LOW INTENSITY PULSED ULTRASOUND TREATMENT INCREASES RABBIT RADIAL FRACTURE HEALING

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ABSTRACT

In order to study the effect of ultrasound (US) on bone healing, a complete transverse fracture was made in the right radius of adult male rabbits by a cutter. 52 rabbits were used: group 1, control (n=17); group 2, test (n=15); group 3, intact (n=1) and group 4 (n=12) who were examined only for evaluation of the cutter effects in detail. 7 animals were operated with a Gigli saw. The test group received US treatment at 0.5 W/cm², 1MHz, 2 msec on-8 msec off for 10 min/day, from the day after surgery until complete fusion was observed. Radiological studies indicated that mean healing duration and rate of healing was significantly more in the test group than in controls. Histological evaluation showed the presence of ossified callus at week 3 in the test group, but in controls fibrous callus was still seen at week 5 after surgery. Bone mineral analyses by stereoelectron microscope showed that the mineral component of the treated bone reverts to normal, similar to the composition of intact bone, and sooner than that of the control. However, our results revealed no deleterious effects of US on treated and untreated ipsilateral and contralateral bones during the experiment or one month after complete fusion of the bones, at which time US was terminated.

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INTRODUCTION

Sound has been used as a physical signal in the detection or alteration of biological effects for many years. While the diagnostic applications of ultrasound (US) require very low intensities (in the range of mW/cm²) in order to avoid excessive heating of the tissues, US intensities of 0.5 to 3 W/cm² have been shown to reduce stiffness, pain and muscle spasm and to improve muscular motility. In 1965, Knoch first demonstrated that US application stimulates callus formation. Ever since, US therapy of fractures, with the use of several different US signals and dosing schedules has been reported to augment repair in various animals. Duarte first reported that low intensity US stimulated the growth of bone and healing of fractures in fibular osteotomies in rabbits. Subsequent studies either did or did not support these findings. Pilla et al. showed that US stimulation enhanced fracture healing as measured by biomechanical testing. However, Tsai et al. observed that US with an intensity of 1W/cm² may be inhibitory or deleterious to the treated fractures. Nevertheless assessment of bone repair in the above studies and in others has been carried out primarily by radiographical analyses. We have already reported that low intensity pulsed US accelerates bone healing. However, the goals of the present study were to assess radial bone repair by low intensity
pulsed US, 0.5 W/cm² with a frequency of 1MHz, pulsed 2 msec on-8 msec off, with a duration of 10 min/day, radiographically and histologically and also to perform bone mineral analyses by a stereoelectron microscope (SEM) to evaluate the above discrepancies in detail.

**MATERIALS AND METHODS**

A total of 52 male white rabbits (obtained from the Animal Center of Shiraz Medical School) were used. Each rabbit (1.5±2.2kg) was caged individually and given standard pellet diet and water ad libitum. Out of them, 7 rabbits were operated by a Gigli saw, one remained intact and the rest were operated by cutter (Stanley or razor knife).

**Inducing fracture by a Gigli saw**

In the preliminary experiments, the radial bones of 7 rabbits were fractured by a Gigli saw.14 It should be mentioned that the entire surgical procedure was exactly the same as when using the cutter (below procedures), except for use of the Gigli saw for bone cutting. However, the results of these bones were compared to those obtained from inducing fracture by a cutter later on.

**Cutter use and final experiment**

45 rabbits were randomly divided into four groups: group one, control (n=17); group two, test (n=15); group three, intact (n=1) and group four (n=12) were examined only for evaluation of the cutter effects in detail. The surgical procedure was carried out under general anesthesia using ketamine injection (100 mg/kg IM) and ether as needed. Under aseptic conditions, the right radius was exposed and a fragment of about 1mm was removed by the cutter from the diaphysis. The incision was then closed and a plaster thigh dressing was applied all over the forelimb. Surgical procedures were the same in all groups. However, control and intact groups did not receive any ultrasonic treatment.

**US duration and intensity**

US was applied to the test group, by Sonopulse 434, through an opened window in the plaster exactly over the fracture site, at an intensity of 0.5 W/cm², repeating at 1 MHz, pulsed 2 msec on-8 msec off for 10 min/day, starting on the first day after surgery up to the end of the experiment when complete fusion of the bone was observed radiologically.

**Radiological evaluation of bone healing**

The progress of repair was assessed radiologically. X-rays were taken by a Siemens Heliophos 45 from right and left forearms in anterior-posterior (AP) and lateral (L) projections before and after surgery and thereafter once a week, throughout the experiment until complete fusion was observed. X rays were read by a radiologist blinded to the treatment groups.

**Assessment of rate of healing**

To have a better idea for healing, we decided to calculate the rate of healing in spite of the different fracture spaces which might have been produced by the cutter. To do this, the space existing in the fracture site was measured by the planimeter in all X-ray images. Then the obtained value from the X-ray taken immediately after surgery was subtracted from the last one (which was almost zero) and divided by the duration of healing (week).

**Histological evaluation**

Two animals from each group were examined histologically weekly. However, 5 animals from test and 7 animals from control groups were also studied one month after complete healing. To do this, fixed bone was sectioned longitudinally through the fracture site. One half of each was decalcified by 10% acetic acid (for 24 hr) and prepared for hematoxylin-cosin staining, the other half was prepared for mineral assessment by the SEM. Evaluation of bone healing was made by two pathologists who were blinded to the treatment groups.

**Bone mineral examination by SEM**

To prepare the bone, samples were dried in a hot oven (150°C) for 8 hours, then in acetone for 30 min. However, 2 cm of bone–1 cm on each side of the fractured area–was taken and glued to the stub by a watery glue. Specimens, which were electrical insulators by silver glue, were coated with a thin conductive film of carbon by a carbon coater (306, Edward, England). The morphology of the samples was studied and bone minerals were analyzed by a Cambridge SEM equipped with an energy dispersive spectrometer (EDS).

**Statistical analysis**

Rate of healing was tested using ANOVA. Student's t-test was used to indicate the differences in average time of healing. Significance was accepted at $p\leq0.05$.

**RESULTS**

Comparing the fracture induced by a Gigli saw and a cutter

In our preliminary study, when a Gigli saw was used, 42.8% of animals developed severe necrosis and died after 2 weeks. In 28.6% ulna and radial bones were broken together and we could carry out our experiment only in 28.6%. However when the cutter was used only 6.8% died around 4 weeks after surgery. In the autopsy, no sign of necrosis or any other abnormalities was observed and
operated arms were completely normal. Therefore in the final experiment, a cutter was used.

A total of 33 rabbits were used to examine the effect of US on fractured bone healing. Animals were divided as mentioned before. Different criteria were used to study the effects of US on bone healing in detail.

Radiological assessment of healing

Results showed that mean duration of the right radial bone healing was 8.7 weeks in controls and 4 weeks in test groups (p<0.05) (Table I, Fig. 1). Nevertheless radiological examination showed that US treated bone was completely normal one month after US termination. Ulnar bones of the right forearm and left radial and ulnar bones did not show any abnormality radiologically.

Evaluation of bone healing by histological studies

There was a periosteal reaction in both groups one week after surgery. However at the end of the second week, there was new bone formation and fibrous callus in the test group, while in controls there was still only a periosteal reaction. By the end of the third week, new bone formation and fibrous callus appeared in the control and mature bone formation and ossified callus in the test group. Even by the end of the 5th week when complete mature bone formation was observed in the test group, there was still only fibrous callus in the control group (Fig. 2). Nevertheless, histological examination of right ulnar and left radial and ulnar bones revealed completely normal features. However, one month after US termination, left and right radial and ulnar bones all revealed normal structure.

Mineral analysis of bones by the SEM

Fig. 3a shows the topography of the longitudinal section and Ca X-ray line scan in an intact bone. However in the test group, 5 weeks after surgery, the X-ray line scan showed a relatively uniform distribution of Ca (Fig. 3b) similar to intact bone. But in the control, Ca X-ray line scan (Fig. 3c) was still non-uniform at this time. A relatively similar spectrum of bone minerals, especially Ca and P, are seen in both control and test groups one week after surgery. However, 5 weeks after surgery, Ca and P were higher in test and intact groups compared with controls (Fig. 4). Obtained EDS results revealed no significant differences in Ca 5 weeks after surgery in test compared to the intact group. However, there was a significant decrease in Ca during the first three weeks after surgery in controls (Table II). Although Ca in controls was less than the test and intact
Ultrasound-Increased Rate of Fracture Healing

Table II. Mean±SE of bone minerals weight percent in intact (G3), control (G1), and test (G2) groups 5 weeks after surgery. Significant differences (p<0.05) are shown by similar small letters between groups and by similar capital letters within one group after 5 weeks.

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Ca</th>
<th>P</th>
<th>Na</th>
<th>Mg</th>
<th>K</th>
<th>Cl</th>
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<td>1</td>
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<td>B</td>
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<tr>
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<td>G2</td>
<td></td>
<td>E</td>
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<td>66.40±2.42</td>
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<tr>
<td></td>
<td>G3</td>
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<td></td>
<td>1.12±0.63</td>
<td>1.09±0.41</td>
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<td>67.07±4.51</td>
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<td>2</td>
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groups during the experiment, these differences were significant at week 3 and 4 (Table II). There was a significant increase in phosphorus (P) during the first 3 weeks after surgery in controls which was significant at week 3 compared with the intact group. Sodium (Na) in the control was more than test and intact groups at weeks 2, 3 and 4 (p<0.05). In the control group magnesium (Mg) was significantly more than in test and intact groups throughout the experiment except for the first week. At weeks 4 and 5, chlorine (Cl) was more in the control than in test and intact groups. At weeks 2 and 3, potassium (K) levels in intact bone were more than control and test groups (Table II). However, the above mentioned results showed that the mineral composition of treated bone normalizes to a composition similar to intact bone, sooner than that of controls.

DISCUSSION

Cutter or Gigli saw

The results of our preliminary experiment showed that after making a fracture with a Gigli saw, only 28.6% of animals could be used. However, when the cutter was used only 6.8% of animals died 4 weeks after surgery, with no sign of necrosis or other pathology. Thereafter, death was certainly not due to the surgical procedure. As far as anatomy is concerned, ulnar and radial bones are in close proximity to each other in the rabbit. For this reason, it is hard to pass the Gigli saw through the bones without fracturing them. Even after successful passage of the Gigli saw, it is impossible to cut the bone without inflicting any damage to the surrounding soft tissues. It was shown that
Using the cutter instead of the Gigli saw provided us with a perfect surgical procedure, no bleeding, no damage, and took less time to cut the bone. For the above reasons, we recommend that the ordinary cutter be used instead of a Gigli saw for small animals and in fine open fractures.

**US treatment**

The present data showed that US accelerates bone healing without having any deleterious effects on the fractured bone or ipsilateral and contralateral intact bones, when examined radiologically and histologically and when mineral analysis was done by SEM. Tsai et al. showed that US treatment at 0.5 W/cm², 1.5 MHz significantly accelerated bone formation at the fracture site when used for 5, 15 and 25 min/day. A higher intensity of 1 W/cm² suppressed bone formation of the treated fractured fibula and could systemically affect the opposite leg and accelerate bone formation on the contralateral bone which received no direct US. Although we agree that US might be deleterious or beneficial depending on its intensity or duration, it should be mentioned that the authors used no internal or external fixation for the broken leg which certainly caused dislocation of the broken leg and a decrement of bone growth on the ipsilateral limb. However abnormal posture and locomotion might increase bone formation in the contralateral bones. But it is unlikely that the systemic effects of US existed as they postulated. Klug et al. treated the tibial bone fracture by US (0.2 W/cm², 3 min/day) from days 14 to 28 after surgery. Although they used US for only 14 days, they showed that treated bones healed after 168 days in test and 203 days in control groups. Dyson and Brookes used different patterns of US treatment (0.5 W/cm², 1.5 MHz, 5 min/day, 4 days/week) and they also showed acceleration of rat fibula healing. It was also shown that US treatment with an intensity of 30 mW/cm² and a frequency of 1.5 and 0.5 MHz for 15 min/day could also accelerate rat femoral bone healing with internal fixation. Acceleration of healing by US (30 mW/cm², 1.5 MHz, 20 min/day) was also demonstrated when the C form fracture (not complete) was made on rabbit fibula or when human tibia was fractured. In the present experiment, the possible deleterious effect of US after its termination was also studied. The results revealed normal appearance of the bones and histological studies also showed no differences between test and control groups. It thus appears that US effects are not deleterious to the bone or its healing. The obtained results of Mortimer and Dyson on chick embryos also confirmed ours. They showed that US treatment (0.25-1.5 W/cm²) increased Ca absorption by chick embryonic tissues and 20 min after termination of US, Ca entrance to the cell decreased. However, the present data also showed that US accelerates bone healing without having any deleterious effects on ipsilateral or contralateral intact bones.

**Fig. 3.** SEM micrographs showing Ca line scan in intact bone (a) and in test (b) and control (c) groups 5 weeks after surgery.

Using a cutter knife made no damage to the soft tissues, and we could also cut the exact part which we wanted and make the desired gap between the two broken ends of the bone. We could also cut the radius without damaging the ulna.
bones. Nevertheless, the exact mechanisms are not clear. Several mechanisms have been proposed. It has been shown that the thermal effects of US (0.2 W/cm²) cause an increase in circulation and oxygen of the bones which affect callus formation. While US with an intensity of less than 100 mW/cm² could not produce significant thermal effects, it has been shown that US with an intensity of 30 mW/cm² at 1.5 MHz accelerates bone healing. This intensity increases temperature by about 0.1°C, which is not capable of producing biological effects. It has also been shown that a US frequency of 1.5 MHz is more effective than 3 MHz on bone healing when other parameters of US remain constant. Then, it could be postulated that other factors are also involved in healing. It has also been shown that US causes stable cavitation, which results in acoustical streaming, vibration of cell membranes and changes in permeability which may cause increased Ca influx and DNA and protein synthesis in fibroblasts and activation of macrophages, both which exist early in fracture repair. However, stable cavitation may cause changes in these cells that could accelerate fracture repair. Nevertheless, using US creates a pressure wave which leads to mechanical perturbation and deformation of the cells which cause a change in membrane permeability and second messenger activity. US can also produce electrical changes and affect ionic binding with membranes indirectly. However, in the present experiment we also showed that the mineral composition of the treated bone returns to normal sooner than that of controls; i.e. US increases mineral influx in the bone cell. Several investigators showed that the healing effects of US are due to its piezoelectrical effects. Recently, PGE has been proposed as a mediator of US effects. It has also been shown that indomethacin (PGE synthesis blocker) decreases callus formation and PGE accelerates bone healing and spongy and compact bone formation. However, PGE and PGI are produced by osteoblasts and the PGE receptor is also found on osteoblasts and osteoclasts. The mechanical perturbation produced by US activates prostaglandin synthesis in the osteoblast. The effects of mechanical force on bone cell culture is PGE production through activation of phospholipase A₂ and recruitment of arachidonic acid. However, US can also produce mechanical forces which increase cAMP through activation of adenylyl cyclase by PGE. It is proposed that PGE might have specific effects on osteoblast precursor division and differentiation to osteoblasts and initiation of collagen formation and can also produce bone remodelling. PGE also activates endosteum and periosteum production.

In summary, the above discussion revealed that US treatment at 0.5 W/cm², 1 MHz, 10 min/day, accelerates bone healing, and is not deleterious to the treated bone or intact ipsilateral and contralateral bones. Several

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**Fig. 4.** EDS results taken 5 weeks after surgery in control (a) and test (b) groups. (c): Taken from intact bone.
mechanisms have been proposed to explain these effects, but the exact mechanism remains to be elucidated in the future.

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