The Effect of Ultrasound on Angiogenesis: An In Vivo Study Using the Chick Chorioallantoic Membrane

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Purpose: *Ultrasound therapy induces clinical healing of irradiated avascular mandibular bone and fractures. In vitro ultrasound in tissue culture has been shown to stimulate bone formation synthesis and bone remodeling factors and to stimulate osteoblast proliferation. Therefore, the aim of the present study was to investigate the effect of short-wave (1-MHz) and long-wave (45-kHz) ultrasound on the vascularity of the chorioallantoic membrane (CAM) of a fertilized egg.* *Materials and Methods:* *The nature of the angiogenic effect was investigated using the CAM of a fertilized egg by: (1) application of sonicated fibroblast media incorporated into methylcellulose disks onto the CAM and (2) direct application of the ultrasound, using both long-wave (45-kHz) and short-wave (1-MHz) frequencies at a range of intensities, to the surface of the egg. Angiogenesis was assessed quantitatively by three independent observers.* *Results:* *Both ultrasound methods showed evidence of an angiogenic effect compared to controls. The most effective results were seen with direct application of a 45-kHz wave at an intensity of 15 mW/cm^2 and indirect application of the media of fibroblasts ultrasonicated at 1 MHz with an intensity of 0.4 W/cm^2.* *Conclusion:* *This model confirms that ultrasound can induce neoangiogenesis in vivo.* INT J ORAL MAXILLOFAC IMPLANTS 2009;24:591–596

Key words: *angiogenesis, bone, chorioallantoic membrane culture, osteogenesis, ultrasound*

Ultrasound is a physical wave form that mechanically perturbs the transmitting tissue. It is used in clinical practice at frequencies of 1 to 20 MHz for diagnostic purposes and lower frequencies (from 45 kHz to 3 MHz) to enhance healing. The 1- to 3-MHz ultrasound range has been shown to stimulate tissue regeneration,[1–3] healing of ischemic varicose ulcers,[1] tendon repair,[4] angiogenesis in full-thickness incisions in the flank skin of adult rats,[5] and to accelerate fracture repair in animals[6,7] and humans.[8] Mandibular osteoradionecrosis is radiation-induced ischemic necrosis of the jaw, which can be a serious intractable problem. In an uncontrolled series of 22 patients with mandibular osteoradionecrosis, the application of ultrasound proved to be effective in inducing healing.[9]

In bone repair, both osteogenesis and angiogenesis are essential. Earlier work has shown that the therapeutic range of ultrasound stimulates bone formation,[10] osteoblast proliferation,[11] and the synthesis of angiogenic vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), and interleukin 8.[12] Further work has suggested that ultrasound stimulates bone formation by increasing the ratio of osteoprotegerin (OPG) to the receptor activator of nuclear factor kappa B ligand (RANKL) in osteoblasts,[13] because the former is osteogenic and RANKL activates osteoclast formation. In addition, ultrasound stimulates the production of nitric oxide in human osteoblasts.[14] This is of clinical interest, as the L-arginine nitric oxide pathway is also involved in the mechanical stimulation of bone formation.[15]

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The present investigation was performed to confirm that ultrasound stimulates angiogenesis in vivo using the chorioallantoic membrane (CAM) of fertilized chicken eggs.

MATERIALS AND METHODS

Two methods were used to induce angiogenesis, and the effect of two ultrasound machines was compared: a "traditional" short-wave 1-MHz machine (Therasonic 1032, EMS) pulsed 2:8 at intensities of 0.1, 0.4, 0.7, and 1.0 W/cm$^{2(SAPA)}$ (spatial average pulse averaged); and a 45-kHz continuous long-wave machine (Phys-Assist Unit, Orthosonics) at intensities of 5, 15, 30, and 50 mW/cm$^{2(SA)}$ (spatial average). The 1-MHz machine was calibrated by the Department of Medical Physics at University College London and the 45-kHz machine was calibrated by the manufacturer prior to use.

Ethical approval was obtained from the Research and Ethics Committee of the Eastman Dental Institute. Home Office Animal Licenses were granted to the three principal investigators (SM, RR, and PR), who also underwent a statutory training program to carry out the CAM culture experiments.

Indirect Ultrasound Treatment
Fibroblast Cultures. Fibroblasts were grown from explants of human gingiva obtained during routine oral surgery, as described by Reher et al.[12] Fibroblasts between passages 6 to 12 were plated out at a density of 1.5×10^5 cells per well in six well plates (Corning).

Stimulation of the Fibroblasts by Ultrasound. The six-well plate containing confluent fibroblasts was placed in a thermostatically controlled water bath set at 37°C. The water bath was lined with ultrasound-absorbing rubber and filled with deionized water. The plate was allowed to float freely on the surface; air bubbles between the plate and the water surface were eliminated. Each well had 5 mL of medium; the distance between the ultrasound transducer head and the cells was 5 mm. The transducer head was held by a stand, placed on a rotating platform (model KL2, Edmund Bühler), and set to 30 rotations/minute to avoid standing wave formation in the media.

As stated, two machines were used: the 1-MHz machine at intensities of 0.1, 0.4, 0.7, and 1.0 W/cm$^{2(SAPA)}$ and the long-wave 45-kHz machine at intensities of 5, 15, 30, and 50 mW/cm$^{2(SA)}$. Before the experiment began, the transducer head was swabbed with 70% isopropyl alcohol (Merck Europe); it was then immersed vertically into the culture well,

just touching the surface of the medium. Each well was sonicated for 5 minutes; the control sham-sonicated wells were treated with the generator switched off. The wells were monitored using a thermocouple to confirm that the temperatures of the ultrasound groups were not different from those of the controls. The plates were incubated for 24 hours at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. The media were then removed and stored at 700°C until required.

Preparation of the Microcellulose Disks. A 20-µL aliquot of sonicated medium was mixed with an equal volume of autoclaved 2% methylcellulose and allowed to dry. The positive control was recombinant human VEGF (rhVEGF) (sf-21; R&D Systems Europe), 0.5 µg per disk. Distilled water was used as a negative control, and sham-sonicated medium was used as the internal control. The methylcellulose disks were carefully lifted from the dish, placed on a 9- to 10-day chick embryo CAM, and incubated for 3 days.[16]

Direct Ultrasound Treatment
Fertilized *Gallus domesticus* Plymouth Rock White Leghorn eggs (Poyndon Farm) were incubated at 37°C in a humidified atmosphere (relative humidity ~70%) for 9 to 10 days and swabbed with 70% isopropyl alcohol; ultrasound gel was then applied (Aquasonic 100, Parker Laboratories). Sonication was carried out for 5 minutes with the 1-MHz machine at intensities of 0.1, 0.4, 0.7, and 1.0 W/cm$^{2(SAPA)}$ and with the long-wave 45-kHz machine at intensities of 5, 15, 30, and 50 mW/cm$^{2(SA)}$. The eggs were then incubated for 3 days and treated as described in the following section. The control groups were treated with the ultrasound generator switched off.

Chick CAM Assay
The angiogenic activities of the sonicated cell media were evaluated by the chick CAM assay as described by Boshoff et al[16] and Oikawa et al.[17] Three days after treatment, the CAMs were examined by a stereoscope (Olympus). A previously prepared window was enlarged for viewing, and a contrast medium (Intralipos, Midori-Juji) was injected into each CAM. This produced a dramatic contrast of red vessels against a white background, facilitating observation.[16] The CAMs were photographed, coded, and labeled randomly for blind evaluation. Angiogenic responses were graded independently by three investigators as negative or positive on the basis of the infiltration of blood vessels.[16] No proliferation of blood vessels was scored 0, and positive proliferation was scored 1. It should be noted that this is a semiquantitative measure of neovascularity.

Table 1 Results of the Indirect Ultrasound Technique

Ultrasound treatment intensities	No. of embryos	Angiogenic response		% angiogenic response	Odds ratio	Risk ratio	Log odds
		+VE	−VE				
1 MHz at 0.1 W/cm^2	9	6	3	67	2.8	1.6	1.096
1 MHz at 0.4 W/cm^2	6	6	0	100	>100	2.4	> 100
1 MHz at 0.7 W/cm^2	8	7	1	87	9.8	2.1	2.22824
1 MHz at 1.0 W/cm^2	6	4	2	67	2.8	1.6	1.0296
45 kHz at 5 mW/cm^2	12	6	6	50	1.4	1.2	0.3365
45 kHz at 15 mW/cm^2	8	4	4	50	1.4	1.2	0.3365
45 kHz at 30 mW/cm^2	12	7	5	58	1.96	1.4	0.6729
45 kHz at 50 mW/cm^2	12	7	5	58	1.96	1.4	0.6729
Water (no ultrasound)	5	2	3	40	0.93	0.96	−0.069
VEGF (0.5 µg/mL)	5	4	1	80	5.6	1.92	1.7228
Sham ultrasound control	12	5	7	42			

Angiogenesis following the application of methylcellulose disks containing water, VEGF, and sham sonicated fibroblast media as controls compared with conditioned media from fibroblasts sonicated by the 1-MHz and 45-kHz machines at the stated intensities. Unanimously positive embryos were scored as 1, unclear or split judgment as 0.5, and unanimously negative embryos as 0. The angiogenic response is presented as the positive score of each group as a percentage of the potential total score for that group. (+VE score/no. of embryos × 100). The highest response was observed with the 1-MHz treatment. Odds ratios, risk ratios, and log odds are versus sham ultrasound control.

Fig 1 Data regarding angiogenesis following indirect ultrasound application to methylcellulose disks (see Table 1), with the 95% confidence interval for the controls shown as horizontal dotted lines. The 1-MHz data lie outside or close to the control confidence region, indicating evidence of a significant effect at this frequency. The confidence intervals for the 1-MHz data (not shown) are reasonably tight and support the evidence that the 0.4- and 0.7-W/cm^2 intensities produce a greater effect than the 0.1- and 1.0-W/cm^2 intensities. The 45-kHz frequency produced a weaker effect and, at these sample sizes, there was no evidence of a significant difference versus the controls. S-US = sham ultrasound; VEGF = vascular endothelial growth factor.
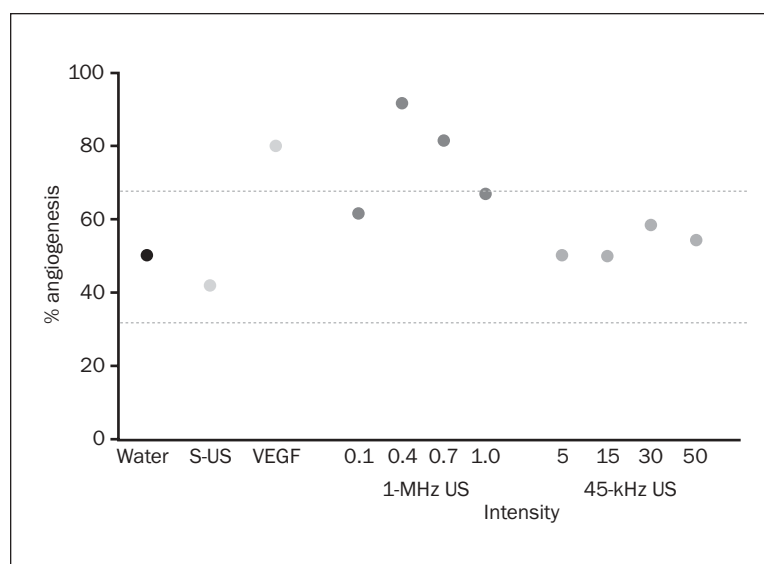
RESULTS

Following application of the fibroblast media/methylcellulose disks on the CAM, the 1-MHz frequency produced a significant angiogenic response at intensities of 0.4 and 0.7 W/cm^2. The angiogenic response ranged from 67% to 100%, whereas the angiogenic response to the positive VEGF control was 80%. The angiogenic response to the 45-kHz frequency was not significant, ranging from 40% to 58%; the response by the sham ultrasound control was 42% (Table 1, Fig 1). Some embryos in the indirect ultrasound group did not survive (initial n = 12 in each treatment group, except for the water and VEGF groups, n = 5).

Direct application of ultrasound on the egg resulted in enhanced angiogenesis in all the test groups (range, 66.7% to 85.7%). The 45-kHz frequency gave better results at intensities of 15 and 30 mW/cm^2 (Table 2, Fig 2).

Table 2 Results of the Direct Ultrasound Technique

Ultrasound treatment intensities	No. of embryos	Angiogenic response +VE	Angiogenic response −VE	% angiogenic response	Odds ratio	Risk ratio	Log odds
1 MHz at 0.1 W/cm^2	12	8	4	66.7	3.667	1.89	1.2993
1 MHz at 0.4 W/cm^2	12	8	4	66.7	3.667	1.89	1.2993
1 MHz at 0.7 W/cm^2	12	9	3	75	5.5	2.125	1.7047
1 MHz at 1.0 W/cm^2	12	8	4	66.7	3.667	1.89	1.2993
45 kHz at 5 mW/cm^2	12	8	4	66.7	3.667	1.89	1.2993
45 kHz at 15 mW/cm^2	12	10	2	85.7	9.167	2.36	2.2156
45 kHz at 30 mW/cm^2	12	10	2	85.7	9.167	2.36	2.2156
45 kHz at 50 mW/cm^2	12	8	4	66.7	3.667	1.89	1.2993
Sham ultrasound control	17	6	11	35.3			

Angiogenesis following direct ultrasound application to the egg surface. Controls were sham sonicated. Unanimously positive embryos were scored as 1, unclear or split judgment as 0.5, and unanimously negative embryos as 0. The angiogenic response is presented as the positive score of each group as a percentage of the potential total score for that group (+VE score/no. of embryos × 100). All showed an overall angiogenic response; this was greater with the 45-MHz machine. Odds ratios, risk ratios, and log odds are versus sham ultrasound control.
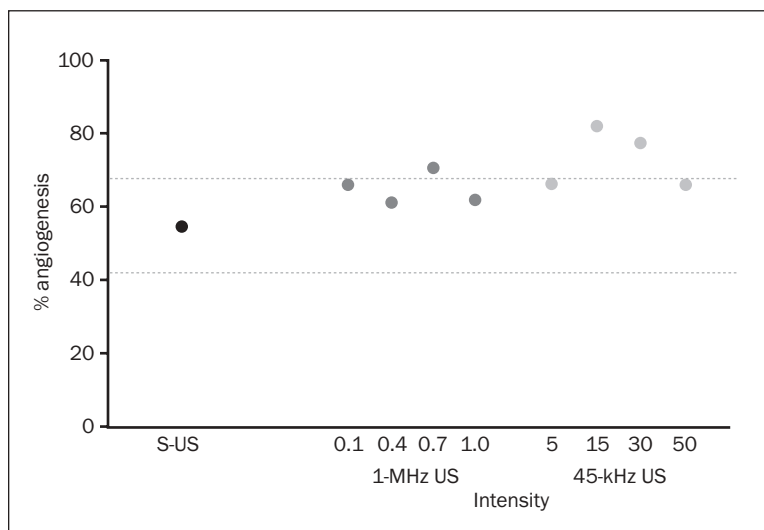

Fig 2 Angiogenesis following direct application of ultrasound. There is strong evidence for a difference between the 45-kHz-frequency treatments and the controls, with the 15- and 30-mW/cm^2 intensities showing the best effect. The 1-MHz-frequency treatments were close to significance. S-US = sham ultrasound.

DISCUSSION

Ultrasound is the generation of sound waves with a frequency above the limit of human audibility of 20 kHz that transfers mechanical energy into the tissues; it is used extensively in sports medicine and physiotherapy. The traditional 1- to 3-MHz frequency has a penetration of up to 2 cm. As penetration is inversely proportional to frequency, the 45-kHz long-wave machine has a theoretical advantage of penetrating tissues up to 10 cm. As shown, ultrasound can induce angiogenic and bone morphogenetic factors and bone formation in vitro.[11] These ultrasound effects would appear to be mediated by core binding factor 1, a key transcription factor that appears to induce the differentiation of the osteoblast and promote osteogenesis.[18,19] Core binding factor 1 also mediates the nitric oxide regulation of matrix metalloproteinase 13, which is responsible for extracellular matrix remodeling.[20] In vitro ultrasound has also been shown to up-regulate the release of the osteogenic cytokine OPG and downregulate RANKL, the ligand of the receptor activator nuclear factor kappa B, which recruits and activates osteoclasts. The net result is enhanced bone formation, as seen by increased osteocalcin and alkaline phosphatase.[13] This complex system of bone regeneration by ultrasound has been reviewed by Claes and Willie[21] and ter Haar.[22]

Fig 3 Examples of the CAM angiogenic response after direct ultrasound application to the egg. (*Left*) Response to sham ultrasound; (*right*) positive response to ultrasound. The pictures were taken 3 days after treatment, when the shell window was enlarged and a 20% fat emulsion was injected to enhance the contrast between the blood vessels and the background.
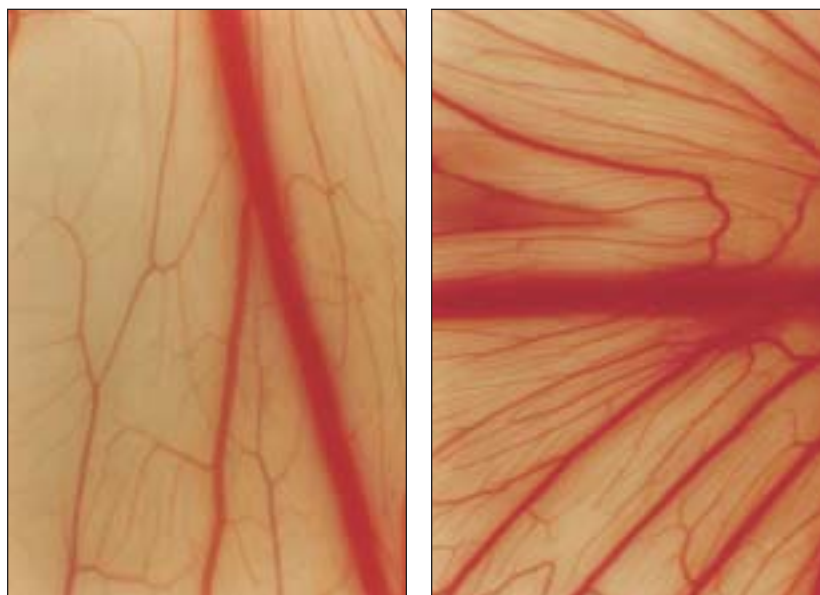
Both ultrasound machines produced significant results, and the best results were observed at 15 and 30 mW/cm$^{2(SA)}$ with the 45-kHz machine and at 0.1 and 0.4 W/cm$^{2(SAPA)}$ with the 1-MHz machine. Previous work by the present authors has shown that ultrasound induces osteoblasts and fibroblasts to synthesize angiogenic factors.[11] Sonicated macrophages have also been shown to play an important role in stimulating angiogenesis with their formation and release of the angiogenic factors FGF and tumor necrosis factor alpha.[23]

The CAM is an ideal assay for the study of angiogenesis in vivo because it is inexpensive and reproducible.[16] Normal vascular growth is rapid in the chick embryo from days 4 through 9, but it slowly decreases and virtually ceases by day 11.[24] This implies that the promotion of angiogenesis is more responsive in the younger embryo. This in vivo experiment clearly showed the potential of ultrasound to promote neoangiogenesis (Fig 3). The greater effect via direct application is not surprising because it does not rely on application of the disk with an unknown optimum concentration of the angiogenic factors to the CAM of unknown permeability. As will be noted, the concentration of the positive control, VEGF, was 0.5 μg/mL, whereas the concentration induced in the fibroblast cultures was only 250 pg/ (3×10^5) cells.

The best overall angiogenic response by the sonicated fibroblast medium incorporated into methylcellulose disks was with the 1-MHz machine at an intensity of 0.4 W/cm$^{2(SAPA)}$, followed by the 1-MHz machine at 0.7 W/cm$^{2(SAPA)}$. The rationale for using three controls was to exclude the possibility of the methylcellulose itself causing a hyperemic response in the CAM. However, each control showed a different angiogenic response: water, 50.0%; VEGF, 80.0%; and the sham-sonicated control, 41.7%. There were no detectable thermal changes in the wells after sonication. In the direct treatment, the best angiogenic response was with the 45-kHz machine at an intensity of 15 mW/cm$^{2(SA)}$, followed by 30 mW/cm$^{2(SA)}$, and with the 1-MHz machine at 0.7 W/cm$^{2(SAPA)}$. Thermal changes were not measured; however, embryo survival at 0.4, 0.7, and 1 W/cm^2 suggested no adverse effects. The authors have published elsewhere the detailed response of a variety of cells treated with ultrasound, which were shown to synthesize a range of angiogenic factors, including nitric oxide measured as nitrite,[12,14] all of which would explain the mechanism of the observed angiogenesis in these experiments.

As stated, ultrasound has proven to be clinically valuable for the revascularization of mandibular osteoradionecrosis[9] and appears to be more effective than hyperbaric oxygen, which is expensive, not readily available, and of disputed relative value.[25] Other potential applications are to improve implant integration in the irradiated mandible or maxilla, diabetic individuals, uncontrolled heavy smokers, or perhaps after intravenous bisphosphonate therapy, when implant integration is at risk as a result of avascular osteonecrosis. Ultrasound is readily available, inexpensive, and acceptable to all patients, even when administered 10 minutes daily for 6 weeks. This has been also been suggested by Li et al.[26]

Animal studies on the use of ultrasound to enhance osseointegration include a study by Tanzer et al,[27] which showed that ultrasound enhanced bone growth in dog femora containing porous titanium implants. Similarly, Hantes et al[28] observed accelerated healing of experimental osteotomies in sheep tibiae that were stabilized with midshaft bicortical steel pins. On this basis, ultrasound should be considered to have angiogenic and osteogenic value both before and after dental implant placement. Hence, these in vivo findings can be considered clinically important.

ACKNOWLEDGMENTS

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