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Low-intensity ultrasound stimulates endochondral ossification \textit{in vitro}

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Summary

Background Animal and clinical studies have shown an acceleration of bone healing by the application of low-intensity ultrasound. The objective of this study was to examine in vitro the influence of low-intensity ultrasound on endochondral ossification of 17-day-old fetal mouse metatarsal rudiments.

Methods Forty-six triplets of paired metatarsal rudiments were resected ‘en block’ and cultured for 7 days with and without low-intensity ultrasound stimulation (30 mW/cm²). At days 1, 3, 5, and 7, the total length of the metatarsal rudiments, as well as the length of the calcified diaphysis were measured. Histology of the tissue was performed to examine its vitality.

Results The increase in length of the calcified diaphysis during 7 days of culture was significantly higher in the ultrasound-treated rudiments compared to the untreated controls (p=0.006). The growth of the control diaphysis was 180 ± 30 µm (mean ± SEM), while the growth of the ultrasound-treated diaphysis was 530 ± 120 µm. The total length of the metatarsal rudiments was not affected by ultrasound treatment. Histology revealed a healthy condition of both ultrasound-treated and control rudiments.

Conclusions In conclusion, low-intensity ultrasound treatment stimulated endochondral ossification of fetal mouse metatarsal rudiments. This might be due to stimulation of activity and/or differentiation of osteoblasts and hypertrophic chondrocytes. Our results support the hypothesis that low-intensity ultrasound activates ossification via a direct effect on osteoblasts and ossifying cartilage.
Introduction

Low-intensity, high-frequency ultrasound is in clinical use for enhancement of fracture healing. Animal studies have shown stimulation of callus tissue and acceleration of bone healing by low-intensity ultrasound. Scintigraphic control of the bone-healing process has shown faster healing in ultrasonically treated animals compared to untreated control animals. In fresh human diaphyseal tibia fractures the application of ultrasound accelerates consolidation of the fractures by 40%. In addition, the time needed for bony healing in fresh fractures of the distal radial metaphysis was decreased leading to diminished loss of reduction in the ultrasound-treated patients. Both studies were prospective, randomized, double-blinded, placebo-controlled clinical trials. The first results of a similar clinical study on the effect of low-intensity ultrasound in osteotomies of the lower extremities have shown an acceleration of bone healing. The stimulatory mechanism of ultrasound on callus tissue healing and bone formation has been shown to be based on non-thermal effects. Faster bone healing as a result of ultrasound treatment may be mediated via increased production of prostaglandin E₂. In a rat femur fracture model, exposure to ultrasound increased the mechanical properties of callus tissue, and stimulated early aggrecan mRNA and procollagen mRNA levels. An in vitro study with mesenchymal cell cultures showed that low-intensity pulsed ultrasound stimulated calcium incorporation. Ultrasound exposure of MC3T3 osteoblastic cells decreased PTH-stimulated adenylate cyclase activity and TGF-β synthesis. The effect of low-intensity ultrasound (30 mW/cm²) on endochondral ossification in vitro has not yet been examined. Fetal mouse long bone rudiments have been used earlier as an in vitro model to test the effect of higher intensity ultrasound (100 mW – 770 mW/cm²), which produces heat.

In the present study, we used this in vitro model of fetal mouse long bone rudiments to examine the effect of low-intensity ultrasound on bone growth and events occurring during fracture healing. The model is extremely suited for these studies because it represents two phases of endochondral ossification. First, cartilage ossification, and second, bone collar formation occur in this model during organ culture. The model shows the process of cartilage calcification in the center of the rudiment and formation of a bone collar around it. Calcification occurs at a reduced rate compared to in utero conditions. Calcification of the fetal long bone rudiments is visible as a dark spot in the center of the rudiment. Its length can be measured during organ culture, and is indicative for the area of tissue calcification. We hypothesize that the ossification process is modulated by ultrasound’s direct effect on calcifying chondrocytes and osteoblasts. Low-intensity ultrasound produces micromechanical forces, which, although the exact mechanism is still unknown, mimic the forces on bone applied by physical loading according to Wolff’s law.
**Methods**

**Tissue culture**

Institutional review board approval was obtained before use of animal subjects. A total of four experiments were performed, with five or six paired metatarsal rudiments per experiment. Sixty-nine paired cartilaginous metatarsal bone rudiments were dissected under sterile conditions from 17-day-old fetal mice. The 2nd, 3rd, and 4th metatarsal together with the 5th metatarsal or 4th phalange for orientation were taken out without disrupting the interpositioned tissue. Rudiments from each embryo were paired, so each animal served as its own control. The metatarsals were cultured in fluid culture medium within a standardized environment. This culture medium consisted of alpha minimum essential medium (alphaMEM) without nucleosides, supplemented with 0.3% fetal bovine serum (FBS), 0.6 mM L-ascorbic acid, 1.25 μg/ml fungizone, 50 μg/ml gentamicine, and 1 mM β-glycerophosphate, and put into six well culture dishes or petri dishes with the metatarsals of one foot in each well. β-glycerophosphate was added at 1 mM to enhance calcification. The culture plates were placed in a humidified incubator (5% CO₂ in air) at 37°C for 7 days. The medium was not renewed during the investigation.

**Ultrasound treatment**

After 24 h of preculture, low-intensity (30 mW/cm²) ultrasonic treatment in a therapy unit (Figure 1) was started for 20 min a day, for a total period of 6 days. The therapy unit consisted of two sonic accelerated fracture healing system (SAFHS®) devices (model 2A; Exogen) and transducers (with coupling gel) connected to a multiwell tissue culture plate filled with 2.54 ml standard tissue culture medium. This amount of culture medium leads to liquid height equivalent to one-quarter of the carrier frequency wavelength. The SAFHS® device provides pulsed ultrasound with a carrier frequency of 1.5 MHz. This setting was used in earlier experiments by Exogen Inc. and has proven to provide ultrasonic waves in the medium. The multiwell tissue culture plate consisting of six wells contained the metatarsal rudiments. The distance between transducer and metatarsal was smaller than 2 mm. The controls were kept under identical conditions but without the ultrasonic stimulation.

**Bone development**

After 24 h of preculture at day 1 (start of treatment), and at days 3, 5, and 7 after the daily ultrasonic treatment the total length and width of the metatarsal rudiments and the length and width of the calcification zone were measured with a linear eye piece micrometer (Zeiss) at 40x magnification.
Histology

Histology of the tissue was performed to examine the vitality of the tissue. A few random cultures were fixed in 4% phosphate-buffered formalin overnight at 4°C. Some cultures were dehydrated and embedded in glycol methacrylate (GMA). From these cultures 3 μm sections were made using an ultracut microtome with a glass knife. These sections were stained with 0.1% toluidine blue and evaluated at 400x magnification using a Zeiss microscope for general morphology and evaluation of resorption. To examine calcification, some other cultures were fixed and embedded in paraffin, and 5 μm sections stained with alizarin red.

Statistical analysis

Statistical analysis of the data was performed using the Student’s paired t-test. Data is expressed as mean ± SEM. A $p$-value of $<0.05$ is considered significant.
Results

All triplets in this experiment showed a gross appearance of growth and calcification within 7 days. Figure 2 shows the diaphyses of randomly chosen paired triplets of metatarsal rudiments without (A) and with (B) stimulation by low-intensity ultrasound at day 3. Each rudiment shows an ossifying zone, consisting of a center of calcified cartilage plus surrounding bony collar, bordered proximally and distally by a zone of cartilage hypertrophy. At day 3 of culture, ultrasound-treated metatarsal rudiments showed some 20% increase in the length of the calcified diaphysis in comparison with non-treated controls.

The diaphyses of another set of paired triplets of metatarsal rudiments at day 7 are shown in Figure 3. The diaphysis of the ultrasound-stimulated rudiments are more clearly

Figure 2. Metatarsal rudiments without (A) and with (B) ultrasound stimulation at day 3: detail of calcified diaphysis. The calcified tissue, consisting largely of calcified hypertrophic cartilage, can be seen as the black spot in the center. Original magnification: x 40. Bar = 20 μm.

Figure 3. Metatarsal rudiments cultured without (A) and with (B) ultrasound stimulation for 7 days: detail of calcified diaphysis. Note that the area of calcification is now sharply delineated by a calcified bone collar (arrows). Original magnification: x 40. Bar = 20 μm.
Figure 4. Effect of low intensity ultrasound on the length of the calcified diaphysis (A) and the total length (B) of the metatarsal rudiments during 7 days of culture. Values are means ± SEM of 69 metatarsal rudiments. *P < 0.05; significant effect of low intensity ultrasound compared to controls.

demarcated, due to newly formed bone. The length of the calcified diaphysis with ultrasonic stimulation was increased by 30% in comparison with controls after 7 days of culture. Low-intensity ultrasound increased the length of the calcified diaphysis of the metatarsal rudiments during 7 days of culture (Figure 4A). The stimulatory effect was already visible after 3 days of ultrasound treatment, continued during the entire treatment period, and reached a maximum after 7 days. The total length of the metatarsal rudiments with and without ultrasound stimulation increased 30% during the entire culture period of 7 days. However, the growth in length of the whole metatarsal rudiments was not changed by ultrasound stimulation (Figure 4B).

The increase in length of the calcified diaphysis from day 1 to day 7 amounted to 194% in the ultrasound-treated metatarsal triplets compared to untreated controls (Figure 5A). The

Figure 5. Increase in length of calcified diaphysis (A) and total length (B) after 7 days of culture. Values are means ± SEM of 69 metatarsal rudiments. *P = 0.006; significant effect of low intensity ultrasound compared to controls.
Figure 6. Histology of metatarsal rudiments with and without ultrasound stimulation after 7 days of culture. Details of the rudiment without ultrasound (A) and after 7 days of stimulation by low-intensity ultrasound (B) are shown. The hypertrophic cartilage cells, with a matrix that has calcified during culture (black arrows), appear quite healthy. A thin bony collar (BC) has been formed around the rudiment which is clearly visible in the ultrasound stimulated rudiment. HC = hypertrophic chondrocytes; OB = osteoblasts; P = periosteum (toluidine blue stained, original magnification x 320); bar = 5 μm.
increase in total length of the metatarsal rudiments from day 1 to day 7 was similar between the ultrasound-stimulated and the control metatarsal rudiments (Figure 5B). No gross effects on the width of the calcified diaphysis, the total width of the metatarsal rudiment or the hypertrophic zone were observed in the control and ultrasound treated rudiments (data not shown).

Toluidine blue-stained plastic sections of metatarsal rudiments without and with ultrasonic stimulation revealed a healthy appearance of the cultured rudiments (Figure 6A,B). Hypertrophic chondrocytes appear as large round cells surrounded by a matrix that has calcified during culture in both ultrasound-treated and control rudiments (Figure 6A,B). The calcified matrix of the hypertrophic cartilage zone seemed more pronounced in the ultrasound-stimulated rudiments than in the controls (Figure 6A,B). A thin bony collar has been formed during culture around the central diaphysis of the rudiment, and seemed more pronounced in the ultrasound-stimulated metatarsal rudiment than in the control rudiment. Many osteoblasts are visible as plump cells lining the bone collar in both control and ultrasound-stimulated rudiments (Figure 6A,B).

Discussion

There is growing evidence that low-intensity ultrasound has an accelerating effect on bone healing. Clinical application of ultrasound using the SAFHS® device is performed worldwide. However, the direct effects of ultrasound treatment on endochondral ossification are still incompletely understood. To examine these effects of the SAFHS® device, we used an in vitro model of endochondral ossification. This in vitro model, consisting of cartilaginous long bone rudiments with a calcifying center, is very suitable for examining the short-term effects of low-intensity ultrasound, since the calcified diaphysis of the metatarsal rudiments can be measured daily. The present study demonstrates that low-intensity pulsed ultrasound applied 20 min daily to mouse metatarsal rudiments in vitro exerts a stimulating effect on the length of the calcified diaphysis within a few days. Both the bony collar and the area of calcified hypertrophic cartilage were increased by low-intensity ultrasound treatment. This stimulatory effect on the bony collar might be due to either increased activity of osteoblasts, or to an increased number of osteoblasts. Other studies are currently underway to address this issue.

The endochondral ossification in vitro model that we used has previously shown stimulation of endochondral ossification by intermittent compressive force.4,5 This effect was similar to the effect we observed in the present study using low-intensity ultrasound. Both intermittent compressive force and ultrasound did not change the total length of the rudiments, indicating that there was no effect on cell (chondrocytes) proliferation by both treatments.
Low-intensity pulsed ultrasound in vitro did not change the total length or the length of the hypertrophic zone of the metatarsal rudiments. This suggests that ultrasound affected only the hypertrophic cartilage cells which produce the calcified matrix, as well as the osteoblasts, which produce the bone collar, but did not affect the non-hypertrophic cartilage cells. An in vivo study using rats has shown that low-intensity ultrasound did not stimulate longitudinal growth of the femur. This observation is in agreement with our results, also showing that low-intensity ultrasound does not change the longitudinal growth of embryonic long bones. Higher intensity pulsed ultrasound (100 mW - 770 mW/cm²) has been shown to increase the length of the proliferative zone of metatarsal rudiments without changing the length of the hypertrophic cartilage zone. In the present study using low-intensity ultrasound, we did not observe an effect on the length of the proliferative zone. Other in vitro studies on the effects of low-intensity ultrasound have shown a stimulatory effect on chondrocyte activity, as well as on bone cell activity. Our in vitro study indicates a direct effect of low-intensity ultrasound on chondrocyte activity. The previous study used cell cultures, whereas in our study we used intact bone organ tissue, through which we determined that the increased calcification of the diaphysis of the rudiments by low-intensity ultrasound suggests that low-intensity ultrasound enhances the physiological process of calcification.

The influence of ultrasound on second messenger activity in rat chondrocyte cultures has been demonstrated using fluorescent markers. Low-intensity ultrasound (0.5 W/cm²) induced a real time increase in intracellular calcium. Stimulation of aggrecan gene expression by low-intensity ultrasound was demonstrated in both animal experiments and in in vitro experiments. Therapeutic ultrasound at an intensity of 0.1 W/cm² stimulates in vitro collagen and non-collagenous protein synthesis, whereas higher intensities (1.0 - 2.0 W/cm²) inhibit the protein synthesis in a mouse calvarial bone organ culture system. These intracellular stimulatory effects of low-intensity ultrasound, as stated above, are a non-thermal effect. These studies mentioned above on cellular effects on chondrocytes show increased matrix production after low-intensity ultrasound treatment. This is in agreement with our findings, i.e. increased endochondral ossification of metatarsal rudiments after low-intensity ultrasound treatment.

In summary, low-intensity ultrasound as used in this study has a direct effect on chondrocytes and/or osteoblasts. A possible effect of ultrasound on vascularization, shown in a dog ulnar osteotomy model, could not be tested since vascularization is excluded using our model. Our study showed that calcification, rather than overall growth is stimulated by low-intensity ultrasound. Abnormal calcifications were not observed in our in vitro experiment. The histology of both stimulated and non-stimulated rudiments showed tissue vitality. The effect on calcification is dependent on the intensity of ultrasound applied, as
showed in earlier experiments by Wiltink et al. The intensity we used in this study was only 10% of the lowest intensity as used in the study by Wiltink et al. Finally, our in vitro study supports and provides a partial explanation for the clinical experiments using ultrasound, which have shown a stimulating effect of ultrasound on fracture healing. Of course, in vivo, other factors like vascularization, hormones, growth factors, etc., also affect the healing of a fractured bone.

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References


