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Review

The enhancement of bone regeneration by ultrasound

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Abstract

Millions of fractures occur every year worldwide, with nearly 6.2 million fractures reported annually in the United States alone. Even though treatment methods have improved over the last few decades, 5–10% of fractures still show delayed healing. A significant subpopulation of these delayed healings do not heal by nine months and are thus termed non-unions. Experimental studies have shown some evidence that low intensity pulsed ultrasound stimulation (LIPUS) results in enhanced bone regeneration during fracture healing and callus distraction. LIPUS treatment has led to increased callus area and accelerated return of bone strength following fracture. Histological studies suggest that LIPUS influences all major cell types involved in bone healing, including osteoblasts, osteoclasts, chondrocytes and mesenchymal stem cells. The affect of LIPUS seems to be limited to cells in soft tissue, whereas cells in calcified bone seem not to be effected. In vitro cell culture studies as well as tissue culture studies have shown some effects on cell differentiation and protein synthesis. Even though the energy used by LIPUS treatment is extremely low, the effects are evident. The most probable source of the therapeutic benefits observed with LIPUS treatment involves nonthermal mechanisms that influence cell membrane permeability and increase cellular activity. Despite clinical and experimental studies demonstrating the enhancing effect of LIPUS on bone regeneration, the biophysical mechanisms involved in the complex fracture healing process remain unclear and requires further research.

Keywords: Fracture; Healing; Ultrasound; Distraction osteogenesis; Review

Contents

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1. The treatment of fracture healing

Millions of fractures occur every year worldwide, with nearly 6.2 million fractures reported annually in the United States alone (Praemer et al., 1992). Fractures in bone result from two general causes, trauma or pathological conditions. A trauma-induced fracture usually occurs when the normal range of loading to which a bone has adapted during growth and development is exceeded. A pathological fracture usually occurs under normal loading conditions after the bone has been weakened by disease, such as osteoporosis or bone tumours. In either case, the continuity of the bone has been disrupted so that the transmission of mechanical loads through the bone becomes impossible. When a fracture occurs, the bone has a self-regulating mechanism in which it heals with the purpose of recovering its mechanical function. Even though treatment methods have improved over the last few decades, 5–10% of fractures still show delayed healing. Many of these delayed healings persist for more than nine months and are thus termed non-unions. Many patients would benefit from improved methods of treating these delayed unions and non-unions. One promising treatment method is the use of low-intensity pulsed ultrasound (LIPUS).

LIPUS began to be widely used clinically to treat fresh fractures in the early 1990s and nonunions or delayed unions in the late 1990s. In the world literature, LIPUS generally entails a 20 min treatment per day of 1 MHz sine waves repeating at 1 kHz, average intensity 30 mW/cm^2, pulse width 200 μs. LIPUS is a form of mechanical energy that is transmitted through and into living tissue as acoustic pressure waves. Ultrasound (US) energy is absorbed at a rate proportional to the density of the tissues in which it passes through. It has been theorized that the micromechanical strains produced by these pressure waves in biological tissues may result in biochemical events that regulate fracture healing.

2. Historical background of ultrasound treatment

Studies as early as 1949 (Buchtala, 1950) first suggested that US might stimulate osteogenesis. In 1950, the relationship between high intensity US and bone healing was first investigated (Maintz, 1950). Maintz (1950) reported that histological and radiographic analysis of rabbit radial fractures showed minimal changes after US (500 mW/cm^2) treatment, but reduced callus formation was observed using higher intensities (1000, 1500, 2500 mW/cm^2). Early reports from the Mayo Clinic (Bender et al., 1954; Herrick et al., 1956; Ardan et al., 1957) in the mid to late 1950s using very high US intensities (5000–25 000 mW/cm^2) showed delayed bone healing, necrosis, and dense fibrous tissue formation in dog femora. High intensity US (200–3000 mW/cm^2) has also been shown to increase callus formation and accelerate healing in rabbit radii (De Nunno, 1952; Corradi and Cozzolino, 1953) and tibiae (Klug et al., 1986) fractures as well as guinea pig ulnae fractures (Murolo and Claudio, 1952) compared to nontreated controls. Additionally, Chang et al. (2002) have shown a 36% increase in new bone formation and an 80% increase in torsional stiffness of limbs stimulated with high intensity US (500 mW/cm^2) compared to untreated limbs. The mixed results using high intensity US led many to begin pulsating the source of the sound head itself, pulsating the source of the sound, or decreasing the output of the ultrasonic generator. This eventually led to the development and use of LIPUS clinically to stimulate bone osteogenesis (Duarte, 1983).

3. Biophysics of ultrasound induced bone osteogenesis

3.1. Thermal effects of LIPUS

The ability of LIPUS to stimulate changes in tissues and cells may be due to the temperature increase, associated with energy absorption (Wu and Du, 1990a, b; Chang et al., 2002). Temperature effects using intensities as high as 1000–3000 mW/cm^2 can cause considerable heating of tissues. However, studies (Chang et al., 2002) have shown that the heating effect from LIPUS (20–50 mW/cm^2) is well below 1 °C (Chang et al., 2002). Duarte (1983) reported negligible temperature variations after LIPUS (50 mW/cm^2, 15 min/day) treatment (0.01 °C ± 0.005 °C) on rabbit fibula osteotomies. Despite only a small increase in temperature (< 1 °C), studies have shown that minimal heating effects may affect some enzymes such as matrix metalloproteinase 1, also known as interstitial collagenase or collagenase 1 (Welgus et al., 1981, 1985). This enzyme has been shown to be very sensitive to small variations in temperature (Welgus et al., 1981, 1985).

3.2. Nonthermal effects of LIPUS

Changes observed in tissues and cells after LIPUS treatment may also be associated with nonthermal processes such as acoustic streaming and cavitation (Hill, 1971; Dyson, 1982). Several studies have suggested that an increase in protein synthesis (Harvey et al., 1975; Webster et al., 1978) and collagen synthesis (Webster et al., 1980) observed in human fibroblasts in vitro after US (500 mW/cm^2) stimulation may involve cavitation mechanisms. Cavitation "involves the pulsation of gas or vapour-filled voids in a sound field" (Webster et al., 1978). During stable cavitation, dissolved gas accumulates in the medium and forms gas bubbles, with the cavity acting to enhance acoustic streaming (Watson, 2000). Unstable cavitation is "the formation of bubbles at the low pressure part of the US cycle," whereby the bubbles collapse quickly releasing large amounts of energy (Watson, 2000). These cavities can act as intense concentrator of acoustic energy that result in shearing and microstreaming fields (Dyson, 1982). Acoustic streaming is "a small scale eddying of fluids near a vibrating structure such as cell membranes and the surface of stable cavitation gas bubble" (Dyson and Suckling, 1978). Stable cavitation and acoustic streaming have been shown to affect diffusion rates and membrane permeability. Several studies (Dyson and Brookes, 1983; Mortimer and Dyson, 1988; Dinno et al., 1989; Ryaby et al., 1989, 1991; Rawool et al., 2003) have suggested that LIPUS may have a direct effect on cell membrane permeability. Additionally, an overall increase in the activity level of the cell has been identified as a potential source of the therapeutic benefits observed after LIPUS treatment (Dinno et al., 1989; Watson, 2000; Leung et al., 2004b). This LIPUS induced changes in cell membrane permeability may result in an increase in micromechanical blood pressure leading to accelerated fracture healing (Rawool et al., 2003). The effect of cavitation could explain the effect of very low energy to cellular reactions. However, cavitation has never been adequately confirmed in tissues.

Several other theories exist concerning the mechanism by which the US stimulates biochemical events at the cellular level. Many studies have suggested that LIPUS may induce micromotion, producing mechanical stimulation. In this way, LIPUS may follow Wolff's Law (Wolff, 1892) by serving as a noninvasive force stimulating bone healing processes. The low energy used for LIPUS treatment can only cause extremely low pressure waves that penetrate at a rate proportional to the density of the tissue. LIPUS is mainly reflected at tissue borders of soft and hard tissue, like connective tissue and cortical bone. This may be supported by the inability of LIPUS to stimulate osteogenesis in intact bone (Elmer and Fleischer, 1974; Spadaro and Albanese, 1998; Wimsatt et al., 2000; Warden et al., 2001) or callus in the remodelling phase (Pilla et al., 1990; Wang et al., 1994; Hantes et al., 2004). The differential absorption of LIPUS may establish a gradient of mechanical strain in the healing callus that stimulates periosteal bone formation (Gross et al., 1997; Rubin et al., 2001). This allows for a better understanding of how LIPUS may influence the inflammatory and soft callus formation phases of fracture healing. Despite extensive clinical and experimental studies examining the enhancing effect of LIPUS on bone regeneration, biophysical mechanisms involved in the complex fracture healing process remain unclear and require further research.

4. LIPUS parameters

In an attempt to determine the optimal LIPUS setting needed to enhance fracture healing, studies have examined several different low intensities and frequencies. Harle et al. (2001) determined that the response of cells in vitro to US is highly dependent on the intensity. Alkaline phosphatase showed progressively increased expression with increasing US intensity (120, 390, and 1490 mW/cm^2). Using diagnostic sonographic equipment, Heybeli et al. (2002) have recently shown that treatment with US intensities as low as 11.8 mW/cm^2 led to improved radiographic fracture healing and increased bone density in rat femora. Yang et al. (1996) directly compared different LIPUS intensities (50 and 100 mW/cm^2) in restoring the mechanical properties of a rat femora following fracture. They (Yang et al., 1996) measured an increased maximum torque and a slightly decreased torsional stiffness using 50 mW/cm^2 compared to 100 mW/cm^2. They also showed that untreated controls had significantly lower maximum torque and torsion stiffness compared to 50 mW/cm^2 treated femora, but not compared to femora treated with 100 mW/cm^2.

In addition to the intensity, the frequency of the US signal has also been investigated in stimulating bone osteogenesis. Dyson and Brookes (1983) showed using higher intensity (500 mW/cm^2) pulsed US that 78.6% of 1.5 MHz and 56.2% of 3.0 MHz treated rat fibulae fractures had more advanced radiographic and histological healing than untreated controls. Wang et al. (1994) demonstrated that there was no significant difference in maximum torque or torsional stiffness in rat femora with LIPUS (30 mW/cm^2) treatment using a frequency of 0.5 MHz compared to 1.5 MHz. After 21 days of healing, both frequencies led to increased maximum torque and torsional stiffness compared to untreated controls. Tsai et al. (1992) showed significantly greater mineral apposition rates at 2 and 3 weeks postfracture in rabbit fibulae that had been treated with 1.5 MHz compared to 3 MHz frequencies, both using a US intensity of 500 mW/cm^2. A carrier frequency of 1.5 MHz has been more commonly used to stimulate osteogenesis experimentally and clinically.

5. The influence of LIPUS treatment on fracture healing

5.1. Phases of fracture healing

During secondary fracture healing, bone forms via two mechanisms: intramembranous bone formation (directly by osteoblasts) and endochondral ossification (indirectly by the calcification of cartilage and subsequent replacement by bone) (Fig. 1). Secondary fracture healing processes can be divided into five overlapping phases: (1) inflammation, (2) angiogenesis, (3) soft callus formation, (4) hard callus formation and (5) remodelling. At the beginning of the inflammatory phase, blood is extravated from blood vessels of the bone, periosteum marrow, and surrounding tissues, forming a haematoma that aids in cell recruitment to the fracture site. Acute edema ensues with inflammatory cells, beginning with polymorphonuclear leukocytes followed by macrophages and lymphocytes, arriving at the fracture site to remove damaged and necrotic tissue. Angiogenesis proceeds with further development of the haematoma and vascular proliferation. The periosteal vessels contribute the majority of the capillary buds. Mesenchymal cells from the haematoma proliferate and differentiate to produce cartilage and woven bone that creates a fracture callus. The intramembraneous bone formation starts periosteally, some distance from the fracture gap (Fig. 2). Additionally, a soft fracture callus made up of a fibrous or cartilaginous callus surrounds the fracture site forming an external callus. Several days later, new bone is formed in addition on a cartilaginous template by endochondral ossification. The hard callus is made up of the periosteal callus and continues to grow as calcification proceeds and the soft callus transforms into woven bone (Fig. 2). During the remodelling stage, woven bone and calcified cartilage are replaced by secondary lamellar bone, either cortical or trabecular, depending on the anatomical site. Along with the remodelling of woven bone into lamellar bone, resorptive processes also actively remove any unnecessary callus. Remodelling has been shown to continue for years after clinical and radiographic union (Wendeberg, 1961); however, the bone mineral density will remain lower in the fractured bone for years.

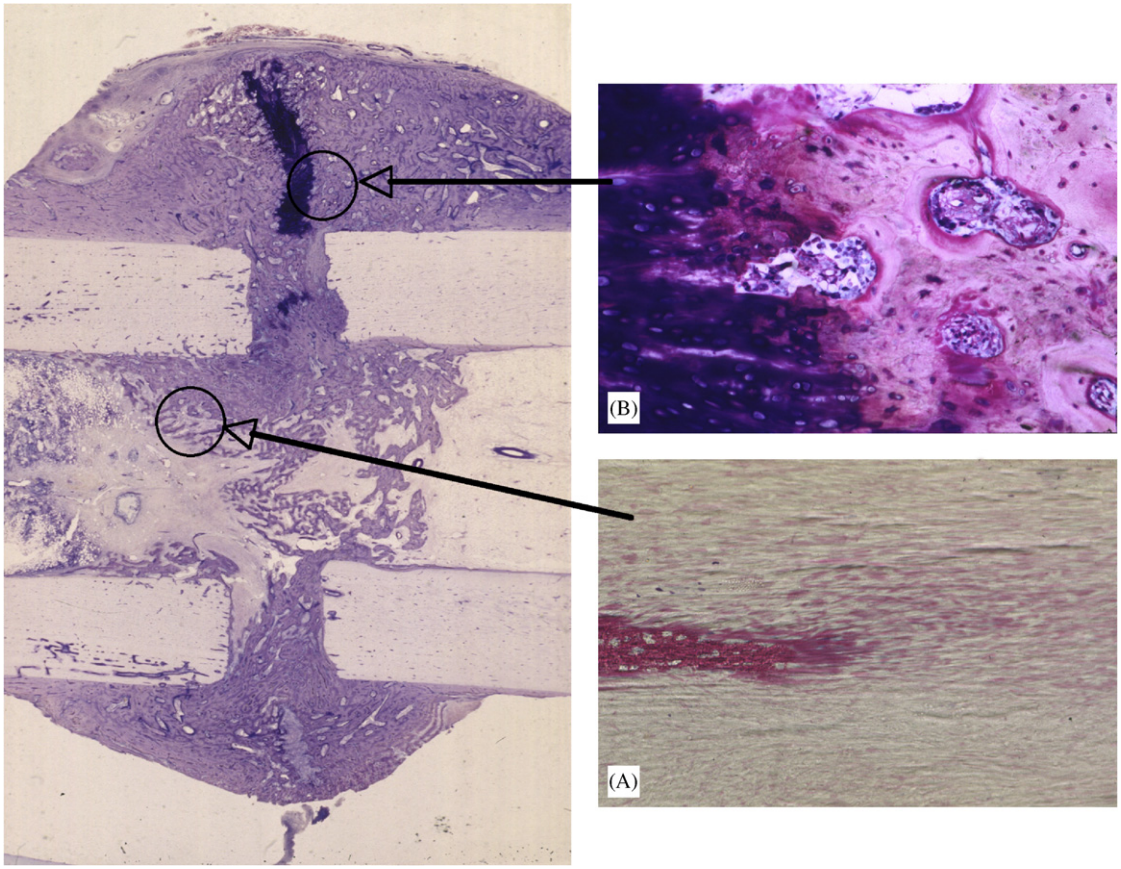

Fig. 1. Bone healing by callus formation around the fracture zone (longitudinal section through a broken sheep metatarsal). Bone is laid down by osteoblasts (intramembraneous bone formation) (A) and also developes through endochondral bone formation by transforming fibrocartilaginous tissues (including chondrocytes and fibroblasts) into calcified bone (B).

5.2. *Timing of LIPUS during fracture healing*

Several experimental studies have attempted to determine which phases of fracture healing are affected by LIPUS. Many in vivo experimental studies (Pilla et al., 1990; Wang et al., 1994; Yang et al., 1996; Hantes et al., 2004) suggest that LIPUS does not affect the remodelling phase of fracture healing, but rather the earlier inflammatory or callus formation phases of healing (Fig. 2a–c). Yang et al. (1996) showed that aggrecan gene expression in LIPUS treated femora was significantly higher at day 7 and significantly lower at day 21 compared to untreated controls. They observed a similar although insignificant trend with the expression of the α1(II) procollagen gene. They observed no significant change in the expression of genes coding for bone-related proteins, including α1(I) procollagen, bone γ-carboxyglutamic acid protein, alkaline phosphatase, and transforming growth factor-β1. In vitro, increased aggrecan gene expression in LIPUS treated cultured chondrocytes has also been observed. This seems to indicate that endochondral ossification is mainly influenced by LIPUS.

Rawool et al. (2003) suggested that LIPUS may affect the angiogenesis phase of fracture healing. Using power Doppler assessment, they observed LIPUS treatment for 10 days resulted in a 33% increase in vascularity at the osteotomy site of dog ulnae. The authors suggested that LIPUS induced mechanical vibrations may have increased the permeability of the cell membrane to calcium ions, leading to increased micromechanical fluid pressure of blood flow.

A report by Azuma et al. (2001) remains one of the only experimental study that has addressed this question directly by measuring mechanical and histological changes after 25 days of continuous LIPUS treatment and

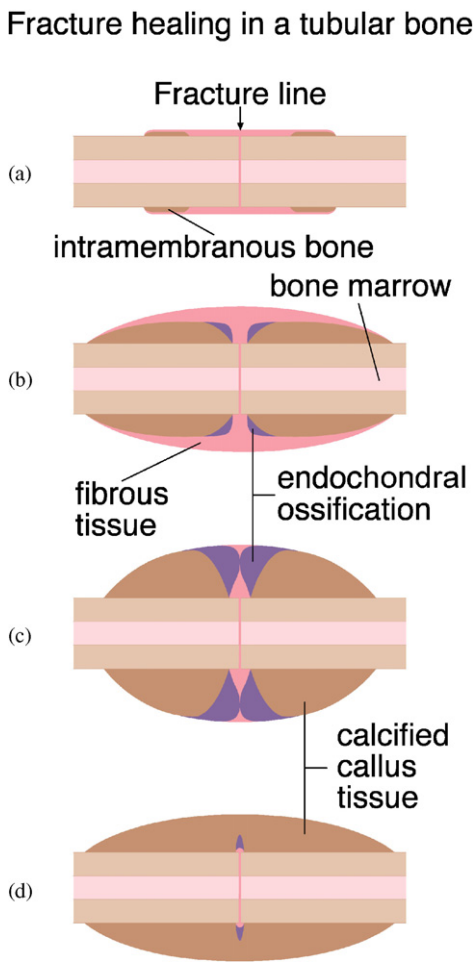

Fig. 2. The phases of callus formation at a fracture. Soon after trauma and haematoma intramembraneous bone formation starts at the periosteum (a), grows in diameter and length (b) and shows the first signs of endochondral ossification. Furthermore, callus grows mainly by endochondral ossification towards the fracture line (c) and bridges peripherally (d).

after 8 days of LIPUS treatment at different time periods during the healing process. The timing or duration had no significant effect on the bone mineral content measured after 25 days. However, because the study only measured bone mineral content after 25 days it was unclear whether LIPUS treatment affected early bone healing. More extensive bone bridging at the fracture site was observed in bones that were continuously treated with LIPUS and those that were treated with LIPUS from postoperation days 17–24, compared to the untreated controls. Additionally, they showed a significant increase in LIPUS treated bone stiffness and maximum torque compared to untreated controls in all groups (Azuma et al., 2001). They also showed that continuous treatment with LIPUS throughout a 24 day healing period resulted in rat femoral bone with higher although not significantly different stiffness and maximum torque compared to bone that underwent LIPUS treatment for only 8 days during healing (Azuma et al., 2001). These data suggest that LIPUS may influence the inflammatory and callus formation phases of the fracture healing process.

5.3. *The influence of LIPUS on callus area during fracture healing*

Studies examining various animal models have indicated that LIPUS (49, 57 mW/cm^2) treatment results in accelerated callus formation compared to untreated controls. Duarte (1983) reported that callus

area increased rapidly in the LIPUS treated bones during the first 12 days of treatment, while callus area increased rapidly in untreated bones after 12 days post-operation. Qualitative radiographic and histological analysis (Duarte, 1983) demonstrated LIPUS treatment resulted in a significant increase in bone growth inside femoral cortical bone defects (36% at day 15) and at a rabbit fibulae osteotomy site (27% at day 18) compared to untreated controls. Tsai et al. (1992) demonstrated significantly greater mineral apposition rates at 2 and 3 weeks postfracture in rabbit fibulae treated with 500 mW/cm^2 of US compared to untreated controls. Larger callus area has often been attributed to increased endochondral bone formation processes after LIPUS treatment. Histological analysis (Wang et al., 1994) showed more advanced endochondral ossification and a smaller fracture gap in LIPUS treated compared to untreated rat femora after 14 days of healing.

5.4. *The influence of LIPUS on callus strength during soft and hard callus formation phases of fracture healing*

LIPUS treatment has been shown in various experimental studies to accelerate restoration of mechanical properties (Table 1), such as maximum torque and torsional stiffness during fracture healing. Using a slightly higher US (50 mW/cm^2) intensity, Yang et al. (1996) measured a significant increase in maximum torque (29%) and torsional stiffness (37%) 21 days following fracture in treated rat femora compared to the untreated controls. Using LIPUS and the identical animal model, the same research group (Wang et al., 1994) again demonstrated a significantly greater maximum torque (22%) and stiffness (67%) in LIPUS treated bones compared to untreated control limbs after 21 days. In the rat model, cell proliferation associated with hard callus formation ceases after 14 days and after 21 days for soft callus formation (Iwaki et al., 1997; Einhorn, 1998; Lee et al., 1998). Due to the mechanical testing taking place 21 days following fracture, the improved mechanical properties (Wang et al., 1994; Yang et al., 1996) suggest that LIPUS treatment affects the earlier phases of fracture healing rather than the remodelling phase. However, it is still unclear from the experimental design of either study whether LIPUS influenced the inflammatory, angiogenesis or callus formation phases of healing.

To better understand how LIPUS affects the different phases of healing, Pilla et al. (1990) investigated the maximum torsional strength and stiffness of mid-shaft tibiae rabbit osteotomies after 28 days of LIPUS treatment. At post-op days 14, 16, 17, 18 and 21 the LIPUS treated bones had significantly greater maximum

Table 1
Experimental fracture healing studies examining a return of mechanical properties following fracture

Author	Experimental model	US intensity (mW/cm^2)	Treatment duration (min/day)	No. of treatments	Days treated postfracture	Torsional stiffness (% increase)	Maximal torque (% increase)
Pilla (2002)	Rabbit fibula	30	20	9	1–9	n/a	29
Pilla et al. (1990)	Rabbit fibula	30	20	14	1–14	25	18
Pilla et al. (1990)	Rabbit fibula	30	20	16	1–16	56	37
Pilla et al. (1990)	Rabbit fibula	30	20	17	1–17	54	67
Pilla et al. (1990)	Rabbit fibula	30	20	18	1–18	58	50
Pilla et al. (1990)	Rabbit fibula	30	20	21	1–21	33	41
Pilla et al. (1990)	Rabbit fibula	30	20	23	1–23	10	38
Pilla et al. (1990)	Rabbit fibula	30	20	28	1–28	−12	−7
Wang et al. (1994)	Rat femur	30	15	10	14	67	22
Yang et al. (1996)	Rat femur	50	15	15	21	37	29
Ito et al. (1998)	Rat femur	30	20	20	20	n/a	46
Azuma et al. (2001)	Rat femur	30	20	8	1–8	44	36
Azuma et al. (2001)	Rat femur	30	20	8	9–16	50	37
Azuma et al. (2001)	Rat femur	30	20	8	17–24	33	38
Azuma et al. (2001)	Rat femur	30	20	24	1–24	40	48

Torsional stiffness and maximal torque are expressed as the percent increase observed in LIPUS treated fractured limbs compared to untreated fractured control limbs.

torque and torsional stiffness than untreated controls. After 28 days, there was no significant difference measured in either the maximum torque or the torsional stiffness of LIPUS treated bones compared to the untreated controls. These results again demonstrate that mechanical properties are improved via LIPUS treatment during early fracture healing phases. However, it remains unclear whether a certain phase in the callus formation in particular is influenced by the LIPUS treatment.

Hantes et al. (2004) investigated the effect of LIPUS treatment at 75 and 120 days postoperation in a sheep tibiae midshaft osteotomy model. Radiography demonstrated that LIPUS treated bones (75 days) healed faster than control (103 days) limbs. At day 75 the LIPUS treated bones had increased bone mineral density and ultimate strength compared to untreated controls. At day 120 there was no significant difference measured in either the bone mineral density or the ultimate strength between the LIPUS treated bones and the untreated controls. With the larger animal model, the data suggest again that early phases of fracture healing are influenced by LIPUS, but not the remodelling phase. The lack of observed differences between LIPUS treated and untreated control group after 120 days of healing is not surprising because of the long treatment time. The untreated control group had enough time to consolidate the bone, thus the accelerating effect of the LIPUS treatment became more and more reduced.

6. The influence of LIPUS on distraction osteogenesis

Distraction osteogenesis is an attractive method to stimulate new bone formation. It has been used to treat length discrepancies, deformities, bone defects after tumour resection, non-union and osteomyelitis (Ilizarov, 1989a, b). For this treatment most often the bones are osteotomized and the two fragments distracted, using an external fixator. The external fixator stabilizes the fragments and allows the distraction of the bone in the longitudinal direction at adjustable values (Fig. 3). During clinical application, the distraction is started about 7 days after osteotomy (latency period) using a distraction rate of about 1 mm/day. This treatment allows one to distract up to 100 mm and to create a corresponding length of new bone. It is believed that the stimulus for the new bone formation is due to the strain induced in the regenerative tissue, which is transferred to the bone forming cells (Ilizarov, 1989a, b). The disadvantage of this method is the very long treatment time. After distraction of the fragments to the needed length, the regenerated bone takes two to three times the distraction period to mature (consolidation period) and allow removal of the external fixator. Long external fixation periods carry the risk of increased complication rates (Paley, 1990; Faber et al., 1991; Forriol et al., 1999). Shortening of the treatment time and in particular of the maturation period would therefore reduce the costs, complications and burden on the patient.

Several studies have tried to stimulate distraction osteogenesis by LIPUS application. Experimental investigations have been performed on various animal models. In most studies the LIPUS treatment of the animals was performed with the same systems as used for the treatment of patients (Exogen, Piscataway, NJ, USA), with the same physical parameters (20 min a day, 1 MHz sine waves repeating at 1 KHz, average intensity 30 mW/cm^2, pulse width 200 μs). The most often used model involves leg-lengthening of the rabbit tibia. However, the main factors for the distraction protocol are often not the same and the results therefore are not directly comparable. The latency period was 7 days in three out of four studies reviewed. The distraction rate was 0.75–3 mm a day and the distraction length 10–21 mm . The LIPUS application was performed in most studies during the maturation period. In two of these investigations the LIPUS treatment was applied exclusively or additionally during the distraction period.

Most groups studied the influence of LIPUS treatment on the maturation period of the bone regenerate because this makes up the longest part of the treatment time and thus the benefit of stimulation would be the greatest. Three groups (Shimazaki et al., 2000; Tis et al., 2002; Sakurakichi et al., 2004) investigated the effect of LIPUS in a rabbit tibia distraction model. They all started distraction 7 days postoperation and distracted approximately 10 mm (10–10.5 mm). They all applied the same LIPUS signal for 20 min per day and sacrificed the rabbits 14–21 days following distraction. All studies found a larger callus formation in the US treated animals compared to the untreated control groups. Two groups (Shimazaki et al., 2000; Sakurakichi et al., 2004) found higher bone density and a corresponding higher biomechanical stiffness and strength in bones treated with LIPUS, whereas the third group (Tis et al., 2002) did not find significant effects for these parameters in comparison to the untreated control group.

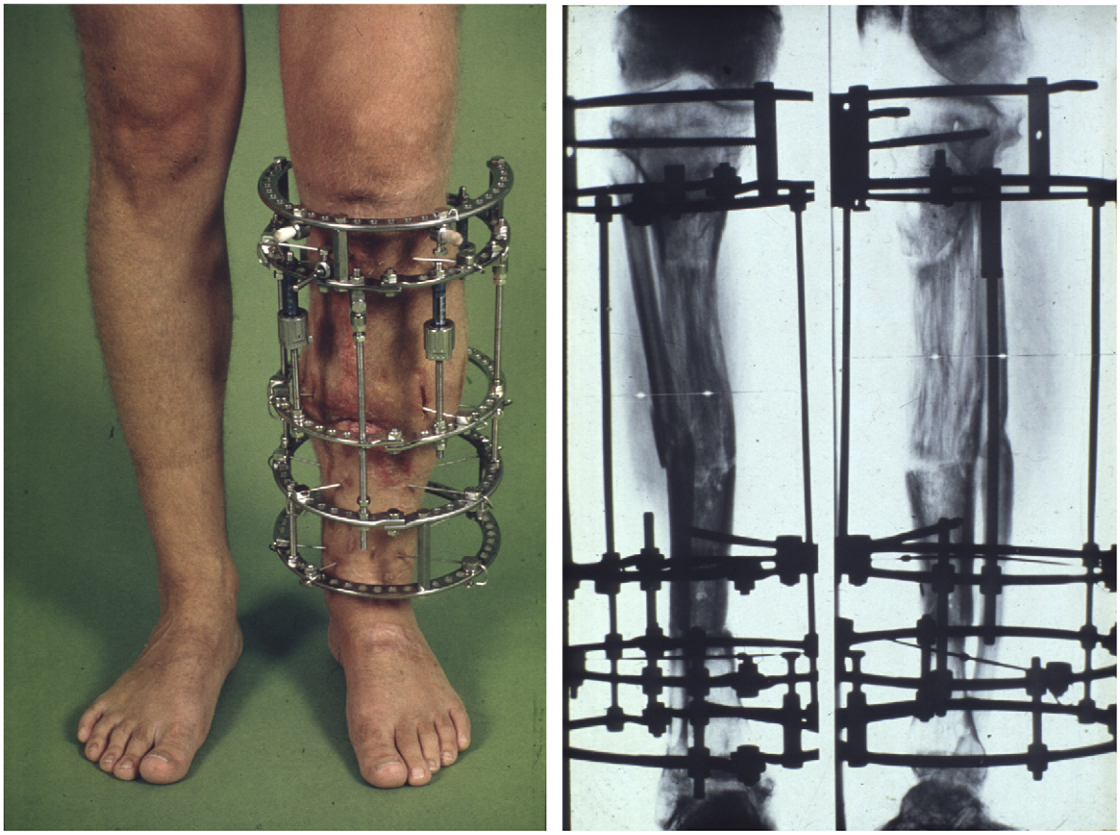

Fig. 3. Closing of a large bone defect by a callus distraction regime, using an external ring fixator. The newly formed bone is mechanically weak and needs a long time for maturation.

Shimazaki et al. (2000) compared a maturation time of 14 days and 21 days and found that the differences between the LIPUS and control groups were reduced with increasing maturation time for all parameters tested (bone area, BMD and mechanical strength and stiffness, Table 2). This might be an explanation for the insignificant differences between US treated and untreated control groups in regard to mechanical parameters and BMD. The publication of Tis et al. (2002), Sakurakichi et al. (2004) and Shimazaki et al. (2000) found significant effects after 14 days of maturation and LIPUS treatment. In the untreated control group, a longer maturation time led to an increase in bone regenerate quality described by BMD and mechanical strength and stiffness. This resulted in a reduced difference between the LIPUS treated and untreated bones, demonstrating that LIPUS treatment has only an acceleratory effect on the maturation of newly formed bone.

Positive effects of LIPUS treatment on the maturation process of the bone regenerate have also been found in a study of rats (Eberson et al., 2003) and in a large animal model of sheep (Mayr et al., 2001; Claes et al., 2005). Eberson et al. (2003) found in a rat femur distraction model (latency 7 days, distraction rate 0.167 mm/day, distraction distance 7 mm and 35 days of maturation) a significantly higher bone volume fraction, faster mineralization and a 33% higher torsional strength for the LIPUS treated group in comparison to the untreated control group.

However, the later differences were not significant, which may be explained by the very long (35 days) maturation period for a rat. This might have reduced the possible differences in earlier time periods. The large animal study was performed on a sheep metatarsal defect model using bone segment transport with a distraction length of 15 mm (distraction rate 1 mm/day, maturation time 63 days, latency period 5 days). The longer maturation time was chosen because of the slower bone-healing rate in sheep in comparison to small animals, like rats and rabbits.

Table 2
The return of stiffness and strength is shown for various experimental studies examining the effect of LIPUS (30 mW/cm^2) treatment for 20 min/day during callus distraction

Author	Experimental model	Latency period (days)	Distraction rate (mm/day)	Distraction distance (mm)	Maturation period (days)	LIPUS treatment following osteotomy (days)	Stiffness (% increase)	Strength (% increase)
Eberson et al. (2003)	Rat femur	7	0.167	7	35	28–63	20	33
Sakurakichi et al. (2004)	Rabbit tibia	7	1.5	10.5	14	1–7	12	5
Sakurakichi et al. (2004)	Rabbit tibia	7	1.5	10.5	14	7–14	98	85
Sakurakichi et al. (2004)	Rabbit tibia	7	1.5	10.5	14	14–21	49	24
Shimazaki et al. (2000)	Rabbit tibia	7	1	10	14	17–31	37	100
Shimazaki et al. (2000)	Rabbit tibia	7	1	10	21	17–38	9	20
Uglow et al. (2003)	Rabbit tibia	1	0.75	10.5	14	7–28	13	23
Uglow et al. (2003)	Rabbit tibia	1	0.75	10.5	14	7–42	−14	7
Tis et al. (2002)	Rabbit tibia	7	1	10	21	17–37	−20	−8
Machen et al. (2002)	Rabbit tibia	7	1	10	20	17–37	−41	−39
Claes et al. (2005)	Sheep metatarsal	4	1	15	63	21–84	136	n/a

Stiffness and strength are expressed as the percent increase observed in LIPUS treated callus distracted limbs compared to untreated callus distracted control limbs. Stiffness and strength were measured using torsional testing, except for Claes et al. (2005) who used nondestructive compression testing and Uglow et al. (2003) who used four point bending.

Mayr et al. (2001) and Claes et al. (2005) reported in two publications radiographic, biomechanical and histological results from the same animal experiment. They found significantly more callus formation, higher bone mineral content, and a higher bone regenerate stiffness for the LIPUS treated group in comparison to the untreated control group.

The only study that could not find any significant influence of the LIPUS treatment was the group of Uglow et al. (2003), which used a rabbit tibia distraction model (1 day latency, 1 mm/day distraction rate, 10.5 mm distraction, maturation period 2 and 4 weeks). They found a trend towards higher bending strength at 2 weeks maturation for the LIPUS treated group but this difference disappeared after 4 weeks of maturation.

The comparison of the results at 2 week maturation period with the other studies on rabbits with 2 week maturation period was limited because of the different latency period using in the study (1 day instead of 7 days) and the slower distraction rate (0.5 mm/day instead of 1 or 1.5 mm/day) used. The authors discussed that the very stiff fixator may have partially inhibited the stimulatory effect of the US treatment.

In addition to the radiographic and biomechanical investigations on the bone regenerate some groups performed histomorphological investigations. During the distraction process, a growth zone of nearly constant thickness in the middle of the lengthened segment with formation of new bone at its proximal and distal ends was observed. At the end of the distraction period this growth zone was filled by fibrous connective tissue and cartilaginous tissue. During the maturation period, soft tissue was replaced via intramembranous bone formation, endochondral ossification, or a process of bone formation by chondrocytes (transchondroid bone formation, Yasui et al., 1997). LIPUS treatment seemed to accelerate this tissue differentiation during the maturation period. Several groups showed that the percentage of fibrous tissue in the maturing callus was

lower and the percentage of newly formed bone higher in the LIPUS treated groups than in the untreated control groups (Tis et al., 2002; Sakurakichi et al., 2004). Sakurakichi et al. (2004) have also demonstrated that the effect of LIPUS treatment was even more pronounced when applied during the distraction period compared to the treatment in the maturation period.

7. The influence of LIPUS on isolated cells

The major cell types for the development of a fracture callus are mesenchymal stem cells, fibroblasts, osteoblasts and chondrocytes. Several studies have demonstrated that US (500 mW/cm^2) stimulation leads to increases in protein synthesis (Harvey et al., 1975; Webster et al., 1978) and collagen synthesis (Webster et al., 1980) observed in human fibroblasts in vitro. The formation of cartilage by chondrocytes is often identified by immunostaining for aggrecan, a major structural macromolecule of cartilage. In vitro studies have demonstrated increased chondrocyte differentiation by increased aggrecan expression (Parvizi et al., 1999; Ebisawa et al., 2004), proteoglycan synthesis (Wu et al., 1996), and upregulation of chondroitin sulphate release (Nishikori et al., 2002) after treatment with LIPUS. These results (Yang, 1996 #115; Wu et al., 1996; Parvizi et al., 1999; Nishikori et al., 2002) suggest that LIPUS may stimulate synthesis of extracellular matrix proteins in cartilage altering chondrocyte maturation and endochondral bone formation. Zhang et al. (2003) in a cell culture study with chondrocytes from chicken embryos after US treatment observed increased proliferation of undifferentiated chondrocytes. However, Parvizi et al. (1999) and Nishikori et al. (2002) found that US had no effect on chondrocyte proliferation. Wang et al. (2004) showed that US stimulation led to significantly increased vascular endothelial growth factor, VEGF-A mRNA and protein levels in human osteoblast cells. VEGF is an important regulator for angiogenesis and endochondral bone formation (Midy and Plouet, 1994; Deckers et al., 2000; Hausman et al., 2001; Mayr-Wohlfart et al., 2002; Uchida et al., 2003).

In vitro investigations seeking to determine whether LIPUS has a direct effect on bone formation have often quantified the bone markers, osteocalcin and alkaline phosphatase. The osteocalcin protein, synthesized by mature osteoblasts, odontoblasts and hypertrophic chondrocytes, is used as a highly specific bone marker for assessing bone turnover (Gundberg, 2001). The bone isoform of the alkaline phosphatase enzyme produced by osteoblasts is a commonly used marker for bone metabolism (Demers, 2001). Warden et al. (2001) showed in vitro that LIPUS stimulated the expression of c-fos and cyclooxygenase-2 genes and elevated mRNA levels for the bone matrix proteins alkaline phosphatase and osteocalcin. US stimulated osteoblast differentiation (Yang et al., 2005) by increasing alkaline phosphatase, osteocalcin and VEGF expression and mineralization (Leung et al., 2004a), with these effects more pronounced using higher US intensities (Harle et al., 2001; Li et al., 2002). Chen et al. (2003) identified the pertussis toxin sensitive $G\alpha i$ protein and ERK to be essential elements in the transduction of LIPUS signal into a cellular response. They (Chen et al., 2003) also found an increased cbfa1 and osteocalcin expression in human osteoblasts in response to LIPUS treatment. Chapman et al. (1980) demonstrated an increase in second messenger activity paralleled by the modulation of adenylate cyclase activity and TGF-β synthesis in osteoblasts after LIPUS treatment. Kokubu et al. (1999) found an increase of prostaglandin E2 release and an increased expression of the PGE synthesis enzyme cyclooxygenase-2 (COX-2) in mouse osteoblasts after US treatment. Prostaglandin E2 has been identified as a mediator in the bone forming response to external stimuli (Forwood, 1996). The increase of both PGE2 release and COX-2 expression could be induced by fluid flow in bone cells (Ajubi et al., 1999; Bakker et al., 2003). Similar to the effects of mechanical stimulation on bone cells (Pavalko et al., 1998), integrins and the organization of the cytoskeleton seem to play an important role in the transduction of US (Yang et al., 2005).

8. The influence of LIPUS on tissue culture

In vitro tissue culture studies on foetal bone have shown that the volume of the calcified diaphysis and the bone collar increased in LIPUS treated versus untreated bones (Nolte et al., 2001; Korstjens et al., 2004). Nolte et al. (2001) suggested LIPUS stimulation caused an increase in osteoblast activity or number as well as stimulating hypertrophic cartilage cells, which resulted in the increased calcified matrix. Korstjens et al. (2004)

suggested that the increased bone collar and calcified cartilage volume was due to the stimulation by LIPUS of bone cell differentiation and calcified matrix production, respectively, but not due to cell proliferation. These studies showed that LIPUS treatment for 20 min a day over 7 days had no effect on the longitudinal growth of the proliferative zone in foetal mouse metatarsal bone rudiments (Nolte et al., 2001; Korstjens et al., 2004).

Contrary to these studies that used LIPUS, Wiltink et al. (1995) investigated the influence of high intensity (100–770 mW/cm^2) pulsed US in developing murine foetal metatarsal bone rudiments. They demonstrated that higher intensity pulsed US treatment for 5 min a day over 7 days resulted in significantly increased longitudinal growth in the proliferative zone in US treated bones while the calcified zone was identical in treated and untreated bones. The authors suggested that the increased longitudinal growth originated from an increased proliferation of undifferentiated chondrocytes. Korstjens et al. (2004) suggested that the increased differentiation and matrix production associated with endochondral bone formation observed with LIPUS treatment is not observed with higher US intensities as used in the study by Wiltink et al. (1995). Higher US intensities may inhibit the synthesis of collagen and non-collagen proteins (Reher et al., 1997), which results in the observed cartilage cell proliferation (Wiltink et al., 1995). These studies suggest that LIPUS treatment stimulates endochondral ossification by affecting calcification and cell differentiation, rather than cell proliferation. LIPUS likely stimulates hypertrophic chondrocytes and osteoblasts, but does not seem to affect nonhypertrophic chondrocytes, observed by the lack of longitudinal growth of the embryonic long bones and the results of studies investigating intact bone in vivo.

9. The influence of LIPUS on intact bone in vivo

In vivo experimental studies (Elmer and Fleischer, 1974; Spadaro and Albanese, 1998; Wimsatt et al., 2000; Warden et al., 2001) have demonstrated that LIPUS does not stimulate osteogenesis in intact bone. Spadaro and Albanese (1998) showed that LIPUS applied for 4 weeks had no effect on the longitudinal growth or bone mineral density of the femur of 4-week-old growing male rats. Similarly, Warden et al. (2001) showed that LIPUS treatment for 20 min per day, 6 days a week for 12 weeks had no effect on bone mineral content or bone mineral density within the distal femur or proximal tibia of ovariectomy rats or normal rats following sham ovariectomy. Wimsatt et al. (2000) reported that LIPUS treatment twice weekly for 5 weeks had no effect on immobilization induced bone mineral loss, associated with wing bandaging in pigeons. The results of these in vivo investigations suggest that LIPUS treatment does not affect bone remodelling.

10. Conclusions

From the review of the literature there is evidence that low intensity pulsed ultrasound (LIPUS) has a positive effect on bone regeneration. LIPUS treatment has been shown to accelerate and stimulate fracture healing and callus maturation after distraction osteogenesis. The stimulatory effects are often observed during the soft callus formation phase and not during the remodelling phase. Even though the energy used by the LIPUS treatment is extremely low, effects on cells in vitro and in vivo have been described. However, the biophysical process by which LIPUS stimulates bone regeneration still remains unknown.

References

Ajubi, N.E., Klein-Nulend, J., Alblas, M.J., Burger, E.H., Nijweide, P.J., 1999. Signal transduction pathways involved in fluid flow-induced PGE2 production by cultured osteocytes. Am. J. Physiol. 276 (1 (Pt 1)), E171–E178.

Ardan Jr., N.I., Janes, J.M., Herrick, J.F., 1957. Ultrasonic energy and surgically produced defects in bone. J. Bone Joint Surg. Am. 39-A (2), 394–402.

Azuma, Y., Ito, M., Harada, Y., Takagi, H., Ohta, T., Jingushi, S., 2001. Low-intensity pulsed ultrasound accelerates rat femoral fracture healing by acting on the various cellular reactions in the fracture callus. J. Bone Miner. Res. 16 (4), 671–680.

Bakker, A.D., Klein-Nulend, J., Burger, E.H., 2003. Mechanotransduction in bone cells proceeds via activation of COX-2, but not COX-1. Biochem. Biophys. Res. Commun. 305 (3), 677–683.

Bender, L.F., Janes, J.M., Herrick, J.F., 1954. Histologic studies following exposure of bone to ultrasound. Arch. Phys. Med. Rehabil. 35 (9), 555–559.

Buchtala, V., 1950. Present state of ultrasound therapy. Dia. Med. 22 (70), 2944–2950.

Chang, W.H., Sun, J.S., Chang, S.P., Lin, J.C., 2002. Study of thermal effects of ultrasound stimulation on fracture healing. Bioelectromagnetics 23 (4), 256–263.

Chapman, I.V., MacNally, N.A., Tucker, S., 1980. Ultrasound-induced changes in rates of influx and efflux of potassium ions in rat thymocytes in vitro. Ultrasound Med. Biol. 6 (1), 47–58.

Chen, Y.J., Wang, C.J., Yang, K.D., Chang, P.R., Huang, H.C., Huang, Y.T., Sun, Y.C., Wang, F.S., 2003. Pertussis toxin-sensitive Galphai protein and ERK-dependent pathways mediate ultrasound promotion of osteogenic transcription in human osteoblasts. FEBS Lett. 554 (1–2), 154–158.

Claes, L., Ruter, A., Mayr, E., 2005. Low-intensity ultrasound enhances maturation of callus after segmental transport. Clin. Orthop. Relat. Res. (430), 189–194.

Corradi, C., Cozzolino, A., 1953. Effect of ultrasonics on the development of osseous callus in fractures. Arch. Ortop. 66 (1), 77–98.

Deckers, M.M., Karperien, M., van der Bent, C., Yamashita, T., Papapoulos, S.E., Lowik, C.W., 2000. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology 141 (5), 1667–1674.

Demers, L.M., 2001. Chapter 5: Bone-specific alkaline phosphatase. In: Eastell, R., Bauman, M., Hoyle, N., Wieczorek, L. (Eds.), Bone Markers: Biochemical and Clinical Perspectives. Martin Dunitz Ltd, London, pp. 57–63.

De Nunno, R., 1952. Effect of ultrasonics on ossification; experimental studies. Ann. Ital. Chir. 29 (4), 211–220.

Dinno, M.A., Dyson, M., Young, S.R., Mortimer, A.J., Hart, J., Crum, L.A., 1989. The significance of membrane changes in the safe and effective use of therapeutic and diagnostic ultrasound. Phys. Med. Biol. 34 (11), 1543–1552.

Duarte, L.R., 1983. The stimulation of bone growth by ultrasound. Arch. Orthop. Trauma Surg. 101 (3), 153–159.

Dyson, M., 1982. Non-thermal cellular effects of ultrasound. Br. J. Cancer Suppl. 45 (5), 165–171.

Dyson, M., Brookes, M., 1983. Stimulation of bone repair by ultrasound. Ultrasound Med. Biol. Suppl. 2, 61–66.

Dyson, M., Suckling, J., 1978. Stimulation of tissue repair by ultrasound: a survey of the mechanisms involved. Physiotherapy 64 (4), 105–108.

Eberson, C.P., Hogan, K.A., Moore, D.C., Ehrlich, M.G., 2003. Effect of low-intensity ultrasound stimulation on consolidation of the regenerate zone in a rat model of distraction osteogenesis. J. Pediatr. Orthop. 23 (1), 46–51.

Ebisawa, K., Hata, K., Okada, K., Kimata, K., Ueda, M., Torii, S., Watanabe, H., 2004. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. Tissue Eng. 10 (5–6), 921–929.

Einhorn, T.A., 1998. The cell and molecular biology of fracture healing. Clin. Orthop. Relat. Res. (355 Suppl), S7–S21.

Elmer, W.A., Fleischer, A.C., 1974. Enhancement of DNA synthesis in neonatal mouse tibial epiphyses after exposure to therapeutic ultrasound. J. Clin. Ultrasound 2 (3), 191–195.

Faber, F.W., Keessen, W., van Roermund, P.M., 1991. Complications of leg lengthening. 46 procedures in 28 patients. Acta Orthop. Scand. 62 (4), 327–332.

Forriol, F., Iglesias, A., Arias, M., Aquerreta, D., Canadell, J., 1999. Relationship between radiologic morphology of the bone lengthening formation and its complications. J. Pediatr. Orthop. B 8 (4), 292–298.

Forwood, M.R., 1996. Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J. Bone Miner. Res. 11 (11), 1688–1693.

Gross, T.S., Edwards, J.L., McLeod, K.J., Rubin, C.T., 1997. Strain gradients correlate with sites of periosteal bone formation. J. Bone Miner. Res. 12 (6), 982–988.

Gundberg, C.M., 2001. Chapter 6: Osteocalcin. In: Eastell, R., Bauman, M., Hoyle, N., Wieczorek, L. (Eds.), Bone Markers: Biochemical and Clinical Perspectives. Martin Dunitz Ltd, London, pp. 65–72.

Hantes, M.E., Mavrodontidis, A.N., Zalavras, C.G., Karantanas, A.H., Karachalios, T., Malizos, K.N., 2004. Low-intensity transosseous ultrasound accelerates osteotomy healing in a sheep fracture model. J. Bone Joint Surg. Am. 86-A (10), 2275–2282.

Harle, J., Salih, V., Knowles, J.C., Mayia, F., Olsen, I., 2001. Effects of therapeutic ultrasound on osteoblast gene expression. J. Mater. Sci. Mater. Med. 12 (10–12), 1001–1004.

Harvey, W., Dyson, M., Pond, J.B., Grahame, R., 1975. The stimulation of protein synthesis in human fibroblasts by therapeutic ultrasound. Rheumatol. Rehabil. 14 (4), 237.

Hausman, M.R., Schaffler, M.B., Majeska, R.J., 2001. Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone 29 (6), 560–564.

Herrick, J.F., Janes, J.M., Ardan Jr., N.I., 1956. Experimental studies relative to the therapeutic use of ultrasound. J. Am. Vet. Med. Assoc. 128 (12), 571–577.

Heybeli, N., Yesildag, A., Oyar, O., Gulsoy, U.K., Tekinsoy, M.A., Mumcu, E.F., 2002. Diagnostic ultrasound treatment increases the bone fracture-healing rate in an internally fixed rat femoral osteotomy model. J. Ultrasound Med. 21 (12), 1357–1363.

Hill, C.R., 1971. Ultrasonic Exposure Threshold for Changes in Cells and Tissues. J. Acoust. Soc. Am. 52 (2 (Pt 2)), 667–672.

Ilizarov, G.A., 1989a. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. Clin. Orthop. Relat. Res. (239), 263–285.

Ilizarov, G.A., 1989b. The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation. Clin. Orthop. Relat. Res. (238), 249–281.

Iwaki, A., Jingushi, S., Oda, Y., Izumi, T., Shida, J.I., Tsuneyoshi, M., Sugioka, Y., 1997. Localization and quantification of proliferating cells during rat fracture repair: detection of proliferating cell nuclear antigen by immunohistochemistry. J. Bone Miner. Res. 12 (1), 96–102.

Klug, W., Franke, W.G., Knoch, H.G., 1986. Scintigraphic control of bone-fracture healing under ultrasonic stimulation: an animal experimental study. Eur. J. Nucl. Med. 11 (12), 494–497.

Kokubu, T., Matsui, N., Fujioka, H., Tsunoda, M., Mizuno, K., 1999. Low intensity pulsed ultrasound exposure increases prostaglandin E2 production via the induction of cyclooxygenase-2 mRNA in mouse osteoblasts. Biochem. Biophys. Res. Commun. 256 (2), 284–287.

Korstjens, C.M., Nolte, P.A., Burger, E.H., Albers, G.H., Semeins, C.M., Aartman, I.H., Goei, S.W., Klein-Nulend, J., 2004. Stimulation of bone cell differentiation by low-intensity ultrasound—a histomorphometric in vitro study. J. Orthop. Res. 22 (3), 495–500.

Lee, F.Y., Choi, Y.W., Behrens, F.F., DeFouw, D.O., Einhorn, T.A., 1998. Programmed removal of chondrocytes during endochondral fracture healing. J. Orthop. Res. 16 (1), 144–150.

Leung, K.S., Cheung, W.H., Zhang, C., Lee, K.M., Lo, H.K., 2004a. Low intensity pulsed ultrasound stimulates osteogenic activity of human periosteal cells. Clin. Orthop. Relat. Res. (418), 253–259.

Leung, M.C., Ng, G.Y., Yip, K.K., 2004b. Effect of ultrasound on acute inflammation of transected medial collateral ligaments. Arch. Phys. Med. Rehab. 85 (6), 963–966.

Li, J.G., Chang, W.H., Lin, J.C., Sun, J.S., 2002. Optimum intensities of ultrasound for PGE(2) secretion and growth of osteoblasts. Ultrasound Med. Biol. 28 (5), 683–690.

Machen, M.S., Tis, J.E., Inoue, N., Meffert, R.H., Chao, E.Y., McHale, K.A., 2002. The effect of low intensity pulsed ultrasound on regenerate bone in a less-than-rigid biomechanical environment. Biomed. Mater. Eng. 12 (3), 239–247.

Maintz, G., 1950. Animal experiments in the study of the effect of ultrasonic waves on bone regeneration. Strahlentherapie 82 (4), 631–638.

Mayr, E., Laule, A., Suger, G., Ruter, A., Claes, L., 2001. Radiographic results of callus distraction aided by pulsed low-intensity ultrasound. J. Orthop. Trauma 15 (6), 407–414.

Mayr-Wohlfart, U., Waltenberger, J., Hausser, H., Kessler, S., Gunther, K.P., Dehio, C., Puhl, W., Brenner, R.E., 2002. Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. Bone 30 (3), 472–477.

Midy, V., Plouet, J., 1994. Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. Biochem. Biophys. Res. Commun. 199 (1), 380–386.

Mortimer, A.J., Dyson, M., 1988. The effect of therapeutic ultrasound on calcium uptake in fibroblasts. Ultrasound Med. Biol. 14 (6), 499–506.

Murolo, C., Claudio, F., 1952. Effect of ultrasonics on repair of fractures. G. Chir. 8 (11), 897–903.

Nishikori, T., Ochi, M., Uchio, Y., Maniwa, S., Kataoka, H., Kawasaki, K., Katsube, K., Kuriwaka, M., 2002. Effects of low-intensity pulsed ultrasound on proliferation and chondroitin sulfate synthesis of cultured chondrocytes embedded in Atelocollagen gel. J. Biomed. Mater. Res. 59 (2), 201–206.

Nolte, P.A., van der Krans, A., Patka, P., Janssen, I.M., Ryaby, J.P., Albers, G.H., 2001. Low-intensity pulsed ultrasound in the treatment of nonunions. J. Trauma 51 (4), 693–702 discussion 702–693.

Paley, D., 1990. Problems, obstacles, and complications of limb lengthening by the Ilizarov technique. Clin. Orthop. Relat. Res. (250), 81–104.

Parvizi, J., Wu, C.C., Lewallen, D.G., Greenleaf, J.F., Bolander, M.E., 1999. Low-intensity ultrasound stimulates proteoglycan synthesis in rat chondrocytes by increasing aggrecan gene expression. J. Orthop. Res. 17 (4), 488–494.

Pavalko, F.M., Chen, N.X., Turner, C.H., Burr, D.B., Atkinson, S., Hsieh, Y.F., Qiu, J., Duncan, R.L., 1998. Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton–integrin interactions. Am. J. Physiol. 275 (6 (Pt 1)), C1591–C1601.

Pilla, A.A., 2002. Low-intensity electromagnetic and mechanical modulation of bone growth and repair: are they equivalent? J. Orthop. Sci. 7 (3), 420–428.

Pilla, A.A., Mont, M.A., Nasser, P.R., Khan, S.A., Figueiredo, M., Kaufman, J.J., Siffert, R.S., 1990. Non-invasive low-intensity pulsed ultrasound accelerates bone healing in the rabbit. J. Orthop. Trauma 4 (3), 246–253.

Praemer, A., Furner, S., Price, O., 1992. Musculoskeletal conditions in the United States. Am. Acad. Orthop. Surg., 85–91.

Rawool, N.M., Goldberg, B.B., Forsberg, F., Winder, A.A., Hume, E., 2003. Power Doppler assessment of vascular changes during fracture treatment with low-intensity ultrasound. J. Ultrasound Med. 22 (2), 145–153.

Reher, P., Elbeshir el, N.I., Harvey, W., Meghji, S., Harris, M., 1997. The stimulation of bone formation in vitro by therapeutic ultrasound. Ultrasound Med. Biol. 23 (8), 1251–1258.

Rubin, C., Bolander, M., Ryaby, J.P., Hadjiargyrou, M., 2001. The use of low-intensity ultrasound to accelerate the healing of fractures. J. Bone Joint Surg. Am. 83-A (2), 259–270.

Ryaby, J., Bachner, E., Bendo, J., Dalton, P., Tannenbaum, S., Pilla, A.A., 1989. Low Intensity Pulsed Ultrasound Increases Calcium Incorporation in Both Differentiating Cartilage and Bone Cell Cultures, vol. 15. Orthopedic Research Society, Las Vegas, NV.

Ryaby, J.T., Mathew, J., Pilla, A.A., Duarte-Alves, P., 1991. Chapter 16: Low-intensity pulsed ultrasound modulates adenylate cyclase activity and transforming growth factor beta synthesis. In: Brighton, C.T., Pollack, S.R. (Eds.), Electromagnetics in Biology and Medicine. San Francisco Press, Inc., San Francisco, pp. 95–99.

Sakurakichi, K., Tsuchiya, H., Uehara, K., Yamashiro, T., Tomita, K., Azuma, Y., 2004. Effects of timing of low-intensity pulsed ultrasound on distraction osteogenesis. J. Orthop. Res. 22 (2), 395–403.

Shimazaki, A., Inui, K., Azuma, Y., Nishimura, N., Yamano, Y., 2000. Low-intensity pulsed ultrasound accelerates bone maturation in distraction osteogenesis in rabbits. J. Bone Joint Surg. Br. 82 (7), 1077–1082.

Spadaro, J.A., Albanese, S.A., 1998. Application of low-intensity ultrasound to growing bone in rats. Ultrasound Med. Biol. 24 (4), 567–573.

Tis, J.E., Meffert, C.R., Inoue, N., McCarthy, E.F., Machen, M.S., McHale, K.A., Chao, E.Y., 2002. The effect of low intensity pulsed ultrasound applied to rabbit tibiae during the consolidation phase of distraction osteogenesis. J. Orthop. Res. 20 (4), 793–800.

Tsai, C.L., Chang, W.H., Liu, T.K., 1992. Preliminary studies of duration and intensity of ultrasonic treatments on fracture repair. Chin. J. Physiol. 35 (1), 21–26.

Uchida, S., Sakai, A., Kudo, H., Otomo, H., Watanuki, M., Tanaka, M., Nagashima, M., Nakamura, T., 2003. Vascular endothelial growth factor is expressed along with its receptors during the healing process of bone and bone marrow after drill-hole injury in rats. Bone 32 (5), 491–501.

Uglow, M.G., Peat, R., Hile, M., Bilston, L., Smith, E., Little, D., 2003. Low-intensity ultrasound stimulation in distraction osteogenesis in rabbits. Clin. Orthop. Relat. Res. (417), 303–312.

Wang, S., Lewallen, D., Bolander, M., Chao, E., Ilstrup, D., Greenleaf, J., 1994. Low intensity ultrasound treatment increases strength in a rat femoral fracture model. J. Orthop. Res. 12 (1), 40–47.

Wang, F.S., Kuo, Y.R., Wang, C.J., Yang, K.D., Chang, P.R., Huang, Y.T., Huang, H.C., Sun, Y.C., Yang, Y.J., Chen, Y.J., 2004. Nitric oxide mediates ultrasound-induced hypoxia-inducible factor-1alpha activation and vascular endothelial growth factor-A expression in human osteoblasts. Bone 35 (1), 114–123.

Warden, S.J., Favaloro, J.M., Bennell, K.L., McMeeken, J.M., Ng, K.W., Zajac, J.D., Wark, J.D., 2001. Low-intensity pulsed ultrasound stimulates a bone-forming response in UMR-106 cells. Biochem. Biophys. Res. Commun. 286 (3), 443–450.

Watson, T., 2000. The role of electrotherapy in contemporary physiotherapy practice. Man. Ther. 5 (3), 132–141.

Webster, D., Pond, J., Dyson, M., Harvey, W., 1978. The role of cavitation in the in vitro stimulation of protein synthesis in human fibroblasts by ultrasound. Ultrasound Med. Biol. 4 (4), 343–351.

Webster, D.F., Harvey, W., Dyson, M., Pond, J.B., 1980. The role of ultrasound-induced cavitation in the 'in vitro' stimulation of collagen synthesis in human fibroblasts. Ultrasonics 18 (1), 33–37.

Welgus, H.G., Jeffrey, J.J., Eisen, A.Z., 1981. Human skin fibroblast collagenase. Assessment of activation energy and deuterium isotope effect with collagenous substrates. J. Biol. Chem. 256 (18), 9516–9521.

Welgus, H.G., Jeffrey, J.J., Eisen, A.Z., Roswit, W.T., Stricklin, G.P., 1985. Human skin fibroblast collagenase: interaction with substrate and inhibitor. Coll. Relat. Res. 5 (2), 167–179.

Wendeberg, B., 1961. Mineral metabolism of fractures of the tibia in man studied with external counting of Sr85. Acta Orthop. Scand. Suppl. 52, 1–79.

Wiltink, A., Nijweide, P.J., Oosterbaan, W.A., Hekkenberg, R.T., Helders, P.J., 1995. Effect of therapeutic ultrasound on endochondral ossification. Ultrasound Med. Biol. 21 (1), 121–127.

Wimsatt, J., Dressen, P., Dennison, C., Turner, A.S., 2000. Ultrasound therapy for the prevention and correction of contractures and bone mineral loss associated with wing bandaging in the domestic pigeon (*Columba livia*). J. Zoo. Wildl. Med. 31 (2), 190–195.

Wolff, J., 1892. The Law of Bone Remodelling (Das Gesetz der Transformation der Knochen, Hirschwald). Springer, Berlin.

Wu, J.R., Du, G.H., 1990a. Temperature elevation generated by a focused Gaussian beam of ultrasound. Ultrasound Med. Biol. 16 (5), 489–498.

Wu, J.R., Du, G.H., 1990b. Temperature elevation generated by a focused Gaussian ultrasonic beam at a tissue–bone interface. J. Acoust. Soc. Am. 87 (6), 2748–2755.

Wu, C.C., Lewallen, D.G., Bolander, M., Bronk, J., Kinnick, R., Greenleaf, J.F., 1996. Exposure to Low Intensity Ultrasound Stimulates Aggrecan Gene Expression by Cultured Chondrocytes, vol. 622. Orthopedic Research Society, Atlanta, GA.

Yang, K.H., Parvizi, J., Wang, S.J., Lewallen, D.G., Kinnick, R.R., Greenleaf, J.F., Bolander, M.E., 1996. Exposure to low-intensity ultrasound increases aggrecan gene expression in a rat femur fracture model. J. Orthop. Res. 14 (5), 802–809.

Yang, R.S., Lin, W.L., Chen, Y.Z., Tang, C.H., Huang, T.H., Lu, B.Y., Fu, W.M., 2005. Regulation by ultrasound treatment on the integrin expression and differentiation of osteoblasts. Bone 36 (2), 276–283.

Yasui, N., Sato, M., Ochi, T., Kimura, T., Kawahata, H., Kitamura, Y., Nomura, S., 1997. Three modes of ossification during distraction osteogenesis in the rat. J. Bone Joint Surg. Br. 79 (5), 824–830.

Zhang, Z.J., Huckle, J., Francomano, C.A., Spencer, R.G., 2003. The effects of pulsed low-intensity ultrasound on chondrocyte viability, proliferation, gene expression and matrix production. Ultrasound Med. Biol. 29 (11), 1645–1651.