Low-intensity pulsed ultrasound accelerates bone maturation in distraction osteogenesis in rabbits

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We investigated the effects of low-intensity pulsed ultrasound on distraction osteogenesis in a rabbit model.

Callotasis of the right tibia was performed in 70 male Japanese white rabbits using mini-external fixators. In the first part of the study in 64 animals using normal distraction (waiting period seven days; distraction rate 0.5 mm/12 hours; distraction period ten days), we evaluated the distraction site by radiography, measurement of the bone mineral density (BMD), mechanical testing, and histology. In the second part in six rabbits using fast distraction (waiting period 0 days; distraction rate 1.5 mm/12 hours; distraction period seven days) the site was evaluated radiologically. Half of the animals (35) had received ultrasound to their right leg (30mW/cm²) for 20 minutes daily after ceasing distraction (ultrasound group), while rigid fixation only was maintained in the other half (control group). With normal distraction, the hard callus area, as shown by radiography, the BMD, and the findings on mechanical testing, were significantly greater in those receiving ultrasound than in the control group. Histological analysis showed no tissue damage attributable to exposure to ultrasound. With fast distraction, immature bone regeneration was observed radiologically in the control group, while bone maturation was achieved in the ultrasound group.

We conclude that ultrasound can accelerate bone maturation in distraction osteogenesis in rabbits, even in states of poor callotaxis.

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Limb-lengthening was originally described by Codivilla, but it was not until the use of distraction by Ilizarov that it achieved worldwide use. Failure, however, results in non-union of the distracted site and prolonged further treatment. Several studies have attempted to resolve these problems, including the use of axial dynamisation and transplantation of fresh autologous bone-marrow cells. According to some of these reports, mechanical stress may stimulate bone maturation.

Webster et al first reported the effects of ultrasound on protein synthesis in human fibroblasts in vitro. Subsequently, low-intensity pulsed ultrasound has been found to stimulate the healing of fractures in animal models and to accelerate the normal repair process in randomised, double-blind, controlled clinical trials involving fractures of the tibia and radius.

Our aim in this study was to investigate the effects of low-intensity pulsed ultrasound on distraction osteogenesis in rabbits.

Materials and Methods

Animal models. We used 70 male immature Japanese white rabbits weighing about 2 kg. Under sterile conditions and general anaesthesia with ketamine (20 mg/kg) and xylazine (1.5 mg/kg), a longitudinal incision was made on the medial aspect of the right tibia. The periosteum was incised longitudinally and carefully stripped with the surrounding soft tissue. Four screws were then inserted into the medial aspect, and a transverse osteotomy of the tibial diaphysis was made distal to the tibiofibular junction between the central two screws. Approximately 5 mm of segmental bone in the centre of the two central screws were removed to release tension after leg lengthening, followed by reduction of both bone fragments. The osteotomised leg was fixed with a unilateral dynamic external fixator (Orthofix M-100, Verona, Italy). All animals received conventional care, feeding, and ambulation. The legs of these animals were lengthened by one of two techniques, either normal distraction or fast distraction.

Normal distraction. In 64 animals after a waiting period of seven days, the right leg was lengthened at a rate of 0.5 mm per 12 hours (1 mm/day) for ten days. From the day after the cessation of distraction (day 0), 32 rabbits...
underwent 20 minutes per day of stimulation by ultrasound. An ultrasound transducer was fitted onto the bone-lengthening site against the lateral side of the right tibia using coupling gel (Acousix; Conmed Corp, Utica, New York). The ultrasound signal consisted of a 200s burst of 1.5 MHz sine waves repeating at 1.0 kHz. The intensity was 30 mW/cm² spatial average and temporal average. Rigid fixation was maintained without exposure to ultrasound in the remaining 32 rabbits (control group).

**Radiological assessment.** Anteroposterior plain radiographs were taken on days 0, 7, 10, 14, 17 and 21 after distraction, under anaesthesia as described above. They were digitised and the hard callus of the distraction gap was determined with a fixed threshold in 256 greyscale using NIH Image (National Institute of Health) with a screw as control. In order to compensate for individual variances, the percentage of the hard callus in the area of the gap (% area) was calculated.

**Assessment of bone mineral density.** We assessed the bone mineral density (BMD) of the distracted tibia using dual-energy x-ray absorptiometry (Hologic QDR-2000, Waltham, Massachusetts). The coefficient of variation for the paired measurement of the BMD of standard samples by this technique was 1.0%. The BMD was measured in the anteroposterior view at weekly intervals, under anaesthesia as described above. Three regions of interest were measured: the cut ends of the proximal (L1) and distal (L3) fragments, not including the screws and the device, and the distracted area (L2). The BMD percentage of the distracted area (L2) versus the average BMD of the proximal (L1) and distal areas (L3) was calculated in order to standardise each of the values (%BMD). The formula is expressed as follows:

\[
\text{%BMD} = \frac{\text{BMD (L2)}}{\left[ \text{BMD (L1)} + \text{BMD (L3)} \right] / 2} \times 100
\]

**Mechanical testing.** For mechanical testing, 30 animals were killed by the intravenous administration of a lethal dose of sodium pentobarbital on days 7, 14 or 21 after cessation of distraction. Specimens were prepared for mechanical testing by removing the soft tissues and external fixators from the tibias, and storing them in gauze soaked in 0.9% saline solution at -20°C. The intact tibiae were treated in the same manner as the normal distraction group. Serial radiographs were taken in each group for 42 days in order to analyse the effect of ultrasound on the tibial area that was fast distracted. This represented a distraction osteogenesis model in adverse conditions for bone maturation. In six animals from the day after the osteotomy, the right leg was lengthened at a rate of 1.5 mm per 12 hours (3 mm/day) for seven days. After distraction, half received ultrasound in the same manner as the normal distraction group. Serial radiographs were taken for each time point in order to analyse %area by the method described above.

**Statistical analysis.** We compared %area, %BMD, %torque and %stiffness, except for the data obtained on day seven in mechanical testing, between the two groups using an unpaired Student t-test for each time point. The data on day seven in mechanical testing was analysed by Fisher’s probability test.

**Results**

**Normal distraction.** The progression of radiographs and %area changes over the course of the study period in the two groups are shown in Figures 1 and 2. The %area in the ultrasound group on day 10 was significantly greater than that of the control group (Student’s t-test; p < 0.01). On days 14 and 17, significant differences were observed between the two groups (p < 0.0001, p < 0.01, respectively), but on day 21 the difference was not significant.

The %BMD of the two groups was not significantly different on day 0 (ultrasound group 36.1 ± 1.8%; control group 38.2 ± 1.6%), while significant differences were seen on day 7 (ultrasound group 69.8 ± 4.9%; control group 47.4 ± 1.1%; p < 0.01) and day 14 (ultrasound group 77.0 ± 6.3%; control group 55.8 ± 6.6%). The two groups did not differ significantly on day 21 (ultrasound group 82.5 ± 6.3%; control group 69.4 ± 4.3%) (Fig. 3).

In mechanical torsional testing, no ultimate failure points were found in the specimens of the control group on day 7, while four of the five in the ultrasound group had such points at that time. There was a significant difference between the two groups in this finding by Fisher’s exact probability test (p < 0.01) (Table I). On day 14, %torque differed significantly between the two groups by Student’s t-test. There was, however, no significant difference between the two groups on day 21 (Table II). The %stiff-
ness of the ultrasound group was also greater than that of the control group on days 14 and 21, although the difference did not reach statistical significance (Table II). The ultrasound group exceeded intact bone strength on day 14 and maintained this level until day 21. The control group reached only the strength level of intact bone on day 21.

Histologically, endochondral bone formation in the distracted callus was observed in the two groups and there were no differences between the groups. No tissue damage attributable to ultrasound was observed (Fig. 4).

Fast distraction. Figure 5 shows the changes in %area during the experimental period for 42 days. Although the %area of the control group had reached approximately 50% on day 24, there was no further increase. The %area in the ultrasound group reached almost the same level as that of the US group with normal distraction on day 28. Analysis by Student’s t-test at each time point showed significant differences from day 21 to the end of this experiment.
Photomicrographs of the ultrasound (a) and the control (b) groups on day 7 after ceasing distraction. The arrows indicate the direction of ultrasound stimuli (Toluidine Blue ×35).

Fig. 4a

Fig. 4b

Table I. Number of specimens ultimately showing a failure point on day seven after discontinuation of distraction

<table>
<thead>
<tr>
<th>Ultimate failure point</th>
<th>US (n=5)</th>
<th>Control (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>4*</td>
<td>0</td>
</tr>
</tbody>
</table>

* p < 0.01 (Fisher’s exact probability test)

Table II. Changes in mechanical strength of distracted callus (normal distraction). Each value (mean ± SEM) is the ratio of maximal torque or stiffness of the treated leg against the values of an intact leg

<table>
<thead>
<tr>
<th>Day</th>
<th>% Torque US group</th>
<th>Control group</th>
<th>% Stiffness US group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>146.9 ± 20.9*</td>
<td>73.3 ± 19.1</td>
<td>102.5 ± 15.4</td>
<td>75.0 ± 21.6</td>
</tr>
<tr>
<td>21</td>
<td>141.6 ± 16.9</td>
<td>118.0 ± 36.1</td>
<td>106.5 ± 9.8</td>
<td>97.5 ± 19.4</td>
</tr>
</tbody>
</table>

* p < 0.05 (unpaired Student’s t-test)

Changes in %area (mean ±SEM) of the ultrasound and control groups with fast distraction (*p < 0.05, **p < 0.01).

Correlation between %area and %torque in normal distraction. %Torque correlated significantly with %area (r = 0.67, p = 0.0005). Bone strength was considered to follow essentially the same time course as that of %area.
Discussion

Dinno et al.\textsuperscript{16} demonstrated that intensities of ultrasound of less than 100 mW/cm\textsuperscript{2} spatial average and temporal average were non-thermal. Duarte\textsuperscript{7} and Pilla et al.\textsuperscript{8} reported that low-intensity ultrasound treatment (30 to 57 mW/cm\textsuperscript{2}) yielded minimal temperature changes when applied to the site of a bone fracture. Histological analysis in our study revealed endochondral formation of bone in the distracted callus in both groups and no tissue damage attributable to ultrasound in the ultrasound group. Application of low-intensity pulsed ultrasound (30 mW/cm\textsuperscript{2}) was considered to have little thermal effect and to produce stable cavitation and streaming, according to our histological observations.

With usual distraction, we showed that the %area, %BMD, %torque and %stiffness of the ultrasound group were greater than those of the control group at each time point. The %area of the ultrasound group was significantly greater than that of the control group on day 10 (p < 0.01), day 14 (p < 0.0001) and day 17 (p < 0.01). The difference between the two groups, however, was not significant on day 21. The %BMD of the ultrasound group was significantly greater than that of the control group on day 7 (p < 0.01) and day 14 (p < 0.05). The %BMD of both groups was essentially unchanged on day 21. This process followed nearly the same course as that of %area. These findings suggest that ultrasound resulted in mineralisation of the callus in the early stages of the process of bone maturation. In mechanical testing, we observed that the values in the ultrasound group were ahead of those of the control group by approximately one week. The final values of the four parameters did not differ significantly between the two groups. Taken together these data suggest that ultrasound may be capable of advancing the endpoint of distraction osteogenesis.

With fast distraction, the %area of the ultrasound group was significantly greater than that of the control group on day 21 (p < 0.05). The %area of the control group ultimately reached approximately 50%, and that of the ultrasound group approximately 80%. %torque and %area were found to be correlated in normal distraction (linear regression analysis: r = 0.67; p = 0.0005; Fig. 6). We assumed that %torque and %area also correlated in fast distraction. Therefore the bone strength of the ultrasound group was considered to be significantly higher than that of the control group. Normal bone maturation was not achieved in the control group, while the US group showed excellent maturation. We demonstrated that treatment with ultrasound was highly effective in achieving maturation of bone of distracted callus even under the most adverse conditions.

The mechanism underlying the ultrasound effect is not yet well understood. Recent reports have proposed certain mechanisms to account for the effects of ultrasound with fracture healing. Ryaby et al.\textsuperscript{17} demonstrated that adenyly cyclase activity decreased after 5 and 15 minutes of treatment with ultrasound in MC3T3-E1 mouse osteoblastic cells and TE85 human osteosarcoma cells. Yang et al.\textsuperscript{10} reported that exposure to low-intensity ultrasound of 50 mW/cm\textsuperscript{2} for 15 minutes increased aggrecan gene expression in a rat model of a femoral fracture. Parviz et al.\textsuperscript{18} reported that low stimulation with intensity ultrasound increased the intracellular concentration of calcium. According to these reports, ultrasound may, via calcium channels, stimulate osteoblastic cells to synthesise extracellular matrix. These mechanisms were thought to be adapted in distraction osteogenesis because endochondral calcification has been observed in several previous studies.\textsuperscript{15} Several clinical studies of ultrasound have demonstrated effects on fresh fractures. Heckman et al.\textsuperscript{13} evaluated the effectiveness of low-intensity ultrasound in closed or Gustilo grade-I open tibial fractures in a multi-institutional prospective, randomised, double-blind and placebo-controlled study. Their results demonstrated a statistically significant decrease in clinical healing time. Kristiansen et al.\textsuperscript{14} reported the benefit of low-intensity ultrasound in fresh Colles’ fractures. Cook et al.\textsuperscript{19} showed that the use of an active ultrasound device accelerated the healing of fractures, substantially mitigated the delayed healing effects of smoking, promoted a return to normal activity, and reduced the incidence of delayed union. Their results are consistent with our study using fast distraction.

We have shown that ultrasound promotes bone maturation in distraction osteogenesis even under adverse conditions. Further study is needed to determine whether this positive finding in normal rabbit bones occurs also in human bones.

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References


