ENDOCHONDRAL CALCIFICATION IS STIMULATED BY LOW-INTENSITY ULTRASOUND IN CULTURED MOUSE METATARSAL RUDIMENTS

Introduction

Animal studies have shown an acceleration of bone healing and more callus formation by applying low-intensity ultrasound. In fresh human diaphyseal tibia fractures and metaphyseal distal radius fractures the application of ultrasound accelerates consolidation of the fractures by 40%. Both studies are prospective, randomized, double-blinded, placebo-controlled clinical trials. The objective of this study was to examine in vitro the influence of low-intensity ultrasound on endochondral calcification in 17-days-old fetal mice metatarsal rudiments. We hypothesize that the calcification process is modulated by ultrasound’s direct effect on calcifying chondrocytes and osteoblasts.

Materials and Methods

Tissue culture

Institutional review board approval was obtained before use of animal subjects. From 17-days-old fetal mice, paired cartilaginous metatarsal bone rudiments were dissected under sterile conditions. 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 that 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 (αMEM) without nucleosides, supplemented with 0.3% fetal bovine serum (FBS), 0.6 mM L-ascorbic acid, 1.25 µg/ml fungizone, 50 µg/ml gentamycin, 100 U/ml penicillin, 50 µg/ml streptomycin sulphate, and 1 mM β-glycerophosphate, and put into 6 well culture dishes with the metatarsals of one foot per well. The culture plates were placed in a humidified incubator with 5% CO₂ in air at 37°C for 7 days. The medium is not renewed during the investigation. Four experiments were performed, using twenty-three triplets of paired bone rudiments.

Ultrasound treatment

After 24 hours of preculture, low-intensity (30 mW/cm²) ultrasonic treatment in a therapy unit was started for 20 min/day during 6 days. The therapy unit consisted of two sonic accelerated fracture healing system (SAFHS® devices (model 2A; Exogen Inc.) and transducer (with coupling gel) connected to the 6-well culture dish containing the rudiments in a total of 2.54 ml standard tissue culture medium (leaving to a liquid height equivalent to ¼ of the carrier frequency wavelength). The SAFHS® device provided pulsed ultrasound at 1.5 MHz frequency. The distance between transducer and metatarsal was 1 mm. The controls were kept under identical conditions but without the ultrasonic stimulation.

Bone development

After 24 h of preculture (day 1, start of treatment), and at days 3, 5, and 7 after ultrasonic treatment the total length and the length of the calcified diaphysis were measured using a microscope with a linear eye piece micrometer at ×40 magnification.

Histology

Histology of the tissue was performed to show qualitatively the vitality of the tissue. Some cultures were fixed in 4% phosphate-buffered formalin, and embedded in glycol methacrylate. Then 3 µm sections were made using an ultracut microtome with a glass knife. Sections were stained with 0.1% toluidin blue and evaluated at 400x magnification using a microscope for general morphology.

Statistical analysis

Statistical analysis of the data was performed using Student’s paired t-test. A p-value of <0.05 was considered significant.

Results

Ultrasound treatment significantly increased the length of the calcified diaphysis from day 3 to day 7 (p< 0.05) (figure 1). The increase in length from day 1 to day 7 was significantly higher in the ultrasound-treated rudiments than in the untreated controls (p< 0.006 (control rudiments, Δ = 180 ± 30 µm (mean ± SEM); ultrasound-treated rudiments, Δ = 530 ± 120 µm)). The total length was not affected by ultrasound treatment. Histologically both ultrasound and control rudiments showed a healthy aspect. Gross histological examination revealed a more pronounced bone collar as well as calcified hypertrophic cartilage in the ultrasound-treated rudiments compared to the untreated controls. Abnormal tissue calcifications were not observed.

Discussion

This study shows for the first time that low-intensity ultrasound stimulates in vitro endochondral calcification of fetal mouse metatarsal rudiments. Values are means ± SEM of 69 metatarsal rudiments.

*Significant effect of ultrasound, p<0.05.

Figure 1. Effect of ultrasound on calcification of the diaphysis in fetal mouse metatarsal rudiments. Values are means ± SEM of 69 metatarsal rudiments.

***Dept Orthopaedics, Hospital Hilversum, Hilversum, The Netherlands.