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Mode & mechanism of low intensity pulsed ultrasound (LIPUS) in fracture repair

Andrew Harrison [a,*], Sheldon Lin [b], Neill Pounder [c], Yuko Mikuni-Takagaki [d]

[a] Bioventus Cooperatief, Amsterdam, Netherlands
[b] Department of Orthopedics, Rutgers, New Jersey Medical School, USA
[c] Bioventus LLC, Durham, NC, USA
[d] Kanagawa Dental University, Graduate School of Dentistry, Yokosuka, Japan

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ABSTRACT

It has been 30 years since the first level one clinical trial demonstrated low intensity pulsed ultrasound (LIPUS) could accelerate fracture repair. Since 1994 numerous investigations have been performed on the effect of LIPUS. The majority of these studies have used the same signal parameters comprised of an intensity of 30 mW/cm^2 SATA, an ultrasound carrier frequency of 1.5 MHz, pulsed at 1 kHz with an exposure time of 20 minutes per day. These studies show that a biological response is stimulated in the cell which produces bioactive molecules. The production of these molecules, linked with observations demonstrating the enhanced effects on mineralization by LIPUS, might be considered the general manner, or mode, of how LIPUS stimulates fractures to heal.

We propose a mechanism for how the LIPUS signal can enhance fracture repair by combining the findings of numerous studies. The LIPUS signal is transmitted through tissue to the bone, where cells translate this mechanical signal to a biochemical response via integrin mechano-receptors. The cells enhance the production of cyclo-oxygenese 2 (COX-2) which in turn stimulates molecules to enhance fracture repair. The aim of this review is to present the state of the art data related to LIPUS effects and mechanism.

Contents

1. Introduction

Fracture healing is a complex process. Clinically, it requires adequate reduction of the displaced fracture and stabilization. The biological process consists of several distinct temporal phases. These

* Corresponding author at: Research & Development, Bioventus, Taurusavenue 31, 2132 LS Hoofddorp, Netherlands.
E-mail address: andrew.harrison@bioventusglobal.com (A. Harrison).

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phases are the inflammatory phase with cell proliferation, the chondrogenic phase with cartilage hypertrophy and angiogenesis, and the osteogenic phase with replacement of cartilage with woven bone and subsequent remodeling. During the fracture healing process, various cell types must interact and deliver adequate and appropriate levels of inflammatory and bioactive molecules. Despite the complexity, most fractures heal without a problem. In the United States, over eight million fractures occur annually, with a delayed or non-union rate of 5–10% [1].

Several risk factors are associated with impaired bone healing. These factors include the presence of systemic disease, such as diabetes [2]; advanced age [3,4]; or external chemical factors, such as alcohol abuse [5], smoking [6], or prescribed medications like chronic steroid use or chemotherapeutics [7]. These risk factors provide an ongoing clinical challenge for the treatment of fractures in our aging, comorbid population.

Several biophysical agents have been proposed and analyzed as therapeutic adjuncts to address this impaired bone healing process. These adjuncts include pulsed electromagnetic fields, combined magnetic fields, direct electrical current and low-intensity pulsed ultrasound. The pulsed electromagnetic systems generate asymmetric, quasirectangular electromagnetic pulses grouped in bursts. Individual pulse duration is approximately 260 μs, burst frequency is 15 Hz and the peak field strength is 20 mT. The nonunion effectiveness rate for patients using one of the pulsed electromagnetic system for an average of 7.1 hours per day was 80% [8]. Another pulsed electromagnetic system demonstrated a nonunion success rate of 63.5% for treatment periods up to 10 hours [9]. The external combined magnetic field device produces a static 20 μT magnetic field and a sinusoidal dynamic field at a frequency of 76.6 Hz and amplitude of ±40 μT. Patients use the device for 30 minutes per day, providing a nonunion heal rate of 60.7% [10]. The implanted direct current stimulator delivers a 20 μA current via a wire cathode continuously for 24 hours a day for at least 6 months, resulting in a long term nonunion success rate between 38.8% and 66.7% [11]. Low-intensity pulsed ultrasound (LIPUS) is indicated for certain fresh fractures and the treatment of nonunions. A 20 minute treatment time results in a 38% acceleration of fresh fractures [12,13] and an 86% nonunion success rate [14]. This review discusses LIPUS exclusively. When defining low intensity for LIPUS we will deal specifically with an intensity of 30 mW/cm^2 SATA (spatial average–temporal average) and a power of 117 mW delivered via an unfocussed ultrasound transducer with an active area of 3.88 cm^2, as measured by the radiation force balance method. The ultrasound carrier frequency is 1.5 MHz, pulsed at 1 kHz. The ultrasound power is measured by the Ohmic Instruments ultrasound power meter.

The use of low intensity ultrasound to accelerate the fracture repair process in humans was first reported by Xavier and Duarte in 1983 [15]. This success led to clinical trials in the United States [12,13] and the 1994 approval of EXOGEN by the U.S. Food and Drug Administration for the accelerated healing of certain fresh fractures. The product was approved for the treatment of established non-unions in 2000 [14,16]. All the LIPUS articles stated in this review have used the clinically approved ultrasound signal. Specifically, 1.5 MHz sinusoidal waves modulated in bursts of 200 ms at a repetition frequency of 1 kHz, and a spatial average–temporal average intensity of 30 mW/cm^2.

The positive effect of low-intensity pulsed ultrasound on the acceleration of bone healing has been demonstrated in nonclinical and clinical studies. Clinical studies have demonstrated significant positive effect of LIPUS in the treatment of fresh fractures [12,13] and non-unions [14,16,17]. The prospective, randomized, multi-center, double-blind level I clinical studies investigating LIPUS treatment of conservatively treated tibia [12] and distal radius [13] both demonstrated 38% acceleration in fracture repair compared to fractures treated with a placebo device ($p < 0.0001$), as assessed radiographically and clinically by blinded physicians. For nonunion fractures, the studies [14,16,17] included nonunion fractures that would not otherwise have healed. The fractures were at least 9 months old, with a minimum of 3 months since the last intervention. The only treatment change was daily LIPUS use. Gebauer et al. investigated the effect of LIPUS in 67 subjects, demonstrating heal rates of 85% after 5.6 months [16], while Nolte et al. studied 29 nonunions that resulted in an 86% heal rate in an average time of 5.5 months [14]. Neither study reported any safety issues.

The diffraction pattern of the ultrasound waves produced by a piston transducer, such as the LIPUS transducer, has two characteristic zones: the near field (close to the transducer) and the far field (farther from the transducer). The interface between the near field and far field is described by the equation:

$$Z = \frac{a^2 f}{c}$$

where Z is the near field length, a is the radius of the transducer after the near field length, f is the frequency, and c is the speed of sound in the medium [18]. The LIPUS transducer has a radius of 11 mm, emitting 1.5 MHz ultrasound wave and propagating in average tissue at 1540 ms^{-1} [19]. The computed value of Z is 118 mm. The ultrasound beam pattern measured in water using a hydrophone shows rapidly changing intensity amplitude across the beam in the near field ($Z = 0$ mm). The complexity (successive axial maxima and minima) of the ultrasound field decreases as the axial distance from the transducer increases. At $Z = 60$ mm (mid-near field), the complexity of the beam decreases and forms less maxima and minima. At $Z = 118$ mm (far field), the ultrasound pressure wave is more uniform. For the experiments described in this review, all the cell culture and in vivo experiments were performed in the near field. This is contrary to recommendations to place the in vitro cell culture within the far field where the pressure levels and temporal variation of intensity are controlled [20]. However, placement within the near field more closely matches the clinical situation as the majority of bones have little soft tissue coverage. The deepest bone treated clinically is the femur. With the far field starting at 118 mm, and assuming the femur is in the center of the thigh, the circumference of a patient's thigh would need to be 74 cm (29 inches) before a femur would be receiving ultrasound in the far field. Anthropometric data indicates that 95% of Americans have a mid-thigh circumference of less than 75 cm (29.5 inches) [21]. Therefore, application of the ultrasound coupled to a petri dish or directly to a rodent's limb provides a more clinically relevant ultrasound field. In the in vivo studies, the animals were sedated daily and the active or placebo transducer coupled to the skin with ultrasound gel directly above the fracture, unless otherwise stated. The in vivo models typically used either rats or mice, with a closed femoral fracture first described by Bonnarens and Einhorn [22]. This model is widely regarded as the standard in vivo model for traumatic fractures. The phases of fracture healing match those observed clinically, albeit on a shorter timescale. The consistency of the fracture allows this in vivo model to be used to investigate different interventions and mechanisms. The purpose of this review is to provide our current understanding regarding the mechanism and mode of action for LIPUS.

Routinely the literature states that 'the mechanism of LIPUS has yet to be elucidated'. We believe that there is evidence supporting both a credible mode and a mechanism behind the LIPUS technology. 'Mode' is described in dictionaries as 'a way in which something occurs or is done' [23]. Therefore the mode of action that we propose for how LIPUS stimulates an enhanced healing process to accelerate fracture repair can be seen as the stimulation of processes and molecules that contribute to heal the fracture is related

to mechanoreceptors at the cellular level. The mechanism of how LIPUS achieves this is quite different from the mode. The dictionary definition of 'Mechanism' is 'the way in which something works or is brought about' [23]. This review will seek to investigate the evidence for both the mode and mechanism behind the LIPUS technology, first by explaining the osteogenic action of LIPUS on fracture repair, then by demonstrating the mechanism by evaluating the LIPUS signal and how this is 'interpreted' by the cells to elicit a healing response, using the best key evidence. This review is an update of a previous review in this journal in 2008 to pull together aspects of the biology to describe a mechanism of action [24]. Previously, LIPUS was believed to work equally in all phases of fracture repair. This update provides more detailed evidence supporting the impact on endochondral ossification and a clear effect on bone resorption during healing. In addition, there is a clear mechanistic link between integrin activation and COX2 upregulation.

2. The mode of action of LIPUS in fracture repair

2.1. Effect of LIPUS in animal models of fracture repair

In order to understand the effect LIPUS has on fracture repair, numerous pre-clinical models have been used to simulate the clinical situation. One such experimental in vivo model was performed treating fractures at different periods of repair. A rat closed femoral fracture was created in both hind limbs with the right femur exposed to LIPUS, and the left femur used as a control [25]. Rats were divided into four groups according to timing and duration of treatment. The animals in each group were treated with LIPUS for 20 minutes per day as follows: LIPUS treatments were performed in the Phase 1 group for 8 days, from day 1 to 8 after fracture; in the Phase 2 group for 8 days, from day 9 to 16 after fracture; in the Phase 3 group for 8 days, from day 17 to 24 after fracture. The last group was treated from days 1 to 24, throughout the healing process. The authors considered this to represent the hematoma, soft callus and mineralization phases of fracture repair. Animals were euthanized on day 25, and radiographs and torsional biomechanical testing were used to assess fracture healing. The maximal torque and stiffness in torsion of the fractured femur on the LIPUS-treated side was significantly higher than that of the contralateral control side in all groups ($p < 0.01$). This indicated that even partial treatment with LIPUS during Phase 1, 2, or 3 improved the mechanical properties of the fracture callus; however, treatment throughout the 24 days was most effective ($p < 0.05$). The histology presented in this study showed an acceleration of endochondral ossification, in that the LIPUS-treated fractures at day 25 resulted in much more bone formed than in controls at the same time point. This observation of accelerated endochondral ossification was reproduced in another in vivo study using a closed transverse mid-diaphyseal femoral fracture also in rats [26]. LIPUS was applied for 10 min daily, with the contralateral control limb receiving an inactive transducer. The formation of the new tissue after fracture was assessed by micro CT each week for three weeks, with phasing thresholds which differentiated unmineralized tissue, less dense bone, calcified cartilage, and dense or cortical bone. This technique provided further evidence of LIPUS influencing the fracture cascade, including enhanced bone formation in the fracture gap and enhanced resorption of the 'old' cortical bone. Using histology, this study also showed that the number of osteoclasts was significantly increased in the LIPUS-treated fractures compared with controls, which suggests that LIPUS can influence the remodeling phase of fracture repair. The stimulation of osteoclastic action has been hypothesized as resulting from increased concentrations of ATP released by LIPUS-treated osteoblasts into culture medium which was associated with increased Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) and decreased osteoprotegerin expression [27].

These data suggest, along with other studies [25,26,28], that the most significant effect of LIPUS on fracture repair is on endochondral ossification, the process of converting soft, cartilaginous callus to hard, mineralized callus, increasing the mechanical stability of the healing fracture. For an efficient process of endochondral ossification, it is accepted that the fracture site needs to be vascularized similarly to that observed in growing bone [29].

2.2. Processes in accelerating endochondral remodeling by LIPUS

As seen in the preclinical models of fracture repair, LIPUS enhances the process of endochondral ossification. One of the key processes in endochondral remodeling is the increase in blood vessels invading the fracture site. LIPUS has been shown to increase key growth factors involved in the process of angiogenesis, the formation of new blood vessels. Vascular endothelial growth factor (VEGF) is a growth factor that causes the formation of new blood vessels [30]. An in vivo study investigated the effect of LIPUS in a type 1 diabetes mellitus (DM) femoral fracture rat model [31]. The number of blood vessels invading the fracture site was assessed by measuring PECAM 1 levels. In the DM group the density of blood vessels at the fracture site was significantly reduced compared with normal control animals ($p = 0.004$). With the addition of LIPUS, the DM animals had a significantly increased blood vessel density compared with DM animals that had not received LIPUS ($p = 0.017$), representing a 77.6% increase. This effect was linked to significant increases in VEGF production in LIPUS-treated fractures ($p = 0.02$).

The impact of LIPUS on the process of endochondral ossification was clearly demonstrated in a mid-diaphysis femur fracture model in aged mice [32]. At day 21 post-fracture in 8 week old mice, the process of endochondral ossification was mostly complete. However, in aged, 40 week old mice, the fracture gap was still present at 21 days post-fracture and the tissue that remained was cartilaginous. The impact of age on the fracture repair process was diminished when the animals were treated with LIPUS. In the 40 week old mice, radiographs showed that the fracture callus after 21 days of LIPUS treatment was fully mineralized, leading to greater stability of the fracture. The onset of endochondral ossification occurred at the same time in the aged animals, but the overall process was accelerated. In a parallel study of aged (1 year old) mice with bilateral femur factures, X-rays and histology showed a similar phenomenon. The mice were treated for 20 minutes per day with an active or placebo ultrasound transducer. The period of endochondral ossification in control aged mice was considerably longer compared to the young mice, approximately 18 days compared to 7 days over the 28 day assessment period, with LIPUS shortening the endochondral ossification in aged mice to approximately 11 days [33].

2.3. Osteogenic response of bone cells

In the process of endochondral ossification, the soft cartilaginous or fibrous callus is converted to a calcified hard callus. In order to complete this process, the cells in the local environment produce factors that are associated with this conversion, such as type X collagen, alkaline phosphatase and osteocalcin [34].

Two papers have demonstrated the enhanced stimulation of osteogenic cells by LIPUS to drive endochondral ossification [35,36]. These two independent research teams both took human osteogenic cells and exposed them to LIPUS in vitro. In both sets of experiments LIPUS was applied through the bottom of the culture plates for 20 minutes daily at 37 °C and therefore the cells

experienced a near field exposure. The same ultrasound transducer that is used clinically, described previously, was used in both sets of experiments. Periosteal cells [35] and isolated cells from fractures in the early phase of fracture repair [36] were used. The results from these two studies were remarkably similar, with alkaline phosphatase levels stimulated by LIPUS increasing with time in culture over controls. In the case of the human periosteal cells [35], 20 minutes exposure of LIPUS over 4 days resulted in a significant difference in alkaline phosphatase over controls of around 10% ($p = 0.01$) and 30% ($p = 0.03$) on days 2 and 4 respectively. With progenitor cells isolated from the fracture hematoma [36], LIPUS was applied daily for 20 minutes over 28 days. Alkaline phosphatase levels were significantly higher after 2, 4, 7, and 14 days of LIPUS exposure ($p < 0.05$) over controls, showing a 20% increase at day 2 through to, 40% on day 14; the same was true with regard to osteocalcin secretion. Two master control genes of the osteogenic lineage, specifically cbfa-1/RUNX2 and osterix, were significantly upregulated compared to controls ($p < 0.05$). The increase in alkaline phosphatase levels, together with osteocalcin secretion and expression of other osteoblast related genes demonstrated that LIPUS promoted osteogenic differentiation of the progenitor cells. The net result of this activity was to increase calcium deposition in the cultures of cells as seen by alizarin red staining. These two studies show strikingly comparable results demonstrating osteogenic differentiation in human cells in response to LIPUS. These results also correspond to what is seen in animal models and other pre-clinical studies, indicating that the responses initiated in the human is similar to that seen in the animal. Increases in bone specific genes was also observed in vivo following the extraction of mRNA from the fracture callus of aged mice at 7 days post fracture [33]. LIPUS treatment led to increases in bone morphogenic proteins BMP-2, BMP-4 and BMP-6 of around 8, 10 and 5-fold higher respectively over controls. This enhanced expression of BMPs in response to LIPUS has also been reported in a study of ROS 17/2.8 osteoblastic cells stimulated in 6 well tissue culture plates [37]. The ultrasound stimulation configuration differed from previous in vitro studies, in that the cells were stimulated from above with the transducer touching the surface of the medium, with the cells 3–4 mm away from the surface of the transducer. Although the cells were stimulated from above, they were still within the near ultrasound field. The cells were stimulated with ultrasound 20 minutes per day for up to 7 days. After 7 days stimulation in culture there were clear increases in protein levels for BMPs 2, 4 and 7 in the LIPUS treated cells versus control cultures, as determined by Western blot analysis.

Another role of enhanced vascularity stimulated by the production of angiogenic growth factors is in recruiting osteogenic progenitor cells to the fracture site from the systemic circulation. To evaluate this effect, parabiotic mice were produced by surgically joining the circulatory systems of two mice, one of which constitutively expressed green fluorescent protein (GFP) in all cells and tissues and a syngeneic wild-type mouse [38]. Femoral fractures were then created in the wild type (non-GFP) animals and treated with LIPUS or a sham device for 20 minutes per day for up to 4 weeks. Any GFP-positive cells in the callus of a wild-type animal would, by definition, be derived from circulating progenitor cells. At two and four weeks, animals were euthanized and the number and localization of GFP-positive cells were assessed. The hard callus area was significantly greater in the LIPUS group at 2 and 4 weeks post fracture ($p < 0.05$), as reported in other studies [25,26,28]. In addition, a significant increase in alkaline phosphatase positive cells in the fractures treated with LIPUS at 2 weeks post fracture was observed ($p < 0.05$), which complements the increased mRNA expression of alkaline phosphatase reported in other studies [39]. Fractures treated with LIPUS had significantly more GFP cells, from the adjacent animals as compared to control fractures, as assessed by histomorphometry, suggesting that LIPUS increased the homing of the progenitor cells to the fracture site. The authors investigated the mechanism for the enhanced recruitment of osteogenic progenitors. Confocal micrographs of the fracture site at 2 weeks showed many CXCR4 positive cells in the LIPUS treated fractures that were virtually absent in control fractures. CXCR4 is expressed on the surface of cells and is the receptor for stromal derived factor 1 (SDF-1 or CXCL12) which plays a key role in the recruiting of circulating cells to sites of repair [40,41]. In a separate study [42] the migration of transplanted mesenchymal stem cells (MSCs) to the fracture site was investigated by fluorescent imaging. MSCs were isolated from a rat femur and stimulated with LIPUS for 20 minutes per day for 3 days. The cells were subsequently injected into rats with a closed femoral fracture. This study demonstrated that LIPUS promoted MSC migration to the fracture site, which was associated with an increase of local and serum SDF-1 level. The in vitro analysis showed that LIPUS upregulated SDF-1 and CXCR4 expressions in MSCs. MSCs migration was promoted by LIPUS. Therefore, two studies independently concluded that LIPUS is having an effect of increasing the recruitment of cells to the fracture site [38,42].

3. Mechanism of action of LIPUS in fracture repair

3.1. Process of LIPUS stimulation

As described earlier the general manner of how LIPUS enhances fracture repair can be explained by the mode of action. The mode is the expression of molecules and actions that need to occur in order to achieve fracture repair, as outlined above. However, to truly understand the process of how LIPUS influences fracture repair we must understand the mechanism of action, which is the description of the events after the signal leaves the transducer, enters the tissue and initiates key events that lead to fracture repair.

Work performed on human cadaveric specimens has demonstrated that the LIPUS signal can produce motion at the fracture site [43]. An osteotomy was performed on the radius of the cadaver, LIPUS applied, and the motion measured using a laser interferometer. Motion was measured at the proximal and distal edges of the bone in the osteotomy, while the LIPUS device was activated (Fig. 1). From these experiments it was found that motion occurred at a frequency of 1 kHz, matching the pulse modulation of the LIPUS signal. The interferometer measured velocity amplitude. Velocity amplitude measurements can be converted to displacement amplitudes using the following equation:

$$v = A\cos\omega t = A\cos[2\pi ft]$$

$$s = \int v \cdot dt = \int A\sin[2\pi ft] = \frac{A}{2\pi f}\sin[2\pi ft]$$

where A = velocity amplitude, s = displacement amplitude, f = measurement frequency. When calculated, it was found that at a measurement frequency of 1 kHz, the velocity amplitude of the bone ends and soft tissue ranged from 1 μm s^{-1} to 3.5 μm s^{-1}, equating to 0.16–0.56 nm displacement, respectively. It is understood that bone responds to mechanical stimulations and the displacement is referred to as micromotion. The normal levels of displacement associated with micro-motion are accepted to be in the range of 0.5–2 mm. Therefore the displacements caused by LIPUS are around 1000 times less than these values, which would suggest that LIPUS has a different effect on bone compared to micro-motion. Micromotion has an anabolic effect on bone mass [44], whereas LIPUS does not. This difference was demonstrated clinically when patients suffering from osteoporosis had their distal radius treated daily with LIPUS for 3 months, and evaluated 3 months after discontinuing the treatment [45]. The results showed that the rate of bone

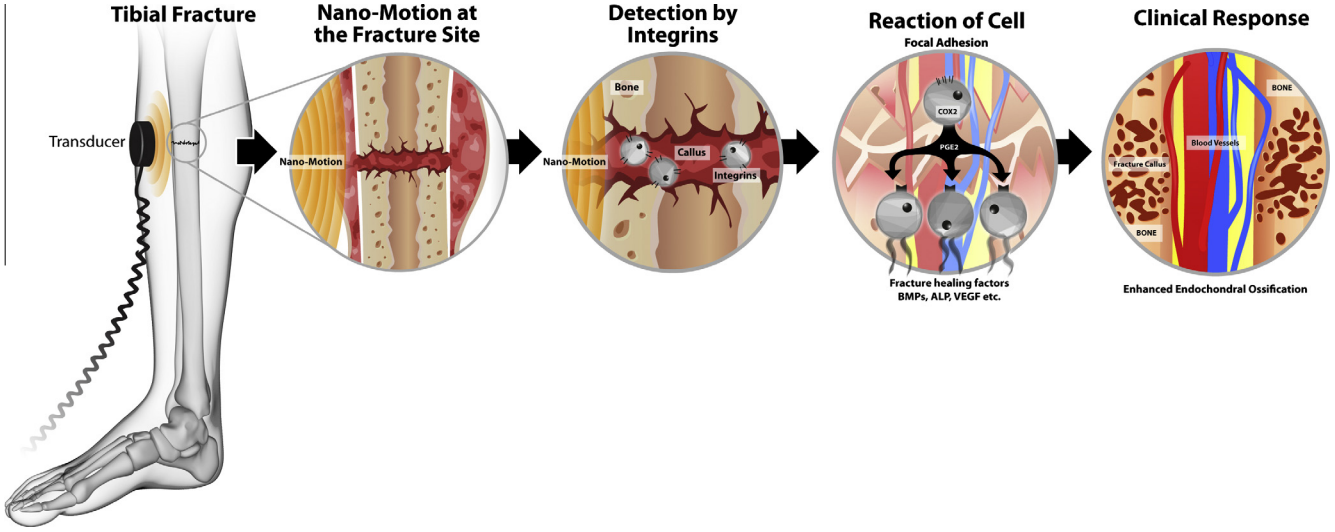

Fig. 1. The transducer is placed on the skin above the fracture and the low intensity pulsed ultrasound produces nano-motion at the fracture site. The nano-motion is detected by integrins and the biomechanical wave is converted into a biochemical wave in the cell. One of the main actions in the cell is the production of cyclo-oxygenase 2 or COX2. The enzymatic action of COX2 is the rate limiting step in the production of prostaglandin E2 or PGE2, which is released from the cells and interacts with surrounding cells through their EP receptors. This action enhances the process of endochondral ossification, which is key for the clinical response.

change (trabecular bone mineral density and integral bone mineral density) did not significantly differ between the site treated with LIPUS and the contralateral control at either 3 months or 6 months ($p = 0.954$ and $p = 0.410$ respectively), suggesting that micro-motion and nano-motion have different effects.

The EXOGEN device, which produces low intensity pulsed ultrasound, was approved by the US Food and Drug Administration in 1994 to accelerate the healing of certain fresh fractures based on the intensity of 30 mW/cm^2 SATA. This intensity of 30 mW/cm^2 has been used for all the clinical studies and the majority of pre-clinical studies when LIPUS has been used. Studies have been performed that have reduced the intensity to see if a threshold could be detected, below which no biological effect occurred. Rat bone marrow stromal cells (rBMSC) were recovered from the femurs of adult animals and cultured in 12 well plates. The culture plates were placed on an ultrasound transducer array, with the LIPUS coming from below. The in vitro cell cultures were treated with ultrasound intensities of 2, 15, and 30 mW/cm^2. Alkaline phosphatase activity was measured in cells that were treated for 20 minutes per day for 3, 5, and 7 days and mineral incorporation over a 24 day period. Interestingly, all intensities, even as low as 2 mW/cm^2, showed increases in alkaline phosphatase activity and mineralization over the sham control [46]. These data indicate that it is important for the cells to receive the LIPUS signal, rather than a specific intensity. The analogy would be similar to a person speaking in a conversation; you can either whisper the message or shout the message, but as long as the recipient receives the message the volume is irrelevant. This in vitro conclusion is supported by an in vivo fracture repair study [47]. Using the closed rat femoral fracture model, the effect of fracture healing of LIPUS treatment at 30 mW/cm^2 (approved clinically) and 150 mW/cm^2 SATA was evaluated by weekly radiographs, micro CT, and biomechanically. The study concluded that LIPUS at I_{SATA} of 150 mW/cm^2 did not further enhance fracture healing over that of 30 mW/cm^2 and was closer to that of control values compared to that of 30 mW/cm^2.

3.2. Conversion of mechanical signal into biochemical signaling

For the ultrasound to have a biological effect, the mechanical wave must be converted to a biochemical signal within the cell. The mechano-receptors, integrins, have been implicated in this process. Independent groups have reported activation of integrin associated signaling pathways through the observation of focal adhesions forming on the surface of cells treated with LIPUS [48,49]. Focal adhesions are conglomerations of integrin molecules which initiate intra-cellular proteins to translocate to inside of the cell where the intra-cellular components of integrins are found. There are many components of the intracellular portion of the focal adhesion that are able to initiate a cell signaling cascade. One of the key focal adhesion proteins implicated with the transduction of the LIPUS signal from a mechanical force to a biochemical signal is focal adhesion kinase (FAK), which is phosphorylated in response to LIPUS. Several groups have also shown a link between the stimulation of integrins and extracellular signal-regulated kinase (ERK) signaling in the transmission of LIPUS, which is considered downstream of FAK [49,50].

3.3. Downstream effect of biochemical signaling: COX2 in the mechanism of LIPUS induced fracture repair

From the best available evidence, it is strongly suggested that a key molecule stimulated by LIPUS through the pathways described above is cyclo-oxygenase 2 (COX2) [51]. COX2 is the rate-limiting enzyme in the production of prostaglandin E2 (PGE2). It has also been demonstrated that LIPUS stimulates prostaglandin H synthase 2 (PGHS-2) expression and PGE2 synthesis [52]. The inhibition of COX2 by non-steroidal anti-inflammatory drugs (NSAIDs) has been strongly implicated in impaired fracture repair in both clinical and pre-clinical studies [53,54]. It is also reported that older mice produce less COX2, leading to reduced capacity of fracture repair [4]. It is therefore unsurprising that a number of publications have shown that COX2 is a key molecule stimulated in cells after the application of LIPUS [50,55]. The secretion of PGE2 was significantly upregulated over 24 h after a single 20 min application of LIPUS [50,55]. One study [50] has linked all aspects of the signaling described above and associated this to the enhanced mineralization caused by LIPUS. This work demonstrated that LIPUS was able to increase the levels of integrin expressed on the cell surface (α2, α5, β1 and β5 sub-units). They also showed that LIPUS caused the phosphorylation of FAK, which led to the phosphorylation of the p85 sub-unit of phospho-inositol 3 kinase (PI3K), then the phosphorylation of AKT, and finally the translocation of the

p65 subunit of NF-κβ to the nucleus. At each stage of this pathway, when inhibitors were used, the enhanced COX2 production was ablated, strongly suggesting that this intra-cellular pathway is stimulated by LIPUS to initiate the production of COX2. These results were corroborated using a COX2 luciferase assay which, after the treatment with LIPUS, significantly increased this activity compared to non-treated controls. When these LIPUS stimulated cells were treated with either chemical pathway inhibitors or dominant negative mutant forms of FAK, PI3K, AKT or ERK, the activity of the COX2 was significantly reduced. Finally, it was demonstrated that, via this pathway, LIPUS could enhance the mineralization of osteoblastic cells. This enhanced mineralization stimulated by LIPUS could be significantly ablated when the cultures were treated with dominant-negative mutant forms of FAK, PI3K or AKT. However, the most compelling evidence that the stimulation by LIPUS through this pathway activates COX2 was shown when these cultures were treated with NS-398, a COX2 inhibitor. Again the enhanced mineralization caused by LIPUS was significantly reduced. This evidence strongly implicates the LIPUS signal is transduced through these specific pathways in the cell, which leads to the production of COX2 and drives mineralization in stimulated osteoblasts.

In vitro cell biology experiments, especially in cells of established cell lines, can only explain so much, and the mechanism of COX2 activation was verified in an in vivo model. In femur fractures of standard aged (one year old) wild type mice, stimulation with LIPUS showed an enhanced healing response compared to untreated contralateral controls, as previously discussed. However, in animals where COX2 had been knocked out, fracture healing via histology was undistinguishable compared to controls. These results demonstrate that the production of COX2 is essential for the induction of gene transcription for both osteogenesis and remodeling, and thereby for the enhanced fracture repair stimulated by LIPUS [33]. In a study of alveolar bone defect as a result of molar tooth removal in rats, involvement of humoral PGE2, which was released from the site of LIPUS stimulation via upregulated COX2, was suggested in the de novo expression of CXCR4 in the remote bone marrow in tibia [56].

COX2 is associated with the initiation of inflammatory responses by producing prostaglandins. PGE2 stimulates osteoclast formation by increasing RANKL mRNA expression via cAMP-protein kinase A (PKA) pathways in osteoblasts. Therefore, a timely COX2 induction by LIPUS inevitably accelerates callus remodeling from cartilage and woven bone into lamellar bone to restore the cortical bone. The activation of resorbing cells (osteoclast and chondroclast) is supported by the increased RANKL expression, both at the protein and mRNA levels in established cell lines and osteoblastic cells [57,58]. Inflammatory conditions may activate multiple pathways in inflammasome preparation as a first step priming through toll-like receptors and TNF or IL-1β receptors that also activate NF-kB [59].

4. Summary

LIPUS waves cause nanomotion at the fracture site. The mechanical signal is converted to a biochemical signal inside the cells and is ultimately transmitted through signaling molecules to drive the production of COX2 in the cell. This cascade requires the involvement of integrins and the formation of focal adhesions. The enhanced COX2 stimulation, likely through the production of PGE2, drives the expression of osteogenic genes. These osteogenic

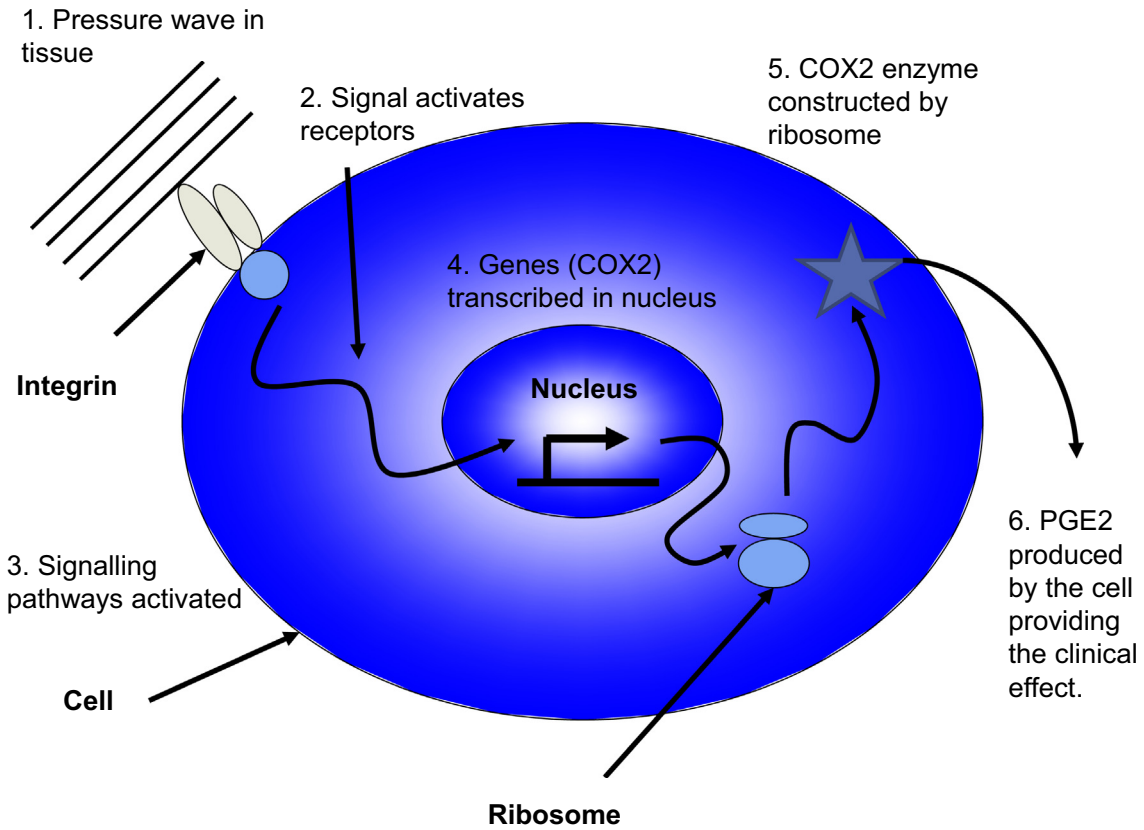

Fig. 2. As the pressure wave comes through the tissues it interacts with cells and is detected by integrins. Multiple pathways are initiated in the cell which leads to the transcription of genes in the nucleus, the main activation is in the transcription of COX2. The mRNA of COX2 is converted to protein that then produces the eicosanoid prostaglandin E2 which is released from the cells to have the clinical effects.

genes stimulate enhanced mineralization, which is seen as enhanced endochondral ossification that heals the fracture (Fig. 2). This enhanced mineralization caused by LIPUS can be observed in cell culture experiments [35,36,50], in vivo models of fracture repair [25,26,33] and in clinical trial data [60].

5. Conclusion

Many intensities and frequencies have been evaluated, but the greatest number of clinical and pre-clinical studies have used a signal comprised of 30 mW/cm^2 I_{SATA}, an ultrasound frequency of 1.5 MHz and pulsed at 1 kHz. It is this signal that was used in the level-I clinical studies demonstrating acceleration of fracture repair in diaphyseal tibial fractures and distal radial fractures respectively [12,13]. Studies have also demonstrated that this signal is highly efficacious in the treatment of recalcitrant non-union fractures [14,16].

A search of the PubMed database of LIPUS finds over 250 articles. However, most articles generally state that the underlying mechanism of action remains unclear. From the data presented here, we hope to convince the reader that there is a rich literature in the science of LIPUS and that these articles indicate that there is a robust mode and mechanism behind the LIPUS technology.

It is commonly understood that fracture repair can be described as different phases. These phases include inflammatory, intramembranous ossification, chondrogenesis, endochondral ossification and finally remodeling. It is not clear at this time exactly which types of cells are involved in each phase; however it has been demonstrated that LIPUS can have positive effects in all these phases [25].

The central molecule in this proposed mechanism of action is cyclo-oxygenase 2 (COX2), the key enzyme in the production of prostaglandins. Prostaglandins have been shown to play an important role in bone fracture repair [53,54]. This evidence could indicate, therefore, that stimulating COX2 might be a good attribute for fracture repair. Studies have shown a positive effect on COX2 and PGE2 levels in response to LIPUS [50,55]. However, the most compelling evidence of this was the near total lack of efficacy of LIPUS in the aged COX2 knockout mouse, which strongly implies that COX2 sits centrally in the mechanism of action of LIPUS [33].

In conclusion, we have set out in this paper to demonstrate that within the scientific literature there is evidence that suggests a mechanism of action of how LIPUS stimulates fracture repair. We have illustrated how the signal is transmitted through tissue to the fracture site, how the bone at the fracture site is made to move at the nano-meter range, and how cells are able to sense this signal by forming focal adhesions. We have shown how signaling pathways are initiated within the cell and how COX2 is produced leading to increased production of PGE2, which when blocked correlates to a lack of effect of LIPUS in enhanced fracture repair. The LIPUS signal has had a long history of safe and efficacious use and with continuing investigation more information will be generated in how this technology can stimulate the healing of tissues.

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