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Concurrent heat and ultrasound treatment does not improve mineralization in bone cell cultures *in vitro*

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ABSTRACT

Several human clinical trials have shown Low Intensity Pulsed UltraSound (LIPUS) to improve bone fracture healing. *In vitro*, this improvement has been shown through improved mineralization in bone cells. Low level heating of bone (*in vivo*) and bone cell cultures (*in vitro*) has also been shown to improve mineralization. This study examines the effect of concurrent LIPUS and heating on MC3T3-E1 bone cell cultures. METHOD: The bone cells were split into four treatment groups: LIPUS, heating, LIPUS + heating, and control. The LIPUS treatment was delivered with the intensity of I_{SATA}=30 mW/cm^2 at the frequency of f=1.5 MHz for 40 minutes each day over 15 days. The heat treatment was applied at 40ºC for 40 minutes each day over 15 days. The LIPUS + heating group received the treatments concurrently. RESULTS: All treatment groups showed statistically significantly improved mineralization when compared to the control cell cultures. Although the LIPUS and LIPUS + heat groups each showed almost a 4 fold increase in mineralization over the control, there was no statistical difference in mineralization between these two groups. CONCLUSION: Early results suggest that concurrent heat and LIPUS exposures on MC3T3-E1 bone cells have no additive effect on mineralization.

INTRODUCTION

Low Intensity Pulsed UltraSound (LIPUS) has been shown to accelerate bone fracture healing. From 1983 to present there have been multiple *in vivo*, *in vitro* and clinical LIPUS studies[1]. There have been several phase-I clinical studies on the effects of LIPUS on bone healing, with up to 40% improvement in bone healing time for fresh fractures (tibia, radius and scaphoid) and up to 85% improvement in bone healing time in the case of non-unions [1-9]. According to Warden et al., LIPUS is now widely available to promote both fresh fracture and non-union bone healing [10].

In 1994 the first therapeutic LIPUS device was approved by the FDA for clinical use with fresh fractures (Exogen® Bone Healing System, Smith & Nephew Inc., Memphis, TN) [11,12]. Further, in 2000, the range of applications increased to include non-unions [12]. Typical LIPUS application is defined as 20 minutes of treatment per day with a 1.5 MHz sine wave ultrasound pulse with intensity (spatial average temporal average) of I_{SATA}=30 mW/cm^2 repeated at 1kHz with a pulse width of 200μs [1,4]. Due to the prevalence of the Exogen® device, these LIPUS settings are often used as standard treatment settings.

In their review article, Pounder and Harrison suggest that the increase in mechanical strength at the fracture site is due to accelerated mineralization of the fracture callus [4]. This has been well modeled in cell culture experiments [13-16]. With clinical LIPUS settings, Unsworth et al. demonstrated that after 10 days of daily ultrasound stimulation, MC3T3 –E1 mouse osteoblast cells had statistically significant increased mineralization when compared with the control [17]. In addition, they found that with the application of LIPUS the production of alkaline phosphotase (ALP) protein peaked at day 6, where as the control peaked at day 10, with LIPUS treated having statistically significantly greater production of ALP from day 6 onward.

Similar to LIPUS, low levels of heat seem to stimulate bone deposition after injury. Leon et al., while studying the *in vivo* temperature distribution in bone, found that after heating bone to 43°C for 45 minutes, treated 4 times over 21 days, the bone was denser [18]. The study found that the heat treated bone shows a significantly thicker callus. Evidence of improved mineralization was also apparent on a microscopic level. According to Flour et al., a temperature increase to 40°C for 24 hours did not significantly change the viability or proliferation of MC3T3, cells[19]. They suggests the critical temperature for cell culture viability and proliferation is between 42°C and 43°C above which cells will not be viable. Shui et al. tested human bone marrow stromal cells (BMSC) *in vitro* for the effect of heating on mineralization [20]. They found that cells heated for 39-41°C for one hour every 3rd day for 21 and cells heated at 39°C for 96 hours that were measured after 10 days of incubation both showed significant increases in calcium mineralization. Although there is not a large volume of research on the effects of low level heating on bone, the research that has been done indicates that increases in temperature of just a few degrees can significantly increase mineralization of both bone and bone cells.

At intensities in the LIPUS range, ultrasound-induced heat is insignificant and does not seem to be a mechanism of action for enhancing bone mineralization [21,22]. More recently Leskinen et al. [23] tested the effects of heat and ultrasound on an osteosarcoma cell line. The study looked at temporal average power ranging from 200 to 2000 mW (I_{SATA}=20-200 mW/cm^2, based on a transducer aperture diameter of 25mm) with frequency of 1.035 MHz, pulse repetition frequency of 1 kHz and duty cycle of 20%. Cell signaling associated with improved bone formation increased at temperatures above 48°C and ultrasound power above 400 mW. The heat and ultrasound treatments were not given concurrently. No examples of LIPUS and low level heat (above 37°C and below 42°C) given concurrently have been found in the literature review. Although concurrent application of low level heating and LIPUS has not been tested; the individual treatments seem to improve mineralization in cell cultures.

The hypothesis for this study is that the addition of LIPUS and low level heat will increase mineralization in bone cell cultures.

MATERIALS AND METHODS

The experimental protocol was developed in collaboration with the R&D department of Smith & Nephew Inc., Memphis, TN. For more details of the protocol, refer to Weidman (2010) [24].

LIPUS and Heating

Bench Mark Testing

The research version of a clinical LIPUS device was used in this study (Exogen® Bone Healing System, Smith & Nephew Inc., Memphis, TN). To establish that the cell line was behaving as previously, the cells were treated with the standard LIPUS treatment for 20 minutes. Two treatment groups were included in this experiment; Control (c) which received no treatment and LIPUS 20 which received 20 minutes of treatment.

LIPUS and Heating

For the concurrent treatment, LIPUS was delivered with the intensity of I_{SATA}=30 mW/cm^2 with an effective radiating area of 3.88 cm^2 at the frequency of f=1.5 MHz for 40 minutes (LIPUS 40). The heat treatment was applied at 40ºC for 40 minutes (H 40). Outside of treatment all groups were kept at 37°C with 5% CO_2 concentration.

Four Treatment groups were included in this study: control (C), LIPUS 40, LIPUS 40 + H 40, and H 40. All treatment groups were grown on polystyrene 6 well plates with a well diameter of 3.5 cm. All cells cultures were treated in a 7-day cycle with 5 days of treatment and 2 days off. Samples were taken on days 5, 10 and 15. The experiment was repeated 3 times to account for possible effects due to variations in seeding and cell passage number. The cells samples were taken from passages 4, 5 and 6. Samples were taken out of treatment groups on day 5 of the cycle.

All wells on the 6 well plate were treated simultaneously and driven by the same power source. For the concurrent treatment (LIPUS 40 + H 40), the incubator and water temperature were increased to 40.5 ±0.5 °C prior to treatment; otherwise the set up was left the same as for LIPUS 40. For H 40 the LIPUS device was disconnected from the power source and the incubator and water temperature were increased to 40.5 ±0.5 °C prior to treatment. The control cell culture group remained in the holding incubator.

The schematic in Figure 1 illustrates the experimental set up. The transducer was placed 13 mm below the cell culture well and coupled to the cell culture well using 37°C water. The cell plate was held in place with a fixture above transducer, so that the bottom of the cell plate was always in contact with the water. The water tank was kept inside an incubator to maintain water temperature.

Cell Culture Technique

The cells were cultured in an ascorbic acid free Minimum Essential Medium Alpha (Gibco® by Invitrogen Carlsbad, California) supplemented with 10% Fetal Bovine Serum and 1% antibiotics. The cells were seeded at approximately 10^5 cells/ml. At the seeding stage, 50µg/ml of ascorbic acid and 3mM/ml of β-glycerol phosphate were added to the cell culture media as sources of nutrients to the cells. A total of 2ml of media was added to each well. In all experiments cells were seeded 72 hours prior to treatment. This allowed the cells time to proliferate, adhere to the well plate surface.

Staining for Mineralization

To prepare the cell culture samples for mineralization, the media was removed from the wells, the cultures were washed 3 times with CaCl2- and MgCl2-free PBS. The culture was then fixed by adding 1ml of 10% formalin at room temperature (20°C) (Sigma Aldrich Inc., Oakville, Ontario) to each well. Once fixed, the wells were rinsed and then stained with 1ml of 1 mg/ml Alizarin red (pH 4.2). The cultures were incubated at room temperature for 20 minutes at 20°C. The cultures were then rinsed 3 more times. The fixed and stained cell cultures were then left to dry for 24 hours.

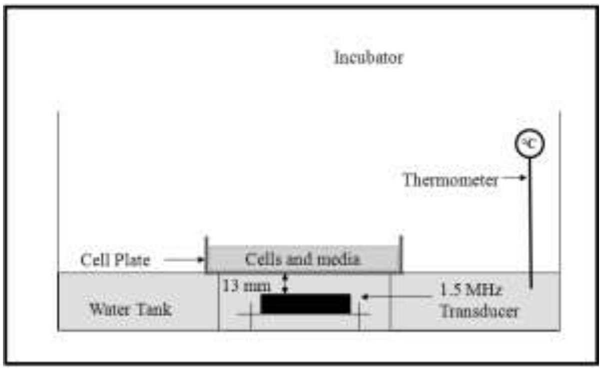

Figure 1: Experimental set up.

To quantify mineralization, the cell cultures were de-stained by adding 1 ml of room temperature 5% perchloric acid to each well. The perchloric acid rehydrated and dissolved the culture stain for 23hours. After 23 hours of incubation at room temperature, five samples of the dissolved stain were taken from each well to measure optical absorbance.

To quantify the degree of staining, the 96 well plate was put through a Thermo Lab Systems Multiskan Ascent plate reader with Ascent software (Thermo Fischer, Franklin, MA) to measure absorbance. Absorbance for each well was read at 405 nm. The average of 5 mini-wells was considered the absorbance for that sample.

Statistics

The samples were compared to the control treatment using a single sided student's t-test.

RESULTS

Bench Mark Testing

When initially testing LIPUS 20 treatment against the control, the results indicated statistically significant differentiation by day 10 (see Table 1).

Although these results are similar to previously published data [17], the cell culture mineralization was weak. To improve mineralization, the LIPUS treatment time was increased from 20 to 40 minutes.

Combined Treatment Effects

Using LIPUS 40 and H 40, by the fifth day after treatment, all cell groups showed significant mineralization when measured against day 0 cells (see Figure 2). The greater degree of mineralization suggests that the cells have begun the cycle of differentiation [25]. This occurred in all cell culture treatment groups over all three trials.

Table 1. The statistical treatment effect for LIPUS 20 treatment. The P value is the probability that the mean mineralization of the treatment is greater than the control. Statistical difference reached by day 10. $P < 0.05$ is considered statistically significant.

	P values
Day 5	0.0669
Day 10	0.0074
Day 15	0.0022

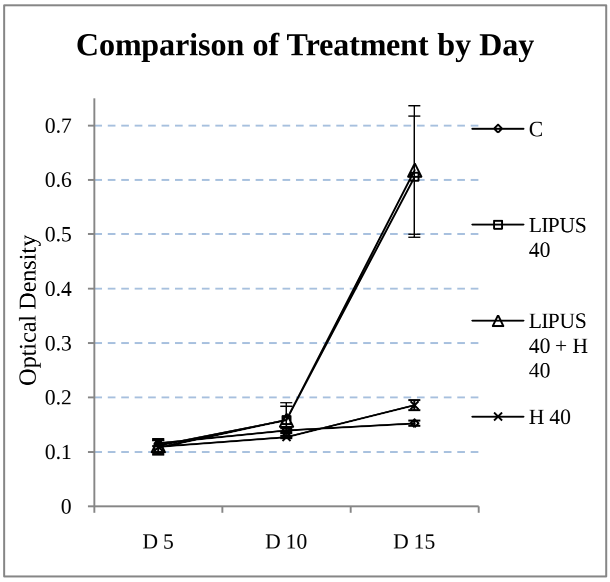

Figure 2. Comparison of treatment groups by treatment day. All samples are differentiation supplement positive. Error bar indicates a standard error of 18 measurements.

By day 15, the mean optical absorbance of LIPUS 40 and LIPUS 40 + H 40 has increased almost 6 fold over the Control and H 40 samples (see Table 2). H 40 showed an increase in mineralization of 1.2 fold over the Control, which is comparable to published values [26]. The results indicate that LIPUS 40, LIPUS 40 + H 40, and H 40 treatment groups all show statistically significantly improved mineralization when compared to the Control (see Table 3). The error bars for the LIPUS 40 and LIPUS 40 + H 40 treatment groups are much larger than the error for the H 40 and the Control treatment groups. In addition, there was no statistically significant difference in mineralization between the LIPUS 40 and the LIPUS 40 + H 40 treatments.

Table 2. Mean optical absorbance of treatment groups.

	Control	LIPUS 40	LIPUS 40 + H 40	H 40
Day 5	0.116	0.107	0.111	0.109
Day 10	0.140	0.159	0.158	0.127
Day 15	0.153	0.606	0.618	0.186

When the treatments are compared within each group, it is clear that there is an increase in mineralization over time (see Figure 3). Both of the LIPUS 40 and the LIPUS 40 + H 40 treatment groups showed distinct mineralization between days 10 and 15. This trend indicates that mineralization seems to begin in this window of time.

Table 3. Treatment effect statistics – P values. The P value is the probability that the mean mineralization of the treatment is greater than the control. All day 5 measurements are statistically significantly greater than day 0 ($P=0.0001$). $P<0.05$ is statistically significant.

	LIPUS 40	LIPUS 40 + H 40	H 40
Day 5	0.0554	0.3019	0.13
Day 10	0.457	0.567	0.0034
Day 15	0.0003	0.0004	0.0031

DISCUSSION

Many adjuvant therapies have been tested with ultrasound; however the combination of low level heating and LIPUS has not been studied. The addition of heat to ultrasound is potentially a low cost and non-invasive technique to improve fracture healing. From practical point of view, combining the two therapies would be quite attractive since at the interface between bone and soft tissue, the ultrasound alone can be used as a non-invasive local heat source. The importance of the individual and combined therapies is that they reduce the time for fractures to heal and increase the functional properties of bone. Both early healing and improved bone function are associated with mineralization.

The results of the experiment showed that there was a 6 fold increase for the LIPUS 40 treatment group when compared to the control. Based on published data, the result for the LIPUS 40 was expected. Leung et al. showed a 4 fold increase in mineralization after 4 weeks of ultrasound treatment when using human periosteal cells [27]. The H 40 treatment group also showed an expected increase of 1.2 fold in mineralization over the control. Shui et al, using an osteosarcoma derived cell line, showed an increase in mineralization of 1.25 fold when the cell cultures were heated to 39°C and 1.69 fold when the cell line were heated to 41°C [20]. An additive effect for the LIPUS 40 + H 40 group might be expected to be in the range of a 4.2 fold increase in mineralization. However, the LIPUS 40 + H 40 showed only a 4% increase over the LIPUS40 treatment group. Due to the large variation of mineralization in the samples, this increase was not statistically significant. Therefore the outcome of our study shows no additive effect in the combined treatment group.

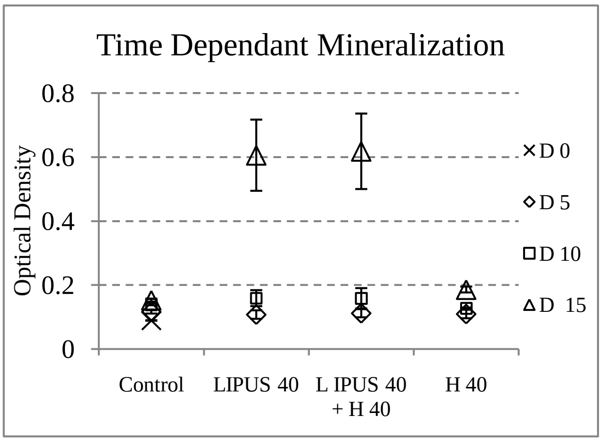

Figure 3. Comparison of treatments over time. Error bar indicates a standard error of 18 measurements.

There are a couple of possibilities to explain why there was no additive effect found for the LIPUS 40 + H 40 treatment group. It is possible that the mechanisms of action of each treatment may have different onset timing, the mechanisms of action of the treatments may not complement each other, and finally the test method may not be sensitive enough to detect a difference between the treatment groups.

Although the exact mechanisms are unknown, certain cellular level responses to ultrasound treatment have been shown to be repeatable. Increased mineralization is a distinct repeatable outcome from the application of ultrasound [4]. The mechanisms of action for ultrasound are thought to be the mechano-sensitization of cell integrins. According to Pounder et al. surface integrins mediate the mechanical signal on the cell surface and cause a cascade of changes throughout the cell [4]. Integrins are a large family of cell adhesion molecules that mediate interactions between the extracellular environment and the cytoplasm [28]. These integrins provide a physical link between the cytoskeleton and the extracellular matrix. According to Tang et al. [29], these integrins are stimulated by the ultrasound signal from the surrounding matrix, and this stimulation causes the integrins to start a cascade of change in the cell causing a series of subsequent expressions eventually causing the cells to express calcium and the collagen matrix to mineralize. The mechano-sensitive integrins stimulation caused by the ultrasound waves is theorized to be the mechanism behind ultrasound-cell interaction [29,30].

Although there are multiple examples of the temperature dependence of bone growth, the mechanisms of action are even more elusive than ultrasound. Shui and Scutt suggest that most likely the mechanism of action is related to the expression of Heat Shock Proteins (HSP); where HSP are molecular chaperones associated with cell survival after an insult [20]. Shui suggests that HSP47 is involved with collagen synthesis and the expression of HSP47 is more likely to be induced in the presence of Transforming Growth Factor (TGF-β1), where TGF-β1 is released by the addition of heat. According to Naruse et al., LIPUS does not stimulate the expression of TGF- β1 in MC3T3 cells [31]. However, ultrasound does stimulate this growth factor in other cell lines or at higher intensities [32,33]. Calderwood and Asea [34] suggest that when cells are exposed to temperatures over 40°C the production or Cyclo-oxygenase 2 (COX-2) and prostaglandin (PGE2) will increase.

The combination of LIPUS 40 + H 40 concurrently may prove not to be additive. Although heat induces HSP and ultrasound induces mechano-sensitivity, both energy sources have a downstream effect of increasing COX-2 and PGE2. It is possible that these expressions are maximized with one energy source and cannot be expressed more with the addition of a second source.

It is also possible that the additive effect of LIPUS 40 + H 40 was missed simply because the testing was not sensitive enough. From day 15 measurements, the standard error in light absorbance of the LIPUS 40 and LIPUS 40 + H 40 treatment groups is 0.1 with an average absorbance of 0.6. H 40 treatment produced an error 10 times smaller than either of LIPUS 40 or LIPUS 40 + H 40. With an error of 0.01 and an average absorbance of approximately 0.2, the error of both LIPUS groups is almost as large as the total absorbance of the H 40 group.

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