are essential for maintain cell viability and motility, and possibly serve as a countermeasure to the catabolic effects of MG. The objective of this study was to examine the effects of high frequency acoustic wave signals on osteoblasts in a simulated microgravity (SMG) environment (created using 1D clinostat bioreactor) using a modified low intensity pulsed ultrasound (mLIPUS). Specifically, we evaluated the hypothesis that osteoblasts [human fetal osteoblastic (hFob) cell line] exposure to mLIPUS for 20 min per day at 30 mW/cm² will significantly reduce the detrimental effects of SMG. Effects of SMG with mLIPUS were analyzed using the MTS assay for proliferation, Phalloidin for F-actin staining, Sirius red stain for collagen and Alizarin red for mineralization. Our data showed that osteoblast exposure to SMG results in significant decreases in proliferation (~ -38% and ~ -44% at day 4 and 6, respectively, $p < 0.01$), collagen content (~ -22%, $p < 0.05$) and mineralization (~ -37%, $p < 0.05$) and actin stress fibers. In contrast, mLIPUS stimulation in SMG condition significantly increases the rate of proliferation (~24% by day 6, $p < 0.05$), collagen content (~52%, $p < 0.05$) and matrix mineralization (~25%, $p < 0.001$) along with restoring formation of actin stress fibers in the SMG-exposed osteoblasts. These data suggest that the acoustic wave can potentially be used as a countermeasure for disuse osteopenia.

Keywords

simulated microgravity; mechanotransduction; countermeasure; bone loss; LIPUS; acoustic streaming; osteoporosis

Introduction

Microgravity (MG) induced during extensive space-flight can significantly reduce bone mineral density (BMD). Overall, 1–2% monthly loss of BMD is observed during a typical

© 2012 World Federation for Ultrasound in Medicine and Biology. Published by Elsevier Inc. All rights reserved.

*Corresponding Author: Yi-Xian Qin, Ph.D., Department of Biomedical Engineering, Stony Brook University, 215 Bioengineering Bldg, Stony Brook, NY 11794-5281, Tel: 631-632-1481, Fax: (631) 632-8577, Yi-Xian.Qin@StonyBrook.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

3–6 month space mission, in which, greater bone loss was observed in the weight-bearing sites (Marie et al. 2000; Vico et al. 2001). Further, ~20–30% total bone loss is expected in a 30-month manned mission to Mars (Hughes-Fulford et al. 2006; Qin 2010), which will significantly hamper the astronauts bone's structural and physiological function. To make long term space exploration possible, it is imperative that an effective countermeasure against the catabolic effects of MG can be developed, ideally noninvasive, low weight, and portable. To address the challenge of microgravity induced musculoskeletal alteration, astronauts usually spend ~2.5 hrs/day of exercising (running and weight lifting). However, this is still not sufficient enough to attenuate bone loss in space. While promising studies have shown anabolic effects using drugs and growth factors, the long-term effects of pharmacological agents are still unclear. Furthermore, drugs have mostly systemic effects, are not as effective in space, and are expensive as a treatment for extended space time (Shapiro 2006).

To understand the underlying biological processes behind the observed bone loss, *in vitro* studies were conducted at the international space station (ISS) using various types of bone cells. For example, osteoblasts cultured in space showed significant reduced glucose utilization, rate of proliferation and mineralization (Van Loon et al. 1995; Hughes-Fulford and Lewis 1996; Hughes-Fulford et al. 1998; Hughes-Fulford et al. 2006). It was also observed a reduction in collagen 1 and fibronectin content in the extracellular matrix (Hughes-Fulford 2002; Hughes-Fulford 2003), and alteration of the cytoskeletal organization and cell adhesion (Nabavi et al.). Conducting *in vitro* studies at the space station is greatly limited by accessibility and can be expensive with many technical difficulties. Therefore, to address these disadvantages, various ground base simulated microgravity (SMG) models have been developed and utilized. Two most commonly used SMG models are the Rotating Wall Vessel (RWV) (Schwarz et al. 1992; Hoson et al. 1997) and Random Position Machine (RPM) (Huijser 2000). Studies conducted using these SMG models showed down-regulation of a number of bone related genes, including alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), parathyroid hormone receptor 1 (PTHr1), bone morphogenetic protein 4 (BMP4), and procollagen and osteoglycin, as well as significant reduction in the formation of calcium nodules and collagen matrix formation (Schwarz et al. 1992; Sheyn et al. 2010). Lastly, osteoblasts grown in SMG also showed similar effects on morphology, cytoskeleton, apoptosis and gene expression as those cultured during space flight (Schwarz et al. 1992; Sheyn et al. 2010).

In the past decade researchers have explored the anabolic effects of various mechanical stimulatory signals (vibration, fluid flow, and ultrasound) on the skeleton. Specifically, low-intensity pulsed ultrasound (LIPUS) was applied *in vivo* and showed reversal of osteopenia and enhancement of bone fractures (Boyd et al. 2002; Carvalho and Cliquet Junior 2004; Ferreri et al. 2011). LIPUS has shown increased anabolic events in bone cells (Binderman et al. 1984; Binderman et al. 1988; Rubin et al. 1997; Uddin et al. 2011), including enhanced cytokine release (Li et al. 2003), increased mRNA expression of RUNX2, ALP, Osterix and collagen 1 (Yang et al. 1996), calcium mineralization of ECM (Nolte et al. 2001), activation of Akt pathway (Takeuchi et al. 2008a), and increase of potassium influx (Chapman et al. 1980), angiogenesis (Kaneko et al. 2002), adenylyl cyclase activity and TGF- β synthesis resulting in increased osteogenesis of bone stroma cells (Naruse et al. 2003). Despite these effects, the exact mechanism through which LIPUS enhances bone cell properties and increases the rate of bone formation remains unknown. As such, we attempted to further elucidate the effects of a modified LIPUS on osteoblasts by testing the hypothesis that mLIPUS serves as an effective countermeasure for the detrimental effects of SMG on osteoblast activity and morphology.

Materials and Methods

Rotation Microgravity Simulation

An *one-dimensional clinostat (1-D Clinostat)* was developed to keep cells in continuous 1-D rotation with a steady speed of 15 rpm around the horizontal axes resulting in averaging out the gravity factor (Fig. 1). Lee Silver (1976) explained in detail the effects of a 1-D rotation on cell and sub-cellular organelles with respect to gravity; continuous rotation along horizontal axis disables cells to respond to gravitational force and induces a lack of orientation. Fig. 1A explains the changing rotation of cells with respect to gravity vector during rotation. The rotation resulted in a radial force that is less than 0.045N. The rotator is controlled by a variable speed motor with the holder space of 80 mm (radius = 40mm), which is capable of holding 8 Opticell® cartridges (Nunc, VWR, Bridgeport, NJ, USA) (Fig. 1B). Each Opticell cartridge is 2 mm thick, and cells are seeded on a gas-permeable polystyrene membrane. Each cartridge was filled with 10 ml of media, and no air bubbles were present in the media to cause fluid shear stress. The clinostat was kept under sterile conditions at 37°C in 5% CO₂ environment in a standard incubator (Thermo scientific, Asheville, NC, USA) for the duration of all experiments.

Ultrasound Exposure

A mLIPUS stimulus was applied using an acoustic device, Sonicator® 740 (Mettler Electronics, Anaheim, CA, USA) with a 10cm² transducer (32mm in diameter) utilizing modified repetitive frequency at 100 Hz pulse, an ultrasound characteristic frequency of 1MHz, and a pulse width of 200 µs, repeated at 100 Hz at an intensity of 30 mW/cm² for 20 minutes/day. The ultrasound signal was modified using 100Hz repetition frequency rather than the 1KHz repetition frequency that is typically used. This type of application of ultrasound at a modified low repetitive comparable dynamic frequency may lead to a new approach for noninvasive stimulation of bone turnover. The region of mLIPUS treatment was marked with a 10cm diameter circle before the start of each experiment. All stimulations targeted the same region of each Opticell and only those cells that were growing in the Region of Stimulation (RoS) were harvested and analyzed. Lastly, the 3–5mm distance between the transducer and Opticell was filled with degassed water.

Cell Culture

Human fetal osteoblasts (Hfob 1.19, ATCC, Manassas, VA, USA) were cultured in DMEM:F12 media supplemented with 15% FBS, 0.3mg/ml G418 with an initial seeding density of 250,000 cells/Opticell. Cells were distributed into four groups (n=4/group): 1) Gravity (G); 2) Gravity + mLIPUS (GU); 3) simulated microgravity (M); and 4) simulated microgravity + mLIPUS (MU). GU and MU samples were stimulated with mLIPUS for 20 min for the duration of the experiments while G and M samples were placed on a switched-off transducer. All cells were maintained at 37°C and 5% CO₂ and the culture medium was changed every other day.

Cell layer Deformation

Hfob cells were cultured to confluency and form a monolayer in Opticells (visually inspected using a light microscope). Cells were kept under SMG for 7 days and the cell layer was observed for structural integrity using bright field microscopy (Axiovert 200, Carl Zeiss, Thornwood, NY, USA).

Proliferation Assay

Hfob Cells were cultured at 250,000 per Opticell cartridge, and were allowed to adhere to the Opticell surface for 24 hr before subjected to the clinostat. Daily mLIPUS was applied

after 24 hr of SMG. Samples were collected at Day 2, 4 and 6 and each RoS was cut out from the Opticell and processed with the MTT reagent (Invitrogen) in serum-less DMEM per manufacturer's instructions. Briefly, the cells were incubated for 4 hr at 37°C, 5% CO₂ with the MTT reagent followed by lysis with DMSO for 10 min. Finally, the supernatant was analyzed at 540nm in a spectrophotometer (*Bio-Tek EL800*, Winooski, VT, USA).

Collagen Staining

Hfob cells were cultured in Opticells at an initial density of 250,000 cells and were allowed to adhere to surface for 24 hr. Opticells were then inserted into rotator and exposed to SMG for 7 days. The culture medium was changed every other day and MU and GU cells were stimulated with mLIPUS every day for 20 min. After 7 days, the RoS was cut out from the Opticell and fixed in 4% paraformaldehyde for 30 min at room temperature. Samples were stained with 1% pico-sirius red for 1 hr and the cells were washed with acidified water (0.5 % acetic acid water) and dehydrated with serial ethanol washes: 70%, 90% and 100% (5 min each). Images were taken by polarized microscope (Nikon Diaphot 200, Melville, NY, USA) at 2.5X

F-Actin Staining

Hfob cells were seeded at initial density of 100,000 cells per Opticell cartridge, and were allowed to adhere for 24 hr. The cells were cultured in SMG for 3 days and then fixed in 10% formaldehyde buffer for 15 min at room temperature and washed with PBS. The cell membrane was permeabilized using 1% Triton-X100 for 10 min followed with three, 5 min PBS washes. The cells were then blocked with 1% BSA and incubated with Rhodamine-conjugated Phalloidin for 20min and mounted using vectashield mounting medium containing DAPI. Finally, the cells were imaged at 100x using an Axiovert 2000M Inverted Microscope (Carl Zeiss, Thornwood, NY, USA).

Matrix Mineralization

Matrix calcification was analyzed using alizarin red staining. SMG and mLIPUS stimulation were initiated after 7 days of culture (initial seeding density of 250,000 cells per Opticell cartridge). SMG and mLIPUS stimulations were applied for an additional 14 days and then the RoS was cut out of the Opticell and the cells were fixed in 70% ethanol for 1 hr at room temperature and stained with 40mM alizarin red (pH 4.2) for 10 min. Samples were then washed with tap water, allowed to dry at room temperature and imaged using an Axiovert 2000M Inverted Microscope (Carl Zeiss, AxioCam MRC, Thornwood, NY, USA). Following imaging, the stain was eluted off the cells in a solution of 10% cetylpyridinium chloride in 10mM sodium phosphate for 15 min. The eluted stain was then measure at 562nm in a spectrophotometer (Bio-Tek EL800, Winooski, VT, USA). All samples were quantified against an alizarin red standard curve in 10% cetylpyridinium and normalized by the total number of cells.

Statistics

The GraphPad Prism 3.0 software was used to run statistical analyses. All data is presented in $\pm$ standard deviation. One-way ANOVA with Newman Keuls Post hoc was used to calculate significance within the different groups and time points. A *p* value of <0.05 was consider to be significant.

Results

Simulated microgravity disrupts the cellular monolayer

As osteoblast function is highly dependent on the ECM, any deficiency in the quality of the ECM will negatively affect osteoblast activity. As such, we investigated the effects of SMG and mLIPUS on the integrity of the cellular monolayer. During SMG simulation cells were observed daily and after 7 days of SMG, samples in M group displayed dark areas that represent disruptions in the cell monolayer (Fig. 2, M, arrows). In contrast, the control samples in gravity group (G) maintained a uniform and intact cell monolayer (Fig. 2, G).

mLIPUS exposure significantly reverses SMG-induced decreases in osteoblast proliferation

The observed non-uniform cell layers in M group samples are indicative of either a reduced rate of proliferation or a compromised ECM. Thus, we decided to examine these possibilities starting with cell proliferation. Results from these experiments showed that the rate of proliferation is significantly reduced in samples exposed to M group by ~38% ($p<0.01$) and 45% ($p<0.01$), on day 4 and 6 samples, respectively (Fig 3). The application of mLIPUS reversed this trend and in fact, it increased cell proliferation by ~24% ($p<0.05$) and 19% ($p<0.001$), when compare to the M group samples at day 4 and 6, respectively (Fig. 3). In contrast, mLIPUS stimulation had no major effect on cell proliferation in the GU as compared to the control (G) (Fig. 3).

mLIPUS exposure significantly reverses SMG-induced decreases in collagen production

As we postulated earlier, disruption of the cell monolayer in the presence of SMG may be also due to a compromised ECM. Thus, we sought to measure the amount of secreted collagen produced by osteoblasts using Sirius red (stains collagen, predominantly I and III fibers). Histological/qualitative analyses, show a reduction in collagen staining in the SMG exposed samples (M) and significant recovery when stimulated with mLIPUS, MU (dark red patches of collagen distributed evenly in extracellular matrix, Fig. 4A). The application of mLIPUS on the GU group did not result in any significant increase in collagen secretion as compared to the control (G) (Fig. 4A). In addition, while all three cultures, G, GU and MU showed variable collagen density (with some areas of low and some denser patches of collagen), the M group cultures showed a weaker and overall a very low collagen density throughout the culture (Fig. 4A). Further, when we quantitatively measured the intensity of the staining in these cultures we detected a significant reduction (~22%, $p<0.05$) of collagen secretion in the presence of SMG in comparison to the control group (G) cells, (Fig. 4B). In contrast, mLIPUS stimulation reversed this trend and in fact, restored collagen levels to those observed with the G and GU groups (Fig. 4B). Specifically, mLIPUS stimulation decreased the loss of collagen in the presence of SMG by ~52% ($p<0.05$) (Fig. 4B).

mLIPUS exposure significantly reverses SMG-induced decreases in cytoskeletal F-actin stress fiber polymerization

Since we observed changes in collagen production, especially in the presence of SMG, we wanted to know if the cells' cytoskeleton was altered. Thus, we monitored F-actin filaments using immunofluorescence. While G group showed formation of dense F-actin stress fibers surrounding the nucleus, this was not the case with cells SMG (Fig. 5); these cells showed reduced F-actin stress fibers and in some cases no fibers at all. Exposure to mLIPUS had no effect on the overall density of F-actin stress fibers, but it did affect the orientation of F-actin fibers in the GU group cells (Fig. 5, GU). In contrast, mLIPUS did restore the formation of F-actin stress fibers in the SMG exposed samples (Fig. 5, MU). We further quantitatively measured these data by examining the average integrated density [(Area $\times$ Mean gray

value)/total number of nuclei] using Image J and the data showed a significant reduction ($\sim 146\%$, $p < 0.05$) in F-actin stress fibers in M cells that is recovered by mLIPUS stimulation (MU) to the levels of the control (G) (Fig. 5B). As expected, no significant changes were observed between the cells in G and GU groups (Fig. 5B).

mLIPUS exposure significantly reverses SMG-induced decreases in calcification

To further probe the positive effects of mLIPUS stimulation on SMG-exposed cells, we investigated matrix calcification using alizarin red staining. Results from these experiments showed the distribution of calcium phosphate in G cultures, but with mLIPUS stimulation, we observed a much denser and wider presence of stained patches of calcified matrix (Fig. 6A, GU). Alizarin red staining was also less evenly distributed in the M cultures with apparent areas of no staining, probably due to the reduced number of cells and collagen (Fig. 6A, M). This was again reversed in the presence of mLIPUS (MU) whereby the formation of calcium phosphate nodules resembled that seen with GU (Fig. 6A, GU). Further, when we quantitatively measured the concentration of the eluted dye, we found a $\sim 37\%$ ($p < 0.05$) decrease in matrix calcification in M as compared to G but with mLIPUS stimulation it was increased by $\sim 25\%$ ($p < 0.01$) in the M cultures. Although there was a slight increase ($\sim 16\%$) in the GU cultures in comparison to G, it was not statistically significant.

Discussion and Conclusion

The experiments, reported herein using the *I-D* clinostat to generate SMG, confirm previous *in vitro* experiments conducted in space and SMG conditions on the ground (Marie et al. 2000; Hughes-Fulford 2002; Hughes-Fulford 2003) that this experimental setup is capable of inducing SMG. Further, it enabled us to test the hypothesis that mLIPUS can serve as a countermeasure for microgravity induced bone loss.

Application of mLIPUS significantly restored the functional and morphological changes in human osteoblasts as induced by SMG. The observed disruption in the cell monolayer in the presence of SMG resulted from both, the reduction in the rate of proliferation and decreased ECMs leading to reduced cell adherence. Initially, we showed that SMG inhibited osteoblast proliferation which was fully restored in the presence of mLIPUS but not to the level of the control cells. It is well known that mechanical stimuli enhance the differentiation of osteoblasts, which can lead to a reduced rate of proliferation (Raucci et al. 2008). The same observation has been made by Pre et al (2009) with low-amplitude high frequency mechanical vibrations (Pre et al. 2009). In contrast, other studies have shown increased osteoblast proliferation during LIPUS exposure (Chen et al. 2007; Alvarenga et al. 2010). While Chen et al., used a signal of 50, 100 and 150 mW/cm² for 3 min per day, our study used 30mW/cm² for 20 min per day, and perhaps it is possible that the difference in intensity and time resulted in the observed changes in cell proliferation. Raucci et al (2008) have argued that the relative activation of the ERK and AKT pathways determines the tendency of osteoblasts towards differentiation or proliferation (Raucci et al. 2008). Specifically, high ERK expression induced osteoblast proliferation and increased expression of AKT enhances differentiation. Ultrasound stimulation is also known to upregulate the ERK1/2-MPAK pathways through activation of integrins (Hou et al. 2009; Papachroni et al. 2009; de Gusmao et al. 2010) and various studies showed activation of the AKT pathway after LIPUS exposure (Tang et al. 2007; Takeuchi et al. 2008b). Thus, it is possible that different intensities and time of treatment may affect different mechanotransductive pathways and furthermore it is still unclear how and if LIPUS regulates the synergy between ERK-MPAK and AKT pathways. Alvarenga et al (2010) also showed increased proliferation at day 5 and 7 in primary osteoblasts, when stimulated with LIPUS (30mw/cm², 20 min per day, 1.5 MHz), and as such it was speculated that the different cell types and frequency can potentially affect the cellular response to LIPUS. More in depth studies

are required to fully understand the exact LIPUS effects on rate of proliferation relative to different signal parameters.

SMG also reduced the amount of ColI in the ECM leading to decreased osteoblast adhesion and even induced cell death (Lynch et al. 1995). As this is consistent with our data where we showed that mLIPUS exposure restored the amount of ColI; the MU cultures showed areas with little or no staining, probably due to reduced number of cells as a result of apoptosis (but this was not experimentally verified). Interestingly, mLIPUS stimulation also increased ColI content in the GU cultures, indicating that the ultrasound signal is capable of stimulating ColI production, under normal conditions.

mLIPUS stimulation also increased the SMG-induced reduction in matrix mineralization. Specifically, the mLIPUS treated SMG samples showed a 25% increase in mineralization in comparison to the untreated M cultures, but this is significantly lower than the controls. Our mineralization study was conducted over 14 days and it is possible that extended exposure of mLIPUS can continue to increase mineralization in the gravity plus ultrasound samples. The mLIPUS-induced increase in mineralization in SMG conditions is probably due to an increase in the number of osteoblasts, higher content of collagen secretion and an overall increase in osteoblastic activity. This study extends the findings of other studies using LIPUS as anabolic stimuli in bone cells. For example, Suzuki et al (2009) showed that daily application of LIPUS for 14 days increased the expression of runx2, ALP, Dlx5, BMP-2 and osterix, as well as the matrix calcification in rat osteoblast cells. The anabolic effects of LIPUS were also reported in various animal models of bone fracture healing and ovariectomy (Cheung et al. 2011a; Cheung et al. 2011b). These studies, along with our data, suggest that LIPUS can potentially be used to reverse the detrimental effects of MG on the mammalian skeleton.

Lastly, we also showed that mLIPUS stimulation induced increased F-actin polymerization, which is known to play an critical role in both the tensegrity and mechanosome concepts of mechanotransduction (Huang and Ogawa 2010). Tensegrity is based on the transmission of applied forces on cell membrane to chromosomes resulting in chromosomal deformations and changes in transcription (Geiger and Bershadsky 2002). This is highly dependent on the continuous transfer of forces from the plasma membrane to chromosomes through intact connections of the cytoskeletal actin stress fibers to the plasma and nuclear membranes (Geiger and Bershadsky 2002; Ingber 2006). The mechanosome theory is based on localized changes on the plasma membrane, specifically, the induction of conformational changes on cell surface proteins leading to activation of different signaling pathways and resulting in transcriptional changes (Pavalko et al. 2003). Thus, actin plays an important role in the conduction of mechanical conformational changes of cell surface receptors to the activation of downstream signaling pathways. Further, it is also known that acoustic vibrations caused by LIPUS lead to conformational changes in integrins receptors and activate downstream mechanotransductive pathways (Li et al. 2008). Yang et al has shown increased integrin expression after ultrasound stimulation in osteoblast cells (Yang et al. 2005), thereby activating the ERK1/2-MAPK downstream pathways (Tang et al. 2006). Kook et al has also shown that the ERK pathway plays an important role in ColI expression (Kook et al. 2009). Since osteoblasts are known to mineralize bone matrix along collagen fibers (Landis 1996; Landis et al. 1996a; Landis et al. 1996b)), any decrease in collagen content will lead to a reduction in osteoblast adhesion to the ECM as well as a reduction in matrix mineralization. On the other hand, reduced numbers of F-actin stress fibers in MG can inhibit the integrin mediated ERK1/2 pathway along with a reduced rate of proliferation, ultimately, resulting in low ColI production and overall matrix mineralization (as observed with the SMG exposed samples). Furthermore, it would also be interesting to observe whether the structural properties of ColI and calcium phosphate crystals are altered in SMG conditions.

The objective of this study was to test the hypothesis that mLIPUS serves as an effective countermeasure for the detrimental effects of SMG on osteoblast activity and morphology. We found that application of mLIPUS significantly increased the rate of osteoblast proliferation and restored F-actin polymerization. Further, the collagen content was fully restored and mineralization showed significant increase with mLIPUS exposure relative to SMG cultures. Taken together, we conclude that mLIPUS can be successfully serve as a countermeasure to the detrimental effects of SMG on osteoblasts mLIPUS can potentially provide a non-invasive and targeted therapy for astronauts in space as ultrasound transducers or arrays can be designed such that they adhere to the region of interest (i.e. limbs and spine) and operated by rechargeable batteries. Regardless, further studies are required to completely understand the molecular mechanism(s) by which mLIPUS induces mechanotransduction in osteoblasts before it can be accepted as a “true” countermeasure for microgravity-induced bone loss.

Acknowledgments

This work is kindly supported by the National Space Biomedical Research Institute through NASA Cooperative Agreement NCC 9-58 (SMST01603, Qin), NIH (AR49286 and AR52379, Qin), and NYSTAR. The authors would like to sincerely thank Yunkai Huang and other members in the Orthopaedic Bioengineering Research Laboratory at Stony Brook University for their kind assistance and technical supports for the study.

References

- Alvarenga EC, Rodrigues R, Caricati-Neto A, Silva-Filho FC, Paredes-Gamero EJ, Ferreira AT. Low-intensity pulsed ultrasound-dependent osteoblast proliferation occurs by via activation of the P2Y receptor:role of the P2Y1 receptor. *Bone*. 2010; 46:355–62. [PubMed: 19781676]
- Binderman I, Shimshoni Z, Somjen D. Biochemical pathways involved in the translation of physical stimulus into biological message. *Calcif Tissue Int*. 1984; 36 (Suppl 1):S82–5. [PubMed: 6331615]
- Binderman I, Zor U, Kaye AM, Shimshoni Z, Harell A, Somjen D. The transduction of mechanical force into biochemical events in bone cells may involve activation of phospholipase A2. *Calcif Tissue Int*. 1988; 42:261–6. [PubMed: 2840180]
- Boyd JL, Holcomb JP, Rothenberg RJ. Physician treatment of osteoporosis in response to heel ultrasound bone mineral density reports. *J Clin Densitom*. 2002; 5:375–81. [PubMed: 12665638]
- Carvalho DC, Cliquet A Junior. The action of low-intensity pulsed ultrasound in bones of osteopenic rats. *Artif Organs*. 2004; 28:114–8. [PubMed: 14720297]
- Chapman IV, MacNally NA, Tucker S. Ultrasound-induced changes in rates of influx and efflux of potassium ions in rat thymocytes in vitro. *Ultrasound Med Biol*. 1980; 6:47–58. [PubMed: 6245493]
- Chen SH, Chiu CY, Yeh JM, Wang SH. Effect of low intensity ultrasounds on the growth of osteoblasts. *Conf Proc IEEE Eng Med Biol Soc*. 2007; 2007:5834–7. [PubMed: 18003340]
- Cheung WH, Chin WC, Qin L, Leung KS. Low intensity pulsed ultrasound enhances fracture healing in both ovariectomy-induced osteoporotic and age-matched normal bones. *J Orthop Res*. 2011a
- Cheung WH, Chow SK, Sun MH, Qin L, Leung KS. Low-intensity pulsed ultrasound accelerated callus formation, angiogenesis and callus remodeling in osteoporotic fracture healing. *Ultrasound Med Biol*. 2011b; 37:231–8. [PubMed: 21257088]
- de Gusmao CVB, Pauli JR, Saad MJA, Alves JM, Belangero WD. Low-intensity Ultrasound Increases FAK, ERK-1/2, and IRS-1 Expression of Intact Rat Bones in a Noncumulative Manner. *Clinical Orthopaedics and Related Research*. 2010; 468:1149–56. [PubMed: 19851814]
- Ferreri SL, Talish R, Trandafir T, Qin YX. Mitigation of bone loss with ultrasound induced dynamic mechanical signals in an OVX induced rat model of osteopenia. *Bone*. 2011
- Geiger B, Bershadsky A. Exploring the neighborhood: adhesion-coupled cell mechanosensors. *Cell*. 2002; 110:139–42. [PubMed: 12150922]
- Hoson T, Kamisaka S, Masuda Y, Yamashita M, Buchen B. Evaluation of the three-dimensional clinostat as a simulator of weightlessness. *Planta*. 1997; 203 (Suppl):S187–97. [PubMed: 9299798]

- Hou CH, Lin J, Huang SC, Hou SM, Tang CH. Ultrasound Stimulates NF-kappa B Activation and iNOS Expression Via the Ras/Raf/MEK/ERK Signaling Pathway in Cultured Preosteoblasts. *Journal of Cellular Physiology*. 2009; 220:196–203. [PubMed: 19288477]
- Huang C, Ogawa R. Mechanotransduction in bone repair and regeneration. *FASEB J*. 2010; 24:3625–32. [PubMed: 20505115]
- Hughes-Fulford M. Physiological effects of microgravity on osteoblast morphology and cell biology. *Adv Space Biol Med*. 2002; 8:129–57. [PubMed: 12951695]
- Hughes-Fulford M. Function of the cytoskeleton in gravisensing during spaceflight. *Adv Space Res*. 2003; 32:1585–93. [PubMed: 15002415]
- Hughes-Fulford M, Lewis ML. Effects of microgravity on osteoblast growth activation. *Exp Cell Res*. 1996; 224:103–9. [PubMed: 8612673]
- Hughes-Fulford M, Rodenacker K, Jutting U. Reduction of anabolic signals and alteration of osteoblast nuclear morphology in microgravity. *J Cell Biochem*. 2006; 99:435–49. [PubMed: 16619267]
- Hughes-Fulford M, Tjandrawinata R, Fitzgerald J, Gasuad K, Gilbertson V. Effects of microgravity on osteoblast growth. *Gravit Space Biol Bull*. 1998; 11:51–60. [PubMed: 11540639]
- Huijser R, Desktop RPM. New Small Size Microgravity Simulator for the Bioscience Laboratory. *Fokker Space*. 2000:1–5.
- Ingber DE. Cellular mechanotransduction: putting all the pieces together again. *FASEB J*. 2006; 20:811–27. [PubMed: 16675838]
- Kaneko T, Kimata H, Sugimoto H, Inoue S, Ito S, Ishiguchi T, Nakao A. Power Doppler ultrasonography for the assessment of vascular invasion by pancreatic cancer. *Pancreatol*. 2002; 2:61–8. [PubMed: 12120009]
- Kook SH, Hwang JM, Park JS, Kim EM, Heo JS, Jeon YM, Lee JC. Mechanical force induces type I collagen expression in human periodontal ligament fibroblasts through activation of ERK/JNK and AP-1. *J Cell Biochem*. 2009; 106:1060–7. [PubMed: 19206162]
- Landis WJ. Mineral characterization in calcifying tissues: atomic, molecular and macromolecular perspectives. *Connect Tissue Res*. 1996; 34:239–46. [PubMed: 9084632]
- Landis WJ, Hodgins KJ, Arena J, Song MJ, McEwen BF. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. *Microsc Res Tech*. 1996a; 33:192–202. [PubMed: 8845518]
- Landis WJ, Hodgins KJ, Song MJ, Arena J, Kiyonaga S, Marko M, Owen C, McEwen BF. Mineralization of collagen may occur on fibril surfaces: evidence from conventional and high-voltage electron microscopy and three-dimensional imaging. *J Struct Biol*. 1996b; 117:24–35. [PubMed: 8776885]
- Li J, Zhao Z, Wang J, Chen G, Yang J, Luo S. The role of extracellular matrix, integrins, and cytoskeleton in mechanotransduction of centrifugal loading. *Mol Cell Biochem*. 2008; 309:41–8. [PubMed: 18026855]
- Li JK, Chang WH, Lin JC, Ruaan RC, Liu HC, Sun JS. Cytokine release from osteoblasts in response to ultrasound stimulation. *Biomaterials*. 2003; 24:2379–85. [PubMed: 12699675]
- Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. *Exp Cell Res*. 1995; 216:35–45. [PubMed: 7813631]
- Marie PJ, Jones D, Vico L, Zallone A, Hinsenkamp M, Cancedda R. Osteobiology, strain, and microgravity: part I. Studies at the cellular level. *Calcif Tissue Int*. 2000; 67:2–9. [PubMed: 10908405]
- Nabavi N, Khandani A, Camirand A, Harrison RE. Effects of microgravity on osteoclast bone resorption and osteoblast cytoskeletal organization and adhesion. *Bone*. 49:965–74. [PubMed: 21839189]
- Naruse K, Miyauchi A, Itoman M, Mikuni-Takagaki Y. Distinct anabolic response of osteoblast to low-intensity pulsed ultrasound. *J Bone Miner Res*. 2003; 18:360–9. [PubMed: 12568414]

- Nolte PA, Klein-Nulend J, Albers GH, Marti RK, Semeins CM, Goei SW, Burger EH. Low-intensity ultrasound stimulates endochondral ossification in vitro. *J Orthop Res*. 2001; 19:301–7. [PubMed: 11347705]
- Papachroni KK, Karatzas DN, Papavassiliou KA, Basdra EK, Papavassiliou AG. Mechanotransduction in osteoblast regulation and bone disease. *Trends Mol Med*. 2009; 15:208–16. [PubMed: 19362057]
- Pavalko FM, Norvell SM, Burr DB, Turner CH, Duncan RL, Bidwell JP. A model for mechanotransduction in bone cells: the load-bearing mechanosomes. *J Cell Biochem*. 2003; 88:104–12. [PubMed: 12461779]
- Pre D, Ceccarelli G, Benedetti L, Magenes G, De Angelis MG. Effects of low-amplitude, high-frequency vibrations on proliferation and differentiation of SAOS-2 human osteogenic cell line. *Tissue Eng Part C Methods*. 2009; 15:669–79. [PubMed: 19257810]
- Qin Y-X. Challenges to the musculoskeleton during a journey to Mars: Assessment and Counter Measures. *Journal of Cosmology*. 2010; 12:3778–80.
- Rauci A, Bellosta P, Grassi R, Basilico C, Mansukhani A. Osteoblast proliferation or differentiation is regulated by relative strengths of opposing signaling pathways. *J Cell Physiol*. 2008; 215:442–51. [PubMed: 17960591]
- Rubin J, Biskobing D, Fan X, Rubin C, McLeod K, Taylor WR. Pressure regulates osteoclast formation and MCSF expression in marrow culture. *J Cell Physiol*. 1997; 170:81–7. [PubMed: 9012787]
- Schwarz RP, Goodwin TJ, Wolf DA. Cell culture for three-dimensional modeling in rotating-wall vessels: an application of simulated microgravity. *J Tissue Cult Methods*. 1992; 14:51–7. [PubMed: 11541102]
- Shapiro JR. Microgravity and drug effects on bone. *J Musculoskelet Neuronal Interact*. 2006; 6:322–3. [PubMed: 17185807]
- Sheyn D, Pelled G, Netanel D, Domany E, Gazit D. The effect of simulated microgravity on human mesenchymal stem cells cultured in an osteogenic differentiation system: a bioinformatics study. *Tissue Eng Part A*. 2010; 16:3403–12. [PubMed: 20807102]
- Takeuchi R, Ryo A, Komitsu N, Mikuni-Takagaki Y, Fukui A, Takagi Y, Shiraishi T, Morishita S, Yamazaki Y, Kumagai K, Aoki I, Saito T. Low-intensity pulsed ultrasound activates the phosphatidylinositol 3 kinase/Akt pathway and stimulates the growth of chondrocytes in three-dimensional cultures: a basic science study. *Arthritis Res Ther*. 2008a; 10:R77. [PubMed: 18616830]
- Takeuchi R, Ryo A, Komitsu N, Mikuni-Takagaki Y, Fukui A, Takagi Y, Shiraishi T, Morishita S, Yamazaki Y, Kumagai K, Aoki I, Saito T. Low-intensity pulsed ultrasound activates the phosphatidylinositol 3 kinase/Akt pathway and stimulates the growth of chondrocytes in three-dimensional cultures: a basic science study. *Arthritis Research & Therapy*. 2008b; 10
- Tang C, Fu W, Lu D, Tan T, Yang R. Ultrasound induces hypoxia-inducible factor-1 activation and iNOS expression through integrin/ILK/Akt pathway in cultured osteoblasts. *Journal of Bone and Mineral Research*. 2007; 22:S360-S.
- Tang CH, Yang RS, Huang TH, Lu DY, Chuang WJ, Huang TF, Fu WM. Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, focal adhesion kinase, phosphatidylinositol 3-kinase, and Akt pathway in osteoblasts. *Mol Pharmacol*. 2006; 69:2047–57. [PubMed: 16540596]
- Uddin SMZ, Cheng JQ, Lin W, Qin YX. Low-Intensity Amplitude Modulated Ultrasound Increases Osteoblastic Mineralization. *Cellular and Molecular Bioengineering*. 2011; 4:81–90.
- Van Loon JJ, Bervoets DJ, Burger EH, Dieudonne SC, Hagen JW, Semeins CM, Doulabi BZ, Veldhuijzen JP. Decreased mineralization and increased calcium release in isolated fetal mouse long bones under near weightlessness. *J Bone Miner Res*. 1995; 10:550–7. [PubMed: 7610925]
- Vico L, Hinsenkamp M, Jones D, Marie PJ, Zallone A, Cancedda R. Osteobiology, strain, and microgravity. Part II: studies at the tissue level. *Calcif Tissue Int*. 2001; 68:1–10. [PubMed: 12037617]

- Yang KH, Parvizi J, Wang SJ, Lewallen DG, Kinnick RR, Greenleaf JF, Bolander ME. Exposure to low-intensity ultrasound increases aggrecan gene expression in a rat femur fracture model. *J Orthop Res.* 1996; 14:802–9. [PubMed: 8893775]
- Yang RS, Lin WL, Chen YZ, Tang CH, Huang TH, Lu BY, Fu WM. Regulation by ultrasound treatment on the integrin expression and differentiation of osteoblasts. *Bone.* 2005; 36:276–83. [PubMed: 15780953]

B **Figure 1.**

Experimental setup to a SMG using *1-D Clinostat*. (A) Schematics explaining orientation of cells and gravity vector during rotation. (B) *1-D Clinostat*.

Figure 2. SMG induces gaps in cellular layer. Brightfield microscopy was conducted on Day 7 of primary osteoblasts in the presence of simulated microgravity (M) and Gravity (G) groups. Images clearly showed gaps within the cell monolayer of the M cultures (arrows) as compared to the G. Scale Bar = 100 μ m.

Figure 3. mLIPUS exposure significantly reverses SMG-induced decreases in Osteoblast proliferation

Osteoblast proliferation (n=4) has shown in the various conditions (G, GU, M, MU) and time (Day 2, 4 and 6). G cultures showed a steady rate of proliferation whereas SMG significantly reduces osteoblast proliferation. mLIPUS exposure increased proliferation rate in SMG (* $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Osteoblasts showed significant growth between day 2 to 4 and 6 ($p < 0.01$). SMG cultured cells showed no significant proliferation between 2–4 and 4–6 days ($p > 0.05$). mLIPUS stimulation also induced significant proliferation between 2–4 and 2–6 days ($p < 0.05$).

A

Figure 4. mLIPUS exposure significantly reverses SMG-induced decreases in Coll production
 (A) Sirius Red Staining: G samples showed evenly distributed Coll fibers. mLIPUS stimulation increased the stained intensity of Coll fibers in the ECM. Cell cultures in simulated microgravity group (M) showed minimal Coll fibers, whereas exposure of mLIPUS increases Coll staining. MU group samples showed uneven Coll distribution. Scale Bar = 500 μ m. (B) Quantitative measurement of Sirius Red stain as a function of Coll content: Significant decreased in Coll in SMG. mLIPUS treatment recovered Coll content in the ECM (* $p < 0.01$).

Figure 5. mLIPUS exposure significantly reverses SMG-induced decreases in F-actin polymerization

(A): F-Actin Stress fibers Polymerization: staining showing that the F-actin filaments were significantly reduced in simulated microgravity group (M) whereas Cells in G showed formation of thick and dense actin fibers. mLIPUS exposure increased polymerization of actin fibers (MU) . Scale Bar = 10 μ m (B) Quantification of stress fibers using Image J showed significant increase in F-actin polymerization in SMG when stimulated with mLIPUS (MU). (* $p < 0.01$)

Figure 6. mLIPUS exposure significantly reverses SMG-induced decreases in Osteoblast matrix calcification

Calcification of ECM –G control cultures showed evenly distributed calcified matrix and with mLIPUS stimulation denser patches (white arrows GU) are visible. In contrast, MG cultures showed a dispersed calcification pattern with areas of no calcification (black arrow M). mLIPUS exposure increased the area of calcified matrix and formation of denser calcium nodules (MU). Scale Bar = 500µm. (B) Quantification of ECM Calcification: SMG

induced osteoblast showed 47% less matrix calcification than G controls. mLIPUS restored matrix calcification significantly by 25%. (*p <0.05, **p<0.01). GU cultures showed positive calcification trends after 14 days of mLIPUS exposure.