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Original Paper

Therapeutic Ultrasound for Osteoradionecrosis: an *In Vitro* Comparison Between 1 MHz and 45 kHz Machines

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Mandibular osteoradionecrosis is a serious chronic complication which may follow radiotherapy. Therapeutic ultrasound is a highly effective, inexpensive and readily available means of promoting revascularisation and healing. 'Long wave' ultrasound increases penetration depth and, therefore, seems to be more appropriate than traditional high frequency ultrasound. The aim of this study was to compare a new treatment using 45 kHz with the current standard 1 MHz machine. A traditional 1 MHz machine, pulsed 1:4, at intensities of 0.1, 0.4, 0.7 and 1.0 W/cm$^{2(SAPA)}$ was compared with a long wave machine, 45 kHz, at intensities of 5, 15, 30 and 50 mW/cm$^{2(SA)}$. The ultrasound was applied to human gingival fibroblasts and mandibular osteoblasts *in vitro*. Cell proliferation (DNA synthesis) and collagen and non-collagenous protein synthesis assays were performed, using radiolabelled thymidine and proline, respectively. Controls were sham-insonated and all readings were given as a percentage of controls. Fibroblast proliferation increased by 47% at 0.7 W/cm^2 (1 MHz) and by 43% at 50 mW/cm^2 (45 kHz), and osteoblast proliferation increased by 52% at 1.0 W/cm^2 (1 MHz), and by 35% at 30 mW/cm^2 (45 kHz). Fibroblast collagen production increased by 48% at 0.1 W/cm^2 (1 MHz), and by 44% at 15 mW/cm^2 (45 kHz) and osteoblast collagen production increased by 55% at 0.1 W/cm^2 (1 MHz) and by 112% at 30 mW/cm^2 (45 kHz). Long wave ultrasound was, therefore, capable of inducing a comparable or even higher enhancement of bone formation compared with traditional ultrasound, which, with its greater penetration, may accelerate the healing effect of ultrasound on osteoradionecrosis. The suggested intensity for 45 kHz ultrasound is 30 mW/cm^2.

Key words: bone healing, cancer, cell proliferation, collagen synthesis, osteoradionecrosis, radiotherapy, radiotherapy effects, therapeutic ultrasound
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INTRODUCTION

RADIOTHERAPY IS an essential treatment modality for oral and head and neck malignant neoplasms. Unfortunately, it induces alterations in the normal tissues, resulting in early and long-term complications. Mandibular osteoradionecrosis is the most serious long-term complication of radiotherapy, with a variable incidence, ranging from 2 to 44.2%. With adequate prevention, the incidence is still around 2–5%. Treatment modalities include curettage and antibiotics [1, 2], hyperbaric oxygen therapy [3, 4], resection and reconstruction and, more recently, revascularisation with therapeutic ultrasound, which was successfully introduced by Harris in 1992 [5].

Ultrasound is the term applied to sound waves, the frequency of which is above the limit of human audibility, which is approximately 20 kHz. Ultrasound is a propagating pressure wave that can transfer mechanical energy into the tissues. Its applications can be divided into diagnostic, surgical and therapeutic [6]. Diagnostic ultrasound employs a

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frequency between 3 and 5 MHz, and very low intensities (1–50 mW/cm^2) are used to avoid tissue heating. Surgical ultrasound (disruptive) uses very low frequencies (20–60 kHz) and very high intensities (above 8 W/cm^2) although other surgical non-disruptive applications use high frequencies (HIFU operates at 0.5–3 MHz and at kW/cm^2). As the term suggests, therapeutic ultrasound is used principally in physiotherapy. This can be through its ability to generate heat, and also the 'activation' of tissues, the so-called non-thermal effects. Several non-thermal applications have been described in the literature, and some soft tissue applications include the stimulation of tissue regeneration [7–10], enhanced blood flow in chronically ischaemic muscles [11], protein synthesis in fibroblasts [12, 13], the healing of ischaemic varicose ulcers [8], tendon repair [14] and angiogenesis in full thickness excised incisions in the flank skin of adult rats [15].

Ultrasound effects in bone have also been evaluated. Accelerated repair in fibula fractures has been reported, with the best results when treated with 1.5 MHz, using 0.5 W/cm^2 [16]. Similarly, Tsai and colleagues [17] showed that low intensities of ultrasound (1.5 MHz, pulsed, 0.5 W/cm$^{2(SAPA)}$) stimulate fracture healing in a rabbit fibula model. However, they also showed a deleterious effect when ultrasound was applied at 1.0 W/cm$^{2(SAPA)}$. For clarity, the intensity measurements used here are spatially averaged intensity (SA), used for continuous ultrasound, and spatial average pulsed averaged (SAPA), for pulsed ultrasound.

The use of much lower intensities (diagnostic range) has been recommended for the acceleration of the normal fracture repair process (1.5 MHz, pulsed, 30 mW/cm$^{2(SAPA)}$) [18–20]. Using a rat femur fracture model, Yang and associates [21] observed greater torque and torsional stiffness after treating the fractures with 0.5 MHz ultrasound, which reached significance at 50 mW/cm$^{2(SAPA)}$. They also noted a shift in the expression of genes associated with cartilage formation: aggrecan gene expression was significantly higher on day 7 after fracture, and significantly lower on day 21. The first controlled clinical trial investigating the effectiveness of low intensity pulsed ultrasound (1.5 MHz, 30 mW/cm$^{2(SAPA)}$), to evaluate the healing of tibial fractures on humans was reported in 1994 [22]. The treated group showed a significant decrease in the time to clinical healing (86 ± 5.8 days) as compared with the control group (114 ± 10.4 days).

As mentioned above, we have established the use of ultrasound as an important means of revascularisation of mandibular osteoradionecrosis [5]. Patients were treated with ultrasound (3 MHz, pulsed 1:4, 1 W/cm$^{2(SAPA)}$) for 40 sessions of 15 min/day. 10 out of 21 (48%) cases showed healing when treated with debridment and ultrasound alone. 11 cases showed lesser healing after ultrasound therapy, but healed completely after debridment and cover with a local flap, and only 1 needed mandibular resection and reconstruction. These results are superior to the conventional treatment with hyperbaric oxygen therapy and surgery [23], where hyperbaric oxygen alone only achieved complete healing of osteoradionecrosis in 15% of cases, and 70% required resection and major reconstruction. Using near infrared spectroscopy, it has been demonstrated that patients with osteoradionecrosis who received ultrasound therapy showed significant improvements in metabolic activity, as measured by an increase in their deoxyhaemoglobin concentrations [24]. Bone formation stimulation was also observed *in vitro*, using 3 MHz pulsed 1:4 ultrasound, with the best results at low intensities (0.1 W/cm$^{2(SAPA)}$) [25].

Recently, a new ultrasound device has been developed, which instead of using the traditional frequencies of 1–3 MHz, uses 'long wave' ultrasound, at 45 kHz [26]. This lower frequency/long wavelength combination gives a widely divergent field shape, with the treated volume effectively in the far field region. This wave penetrates much deeper into the tissues, reaching areas as deep as several centimetres, instead of millimetres as with the megahertz machines. In order to minimise heating effects, it uses low intensities (5–50 mW/cm$^{2(SA)}$).

The purpose of this study was to evaluate *in vitro* this long wave machine, comparing it with a traditional 1 MHz ultrasound machine. The parameters evaluated were cell proliferation (DNA synthesis), and collagen/non-collagenous protein synthesis, in fibroblasts and osteoblasts.

MATERIALS AND METHODS

Cell cultures

The cell types used in the experiments were human gingival fibroblasts and mandibular osteoblasts. The fibroblasts were cultured from gingival tissue specimens obtained from patients admitted for planned dental extractions and/or surgical removal of wisdom teeth. The osteoblasts were cultured from bone obtained from mandibular osteotomies performed for surgical removal of wisdom teeth. All patients had no known diseases, and were 20–30 years of age. The specimens were rinsed several times with phosphate buffered saline (PBS, Gibco, Paisley, U.K.), minced and cultured in 75 cm^2 culture flasks using Dulbecco's modified Eagle medium (DMEM), complemented with heat-inactivated fetal bovine serum (HIFBS) 10% v/v (Sigma, Dorset, U.K.), freshly prepared L-ascorbic acid 50 μg/ml (Sigma), L-glutamine 2 mM (Sigma), and penicillin/streptomycin 100 U/ml each (Gibco). The flasks were transferred into a humidified 5% CO_2/95% air incubator at 37°C. After approximately 10 days, the cells started to grow out of the explants, and the media was changed twice a week. When the cells were confluent, they were trypsinised (0.25% w/v trypsin in PBS) and divided 1 in 3. The cells were used between the sixth and 10th passage for the fibroblasts and between the fourth and eighth passage for the osteoblasts. For the proliferation assays, they were plated in 6 well plates (Corning, New York, U.S.A.) at 1.5×10^5 cells/well and for the collagen assays at 3×10^5 cells/well. Each well was filled with 5 ml of the media used for the cell cultures, but with different concentrations of HIFBS. For the cell proliferation assays (DNA synthesis), the media used contained 2% HIFBS, the positive control group had 10% HIFBS. In the collagen assays, the media used contained 10% HIFBS. The cells were insonated the following day.

The ultrasound machines evaluated

A 'traditional' ultrasound machine, that uses a frequency of 1 or 3 MHz, and a 'long wave' machine, that uses a frequency of 45 kHz, were used. The 'traditional' ultrasound machine was a Therasonic 1032 unit, produced by E.M.S., Oxfordshire, U.K. This apparatus can be set to work with 1 or 3 MHz, and can deliver an intensity ranging from 0.1 to 2.0 W/cm^2. It also has a pulsing facility, and can be set to continuous or pulsed mode, pulsing 1:2, 1:4 or 1:9. The machine has an electronic control panel, a facility to do an electronic check each time it is switched on, and an alert signal

if there is no coupling gel or liquid. The handset head has a flat surface and an effective radiating area of approximately 2.0 cm^2. The apparatus was set to 1 MHz, pulsed 1:4 (2 msec 'on' and 8 msec 'off') and the intensities evaluated were 0.1, 0.4, 0.7 and 1.0 $W/cm^{2(SAPA)}$. Several calibrations were performed during the experiments, but at least once before and after each set of assays. The calibration was performed at the Department of Medical Physics, University College London. At each calibration, a full electronic check-up was performed, according to the manufacturer's manual. The acoustic output power was measured/calibrated using a precision ultrasound balance (E.M.S. model 67). After setting up the balance and warming up the ultrasound machine, the measurements were taken at 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 W/cm^2. The calibration was considered in the admissible range if the error in accuracy of the output readings was $\leq 10\%$.

The 'long wave' ultrasound machine (45 kHz) used in this study was a Phys-Assist unit, produced by Orthosonics (Ashburton, Devon, U.K.). This apparatus has a fixed frequency of 45 kHz, and can deliver an intensity ranging from 5 to 50 $mW/cm^{2(SA)}$. It does not have a pulsing facility, working in the 'continuous' mode, which at low intensities does not produce harmful tissue damage. The machine also has an electronic control panel, with a liquid crystal display, and a facility to calibrate itself each time it is switched on. The handset head type is conic and has an effective radiating area of approximately 12.8 cm^2. The intensities evaluated were 5, 15, 30 and 50 $mW/cm^{2(SA)}$. Calibration was performed several times during the experiments, but at least once before and after each set of assays. The calibration was performed at Orthosonics Ltd, and was considered satisfactory if the acoustic intensity ranged from 45 to 55 mW/cm^2, at power 4, and corresponding values at the other settings.

The ultrasound application model

A thermostatically controlled water bath (Electrothermal, London, U.K.) was used to maintain a constant temperature of 37°C during the assays. The tank had an internal diameter of 20 cm and a depth of 5 cm and was covered on the inferior and side walls with ultrasound-absorbing rubber. The water-bath was filled with distilled, de-ionised, demineralised water which was changed before each experiment. The cells used in the experiments were prepared in 6 well culture plates, which had a diameter of 35 mm, and a plate thickness of 1 mm. The plates were placed floating directly over the water surface, taking care not to let any air bubbles form between the plate and the water surface (Figure 1). The transducer was held by a microscope stand, which was placed over a rotating platform/shaker (Edmund Bühler, Tübingen, Germany, model KL2), set to 30 rotations/min. In this way, the transducer was constantly moved while the ultrasound was applied, avoiding the production of standing waves. The whole apparatus (water bath, transducer head and the rotating platform) was set up in a sterile air flow cabinet (Microflow Pathfinder, Intermed, Andover, U.K.). The transducer head was swabbed with 70% isopropyl alcohol BB (Azowipe, Vernon Carus, Lancashire, U.K.), left to dry, and immersed vertically into the culture well, just touching the surface of the medium. Each well of the culture plate had 5 ml of medium, and in this way, the distance between the transducer head and the cells/bones was approximately 5–6 mm. The transducer head from the 45 kHz ultrasound machine has a conic shape, therefore, only approximately 2–3 cm^2 of the total area of 12.8 cm^2 was immersed. However, since most of the energy comes through the centre of the head, we believe that there was not much energy loss. The ultrasound was applied to five wells ($n = 5$) for each evaluated intensity. Each well was insonated for 5 min, and the control group was treated in the same way, but with the ultrasound generator switched off. After insonation, the plates were cultured for a further 18 h in the 37°C incubator.

Cell proliferation assay (DNA synthesis)

After 18 h in the incubator, 3.5 ml of the media was removed, the cells were radiolabelled with 5-^{3}H thymidine (specific activity 14.3 Ci/mmol, 1 ml/1 mCi, Amersham, Buckinghamshire, U.K.) to a final concentration of 0.5 μCi/ml in 1.5 ml of culture medium. The cells were re-incubated for a further 6 h, when the incubation was terminated. The media was removed, 1 ml of 5% trichloroacetic acid (TCA) was added to the wells, and the plates were kept in the fridge (4°C) for 2 h. The TCA was then removed, and the cells washed three times with PBS. Then, 300 μl of 0.5 M NaOH was added to each well, and left for 20–30 min at 4°C. This was removed and transferred to scintillation vials (Minitubes, Hughes and Hughes Ltd, Lancashire, U.K.) containing 200 μl of 0.5 M acetic acid. Scintillation fluid (NBS Biologicals, Cambridge, U.K.) was added to each tube, and radioactivity measured with a beta counter (Wallac 1409, Turku, Finland) with external standardisation, expressed in disintegrations per minute (DPM).

Collagen/non-collagenous protein synthesis assay

After 18 h in the incubator, 3.5 ml of the media was removed, the cells were radiolabelled with 5-^{3}H proline (specific activity 31.0 Ci/mmol, 1 ml/1 mCi, Amersham) to a final concentration of 2 μCi/ml in 1.5 ml of culture medium. The cells were re-incubated for a further 6 h, and thereafter 700 μl of the media was transferred to Eppendorf tubes containing 700 μl of 10% TCA (final concentration 5% TCA), and left at 4°C for at least 2 h. The tubes were centrifuged at 4°C (2500 rpm for 30 min) to remove unbound isotope and small peptides from the cells, and the supernatant discarded. The pellets were suspended in 1 ml 0.5 M acetic acid containing pepsin (0.5 mg/ml; EC 3.4.4.1, Sigma) and left at 4°C. Collagen was extracted using limited pepsin digestion (16 h). Rat acid soluble collagen was added (100 μl of collagen at 5 mg/ml in 0.5 M acetic acid) to act as a carrier for the newly

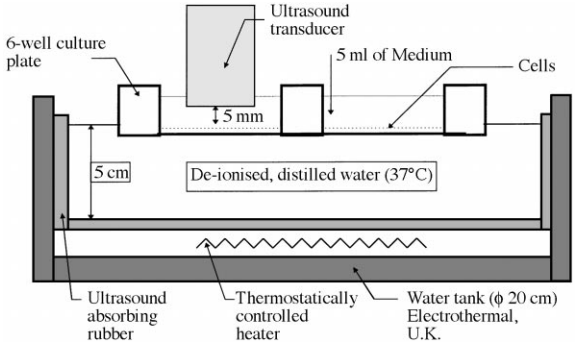

Figure 1. Schematic representation of the ultrasound insonation model used during the assays. The transducer was kept in motion to avoid the formation of standing waves, and inserted into the culture medium above the cells. These were plated into 6 well plates that were placed floating over the water tank (37°C). The whole system was placed in a sterile air flow cabinet, and insonation was applied for 5 min for each well.

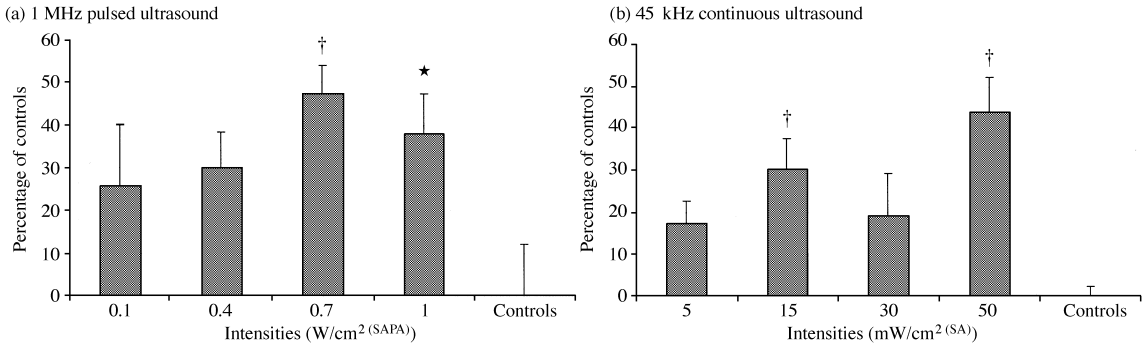

Figure 2. Fibroblast DNA synthesis (cell proliferation) induced by 1 MHz (a) and 45 kHz (b) ultrasound. Controls received the same treatment but with the ultrasound generator switched 'off'. Values are given as percentages of the controls. Standard error bars are shown. Significance of results: *$P < 0.05$, †$P < 0.01$.

formed collagen. Collagen was precipitated by the addition of NaCl to a final concentration of 5% (w/v) in 0.5 M acetic acid, for 3 h at 4°C. The tubes were mixed gently and centrifuged at 4,000 rpm for 30 min at 4°C. The supernatant containing the non-collagenous protein was stored in scintillation vial inserts (Minitubes, Hughes and Hughes Ltd). The pellets were redissolved in 1 ml of 0.5 M acetic acid, and the collagen reprecipitated with NaCl as described, for 2–3 h. The tubes were centrifuged again, at 5,000–8,000 rpm for 30 min at 4°C, and the second supernatant (non-collagenous protein) added to the first supernatant. The final precipitates with the purified collagen were resuspended in 400 µl of 0.5 M acetic acid and transferred into another disposable scintillation vial insert. Each scintillation vial was filled with 3 ml of scintillation fluid (NBS Biologicals) and radioactivity was measured as for the proliferation assays.

Statistical analysis

Each experiment was repeated at least twice. The number of observations for controls and for each intensity evaluated was five ($n = 5$). The values obtained in DPMs were transformed into percentages of the controls (sham-insonated), which were considered as 0%. All test values were compared with, and graphically presented as a percentage of control values. The results obtained were analysed using ANOVA single factor and Student's t test for unpaired samples.

RESULTS

Cell proliferation (DNA synthesis)

The cell proliferation assays showed an increase in DNA synthesis with both ultrasound machines. In the fibroblasts group treated with 1 MHz ultrasound (Figure 2a), the most significant results were an increase of 47% at 0.7 W/cm^2 ($P < 0.01$) and of 37% at 1.0 W/cm^2 ($P < 0.05$). In the group treated with 45 kHz (Figure 2b), increases of 30 and of 43% were observed, with 15 and 50 mW/cm^2, respectively ($P < 0.01$). When osteoblasts were treated with 1 MHz ultrasound (Figure 3a), again an increase in DNA synthesis at the higher intensities was observed. This was in the order of 34% at 0.7 W/cm^2 ($P < 0.01$) and of 52% at 1.0 W/cm^2 ($P < 0.001$). In the 45 kHz treated group (Figure 3b), a more uneven distribution occurred, showing an increase of 32% at 5 mW/cm^2, and of 35% at 30 mW/cm^2 ($P < 0.05$ and $P < 0.01$).

Collagen/non-collagenous protein synthesis

In these assays, there was a clear tendency for increased collagen production in the lower intensities when using 1 MHz ultrasound. The fibroblasts group treated with 1 MHz (Figure 4a) showed increases of collagen production of 48, 57 and 52%, at intensities of 0.1, 0.4 and 0.7 W/cm^2, respectively ($P < 0.01$, 0.05 and 0.01, respectively). When these cells were treated with 45 kHz ultrasound (Figure 4b), the last three intensities showed increases in collagen ranging from 37 to 44%, although significant only at 15 and 50 mW/cm^2 ($P < 0.01$ and $P < 0.05$). The collagen/non-collagenous protein production by osteoblasts were probably the most significant results, since these are the target cells involved in repair in osteoradionecrosis. In this case, a clear superiority of the 45 kHz ultrasound was observed. In the 1 MHz treated cells, the previously observed tendency to increase collagen production at the lower intensities continued (Figure 5a).

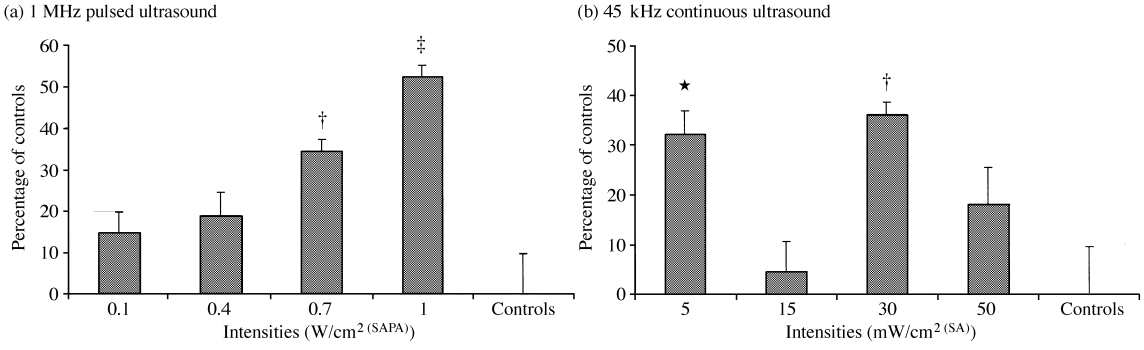

Figure 3. Osteoblast DNA synthesis (cell proliferation) induced by 1 MHz (a) and 45 kHz (b) ultrasound. Controls received the same treatment but with the ultrasound generator switched 'off'. Values are given as percentages of the controls. Standard error bars are shown. Significance of results: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

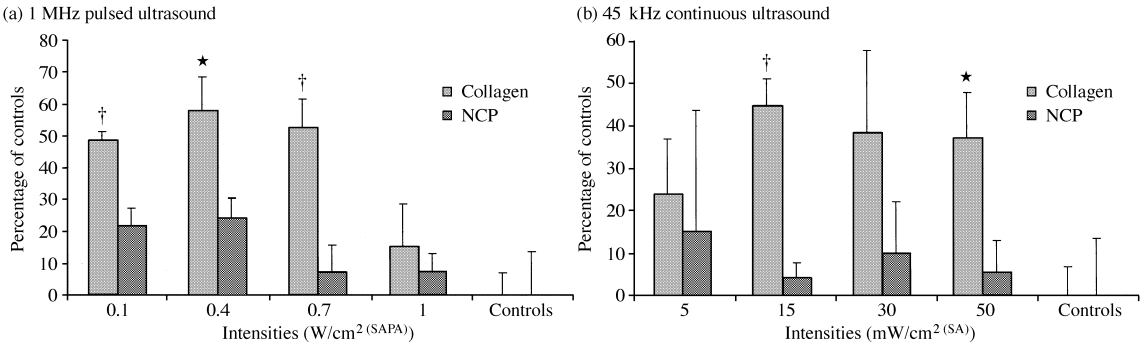

Figure 4. Fibroblast collagen and non-collagenous protein (NCP) synthesis induced by 1 MHz (a) and 45 kHz (b) ultrasound. Controls received the same treatment but with the ultrasound generator switched 'off'. Values are given as percentages of the controls. Standard error bars are shown. Significance of results: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

Increases of 55 and 38% at 0.1 and 0.4 W/cm^2, respectively, were observed in collagen synthesis ($P < 0.05$), and an increase in non-collagenous protein synthesis could also be detected, but significant only at 0.4 W/cm^2 ($P < 0.05$). With the 45 kHz ultrasound (Figure 5b), the amount of increased osteoblast production of collagen was much higher, at a magnitude of 112% at 30 mW/cm^2 ($P < 0.05$). Furthermore, the non-collagenous protein synthesis was also significantly increased in all intensities evaluated, ranging from 59 to 88% ($P < 0.01$).

DISCUSSION

After radiotherapy, the irradiated area is hypoxic–hypocellular and hypovascular [27]. The tissues have a complex metabolic/homeostatic deficiency, bordering an ischaemic necrosis, and are prone to breakdown, leading to a chronic non-healing wound, i.e. osteoradionecrosis [27]. Therefore, the treatment or prevention of this complication should aim to restore the normal soft tissue and bone vascularity. Ultrasound therapy has the capacity of inducing angiogenesis [15], blood flow in muscles [11], and healing of ischaemic varicose ulcers [8]. This facilitates repair and can stimulate soft tissue regeneration [7, 8, 10], healing of pressure sores [9], as well as accelerating bone healing [16–22].

Having successfully used high frequency ultrasound clinically and confirmed its value on bone healing *in vitro* [25], we decided to compare the effect of the low frequency, low intensity machine [26] with our conventional machine on parameters related to connective tissue healing. The proliferation assays (DNA synthesis) showed that both machines were able to induce proliferation, in fibroblasts and osteoblasts (Figures 2 and 3). This is a crucial effect, since osteoradionecrosis is a hypocellular tissue. However, this can also be interpreted as having a deleterious affect, since the cells may be involved in cellular division, and not in the production of collagen and other physiological proteins. This was observed when the cells were stimulated with the 1 MHz machine, when increased proliferation was observed for the same intensities that caused reduced collagen production (Figures 3a, 5a). The 45 kHz ultrasound revealed similar observations in fibroblasts, but on osteoblasts the proliferation was high at the same intensities which increased collagen synthesis.

The procedure for extraction and purification of the radiolabelled collagen was a modification of the method of Webster and Harvey for monolayer cultures [28]. The pepsin assay was based on the measurement of radioactively labelled collagen present in cells and culture medium after a 6 h pulse with ^{3}H-proline. This is achieved by extraction of native collagen using limited pepsin digestion, purification by salt precipitation and measurement of the radioactivity in the final preparation [29]. Non-collagenous protein is largely susceptible to degradation by pepsin and does not coprecipitate with the collagen. This assay, therefore, gives an estimate of the 'net' rates of collagen and non-collagenous protein synthesis over the duration of the proline pulse. One advantage of this assay is its simplicity, allowing large numbers of samples to be analysed, when compared with the established bacterial collagenase extraction assay of Peterkofsky and Diegelman [30], and is equally efficient [29].

In the current collagen/non-collagenous protein synthesis assays, both ultrasound machines induced collagen production.

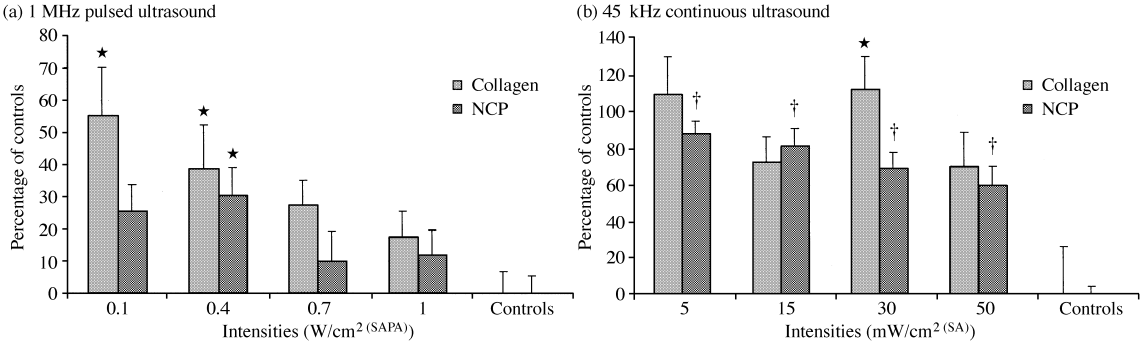

Figure 5. Osteoblast collagen and non-collagenous protein (NCP) synthesis induced by 1 MHz (a) and 45 kHz (b) ultrasound. Controls received the same treatment but with the ultrasound generator switched 'off'. Values are given as percentages of the controls. Standard error bars are shown. Significance of results: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

With the 1 MHz machine, this was more evident with lower intensities, both in fibroblasts and in osteoblasts (Figures 4a, 5a). However, in osteoblasts, the 45 kHz ultrasound machine produced much higher increases in collagen synthesis (up to 112%) than those for the 1 MHz machine (maximum of 55%) (Figure 5). These results are higher than those observed by Harvey and colleagues [12], who found an increase of both collagen and non-collagenous protein synthesis, which was intensity dependent, using human skin fibroblasts insonated in suspension and subsequently cultured *in vitro*. Fibroblasts exposed to continuous ultrasound ($0.5\,W/cm^{2(SA)}$) showed a 20% increase in collagen secretion, which was increased to 30% when the ultrasound was pulsed ($0.5\,W/cm^{2(SAPA)}$). Webster and associates [13] also observed lower increases in protein synthesis by fibroblasts of 29% using a 3 MHz signal at $0.5\,W/cm^2$. Reher and colleagues [25] using a mice calvaria model showed that 3 MHz pulsed ultrasound stimulates bone formation (collagen and non-collagenous protein production), with the best results at 0.1, 0.25 and $0.5\,W/cm^2$.

Non-collagenous protein synthesis was also stimulated, but it could not be correlated to the collagen synthesis in the majority of the assays. Furthermore, the non-collagenous protein synthesis increase reached significance only in osteoblasts, and mainly with the 45 kHz ultrasound (Figure 5b), where all intensities significantly stimulated the cells. This may be an important observation, as the non-collagenous protein contains many cytokines, growth and angiogenic factors and enzymes which may enhance healing and angiogenesis.

The molecular mechanisms by which ultrasound alters cell function or protein synthesis are still not known, although some mechanisms have been mentioned [21]. The compression of microtubules, or cavitation, producing oscillatory movement of microbubbles and acoustic streaming, could have a direct effect on the permeability of the cell membrane and enhance second messenger activity. Such changes in ion or protein transport could consequently modify intracellular signals for gene expression [13, 31–33]. The effects of mechanical pressure at the cell surface could also activate the 'stretch receptor' type of cation channel [34], and changes in cation concentrations could also modify intracellular signals regulating gene expression. Alternatively, mechanical energy transferred by the ultrasound might activate changes in the attachments of cytoskeleton to the extracellular matrix, affecting cell metabolism and gene expression [21]. Electrical currents in bone may be potentiated by exposure to ultrasound energy. Investigators have reported increased potentials as a function of ultrasound intensity, frequency and burst pattern [35–37]. Finally, a rise in temperature during ultrasonic exposure may have an effect on cell metabolism. The use of low intensity ultrasound reduces tissue heating, and also reduces the possibility of cavitation phenomena, i.e. the pulsation of gas or vapour filled voids in a sound field [38]. We have shown a maximum temperature rise of 1.8°C at $2.0\,W/cm^{2(SAPA)}$, but no measurable rise was observed at the best stimulatory dose of $0.1\,W/cm^{2(SAPA)}$ [25]. The use of 45 kHz ultrasound machines has been shown to produce less heating than the 1 MHz machine [39].

The intensity of ultrasound used in the clinical treatment of osteoradionecrosis [5] was relatively high (3 MHz, pulsed 1:4, $1\,W/cm^{2(SAPA)}$). The favourable results observed could, therefore, be explained in terms of ultrasound promoting heating or angiogenesis [15], rather than due to the effects on collagen protein synthesis, which is higher at lower intensities. This was supported by the use of near infrared spectroscopy scans of irradiated mandibles [24], which showed higher levels of deoxyhaemoglobin concentrations in the osteoradionecrotic mandibles of patients after treatment with this ultrasound regimen. This suggests significant improvements of the metabolic activity of the mandibular tissue, probably due to neoangiogenesis. However, since collagen and non-collagenous protein synthesis are also important in the reorganisation of the bone matrix, the lower intensities should be used in the treatment of osteoradionecrosis. With 45 kHz ultrasound, intensities between 15 and 50 $mW/cm^{2(SA)}$ seem appropriate as the best results in our series were achieved with $30\,mW/cm^{2(SA)}$.

The *in vitro* assays reported here demonstrated comparable effects on cell proliferation and collagen/non-collagenous protein synthesis for 1 MHz and 45 kHz. The best effects are achieved by the 45 kHz ultrasound, mainly in collagen/non-collagenous protein synthesis by osteoblasts. These results support the use of low intensity regimens. Our recommended ultrasound regimens are $0.1\,W/cm^2$ pulsed 1:4 if using 1 MHz, and $30\,mW/cm^2$ continuous insonation if using 45 kHz. However, we recommend the use of 'long wave' ultrasound for the treatment and prevention of osteoradionecrosis, due to its overall clinical and *in vitro* advantages.

In conclusion, 'long wave' ultrasound appears to have significant therapeutic advantages, which are that it has a much higher penetration depth, allowing treatment of thick tissues, penetrating centimetres rather than millimetres as with traditional ultrasound. It uses low intensity energy levels, causing less heat production, and is used in continuous mode, reducing the treatment time, with a spherical head, giving a large effective treatment area.

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