Ultrasound stimulation of mandibular bone defect healing

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Chapter 1

General introduction

Introduction

As long as humans exist, their bones have been at risk for fracturing by either injury or disease.[1] As a consequence, various strategies have been developed to ensure an undisturbed healing of the bone after a fracture has occurred. In Hippocrates' time, the principle of closed reduction and fixation of fractures was already known and applied,[2] and in the last century the principle of open (operative) reduction and internal fixation became well-established.[3] Nevertheless, bone healing does not always occur after the initial treatment of the fracture. To stimulate bone repair in these cases, different surgical and non-surgical strategies have been explored. A widely used surgical strategy is the secondary surgical intervention in which bone grafts are used to stimulate the healing process.[4] Non-surgical ways to stimulate fracture healing, by using for example electric and electromagnetic fields, can be found in early[5] and recent literature.[6] One relatively unknown way to influence bone healing is the use of ultrasound. Ultrasound therapy is based on the application of (micro)mechanical vibrations to the bone and bone cells. This treatment, using high frequency pressure waves, dates back more than 60 years and might eventually be used to stimulate maxillofacial bone healing.

Historical development

In France, at the beginning of World War I, the first ultrasound devices were constructed for military purposes to produce a high frequency sound wave for echo-location of submarines and measuring the depth of the sea.[7] It was found that fish died when exposed to a strong ultrasound field, and this lead to the investigation of other biological effects.[8,9] Therapeutic applications of ultrasound were initiated by Pohlman in 1938. In his opinion, the "root of disease lies in a stasis of metabolism" and ultrasound could eliminate this stasis by sending intense mechanical pulses through the bodily tissues. He constructed an ultrasound device that heated tissue locally (Figure 1). Empirically, ultrasound had been found beneficial in the treatment of various soft tissue disorders such as neuralgia's and myalgia's[10] and this started a widespread use of ultrasound therapy to treat almost any physical disorder. Because ultrasound treatment of soft tissues occasionally involved the irradiation of bony structures, there was need to study the influence of ultrasound on bone. Since bone has a higher density than muscle or fat, ultrasound energy is more easily absorbed in bone and this would lead to bone overheating and possible damage.

Table of contents

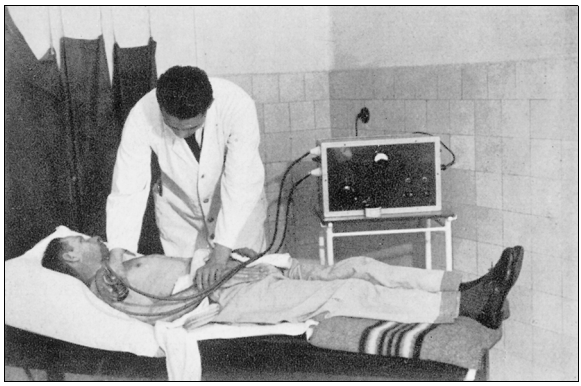

Figure 1. One of the first ultrasound treatments, conducted at the Martin-Luther-Krankenhaus in Berlin-Grunewald (1938). Most likely, the therapist is Dr. Pohlman.

Indeed, it was found in animal experiments that ultrasound could lead to bone sclerosis, growth retardation, cyst formation and spontaneous fractures.[11,12] Although the research on ultrasound and bone initially focused on bone damage,[11-14] Maintz (1950)[15] decided to focus on bone healing. In rabbits he attempted to stimulate fracture healing with ultrasound, but the ultrasound intensity was too high and resulted in bone damage. Later it became clear that ultrasound could in fact stimulate bone healing in certain cases when lowering the ultrasound intensity and by spreading the ultrasound energy over the tissue by moving the transducer across the skin during treatment.
After a few human case series in which ultrasound appeared to promote the healing of fractures in cases with a disturbed healing pattern,[16-18] Hippe and Uhlman (1953)[19] presented one of the first large series of 181 slow uniting fractures that were treated with ultrasound. In 154 cases (85%), healing was obtained by using 800 kHz ultrasound with an intensity of 1 - 1.5 Watts per square centimetre (W cm^{-2}) for five minutes every other day, in total for 10 - 12 times. Treatment was applied under water or using a viscous gel. The ultrasound was administered through a moving transducer. An example was presented of a 41-year-old crane driver who suffered from a pseudarthrosis of his left humerus (Figure 2a). After 16 ultrasound treatments, the fracture was clinically stable. At this time, the radiograph showed bridging of the fracture gap (Figure 2b) and complete union remained in the following years (Figure 2c).

non-union site. This low intensity pulsed ultrasound field was later used in the development of the Sonic Accelerated Fracture Healing System (SAFHS, Smith & Nephew, Exogen, TN, USA) (Figure 3).

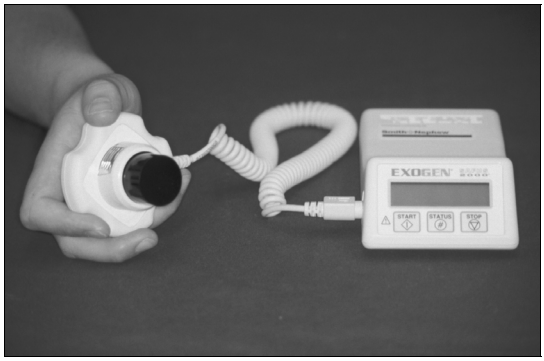

Figure 3. The Sonic Accelerated Fracture Healing System (SAFHS Model 2000®).

This is a battery-operated device, which emits a high frequency, pulsed, low intensity, ultrasound field. This ultrasound therapy is applied onto the skin overlying the fracture through a window made in the immobilisation plaster. In 1986 and 1987, two double blind randomised clinical trials were started with SAFHS ultrasound to treat fresh radial[23] and tibial fractures.[24] It was found that the time to union could be reduced up to 38%. The 20 minutes daily ultrasound treatment was also studied in large series of non-unions.[25] A non-union was defined as a fracture that did not show clinical or radiographic signs of healing for more than 256 days (9 months). The average fracture age in this group was 692 days. Overall, healing was obtained in 83% of 1546 cases of non-union. After an average of 136 days of ultrasound treatment, these fractures were healed. In further studies, the positive effect of ultrasound on bone healing was found in different species such as the rat,[26] rabbit,[27] dog,[28,29] and homo sapiens,[23,24] and in different circumstances such as fresh fractures,[23,24,30] delayed unions, non-unions,[25,30-32] osteotomies,[32] osteodistractions,[33-35] and in cases of osteoradionecrosis.[36] It has been reported that the pressure wave serves as a surrogate for physiological stresses in bone, which normally would stimulate bone formation.[37] On a more basic level, the therapeutic effect of ultrasound may be related to piezo-electric[38-41] and cell membrane effects,[42-44] or to effects on the angiogenesis.[45-48]
In 1994, the SAFHS device was approved by the American Food and Drug Administration (FDA) for 'the acceleration of the time to a healed fracture for

fresh, closed, posteriorly displaced, distal radius (Colles') fractures and fresh, closed or grade I open tibial diaphysis fractures in skeletally mature individuals when these fractures are orthopedically managed by closed reduction and cast immobilization',[49] and later also for 'non-unions excluding skull and vertebra'.[50] In the Netherlands, the SAFHS device has been approved by the Dutch Medical Council (Ziekenfondsraad) only for the treatment of fractures without a tendency to heal.[51] This approval was based on an estimated cost reduction in the management of these fractures of about Fl 10.000,- (Euro 4550,-) per patient when treated with ultrasound instead of surgical intervention. The treatment costs are, therefore, reimbursed by the Dutch health care providers.[51]

Therapeutic ultrasound in the maxillofacial region: focus on soft tissue disorders

In dentistry and maxillofacial surgery, ultrasound has largely been applied to treat various soft tissue and temporomandibular joint disorders. In the early years (1938 - 1949) ultrasound therapy of sinusitis, parotitis, trismus and trigeminal neuralgia had been advocated,[52] but little notice was given to it within the maxillofacial surgery profession outside Germany. In fact, Erickson's report published in an international journal (1964) to promote the use of ultrasound as a useful adjunct in temporomandibular joint therapy received little attention.[53] Apart from one report in the seventies,[54] it was not until the nineteen eighties that further research on maxillofacial therapeutic ultrasound was conducted. This research mainly concerned the reduction of postoperative edema[55-57] and the treatment of musculoskeletal disorders such as temporomandibular joint pain and myofascial pain in the head and neck region.[58-60] A definite effect of ultrasound could not be established. Ultrasound for the treatment of healing disturbances in the maxillofacial skeleton remained a curiosity. The few available reports concern the treatment of mandibular fractures in rabbits[61] and humans,[18] and the treatment of osteoradionecrosis of the mandible in humans.[36]

So, despite the reported positive effects of ultrasound on bone healing of the long bones, it may be concluded that little attention has been given to the possible effects of ultrasound on bone healing of the maxillofacial skeleton. If bone healing of the facial skeleton can be stimulated with ultrasound, several fields in maxillofacial surgery might benefit from this non-invasive therapy such as traumatology (accelerated fracture healing), oncology (treatment/prevention of osteoradionecrosis), implant surgery (accelerated implant osseointegration), and reconstructive surgery (bone defect healing, accelerated callus maturation after osteodistraction). However, this potential has not been investigated. Of the facial bones, the mandible is most frequently subject to fracture, osteoradionecrosis and in need of reconstructive pre-prosthetic surgery.

Especially the treatment of bone defects pose considerable challenges to the maxillofacial surgeon. It is therefore that the experiments presented in this thesis focus on the healing of bone defects of the mandible.

Aim of thesis

The aim of this study was to decide whether mandibular bone defect healing can be stimulated with low intensity pulsed ultrasound. This was done by:

1. Investigating the potential of ultrasound to stimulate maxillofacial bone healing in a literature review (Chapter 2),
2. Evaluating microradiography for the identification of bone/no-bone boundaries of rat mandibular defects (Chapter 3),
3. Using this technique to measure areas of bone growth into defects when exposed to low intensity pulsed ultrasound;
   - in plain mandibular defects in rats (Chapter 4.1),
   - in rat mandibular defects covered with expanded polytetrafluoroethylene (e-PTFE) membranes (Chapter 4.2),
   - and in rat mandibular defects covered with collagen membranes (Chapter 4.3),
4. Assessing if ultrasound can stimulate early bone formation in a distraction gap in the severely resorbed edentulous mandible in humans (Chapter 5).

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Chapter 2

Ultrasound stimulation of maxillofacial bone healing

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Abstract
A substantial part of the maxillofacial surgery practice deals with maxillofacial bone healing. In the past decades, low intensity ultrasound treatment has been shown to reduce the healing time of fresh fractures of the extremities up to 38% and to heal delayed and non-unions up to 90% and 83%, respectively. Based on the assumption that the process of bone healing in the bones of the extremities and maxillofacial skeleton is essentially the same, the potential of ultrasound to stimulate maxillofacial bone healing was investigated. Although limited evidence is available to support the susceptibility of maxillofacial bone to the ultrasound signal, ultrasound may be of value in the treatment of delayed unions, in callus maturation after distraction, and in the treatment of osteoradionecrosis.

Introduction

Disturbed bone healing is generally considered a serious medical problem because of the resulting impairment of function. Traditionally, bone healing disturbances have been associated with fractures. Nowadays, however, it may occur as an unwanted side-effect of several therapies, such as osteotomies, bone grafting, bone distraction, and therapeutic irradiation. Insight in the (patho)physiologic mechanisms of bone healing has lead to several interventions essentially intended to stimulate the healing process.[1] Of all the means to influence fracture healing, sound distinguishes itself by being non-invasive and easy to apply. Ultrasound as a treatment modality is traditionally used in the field of physiotherapy to treat soft tissue disorders by deep-heating of the tissue. This is accomplished by using intensities of 0.5 - 3.0 watts per square centimetre (W cm^{-2}). Despite its medical use for more than half a century, the efficacy of ultrasound in the treatment of various musculoskeletal disorders, such as temporomandibular disorders and myofascial pain, still remains to be established.[2] In contrast, the effect of ultrasound on the bone healing has become well established during the past decades. The intensities used in the treatment of fractures is considerably lower than those used in physiotherapy because of the risk of over-heating of bone. Currently, the most widely used device to treat patients with compromised fracture healing is the Sonic Accelerated Fracture Healing System (SAFHS) (Smith and Nephew, Exogen, Memphis, TN, USA). This device emits a pulsed sound wave of 1.5 MHz, with an average intensity over space and time of 30 milliwatts per square centimetre (mW cm^{-2}).

Since bone healing and regeneration are of particular interest in maxillofacial surgery, it is the aim of this manuscript to review current knowledge of the stimulation of bone healing by means of ultrasound, and to investigate the potential of its application in maxillofacial surgery.

Materials and methods

This review is based on a Medline search (Medline 1967-2001) using the following key words as free text, without language restrictions: bone healing, therapeutic, ultrasound, maxillofacial, TMJ, temporomandibular, myofascial. In addition, the register books of Strahlentherapie covering the years 1912-1967 (key-words: Ultraschall, Knochen, Kiefer, Mund, Schaden), and the text books of Cady,[3] Pohlman,[4] Wiedau and Röher,[5] Kolář et al.,[6] Urick,[7] Knoch and Knauth,[8] Wells,[9] Suslick,[10] and Knoch and Klug[11] were used. The reference lists in the obtained literature were traced for relevant additional publications. Furthermore, information was obtained from the Archives of the "Commission de la Défense Nationale", Academy of Sciences, Paris (France) and from the

internet site of the American Institute of the History of Physics. Publications that could not be traced were not included in this survey.

Review of current knowledge

Historical survey

The foundation of ultrasound was laid in 1880, when the brothers Jacques and Pierre Curie observed that certain crystals generated electricity when submitted to pressure at specific angles.[12] This observed piezo-electric effect can be reversed: by submitting the crystal to an alternating current at the resonance frequency of the crystal, it expands and contracts at this frequency. Thus, a high frequency sound wave is emitted. At the beginning of World War I, in France, Paul Langevin used this principle to perfect a submarine detector.[13] He was also the first to notice biological effects of ultrasound: fish put within a strong ultrasound field died after a period of violent movements, and pain of considerable severity was experienced when the hand was thrust into the water in the tank.[14] In 1938, in Germany, Pohlman expressed the opinion that ultrasound could be therapeutically, since the "root of disease lies in a stasis of metabolism" and that ultrasound could eliminate this stasis by sending intense mechanical pulses through tissues.[4] He was the first to construct a device for the treatment of patients. Soon after treating the first patients, it became clear on an empirical basis that lower back pain, neuralgia's, myalgia's and corresponding diseases responded favourably to the treatment.[15] The treatment regime involved exposure of the affected area for five to 10 minutes daily, for about 10 days. Because ultrasound hardly propagates through air, paraffin oil was applied on the skin to ensure an air free contact between the ultrasound treatment head and the skin. Ultrasound with a frequency of 800 kHz and an intensity of 4 - 5 W cm^{-2} was used. This intensity was high enough to cause tissue heating and just low enough to prevent burning pain. In Europe, the first reported successes led to a widespread medical use of ultrasound therapy in which virtually any disorder was considered to benefit from ultrasound treatment. However, the influence of ultrasound on bone received little attention at first, because bone was considered a limitation for the use of ultrasound. Studies concerning the influence of ultrasound on bone mainly focused on bone damage.

In 1949, it was proposed that bones of children should not be exposed to ultrasound.[16] Therapeutic doses were found to produce extensive bone-damage in young dogs, such as a decrease in size of the epiphyseal line, growth retardation and arrest, irregular bone structure, and spontaneous fracture with reactive periosteal bone formation.[16] These findings were not in agreement with that reported by Barth and Bülow who found that a painless dose did not

produce bone-damage.[17] Furthermore, bone-damage was not observed in clinical practice.[17] Others reported the same, but only on the condition that a moving ultrasound transducer was used.[18] However, a case of bone-damage involved the treatment of a lesion of the lower lip in a young woman with ultrasound with unknown intensity and which resulted in osteomyelitis of the alveolar bone.[19] Other experiments confirmed the damaging capabilities of ultrasound to bone.[20,21]

Interestingly, indications that ultrasound may stimulate bone growth can actually be found in studies that mainly concern bone damage.[16,22,23] A slight growth acceleration occurred when a young dog's leg was exposed to a fast moving ultrasound transducer.[16] A growth acceleration of the heel bone in a child when the apophysis was treated, as well as a growth acceleration after exposing roots of plants to ultrasound has been observed. In another study, the radiographic and histologic changes in bone exposed in water to 1 MHz ultrasound at different intensities has been described.[22] Limbs of young dogs and rabbits were treated for 5 minutes at a time. It was claimed that 1.2 W cm^{-2} for 5 minutes a day for 4 days was capable of producing bone changes leading to deformation of limbs. Epiphysis exposed to low intensity ultrasound responded with an acceleration of growth (i.e., widening of the epiphyseal line). Higher intensities slowed down the growth, whereas the highest intensities arrested bone growth. Another observation was that articular cartilage exhibited considerable hypertrophy following ultrasound treatment. The intensities used, however, were not specified.

In 1950, Maintz published the first study in which the relationship between ultrasound and bone healing was investigated.[24] This study marked a turning point in this research arena because the study focused on the possible stimulatory effects of ultrasound on bone, rather than on its harmful effects. At that time, it was known that callus formation could be accelerated by inducing a more intensive and longer reactive fracture hyperaemia. This was accomplished by, for example, sympathectomies and manual manipulation of the fracture site. The concept that all bone regenerations were accompanied with hyperaemia had been stated earlier.[25] Since ultrasound induces tissue-hyperaemia, Miantz decided to investigate the potential of ultrasound to accelerate bone healing.[24] In 3 months old rabbits, a piece of the radius was resected bilaterally. The procedure was not further detailed. The treatment regime involved 5 ultrasound treatments of the right leg, starting at the third post-operative day. The treatments were either done on a daily basis or every other day. Eight groups were exposed to 0.5, 1.0, 1.5, and 2.5 W cm^{-2} ultrasound energy, for 1 or 5 minutes duration. Ultrasound of 800 kHz was used. The fractures were examined histologically and radiographically. No effect was observed using the 1 and the 5 minute

ultrasound treatment at 0.5 W cm^{-2}. Exposures to ultrasound at higher intensities showed a reduction and arrest of callus formation and a detachment of the epiphysis. The non-treated legs healed without complications. Interestingly, lower doses did cause osteogenesis at a site distant from the fracture site and ultrasound application. Also, the simultaneous exposed ulnae showed subperiosteal osteogenesis. Maintz concluded that periosteal new bone formation could be produced by ultrasonic energy, but only in intact normal bone, and that the required dose was close to the destructive level, so that this formation of new bone often was followed by atrophy of bone, with or without fracture. Unfortunately, the study did not show an accelerated healing of bone, but basically confirmed the already known destructive effects of ultrasound on bone. Later, similar results were reported.[21] However, similar treatment regimes did show a positive effect on callus formation in another study which involved bilateral femoral fractures in rabbits.[26] The fractures on one side were treated daily, during 5 days, for 10 minutes, with 1 MHz ultrasound at 2.0 W cm^{-2}. The fracture on the other side served as a control. At post-operative days 4, 6, 8, and 10, animals were sacrificed and histological evaluation was performed. The results showed, in contrast to those reported by Maintz, that callus formation was more abundant in the ultrasound treated legs, and only observed during the early phases of healing. The histological changes seen in callus after 10 days in the non-treated leg, were similar to the changes seen in the ultrasound treated callus at day 6.

In a controlled study, continuous wave 800 kHz ultrasound of 1.5 W cm^{-2} was found to stimulate the formation of callus in radial fractures in rabbits.[27]

Soon after the study of Maintz, it became clear that the intensity of ultrasound had to be decreased in order to stimulate fracture healing[28] and bone growth.[29] Using 0.5 W cm^{-2} ultrasound which was pulsed 1:5 (i.e., period "on" : period "off" = 1:5), a histological and radiographic acceleration of ulnar fracture healing was observed in guinea pigs.[28] The treatment regime consisted of a maximum of 25 treatments for 2 minutes daily. By administrating ultrasound in short bursts, heat accumulation is limited, and the average amount of administered energy per second is less (Figure 1).

Ultrasound stimulation of bone growth was observed in the proximal end of the tibia in young rabbits.[29] Ultrasound with a low intensity (0.2 W cm^{-2}), two times for five minutes, was used. Histologically, osteo-chondroblastic activity was found to be greater than in the non treated controls.

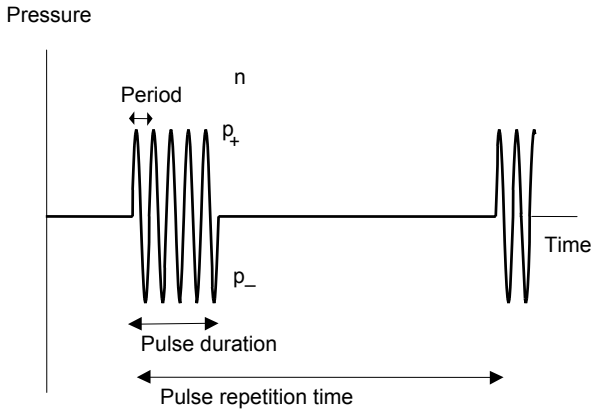

Figure 1. Graphic representation of a pulsed ultrasound signal. A series of n pulses with a period which is determined by the frequency is presented. The pulse duration equals n times the period. The pulse sequence is repeated after the pulse repetition time. The maximal and minimal pressure during a pulse train are indicated by p+ (peak positive pressure) and p- (peak negative pressure). The mechanical effects are mainly determined by the pressure of the pulse train. The pulse duration: pulse repetition time ratio modulates the heat load. By administrating ultrasound in short bursts, heat accumulation is limited.

Human studies

An indication that ultrasound could positively influence bone repair processes in humans was found in an early report by Strauß (Table 1).[30] He treated various infectious conditions in his surgical practice with ultrasound because of the reported bactericidal action of ultrasound. Ultrasound of 0.8 W cm^{-2} intensity was used, with frequencies of 800 and 2400 kHz. The transducer was applied to the skin in a stroking motion to prevent overheating. Although no data were presented, the author reported an accelerated healing of chronic osteomyelitis due to, for example, gun shot wounds. Another report described the treatment of two cases of osteoradionecrosis, in which ultrasound treatment led to the covering of the non-healing bone with fresh granulation tissue.[19] After a period, a sequester formed, exfoliated and the defects healed. The earliest studies concerning ultrasound treatment of fractures can be traced back to the 1950s. In 1953, the treatment of 181 slow- and non-uniting fractures with ultrasound was reported.[31] In 154 cases (85%), healing was obtained by using 800 kHz ultrasound of 1 - 1.5 W cm^{-2} for five minutes every two days, with a

Table 1. Chronological overview of reports concerning ultrasound stimulation of bone healing in humans.

Year, Author	Indication	Study design	Number of patients	Ultrasound intensity* I_{SATA}; I_{SAPA} (mW cm^{-2})	Observed effect
1948, Strauß[30]	Osteomyelitis	O	1	800 (cw)	Healed
1949, Halsscheidt et al.[19]	Osteoradionecrosis	O(CS)	2	600-1300 (cw)	Healed
1953, Corradi and del Moro[33]	Lunate necrosis	O(CS)	3	Unknown	Less pain, improved function
1953, Corradi and Cozzolino[32]	Slow uniting fractures	O(CS)	6	Unknown	Healed
1953, Hippe and Uhlman[31]	Non-unions	O(CS)	181	1000-1500 (cw)	85% healed
1957, Cavaliere[34]	Mandibular fractures (3 fresh and 1 delayed union)	O(CS)	4	1000-2000** (p)	Less pain, more callus
1965, Knoch[35]	Slow uniting fractures	O(CS)	31	300-800 (cw)	100% healed
	Fresh radial fractures	O(CC)	200	300 (cw)	41% reduction in disability time
	Fresh navicular fractures	O(CC)	28	300 (cw)	60% reduction in disability time
1983, Xavier and Duarte[36]	Non-unions	O(CS)	27	30;150 (p)	70% healed
1992, Harris[87]	Mandibular osteoradionecrosis	O	24	1000** (p)	48% spared surgery
1994, Heckman et al.[37]	Fresh tibial fractures	DBRCT	67	30;150 (p)	38% reduction in healing time
1997, Kristiansen et al.[38]	Fresh distal radial fractures	DBRCT	61	30;150 (p)	38% reduction in healing time
1997, Cook et al.[39]	Fresh tibial fractures	DBRCT	67	30;150 (p)	41% reduction in healing time in smokers
	Fresh distal radial fractures	DBRCT	61	30;150 (p)	51% reduction in healing time in smokers
1998, Nolte et al.[93]	Osteotomies of lower extremity	DBRCT	20	30;150 (p)	24% reduction in healing time
1999, Emami et al.[43]	Fresh tibial fractures	DBRCT	30	30;150 (p)	No effect on healing rate
1999, Sato et al.[94]	Leg distraction	O	1	30;150 (p)	Increased bone mineral density
2000, Mayr et al.[40]	Delayed unions	O(CS)	26	30;150 (p)	85% healed
	Non-unions	O(CS)	16	30;150 (p)	94% healed
2000, Mayr et al.[113]	Fresh scaphoid fractures	RCT	30	30;150 (p)	30% reduction in healing time
2000, Fujioka et al.[114]	Hamate non-union	O	1	30;150 (p)	Healed
2001, Nolte et al.[41]	Nonunions	O(CS)	29	30;150 (p)	86% healed

*: When continuous wave (cw) ultrasound was used, the average intensity over space and time is given (I_{SATA}). When pulsed (p) ultrasound was used, both the I_{SATA} and the average intensity of the "on" period (I_{SAPA}) is

given. **: not clear. Abbreviations: cw: continuous wave, p: pulsed, I_{SATA} = Space average time average intensity, I_{SAPA} = Space average peak average intensity, O: observational, O(CS): observational case series, O(CC): observational case control, RCT: randomised clinical trial, DBRCT: double blind randomised clinical trial

total of 10 - 12 treatments. Treatment was applied with the limb and ultrasound transducer under water or with the use of a viscous gel. The ultrasound was administered through a moving or stationary transducer. In the same year, there were reports of an increase in callus formation using ultrasound therapy.[32,33] Later, it was reported that the use of ultrasound on mandibular fractures leads to less pain and increased callusformation.[34] In one case of delayed mandibular union, ultrasound therapy resulted in union.[34] One to two W cm^{-2} ultrasound with a frequency of 0.7 - 1 MHz was used, which was administered in pulses of 1 ms. 10 - 15 treatments of 5 - 10 minutes duration were given. The ultrasound transducer was applied to the skin using circular movements. Knoch, reported successfully treating 31 patients with different non uniting fractures (malleolar, patellar, clavicular, humeral, olecranon, radial and navicular fractures) with ultrasound. 800 kHz ultrasound of 0.3 - 0.8 W cm^{-2} intensity was used for 5 - 8 minutes every other day.[35] After 10 - 20 sessions, all fractures had united clinically. In another study, the influence of the same ultrasound regime on the healing time in fresh radial and navicular fractures was described.[35] It was not until the 1980s, that ultrasound stimulation of bone healing received more attention and more studies involving human subjects were published. Xavier and Duarte reported successful application of low intensity pulsed ultrasound (30 mW cm^{-2}) in the treatment of 27 recalcitrant non-unions.[36] In 70% of the cases, complete healing was obtained by daily 20 minutes ultrasound exposure of the non-union site. Although these results were very promising, the most compelling evidence that ultrasound accelerates fresh fracture healing was presented in prospective, double blind, placebo-controlled clinical trials. The first double blind trial of ultrasound focused on the healing rate of fresh closed or grade I open tibial fractures.[37] Ultrasound, based on the sound used by Xavier and Duarte, was administered by the Sonic Accelerated Fracture Healing System (SAFHS) (Smith and Nephew, Exogen, Memphis, TN, USA). The ultrasound consists of a 1.5 MHz sine wave, which is administered in bursts of 200 μs, followed by a pause of 800 μs (pulsed 1:4). This is repeated 1000 times per second (repetition rate: 1 kHz). The average intensity over space and time is 30 mW cm^{-2}, and the average intensity during the "on" period is 150 mW cm^{-2}. Ultrasound was administered for 20 minutes daily through a non-moving transducer, and led to a 24% reduction in the time for clinical healing to occur (86 ± 5.8 days in the ultrasound treatment group compared to 114 ± 10.4 days in the placebo group, $p = 0.01$). Based on clinical and radiographic criteria, a

38% decrease in time to overall healing was apparent (96 ± 4.9 days in the ultrasound treatment group compared to 154 ± 13.7 days in the control group, $p = 0.0001$). Another double blind trial using SAFHS devices concerned the healing of 61 dorsally angulated fractures of the distal radius. Using the same ultrasound treatment protocol, a reduction in time to union of 38% (61 ± 3 days for the treatment group, compared to 98 ± 5 days for the placebo group, $p < 0.0001$) was found.[38] To assure quality control, these studies excluded patients with conditions that may influence the fracture healing process. However, in reality, patients present with conditions which may disturb fracture healing, such as diabetes, smoking, and certain medications such as calcium-blockers and steroids. These patients are at a higher risk of developing delayed unions and non-unions. A few studies addressed the influence of ultrasound therapy on medically compromised patients.[39,40] By stratifying the results of the two aforementioned studies to patient smoking habits, it became clear that SAFHS ultrasound could overrule the negative effects of nicotine on the fracture-healing process.[39] Nevertheless, patients who smoke during therapy have lower healing rates than those who have never smoked.[40] This means that ultrasound therapy can not only accelerate fracture healing, but also helps to ensure an undisturbed fracture healing.

In disturbed fracture healing (delayed union, non-union), ultrasound can produce healing at a high success rate.[41] Furthermore, the world-wide prescription use registry of the SAFHS device shows that ultrasound is of value in the non operative treatment of fresh fractures, delayed unions and non-unions located at various sites (Table 2).[42]

Table 2. Data from the world-wide prescription use of the SAFHS device in the treatment of fresh fractures, delayed unions and non unions (completed cases as of June 15, 2000). A delayed union is defined as no clinical or radiographical healing observed between 91 and 255 days, a non-union is defined as no clinical or radiological healing for more than 256 days. The fractures were located at different sites in the extremities (humerus, radius, ulna, scaphoid, femur, tibia, fibula, and metatarsus).

Fracture Age (Range)	Healed/Treated	Heal Rate	Heal Time (days)	Fracture Age (days)
Fresh Fracture (0-90 days)	4761/5058	94%	112	39
Delayed Union (91-255 days)	2852/3173	90%	120	149
Non Union (≥ 256 days)	1283/1546	83%	136	692

It should be noted that the positive effects of SAFHS ultrasound are not always evident. During a 75 day course of SAFHS ultrasound of tibial fractures fixed with a locked intramedullary nail, there were no differences in the healing rate between the placebo and the treatment groups.[43] In contrast, a longer period of ultrasound treatment of tibial fractures which were treated by closed reduction and cast immobilisation resulted in a 38% reduction in healing time.[37] This illustrates that ultrasound does not always work in all orthopaedic conditions.[44]

Insight in the mechanism of ultrasound

The basis of the biological effect of ultrasound is an altered, albeit unknown, cell response. Despite the complexity of the fracture healing process[45] and the complexity of the interaction of ultrasound with living tissue, many effects of ultrasound on living cells and tissues are known. These effects provide insights on the biological effects of ultrasound. It seems that the biological effect of ultrasound on bone is the result of a combination of physical and piezo-electric effects leading to cellular responses in which the cell membrane plays an important role (Figure 2).

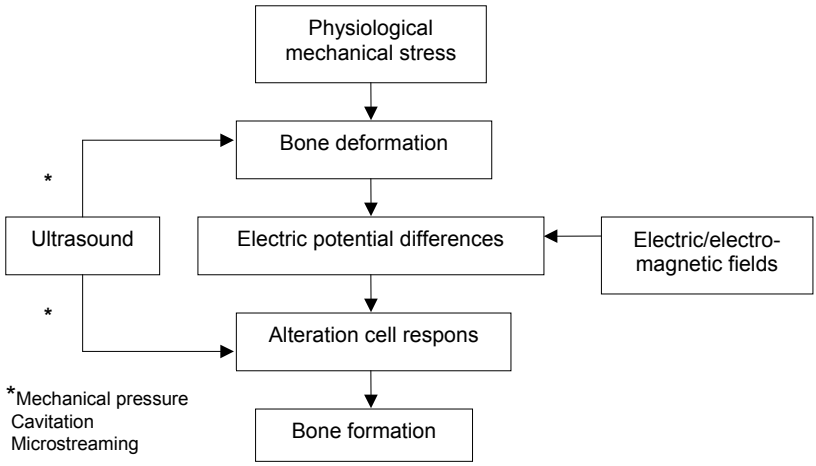

Figure 2. The basis of biophysical stimulation of bone healing according to Wolff's Law in a simplified scheme is depicted. In the case of injury, bone stimulating signals through physiological loading are absent. Ultrasound and electric/electromagnetical stimulation provide a substitute for these signals.

Physical effects

When ultrasound traverses through a tissue, vibrating forces are applied on every tissue component, such as intra- and extracellular fluids and cell membranes. For example, 1.5 MHz, 150 mW cm^{-2} ultrasound displaces particles in the tissue over a distance of 4.6 nm (i.e., some 30 diameters of the hydrogen ion), with a peak velocity of 4.6 cm s^{-1} and a peak acceleration of 410.000 m s^{-2}. The peak pressure applied to the medium equals 70 kPa.[46] The direction of particle displacement is reversed 1.5 million times per second, according to the frequency. Because of these fast vibrations, ultrasound treatment is described in terms of "internal tissue massage" or "micromassage". The physical effects of these motions can be grouped into those which are predominantly thermal in origin and those which are predominantly non-thermal.[47] When an ultrasound wave traverses through tissue, the wave energy is absorbed and can result in heating. This process of energy loss (attenuation) is related to the density of the tissue,[48] making bone sensitive to heat. In physiotherapy, this selective tissue heating is considered beneficial in the treatment of soft tissue disorders (using intensities of 0.5 - 3.0 W cm^{-2} with a moving transducer). The observed thermal effects include an increased blood flow, increased extensibility of collagenous tissues, decreased pain and decreased muscle spasm.[49] However, thermal effects are not considered to play a role in the ultrasound treatment of bone because the intensities currently used are low. For example, the pulsed ultrasound of 30 mW cm^{-2} (SAFHS device) is considered incapable of heating bone.[50] This suggests that non-thermal mechanisms must account for the observed effects on the bone healing process. Non-thermal effects that could explain some observed effects include stable cavitation, microstreaming, acoustical streaming and direct mechanical effects on the cell membrane.

Stable cavitation is the formation of very small gas or vapour filled bubbles in fluids as a result of ultrasonically induced pressure changes. Cavities may be present in aerated media or may develop through the process of rectified diffusion. The bubbles grow and may oscillate in the sound field, increasing and decreasing in volume. This motion gives rise to a localised liquid flow in the fluid around the vibrating bubble, called microstreaming, which in turn may alter cellular processes.[47] Cavitation may occur in living tissue, even when it is subjected to ultrasound of low intensities. There is evidence that 0.75 MHz ultrasound at peak intensities of 240 mW cm^{-2} and higher can produce bubbles greater than 10 μm in guinea pigs hind legs.[51] For the SAFHS device, it has been calculated (data from manual SAFH 2000® system) that peak intensities of 350 mW cm^{-2} are present in the ultrasound field, which may increase further due to reflections at the bone surface. Consequently, cavitation may play a role in the treatment of fractures using the SAFHS ultrasound field. Cavitation is probably

likely to produce cellular change because certain cellular effects could not be observed under elevated ambient pressure which prevents the process of cavitation. It has been reported that collagen synthesis by human fibroblasts was stimulated by 5 minutes exposure to 3 MHz ultrasound at a space-time peak intensity of 0.5 W cm^{-2} and at ambient pressure, but not at a positive pressure of 2 atmospheres.[52] However, the extent to which cavitation plays a role in vivo is not well understood and still needs to be determined.[53]
Acoustic streaming is caused by absorption of kinetic energy of the ultrasonic field in a fluid due to absorption or scattering. This causes a motion of the fluid which is referred as 'a sonic wind'. This motion, at least in theory, may facilitate the movement of intra- and extracellular ions and metabolites.

Ultrasound is capable of producing changes within the cell membrane.[54] This is illustrated by ultrasound's capacity to alter cell membrane permeability to ions and to alter cell membrane electrophysiological properties. Ultrasound can cause an immediate decrease in intracellular potassium content in thymocytes,[55] a reversible increase in the intracellular level of calcium in chondrocytes,[56] and an increase in calcium incorporation in differentiating cartilage and bone cell cultures.[57] In addition to these changes in membrane permeability, ultrasound is also capable of changing the electrophysiological properties of cell membranes. This was found in frog skin which resembles human skin. Following exposure of frog skin (bathed in amphibian sodium chloride Ringer's solution) to 1 MHz continuous wave ultrasound at 60 - 480 mW cm^{-2}, a 5 - 50% intensity dependent decrease in transepidermal potential and resistance was measured in open circuit conditions. In short circuit conditions, an intensity dependent increase of 20 - 220% in total ionic conductance was found.[54] This suggests that ultrasound reduces the electromotive force of the sodium-potassium ATP-ase pump. Because no effect was observed in degassed solution, cavitation seemed to be involved in producing these effects.
Depending on the cell type, the result of changes in intracellular calcium ions can be synthesis, secretion or motility changes, all of which could promote healing.[49] With respect to bone healing, there are also indications that ultrasound influences the adenylate cyclase cascade in the cell membranes of osteoblasts,[58] a finding which is similar to that observed following an application of static mechanical load.[59] The changes in the cell membrane may be the most important mechanism by which the ultrasound signal influences cellular changes and responses. It is not clear if these changes are brought about by a direct mechanical deformation of the cell membrane, deformation of cell receptors or indirectly as a consequence of cavitation, microstreaming or a combination of these or other effects.

Piezo-electric effects

It has been argued that the beneficial effect of ultrasound on bone healing is due to the piezo-electric phenomenon.[60-62] Bone is piezo-electric, which means that electric potentials are produced in bone when it is submitted to mechanical stress.[63] Since Wolff's law[64] basically stated that bone remodels according to functional demands, it is assumed that the stress generated potentials in bone serve as a signal which controls bone remodeling.[65] Much research has been conducted to gain insight in the nature of these stress generated potentials and this eventually has lead to the development of successful therapies to stimulate fracture healing with electromagnetic and electric fields.[66,67] It has been stated that this biophysical intervention serves as a surrogate for the regulatory signals that normally arise as a result of functional loading of the skeleton, but which are absent following bone injury (Figure 2).[68] Ultrasound is a biophysical intervention that is capable of generating piezo-electric effects in bone,[69] and increasing electric potentials in bone.[61] Using 1.27 MHz ultrasound with a very low intensity of 0.00383 W cm^{-2} on bone, an electric potential of 64 μV at the ultrasound frequency was measured in vivo.[69] In a 21-day-old rabbit tibial fracture, there is a reported increase in callus potential of 0.9 mV during application of 880 kHz low intensity ultrasound of 0.01 W cm^{-2}.[61] A major problem with the theory that locally developed potential differences are responsible for cellular change is that the generated potentials using ultrasound are very small compared with potential changes generated by muscle activity. In rabbits, the potential difference generated at a bone surface by bone deformation (2.2 mV) was considerably less than the electric potential difference measured on the bone surface which was generated by muscle activity (100 mV).[70] So, it may be concluded that the extent to which ultrasonic induced potentials exert an effect still remains to be determined.

Ultrasound and the process of bone healing

The physical and piezo-electric mechanisms through which ultrasound may exert an effect, are not limited to one single process during healing. The acceleration of fracture repair seems to be the result of the stimulation of different steps in the fracture healing process.

Combining the results of in vitro studies utilising cell lineages associated with inflammation and other studies concerning the ultrasound effects on inflammation, the influence of ultrasound on the inflammatory, reparative and remodelling phase of the fracture healing process has been described in detail.[71] In the inflammatory phase, ultrasound is capable of increasing mast cell degranulation,[72] augmenting leukocyte adhesion to endothelium,[73] stimulating collagen production by fibroblasts,[74,75] and increasing the release of the

macrophage fibroblast growth factor[76] and vascular endothelial growth factor.[75] Thus, applying ultrasound to a fresh fracture may result in an earlier resolution of the inflammatory phase and earlier commencement of the reparative phase. This may explain why the restoration of mechanical strength in animals following fracture is accelerated by a factor of 1.4 - 1.6 using ultrasound.[77-79] It also may explain why the period of aggrecan gene expression seems to occur earlier in the fracture healing process following ultrasound treatment. In bilateral rat femur fractures, aggrecan gene expression was significantly higher on day 7 post-fracture and lower on day 21 as compared to the non treated side. In these animals, ultrasound of 0.5 MHz, 50 mW cm^{-2}, pulsed 1:4, and a repetition rate 1 kHz for 15 min daily was used.[79] However, there are indications that ultrasound directly stimulates chondrocytes to increase aggrecan gene expression.[80]

Apart from the inflammatory phase, ultrasound seems to influence the reparative phase as well. This stage is characterised by an union through the formation of a primary or provisional callus which stabilises the fracture fragments. This primary callus is formed through the process of chondrogenesis and osteogenesis. There is evidence that ultrasound directly stimulates both processes. In the process of chondrogenesis, ultrasound seems to stimulate chondrocyte proliferation,[81] and chondrogenesis associated gene expression.[79,80] Also, ultrasound is capable of increasing the intracellular concentration of the second messenger, calcium, in chondrocytes.[56] Evidence that osteogenesis is stimulated by ultrasound can be found in in vitro studies. Osteoblasts can be stimulated to increase collagen production,[74,82] and increase the production of prostaglandin E_2, an important bone healing mediator.[83]

Following union, the secondary or definite callus is formed by replacing the cartilage by bone through the process of endochondral ossification. The influence of ultrasound on this process has been investigated in in vitro growing bone. Ultrasound can stimulate this process in vitro in neonatal mouse tibial epyphises[84] and in fetal mice metatarsal rudiments.[81,85] In 17 day-old fetal mice metatarsal rudiments, the influence of pulsed ultrasound at 30 mW cm^{-2} on the process of endochondral ossification was measured. The increase in length of the calcified diaphysis during 7 days of culture was higher in the ultrasound treated rudiments as compared to the untreated controls. The total length was not affected by ultrasound.[85] These results are in accordance with another study, which indicated that DNA synthesis in neonatal mouse tibial epiphyses was increased after exposure to 1.8 W cm^{-2} ultrasound, but not the total length.[84] In a similar study which investigated the effect of different intensities of ultrasound on endochondral ossification on fetal mice metatarsal rudiments, histology revealed a significant increased length of the proliferative zone, whereas the length of the hypertrophic cartilage zone was unaltered. This suggested that the

proliferation of cartilage cells is stimulated without influence on cell differentiation.[81]
Although the above indicates that ultrasound stimulates endochondral ossification, there are also indications that ultrasound stimulates intramembranous ossification. This is illustrated by the accelerated healing of radial fractures using SAFHS ultrasound,[38] which are believed to heal primarily by intramembranous ossification. This finding is of interest because maxillofacial bone healing can involve both intramembranous and endochondral bone healing processes.
In summary, ultrasound stimulation of bone healing may be mediated through cavitation, piezo-electric phenomena, and effects on the cell membrane. This stimulation appears to be multilevel, involving different cell types in and during the healing process.

The potential of ultrasound to stimulate maxillofacial bone healing
Stimulation of maxillofacial bone healing by ultrasound may be possible if the maxillofacial bone is susceptible to the ultrasound signal. In the literature, only limited evidence is available that supports the susceptibility of this bone to ultrasound signals. Evidence that the cells of the mandibular bone respond to ultrasound was reported in an in vitro study which showed that human mandibular osteoblasts could be stimulated by ultrasound to proliferate and produce angiogenesis-related cytokines.[74] In mandibular fractures in rabbits, eight days of ultrasound treatment (five minutes each day, 0.2 - 0.6 W cm^{-2}) stimulated fracture consolidation, as compared to non-treated controls.[86] In a paper concerning the treatment of four mandibular fractures in humans, ultrasound treatment appeared to decrease pain and promote callus formation.[34] Another study found that osteoradionecrosis of the mandible could be treated with some success using 3 MHz ultrasound at 1.0 Wcm^{-2} .[87] In several fields of maxillofacial surgery, ultrasound may be applied to benefit bone healing. These will be discussed below.

Traumatology
Normalisation of occlusion and function are the most important aims in the treatment of maxillofacial fractures. Although rigid fixation has largely replaced intermaxillary fixation in the treatment of many fractures of the maxillofacial bones, there may still be many circumstances that make closed reduction preferable to open reduction and rigid fixation.[88] Prolonged intermaxillary fixation has adverse effects on the masticatory system and poses additional problems such as difficulties in maintaining nutritional status due to difficulties with eating. Therefore, the period of intermaxillary fixation should be limited.

Therapeutic ultrasound has been shown to reduce the time to union in fracture healing by 38% in the tibia which predominantly consists of cortical bone,[37] and by 38% in the radius which predominantly consists of cancellous bone.[38] If these results would be obtained in fractures of the cortical bone of the mandible and the cancellous bone of the maxilla, ultrasound might be helpful in reducing postoperative intermaxillary fixation in fresh fractures. However, when multiple fractures are present, ultrasound treatment of all fractures may not be practical.
Apart from possible benefits of ultrasound treatment in fresh maxillofacial fractures, ultrasound may be helpful in the treatment of compromised maxillofacial fractures. Ultrasound is indicated for the treatment of fractures in the extremities that do not heal with conservative treatment. In the treatment of various delayed unions (defined as failure of healing of a fracture between 3 - 9 months post fracture) and non-unions (defined as failure of healing after more than 9 months), ultrasound treatment resulted in an overall success rate of 88%.[40] Although delayed unions of the facial skeleton are relatively uncommon (1 - 2%) and non-unions are rare,[89] their occurrence can increase up to 43% and 12%, respectively, during war time.[90] In the management of fractures of the edentulous mandible, non-union may be as high as 20%.[91] Also, when endosseous implants are inserted in an atrophic mandible, fracture may occur during or after surgery resulting in non-union.[92]
In these cases, ultrasound treatment may offer a non-invasive treatment alternative. This may especially be of value in medically compromised patients where surgery is not preferred.

Reconstructive surgery
Although most studies concerning the successful clinical application of ultrasound on bone describe bone discontinuities which were accidental in nature (i.e., fresh fractures, delayed and non-unions), the positive influence of ultrasound in the healing of non-accidental bone discontinuities have been described as well. These include osteotomies[93] and osteodistraction.[94] This indicates that ultrasound can influence bone healing processes in general, both accidental and intentional in nature. In the reconstruction of maxillofacial bone, different techniques such as osteotomies, bone grafting, and osteodistraction may be used to optimise treatment.
In fibula osteotomies in rabbits, low intensity ultrasound treatment for 20 minutes a day was capable of increasing the strength of the fibulas. From day 17 to 28, all ultrasound-treated osteotomies were as strong as intact bone, whereas the ultimate strength of the control osteotomies attained intact values only by day 28.[77] In the case of fresh human osteotomies of the lower extremities (femur, tibia, fibula), preliminary results of a double blind trial indicate a 24%

shortening of clinical and radiological time to consolidation using the SAFHS device.[93] In the case of osteotomies that progress to delayed union or non-union, ultrasound treatment resulted in healing in 88% and 89%, respectively.[40] If these results would be applicable to osteotomies of the facial bones, ultrasound would benefit both fresh and compromised consolidation.
Although no specific studies concerning the influence on ultrasound on the healing of bone grafts have been published, some information is available indicating a 91% healing rate of ultrasound treatment for delayed unions after surgical intervention involving bone grafts.[40]
Although the osteodistraction technique as a pre-implantological procedure has the advantage that it limits the need for bone grafting, poor callus formation can be observed during the distraction phase. Moreover, after distraction, a substantial consolidation time has to occur to ensure that enough bone has formed to provide implant stability. This means that the distraction devices must remain in situ during this time. Ultrasound may have the potential to present a solution to these shortcomings. In the field of distraction osteogenesis, ultrasound stimulated callus formation has been described in rabbits,[95] sheep,[96,97] and in the human leg.[94] In rabbits, ultrasound therapy 20 minutes a day after distraction of the right tibia, resulted in a more mature callus as measured by radiography, bone mineral density and mechanical testing. In a situation of poor callus formation, i.e., at a faster rate of distraction (1.5 mm/12 hours instead of 0.5 mm/12 hours), and no postoperative waiting time before active distraction (instead of seven days), ultrasound therapy was still capable of achieving bone maturation. The control group showed only immature bone regeneration.[95] In distracted sheep metatarsus, the influence of SAFHS ultrasound on the callus maturation was studied.[96] Daily application of low intensity ultrasound for 20 minutes resulted in an increased bone mineral content, increased stiffness and homogeneity of the regenerated tissue in the treatment group as compared to the control group. Radiographically, an accelerated maturation of the regenerated tissue was found in the ultrasound stimulated group.[97] In humans, ultrasound stimulation of callus formation during distraction was reported in a 22-year-old woman who was treated for short stature by means of a nine centimetre bilateral leg lengthening.[94] During distraction at a rate of 1 mm/day, poor callus formation was observed at one month. Shortening of the bone gap, increasing the distraction frequency and lowering the daily distance did not improve the callus formation in the following six months. In the eighth month, it was decided to use ultrasound in an attempt to stimulate the callus formation. It was administered for 20 minutes daily until pin removal. The bone mineral content as determined by dual energy x-ray absorptiometry showed a marked increase after commencement of ultrasound treatment (from 0.003 g/day to

0.016 g/day). During the consolidation phase, the bone mineral density increased up to 0.052 g/day and healing progressed uneventful. In summary, the above reports indicate that ultrasound treatment can stimulate callus maturation, even in compromised situations. If this would apply to the facial bones, ultrasound might have a beneficial effect in shortening consolidation time and in ensuring callus maturation.

Oncology

In maxillofacial surgery, therapeutic success often depends on the successful healing of bone under different, sometimes challenging, circumstances. This is illustrated by mandibular osteoradionecrosis, where the healing tendency is severely compromised. In head and neck oncology, the current curative treatment modalities include surgery and radiotherapy. In advanced cases, a combination of these treatments is necessary. The dose of radiotherapy is limited by the toxicity to normal tissues. Because bone is particular sensitive to the radiotherapy dosages, osteoradionecrosis is seen regularly despite the elimination of dental focal infections prior to radiotherapy. When osteoradionecrosis occurs, removal of necrotic bone under antibiotic treatment is indicated. Also, treatment can be supported by hyperbaric oxygen, which is of value in establishing revascularisation.[98,99] Very little information is available concerning the treatment of osteoradionecrosis by means of ultrasound. In 1949, the treatment of "X-ray burns", most likely osteoradionecrosis, was reported.[19] In two cases exposure to ultrasound led to the covering of the non-healing bone with fresh granulation tissue. After a while, a sequester was formed, removed and the defects healed. In these cases, ultrasound with an intensity, at the most, of 1.3 W cm^{-2} was used. Another study examined the conservative management of osteoradionecrosis of the mandible with ultrasound therapy.[87] This study was based on ultrasound's capability to promote neovascularity and neocellularity in ischaemic tissues.[100] Of 24 patients with osteoradionecrosis, 20 received long-term antibiotic therapy with local surgery for at least a year prior to ultrasound therapy. According to the treatment protocol, retained roots and infected teeth were first removed under antibiotic coverage. Subsequently, ultrasound was applied with the transducer stroked for 10 - 15 minutes daily onto the skin overlying the ischaemic mandible (3 MHz, 1 W cm^{-2}, pulsed 1:4), for 40 days. When healing was progressive, but not complete, additional 20 day courses were given up to 100 treatments in total (mean: 55 treatments). Antibiotics were given throughout the treatment period. When healing was slow with persistent bone exposure, the exposed bone was debrided and covered with a local flap. Ten out of 21 patients were successfully treated with debridement and ultrasound alone. Eleven out of the 21 patients

remained unhealed after ultrasound therapy and needed additional debridement and coverage with local flaps. The remaining three patients received conservative debridement without ultrasound and coverage with local flaps prior to ultrasound therapy. Overall, in two patients, the mandible could not be preserved and they were treated by means of combined grafts. It was concluded that 48% of the patients (10/21) were spared surgery due to the ultrasound treatment. Although no data were presented, there was the suggestion that ultrasound is invaluable in the management of chronic osteomyelitis in those cases which failed to respond to antibiotics. Later, another ultrasound regime of 45 kHz continuous wave ultrasound with intensities of 27 and 39 mW cm^{-2} was proposed for the treatment of osteoradionecrosis.[101] This long wave ultrasound is capable of increasing fibroblast and osteoblast proliferation and collagen production comparable to pulsed 1 MHz ultrasound and is capable of deeper bone penetration.[102] The protocol consists of 40 - 50 times 10 minutes sessions until complete healing. It was also recommended that ultrasound should also be used as a prophylactic measure prior to post-radiotherapy extractions, although data to support this are lacking.

Osteoradionecrosis can be considered as an ischaemic necrosis with superficial infection of the exposed bone characterised by hypocellularity, hypoxia and hypovascularity.[103] Thus, improving vascularisation is an important goal. This can be accomplished by, for example, hyperbaric oxygen treatment.[99] By re-establishing or increasing blood flow, cell delivery and tissue oxygenation will improve, and so should healing. It seems that ultrasound can heal osteoradionecrotic bone by countering the negative effect of hypocellularity, hypoxia and hypovascularity. In ulnar osteotomies in dogs, a ten-day period of low intensity ultrasound increased blood flow at the osteotomy site, during and after treatment, and increased the amount of callus.[104] This suggests that oxygen delivery could be improved through a direct dilatory effect on the vessels. Ultrasound can also stimulate the production of angiogenesis related cytokines (Interleukin-8, fibroblast growth factor and vascular endothelial growth factor) in human mandibular osteoblasts,[102] which indicates that it can promote the formation of vessels.

Comparing ultrasound to the other forms of treatment such as surgical intervention or hyperbaric oxygen treatment, adjunct ultrasound treatment of osteoradionecrotic bone seems to be more patient friendly and economically viable.[87]

Other potentials

Apart from traumatology, reconstructive surgery, and oncology, other fields not necessarily limited to maxillofacial surgery may benefit from ultrasound's potential. A recent study indicated that cartilage repair can be improved using SAFHS low intensity ultrasound.[105] In rabbits, osteochondral defects of the patella healed earlier and with less degenerative changes at follow up when treated with ultrasound. Furthermore, it was found that doubling the treatment time to 40 minutes per day increased the histological quality of the repair cartilage. This finding could be of value in the treatment of cartilage defects in other joints, such as the temporomandibular joint.

Endosseous implants are widely used in maxillofacial surgery to support crowns, bridges, overdentures and facial prosthesis. These implants are mostly made out of titanium and bone forms directly against the implant. This process of osseointegration takes a substantial time.

In implant dentistry, it has been stated that implants require, for successful osseointegration, a healing period of at least 3 months in the mandible and 6 months in the maxilla.[106,107] In dogs, it was found that low intensity ultrasound could stimulate bone growth in small porous titanium cylinders.[108] The cylinders were made of 187 - 250 micrometer diameter sintered titanium beads, and had a pore size of 100 - 350 micrometer. In 12 dogs, 22 pairs of these cylinders were placed in holes drilled in the lateral femoral cortex, bilaterally. Ultrasound (SAFHS) was applied daily to one leg. In each dog, one femur served as a control and the other was subjected to daily ultrasound stimulation for 2, 3, or 4 weeks. Overall, the ultrasound-stimulated implants demonstrated an 18% increase in bone ingrowth as compared to the contralateral controls. Ultrasound had its greatest effect in the first three weeks of stimulation. At two and three weeks, the ultrasound-stimulated implants showed 21 and 16% more ingrowth than their respective contralateral controls. This study indicates that the amount of bone formation that is in contact with the surface of the titanium implant can be increased using ultrasound. If these results could be obtained in humans, more bone would be formed against endosseous implants. As a consequence, the osseointegration period could be facilitated by ultrasound therapy. In the case of poor bone quality and/or quantity and in irradiated, resorbed and atrophic bone, ultrasound might help in assuring osseointegration when inserting implants. However, at the present time, this has not been established.

Finally, some attempts were published concerning the treatment of periodontitis.[4] The ultrasound was applied extraorally, on the skin overlying the alveolar bone. Others did not recommend ultrasound treatment of periodontitis because the disease itself was poorly understood at that time.[19] However, nowadays, the process of periodontitis and associated bone loss is less obscure

and it may be noted that ultrasound treatment might contribute to stimulating bone healing around periodontal defects.

General discussion

Ultrasound in the presence of surgical metallic implants

The widespread use of surgical metallic implants in maxillofacial surgery, such as osteosynthesis material, distraction devices, and endosseous implants, makes it necessary to investigate the influence of ultrasound on the tissues nearby these devices. Theoretically, the ultrasound is reflected by metallic implants, which may lead to a more than a double increase of ultrasound intensity in front of the implant. This may cause a rise in temperature inside the body, possibly leading to destructive effects. However, this does not appear to occur in vivo. In two similar studies that investigated the influence of 2.0 W cm^{-2} ultrasound on surgical metal implants, ultrasound caused no extra rise in temperature in front of the metal,[109] and was not associated with evidence of burns or delayed healing of bone or soft tissue.[110] The results were attributed to the metal's thermal conductivity.

The effects of the influence of ultrasound on the internal fixation of osteosynthesis plates has also been studied. Ultrasound did not affect the internal fixation of osteosynthesis plates.[111] In dogs, three-hole AO plates were fixed on femura and humera using tight fitting cortical screws. Low dose (0.5 W cm^{-2}) and high dose (3.0 W cm^{-2}) 1 MHz ultrasound was administered to the legs five minutes daily for 14 consecutive days. Screw torque measurements on insertion and at removal a 4 weeks postoperatively, were not influenced to a significant degree by the ultrasound treatment.

In humans, it has been reported that fractures that were stabilised with metallic implants, such as marrow-nails, Kirschner wires or wire responded with a fast callus formation when exposed to ultrasound.[35] In humans, no harmful effects were observed using the lower ultrasound intensities of the SAFHS device in the presence of metallic surgical implants.[40,43] Thus, the presence of metallic implants do not appear to be a contraindication for ultrasound treatment.

Safety

The head region contains delicate tissues, such as the tissues of the senses and the brain. Therefore, care should be taken when applying ultrasound to this region. Reported adverse effects in the maxillofacial region following the treatment of soft tissue and temporomandibular disorders were associated with the use of high intensities of ultrasound in the order of magnitude of several W cm^{-2}. Haemorrhages in the masticatory muscles, dizziness, nausea, and headaches have been reported.[19,112] Also life-threatening complications occurred

after treating acute inflammations associated with impacted third molars and perimandibular abscesses.[19] However, the low intensity used nowadays to stimulate bone healing has not shown to cause any harmful side effects.

Conclusion

Although ultrasound treatment has been used since 1938, the ultrasound stimulation of both fresh and compromised fracture healing of the long bones has become established only in the past few decades. The question remains, however, whether ultrasound can stimulate bone healing in the maxillofacial skeleton in healthy individuals. In the treatment of mandibular osteoradionecrosis, ultrasound has shown beneficial effects. Although limited evidence is available to support the susceptibility of maxillofacial bone to the ultrasound signal, ultrasound may be of value in the treatment of delayed unions, in callus maturation after distraction, and in the treatment of osteoradionecrosis. Given the successes in the stimulation of bone healing in other parts of the body, it seems that additional research in this field may lead to promising results which will determine the feasibility and potential of ultrasound treatment in maxillofacial surgery.

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Chapter 3

Microradiography to evaluate bone growth into a rat mandibular defect

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Abstract
Microradiography has been evaluated to measure bone healing into a 5.0 mm outer diameter mandibular defect in the rat. This method provides high resolution radiographs of the defects that can be used for an accurate measurement of bone defect healing. In 12 rats, the defect widths of 42-day-old mandibular defects have been measured both using microradiographs and histological sections. The defect width ± SD measured 3.42 ± 0.98 mm microradiographically and 3.47 ± 1.11 mm histologically. Both methods were accurate in determining defect widths but microradiography has the advantage over histology that an image is obtained from the entire defect, making it possible to measure areas of bone growth.

Introduction

Animal models are often used to evaluate new ways of inducing or influencing bone growth. In the maxillofacial skeleton, a frequently used animal model is the mandibular 'critical size' defect in the rat.[1,2] This model consists of a circular through-and-through defect of a diameter varying from 4 to 7 mm drilled into the mandibular ramus. The term 'critical size' implies that the defect will not heal spontaneously,[3-5] so that healing, if obtained, is caused by the experimental intervention. The rat mandibular defect model has been used to evaluate ingrowth of bone substitutes[1,2] and osteoconductive properties of membranes with[6-10] or without[11-13] growth-stimulatory factors. To evaluate the treatment effect, bone growth inside the defect traditionally has been measured histologically using slices through the centre of the defect. Although histological evaluation of bone growth inside the defect is considered the 'golden standard', there are limitations to this technique. An important limitation is that the histological slice represents one specific area of the defect, which does not necessarily represent the entire defect. Furthermore, the diversity in histological scoring systems makes comparison between studies difficult.

Quantitative microradiography is a commonly used technique to measure mineral distributions (calcium, phosphate) and mineral amounts of carious lesions in enamel and dentin.[14,15] The technique has also been used to measure mineral distributions in bone.[16-18] It provides high-resolution radiographs, which may also be used to provide a better overall picture of bone growth into a mandibular defect. To evaluate this, defect widths in 42-day-old rat mandibular defects were measured using both microradiography and histology, and subsequently the techniques were compared to each other.

Materials and methods

The study protocol was approved by the Animal studies review committee, and in accordance with Institutional Guidelines (University of Groningen, The Netherlands).

Operative procedure. In 12 rats (Sprague Dawley, male, age 15 - 17 weeks, mean weight 350 ± 13 (SD) grams, range 330 - 367 grams) a standardised 5.0 mm circular mandibular defect was created into the right-half ramus of the mandible: Under nitrous-oxygen-halothane inhalation anaesthesia, the mandibular and hemicervical areas were shaved. After disinfection of the skin, a submandibular incision was made and the masseter muscle was exposed. After incision of the muscle along the submandibular border, a muscle flap was raised on the buccal and lingual side. Care was taken not to injure the facial nerve and parotid duct. Using a 5.0 mm outer diameter trephine drill (22RF050, Hagar & Meisinger,

Düsseldorf, Germany) mounted in a dental technician drill, a through-and-through defect was drilled into the mandibular ramus (Figure 1). During drilling, the surgical field was continuously irrigated with saline to reduce thermal damage. After the defect was drilled the wound was rinsed with saline. Subsequently, the wound was closed in layers using 4-0 resorbable sutures. For postoperative pain relieve, a single dose of buprenorphine 0.03 mg/kg was given. The rats were housed in groups, and received standard laboratory food and water ad libidum. After 42 days, the rats were anaesthetised by inhalation anaesthesia and sacrificed by an intracardial injection of an overdose pentobarbital. Subsequently, the right mandible was explanted and fixed in phosphate buffered formaline solution. After 48 hours, the specimens were rinsed with saline and put in 70% denatured ethanol solution. Excess of muscle was removed from the specimens by means of a scalpel.

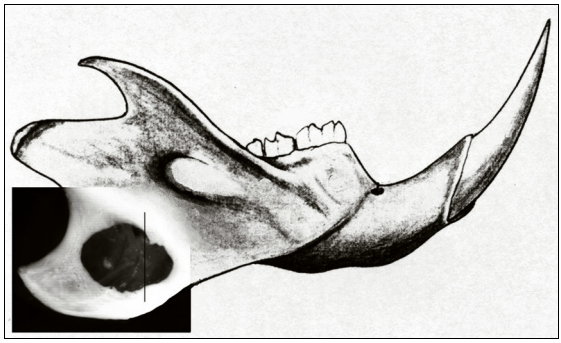

Figure 1. Schematic representation of right side of the rat mandible. The location of the defect is represented by a superimposed microradiograph of a 42-day-old mandibular defect. The vertical line represents the place where the histological section has been made (Figure 2).

Microradiography. An X-ray source (Philips PW 1730, Eindhoven, The Netherlands) was used that produced monochromatous radiation with a specific wavelength of 1.537 Å. The X-ray radiation used is CuKα radiation with a Cu (Copper) X-ray tube and a Nickel filter. The wavelength produced is especially sensitive to be absorbed by calcium. The explanted parts of the mandible were placed between the 35 mm film (Fuji B&W POS/71337, Rotterdam, The Netherlands) 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 minimise magnification effects, the distance was kept small (0.3 mm) between the specimen and the film and large (300 mm) between

the X-ray source and the specimen. Film was used instead of radiographic plates because of a much higher resolution of the film. After development of the film with a D-19 developer (Kodak, Amsterdam, The Netherlands) 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 (Wild/Leitz M7 S, Heerbrugg, Switzerland) with a magnification 10 x and a CCD camera (Teli CS 8310, Tokyo, Japan). The camera was linked to a personal computer equipped with a framegrabber. The magnified microradiographs were stored as images with a size of 640 x 480 pixels and with a resolution of 256 grey values. In addition, a separate image of a microruler was recorded for calibration (Figure 2a).

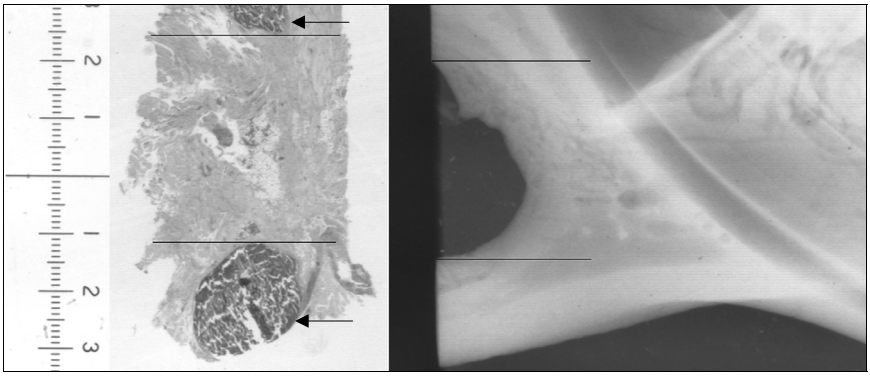

Figure 2. Bone defect width measured using image analysis software on both the histological section (b) and the corresponding microradiograph (c). Two horizontal parallel tangents were drawn at the inner bony edges of the defect. The perpendicular distance between these two lines was measured. A separate image of a microruler was used for calibration (a). Magnification x 10. The arrows in b indicate bone.

Histology. The mandibles were dehydrated in series of ethanol and embedded in methylmethacrylate under negative pressure without decalcification. After the middle of the defect had been determined by placing the mandible imbedded in PMMA on top of the corresponding radiograph on a light box, the specimen was sawn into halves. Sections of 4 µm thickness were cut at the cutting edge from one half of the embedded specimen using a microtome (Jung-K, Heidelberg, Germany). The sections were stained according to the Goldner trichrome method. The histological sections were placed on a light box and digital images were recorded and stored in the same way as the microradiographs (Figure 2b).

Comparison between microradiography and histology. After sectioning, a microradiographic image of the remaining imbedded part of the mandible was made (Figure 2c). The cutting edge of this radiograph exactly shows where the last histological section was made. Both on the histologic specimens and the microradiographs the defect width was measured using image analysis software (Scion Image, version beta 4.0.2, Scion Corporation, Frederick, MD, USA). The image analysis software rather than direct measurement was used because extensive experience was already present using this convenient method. Parallel tangents were drawn at the defect rims and the perpendicular distance in number of pixels was measured automatically between these tangents. A defect rim was histologically defined as the most inner point of bone growth inside the defect. The distances measured in millimetres on the microradiograph and the corresponding histological sections were compared to each other. Each measurement on the histological section and on the microradiograph was repeated three times and then averaged.

Results

The surgical procedure was uneventful and all rats recovered well. No wound infection or dehiscence did occur. All animals gained weight. The defect widths as measured on the histological specimens and on the microradiographs are presented in table 1. The pixel size measured 0.0172 mm^2. In one defect, the embedded specimen had been sectioned deeper from the cutting surface that had been histologically measured. In another defect, a very thin rim of bone could be histologically detected, but not on the microradiograph. Excluding the first case, the results show that the defect width ± SD as measured by histology (3.47 ± 1.11 mm) was 6.8 % larger than the width as measured using microradiography (3.42 ± 0.98 mm).

Discussion

A new promising method of evaluating bone growth into the rat mandibular defect using microradiographs was described.

In two mandibles, the measurements could not be fully compared. In one defect, the embedded specimen had been sectioned deeper from the cutting surface that had been histologically measured. This means that the width as measured histologically did not represent the site where the width has been measured by microradiography. In the other case, a very thin rim of bone could be detected histologically, but not on the microradiograph. This rim was less than 0.09 mm thick. Excluding these two cases, the results show that the defect

width as measured by histology was about 5 % larger than the width measured using microradiography.

Table 1. Defect width as measured by microradiography and histology.

Defect number	Mean defect width (mm) ± SD (mm)		Difference of means (mm)	%
	Microradiography	Histology		Microradiography
1	2.69 ± 0.03	3.89 ± 0.01	1.20	+ 44.6 [a]
2	3.97 ± 0.02	4.00 ± 0.01	0.03	+ 0.8
3	2.56 ± 0.01	2.67 ± 0.01	0.11	+ 4.3
4	3.47 ± 0.02	3.60 ± 0.01	0.13	+ 3.7
5	2.12 ± 0.01	2.45 ± 0	0.33	+ 15.6
6	1.81 ± 0.01	1.83 ± 0.01	0.02	+ 1.1
7	3.07 ± 0.03	2.07 ± 0.01	1.00	- 32.6 [b]
8	4.43 ± 0.01	4.73 ± 0.03	0.30	+ 6.8
9	3.02 ± 0.01	3.04 ± 0	0.02	+ 0.7
10	4.50 ± 0.02	4.51 ± 0.01	0.01	+ 0.2
11	4.41 ± 0.02	4.82 ± 0.03	0.41	+ 9.3
12	4.29 ± 0.01	4.47 ± 0.01	0.18	+ 4.3
Mean excl. 1	**3.42 ± 0.98**	**3.47 ± 1.11**	**0.23**	**(+) 6.8**

[a] The embedded specimen has been sectioned deeper after cutting the slice which has been histologically measured.
[b] This specimen showed histologically a very thin rim of bone (< 0.09 mm thickness) growing inside the defect, which could not be detected on the microradiograph.

An explanation for the consistent slightly larger dimensions as measured histologically may to be due to artefacts in the preparation of the sections. As can be observed in figure 3, space is evident between the muscle fibres, indicating that the histological specimen probably has been torn during the preparation process. These preparation artefacts were seen to some extent in most of the preparations. Due to this, the overall length is slightly larger than the original length (as measured by microradiography), which may account for the observed differences. However, it must be noted that the specimens may shrink during the dehydration process, which may counter the aforementioned increase in length. In any case, the results show that microradiography as compared to histology can accurately distinguish the bony edges of the defect.

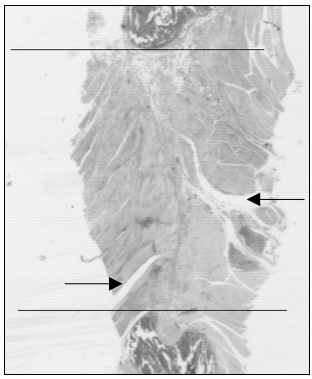

Figure 3. Histological section though a mandibular defect. Space is evident between the muscle fibres (arrows), indicating that the histological specimen has been torn during the preparation process. Magnification x 10.

Although histology is usually considered the 'golden standard' in evaluating bone healing in experimental defects, there seems to be no real 'standard' scoring system. Mostly, a modification of Heiple's[19] semi-qualitative scoring system is used,[6,7,13,20] but these modifications differ from each other, making comparison between studies difficult. Furthermore, because bone growth inside a defect is more or less irregular, histological evaluation of bone growth using sections through the centre of the defect[10,21] may not represent bone growth in other regions of the defect. Although evaluation of defect healing using conventional radiographs has been attempted, giving a more complete picture of defect closure, it was only scored semi-quantitatively, e.g. no, partial or complete healing/closure,[22,23] probably due to lack of radiograph quality.

Microradiography can provide a solution to the limitations of the present techniques in evaluating experimental bone defect healing. The results show that bone boundaries can be detected with accuracy in the plane of the defect. This means that, using microradiographs, not only distances can be measured (one dimension), but areas of bone growth into the defects as well (two dimensions). This seems more appropriate in evaluating bone defect healing than measuring the diameter in the middle of the defect using histology. Furthermore, by providing high-resolution microradiographs in the plane of the defect, patterns of bone growth can be visualised. Another advantage of the microradiograph technique over histology is that the microradiographs can be easily obtained, in a relative short period of time, and at minimal cost.

Nevertheless, microradiography does not allow evaluation of bone growth on the cellular level (in contrast with histology), and only calcified tissue can be detected. In one case a thin calcified bone rim (< 0.09 mm) could not be detected on the microradiograph while it could be seen histologically. Despite the infrequent occurrence and the debatable significance of a very thin sheet of bone, it stresses that microradiography does have limitations in detecting bone. Also, although microradiography is capable of determining whether bone is present (qualitative), a lateral microradiograph does not allow calculating bone volume (quantitative) that is present in the defect.

Summarising, microradiography has some apparent advantages in the evaluation of bone healing of experimentally created defects as compared to histology or conventional radiography. Thus, the microradiography technique seems promising to evaluate bone growth into defects that do not contain any radiopaque material. This is the case with bone morphogenetic proteins, growth factors and non-radiopaque osteoconductive membranes or tissue scaffolds. Future studies are needed to determine whether this technique can be applied to measure bone formation in defects when radiopaque material is present, such as bone grafts or bone substitutes. Although microradiography was evaluated on the rat mandibular defect, it seems that it can also be used in other animal bone defect models such as the calvarial[24] and the nasal defect.[25]

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Chapter 4

Ultrasound and mandibular bone defect healing

Chapter 4.1

Ultrasound to stimulate mandibular bone defect healing. A placebo controlled single blind study in rats

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Abstract
Because of the limitations of the body to heal large maxillofacial bone defects, an attempt was made to stimulate mandibular defect healing with low intensity pulsed ultrasound in rats. This ultrasound consists out of a 1.5 MHz pressure wave administered in pulses of 200 μs, with an average intensity over space and time of 30 mW cm^{-2}. In 72 rats, a 5.0 mm diameter circular mandibular defect was created. Three groups were studied: an ultrasound treatment group, a placebo treatment group and a control group. Ultrasound and placebo treatment involved a daily treatment for 20 minutes at the site of the defect under general anaesthesia. At two and four weeks, the area of bone growth within the defect was measured using microradiographs and the amount of defect healing was expressed as the percentage of defect closure. At two and four weeks, there was no statistical significant difference in the percentage of defect closure between the groups. In conclusion, low intensity pulsed ultrasound does not stimulate bone defect healing in the case of a large mandibular defect in the rat.

Introduction

The restoration of large bone defects in the maxillofacial skeleton remains a challenge because of the body's limitations in healing large bone defects by itself. Such defects may be congenital, traumatic, or iatrogenic due to bone resections in oncologic procedures. The reason for this impaired defect healing seems to relate to the fast soft tissue ingrowth, blocking the bone formation at the defect rims and thus preventing bone healing. A relative lack of certain tissue factors in the centre of the defect, which originate from the edge of the defect, is believed to limit the bone healing process.[1] Research in the field of maxillofacial bone regeneration has yielded several ways of dealing with bone defects, such as the use of bone grafts[2] and/or barrier membranes.[3,4] A relatively unknown treatment that may have an effect on bone defect healing is ultrasound. Recent randomised double blind clinical trials have shown that low intensity pulsed ultrasound treatment of certain fresh fractures can reduce the time to healing.[5,6] In these studies, the Sonic Accelerated Fracture Healing System (SAFHS, Smith & Nephew, Exogen, TN, USA) was used that emits a pulsed ultrasound wave with a high frequency and low intensity. Other reports indicate that ultrasound seems beneficial in the treatment of non-union of the extremities in humans,[7,8] and in the regenerate maturation in distraction osteogenesis in dogs[9] and the human leg.[10] Only one study investigated the influence of ultrasound on healing of large bone defects of the long bones.[11] It was found that daily treatment with low intensity pulsed ultrasound increased new bone formation in small and large ulnar defects in dogs.

Combining the reported positive influences of ultrasound on the bone healing processes in different circumstances with the limitation of the body to heal large maxillofacial bone defects, it was decided to investigate in a single blind placebo controlled study, whether low intensity pulsed ultrasound can stimulate bone defect healing in the rat mandible.

Materials and methods

The study protocol was approved by the Animal studies review committee, and in accordance with Institutional Guidelines (University of Groningen, The Netherlands).

Operative procedure. In 72 rats (Sprague-Dawley, male, age 15 - 17 weeks, mean weight 319 ± 15 g SD, range 282 - 349 g) a standardised circular mandibular defect (5.0 mm outer diameter) was created in the right-half ramus of the mandible according to Kaban.[12] Under 2% Isoflurane inhalation anaesthesia, the mandibular and hemicervical areas were shaved. After disinfecting the skin, a submandibular skin incision was made and the masseter muscle was exposed.

After incision of the muscle along the submandibular border, a muscle flap was raised on the buccal and lingual side. Care was taken not to injure the facial nerve and parotid duct. Using a 5.0 mm outer diameter trephine drill (22RF050, Hagar & Meisinger, Düsseldorf, Germany) mounted in a dental technician drill, a through-and-through hole was drilled in the mandibular ramus. During drilling, the surgical field was continuously irrigated with saline to reduce thermal damage. After the defect was drilled, the wound was rinsed with saline. Subsequently, the wound was closed in layers using 4-0 resorbable sutures for the muscle (Vycril Rapid, Johnson & Johnson, Brussels, Belgium) and skin (Polysorb, Tyco Healthcare, Gosport, United Kingdom). 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 rats received standard laboratory food and water ad libidum.

Experimental groups. The experiment involved two sessions of 36 rats. One session evaluated the influence of ultrasound treatment at two weeks, the other at four weeks. Each session consisted of three experimental groups, each comprising 12 rats, i.e.,

- an ultrasound group, receiving daily ultrasound treatment (except for weekends) for 20 minutes under inhalation anaesthesia;
- a placebo ultrasound group, receiving the same treatment using placebo transducers;
- a control group, only receiving the surgical procedure.

The control group was included to find indications of possible negative physical effects of repeated anaesthesia. The placebo group was included to correct for possible manipulation effects during the ultrasound treatment.

Ultrasound treatment. A custom-made ultrasound device was made because the transducer of the clinical available SAFHS device was too large to treat a rat. The custom made device consisted of a main operating unit (ICT Technical services, University Hospital Groningen, The Netherlands) and four attached ultrasound transducers (Röntgen Technische Dienst, Rotterdam, The Netherlands). The transducers were calibrated to emit the same pulsed ultrasound signal as the SAFHS device. Comparison between the ultrasound field variables of the customised device and the SAFHS device are summarised in table 1 and in Appendix 1, table 3. For the customised device, the effective radiation area and beam non-uniformity ratio were at one time determined according to IEC 61689.[13] To check for the stability of the device, the ultrasonic power was repeatedly determined with a balance according to IEC 61161.[14] In addition, four stainless steel placebo transducers with equal weight and size as

the ultrasound transducers were constructed. In these placebo transducers, the area in contact with the skin was made out of plastic.

Table 1. Comparison of ultrasound field variables between the Sonic Accelerated Fracture Healing System device (SAFHS, Smith & Nephew, Exogen, TN, USA) and the customised ultrasound transducers for the treatment of the rat.[35]

Specification	SAFHS device	Customised transducers
Frequency	1.5 MHz	1.5 MHz
Effective radiating area	3.88 cm^2	1.42 cm^2
Spatial Average Temporal Average intensity (I_{SATA})	30 mW cm^{-2}	30 mW cm^{-2}
Spatial Average Temporal Maximum intensity (I_{SATM})	161 mW cm^{-2}	150 mW cm^{-2}
Pulse duration	200 µs	200 µs
Repetition rate	1 kHz	1 kHz
Beam non-uniformity ratio	2.16	2.32

Frequency: the number of pressure cycles per second
Effective radiation area: the area of the transducer that emits ultrasound
I_{SATA}: the average intensity of the ultrasound field over space and time
I_{SATM}: the maximum intensity of the ultrasound field in time
Pulse duration: the duration of an ultrasound burst
Repetition rate: the number of ultrasound bursts in one second
Beam non-uniformity ratio: ratio between the maximum intensity and the average intensity over the effective radiating area

Eight rats were treated simultaneously. Before ultrasound treatment, the rats were placed in a box into which 2% Isoflurane inhalation anaesthesia was administered. After the rats were anaesthetised, they were taken out of the box and the heads were placed on 8 custom-made silicon pillows in such a way that the right side of the mandible was faced upward. Into the pillows, a syringe was mounted through which 2% Isoflurane inhalation anaesthesia was administered. In this way, 8 rats could be treated simultaneously. Standard aqueous ultrasound coupling gel 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 trapping of air which could block ultrasound transmission. The bodies of the rats lied on a pre-heated rug to prevent hypothermia. The rats were treated with ultrasound for 20 minutes daily, except for weekends. Every day, each rat would be treated by another transducer, limiting the influence of possible ultrasound field variations

between the transducers. During the experiment, the body weight of each animal was scored weekly.
Depending on the session, the rats were anaesthetised and sacrificed by an intracardial injection of an overdose pentobarbital after 2 and 4 weeks. Subsequently, the right mandible was explanted and fixed in buffered formaline solution. After 48 hours, the specimens were rinsed with saline and put in 70% denatured ethanol solution. Excess muscle was removed from the specimens using a scalpel. 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 (Philips PW 1730, Eindhoven, The Netherlands) was used that produced monochromatous radiation with a specific wavelength of 1.537 Å. The X-ray radiation used is CuKα radiation with a Cu (Copper) X-ray tube and a Nickel filter. The wavelength produced is especially sensitive to be absorbed by calcium. The explanted parts of the mandible were placed between the 35 mm film (Fuji B&W POS/71337, Rotterdam, The Netherlands) 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 minimise magnification effects, the distance was kept small (0.3 mm) between the specimen and the film and large (300 mm) between the X-ray source and the specimen. Film was used instead of radiographic plates because of a much higher resolution of the film. After development of the film with a Kodak D-19 (Amsterdam, The Netherlands) developer 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 (Wild/Leitz M7 S, Heerbrugg, Switzerland) with a magnification 10 x and a CCD camera (Teli CS 8310, Tokyo, Japan) (Figure 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 x 480 pixels and with a resolution of 256 grey values. In addition, a separate image of a microruler was recorded in the same way as the specimens for calibration.

Measurement of osteoconduction. Rats who died or obtained wound infection were excluded from analysis. The principal investigator was blinded to the treatment group and number of the rat by coding the microradiographs. The amount of defect healing was expressed as the percentage of defect closure using image analysis software (Scion, Scion Corporation, Frederick, MD, USA) (Figure 1). First, based on the difference in grey values, the individual threshold of the bone/no-bone boundary was determined for each digitised microradiograph.

Second, this threshold was applied to the 5.0 mm diameter defect as a whole and the remaining defect area was measured automatically. Finally, this remaining defect area was expressed as percentage of the original defect size (with a diameter of 5.0 mm). After the measurements were completed, the code was broken and the percentage of average defect closure was calculated for the three experimental groups.

Statistical analysis. Between-group differences of the average percentage of defect closure were compared using one-way ANOVA at 0.05 significance. Where appropriate, differences between two groups were assessed with multiple comparison tests (according to Tukey).

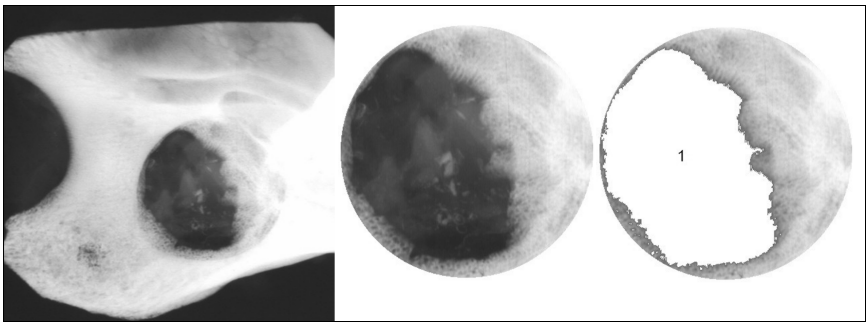

Figure 1. Microradiography pictures of a 2-week-old rat mandibular defect. Of the original microradiograph (a) a digitised image is obtained of the mandibular defect (b). The original outline of the 5.0 mm defect is clearly visible, as well as irregular bone formation into the defect. Using image analysis software the remaining defect area (c, 1) measured 10.11 mm^2, which corresponds to a percentage defect closure of 48.5 %.
Magnification a: x 6, b and c: x 10.

Results

The percentages of defect closure in each group at two and four weeks are presented in table 2. No significant differences could be demonstrated between the groups at two and at four weeks. All animals recovered well from the surgical procedure. The ultrasound treatments were uneventful. During the course of the experiment, one rat had died for unknown reason. No wound infection did occur. All other animals had gained weight. No significant difference of the average body weight between the groups at two and at four weeks was apparent (data not shown). The ultrasound fields as emitted by the customised ultrasound device did not change during the course of the experiment (Appendix 1, Table 4).

Table 2. The amount of bone defect healing reflected as the percentage of defect closure ± SD at two weeks and at four weeks in the ultrasound group, the placebo ultrasound group, and the control group.

	2 wk (%)	**4 wk (%)**
Ultrasound	26.1 ± 11.2, n=12	31.7 ± 16.3, n=11
Placebo	22.5 ± 9.3, n=12	31.5 ± 15.5, n=12
Control	35.5 ± 16.8, n=12	31.2 ± 9.6, n=12

Discussion

Reviewing the literature, the effects of ultrasound on the bone healing process in different circumstances on the cellular, animal and clinical level would suggest that the bone defect healing in the rat mandible would be influenced by the ultrasound treatments. However, this study indicates that low intensity pulsed ultrasound treatment did not influence bone defect healing in this specific case.

The ultrasound regime as used in this study has shown clinically to accelerate the healing of fresh radial,[6] tibial,[5] and scaphoid fractures.[15] Also, high healing success rates have been obtained in the treatment of various delayed- and nonunions,[7,8] indicating that ultrasound can be used in compromised healing situations. In animal studies involving rabbit fibula osteotomies[16] and rat femoral fractures,[17,18] different ultrasound regimes produced an acceleration in the restoration of mechanical strength by a factor of 1.4 - 1.6.

Evidence that the cells of the mandibular bone respond to ultrasound was reported in an in vitro study that showed that human mandibular osteoblasts could be stimulated by ultrasound to proliferate and produce angiogenesis-related cytokines.[19,20] In mandibular fractures in rabbits, eight days of ultrasound treatment (five minutes each day, 0.2 - 0.6 W cm^{-2}) stimulated fracture consolidation, as compared to non-treated controls.[21] In a paper concerning the treatment of four mandibular fractures in humans, ultrasound treatment appeared to decrease pain and promote callus formation.[22] Another study found that osteoradionecrosis of the mandible could be treated with some success using 3 MHz ultrasound at 1.0 Wcm^{-2} .[23]

Only two studies involved the healing of bone defects. In small holes (diameter 1.5 mm) drilled in the cortex of the femur in rabbits, daily 15 minutes ultrasound treatment for two weeks stimulated the callus formation inside the holes as compared to the contralateral non-treated holes.[24] Low intensity pulsed ultrasound was used with frequencies of 4.93 MHz and 1.65 MHz, and intensities of 49.6 and 57 mW cm^{-2}. In another study, it was found that daily

treatment with low intensity pulsed 1.0 MHz ultrasound at 50 mW cm^{-2} increased new bone formation in small and large ulnar defects in dogs, and decreased the incidence of non-union in the large defect model.[11]

Nevertheless, daily 20 minutes ultrasound treatment of the rat mandibular defect did not show an effect on the degree of bone defect closure. Untreated, the rat mandibular defect, as well as the rat calvarial defect, will heal predominantly by soft tissue ingrowth. In the case of rat calvarial defects it has been proposed that the reason for this is the lack of sufficient tissue factors at the defect centre.[1] The release of certain tissue factors from the edge of the wound would cause differentiation of cells within the defect into osteoblasts and chondroblasts that in turn create and mineralise extracellular matrix. This would not occur in the centre of a sufficiently large defect because of a relative lack of these tissue factors.[1] Since ultrasound can stimulate human mandibular osteoblasts to proliferate[20] and produce angiogenesis-related cytokines, such as interleukine 8, basic fibroblast growth factor, and vascular endothelial growth factor in vitro,[19] it may be expected that an increase in bone cell proliferation and release of these factors would facilitate the bone healing of the defect. Also, ultrasound is capable of causing a reversible increase in the intracellular level of second messenger calcium in chondrocytes,[25,26] and an increase in calcium incorporation in differentiating cartilage and bone cell cultures,[27] all of which could eventually stimulate the defect healing process.

Because of the complexity of the bone healing process and the interaction between ultrasound pressure waves and tissues, it is difficult to explain why no effect was seen.

It may be that in all groups studied, the competition between soft tissue ingrowth and bone growth into the defect still was won by the soft tissue ingrowth, despite stimulation of the mandibular bone cells. An important difference of this study as compared to other studies investigating the effect of ultrasound on bone healing is the animal model used. In this study, a large mandibular defect was the subject of investigation (a so called 'critical size defect'). Untreated, large defects will heal by connective tissue ingrowth; complete healing by bone does not occur. This healing characteristic is in contrast to other animal models used (fractures, osteotomies, defects) in which complete bone healing will occur in most instances, and is stimulated using ultrasound.[11,16-18,24] Another difference of this study was that another ultrasound regime was administered than that used in the studies involving mandibular bone and bone cells. Thus, it may also be that the mandibular bone is not susceptible to the low intensity pulsed ultrasound signal used in this study.

It may also be noted that the ultrasound signal may have had an effect on the soft tissue formation. In vitro studies have indicated that fibroblastic activity can

be stimulated using ultrasound.[28] If this would be the case in this animal model, soft tissue formation inside the defect would be stimulated as well, thus limiting bone union.

To our knowledge, no other published studies investigated the effect of ultrasound treatment on the healing of experimental maxillofacial defects. The bone defect model that was used in this study is often referred to as the rat mandibular "critical size" defect. The term "critical size" implies that spontaneous healing will not occur, so that if healing occurs, it is caused by the experimental intervention. When the term was first introduced, a critical size defect was defined as "the smallest intraosseous wound that would not heal by bone formation during the lifetime of the animal".[29] Later, the definition was further specified by stating that a critical size defect "is a defect which has less than 10 percent bony regeneration during the lifetime of an animal".[30] In dental and maxillofacial research, the rat mandibular defect is considered to behave like a critical size defect and is frequently used to evaluate bone regeneration techniques (Table 3.). In the control groups of others, no or very limited bone formation at the defect rims in rat mandibular defects was found (Table 3).

However, in this study, a 35.5% and 31.2% bony defect closure was observed in the control group at two and four weeks, respectively. This indicates that the 5.0 mm diameter rat mandibular defect cannot be considered a critical size defect according to the definition. An explanation could be that in this study, a different, more sensitive, method was used to measure defect healing. Defect closure was previously determined by "gross observation", histological evaluation using sections through the middle of the defect, or by plain radiographs. The amount of defect healing was mostly classified semi-quantitatively (e.g. no, partial or complete healing) or using a histological scoring system based on that of Heiple (Table 3).[31] Only one other study measured the actual defect area by using planimetry on photographs. The results were in accordance with those of our study: 4.0 mm diameter rat mandibular defects healed for 32.4% after 4 weeks (n = 27).[32]

In our study, digitised high-resolution microradiographs were used to measure the area of mineralised bone within the defect. Because bone formation into a defect occurs in an irregular fashion, we felt that measuring bone defect areas using microradiography would be more accurate than measuring defect widths using histology. In a previous study using the same rat mandibular defect model as in our experiment, it appeared that both microradiography and histology were accurate and comparable in measuring defect widths after 6 weeks follow-up.[33]

Table 3. Overview of circular defects made in the mandibular ramus that served as control (no treatment).

Author, year	Rat species, control rats (n)	Sex (m/f), weight (g), age (mo)	Defect size (mm)	Number of control defects (n)	Maximum follow-up (weeks)	Method of measurement	Defect healing in controls
Kaban[36], 1979	Charles-River, 22	-, 250-300, 3	4	44	16	-	No bone formation.
Kaban[12], 1981	Charles-River, 22	m, -, 3	4	44	16	Gross observation, histology	No bone formation.
	Charles-River, 16	m, -, 28 days	4	32	24	Gross observation, histology	No bone formation.
Gongloff[37], 1985	Sprague-Dawley, 3	m, 500-600, outbred	5-7	3	16	-	No osseous healing.
Dahlin[38], 1988	Sprague-Dawley, 24	m, 450-500, 5	5	24	9	Gross observation, histology	5 out of 8 defects: some marginal bone formation.
Lorente[32], 1992	Sprague-Dawley, 27	m, 400-500, retired	4	27	4	Planimetry by photography	1 out of 27 defects: more than 50 % fill of defect. Control defect size: 8.5 ± 3.9 (SD) mm^2.
Dahlin[39], 1994	Sprague-Dawley, 13	m, 450-500, -	5	26	12	Photography	1 completely healed, 1 defect < 3 mm, 18 defects 3-5 mm, 6 defects 5 mm diameter.
Linde[40], 1995	Sprague-Dawley, 8	m, 450-500, -	5	7	1.7	Histology (modified Heiple-score)*	Bone formation: 0.4 ± 0.3.
Same study				7	3.5	Histology (modified Heiple-score)*	Bone formation: 0.6 ± 0.4.
Jones[41], 1996	Sprague-Dawley, 18	m, -, -	4	6	2	Gross observation, radiography	Gross observation (n=6): no bone healing. Radiography (n=2): no bone healing.
Same study				6	4	Gross observation, radiography	Gross observation (n=6): 1 no bone healing, 5 partial. Radiography (n=1): 1 partial
Same study				6	6	Gross observation, radiography	Gross observation (n=6): 4 no bone healing, 2 partial Radiography (n=2): 2 partial

-: not stated, m: male, f: female, g: grams, mo: months. * Modified Heiple-score: 0=no signs of newly formed bone, 1=small amount at defect rims

Because microradiography only visualises mineralised bone, and not the non-mineralised part, it may be that histological measurements of defect widths including non-mineralised bone would yield different results at 2 and 4 weeks follow-up. However, this possible difference between histology and microradiography was not observed at 6 weeks follow-up.[33] Furthermore, if a difference between microradiography and histology measurements at 2 and 4 weeks would exist, this difference would affect all three groups studied, and therefore not influence comparison.

Although it has been reported in an animal experiment that repeated anaesthesia may have a negative influence on the bone healing process,[34] our results do not support this. At two weeks, the ultrasound group and placebo group tend to show less bone formation than the control group (26.1% and 22.5% vs. 35.5%), which may indicate a negative influence of repeated anaesthesia in combination with the treatments. However, this difference was not significant. Moreover, at 4 weeks, there was no difference between bone healing in all groups. Also, there was no difference in the bodyweight of the animals (data not shown).

Because this is one of the first attempts to stimulate mandibular bone defect healing with ultrasound in a standardised experimental setting, we feel that additional research should be encouraged to determine whether or not ultrasound may stimulate mandibular bone defect healing in other circumstances. Furthermore, the reported positive effects of ultrasound on bone healing in other parts of the body should stimulate further research into the mechanism of action. In this way, it may be explained more clearly why no effect was seen in our study and perhaps predict if ultrasound may or may not be applicable to stimulate maxillofacial bone healing.

In conclusion, low intensity pulsed ultrasound treatment does not stimulate bone defect healing in the rat mandible. Future attempts to stimulate maxillofacial bone defect healing using ultrasound may focus on the use of other ultrasound regimes, or on the use of another maxillofacial bone defect model in which soft tissue ingrowth is prevented.

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Chapter 4.2

Therapeutic ultrasound to stimulate osteoconduction. A placebo controlled single blind study using e-PTFE membranes in rats

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Abstract
To decrease healing time of bone defects covered with osteoconductive membranes, an attempt was made to stimulate the osteoconductive process with therapeutic ultrasound. In 72 rats, a circular mandibular defect was created and covered on both sides with an e-PTFE membrane. A control group, an ultrasound treatment group and a placebo treatment group were studied. At two and four weeks, the osteoconduction was expressed as the percentage of defect closure using digitised microradiographs. At two weeks, there was no significant difference in the percentage of defect closure between the groups. At four weeks, there was significantly more bone defect closure in the placebo group (77.9%) as compared to the control group (59.3%). Membrane ultrasound attenuation measurements indicated that the membrane blocks most of the applied ultrasound. In conclusion, low intensity pulsed ultrasound does not appear to significantly stimulate osteoconduction into a bone defect in the rat mandible that is covered by an e-PTFE membrane.

Introduction

The complex treatment of bone defects caused by congenital defects, disease, and injury 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. In practice, the principle of guided tissue regeneration is made possible by using osteoconductive membranes such as the e-PTFE (expanded polytetrafluoroethylene) membrane (Gore-Tex® Regenerative Membrane, W.L. Gore & Associates, Flagstaff, USA). 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 and fill it with bone. Clinically, these membranes are used in implantology to cover exposed implant treads with bone,[1] and to prevent bone 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] Other methods that have been attempted to stimulate the maxillofacial bone healing process in other circumstances are electromagnetic and electric fields.[8-10]

A relatively unknown means to stimulate bone healing processes is ultrasound. In the past years, randomised double blind clinical trials have shown that ultrasound treatment of certain fractures can reduce the time to healing up to 38%.[11,12] In these trials, the treatment regime consists of daily ultrasound self-treatment for 20 minutes onto the skin above the fracture until healing. The ultrasound is administered using the SAFHS-device (Sonic Accelerated Fracture Healing System, Smith & Nephew, Exogen, Memphis, TN, USA) which emits a pulsed ultrasound wave with a high frequency and low intensity. The ultrasound waves exert a small mechanical pressure onto the bone, which in turn seem to serve as a signal for the bone to heal faster.[13] Although the stimulating effect of ultrasound on the bone healing process has been investigated almost exclusively in the bones of the extremities, this has not been established in the bones of the maxillofacial skeleton.[14]

Because of the substantial healing time of bone defects treated according to the principle of osteoconduction, we decided to investigate in a single blind controlled study whether low intensity pulsed ultrasound can stimulate osteoconduction into a bone defect in the rat mandible that is covered by an e-PTFE membrane.

Materials and methods

The study protocol was approved by the animal studies review committee, and in accordance with institutional guidelines (University of Groningen, Groningen, The Netherlands). The sample size was determined by a power analysis based on a 90% power with a 0.05 two 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 72 rats (Sprague-Dawley, male, age 15 – 17 weeks, mean weight 325 ± 14 g SD, range 295 - 354 g) a standardised 5.0 mm circular mandibular defect was created in the right-half ramus of the mandible according to Kaban and Glowacki (Figure 1).[15] Under 2% Isoflurane inhalation anaesthesia, the mandibular and hemicervical area were shaved. After disinfection of the skin, a submandibular incision was made and the masseter muscle was exposed. After incision of the muscle along the submandibular border, a muscle flap was raised on the buccal and lingual side. Care was taken not to injure the facial nerve and parotid duct. Using a 5.0 mm outer diameter trephine drill (22RF050, Hagar & Meisinger, Düsseldorf, Germany) mounted in a dental technician drill, a through-and-through hole was drilled in the mandibular ramus. During drilling, the surgical field was continuously irrigated with saline to prevent thermal damage. In addition, a 1.0 mm hole was drilled next to the defect using a carbide 1.0 mm round dental drill (Figure 1a). After the holes were drilled, the wound was rinsed with saline. One e-PTFE membrane was placed lingually and one buccally onto the defect, covering a minimum of 2 mm bone margin outside the defect. The membranes were kept in place using a transosseous 4-0 suture (Vycril Rapid, Johnson & Johnson Intl., Bruxelles, Belgium) through the 1.0 mm hole (Figure 1b). Subsequently, the wound was closed in layers using 4-0 resorbable sutures for the muscle (Vycril Rapid, Johnson & Johnson Intl., Bruxelles, Belgium) and skin (Polysorb, Tyco Healthcare, Gosport, United Kingdom). 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 rats received standard laboratory food and water ad libidum.

Experimental groups. The experiment involved two sessions of 36 rats. One session evaluated the influence of ultrasound treatment on osteoconduction at two weeks, the other at four weeks. Each session consisted of three experimental groups, each comprising 12 rats, i.e.,

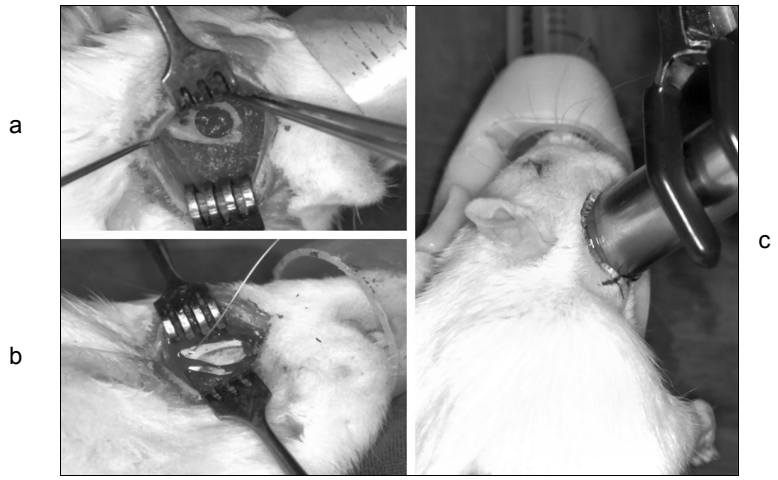

Figure 1. Intraoperative photographs of the rat mandibular defect and postoperative ultrasound treatment. A 5.0 mm diameter through-and-through defect is drilled into the right mandibular ramus and a 1.0 mm hole is drilled next to the defect (a). The lingual and buccal e-PTFE membranes are held in place by a transosseous suture through the 1.0 mm hole (b). During ultrasound treatment, the anaesthetised rat lies on a custom made silicon pillow. The coupling gel is apparent between the ultrasound transducer and the skin to block air (c).

- a control group, only receiving the surgical procedure including placement of e-PTFE membranes;
- an ultrasound group, receiving daily ultrasound treatment (except for weekends) for 20 minutes under inhalation anaesthesia;
- a placebo ultrasound group, receiving the same treatment using placebo transducers.

The control e-PTFE group was included to find indications of possible negative physical effects of repeated anaesthesia. The placebo group was included to correct for possible manipulation effects during the ultrasound treatment.

Ultrasound treatment. A custom made ultrasound device was made because the transducer of the SAFHS device was too large to treat the rat. The device consisted of a main operating unit (ICT Technical services, University Hospital Groningen, The Netherlands) and four attached ultrasound transducers (Röntgen Technische Dienst, Rotterdam, The Netherlands). The transducers

were calibrated to emit the same pulsed ultrasound signal as the SAFHS device. Comparison between the ultrasound field variables of the customised device and the SAFHS device are summarised in appendix 1, table 3. For the customised device, the effective radiation area and beam non-uniformity ratio were at one time determined according to IEC 61689.[16] To check for stability of the device, the ultrasonic power was repeatedly determined with a balance according to IEC 61161.[17] In addition, four stainless steel placebo transducers were constructed with weight (100 grams) and size equal to the ultrasound transducers. In these placebo transducers, the area in contact with the skin was made out of plastic.
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 was faced upward. Into the pillows, a syringe was mounted through which 2% Isoflurane inhalation anaesthesia was administered. In this way, 8 rats could be treated at the same time. Standard aqueous ultrasound coupling gel was applied to the skin and the transducers were placed on the skin on top of the defect (Figure 1c). The skin was shaved weekly to prevent trapping of air which could block ultrasound transmission. The bodies of the rats lied on a pre-heated rug to prevent hypothermia. The rats were treated with ultrasound for 20 minutes daily, except for weekends. Every day, each rat would be treated by another transducer, limiting the influence of possible ultrasound field variations between the transducers. During the experiment, the body weight of each animal was scored weekly. After 2 and 4 weeks, each rat in the session was anaesthetised and sacrificed by an intracardial injection of an overdose pentobarbital. Subsequently, the right-half mandible was explanted and fixed in buffered formalin solution. After 48 hours, the specimens were rinsed with saline and put in 70% denatured 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 (Philips PW 1730, Eindhoven, The Netherlands) was used with a chopper anode that produced monochromatous radiation with a specific wavelength of 1.537 Å. This wavelength is especially sensitive to be absorbed by calcium. The explanted parts of the mandible were placed between the 35 mm film (Fuji B&W POS/71337) and the X-ray source and were 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. After development of the film with a Kodak D-19 developer for 10 minutes, fixating, rinsing, and drying, the film was placed on a light box. A digital image of the original

microradiograph of the mandibular defect was recorded with a stereo microscope (Wild/Leitz M7 S, Heerbrugg, Switzerland) with a magnification 10 x and a CCD camera (Teli CS 8310, Tokyo, Japan) (Figure 2). The camera was linked to a personal computer equipped with a frame grabber. The magnified microradiographs were stored as images with a size of 640 x 480 pixels and with a resolution of 256 grey values. In addition, a digitised image of a micro-ruler was recorded for calibration.

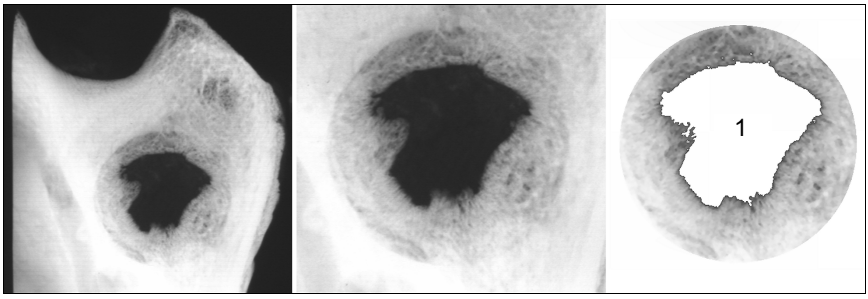

Figure 2. Microradiography pictures of a 4-week-old rat mandibular defect. Of the original microradiograph (a) a digitised image is obtained of the mandibular defect (b). The original outline of the 5.0 mm defect is clearly visible, as well as irregular bone formation into the defect. Using image analysis software the remaining defect area (c, 1) measured 7.91 mm^2 which corresponds to a percentage defect closure of 59.7 %.
Magnification a: x 6, b and c: x 10.

Measurement of osteoconduction (Figure 2). Rats who died or obtained wound infection were excluded from analysis. The principal investigator was blinded to the treatment group and number of the rat by coding the microradiographs. The amount of osteoconduction was expressed as the percentage of defect closure using image analysis software (Scion, Scion Corporation, Frederick, MD, USA). First, based on the difference in grey values, the individual threshold of the bone/no-bone boundary was determined for each digitised microradiograph. Second, this threshold was applied to the 5.0 mm defect as a whole and the remaining defect area was measured automatically. Finally, this remaining defect area was expressed as percentage of the original defect size (diameter 5.0 mm). After the measurements were completed, the code was broken and the percentage of average defect closure was calculated for the three experimental groups.

Ultrasound transmission through e-PTFE membranes. An ultrasound beam was set up in water between a 15 mm diameter 1.5 MHz transducer and a ceramic hydrophone (MKII Active Hydrophone, Medisonics, Watford, England) with a diameter of 1 mm. The hydrophone was placed at a distance of 52 mm from the emitting transducer in the centre of the beam. The Gore-Tex® sample was placed just in front of the hydrophone with its plane perpendicular to the ultrasound beam. The sample was moved over a two dimensional array in steps of 1 mm. For each position of the sample the pressure wave on the hydrophone was registered on a digital sampling oscilloscope (Philips PM3394, Eindhoven, The Netherlands). A semi-continuous wave was used. From the steady state signal the sum of the squares of the signal S was calculated. For comparison also the sum of squares for the signal without sample in place was determined: S_0. Attenuation A (in decibels) of the sample was calculated from $A = 10 \log (S_0/S)$. The sample was prepared by degassing it in demineralised water for 24 h at a pressure of less than 0.1 atm. Thereafter it was transferred to the measuring position in the ultrasonic beam, without exposing it to air. In analysing the data results were ignored that were within 1 mm from the borders of the homogeneous regions of the sample.

Statistical analysis. Between-group differences of the average percentage of defect closure were compared using one-way ANOVA. Further multiple comparison analyses (Tukey's tests) were carried out in cases of significance at the 0.05 level.

Results

The percentages of defect closure in each group at two and four weeks are presented in table 1. After 2 and 4 weeks, there was more defect healing in both the ultrasound and the placebo treatment group as compared to the controls, but the difference was significant only in the placebo group at 4 weeks. During surgery, 2 animals had died. All other animals recovered well from the surgical procedure. The ultrasound treatments were uneventful. During the course of the experiment, 5 rats had died because of wound infection. On autopsy, pus was apparent in these 5 rats. All other animals had gained weight. No significant difference of the average body weight between the groups at two and at four weeks was apparent (data not shown).

The oval e-PTFE membrane consist of an inner part and an outer rim. The ultrasound attenuation of the inner part was inhomogeneous (range 5 to 12 dB), the average attenuation was 8.3 ± 0.4 dB (mean and 95% confidence interval). This corresponds to a transmission of ultrasound energy through the membrane of 15 %. The ultrasound attenuation of the outer part was more than 20 dB,

which corresponds to a transmission of ultrasound energy through the membrane of less than 1%.
The ultrasound fields as emitted by the customised ultrasound device did not change during the course of the experiment (Appendix 1, Table 4).

Table 1. The amount of osteoconduction reflected as the percentage of defect closure ± standard deviation (SD) at two weeks and at four weeks of bone defect healing in the control, the ultrasound group and the placebo ultrasound group. At four weeks, there was significant more defect closure in the placebo group as compared to the control group ($p<0.01$)*.

	2 wk (%)	**4 wk (%)**
Control	41.3 ± 9.4, n = 9	59.3 ± 14.1, n = 12 *
Ultrasound	47.1 ± 13.7, n = 9	70.7 ± 16.5, n = 11
Placebo	46.4 ± 16.1, n = 12	77.9 ± 13.5, n = 12 *

n = number of rats, * $p< 0.05$, one-way ANOVA with post test (Tukey)

Discussion

This study indicates that low intensity pulsed ultrasound does not significantly stimulate osteoconduction into a bone defect in the rat mandible that is covered by an e-PTFE membrane. There may be two reasons for this finding.
The first may be that not enough ultrasound pressure reaches the tissue behind the e-PTFE membrane. The e-PTFE membranes used in this study are porous in nature (Instructions for use, Gore-Tex® Regenerative Membrane). Due to this porosity, air may be trapped inside the membrane which, in turn, blocks ultrasound transmission. It was found that exposing to vacuum during 24 hours does not remove the air (air is visible as white spots), thus the air is probably in closed cells. As a consequence, less ultrasound energy is transmitted through the membrane. This means that areas of the mandible received only up to 15% of the applied ultrasound energy, depending on the location of the membrane centre and the membrane rim. Because low intensity pulsed ultrasound at higher intensities seems to facilitate the bone healing process in different species (rat[18], rabbit[19], sheep,[20] dog,[21]) and in different clinical situations (fresh fractures,[11,12,22] delayed unions, non-unions,[23,24] osteotomies,[25] and distraction osteogenesis[26]), it may be expected that the ultrasound would have an effect on the osteoconductive process in the mandible as well. Furthermore, ultrasound can stimulate mandibular osteoblasts in humans to proliferate and produce angiogenesis related cytokines, such as interleukine 8, basic fibroblast growth factor, and vascular endothelial growth factor in vitro,[27] indicating that human mandibular bone may react to the ultrasound pressure.

However, the second reason that no significant effect of ultrasound therapy was measured may be that the mandibular bone of rats is not responsive to the ultrasound signal, not even in the case the osteoconductive membrane would not partially block the signal. This is an important note because ultrasound does not necessarily stimulate bone healing in all circumstances.[28,29]
The difficulty in predicting in which cases ultrasound may or may not stimulate bone healing is partly due to the highly complex nature of the bone healing process[30] and the complex nature of the interaction of ultrasound with tissue. Ultrasound is a high-frequency pressure wave, of which energy is absorbed by deforming tissue on the microscopic level. Although the exact mechanism as to how this ultrasound pressure signal is transduced to stimulate the bone healing process is unknown, there are indications that ultrasound has a direct effect on the cellular level.[14]
An unexpected finding was that, at 4 weeks, the rats treated with the placebo devices showed significantly more defect closure as compared to the controls (placebo 77.9% Vs control 59.3%, $p < 0.05$) (Table 1). The placebo treatment involved daily anaesthesia, application of coupling gel to the skin, and the placement of the placebo transducer in close contact with the skin on top of the defect. Because of the reported possible negative influences of repeated anaesthesia on the bone healing process,[31] it was expected that the placebo group would show less bone healing than the control group. However, the placebo group at 4 weeks received a total of 400 minutes of anaesthesia, and it does not seem that the placebo treatment influenced the bone healing process negatively. Also, repeated anaesthesia did not seem to influence the general health of the animal, because there was no difference in the body weight between the placebo and control groups at two and four weeks (data not shown). The finding that more defect healing did occur in both the ultrasound and the placebo group at 4 weeks (70.7% and 77.9%) as compared to the controls (59.3%), suggests that the placement and pressure of the ultrasound and placebo transducer may be related to the increase in bone formation in these groups. In a previous ultrasound study, the same mandibular defect model was used, but without an e-PTFE membrane.[32] Using the same microradiography technique, it was found that the 5.0 mm diameter defects healed for an average of 28.0 ± 12.4 % at two weeks (n=36), and for an average of 31.5 ± 13.8 % at four weeks (n=35) regardless of ultrasound therapy, placebo therapy, or absence of therapy. Comparing these results to the present study, it confirms that the presence of an e-PTFE membrane itself facilitates bone growth into the mandibular defect, and that ultrasound does not seem to do so.

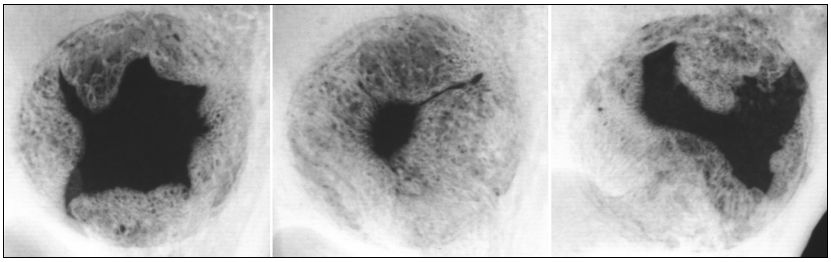

Figure 3. Microradiographs of 4-week-old rat mandibular defects illustrating the irregular pattern of bone formation into the defect. The defects were covered on both sides with e-PTFE membranes. Magnification x 10.

In this study, a microradiography technique[33] was used as an approach to measure bone defect healing in the rat mandible. Because bone formation inside the mandibular defect occurs in an irregular fashion (Figure 3), we felt that measuring bone growth inside the defect in two dimensions using a microradiograph would be more accurate than measuring it in one dimension using histological sections as done previously by others.[7,34]

In conclusion, low intensity pulsed ultrasound does not significantly stimulate osteoconduction into a bone defect in the rat mandible that is covered by an e-PTFE membrane, either because the bone surrounding the defect is exposed to an insufficient amount of ultrasound energy or because the mandibular bone of rats is not responsive to low intensity pulsed ultrasound. Future attempts to stimulate osteoconduction using ultrasound may focus on establishing higher amounts of ultrasound energy behind osteoconductive membranes. This could be accomplished by using higher ultrasound intensities or different types of membranes.

Acknowledgements

The authors would like to thank Mr. J. Lubbers for the calibration of the ultrasound device, the attenuation measurements of the e-PTFE membrane, and his advice during the preparation of this manuscript. Mr. A. Wietsma is acknowledged for the fabrication of the custom made silicon pillows, and the animal laboratory staff for their help in conducting the experiment. Gratitude is expressed to W.L. Gore & Associates, Inc. for the provision of the Gore-Tex® Regenerative Membrane. (Gore-Tex® is a registered trademark of W.L Gore & Associates).

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Chapter 4.3

Does ultrasound stimulate osteoconduction? A placebo controlled single blind study using collagen membranes in the rat mandible

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Submitted

Abstract
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. This ultrasound consists of a 1.5 MHz pressure wave administered in pulses of 200 μs, with an average intensity over space and time of 30 mW cm^{-2}. In 64 rats, a 5.0 mm diameter circular mandibular defect was made in the ramus and, subsequently, covered on both sides with a collagen membrane. Two groups were studied: an ultrasound treatment group and a placebo treatment group. At two and four weeks, the remaining defect area was measured using microradiographs and the amount of osteoconduction was expressed as the percentage of defect closure. At two and four weeks, there was no significant difference in the percentage of defect closure between the groups. An explanation may be that ultrasound does not exert an effect in an area where wound healing is expected to be already at an optimal level. In conclusion, there was no evidence that low intensity pulsed ultrasound stimulates osteoconduction into a bone defect in the rat mandible that is covered by a collagen membrane.

Introduction

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. In practice, the principle of 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 and fill it with bone. Clinically, these membranes are used in implant surgery to cover exposed implant treads with bone,[1] and to prevent bone 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.[5,6,7]

A relatively unknown way that may stimulate bone healing processes is ultrasound. Ultrasound is a mechanical pressure wave with a frequency above the human ear threshold (i.e., >20.000 Hz).[8] In medicine, ultrasound is used to diagnose (pulse-echo), and treat. In the last 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 non-unions, 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, there are suggestions that ultrasound may stimulate maxillofacial bone healing as well.[14] Therefore, it was decided to investigate whether low intensity pulsed ultrasound stimulates osteoconduction into 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 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 0.05 two 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 - 17 weeks, mean weight 310 ± 17.6 g SD, range 265 - 348 g) a standardised 5.0 mm circular mandibular defect was made in the right ramus according to Kaban and Glowacki.[15] Under 2% Isoflurane inhalation anaesthesia, the mandibular and hemicervical area were shaved. After disinfection of the skin, a submandibular incision was made and the masseter muscle exposed. After cleaving the muscle along the submandibular border, a muscle flap was raised on the buccal and lingual side. Care was taken not to injure the facial nerve and parotid duct. Using a 5.0 mm outer diameter trephine drill (22RF050, Hagar & Meisinger, Düsseldorf, Germany) mounted in a dental technician device, a through-and-through hole was made in the 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 collagen membrane (Bio-Gide® Resorbable bilayer membrane, Geistlich Biomaterials, Wolhusen, Switzerland) was placed lingually and one buccally onto the defect, covering a minimum of 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 relieve, a single dose of buprenorphine 0.03 mg/kg was given. The rats were numbered and housed in groups of 4. The first four operated rats were allocated to the ultrasound group, the second four to the placebo treatment group, the next four to the ultrasound group, etc. The rats received standard laboratory food and water ad libidum.

Experimental groups. The experiment involved two sessions with 32 rats. One session evaluated the influence of ultrasound treatment on osteoconduction at two weeks, the other at four weeks. Each session consisted of two experimental groups, each comprising 16 rats, i.e.,

- an ultrasound group, receiving daily ultrasound treatment (except for weekends) for 20 minutes under general inhalation anaesthesia;
- a placebo ultrasound group, receiving 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 made ultrasound device was made because the transducer of the SAFHS device was too large to treat the rat. The device consisted of a main operating unit (ICT Technical services, University Hospital Groningen, The Netherlands) and four 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 customised device and the SAFHS device have been presented elsewhere.[17] For the customised device effective radiation area and beam non-uniformity ratio were at one time determined according to IEC 61689.[18] To check stability of the device the ultrasonic power was repeatedly determined with a balance according to IEC 61161.[19] In addition, four stainless steel placebo transducers with equal weight and size as 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 was faced upward. Into the pillows, a syringe was mounted through which 2% Isoflurane inhalation anaesthesia was administered. In this way, 8 rats could be treated at the same time. Standard aqueous ultrasound coupling gel 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 trapping of air which could block ultrasound transmission. The bodies of the rats lied on a pre-heated rug to prevent hypothermia. The rats were treated with ultrasound for 20 minutes daily, except for weekends. Every day, each rat would be treated by another 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.

After 2 and 4 weeks, each rat in the session was anaesthetised and then sacrificed by an intracardial injection of an overdose pentobarbital. Subsequently, the right half mandible was explanted and fixed in buffered formaline solution. After 48 hours, the specimens were rinsed with saline and put in 70% denatured 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 (Philips PW 1730, Eindhoven, The Netherlands) was used that produced monochromatous radiation with a specific wavelength of 1.537 Å. The X-ray radiation used is CuKα radiation with a Cu (Copper) X-ray tube and a Nickel filter. The wavelength produced is especially sensitive to be absorbed by calcium. 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 minimise magnification effects, the distance was kept small (0.3 mm) between the

specimen and the film and large (300 mm) between the X-ray source and the specimen. Film was used instead of radiographic plates because of a much higher resolution of the film. After development of the film with a Kodak D-19 developer 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 (Wild/Leitz M7 S, Heerbrugg, Switzerland) with a magnification 10 x and a CCD camera (Teli CS 8310, Tokyo, Japan) (Figure 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 x 480 pixels and with a resolution of 256 grey values. For calibration, a separate image of a microruler was recorded in the same way as the specimens.

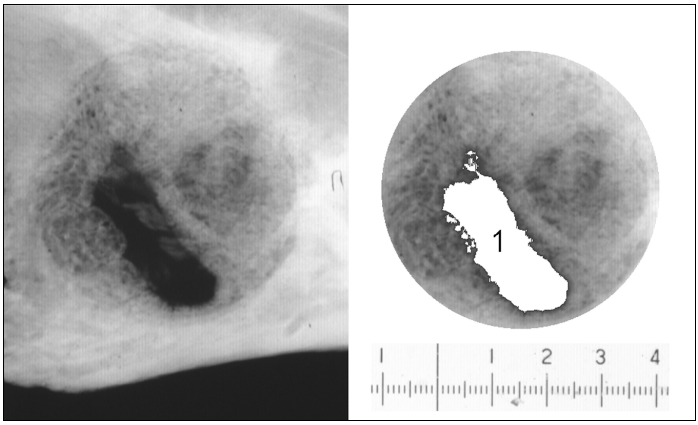

Figure 1. Microradiography pictures of a 4-week-old rat mandibular defect covered on both sides with a collagen membrane. The original outline of the 5.0 mm defect is clearly visible, as well as bone formation into the defect (a). Using image analysis software the remaining defect area (b, 1) measured 2.47 mm^2 which corresponds to a percentage defect closure of 87.4%. Magnification x 10.

Measurement of osteoconduction (Figure 1). Rats who died were excluded from analysis. The principal investigator was blinded to the treatment group and number of the rat by coding the microradiographs. The amount of osteoconduction was expressed as the percentage of defect closure using image analysis software (Scion, Scion Corporation, Frederick, MD, USA). First, based on the differences in grey values, the individual threshold of the bone/no-bone boundary was determined for each digitised microradiograph. Second, this threshold was applied to the 5.0 mm diameter defect as a whole and the remaining defect area was measured automatically. Finally, this remaining defect

area was expressed as percentage of the original defect area ($\pi r^2 = 19.63$ mm^2). After the measurements were completed, the code was broken and the percentage of average defect closure was calculated for the two experimental groups. The differences between the groups were compared using a t-test with a 0.05 significance level.

Results

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

Table 1. The amount of osteoconduction reflected as the percentage of defect closure ± SD at two weeks and at four weeks of bone defect healing in the ultrasound group and the placebo group. The 95% confidence interval (CI) of the difference between the groups is provided.

	2 wk (%)	**4 wk (%)**
Ultrasound	73.3 ± 17.7, n =16	88.0 ± 23.6, n = 16
Placebo	69.4 ± 24.7, n = 15	93.4 ± 5.9, n = 15
95% CI	[-11.8; 19.6]	[-18.2; 7.4]

n = number of rats

Discussion

This study indicates that low intensity pulsed ultrasound does not stimulate osteoconduction into a bone defect in the rat mandible that is 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 different species such as the rat,[20] rabbit,[21] dog,[22,23] and homo sapiens,[9,10] and has been observed in different circumstances such as fresh fractures,[9-11] delayed unions, non unions,[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 case ultrasound will or will not stimulate bone healing. It has been reported that the pressure wave serves as a surrogate for physiological 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 ischaemic tissues, where blood perfusion is limited, ultrasound can promote neovascularity and neocellularity.[37] In dogs with an ulnar osteotomy, daily 20 minutes ultrasound treatment with the SAFHS device for 8 weeks produced an increase in blood flow around the osteotomy site after 2 - 3 days and this increase lasted for two weeks as compared to the non treated 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 and non-unions 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 relative poor blood supply due to anatomical 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 (read: 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 blood perfused and can, therefore, 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 that osteoconduction could not be stimulated 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 reports in the past concerning mandibular fractures in rabbits,[41] humans[42] and mandibular osteoradionecrosis in humans[28] described other ultrasound fields than the SAFHS field. In these reports, a positive effect of ultrasound on mandibular bone healing was described. 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 our experiment because bone healing seemed particularly sensitive to this signal in other circumstances, and the device is approved for clinical use.[43]

In this study a collagen membrane was used on both sides of the defect to provide a secluded space that can be filled with bone according to the guided tissue regeneration principle. Although it has been reported that certain resorbable membranes (including collagen membranes) have a tendency to collapse and, therefore would inhibit bone formation into 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, Appendix 2). This means that the ultrasound dose as used clinically, reaches 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 for an average of 28.0 ± 12.4 % at two weeks (n=36), and for an average of 31.5 ± 13.8 % at four weeks (n=35) regardless of ultrasound therapy, placebo therapy, or no therapy. Comparing these results to the present study, it confirms 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 formed bone inside the defect. Because bone formation inside the mandibular defect is irregular, measuring the area of bone inside the defect using a microradiograph seems more accurate than measuring the diameter of the defect using a histological section through the middle of the defect as done previously 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, we feel that measuring the area of mineralised bone inside the defect should suffice, since this reflects the amount of bone formation.

Summarising, this study presents no evidence that low intensity pulsed ultrasound stimulates osteoconduction into a bone defect in the rat mandible that is covered by a collagen membrane. This result may be related to an already optimal healing tendency in the head and neck region because of a good blood supply and perfusion. Future attempts may focus on stimulating mandibular bone healing using low intensity pulsed ultrasound in relative compromised healing situations.

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Chapter 5

Ultrasound to stimulate early bone formation in a distraction gap: a double blind randomised clinical trial in the edentulous mandible

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Submitted

Abstract
In a double blind randomised clinical trial, it was investigated whether low intensity pulsed ultrasound stimulates early bone formation in a distraction gap created in a severely resorbed mandible. Eight patients underwent a mandibular vertical distraction over an average distance of 6.6 ± 1.1 mm. Ultrasound self-therapy or placebo therapy was started at the first day of distraction and continued daily until the implants were inserted. After 31 ± 3.8 days of consolidation, the distraction device was removed, a transmandibular biopsy was taken, and two endosseous implants were inserted. All patients complied well with ultrasound therapy. During an average of 18.1 ± 4.1 months follow-up, no complications did occur. Microradiographic examination of the biopsies revealed a comparable mean area of mineralised tissue in the distraction gap of 1.9 ± 1.7 mm^2 in the ultrasound treatment group and 1.9 ± 1.3 mm^2 in the placebo treatment group. Histological examination indicated that active woven bone was present within the distraction gap just adjacent to the osteotomy plane, with no apparent differences between the treatment groups. The lamellar bone formation outside the distraction gap appeared to have started as well. The results of this trial indicate that ultrasound treatment does not significantly promote early bone formation in the distraction gap during a 31-day consolidation period. It appeared that bone formation within and outside the distraction gap had just started, so that a possible beneficial effect of ultrasound therapy on bone formation can not be excluded. Therefore, a longer consolidation period has to be studied.

Introduction

Due to the continuing bone resorption of the mandible after loss of teeth, a limit is sometimes reached, making wearing of a lower denture too heavy a burden for the patient. Patients with a severely resorbed mandible frequently suffer from pain and difficulties with eating and speech. The key to a solution is to provide sufficient stability, especially for the lower denture. Nowadays, this is accomplished by using dental implants to support the denture.[1] However, in the severely atrophic mandible (i.e., bone height less than 8 mm), there is insufficient bone to reliably insert implants, which might result in increased loss of implants in the long run. In these cases, bone augmentation procedures have to be performed before implants can be inserted.[1] A pre-implant procedure that is increasingly applied to enable placement of implants in the severely resorbed mandible is the vertical distraction osteogenesis.[2] After distraction, a bone healing time of 8 - 12 weeks is generally taken into account before implant insertion. After implant insertion, another 12 weeks should be allowed to ensure full osseointegration. During this period, the patient cannot wear a denture. Because the bone-healing period is a major factor that determines the total treatment time, ways of shortening the bone healing process may be of substantial benefit to the patient.

Ultrasound is a relatively unknown therapy that can stimulate the bone healing process. This has been investigated in the case of fresh tibial[3] and radial[4] fractures and in various delayed and non-unions.[5,6] In the case of osteodistraction, there are indications that ultrasound therapy may stimulate bone formation within the distraction gap (the regenerate- or callusmaturation).[7-12] These studies indicate that ultrasound therapy may accelerate the mineralisation of the tissue within the distraction gap (the consolidation). In case of osteodistraction, it seems that the primary effect of ultrasound occurs early in the treatment process[7] and that the overall bone healing process occurs, therefore, relatively early. Although the potential of ultrasound to stimulate maxillofacial bone healing has been investigated before,[13] no experiments have been published about ultrasound therapy and human mandibular distraction. To investigate this further, it was decided to evaluate whether therapy with low intensity pulsed ultrasound can stimulate early bone formation in the vertical distracted mandible in humans.

Materials and methods

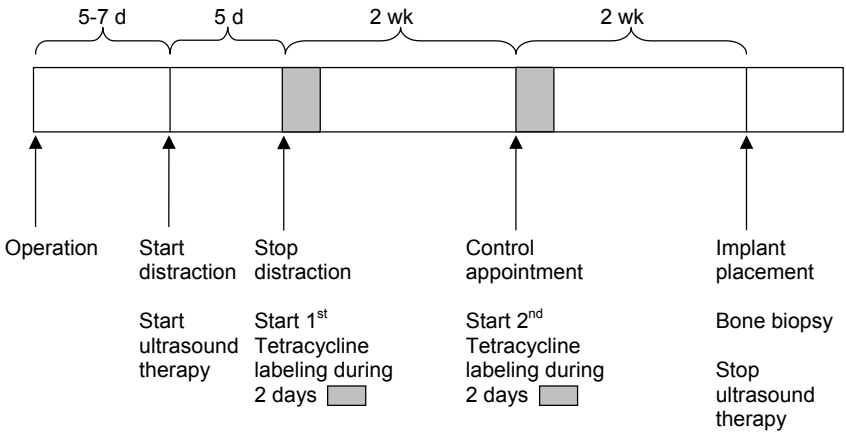
Patient selection. Patients referred to the Department of Oral and Maxillofacial Surgery of the University Hospital Groningen between May 1 until November 30, 2001, were selected to participate in the study when they met the following criteria:

- having complaints related to insufficient retention and stability of their dentures,
- the presence of a severely resorbed edentulous mandible with a mandibular height at the canine region of less than 8 mm,
- unsatisfactory relief of complaints with conventional dentures.

Patients who smoked or used medications were not excluded to participate.
All patients approved of the study and signed a written informed consent statement to participate. The study protocol was approved by the Medical Ethics Committee, University Hospital Groningen, The Netherlands (METc 2001/046).

Operative procedure. The severely resorbed mandible underwent a vertical distraction using the Groningen Distraction Device (GDD, Martin Medizin Technik, Tuttlingen, Germany) according to the procedure described by Raghoebar et al.[2] After a latency period of 5 days after insertion of the distraction device, active vertical distraction was started at a rate of 1 x 1.0 mm/day until the appropriate height was obtained to insert two implants with an implant length of 12.0 mm. At the first day of active distraction, ultrasound treatment was started (Figure 1).

Figure 1. The sequence of the study protocol depicted in a time schedule (d = day, wk = weeks).



Ultrasound treatment. In this study, placebo and active Sonic Accelerated Fracture Healing System devices were used for ultrasound treatment (SAFHS model 2000®, Smith & Nephew, Memphis, TN, USA). This battery operated device consists of a main operating unit to which the ultrasound transducer is attached. The active devices emitted an ultrasound signal consisting of a 1.5 MHz pressure wave that is emitted in pulses of 200 µs. Between pulses a 800 µs pause was present (on:off period = 1:4). The average intensity over space and time was 30 mW cm^{-2}. The placebo-devices did not emit an ultrasound pulse. Prior to the commencement of the study, the placebo and active devices were identified and blinded by coding the devices by subsequent numbering using randomisation software (B.S). The placebo and active devices were indistinguishable from each other by appearance and during function. Next, the devices were allocated to the patients in subsequent order (J.S).

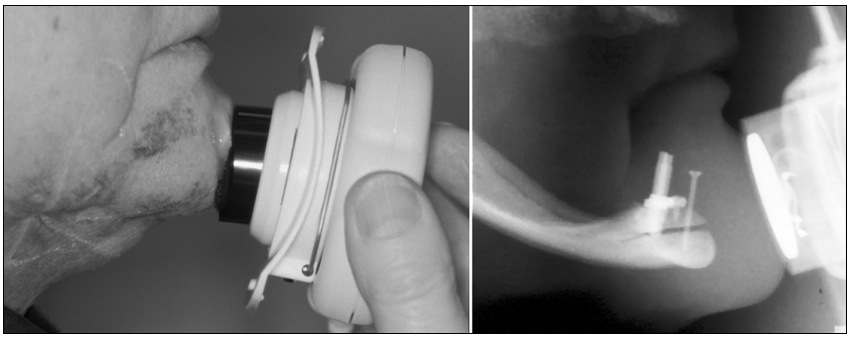


Figure 2. Ultrasound self treatment. The transducer is placed on the chin ventral of the distraction gap (a). A lateral skull radiograph was taken to assure this proper positioning of the transducer (b) so that ultrasound is directed towards the distraction gap.

The ultrasound self-treatment involved a daily treatment with ultrasound for 20 minutes on the skin of the chin covering the distraction gap (Figure 2a). During treatment, the patients were instructed not to move the transducer but keep the transducer stationary on the skin with light pressure. A lateral Tele-X radiograph with the transducer on the chin was made to verify proper positioning of the transducer in such a way that the ultrasound is directed towards the distraction gap (Figure 2b). To monitor patient compliance, the devices have an internal memory chip which records the treatment day and time, and possible errors of the device such as low battery, disconnected cables, and improper coupling between transducer and skin. The coupling alarm was only active in the active devices. The patients were instructed to keep a logbook to record the treatments

and possible problems as well. Comparing the memory chip readouts with the self-kept logbooks assessed patient compliance.

Assessment of bone formation. Four weeks post distraction, the distraction device was removed, two ITI Bonefit® implants (Straumann AG, Waldenburg, Switzerland) with an implant length of 12.0 mm were inserted, and a transmandibular bone biopsy was obtained using a trephine burr (2.0 mm inner diameter). The biopsy was fixed in buffered formaline for 24 hours and used for microradiographic and histological analysis. To assess lamellar bone formation within time, patients were asked to take a two-day course of tetracycline (250 mg, four times daily, for two days) after the last day of distraction and again after two weeks. The total treatment protocol is presented in figure 1.

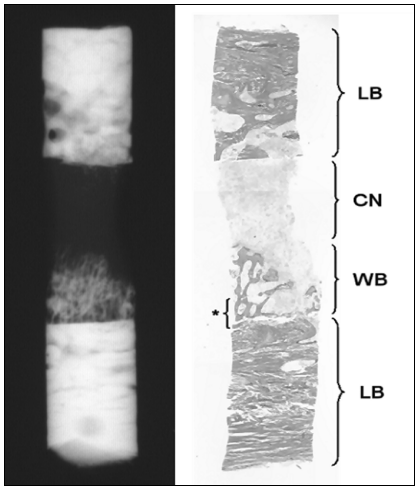


Figure 3. Microradiograph (a) and histological image (b) of a transmandibular bone biopsy, taken 4 weeks after the active distraction. As can be observed on the microradiograph, the native lamellar (cortical) bone (LB) is clearly visible, and only partial bridging of the distraction gap by mineralised tissue. Histologically, the gap is bridged by collagenous fibres (CN) arranged in the distraction direction. Woven bone (WB) is beginning to appear inside the distraction gap. Magnification x 100.

Microradiographical analysis of bone biopsies. Subsequently, a standardised, high-resolution microradiograph of the fixed biopsy was taken by a method described previously and digitised[14] (Figure 3). Inside the distraction gap, the following variables were scored by the principal investigator using the digitised microradiographs and image analysis software (Scion, Frederik, USA): the gap

fill area, defined as the area in square millimetres of the microradiograph that encloses the calcified tissue within the distraction gap, next to the osteotomy plane; and the gap grey percentage, defined as the mean grey value of the gap fill area (0% = absolute black; 100% = absolute white).

Histological analysis of bone biopsies. After the microradiograph was taken, the biopsy was dehydrated in series of ethanol and subsequently embedded in methylmethacrylate under negative pressure without decalcification. Sections of 4 µm thickness were cut parallel to the long axes of the biopsy using a microtome (Jung-K, Heidelberg, Germany). The sections were stained according to the Goldner trichrome method (Figure 3), and some sections were left unstained for fluorescent microscopy. Histological assessment took place on both sides next to one osteotomy plane (Figure 4):
Within the distraction gap, because early bone formation, if present, can be detected here; and outside the distraction gap in the remaining mandibular bone, where lamellar bone formation was expected to occur. Within and outside the distraction gap, assessment was both quantitative and qualitative.
Within the distraction gap, the gap fill length, defined as the maximum distance between the osteotomy plane and bone formation front at right angles to the osteotomy plane, was measured quantitatively using digitised images at 200 x magnification of the histological sections (Leica DM RA microscope, Leica DC 200 digital camera, Leica QWin® Software, Leica, Germany).
Qualitatively, histological scoring was performed next to the osteotomy plane inside the distraction gap to identify:
Whether new bone formation was present or not as indicated by the presence of osteoid (score 0 = no bone formation).
If present, whether the appearance of the newly formed bone itself was that of woven (score = 1), or lamellar bone (score = 2).
Also the type of bone formation was assessed (i.e., endochondral versus intramembranous).
Outside the distraction gap (i.e., within the remaining mandibular bone), the mineral apposition rate of the lamellar bone was measured quantitatively using the unstained histological sections excitated by light of 354 to 425 nm wavelength. In this way, two fluorescent tetracycline bands appeared in the sections. The average distance between the two labels was calculated by measuring the total area between two bands and dividing it by their average length. A minimum of eight consecutive labels per biopsy was measured and averaged (Leica QWin® Software, Leica, Germany).

Qualitatively, histological assessment of the remaining mandibular bone outside the distraction gap was performed to determine possible newly formed bone as assessed by the presence of osteoid.
After all measurements were completed, the code was broken and the quantitative variables were averaged for the ultrasound treatment group and the placebo treatment group.

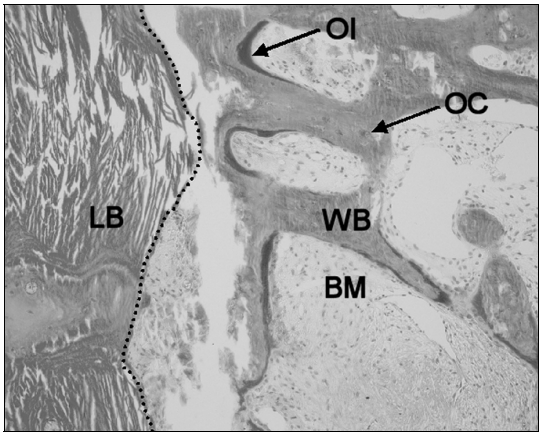


Figure 4. Histological image of woven bone next to the osteotomy plane (* in Figure 3)(Goldner Trichrome stain). The dotted line represents the osteotomy plane. At 4 weeks, calcified woven bone (WB) is beginning to appear within the distraction gap next to the lamellar bone (LB). Osteoid (OI), the non-mineralised bone matrix, is formed next to the woven bone indicating active bone formation. Inside the calcified woven bone, osteocytes (OC) are present indicating that the bone is vital. The space between the woven bone is filled with bone marrow (BM). Magnification x 200.

Follow-up. After implantation, the patients were requested to complete a short questionnaire on their experiences with the ultrasound treatment. The two most important questions were whether the handling of the device was easy or difficult, and whether 20 minutes continuous treatment was easy or difficult to maintain. Also, the following clinical complications were scored during treatment and follow-up: inflammation around the screws, loss of distraction screws, loss of implants, mandibular fracture, wound infection, wound dehiscence, instability of the cranial bone fragment and sensory disturbances of lip and chin.

Results

Eight patients (2 males, 6 females, mean age 65 ± 8.8 years, range 50 - 79 years) were selected to participate in the study. All patients completed the study protocol. The vertical distractions and implantations were uneventful. In total, sixteen implants were inserted. The overall average height of the mandible in the canine region prior to surgery was 7.1 ± 1.1 mm, and the amount of distraction averaged 6.6 ± 1.1 mm (Table 1). The average latency time was 5.5 ± 0.8 days and the average consolidation time was 31.1 ± 3.8 days. The patients were exposed to either ultrasound or placebo treatment for an average of 12.4 ± 1.2 hours (Table 1).

Table 1. Differences between the ultrasound treatment group and the placebo group (average ± SD).

	Ultrasound (n=4)	Placebo (n=4)	Overall (n=8)
Age (yr)	61.5 ± 8.3	69 ± 8.5	65 ± 8.8
Initial mandibular canine height (mm)	7.3 ± 1.5	7.0 ± 0.8	7.1 ± 1.1
Latency time (dy)	5.3 ± 0.5	5.8 ± 1.0	5.5 ± 0.8
Distraction distance (mm)	6.8 ± 1.0	6.3 ± 1.5	6.6 ± 1.1
Consolidation time (dy)	32.8 ± 4.3	29.5 ± 2.9	31.1 ± 3.8
Total time ultrasound/placebo exposure (hr)	13.0 ± 1.5	11.9 ± 1.7	12.4 ± 1.2
Microradiography gap fill area (mm^2)	1.9 ± 1.7	1.9 ± 1.3	1.9 ± 1.3
Gap grey percentage (%)	29.7 ± 31.7	40.8 ± 13.4	36.0 ± 21.4
Histology gap fill length (mm)	0.9 ± 1.1	1.4 ± 0.8	1.1 ± 0.9
Histological score	0.75 ± 0.5	1.0 ± 0.0	0.88 ± 0.35

Latency time = number of days from operation until active distraction
Consolidation time = number of days from end of distraction until insertion of implants/bone biopsy
Microradiography gap fill area (mm^2): area enclosing calcified (radiopaque) tissue in distraction gap, measured only from osteotomy side with maximum bone formation.
Gap grey percentage: average grey percentage of calcified tissue area (0% = black, indicating no tissue calcification; 100% = white, indicating high degree of tissue calcification)
Histological gap fill length (mm): maximum distance between osteotomy plane and bone formation front (measured at right angles to the osteotomy plane).
Histological score: 0 = no bone formation next to osteotomy plane within gap
1 = woven bone formation next to osteotomy plane within gap
2 = lamellar bone formation next to osteotomy plane within gap

Four weeks after distraction, it was difficult to obtain an intact transmandibular biopsy. Only three biopsies remained intact, and of these, the gap area was compressed in two (Table 2). All other biopsies broke, or only half or less of them could be recovered.
Microradiographical analysis of the biopsies revealed no difference in the area of mineralised tissue inside the distraction gap or next to the osteotomy plane. The gap fill area in the ultrasound treatment group measured 1.9 ± 1.7 mm^2, and in the placebo ultrasound group measured 1.9 ± 1.3 mm^2. Also, there were no significant differences or a trend seen in the gap grey percentage.
Histologically, within the distraction gap there was no significant difference in gap fill length. Qualitatively, all but one biopsy showed new bone formation towards the middle of the distraction gap next to the osteotomy plane (Figure 3). In the intact biopsy, the new bone was located only at one osteotomy plane, but not at the other (Figure 3). This newly formed woven bone appeared to be formed by intramembranous ossification. There were no apparent differences between the ultrasound and the placebo group. There were no signs of endochondral ossification.
Outside the distraction gap in the remaining mandibular bone, only one biopsy had a clear distinct tetracycline double label. The lamellar bone formation (mineral apposition rate) in this biopsy measured 2.63 μm/day. All other tetracycline labels were diffuse, and could not be measured. Histologically, locations of osteoid formation could be distinguished in the remaining mandibular bone. There were no apparent differences between the ultrasound and the placebo group.

The readouts of the SAFHS memory chips matched the logbooks of the patients. All patients had been treating themselves on a daily basis. According to the memory chip readouts, a total of 351 treatments had been administered; every one of which corresponded to the logbook administration. The treatment was interrupted 39 times (11%) due to disconnected cables, an improper contact between transducer and skin, or a low battery. In these cases, treatment could be resumed after correcting the error. Only one time a patient forgot a treatment.
All questionnaires were completed. All 8 patients judged the handling of the device as being easy, and the 20 minutes treatment as being convenient.
The clinical follow-up after successful insertion of the implants was 18.1 ± 4.1 months (as on July 1, 2003). During this period no complications occurred. No inflammation around the screws, no loss of distraction screws, no loss of implants, no mandibular fracture, no wound infection, no wound dehiscence, no instability of the cranial bone fragment and no sensory disturbances of lip and chin were encountered.

Table 2. Gross, microradiographic and histological analysis of the distraction biopsies.

No.	Biopsy	Distraction distance (mm)	Treatment	Microradiography gap fill area (mm^2)	Gap grey percentage (%)	Histology gap fill length (mm)	Tetracycline bands	Mineral apposition rate (µm/day)	Histological score
1	Broken, two parts	4.5	Placebo	0.95	24	-	Diffuse	-	1
2	Intact, gap compressed	8.0	Placebo	3.06	51	1.38	Clear double	2.63	1
3	Broken, one part	6.0	Ultrasound	2.74	26	0.83	Diffuse	-	1
4	Broken, two parts	8.0	Ultrasound	0	0	0	Diffuse	-	0
5	Intact, gap compressed	5.5	Ultrasound	2.99	63	2.50	Diffuse	-	1
6	Broken, two parts	5.5	Placebo	0.56	52	0.61	Diffuse	-	1
7	Broken, two parts	7.0	Ultrasound	-	-	0.34	Diffuse	-	1
8	Intact, gap intact	6.0	Placebo	2.90	36	2.20	Diffuse	-	1

-: unable to measure
Distraction distance: amount of bone lengthening according to screw torques.
Microradiography gap fill area (mm^2): area enclosing calcified (radiopaque) tissue in distraction gap, measured only from osteotomy side with maximum bone formation.
Gap grey percentage: average grey percentage of calcified tissue area (0% = black, indicating no tissue calcification; 100% = white, indicating high degree of tissue calcification)
Histological gap fill length (mm): maximum distance between osteotomy plane and bone formation front (measured at right angles to the osteotomy plane).
Histological score: 0 = no bone formation next to osteotomy plane within gap
1 = woven bone formation next to osteotomy plane within gap
2 = lamellar bone formation next to osteotomy plane within gap

Discussion

This study suggests that ultrasound treatment does not stimulate early bone formation in the severely resorbed mandible during and after a vertical distraction procedure. This was not expected because others did find a stimulating effect of the same daily 20 minutes ultrasound treatment on bone healing within a distraction gap. This was found in femur bones of rats,[7] tibial bones of rabbits,[12,15] metatarsal bones of sheep,[9,10] and in the human leg.[11] Moreover, a positive effect of the same ultrasound treatment as used in our study was found on bone formation within a mandibular distraction gap in rabbits.[8] On the cellular level, other ultrasound fields have been capable of stimulating human mandibular osteoblasts to proliferate[16] and produce angiogenesis-related cytokines, such as interleukine-8, basic fibroblast growth factor, and vascular endothelial growth factor in vitro.[17] Ultrasound can cause a reversible increase in the intracellular level of second messenger calcium in chondrocytes,[18,19] and an increase in calcium incorporation in differentiating cartilage and bone cell cultures[20] as well, which could stimulate the regenerate maturation after distraction.

Although only a limited number of patients was included in our study, making strong statements impossible, our results do suggest that ultrasound did not seem to have an effect on the amount of calcified tissue formation during the consolidation period of 31 days. The two groups studied are comparable to each other (Table 1) limiting bias of age, initial mandibular height, distraction distance, and consolidation time. Furthermore, the ultrasound treatment was administered with a high compliance rate as indicated by the internal memory chip readouts and logbooks, so that the distraction gap was exposed to the ultrasound pressure waves.

An explanation that no effect was seen may be related to the differences between the study model used (severely resorbed mandible versus 'healthy' mandibular bone and long bones), to the timing of the biopsy (the period of consolidation) or to the medications used by the patients.

First, there are perhaps differences between regenerate maturation of the long bones and the severely resorbed mandible that may serve as an explanation. In previous studies concerning osteodistraction and ultrasound, rabbit tibia,[12,15,21] rat femora,[7] sheep metatarsus[9,10] and sheep mandibles,[8] have been used as study model. Here, the stimulating effect of ultrasound on regenerate maturation has been described. In these studies, healthy animals were used with no known pathology that could influence bone healing. By contrast, the severely resorbed mandible is characterised by dense cortical bone, with a poor vascularisation, and almost no bone marrow space. However, it must be noted that a positive

effect of ultrasound on bone healing in poor vascular conditions has been observed in cases of scaphoid fractures[22] and mandibular osteoradionecrosis.[23]
Second, a reason why no effect was seen may be related to length of the consolidation period. Unfortunately, it was not possible to determine accurately how much volume of bone had grown within the complete distraction gap, because it was difficult to obtain an intact transmandibular biopsy. Most biopsies were very fragile and tended to break in half. Therefore, the microradiographic and histological measurements were performed only on the part of the biopsy that showed the most bone growth within the distraction gap. Perhaps a more accurate measurement would alter the results of this study. The microradiograph and the histological section of the intact non-compressed biopsy shows that, at 4 weeks post distraction, the distraction gap is not yet bridged by bone (Figure 3). The other biopsies do not indicate complete bridging of the distraction gap either. Comparing the intact 4-week biopsy with a previously published 8-week transmandibular biopsy,[24] the mineralisation zones in the intact 4-week biopsy are considerably smaller. Histologically, at 4 weeks, woven bone can be distinguished next to the osteotomy plane, but not yet the more mature lamellar bone (Figure 4). The soft tissue within the gap consists of collagen fibres, arranged according to the distraction direction. Because collagen is much weaker than bone, this may be the reason of breakage of the biopsies. Thus, the consolidation period may have been too short to evaluate the effect of ultrasound therapy on mandibular regenerate maturation.
The finding that 7 out of 8 tetracycline labels were diffuse, and not clear, suggests this as well. As can be observed in table 2, only one tetracycline double label could be measured, the other labels were diffuse. It may be that the tetracycline tablets were not taken according to the protocol leading to improper labels. However, the patients were highly compliant with ultrasound treatment, making non-compliance for the tetracycline labelling in 7 out of 8 patients unlikely. A more reasonable explanation may be that the timing of bone labelling was inappropriate. The labelling was done directly after the active distraction at approximately postoperative day 11 - 12 and again at day 25 - 26. It seems that the lamellar bone formation in the mandibular bone is not yet fully active at this time because no clear labels were seen in most specimens. This may also account for the fact that no difference could be found in the amount of calcified tissue inside the distraction gap after 31 days between the ultrasound and placebo group.
Thus, during the first 31 days of regenerate maturation, the bone formation within the distraction gap appears to be just beginning and it seems that this period is too short to state whether or not ultrasound may be of value in accelerating mandibular regenerate maturation.

A third reason why no effect was seen may be related to the medications of the patients. Because most elderly patients have co-morbidity that requires medical treatment, it was decided not to exclude patients that use medications. Illnesses that may influence bone healing are alcohol/drugs abuse, diabetes, osteoporosis, cancer, renal insufficiency and vascular disease. Drugs that may influence bone healing are calcium channel blockers, non-steroid anti-inflammatory drugs (NSAID's), anticoagulants, and systemic steroids. The smoking status may also influence bone healing. In the placebo group, one patient used calcium channel blockers (no. 1 in Table 2), one patient used an anti- osteoporosis drug that prevents bone resorption by osteoclasts (no. 2), the other used a low dose NSAID for cardiac reasons (no. 6) and number 8 used inhalator steroids. In the ultrasound group, the drugs used were a low dose NSAID for cardiac reasons (no. 3), and inhalator steroids (no. 4). Patient number 5 used no medications; patient number 7 smoked six cigarettes a day.
Although ultrasound can compensate for the negative effect smoking has on fracture healing,[25] and ultrasound can achieve healing of delayed unions and non-unions in healing disorders related to disease or medications,[26] the results in our study may be biased by the drugs used.

Although the finding that the distraction gap is not yet bridged by bone may suggest that the consolidation period before implant insertion was too short, no complications did occur during or after the insertion of the implants at 4 weeks. No implant failure was observed, and no complications were encountered during further treatment. After implant insertion, an osseointegration period of 12 weeks is considered before the dentures can be made. During this time, it is presumed that sufficient bone will be formed to ensure further implant stability. The finding that successful implantation at 4 weeks post distraction can be accomplished, even without bony bridging of the distraction gap between the fragments, raises the question whether the bone healing process is the limiting factor in determining the time before implant surgery in these patients. On a theoretical basis, one could speculate that the time in which the soft tissue can be stretched to cover the distracted bone seems to be the limiting factor that determines the time to implantation in these patients, but only on the condition that sufficient stability of the cranial fragment is maintained during implant surgery. This was accomplished by removing only one screw of the distraction device and insertion of a threaded implant at the time. The cranial fragment maintained stability at least by one distraction screw and the guide screw.

Based on the findings of this study, the following can be summarised:

1. During a 31-day consolidation period, ultrasound does not appear to stimulate bone formation in the severely resorbed vertical distracted mandible and it seems that this period is too short to evaluate properly if there is an effect.
2. It is possible to insert implants after 31 days of consolidation with clinical success, although the distraction gap does not appear to be bridged by bone at that time.

We feel that the trial should be continued with a longer consolidation period. It is hoped that more transmandibular biopsies will remain intact, the tetracycline labelling will be better, and that a firmer statement can be given whether or not ultrasound therapy may stimulate regenerate maturation in the severely resorbed mandible.

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Chapter 6

General discussion

The central question of this thesis was whether ultrasound therapy can be used to stimulate bone defect healing in the mandible. The literature provides information suggesting that low intensity ultrasound pressure waves can stimulate the bone healing process in different circumstances such as fresh fractures,[1-3] delayed unions,[4] non unions,[5,6] osteotomies,[7] osteodistraction,[8-10] and osteoradionecrosis.[11] This has been observed in both animal[12-15] and human studies.[1-9] However, these investigations mostly concern fractures of the long bones of the extremities. Stimulation of maxillofacial bone healing by ultrasound may be possible if the maxillofacial bone is susceptible to the ultrasound signal. However, only limited evidence is available that supports the susceptibility of this bone to ultrasound signals. Evidence that the cells of the mandibular bone respond to ultrasound was reported in an in vitro study which showed that human mandibular osteoblasts could be stimulated by ultrasound to proliferate and produce angiogenesis-related cytokines.[16] In mandibular fractures in rabbits, eight days of ultrasound treatment (five minutes each day, 0.2 - 0.6 W cm^{-2}) stimulated fracture consolidation, as compared to non-treated controls.[17] In a paper concerning the treatment of four mandibular fractures in humans, ultrasound treatment appeared to decrease pain and promote callus formation.[18] Another study found that osteoradionecrosis of the mandible could be treated with some success using 3 MHz ultrasound at 1.0 W cm^{-2}.[11] In case of mandibular lengthening by distraction osteogenesis in rabbits, it was possible to stimulate the regenerate maturation with daily ultrasound treatment.[19]
Based on the assumption that bone healing involves the same processes in the long bones as in the maxillofacial skeleton, it was investigated whether mandibular bone defect healing can be stimulated with low intensity pulsed ultrasound. The ultrasound field variables and treatment regime used in the animal experiments were identical as used in the commercially available SAFHS device, because favourable results have been obtained by its treatment.[6] Furthermore, the device is approved by the American Food and Drug Administration (FDA) for the treatment of certain fractures of the extremities.[20,21]
The pressure field as used to stimulate bone healing is characterised by being of high frequency, low intensity, and pulsed. Because of the convenience of treatment, the safety record, and self treatment possibility, the therapeutical application does not seem to be a problem in the maxillofacial area.

The animal experimental work described in this thesis suggests that daily low intensity pulsed ultrasound treatment does not stimulate bone growth into a through-and-through circular mandibular defect in rats, both in 'plain' defects and in defects covered with osteoconductive membranes.

The results from the human clinical trial suggest that daily ultrasound given during a consolidation period of 31 days does not produce a difference in early bone formation within the distraction gap between the ultrasound and the placebo group.
Because no stimulating effect of ultrasound on bone defect healing in the mandible could be established, it is tempting to suggest that ultrasound therapy has no value in maxillofacial surgery. However, reviewing the literature, there are possible explanations that would fit the 'negative' results presented in this thesis with the predominantly 'positive' results in the existing literature. These explanations may offer directions for further research efforts that would more clarify the value of ultrasound to stimulate maxillofacial bone healing.

The first explanation that would match the results of this thesis with the existing literature may relate to the susceptibility of mandibular bone to the type of ultrasound and treatment regime. The ultrasound field variables and treatment regime used in the animal experiments presented in this thesis were identical as those used in the commercially available SAFHS device. These variables were chosen because bone appeared sensitive to this type of ultrasound field. However, differences in treatment regimes (treatment time, treatment period) or differences in field variables (frequency, intensity, pulse duration) may be related to differences in the effect of ultrasound on bone healing or the cells involved in bone healing. For example, in rabbits, osteochondral defects of the patella healed earlier and with less degenerative changes at follow up when treated with low intensity pulsed ultrasound. It was found that a treatment time of 40 minutes per day increased the histological quality of the repair cartilage as compared to a treatment time of 20 minutes a day.[22] When the same low intensity ultrasound is applied in cases of mandibular lengthening by distraction in rabbits, daily 20 minutes treatment on one side of the mandible produced more regenerate maturation than alternating daily 20 minutes treatment on both sides.[19] Differences in ultrasound field variables may alter the tissue response as well.[23] It must be noted that, despite the differences in treatment regimes or ultrasound field variables, the overall picture in the literature is one of bone healing stimulation, with a relatively minor emphasis on the importance of differences in field variables. However, this does not exclude that there may be an optimal combination of variables for mandibular bone, but this has not been established yet.

The second reason may be related to the perfusion and healing capacity of the head and neck region. Because the mechanism as to how ultrasound stimulates bone healing is not entirely clear, it is difficult to predict in which case

ultrasound will or will not stimulate bone healing. It has been reported that the pressure wave serves as a surrogate for physiological stresses in bone, which stimulate bone formation.[24] Apart from piezo-electric[25-28] and membrane effects,[29-32] part of the ultrasound effect seems to be related to angiogenesis.
In ischaemic tissues, where blood perfusion is limited, ultrasound can promote neovascularity and neocellularity.[33] In dogs with an ulnar osteotomy, daily 20 minutes ultrasound treatment with the SAFHS device for 8 weeks produced an increase in blood flow around the osteotomy site after 2 - 3 days and this increase lasted for two weeks as compared to the non treated controls.[15] Ultrasound can also stimulate the production of angiogenesis-related cytokines (Interleukin-8, fibroblast growth factor and vascular endothelial growth factor) in human mandibular osteoblasts,[23] which indicates that ultrasound may stimulate angiogenesis.
This may explain why the stimulation of bone healing with ultrasound is apparent in compromised healing situations such as delayed and non-unions of the extremities,[4,6,7] the healing of scaphoid fractures,[3] and osteoradionecrosis of the mandible.[11] These compromised healing situations are thought to be related to a relatively poor blood supply due to anatomical predisposition, vascular disease, treatment (medication, radiation) or habit (smoking).[34] 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 (read: optimal blood perfusion) can be influenced by ultrasound. The head and neck area of the body is well blood perfused and can, therefore, 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 presented studies. The rats used were mature, healthy, and had no known disorders that could compromise angiogenesis/bone healing. However, there are contra arguments to this theory. In healthy rabbits with an apparent optimal perfusion of the head and neck region subjected to mandibular lengthening by osteodistraction, low intensity pulsed ultrasound therapy seemed to accelerate the regenerate maturation in the distraction gap.[19]

The third explanation that would place most of the results of the experimental work presented in this thesis into perspective, may be related to the types of animal and human models used. It has been explained that bone remodels according to functional demands (Wolff's law)[35] which means that bone exists by virtue of mechanical loading. When a fracture occurs, the bone will not be used as it would have been used before and, therefore, this stimulus by

physiological loading will be absent. The healing of a fracture is largely dependent on blood supply and stability.[36] Recent insights indicate that the gap size, hydrostatic pressure and micro-movement between the fractured bone parts are fundamental factors influencing fracture healing.[37,38] It has been proposed that ultrasound therapy serves as a substitute for the physiological loading,[24] and therefore would give an additional incentive for a broken bone to heal. It seems that ultrasound waves exert pressure on the cellular level where bending of the bone-cells membrane alters its ionic permeability and, eventually, its metabolism.[29,30,32] Thus, in case of a bone fracture, there is absence of physiological loading and presence of a certain micro-movement at the fracture site.

In case of a bone defect without bone discontinuity, the situation is reversed. In the mandibular defect-model described in this thesis, physiological use of the mandible is preserved, because there is no discontinuity (read: fracture) of the mandibular bone. Accordingly, there is no micro-movement that may be influenced by ultrasound. This may explain why ultrasound treatment may not have had an effect on the bone healing in the rat mandibular defects with or without the use of osteoconductive membranes. This reasoning may also be applied to the human distraction experiment. During and after the operative procedure, the continuity of the mandible remains preserved. After the operative procedure, the patients can still use their mandible for eating and speaking, albeit limited. This means that the mandible remains subjected to mechanical forces, similar to physiological use of it. In other words, the differences between a bone injury with (fracture) or without (defect) loss of continuity may account for the fact that no effect was seen of ultrasound therapy on bone defect healing.

It is difficult to suggest which of the above explanations may be the most applicable and it may also be that there are unknown factors involved. For now, it is reasonable to assume that ultrasound pressure waves do influence the cells involved in the bone healing process, but that this influence may be related to mechanical and circulatory conditions at the site of bone injury.

Conclusions

The conclusions of the experimental work presented in this thesis are:

1. Low intensity pulsed ultrasound is not effective in stimulating bone growth into a rat mandibular defect, with or without the use of osteoconductive membranes.
2. Low intensity pulsed ultrasound does not seem to stimulate early bone healing in the severely resorbed vertical distracted mandible.

Future perspectives

This thesis focused on a small area in the field of ultrasound and bone healing that had not been explored before. The animal experimental work indicates that ultrasound does not stimulate mandibular bone defect healing with or without the use of osteoconductive membranes in healthy animals. This may be related to the ultrasound field variables used, to an optimal healing tendency of the head and neck region, or to limitations of the animal model. To differentiate between these possibilities, additional animal experiments may be persued repeating the experiments using other ultrasound field variables, using a compromised bone healing model such as for example, irradiated mandibular bone, or developing other maxillofacial bone healing models with the emphasis on bone discontinuities (for example developing a mandibular fracture model). More importantly, unravelling the mechanism of action as to how ultrasound stimulates bone healing in certain cases may eventually predict if, and if so, when, ultrasound may be of value in maxillofacial surgery.

The human experimental work indicates that in case of the severely resorbed distracted mandible, a longer consolidation period before the biopsy may be taken into account to ensure an intact biopsy, or a different method should be used to measure the amount of bone fill in the distraction gap. In this way it may be possible to assess more accurately if ultrasound can accelerate regenerate-maturation in the vertical distracted severely resorbed mandible.

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Chapter 7

Summary

Ultrasound is a high frequency sound wave (> 20.000 Hz) that is used in medicine for diagnostic and therapeutic purposes. When applied to tissue, the high frequency pressure waves will exert a vibrating force onto the tissue. Higher ultrasound intensities may cause heating of tissue because the energy of the vibrations is converted into heat. These vibrations and local heating are considered stimulate healing of damaged tissue. Since the first therapeutic ultrasound application in 1932, ultrasound therapy has evolved within the physiotherapy practice mainly to treat soft tissue disorders. Until today, controversy remains with regard to the effects of ultrasound on soft tissue healing.

In the past, there have also been attempts to stimulate bone fracture healing with ultrasound. Since bone exists by virtue of physiological mechanical loading, ultrasound pressure waves would act as an alternative for this physiological loading in case of a fracture. Ultrasound would, therefore, act as an extra stimulus for a fracture to heal.

The first experiments to stimulate bone fracture healing with ultrasound failed, however, because the ultrasound intensities that were used were too high. Bone has a higher density than soft tissue, and is easily overheated during ultrasound application. Later (1950 - 1970), it became clear that ultrasound could stimulate bone fracture healing by using ultrasound with lower intensities that would prevent overheating. After animal experimental work, indicating that ultrasound could stimulate fracture healing under certain circumstances, the first prospective randomised double blind clinical trials were published in the nineties. The results of these studies indicated that the healing time of fresh tibial and radial fractures could be reduced by 38% after ultrasound was applied. Here, low intensity pulsed ultrasound was used with an intensity of 30 milliwatts per square centimetre, 20 minutes a day, by placing a transducer onto the skin across the fracture. It also became clear that slow or non-uniting fractures could be healed by the application of ultrasound. Furthermore, it seemed that the effect of ultrasound is not limited to fracture healing, but that bone healing after osteotomy or osteodistraction could be stimulated as well.

A substantial part of the maxillofacial surgery practice deals with bone and bone healing. It is, therefore, interesting to assess whether ultrasound can stimulate bone healing in the maxillofacial region. If so, ultrasound could be beneficial in the healing of facial fractures, healing of bone defects after reconstructive surgery or osteodistraction, and in the osseointegration of dental implants. Based on the assumption that the process of bone healing of the facial bones and the extremities is essentially the same, the potential of ultrasound to stimulate maxillofacial bone healing was investigated in a literature review *(Chapter 2)*. Although most of the existing literature deals with bone of the

extremities, there are indications that mandibular bone is responsive to ultrasound as well. It was, therefore, concluded that there may be a potential for ultrasound to stimulate maxillofacial bone healing.

Maxillofacial procedures often involve the mandible. Especially the reconstruction of bone defects can pose challenging technical problems. Therefore, the experimental work described in this thesis has been carried out to explore whether ultrasound therapy can be used to stimulate bone defect healing in the mandible. The research comprised an animal experimental and a clinical part.
The model used to conduct the animal experiments was the rat mandibular defect. This model consists of a round bicortical defect, drilled through the ramus of the mandible. The model was chosen because of its obvious advantages: the operative procedure is relatively simple, and the alveolar nerve as well as chewing ability remain preserved. Furthermore, the mandibular defect model has been extensively used and described in the literature.
Microradiography was used as the method to measure bone growth into the rat mandibular defect. The reason for using this technique rather than histology was that histological sections only represent one part of the defect. Moreover, histology is relatively time consuming and expensive. Microradiography has been described previously, but it had not yet been applied to measure areas of bone growth into rat mandibular defects. In c*hapter 3*, high resolution microradiography was compared to traditional histology and it was found that both were accurate in measuring defect widths. The microradiography technique may also be used to measure a defect's area in addition to its width. Therefore, microradiography was used in the subsequent three experiments to measure the area of bone growth into a rat mandibular defect.
In *chapter 4.1* a placebo controlled single blind study is described in which it was assessed if ultrasound can stimulate bone growth into a rat mandibular defect. Either low intensity pulsed ultrasound or placebo was applied 20 minutes a day on the skin across the defect under general anaesthesia. An additional control group with only the defect was included. After two and four weeks of ultrasound/placebo application, no additional bone growth into a "plain" rat mandibular defect as an effect of ultrasound could be demonstrated. Presumably, the fast soft tissue ingrowth into the defect had blocked the slow bone growth from the defect rims. This phenomenon may have masked an ultrasound effect. Therefore, an attempt was made to stimulate bone growth in a different model, where soft tissue ingrowth was prevented. First, it was attempted to stimulate bone growth with ultrasound into a rat mandibular defect covered by an expanded polytetrafluoroethylene (e-PTFE) membrane

(Chapter 4.2). The experimental design was similar to the previous experiment. After two and four weeks, no effect of ultrasound on the amount of bone defect healing was observed. Since the membranes appeared to block most of the ultrasound, it was concluded that this was the reason why no effect was seen.
Therefore a second attempt was undertaken, by using collagen membranes that do not block ultrasound *(Chapter 4.3)*. However, still no effect of ultrasound on the amount of bone formation into the mandibular defects was seen.
The maxillofacial region is very well blood perfused, and, therefore, is considered to have an optimal healing capacity. This might explain why ultrasound did not have an additional effect on the bone defect healing. As a consequence, a randomised clinical trial was set up to evaluate if bone healing of the mandible could be stimulated in a relatively compromised healing situation. In the severely resorbed, atrophic mandible it was investigated whether ultrasound could stimulate early bone formation within a bone gap after an osteodistraction procedure *(Chapter 5)*. The results (after 31 days) indicate that ultrasound does not appear to affect the early bone formation, which seemed to be in an initial state, within the distraction gap. Possibly an effect would have been found after a longer period of bone healing.

The results of the experimental work presented in this thesis suggest that ultrasound does not stimulate bone growth into mandibular defects, with or without the use of osteoconductive membranes. This finding is not in accordance with the literature concerning ultrasound, fracture healing, and the healing of bone defects. In the literature, in most instances a positive effect is reported. There are, however, explanations that might fit the 'negative' results of the experimental work into the 'positive' results of the current literature.
A first explanation may be that the bone of the mandible is not responsive to the field characteristics of the ultrasound that is usually applied to the bone of the extremities. Variations in ultrasound frequency, intensity, pulse duration, and applied scheme may result in different effects than those found with the applied ultrasound field.
A second explanation may be related to the perfusion of the head and neck region. Ultrasound pressure waves have piezo-electric effects and effects on the cell membrane. Also, the formation of vessels is influenced by stimulating the production of certain proteins excreted by bone cells. When vessel formation is stimulated, transport of blood (and hence of oxygen and nutrients) is facilitated and healing is, therefore, stimulated as well. The head and neck region is well perfused and is considered to have an optimal healing capacity. This raises the question as to whether ultrasound can stimulate bone healing in a region where the healing capacity is already optimal. The clinical trial was set up with the idea

that ultrasound might stimulate bone healing under suboptimal circumstances. In the severely resorbed mandible in the elderly, blood perfusion is considered to be compromised. The results indicate that ultrasound does not affect the early bone formation inside the distraction gap in the severely resorbed mandible. After 31 days, the bone formation seems to be just beginning and it may be that the effect of ultrasound was evaluated too early.
A final explanation may be related to the characteristics of the animal and human model used in the experiments. It has been explained in the literature that bone exists by virtue of mechanical loading (Wolff's Law). When a fracture occurs, the affected body part will not be used, and thus mechanical loading will be virtually absent. In the animal experiments presented in this thesis, however, there was no fracture of the mandible. Instead, the model consisted of a bone defect. By using this model, the functional capacity of the mandible remains preserved and, as a consequence, the mandible remains mechanically loaded during defect healing. Similarly, during the mandibular distraction procedure in the clinical study, no discontinuity of the mandible has been introduced. The mandible was still loaded to some extent during the healing phase. For this reason, ultrasound may not have shown to be effective in these cases.
It is difficult to reveal which explanation would be most feasible. It may also be that unknown factors are of influence.
Based on the literature, it seems to be reasonable to assume that ultrasound has an effect on bone cells during bone healing, but that a possible observed effect may be related to the mechanical and circulatory conditions at the site.

The conclusions of the experimental work presented in this thesis are:

1. Low intensity pulsed ultrasound is not effective in stimulating bone growth into a rat mandibular defect, either with or without the use of osteoconductive membranes.
2. Low intensity pulsed ultrasound does not seem to have an effect on the early bone formation in the vertically distracted, severely resorbed mandible.

This thesis focused on a small area in the field of ultrasound and bone healing that had not been explored before. The animal experimental work indicates that ultrasound does not stimulate mandibular bone defect healing with or without the use of osteoconductive membranes in healthy animals. This may be related to the ultrasound field variables used, to an optimal healing tendency of the head and neck region, or to limitations of the animal model. To reveal which of these possibilities is the most plausible, additional research is needed. For now, it is not recommendable to apply ultrasound in maxillofacial surgery to stimulate bone defect healing. In situations where mechanical loading or blood perfusion

is limited, as for example in the case of mandibular fractures or osteoradionecrosis, ultrasound might have an effect. More importantly, unravelling the mechanism of action as to how ultrasound stimulates bone healing in certain cases may eventually predict if, and if so, when, ultrasound may be of value in maxillofacial surgery.

Chapter 8

Samenvatting

Ultrageluid is een hoog frequente geluidsgolf (> 20.000 Hz) die in de geneeskunde gebruikt wordt voor diagnostische en therapeutische doeleinden. Ultrageluid als therapie is gebaseerd op het fenomeen dat de hoogfrequente drukgolven het te behandelen weefsel in trilling brengen en dat bij hogere geluidsintensiteiten de energie van de vibratie het weefsel opwarmt. Er zijn aanwijzingen dat deze vibratie en opwarming de genezing van beschadigd weefsel zouden bevorderen. Sinds de eerste therapeutische toepassing van ultrageluid in 1932, heeft therapie met ultrageluid zich vooral binnen de fysiotherapie sterk ontwikkeld, met name voor de behandeling van de weke delen, zoals bijvoorbeeld de spieren. Het effect van ultrageluid op het herstel van weke delen letsel is tot op heden evenwel onderwerp van discussie.
Kort na de eerste toepassing van ultrageluid ontstond het idee dat hoogfrequente vibraties de genezing van botbreuken (fracturen) zouden kunnen stimuleren. De gedachte hierachter was (en is) dat bot bestaat bij de gratie van fysiologische mechanische belasting en dat hoogfrequente geluidsgolven een alternatief vormen voor deze belasting bij een fractuur. Ultrageluid zou dus een extra mechanische stimulans zijn bij de fractuurgenezing.
De eerste in de literatuur beschreven experimenten om botgenezing met ultrageluid te stimuleren liepen spaak, omdat er te hoge ultrageluidintensiteiten werden gebruikt. Door de hogere dichtheid van bot in vergelijking met weke delen geraakte het bot oververhit. In de loop der jaren (1950 - 1970) werd duidelijk dat fractuurgenezing gestimuleerd kon worden door toepassing van ultrageluid, zolang er maar een lagere intensiteit werd gebruikt die het bot niet verhit. Na dierexperimenteel onderzoek waaruit bleek dat ultrageluid de genezing van fracturen inderdaad kan versnellen, werden in de jaren negentig enkele prospectieve dubbelblinde gerandomiseerde klinische onderzoeken uitgevoerd. Hieruit bleek dat de toepassing van ultrageluid de genezingsperiode van verse tibia- en radiusfracturen met 38% kon verkorten. Hierbij werd lage intensiteit gepulseerd ultrageluid gebruikt met een intensiteit van 30 milliwatt per vierkante centimeter, dat middels een transducer 20 minuten per dag op de huid boven de fractuur werd geappliceerd. Ook werd duidelijk dat langzaam of niet genezende fracturen alsnog kunnen genezen door toepassing van ultrageluid. Tevens is gebleken dat het effect op de botgenezing zich niet tot fracturen beperkt, maar dat botgenezing na bijvoorbeeld osteotomieën of botdistracties mogelijk ook door ultrageluid gestimuleerd kan worden.
De kaakchirurg is een aanzienlijke hoeveelheid van zijn tijd bezig met bot en botgenezing. Het was daarom de moeite waard om te bestuderen of de botgenezing in het aangezicht eveneens gestimuleerd kan worden met ultrageluid. Zo kan men denken aan de versnelde genezing van aangezichtsfracturen, de genezing van botdefecten na reconstructies of

botdistracties, en het vastgroeien van tandheelkundige implantaten. Aangenomen dat het proces van botgenezing in het aangezicht overeenkomt met de botgenezing in de extremiteiten, zijn in een literatuurstudie de mogelijkheden van ultrageluid om botgenezing in het aangezicht te stimuleren onderzocht *(Hoofdstuk 2*). In de literatuur bleek hierover nog weinig bekend; de beschreven studies betreffen veelal het bot van de lange pijpbeenderen. De resultaten van een beperkt aantal in vitro en klinische studies suggereren dat ook kaakbot gevoelig is voor ultrageluid. Op grond hiervan werd geconcludeerd dat het stimuleren van botgenezing in het aangezicht met ultrageluid de moeite van het bestuderen waard is.

Kaakchirurgische ingrepen betreffen vaak de onderkaak, waarbij het sluiten van botdefecten een groot probleem vormen. Het onderzoek, dat in dit proefschrift wordt beschreven, is dan ook uitgevoerd om een antwoord te krijgen op de vraag of toepassing van hoogfrequente geluidsgolven (ultrageluid) de genezing van botdefecten in de onderkaak kan stimuleren. De experimenten betreffen een dierexperimenteel *(Hoofdstukken 3 en 4)* en een klinisch deel *(Hoofdstuk 5).*
Het model dat werd gebruikt voor de dierproeven was een rond defect, dat door de onderkaak van de rat heen werd geboord. Dit model heeft als voordeel dat de operatie relatief gemakkelijk is uit te voeren, een belangrijke kaakzenuw gespaard blijft en de rat na de operatie kan blijven eten. Het model is uitgebreid beschreven en gebruikt in de literatuur.
Om de botgroei in het kaakdefect van de rat te meten werd gebruik gemaakt van microradiografie. Microradiografie werd reeds beschreven in de literatuur, maar niet met het doel botgroei in een kaakdefect te meten. Er is voor microradiografie en niet voor histologie gekozen omdat histologie over slechts een klein deel van het defect informatie verschaft en de techniek bovendien relatief tijdrovend en duur is. In de in *hoofdstuk 3* beschreven studie werden daarom hoge resolutie microradiografie opnames van kaakdefecten vergeleken met overeenkomstige histologische secties. Het bleek dat beide methoden nauwkeurig zijn voor het meten van defectbreedtes; met microradiografie kunnen evenwel ook oppervlaktemetingen worden gedaan. Op grond van deze resultaten werd microradiografie vervolgens gebruikt in drie ultrageluid experimenten waarbij de oppervlakte van botgroei in de kaakdefecten werd gemeten.
In *hoofdstuk 4.1* wordt een placebo gecontroleerd, geblindeerd dierexperiment beschreven waarbij is gekeken of lage intensiteit gepulseerd ultrageluid de botgroei in een kaakdefect in de onderkaak van de rat kan stimuleren. In algehele narcose werd gedurende 20 minuten per dag of ultrageluid of placebo toegepast door middel van een transducer op de huid boven het defect. In een

extra controlegroep werd alleen een defect aangebracht. Na twee en na vier weken bleek dat ultrageluid geen invloed had op de hoeveelheid bot dat in een kaakdefect groeit. Het is aannemelijk dat het omgevende bindweefsel snel het defect ingroeit en zo de botgroei aan de rand tegenhoudt. Aangezien dit fenomeen van snelle bindweefsel-ingroei een mogelijk effect van ultrageluid zou kunnen maskeren, werd in een volgende experimentele studie de botgroei in het kaakdefect gemeten, nadat er voor was gezorgd dat ingroei van bindweefsel in het defect werd verhinderd.
In een eerste opzet werd getracht de botgroei met ultrageluid te stimuleren in een kaakdefect dat was bedekt met een geëxpandeerd polytetrafluoroethyleen (e-PTFE) osteoconductief membraan *(Hoofdstuk 4.2)*. De experimentele opzet was dezelfde als bij het voorgaande experiment. Na twee en vier weken werd geen effect gemeten van ultrageluid op de botgroei in het defect. Uit hierop gerichte metingen bleek het membraan het grootste deel van het ultrageluid tegen te houden. Op grond hiervan werd geconcludeerd dat dit de meest voor de hand liggende reden was waarom er geen effect werd waargenomen.
Daarom werd vervolgens een tweede opzet gekozen, waarbij gebruik werd gemaakt van een collageen membraan dat wel doorgankelijk is voor ultrageluid *(Hoofdstuk 4.3)*. Ook in deze situatie bleek echter geen effect van ultrageluid op de genezing van een botdefect in de onderkaak waarneembaar te zijn.
De goede doorbloeding van de hoofdhals regio en een daarmee samenhangende optimale genezingstendens, kan mogelijk verklaren dat van ultrageluid geen effect kon worden aangetoond. Om hierin meer inzicht te krijgen werd een dubbelblinde klinische studie opgezet om de invloed van ultrageluid op de kaakbotgenezing in een relatief suboptimale situatie te bestuderen.
Bij patiënten met een sterk geresorbeerde, atrofische onderkaak werd gekeken of ultrageluid de vroege verbening in een botspleet na een kaakverhogingsprocedure door distractie kan versnellen *(Hoofdstuk 5)*. De resultaten uit deze suggereren dat ultrageluid geen effect heeft op de vroege verbening in de distractiespleet. Na 31 dagen lijkt de verbening echter net op gang te zijn gekomen, zodat na een langere periode wellicht wel een effect waarneembaar zou kunnen zijn.

De resultaten van het in dit proefschrift beschreven dierexperimenteel werk suggereren dat ultrageluid de botgroei in defecten in de onderkaak niet stimuleert, onafhankelijk van het gebruik van osteoconductieve membranen. De bevindingen komen niet overeen met de literatuur over het effect van ultrageluid op fractuurgenezing en genezing van een botdefect in het algemeen; de in de literatuur beschreven tendens is steeds een gunstige. Er zijn evenwel verschillende verklaringen mogelijk die de 'negatieve' resultaten van dit

dierexperimentele werk passend maken met de 'positieve' resultaten binnen de huidige literatuur.
Een eerste is dat het bot van de onderkaak niet gevoelig is voor de eigenschappen van het ultrageluidveld dat bij studies aan de extremiteiten wordt gebruikt. Variaties in geluidsfrequentie, intensiteit, pulsatieduur en het toegepaste schema zouden een ander effect kunnen sorteren, dan het effect dat bij toepassing van het in deze studie gebruikte ultrageluidveld werd geconstateerd.
Een tweede verklaring kan met de bloedvoorziening van het hoofdhals gebied te maken hebben. De hoogfrequente drukgolven hebben invloed op vaatvorming door het stimuleren van de productie van vaatgroei-stimulerende eiwitten door botcellen. Door een betere vaatvoorziening wordt transport van bloed (en daarmee van zuurstof en bouwstoffen) van en naar het aangedane gebied bevorderd, hetgeen de genezing ondersteunt. Het hoofdhals gebied is al zeer goed doorbloed en wordt verondersteld een optimale genezingstendens te hebben. Het is daarom de vraag of botgenezing wel extra gestimuleerd kan worden door ultrageluid in een gebied waarin de genezing al optimaal verloopt. De klinische experimentele studie werd opgezet om te bestuderen of ultrageluid de botgenezing in een suboptimale situatie wellicht wel zou kunnen bevorderen. Bij een sterk geresorbeerde onderkaak in een oudere patiënt is de bloedvoorziening van de onderkaak verminderd. Niettemin bleek ultrageluid geen effect heeft op de vroege verbening in een distractiespleet bij patiënten met een sterk geresorbeerde onderkaak. De resultaten suggereren dat de botvorming in de distractiespleet na 31 dagen nog maar net begint en dat er wellicht te vroeg is gemeten om een effect uit te kunnen sluiten dan wel te constateren.
Ten slotte kan een verklaring worden gezocht in de aard van het model dat is gebruikt voor de experimenten. In de literatuur is beschreven dat bot zich vormt op geleide van de mechanische belasting (Wet van Wolff). Bij een botbreuk wordt het aangedane lichaamsdeel niet gebruikt waardoor de fysiologische mechanische belasting nagenoeg afwezig is. In het diermodel dat in dit onderzoek is gebruikt, was echter sprake van een defect, en niet van een breuk in de kaak. Hiermee blijft de kaakfunctie behouden en blijft de kaak tijdens de defectgenezing dus mechanisch belast. Ook bij de klinische experimentele studie blijft de onderkaak in zekere mate belastbaar omdat tijdens de distractiebehandeling geen discontinuïteit van de onderkaak optreedt. In deze gevallen zou ultrageluid mogelijk geen additioneel effect hebben op de botgenezing.
Het is lastig te bepalen welke verklaring het beste past. Bovendien kunnen nog onbekende factoren meespelen. Het is op grond van de literatuur aannemelijk dat ultrageluid wel een effect heeft op botcellen tijdens botgenezing, maar dat

het waarneembare effect afhankelijk lijkt te zijn van de mechanische en circulatoire omstandigheden op de plaats van de genezing.

Uit het in dit proefschrift beschreven onderzoek, kunnen de volgende conclusies worden getrokken:

1. Lage intensiteit gepulseerd ultrageluid is niet effectief om botgroei te stimuleren in een botdefect in de onderkaak van de rat, met of zonder het gebruik van osteoconductieve membranen.
2. Lage intensiteit gepulseerd ultrageluid lijkt geen effect te hebben op de verbening in de verticaal gedistraheerde menselijke onderkaak in de eerst 31 dagen na de operatie.

In het onderzoek van dit proefschrift wordt een nog niet eerder beschreven aspect van ultrageluid en botgenezing belicht. Het dierexperimenteel werk heeft laten zien dat ultrageluid de botgroei in defecten in de onderkaak niet stimuleert. Dit kan te maken hebben met het ultrageluidveld, de bloedvoorziening van het hoofdhals gebied, of met het gekozen onderzoeksmodel. Verder onderzoek is noodzakelijk om een juiste verklaring voor de resultaten te kunnen geven. Het is vooralsnog niet aan te bevelen ultrageluid toe te passen in de kaakchirurgie om de genezing van botdefecten in het aangezicht te stimuleren. In situaties waarbij mechanische belasting afwezig is of de bloedvoorziening gecompromitteerd, zoals bij fracturen, respectievelijk osteoradionecrose, is het niet ondenkbaar dat met ultrageluid wel een relevant effect kan worden bewerkstelligd. Het verdient aanbeveling komend onderzoek primair te richten op het werkingsmechanisme van ultrageluid op de botgenezing. Als bekend is op welke manier ultrageluid de botgenezing in andere situaties stimuleert zou wellicht beter voorspeld kunnen worden of, en zo ja in welke gevallen, ultrageluid toegepast zou kunnen worden in de kaakchirurgie.

Appendix 1

The weight of 1.5 MHz ultrasound. Calibration of a therapeutic unit for small animals

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This appendix is an adapted version of Lubbers[1], in which additional details can be found.

Abstract
A 4-channel customised ultrasound unit for treating small animals with therapeutic ultrasound at 1.5 MHz has been set to specified output similar to that of the Sonic Accelerated Fracture Healing System device (SAFHS, Smith & Nephew, Exogen, TN, USA). Methods to measure the properties of an ultrasonic field according to IEC 61161 and IEC 61689 have been implemented. Specifications of the customised unit are: pulse repetition frequency: 1 kHz, duty cycle: 20%, effective radiating area of the transducer: 1.42 ± 0.11 cm^2, collimated beam. The temporal average power was set to 43 ± 6 mW, giving an effective intensity of 30 ± 6 mW cm^{-2}. The beam non- uniformity ratio is 2.3. During the course of animal experiments, no significant change was observed in the ultrasound intensity of the transducers.

1 Introduction

Ultrasound to stimulate the healing of fractures has been applied to the bones of the leg and the arm.[2] In order to investigate whether this method applies also to the mandible, a series of experiments was planned on rats. As the ultrasound transducer used for human treatment (SAFHS) with a diameter of 31 mm is too large to use on rats, 4 smaller customised transducers with an external diameter of 18 mm were made (RTD, Röntgen Technische Dienst, Rotterdam, The Netherlands). The 4 nominally identical transducers were energised by a 4 channel generator (ICT-networks, University Hospital Groningen, Groningen, The Netherlands). It was desired to use the same ultrasound intensity as in human treatment which uses I_{SATA} = 30 mW cm^{-2} (spatial average-temporal average intensity). This intensity is high enough to promote healing and low enough so that no damage is done.

In order to measure intensities methods described in documents of the International Electrotechnical Commission (IEC) 61689 and IEC 61161 have been implemented.[3,4] In short two measurements are performed. First, the power of the ultrasound beam emitted from the transducer is measured with a balance. Second, the distribution of the intensity in the ultrasound beam was determined by scanning the beam with a hydrophone, from which the effective area of the beam is derived. The quotient of power and effective area gives the intensity. The measurements were aimed at obtaining the parameters of the instrument as required by IEC 60601-2-5 Ed 2.[5]

1.1 Signal generation and electronic measuring instruments

The 4 transducers (RTD) have a piezo-electric crystal with a diameter of 15 mm. This is the active element. The housing is made from a stainless steel cylinder, outer diameter 18 mm and length 80 mm, with a cable of length 1 m, ending in a BNC-connector. A thin (3 mm) coaxial cable is used to insure flexibility. The transducer is designed for operation at 1.5 MHz.

Each transducer is intended to be driven by the corresponding channel of the 4 channel ultrasound generator. This generator gives waveforms with an amplitude of 15 to 30 V_{pp}, adjustable by a potentiometer on the front of the unit. The waveform exists out of pulse trains with a fundamental frequency of 1500 kHz, and a length of 200 μs. The repetition time is 1000 μs. The four transducers are sequentially active. The voltages on the transducer are measured when coupled to water and in the absence of standing waves. For test purposes the transducer has also been driven by a function generator (model 3314A, Hewlett Packard, Everett, Washington, USA). This generator was generally set at its sinusoidal drive, maximum voltage ($10V_{pp}$ in 50 ohm) and continuous wave mode. Pulse trains of various lengths and intervals could also be obtained.

Voltages were measured on a 4 channel digital oscilloscope (PM3394, Philips, Eindhoven, The Netherlands) with delayed triggering and an input impedance of 1 MΩ // 25 pF. Hydrophonic measurements of the ultrasound wave revealed a second harmonic of -38 dB, a third harmonic of -41 dB and a fourth harmonic of -49 dB. Thus harmonic distortion of the sound field is not considered.

2 Power measurement

2.1 Theory

When an ultrasonic beam of power P in a medium with sound velocity c is completely absorbed by an absorber, a force F is exerted on the absorber which satisfies

$$F = P / c$$

In the balance which measures vertical forces we have a flat horizontal absorber. The beam impinges under an angle θ with the normal. Then the force in the direction of the vertical is given by

$$F_v = P \cos(\theta) / c$$

Of the beam a fraction R is reflected (Figure 1), of course also under an angle θ with the normal. The recoil of this beam adds a force F_R

$$F_R = P R \cos(\theta) / c$$

The total vertical force $F = F_v + F_R$, hence we find

$$P = c F / (1+R) \cos(\theta) \qquad (1)$$

The radiation pressure exerts also a horizontal force on the balance. A balance is constructed in such a way that it is insensitive to small horizontal forces. Therefore, the horizontal component of the radiation force can be ignored.

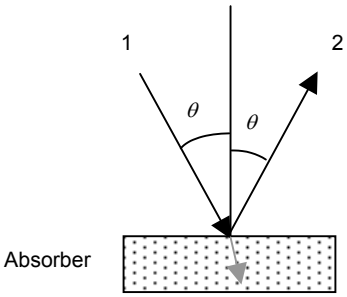

Figure 1. Ultrasonic beam 1 falls under an angle θ with the normal on the absorber. A small part of the energy is reflected as beam 2. Most energy of the beam enters the absorber.

The intensity of the reflected beam is calculated from formulae derived from Wells.[6] Only longitudinal waves are considered, this is justified for beams not far from the normal. If an ultrasonic beam impinges on a boundary under an angle θ with the normal, a part of the beam is reflected and a part of the beam is transmitted under an angle θ_t. If the sound velocity in the first medium is c_1 and in the second medium is c_2 the angle θ_t is calculated from

$$\theta_t = \arcsin (c_2 * \sin (\theta) / c_1)$$

The reflection coefficient is given by

$$R = ((Z_2 \cos (\theta) - Z_1 \cos (\theta_t)) / (Z_2 \cos (\theta) + Z_1 \cos (\theta_t)))^2 \qquad (2)$$

in which Z is the acoustic impedance (the product of c and density ρ) of the two media.

2.2 Target

For the absorbing target a piece of black rubber with a thickness of 6.4 mm was used in the shape of an octagon. The distance between the sides of the octagon was 45 mm. The density was determined by weighing the target in air (13.513 g) and under water (3.016 g), with a reading accuracy of 0.005 g. Using 999 kg/m^3 for the density of water, the density of the rubber was found to be 1286 ± 1 kg/m^3. The acoustic properties were determined at 1.5 MHz by insertion of the absorber in an ultrasound path in water.[7] The change of transmission was –22.5 dB and the change in arrival time –0.50 μs. From the change in arrival time and

the sound velocity in water (1482 m/s at 20 °C) the sound velocity in rubber was derived as 1670 m/s. From formula 2 the reflection coefficient at θ =0 is calculated as R = 0.0341. Thus the loss of intensity by reflection at two boundaries is 2 * 10 * log (1-0.0341) = -0.3 dB. Hence the attenuation by the rubber is (22.5-0.3)/0.64 = 34.7 dB/cm at 1.5 MHz.
With an attenuation of 22 dB only 0.7% of the energy which enters the absorber reaches the back side. As the absorber is placed on a brass plate most of this energy is reflected, and will be absorbed on its way back. Thus the target is considered as perfectly absorbing for the energy which enters it.

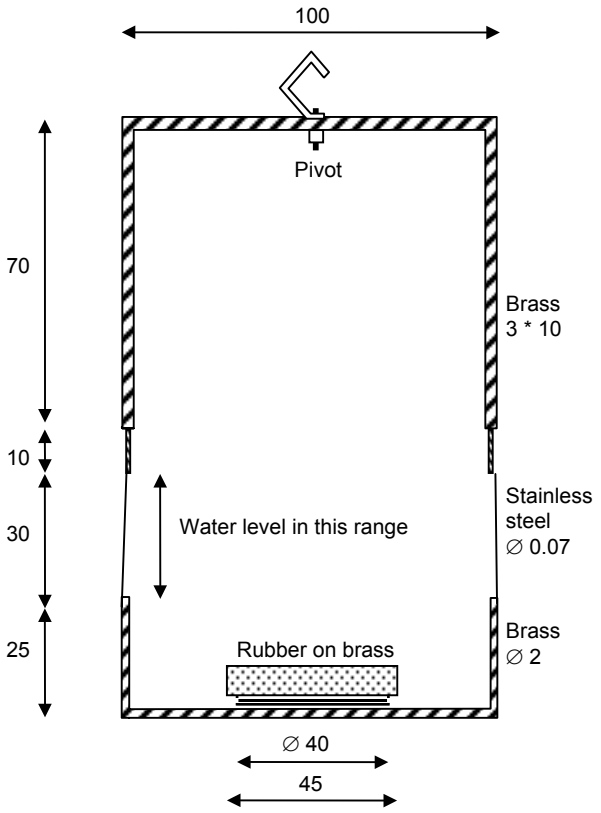

Figure 2. The frame for suspending the underwater target in the balance. The figure is to scale, dimensions in mm.

2.3 Preparation of the balance

A balance from Mettler (Type H10Tw, Zürich, Switzerland) was used. Its range is 160 g and the smallest readable division 0.1 mg. The subdivisions of grams are read on a mirrored image, of which the last two figures are read by adjusting a vernier scale (range 10 mg). The scale was removed and replaced by a specially constructed scale for underwater weighing (Figure 2).

The scale could be rotated around its suspension point. Where the part crosses the water surface two thin wires from stainless steel (diameter 70 μm, length 33 mm) were present in order to reduce the influence of surface tension on the weighing. The wires are cleaned with a brush with acetone, to remove possible fat. The target consisted of a hollow brass plate with a thickness of 2 mm, the middle mm was over a large part of the area replaced by air. The brass plate (diameter 40 mm) was covered by the rubber target. The target was inspected for horizontal position (tolerance 3 mm on 100 mm). The target will be surrounded by a water bath of with a depth of 69 mm, filled to 3 mm under its rim, and an area of 112 x 144 mm (Figure 3).

At the bottom of the bath the same rubber as for the target was used for an absorbing layer, in the direction of the reflected beam also a curved vertical sheet of the same rubber was present. Taking care that no sudden force is applied to the wires, the target is hung in the balance. The empty water bath is put under the target, lifted and placed on a frame constructed from plastic building blocks (Fisher Scientific, 's Hertogenbosch, The Netherlands). The bath is filled with demineralised water of 22 °C which is degassed by exposing 3 l in a 5 l flask at least 24 hours to vacuum (starting with a pressure at which water boils at room temperature, the vacuum-pump is then shut off and the flask with the water is left alone). The target is inspected for gas bubbles; if present they are removed with a brush or a tweezer.

2.4 Surface tension

The surface tension of the water acts on the wires as an additional force. To indicate the importance of this factor we calculate the value of the surface tension for pure water. For two wires of a diameter of 0.07 mm diameter the length of the circumference is $L = 2*\pi*0.07$ mm = 0.4 mm. The surface tension of water $\sigma = 76\ 10^{-3}$ N/m. The force exerted by surface tension is $L*\sigma = 3.\ 10^{-5}$ N = 3 mg. In itself this force does not matter, as it is present both for the activated and for the not activated transducer. However, if the surface tension varies between the two situations the outcome of the measurement is influenced. As our balance can be read to 0.1 mg, it can be seen that surface tension might be important. To minimise this influence, the surface tension of the water is lowered by adding a detergent, only straight suspension wires are

used, and changes in surface structure by contamination of the wires with fat from fingers is avoided.

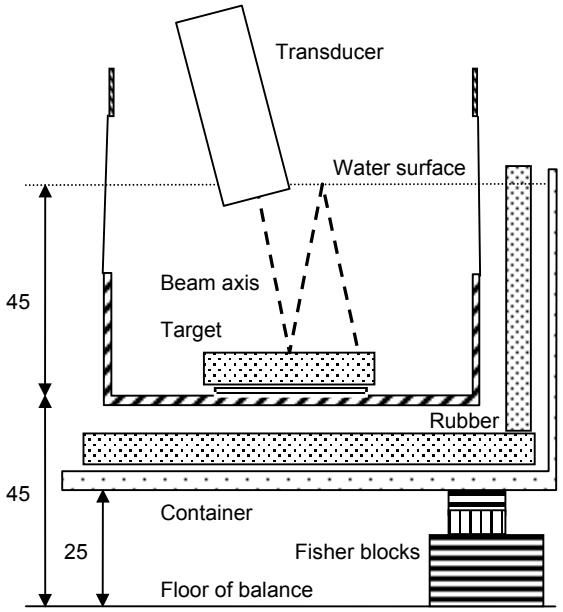

Figure 3. The geometry of the under water target in the balance. The drawing is to scale, dimensions in mm. The left side of the container is not drawn. The suspension wires of the target were rotated out of the plane of the drawing. The beam axis and its two reflections in the target and the water surface indicate that the reflected beam hits the target partially for the second time.

2.5 Procedure for weighing, linearity tests, and statistical analysis

The transducer was mounted in a laboratory support under an angle with the vertical. The angle was measured with a graduated arc provided with a weight of 50 g on a thin (0.2 mm) copper wire indicating the vertical line. Angles could be read with a repeatability of 1°. A reading run on the balance consisted out of five readings. Alternating, the transducer was not activated and activated. After each change of activation the scale for fine reading was adjusted to its fiducial mark. The reading was done to 0.1 mg. This corresponds to 1 balance unit (bu). The difference in the average position of 3 readings with non-activated transducer and 2 readings with activated transducer were taken as the outcome of a measurement. Runs wherein readings for the same activation state of the transducer differed more than 5 bu were rejected. These runs were repeated. For observations which were not part of a series of related runs, four separate runs

were performed and agreement of the runs within 3 bu was required. A measurement angle θ of 15° was chosen, to avoid the occurrence of standing waves between transducer and absorber. From experiments described elsewhere[1] it can be concluded that the effect of standing waves is less than 2%, which can be ignored. In addition, two linearity tests were performed on the balance in air and in water. The results indicated an uncertainty due to non linearity of the vernier scale of 1 bu (systematic error), while the standard deviation (SD) of a run is set at 1 bu. Statistical analysis were carried out in the spreadsheet Excel. The results were interpreted using Barnes.[7]

2.6 Summary of uncertainties in power measurement

Estimates of uncertainty are derived following chapter 7.1 in amendment A1 to IEC 61161.[4] Numbers refer to the subclauses of that document (Table 1).

1. Balance system. For the dry balance the calibration by Mettler is trusted. The vernier scale introduces a systematic uncertainty of 1 bu.
2. Linearity. From the linearity test we estimate the random uncertainty of a run to be 1 bu. The margin of uncertainty (95% confidence level) is set at 2.0 bu.
3. Extrapolation to moment of switching was not used. Its error is included in 2.
4. Target imperfections. The transmitted wave is smaller then 0.7%. The reflected wave is 3.6 % and is accounted for in formula (1). Further reflections from the target or lateral absorbers are not considered.
5. The flat geometry of the target makes errors from geometrical factors small.
6. The reflected wave will hit the surface of the water, and may impinge partially on the target (Figure 3). The contribution of this secondary reflection is set at 1.8 ± 1.8 %. Other contributions from reflected beams can be ignored.
7. Target angle. The target is aligned to within ± 2°. The target angle in formula (1) is thus 15 ± 2°. This leads to a value of $\cos\theta = 0.965 \pm 0.009$: uncertainty is 0.93 %
8. Transducer misalignment. The angle of the transducer is measured to ± 1°. This gives an additional uncertainty in $\cos\theta = 0.965 \pm 0.005$, uncertainty is 0.46 %.
9. Water temperature. Temperature is maintained within 3 °C from 22 °C. The resulting uncertainty due to change of sound velocity is 0.6 %.
10. Ultrasonic attenuation and acoustic streaming. Over a distance x of 4 cm the power at 1.5 MHz is attenuated by a factor $\exp(2\alpha x) = 1.004$. In which

$\alpha = 2.3\ 10^{-4} f^2 = 5.2\ 10^{-4}$ cm^{-1}. The applied correction factor for attenuation and acoustic streaming is thus 1.002 ± 0.002.

11. Coupling foil is not applied.
12. The transducer diameter is 15 mm. Therefore the target diameter should be at least 1.5 * 15 mm = 22.5 mm. To intercept at least 98% of the energy the radius of the target should be at least 21.8 mm.[1] The used target just satisfies this condition, even if we take into account that the beam is widened by a factor $1/\cos\theta = 1.036$. The required diameter in this direction is 45 mm.
13. Plane wave assumption ($ka > 35$) is satisfied.
14. Environmental influences. Measurements should be repeated at least three times to account for random influences of air flows and vibrations. These sources of errors have been accounted for in the linearity assessment (2)
15. An excitation voltage of circa 10 V can be read on the oscilloscope to ± 0.02 V, using the maximal feature. This would imply a repeatability of power settings to 0.4 %. We did not try to obtain absolute calibrations. This would mean calibrating the oscilloscope (to 2 %) and accounting for the influence of all connecting cables. These errors are only relevant in case a comparison with another laboratory is planned. It will be difficult to reach an error margin of ± 4% for the power due to reading of the voltage.
16. The influence of ultrasonic transducer temperature was not tested. No difference was found between the power of a transducer activated during 1 or 15 min prior to taking a reading. The temperature of the measuring bath (22 °C) is relevant for the conditions of use (application to the skin, in a laboratory at room temperature).
17. Non-linear effects were not considered because of modest output power and use of degassed water.
18. An overall measurement accuracy cannot be higher as the ± 10 % (95% confidence level) obtained in a careful setting.[9]

The overall calibration factor is 0.993. The total absolute error is 0.3 mg, the relative error is 0.054. The main contributions to the relative error are (6): the reflected wave hits the target for the second time, (12): part of the beam misses the target, and (7, 8): the angles between transducer, target and balance. Errors 6 and 12 are complementary; reducing one will increase the other. All relative errors are of a systematic nature for a particular mounting of the transducer. Thus we allow for a systematic uncertainty of 6%.

Table 1. Accounted factors to be applied to P/cF and their uncertainties (relative uncertainty unless *mg* is stated).

Item	Factor	Uncertainty
1. Vernier scale of balance	-	*0.1 mg*
2. Balance reading	-	*0.2 mg*
4. Reflection from target $1/(1+R)$	0.964	-
6. Second hit by reflected wave	0.982	0.018
7. Target angle 15 ± 2°	1.035	0.009
8. Transducer misalignment 1°	-	0.005
9. Temp effect on sound velocity	-	0.006
10. Attenuation/streaming	1.002	0.002
12. Part of beam misses target	1.01	0.010
15. Repeatability of power setting	-	0.004

At an average reading of 3 mg (30 bu) the repeatability error is given by item 2, (2 bu) leading to an error of 6 %. A systematic error in force is 1 bu: 3%. Thus the estimate for total uncertainty in power is 6+6+3 = 15 %. Remark that if four readings are averaged the random error (2 due to balance) is halved. The total uncertainty will than be 6+3+3 = 12 %.

3 Scanning of the field

The scans were performed for one transducer (marked in the surveys as transducer A). Because of the identical construction of the transducers the data for the beam shape are used for all transducers.

3.1 Instrumentation

The procedures for measuring sound fields haven been detailed elsewhere.[10] The transducer is placed in a water bath filled with 50 l demineralised water. The water is degassed partially by replacing 2 l water each day by under vacuum degassed water. At the beginning of the measuring day the bath was heated to 23°C by adding 3 - 5 l boiled demineralised water, depending on the initial temperature (15 - 18 °C). In the course of the day the bath cooled to 19 °C. Gas-bubbles on surfaces were not seen. The transducer was energised with a pulse train of 60 sinus waves of 1500 kHz from the function generator. The amplitude was set to 10 V (i.e. 20 V_{pp} when not loaded). The voltage on the transducer was 9.52 V_{pp}. In case this voltage is used in continuous wave mode, the force on the balance, following the procedure in paragraph 2.5, was found to

be 60 bu. The effective intensity of the beam is 0.06 W cm^{-2} (as can be derived from the calculation which will be presented in paragraph 3.4). This value is far below the limit of cavitation 0.5 W cm^{-2}.[3]

The ultrasound field was measured with an active hydrophone (MKII, Medisonics, Watford, England) with a ceramic transducer, diameter 1 mm, and preamplifier (gain 30 dB).
The hydrophone could perform rectangular scans in the x-y plane perpendicular to the beam axis (= z-axis). The transducer was driven by two stepper motors under computer control. All scan lines were taken in the same direction, thus avoiding hysteresis in positions. Readings were done after the hydrophone was come to rest at its position.
The centre of the field was found by trial scans, considering symmetry at the -6 to -12 dB level with respect to the maximum. At this position the distance to the transducer was measured from the time difference between the trigger pulse of the scope, derived from the start of the driving voltage on the transducer, until the first pulse arriving from the hydrophone. Distance was calculated using the sound velocity of water (1488 m/s) at 22 °C. No corrections for temperature were applied.

Signal processing was as follows. The preamplifier was loaded with a resistor of 100 Ω. Voltage U was read on the oscilloscope, triggered on the emitted signal. The delayed time base was used, the delay depending on the distance between hydrophone and transducer. The setting of the vertical gain G of the oscilloscope was 0.2 V/division, time base 2 μs/division. A typical maximal amplitude of the signal was 1 V_{pp} . The image was transferred to a personal computer, which calculated the power of the waveform ($\int U^2 dt$) in the observation window. Care was taken to fill the window (length 20 μs) with a CW-like signal, containing circa 30 periods of the signal waves, thus keeping inaccuracies due to the use of incomplete waveforms below 1.6%. Occasionally a comparison of absolute values was needed. The following framework of formulae was then used. In most cases measurements were relative, and then ratios of values were calculated omitting constants.

Local intensity I is calculated from the Root Mean Square pressure p_{rms}, and acoustic impedance Z, using the plane wave assumption:

$$I = p_{rms}^2 / Z \qquad (3)$$

The pressure is found from the voltage U on the oscilloscope using the end of cable loaded sensitivity of the hydrophone (including its preamplifier): M_L

$$p_{rms} = U_{rms} / M_L \quad (4)$$

If the gain of the oscilloscope is G (volts/division) the signal offered to the computer is D divisions:

$$D = U / G \quad (5)$$

The computer calculates the sum S_t over N_t samples in the time window

$$S_t = N_t D_{rms}^2 \quad (6)$$

Power of the beam P is found by integration of intensity over the beam area A. In practice integration is performed by summing intensities obtained in a raster scan with step size s. Using all the factors introduced above the actual calculation of power is obtained from:

$$P = \int I \, dA = \sum p_{rms}^2 s^2 / Z = G^2 s^2 \sum S_t / Z M_L^2 N_t \quad (7)$$

Unless otherwise indicated the following values were used:
G = 0.2 V/division
Z = 1000 * 1488 = 1.488 10^6 kg m^{-2} s^{-1}
N_t = 500
M_L = 9.63 10^{-6} V/Pa

Noise level was measured at the same setting of the oscilloscope, while the transducer was not energised. Occasionally it was checked that energising the transducer did not cause electrically transmitted crosstalk. Data from the computer were analysed with the spreadsheet Excel. Noise level was subtracted from measured data.

Raster scans were performed in a 31 x 31 array of equidistant points. As these scans went not far enough out to satisfy the condition of -32 dB with respect to the maximum also linear scans through the centre of the pattern in an array of 1 x 31 or 31 x 1 points were made. In these linear scans the distance between points was taken 2 or 3 times as large as in the raster scans. Also the sensitivity of the oscilloscope was increased to 50 mV/div, thus increasing the sensitivity

by a factor of 16 with respect to the raster scan. The linear scans were saturated in the central region; this part is omitted from the data.

3.2 Results

3.2.1 Raster scans

The planes of the raster scans were positioned according to paragraph 8.3.2 of IEC 61689.[3] The farthest scan was made at the distance of the last axial maximum from the transducer. Experimentally this was found at 53 mm from the transducer. It was expected to be at a^2/λ = 56 mm (transducer radius a = 7.5 mm and wavelength λ = 1.00 mm at 1.5 MHz). The scan results at 10, 24, 38 and 52 mm indicate a good circular symmetry of the ultrasound beam. At distances of 52 and 38 mm a single intensity peak is observed. At 24 mm high intensity is seen on a ring around the centre, at 10 mm many oscillations occur in the central region. This can be expected for the near field of an ultrasonic transducer. Earlier scans were made at z = 60, 20 and 5 mm, at a different setting of the oscilloscope. The raster scan at 5 mm from the transducer is presented in figure 4 as this distance is relevant for the insonation of the mandible of the rats.

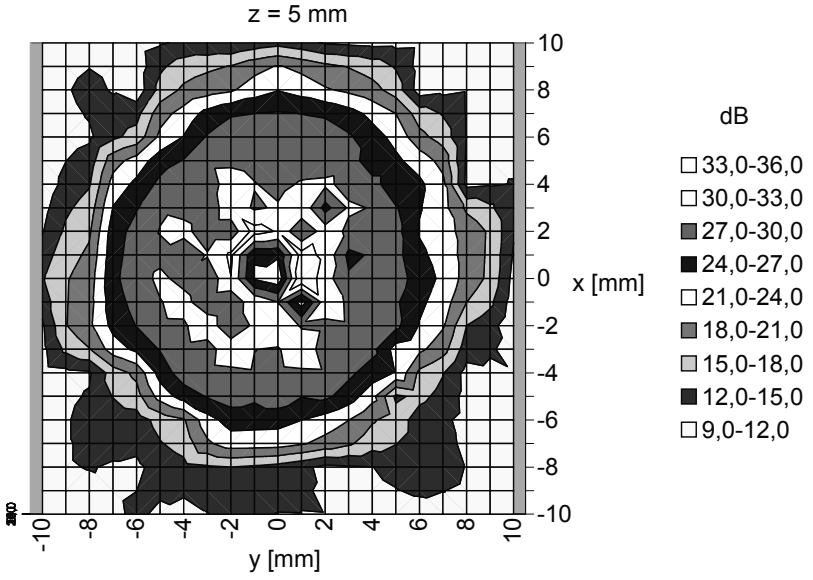

Figure 4. Rectangular raster scan for a scanning plane at a distance of 5 mm from the transducer.

3.2.2 Beam area

Beam area was calculated from the performed raster scans, according to IEC 61689 (annex A).[3] The effective radiating area A_{ER} = 1.42 ± 0.11 cm^2. The effective radius of the transducer $a_1 = \sqrt{(A_{ER} / \pi)}$ = 0.67 cm. From the slope of the regression line Q = 0.044 cm^{-1} we conclude that the beam type is 'collimated' (not divergent, nor convergent).

3.2.3 Beam non-uniformity ratio

The beam non-uniformity ratio R_{BN} is calculated from (adapted from par 9.2.5 in IEC 61689)[3]

$$R_{BN} = S_{t\,max} A_{ER} / \sum S_t . s^2$$

in which $S_{t\,max}$ is the highest occurring time sum and $\sum S_t . s^2$ is the sum of all time sums in a raster scan with step size s. As we have 4 raster scans the average of the four values was taken. This resulted in a beam non-uniformity ratio of R_{BN} = 2.31. Note that this is a very low value, for physiotherapy ultrasound transducers. The requirement is $R_{BN} < 8$.

3.3 Setting of voltage with the balance

The desired effective intensity is 30 mW cm^{-2}. In view of the effective radiating area A_{ER}=1.42 cm^2 the power P should be 30 *1.42 = 42.6 mW. With sound velocity c = 1488 m/s we find that the desired force on the balance is $F = P / 0.993\,c$ = 2.88. 10^{-5} N = 29.4 bu.

The final adjustment of the 4 channel generator for the customised ultrasound transducers was done at a bath temperature of 18.2 °C. Transducer A was checked for a second time (A1), subsequently this transducer was measured in a bath of 22.9 °C (A2). The following results were found (Table 2):

As the number of observations per transducer is small the differences between standard deviations (SD) of individual transducers have no meaning. The SD's per column were quadratically averaged leading to an overall SD of 0.73. The uncertainty due to random errors (at 95% confidence level) is set to 2 SD / $\sqrt{(N-1)}$, in which N is the number of observations on a transducer. The mean value for the 4 transducers A, B, C, and D is 29.0 ± 0.40 (SD). As no transducer falls outside the 95% confidence limit (0.8) this result is valid for all transducers.

Table 2. Force exerted by four transducers (balance units, 1 bu = 0.1 mg).

Transducer	**A**	**B**	**C**	**D**	**A(1)**	**A(2)**
Voltage (V_{pp})	20.5	22.4	19.5	20.3	20.7	20.4
1st reading	29.0	29.7	30.2	29.0	28.8	29.8
2nd reading	28.8	27.2	29.3	28.8	30.2	28.2
3rd reading	29.8	29.3	28.7	29.2	-	27.7
4th reading	29.2	27.5	29.3	28.5	-	-
5th reading	-	-	29.0	-	-	-
SD	0.43	1.26	0.56	0.30	1.00	1.10
Mean	29.2	28.4	29.3	28.9	29.5	28.6
Uncertainty (mean)	0.8	0.8	0.7	0.8	1.5	1.0

The final result is $F = 29.0 \pm 2.0$ bu. The uncertainty is estimated as follows:

- random uncertainty this series 0.8 bu
- random uncertainty expected from validation 1.0 bu
- random uncertainty use maximum 1.0 bu
- systematic uncertainty due to non-linearity of balance 1.0 bu
- total uncertainty 2.0 bu = 6.9%

3.4 Calculation of power and intensity

The power is calculated as $P = 0.993\ F.c. = 42.8$ mW. The factor 0.993 has been derived in paragraph 2.8. To the uncertainty in the force (6.9 %) we have to add the uncertainty in the conversion factor (6%). As the latter uncertainty is systematic, we add the uncertainties to 12.9%. Hence the power $P = 42.8 \pm 5.5$ mW. The effective intensity is $I = P / A_{ER.} = (42.8 \pm 5.5) / (1.42 \pm 0.11) = 30.1 \pm 6.2$ mW cm^{-2}.

3.5 Overview of variables

An overview of the ultrasound field variables of the customised ultrasound unit and the SAFHS device is presented in table 3.

3.6 Control measurements during the course of the animal experiments

To determine whether the ultrasound intensity varied over the course of the rat-experiments presented in this thesis, the force of the ultrasound field was repeatedly determined between the experiments over a period of one year. The results in table 4 indicate that the ultrasound intensity did not change to a

significant degree during the experimental period. There was a slight increase in the average force over the experimental period of 1.9 %.

Table 3. Overview of ultrasound field variables.

	Customised transducers	**SAFHS device**
Ultrasound frequency [MHz]	1.50	1.5
Modulating signal burst width [μs]	200	200
Repetition rate [kHz]	1	1
Effective radiating area [cm^2]	1.42 ± 0.11	3.88
Temporal average power [mW]	43 ± 6	117
Temporal maximum power [mW]	--	625
Peak power [W]	--	1.25
Spatial average - temporal average intensity (I_{SATA})[mW cm^{-2}]	30 ± 6	30
Spatial average - temporal maximum intensity (I_{SATM}) [mW cm^{-2}]	> 150	161
Beam non-uniformity ratio	2.32	2.16

Table 4. Repeated force measurements of the 4 customised ultrasound transducers during the experimental period (bu = balance unit).

Reading	**A (bu)**	**B (bu)**	**C (bu)**	**D (bu)**	**Average (bu)**
1st reading	31.1	28.5	31.5	28.5	29.9
2nd reading	29.8	27.9	29.2	30.1	29.3
3rd reading	29.3	29.5	29.3	29.1	29.3
4th reading	30.8	29.9	31.6	29.4	30.4
Difference 1st - 4th reading	-0.3	1.4	0.1	0.9	0.5
% 1st - 4th reading	99.0	104.9	100.3	103.2	101.9

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Appendix 2

Ultrasound attenuation of an expanded polytetrafluoroethylene (e-PTFE) and a collagen membrane

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Introduction

Ultrasound attenuation at 1.5 MHz has been determined for an expanded polytetrafluoroethylene membrane (Gore-Tex® Regenerative Membrane, W.L. Gore & Associates, Flagstaff, USA), and for a collagen membrane (Bio-Gide® Resorbable bilayer membrane, Geistlich Biomaterials, Wolhusen, Switzerland).

Materials and methods

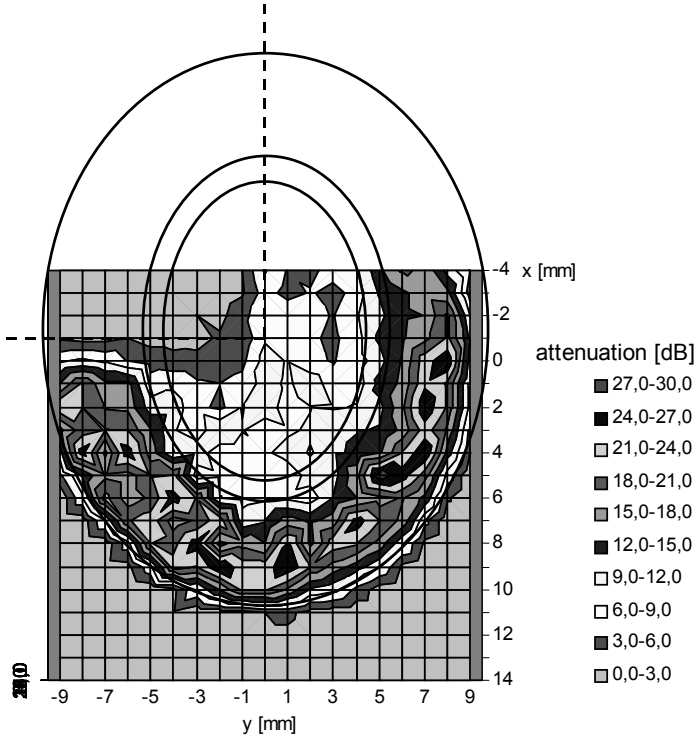
An ultrasound beam was set up in degassed demineralised water between a 15 mm diameter 1.5 MHz transducer and a ceramic hydrophone (MKII Active Hydrophone, Medisonics, Watford, England) with a diameter of 1 mm. The hydrophone was placed at a distance of 52 mm from the emitting transducer in the centre of the beam. A membrane sample was placed just in front of the hydrophone with its plane perpendicular to the ultrasound beam. The sample was moved by stepper motors in two directions perpendicular to the beam. For each position of the sample the pressure wave on the hydrophone was registered on a digital sampling oscilloscope (Philips PM3394, Eindhoven, The Netherlands). A semi-continuous wave was used and care was taken to register the steady state signal. From this steady state signal the sum of the squares of the signal S was calculated. For comparison also the sum of squares for the signal without sample in place was determined: S_0. Attenuation A (in decibels) of the sample was calculated from $A = 10 \log (S_0/S)$. The sample was prepared by degassing it in demineralised water for 24 h at a pressure of less than 0.1 atm. Thereafter it was transferred to the measuring position in the ultrasonic beam, without exposing it to air. In analysing the data results were ignored that were within 1 mm from the borders of the homogeneous regions of the sample. The e-PTFE sample had an homogenous central area in the form of an ellipsoid with axis of a length of 9 x 13 mm. One quarter of the ellipse had been cut away. This sample was scanned in steps of 1.0 mm. The square collagen membrane sample measured 10 x 11 mm and was homogenous in appearance. The central part of this sample (2.5 x 2.5 mm) was scanned in steps of 0.25 mm.

Results

The oval e-PTFE membrane consist of an inner part and an outer rim. The ultrasound attenuation of the inner part was inhomogeneous (range 5 to 12 dB), the average attenuation was 8.3 ± 0.4 dB (mean and 95% confidence interval). This corresponds to a transmission of ultrasound energy through the membrane of 15 %. The ultrasound attenuation of the outer part was more than 20 dB, which corresponds to a transmission of ultrasound energy through the membrane of less than 1% (Figure 1).

The average attenuation of the collagen membrane was 0.02 ± 0.07 dB. This corresponds to a transmission of ultrasound energy through the membrane of more than 98%. This attenuation is so small that local variations caused by the sample could not be separated from instrumental effects.

Figure 1. Attenuation raster scan of the expanded polytetrafluoroethylene membrane. A schematic drawing of the e-PTFE membrane is superimposed. One quarter of the e-PTFE membrane (dotted line) was not present during the measurements.



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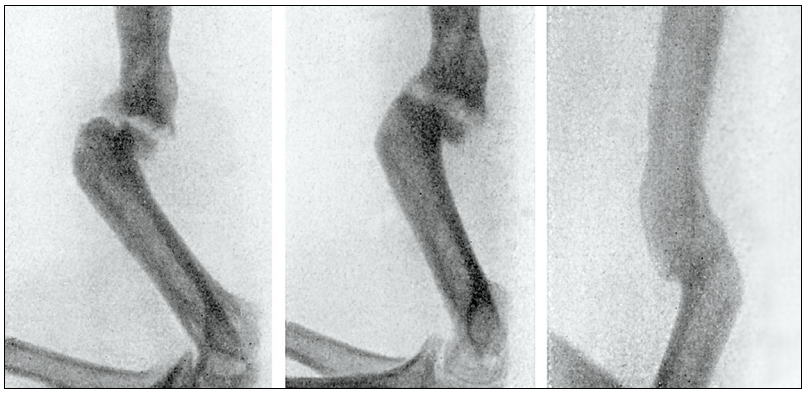

Figure 2. A case of pseudarthrosis of the left humerus (a) successfully resolved with ultrasound therapy (b, c).

Despite the high success rate, ultrasound therapy did not show any effect in 4 cases. One of these cases involved a 51-year-old lady with a non-union of the femoral shaft. After 19 ultrasound treatments, no callus formation could be observed. A further attempt of open reduction and internal fixation using steel wires was unsuccessful, so the leg eventually had to be amputated.
Another large study has been conducted by Knoch (1965).[20] More than 250 patients with fractures were treated with ultrasound. One series involved 31 slow uniting fractures at different locations (malleolar, patellar, clavicular, humeral, olecranon, radial and navicular fractures). 800 kHz ultrasound of 0.3 - 0.8 W cm^{-2} intensity was used for 5 - 8 minutes every other day. After 10 - 20 sessions, all fractures had united clinically. In another series, 100 fresh radial fractures were treated with ultrasound, and another 100 fresh radial fractures were not. The disability time, defined as the period from fracture until the patient resumed working again, was measured. Using ultrasound, a 41% reduction in disability time was observed. In another series of 28 fresh navicular fractures, a 60% reduction in disability time was noted. Despite these promising results, these studies on ultrasound and bone healing remained isolated in the literature and in the seventies there seemed to be little interest in this area.

Renewed interest in ultrasound treatment of bone

In South-America, research on ultrasound and bone healing was initiated by Duarte.[21] After his thesis about 'ultrasound stimulation of callus',[21] Xavier and Duarte reported the successful application of low intensity pulsed ultrasound (30 mW cm^{-2}) in the treatment of 27 non-unions.[22] In 70% of the cases, complete healing was obtained by daily 20 minutes ultrasound exposure of the

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