Does Ultrasound Stimulate Osteoconduction? A Placebo-Controlled Single-Blind Study Using Collagen Membranes in the Rat Mandible

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Purpose: *To investigate whether ultrasound can stimulate osteoconduction in the mandible, an attempt was made to stimulate the osteoconductive process with low-intensity pulsed ultrasound in rats.* ***Materials and Methods:*** *In 64 rats, a 5.0-mm diameter circular mandibular defect was made in the ramus and, subsequently, covered on both sides with collagen membranes. Two groups were studied, an ultrasound treatment group and a placebo treatment group. At 2 and 4 weeks, the remaining defect area was measured using microradiographs, and the amount of osteoconduction was expressed as the percentage of defect closure.* ***Results:*** *At 2 and 4 weeks, there was no significant difference in the percentage of defect closure between the groups.* ***Discussion:*** *An explanation may be that ultrasound does not exert an effect in an area where wound healing is already expected to be at an optimal level.* ***Conclusion:*** *There was no evidence that low-intensity pulsed ultrasound stimulates osteoconduction in a bone defect in the rat mandible that is covered by a collagen membrane.* Int J Oral Maxillofac Implants 2005;20:181–186

Key words: *animal model, collagen membranes, microradiography, osteoconduction, ultrasound*

The complex treatment of bone defects caused by congenital defects, inflammatory disease, injury, and oncologic procedures has initiated much research in the field of bone regeneration. In the maxillofacial skeleton, a widely used technique to regenerate bone is based on osteoconduction. Osteoconduction refers to bone formation by guided tissue regeneration. Guided tissue regeneration is made possible by using osteoconductive membranes. By covering a bone defect with an osteoconductive membrane, soft tissue ingrowth into the defect is prevented. In this way, a confined space is obtained into which bone cells are allowed to migrate. Clinically, these membranes are used in implant surgery to cover exposed implant treads with bone[1] and to prevent the resorption of bone grafts.[2] In periodontology, osteoconductive membranes are used to regenerate periodontal defects.[3,4] However, the process of osteoconduction takes a substantial amount of time and is not always successful. To provide a solution, much research has been undertaken to promote bone formation beneath osteoconductive membranes by combining them with different bone growth–stimulating factors, such as bone morphogenetic proteins.[5–7]

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A relatively unknown method that is being used experimentally to attempt to stimulate bone healing processes is ultrasound. Ultrasound is a mechanical pressure wave with a frequency above the threshold of human hearing (ie, > 20,000 Hz).[8] In medicine, ultrasound is used in diagnosis (pulse-echo) and treatment. In recent decades, the stimulation of certain fractures of the extremities with low-intensity pulsed ultrasound has become more established,[9–11] and in certain cases of delayed unions and nonunions, ultrasound therapy has yielded high success rates.[12,13] Although the effect of ultrasound treatment on bone healing has traditionally been investigated in the extremities, ultrasound may stimulate maxillofacial bone healing as well.[14] Therefore, it was decided to investigate whether low-intensity pulsed ultrasound stimulates osteoconduction in a bone defect in the rat mandible that is covered by a collagen membrane.

MATERIALS AND METHODS

The study protocol was approved by the Animal Studies Review Committee and was conducted in accordance with institutional guidelines (University of Groningen, The Netherlands). The sample size was determined by a power analysis based on a 95% power with a .05 2-sided significance level, given a difference in amount of bone formation between groups of 20% and a standard deviation of 14%.[6]

Operative Procedure

In 64 rats (Sprague-Dawley, male, age 15 to 17 weeks, mean weight ± SD 310 ± 17.6 g, range 265 to 348 g), a standardized 5.0-mm circular mandibular defect was made in the right ramus in accordance with the method of Kaban and Glowacki.[15] Under 2% isoflurane inhalation anesthesia, the mandibular and hemicervical areas were shaved. After disinfection of the skin, a submandibular incision was made, and the masseter muscle was exposed. After cleaving the muscle along the submandibular border, a muscle flap was raised on the buccal and lingual sides. Care was taken not to injure the facial nerve and parotid duct. Using a trephine drill with an outer diameter of 5 mm (22RF050; Hagar & Meisinger, Düsseldorf, Germany) mounted in a dental handpiece, a hole was made through mandibular ramus. During drilling, the surgical field was continuously irrigated with sterile saline to prevent thermal damage.

After the hole was drilled, the wound was rinsed with saline. One resorbable bilayer collagen membrane (Bio-Gide; Geistlich Biomaterials, Wolhusen, Switzerland) was placed lingually and one buccally onto the defect, covering a minimum 2-mm bone margin outside the defect. Subsequently, the wound was closed in layers using 4-0 resorbable sutures. Care was taken not to displace the membranes. For postoperative pain relief, a single dose of buprenorphine 0.03 mg/kg was given. The rats were numbered and housed in groups of 4. The first 4 rats operated on were allocated to the ultrasound group, the second 4 to the placebo treatment group, the next 4 to the ultrasound group, and so on. The rats received standard laboratory food and water ad libitum.

Experimental Groups

The experiment involved 2 sessions, each with 32 rats. One session evaluated the influence of ultrasound treatment on osteoconduction at 2 weeks, the other at 4 weeks. Each session consisted of 2 experimental groups of 16 rats, the ultrasound group and the placebo group. The ultrasound group received ultrasound treatment daily (except for weekends) for 20 minutes under general inhalation anesthesia. The placebo ultrasound group received the same treatment using placebo transducers. The placebo group was included to correct for possible manipulation effects during the ultrasound treatment.

Ultrasound Treatment

A custom ultrasound device was made because the transducer of the Sonic Accelerated Fracture Healing System (SAFHS) device (Smith & Nephew, Memphis, TN) was too large to treat the rat. The device consisted of a main operating unit (ICT Technical Services, University Hospital Groningen, The Netherlands) and 4 attached ultrasound transducers (Röntgen Technische Dienst, Rotterdam, The Netherlands). The transducers were calibrated to emit the same pulsed ultrasound signal as the SAFHS device.[16] Comparison between the ultrasound field variables of the customized device and the SAFHS device has been presented elsewhere.[17] For the customized device, effective radiation area and beam nonuniformity ratio were determined according to International Electrotechnical Commission (IEC) guideline 61689.[18] To check stability of the device, the ultrasonic power was repeatedly determined with a balance according to IEC 61161.[19] In addition, 4 stainless steel placebo transducers equal in weight and size to the ultrasound transducers were constructed.

Before ultrasound treatment, the heads of the rats were placed on 8 custom-made silicon pillows in such a way that the right side of the mandible faced upward. Into the pillows, a syringe was mounted through which 2% isoflurane inhalation anesthesia was administered. In this way, 8 rats could be treated at the same time. Aqueous ultrasound coupling gel

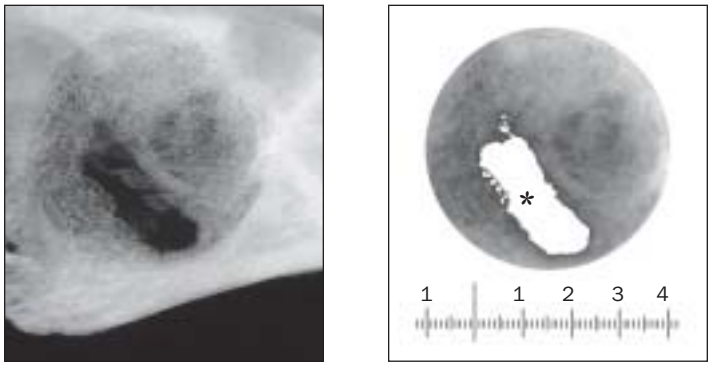

Fig 1 *(a)* Microradiograph of a 4-week-old rat mandibular defect covered on both sides with a collagen membrane. The original outline of the 5.0-mm defect is clearly visible, as well as irregular bone formation in the defect. *(b)* Using image analysis software, the remaining defect area* was measured. The defect was 2.47 mm^2, which corresponds to a percentage defect closure of 87.4% (original magnification ×10).

was applied to the skin, and the transducers were placed on the skin on top of the defect. The skin was shaved weekly to prevent the trapping of air, which can block ultrasound transmission. The bodies of the rats were placed on a pre-heated rug to prevent hypothermia. Every day, each rat would be treated by a different transducer, thus limiting the influence of possible ultrasound field variations between the transducers. During the experiment, the body weight of each animal was measured weekly.

At the end of each session, all the rats in the session were anesthetized and then sacrificed by an intracardial injection of an overdose of pentobarbital. Subsequently, the right hemimandible was explanted and fixed in buffered formalin solution. After 48 hours, the specimens were rinsed with saline and put in 70% denaturated ethanol solution. Excess muscle was removed from the specimens by hand.

At the end of the experiment, the ultrasound emission of the transducers was measured again to ensure that the ultrasound field had remained stable throughout the experimental period.

Microradiography

An x-ray source (PW 1730; Philips, Eindhoven, The Netherlands) was used that produced monochromatous radiation with a specific wavelength of .1537 nm. The x-ray radiation used was CuKα radiation with a copper x-ray tube and a nickel filter. The wavelength produced is especially sensitive to calcium absorption. The explanted parts of the mandible were placed between 35-mm black-and-white film (Fuji B&W POS/71337) and the x-ray source and exposed for 25 seconds, with a tube charge of 25 kV and 25 mA. Care was taken to place the plane of the defect parallel to the film. To minimize magnification effects, the distance between the specimen and the film was kept small (0.3 mm), and the distance between the x-ray source and the specimen was kept large (300 mm). Film was used instead of radiographic plates because film has a much higher resolution. After development of the film with a Kodak D-19 developer (Eastman Kodak, Rochester, NY) for 10 minutes, fixating, rinsing, and drying, the film was placed on a light box. A digital image of the mandibular defect on film was recorded with a stereo microscope (M7 S; Wild/Leitz, Heerbrugg, Switzerland) with a magnification of 10× and a charge-coupled device camera (CS 8310; Teli, Tokyo, Japan) (Fig 1). The camera was linked to a personal computer equipped with a framegrabber. The magnified microradiographs were stored as images with a size of 640 × 480 pixels and a resolution of 256 gray values. For calibration, a separate image of a microruler was recorded in the same way as the specimens.

Measurement of Osteoconduction

Rats who died before sacrifice were excluded from analysis. The microradiographs were coded so that the principal investigator was blinded to the treatment group and number of each rat during measurement. The amount of osteoconduction was expressed as the percentage of defect closure using image analysis software (Scion, Frederick, MD). First, based on the differences in gray values, the individual threshold of the bone/no-bone boundary was determined for each digitized microradiograph. Second, this threshold was applied to the 5.0-mm-diameter

Table 1 Amount of Osteoconduction (Mean ± SD) Reflected as the Percentage of Defect Closure

	2 wk			4wk		
	%	n	95% CI	%	n	95% CI
Ultrasound	73.3 ± 17.7	16	−11.8; 19.6	88.0 ± 23.6	16	−18.2; 7.4
Placebo	69.4 ± 24.7	15		93.4 ± 5.9	15	

defect as a whole, and the remaining defect area was measured automatically. Finally, this remaining defect area was expressed as a percentage of the original defect area (πr^2 = 19.63 mm^2). After the measurements were completed, the percentage of average defect closure was calculated for the 2 experimental groups. The differences between the groups were compared using a *t* test with a .05 significance level.

RESULTS

The percentages of defect closure in each group at 2 and 4 weeks are presented in Table 1. No significant differences could be demonstrated between the ultrasound treatment group and the placebo treatment group at either 2 or 4 weeks. All animals recovered well after the surgical procedure. The ultrasound treatments were uneventful. During the course of the experiment, 2 rats died for unknown reasons. All other animals gained weight. No significant difference was found in regard to average body weight between the groups at either 2 or 4 weeks (data not shown). The ultrasound fields as emitted by the customized ultrasound device did not change during the course of the experiment.

DISCUSSION

This study indicated that low-intensity pulsed ultrasound did not stimulate osteoconduction in bone defects in rat mandibles that were covered by a collagen membrane. This finding does not seem to be in accordance with reports indicating that ultrasound can stimulate bone healing. This positive effect has been observed in various species, such as the rat,[20] rabbit,[21] dog,[22,23] and homo sapiens,[9,10] and has been observed in various circumstances, such as fresh fractures,[9–11] delayed unions, nonunions,[12,13,24] osteotomies,[13] osteodistraction,[25–27] and osteoradionecrosis of the mandible.[28]

Because the mechanism as to how ultrasound stimulates bone healing is not entirely clear, it is difficult to predict in which cases ultrasound will or will not stimulate bone healing. It has been reported that the pressure wave serves as a surrogate for physiologic stresses in bone, which stimulate bone formation.[29] Apart from piezo-electric[30–33] and membrane effects,[34–36] part of the ultrasound effect seems to be related to angiogenesis.

In ischemic tissues, where blood perfusion is limited, ultrasound can promote neovascularity and neocellularity.[37] In dogs with an ulnar osteotomy, 20 minutes of daily ultrasound treatment with the SAFHS device for 8 weeks produced an increase in blood flow around the osteotomy site after 2 to 3 days, and this increase lasted for 2 weeks as compared to the nontreated controls.[38] Ultrasound can also stimulate the production of angiogenesis-related cytokines (interleukin-8, fibroblast growth factor, and vascular endothelial growth factor) in human mandibular osteoblasts,[39] which indicates that it helps the formation of vessels. This may explain why the stimulation of bone healing with ultrasound is apparent in compromised healing situations such as delayed unions and nonunions of the extremities,[12,13,24] the healing of scaphoid fractures,[11] and osteoradionecrosis of the mandible.[28] These compromised healing situations are thought to be related to a relatively poor blood supply because of anatomic predisposition, vascular disease, treatment (medication, radiation), or habit (smoking).

Thus, an important factor in the ultrasound stimulation of bone healing seems to be related to angiogenesis. This raises the question as to whether an already optimal healing tendency (optimal blood perfusion) can be influenced by ultrasound. It has been suggested that normal tissue may not be as responsive as damaged tissue to ultrasound treatment.[40] The head and neck area of the body is well-perfused with blood and therefore can be considered to have an optimal healing capacity. This would imply that the additional effect of ultrasound treatment of mandibular bone in healthy individuals is expected to be minimal. This may explain why no effect of the ultrasound treatment on osteoconduction was measured in the present study. The rats used were mature, healthy, and had no known disorders that could compromise angiogenesis/bone healing.

Another explanation of the failure to stimulate osteoconduction in this experiment may be that mandibular bone in rats is not susceptible to the specific characteristics of the low-intensity pulsed ultrasound field. The few past reports concerning mandibular fractures in rabbits,[41] humans,[42] and mandibular osteoradionecrosis in humans[28] described ultrasound fields other than the SAFHS field. In these studies, ultrasound was reported to have a positive effect on mandibular bone healing. An ultrasound pressure field can be altered in frequency, intensity over space and/or time, pulse durations, and wave shapes, all of which may alter the tissue response to the pressure wave. However, the signal characteristics of the SAFHS device (30 mW cm^{-2}, 200-μs pulse) was used in the present experiment because bone healing seemed particularly sensitive to this signal in other circumstances, and the device is approved for clinical use.[43]

In the present study, a collagen membrane was used on both sides of the defect to provide a secluded space that could be filled with bone according to the principles of guided tissue regeneration. Although it has been reported that certain resorbable membranes (including collagen membranes) have a tendency to collapse and therefore would inhibit bone formation in a defect,[44] this has not been observed in the model used in this experiment. Furthermore, the collagen membrane is more than 99% transparent to the ultrasound pressure wave (attenuation 0.02 ± 0.07 dB). This means that the ultrasound dose as used clinically did reach the tissue behind the membrane.

In a previous ultrasound study, the same mandibular defect model was used, but without a collagen membrane.[17] Using the same microradiography technique, it was found that the 5.0-mm-diameter defects healed an average of 28.0% ± 12.4% at 2 weeks (n = 36) and an average of 31.5% ± 13.8% at 4 weeks (n = 35) regardless of whether the subjects received ultrasound therapy, placebo therapy, or no therapy. Comparing these results to the present study, it confirmed that the presence of a collagen membrane itself facilitates bone growth into the mandibular defect and that ultrasound does not seem to do so.

A microradiography technique[45] was used to measure the area of the bone formed inside the defect. Because bone formation inside the mandibular defect was irregular, measuring the area of bone inside the defect using a microradiograph seemed more accurate than measuring the diameter of the defect using a histologic section through the middle of the defect, as has been done by others.[7,46] However, a limitation of microradiography is that evaluation of bone healing at the cellular level is not possible,[47] so that a cellular effect of the ultrasound in the model used in this study may be overlooked. Despite this disadvantage, the authors assumed that measuring the area of mineralized bone inside the defect would suffice, since this method reflected the amount of bone formation.

In summary, this study presented no evidence that low-intensity pulsed ultrasound stimulated osteoconduction in bone defects in the rat mandible that were covered by collagen membranes. This result may be related to an already optimal healing tendency in the head and neck region because of good blood supply and perfusion. Future research may focus on the stimulation of mandibular bone healing using low-intensity pulsed ultrasound in relative compromised healing situations.

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