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Review:

Low-intensity pulsed ultrasound therapy: a potential strategy to stimulate tendon-bone junction healing*

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Abstract: Incorporation of a tendon graft within the bone tunnel represents a challenging clinical problem. Successful anterior cruciate ligament (ACL) reconstruction requires solid healing of the tendon graft in the bone tunnel. Enhancement of graft healing to bone is important to facilitate early aggressive rehabilitation and a rapid return to pre-injury activity levels. No convenient, effective or inexpensive procedures exist to enhance tendon-bone (T-B) healing after surgery. Low-intensity pulsed ultrasound (LIPUS) improves local blood perfusion and angiogenesis, stimulates cartilage maturation, enhances differentiation and proliferation of osteoblasts, and motivates osteogenic differentiation of mesenchymal stem cells (MSCs), and therefore, appears to be a potential non-invasive tool for T-B healing in early stage of rehabilitation of ACL reconstruction. It is conceivable that LIPUS could be used to stimulate T-B tunnel healing in the home, with the aim of accelerating rehabilitation and an earlier return to normal activities in the near future. The purpose of this review is to demonstrate how LIPUS stimulates T-B healing at the cellular and molecular levels, describe studies in animal models, and provide a future direction for research.

Key words: Low-intensity pulsed ultrasound, ACL reconstruction, Ultrasound, Anterior cruciate ligament reconstruction, LIPUS, Tendon-bone interface healing, Fracture healing

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1 Introduction

Grafted tendon healing within the bone tunnel after anterior cruciate ligament (ACL) reconstruction is a complicated, poorly clarified biological process. The osteointegration of tendon grafts used for ACL replacement may be unsatisfactory and may be associated with postoperative anterior-posterior laxity. Tendon-bone (T-B) healing in bone tunnel occurs through bone incorporation into the fibrovascular interface tissue that initially forms between the tendon and bone. Increasing the integrity of the healing of T-B interface has been attempted by adopting a number of different augmentation strategies (Fig. 1) with the use of bone mesenchymal stem cells (MSCs) (Soon *et al*., 2007), calcium phosphate (Mutsuzaki *et al*., 2011), bone marrow or periosteum (Chen, 2009; Karaoglu *et al*., 2009), bone morphogenetic protein-2 (Hashimoto *et al*., 2007), synovial MSCs (Ju *et al*., 2008), injectable tricalcium phosphate (Huangfu and Zhao, 2007), brushite calcium phosphate cement (Wen *et al*., 2009), transforming growth factor-β3 (Kovacevic *et al*., 2011), granulocyte colony-stimulating factor (Sasaki *et al*., 2008), hyperbaric oxygen treatment (Young and Dyson, 1990), magnesium-based bone adhesive (Gulotta *et al*., 2008), and shock-wave therapy (Wang *et al*., 2011). However, shock-wave therapy is the only currently reported method of exogenous stimulation to enhance

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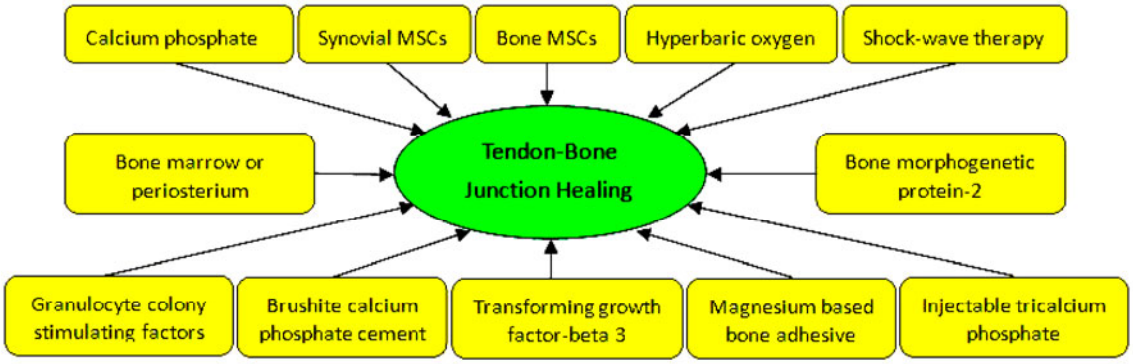

Fig. 1 Various strategies employed for stimulating T-B junction healing

early healing between the grafted tendon and bone tunnel. These augmentation approaches are time-consuming, inconvenient, and expensive, and a profound knowledge is required for the surgery. Furthermore, some of these approaches are still at the laboratory research stage, and have not been implemented in routine clinical practice. Low-intensity pulsed ultrasound (LIPUS), with stimulation at 30 mW/cm^2, is an established, widely applied, and Food and the Drug Administration (FDA) approved intervention for enhancing bone healing in fractures and non-unions (El-Mowafi and Mohsen, 2005; Angle *et al*., 2011). LIPUS is a nondestructive modality in which mechanical energy is transmitted transcutaneously as high-frequency acoustical pressure waves into biological tissues (Qin *et al*., 2006a). Absorption of the ultrasound energy depends on the attenuation and absorption of the tissue as the ultrasound waves pass through. LIPUS provides an immediate mechanical stimulation for osteoblast proliferation, endochondral ossification, and enhancement of mineralization in vitro (Doan *et al*., 1999; Reher *et al*., 2002; Leung *et al*., 2004). Better T-B renovation at the interface has been found experimentally due to progressive ingrowths of collagen fibers, mineralization, and maturation of the healing tissue at the T-B reattachment site (Rodeo *et al*., 1993; Qin *et al*., 1999; Leung *et al*., 2002). Accordingly, we conclude that LIPUS is not only capable of enhancing osteogenesis and maturation of tendon scar tissue, but also of restoring the fibro cartilage zone at the tendon graft-bone junction after ACL reconstruction.

2 Overview of T-B healing

The T-B junction has been described in humans and many animal models as healing between tendon and bone (Rodeo *et al*., 1993; Clark and Stechschulte, 1998; Demirag *et al*., 2005; Kanazawa *et al*., 2006). The T-B interface has been divided into direct and indirect categories. The direct interface is composed of tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone; the indirect interface consists of tendon, Sharpey's fibers, and bone. Several studies have investigated T-B histology in human patients after revisional ACL surgery (Petersen and Laprell, 2000; Robert *et al*., 2003). Fracture healing progress is generally divided into inflammatory, soft callus, hard callus, and remodeling stages. At the T-B junction, the bone tunnel serves as the "fresh fracture" site, and the repair process resembles the first three of four typical bone healing steps, including an initial inflammatory stage, a stage of newly formed bone with regenerated fibrocartilage zone-like structure, and a stage characterized by woven bone remodeling and maturation of the fibrocartilaginous junction (Wong *et al*., 2003). The reparative phase begins within 4–5 d after the fracture. Pluripotent MSCs invade the area and differentiate into fibroblasts, chondroblasts, and osteoblasts. These cells are responsible for the formation of a soft fracture callus and subsequently of woven bone penetrating into the graft. Many factors, both local and systemic, have been associated with the process of T-B healing.

3 Bioeffects of LIPUS

3.1 Angiogenesis

Sufficient vascular invasion is a fundamental prerequisite for endochondral bone formation, fracture healing, and soft tissue repair (Bolander, 1992). LIPUS increases local blood circulation and stimulates angiogenesis both in vivo and in vitro. Therapeutic ultrasound, at an intensity of 0.1 W/cm^2 special average temporal average (SATA), directly stimulates the formation of new blood vessels in full-thickness excised lesions in the flank skin of adult rats (Young and Dyson, 1990). By 5 d after injury, there were more blood vessels in equivalent areas of the granulation tissue of the ultrasound-treated wounds compared with the control ones. It is reported that therapeutic ultrasound can significantly stimulate the production of angiogenesis-related cytokines such as interleukin-8, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). Both ultrasound machines produced significant results: the best intensities were 15 and 30 mW/cm^2 SATA with 45 kHz ultrasound, and 0.1 and 0.4 W/cm^2 SATA with 1 MHz ultrasound (Reher *et al*., 1999). Further studies show that LIPUS significantly elevates VEGF-A expression in human osteoblasts mediated by nitric oxide (NO) and hypoxia-inducible factor-1α (Wang *et al*., 2004). Therefore, LIPUS stimulates healing at the T-B junction by improving tissue perfusion and angiogenesis (Figs. 2a–2d).

3.2 Cartilage maturation

Several investigations have confirmed that LIPUS stimulates osteogenesis during bone growth and repair (Warden *et al*., 2000; Nolte *et al*., 2001; Rubin *et al*., 2001). A significant increase in bone collar volume and percentage of calcified cartilage is found as a result of ultrasound treatment with LIPUS (30 mW/cm^2; 1.5 MHz) for 20 min/d for a period of 3 or 6 d; however, the number of cells does not change significantly. These results indicate that the stimulatory effect of LIPUS on endochondral ossification is due to stimulation of bone cell differentiation and calcified matrix production, but not to change cell proliferation (Korstjens *et al*., 2004). These findings are in agreement with reports on ultrasound stimulation of fracture healing (Shimazaki *et al*., 2000). The hard callus area, the bone mineral density (BMD), and mechanical testing results, were significantly greater in those receiving LIPUS (30 mW/cm^2, 20 min daily) compared with the control group. The bone tunnel in the surgery of ACL reconstruction acts as a fresh fracture site drilling during cruciate ligament reconstruction, may be the most sensitive area for LIPUS treatment to stimulate cartilage maturation.

3.3 Osteoblasts growth

The specific mechanisms by which ultrasound stimulation works on bone cell activities are still unknown. However, in terms of the physical mechanism, ultrasound provides a mechanical force to the

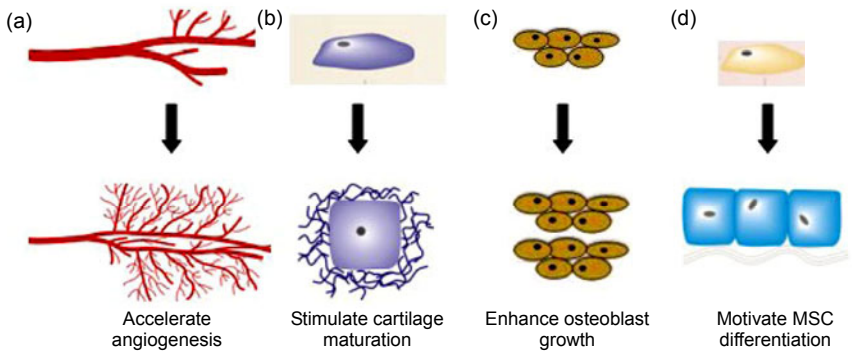

Fig. 2 Bioeffects of the LIPUS enhancing the healing of the T-B interface

(a) Accelerating angiogenesis through stimulating the production of angiogenesis-related cytokines; (b) Stimulating cartilage maturation by bone cell differentiation and calcified matrix production; (c) Enhancing osteoblast growth through nutrient exchange and regulating correlative signal molecules; (d) Motivating MSCs osteogenic differentiation by upregulating chondrogenic differentiation of MSCs

cellular system. It is shown that the acoustic cavitations induced by ultrasound are considered to be strongly dependent on ultrasound frequency (Yoichiro *et al*., 2005). Through the cavitation effect and mechanical perturbation, LIPUS increases cell membrane permeability (Liu *et al*., 2006; Cai *et al*., 2007) either directly by mechanical deformation of the cell membrane, or indirectly by an electrical effect caused by cell deformation (Sun *et al*., 2001). The exact physical mechanisms underpinning cell membrane change are not fully understood. The effects of ultrasound alone or in combination with externally administered microbubbles are likely associated with the generation of shockwaves and microjets (Ohl *et al*., 2006; Juffermans *et al*., 2008) and with acoustic microstreaming (Karshafian *et al*., 2009). Furthermore, LIPUS stimulates the growth of bacteria and physiological cells by increasing the rate of oxygen and nutrient transport to the cells and by increasing the rate of transport of waste of products away from cells (Pitt and Ross, 2003). Another report indicates that ultrasound exposure increases prostaglandin E2 (PGE2) synthesis by upregulating cyclooxygenase-2 messenger RNA (mRNA) in osteoblasts (Kokubu *et al*., 1999), where PGE2 is a potent inflammation mediator and is also involved in bone remodeling. It is reasonable that LIPUS also has the potential to boost osteoblast permeability, promote the transportation of oxygen and nutrients into osteoblasts, and enhance osteoblast growth.

3.4 MSC osteogenic differentiation

MSCs are multipotent stem cells that differentiate into a variety of cell types including osteoblasts, chondrocytes, and adipocytes. However, the degree to which the culture will differentiate varies among individuals and according to how differentiation is induced, e.g., chemically vs. mechanically, or physically. Lim *et al*. (2004) reported that coating hamstring tendon grafts with MSCs resulted in healing through an intervening zone of cartilage resembling the chondral enthesis of normal ACL insertions, rather than collagen fibers and scar tissue. It has been reported that LIPUS could provide a promising micromechanical signal for MSC chondrogenesis in vivo and it strongly indicates that LIPUS preconditioning in vitro could be an effective cue to upregulate chondrogenic differentiation of MSCs in vivo (Cui *et al*., 2007). The LIUS treatment of MSCs increased matrix formation, chondrogenic markers' expression such as collagen type II, the expression of tissue inhibitor of metalloprotease-2, and the capacity to maintain the chondrogenic phenotypes in a monolayer culture (Lee *et al*., 2006). Therefore, it can be concluded that LIPUS increases MSC osteogenic differentiation, enhances viability, and ultimately accelerates remodeling of T-B healing.

4 Physical mechanisms of LIPUS

LIPUS is recommended for a daily application of about 20 to 30 min for acceleration of fracture healing, treatment of delayed or nonunion and bone lengthening (Busse *et al*., 2009). The potential biological actions of ultrasound result from two major mmechanisms: thermal effect and mechanical effect (non-thermal).

4.1 Thermal effect

When ultrasonic waves propagate in the body, ultrasonic energy is absorbed at a rate proportional to the density of the tissue. Absorption of the ultrasound signal results in an increase in the temperature of the body tissue (Liu *et al*., 2010). While this thermal effect is rather small for low frequency ultrasonic waves, some enzymes, such as matrix metalloproteinase-1 and collagenase, are exquisitely sensitive to small changes in temperature (Welgus *et al*., 1981). Thermal deactivation is one of the important mechanisms in the denaturation of enzymes.

4.2 Acoustic cavitation

The cavitation phenomenon is the largest nonthermal effect created by ultrasound energy (Doktycz and Suslick, 1990; Prozorov *et al*., 2004). Cavitation is considered to be a main mechanism for causing alterations to biological tissues, especially increased membrane permeability (Sivakumar *et al*., 2005). There are two different types of cavitations: stable cavitation and transient cavitation. The former produces bubbles, which present for a great number of acoustical cycles and the radius of every bubble varies about an equilibrium value. The later forms bubbles which oscillate in an unstable manner about

their equilibrium, expanding to two or three times their resonant size before collapsing violently during a single compression half cycle (Feril and Kondo, 2004). Transient cavitation actions are supposed to be the primary mechanism of damage to intact cells.

4.3 Mass transfer enhancement

Ultrasound enhances the movement of the liquid medium which precipitates mass transfer and reaction rates in both multiphase and homogeneous systems (Bar, 1988). The boundary layer, the membrane and/or cell wall, and the cytosol are the three main areas where this process takes place. It has been accepted that the vibratory gas bubble generated around an acoustic field a circulatory liquid motion referred to as a microstreaming. This fact leads to the flux of reagents to the active site of the enzyme or to the cell and the flux of the reaction products to the medium respectively, ultimately increasing the turnover quantity.

5 In vivo evidence of LIPUS for T-B healing

How to stimulate bone formation and enhance mineralization at the T-B junction is of clinical importance. One of the possible approaches is to use biophysical intervention, such as LIPUS, mechanical stimuli, and electromagnetic fields. These modalities serve to accelerate healing of bone fractures and soft tissue. LIPUS exerts its effect on fracture healing (Einhorn, 1995; Hadjiargyrou *et al*., 1998) by mechanical signal transduction and the induction of gene expression, the activation of enzymes in response to heat energy, increased vascularity at the fracture site, modulation of intracellular calcium signaling, and enhanced cartilage calcification and maturation. Most studies to date have investigated the effect of LIPUS on T-B healing using the standard partial patellectomy rabbit model, but very few studies have used the rabbit model of ACL reconstruction (Table 1). Furthermore, there is no clinical evidence evaluating the effect of LIPUS on T-B healing. However, all of the animal researches to date have showed that LIPUS significantly enhances T-B healing at different early stages, compared with results in a control group (Lu *et al*., 2006; Qin *et al*., 2006a; 2006b; Walsh *et al*., 2007; Lu *et al*., 2008; Lu C.C. *et al*., 2009; Lu M.H. *et al*., 2009; Lovric *et al*., 2012).

6 Limitations of the current study and future perspectives

ACL reconstruction using semitendinosus and gracilis tendons has become popular in recent years. However, failure to incorporate the biological graft into the bone tunnel continues to occur (George *et al*., 2006). ACL reconstruction requires that the tendon grafts heal in a surgically created bone tunnel. LIPUS, unlike shock waves that may be detrimental to capillaries and the direct current that may cause a skin reaction if planned inappropriately (Chung and Wiley, 2002), shows no adverse effects and good patient compliance (Yan *et al*., 2011). If clinical trial results show a benefit for stimulating the T-B healing, patients could receive physical therapy at home with a portable LIPUS. Moreover, it is rational to extrapolate the LIPUS application to other T-B junction repairs, such as a patellar tendon-patella complex rupture in a comminuted fracture of the patella, Achilles tendon rupture and rotator cuff injuries.

Researches, including in vitro and in vivo have shown encouraging results, with LIPUS able to promote healing at the interface of T-B. The effect on the bone-tendon junction, however, may be primarily on bone. However, current animal model studies do not reproduce the complex intra-articular environment that occurs during the ACL reconstruction in humans, due to the presence of synovial fluid. Furthermore the optimal LIPUS treatment modality for patients undergoing ACL reconstruction surgery is still to be determined. Future studies are not only needed to establish the indications for applying LIPUS but also to identify the effects and appropriate duration of LIPUS in animal model experiments and clinical trials. Adequately high quality evidence in human studies with standardization of intensities and dosages of LIPUS for the T-B junction are needed. It is conceivable that LIPUS could be used to stimulate T-B tunnel healing at home, with the aim of accelerating rehabilitation and an earlier return to normal life.

Table 1 In vivo studies evaluating the effectiveness of LIPUS for T-B junction healing

Study	Animal model	Treatment	Outcome
Lovric *et al*. (2012)	Wethers (the infraspinatus tendon was repaired with a transosseous-equivalent suture-bridge construct using medial row and lateral push-in suture anchors)	20 min/d for 28 d	Histology: generally a thicker region of newly formed woven bone, morphologically resembling trabecular bone, with increased osteoblast activity along the bone surface, was noted at the T-B interface in the LIPUS-treated group compared to the controls Immunohistochemistry: expression patterns of VEGF and RUNX2 both showed a significant difference between the control and the LIPUS-treated groups
Lu C.C. *et al*. (2009)	Rabbits (the extensor digitorum longus tendons of rabbits were transplanted into bone tunnels in both proximal tibias)	20 min/d for 12 weeks	Biomechanical test: at two weeks postoperatively, the mean maximal tensile strength of the LIPUS group was significantly higher than that in the control group Histological findings: at 12 weeks the T-B interface presented a transition zone of new bone formation from bone to mineralized cartilage and non-mineralized fibrocartilage in the LIPUS group
Lu M.H. *et al*. (2009)	Rabbits (established transverse partial patellectomy was performed in rabbits)	20 min/d for 6 weeks	New bone size measured on radiographs: the size of radiographic new bone from the remaining patella showed that significantly more new bone was formed in the LIPUS group compared with that in controls at Week 18. BMD measured by pQCT: the LIPUS treatment group showed significantly higher volumetric BMD in the new bone at Week 6 than that did in the controls Stiffness measured by ultrasound water-jet indentation system: the stiffness of patellar cartilage of LIPUS group was found to be significantly higher than that in controls at postoperative Week 6
Lu *et al*. (2008)	Rabbits (standard partial patellectomy was conducted in rabbits)	20 min/d for 16 weeks	VEGF expression: at Week 4, chondrocytes and osteoblasts expressed significantly more VEGF in the LIPUS group than that in the control group Cartilage formation: an LIPUS treatment group showed significantly thicker fibrocartilage zone when compared with that in the control group at Week 16
Walsh *et al*. (2007)	Sheeps (single digital extensor tendon autografts from the right hoof of the sheeps were transplanted into both tibial and femoral bone tunnels)	20 min/d for 3, 6, and 12 weeks	Mechanical testing: LIPUS treatment resulted in a significantly greater peak load and stiffer compared with the controls at Week 26 Histology: evidence of new bone at the interface in the LIPUS-treated group at Week 26 revealed significant differences compared with that in controls
Lu *et al*. (2006)	Rabbits (standard partial patellectomy was conducted in rabbits)	20 min/d for 2, 4, 8, and 16 weeks	Biomechanical testing: LIPUS significantly improved the tensile mechanical properties of the T-B junction compared with that in the control group Histologic analysis: a fluorescence microscopic evaluation revealed earlier and increased newly formed bone at Weeks 8 and 16 in LIPUS treatment specimens compared with that in the control group New bone formation: significantly more newly formed bone was found in the LIPUS group when compared with that in the controls at both Weeks 8 and 16
Qin *et al*. (2006a)	Rabbits (standard partial patellectomy was conducted in rabbits)	20 min/d for 8 and 16 weeks	New bone area measured on radiographs: the LIPUS group induced significantly more new bone formation when compared with the control at both Weeks 8 and 16 Vickers hardness obtained from micro-indentation: compared with the control group, the Vickers hardness of the newly regenerated fibrocartilage zone, healing tendon, and cartilaginous metaplasia in the LIPUS group was found to be significantly higher at Week 16
Qin *et al*. (2006b)	Rabbits (standard partial patellectomy was conducted in rabbits)	20 min/d for 8 and 16 weeks	New bone size measured on radiographs: significant more new bone was formed in the LIPUS group compared with non-treated controls both at Weeks 8 and 16 BMD measured by pQCT: LIPUS treatment group showed significantly higher volumetric BMD in new bone at Week 8 compared with the control group Descriptive histology: fluorescence microscopic observations revealed more xylenol orange labeling compared with calcein green labeling at Week 8 LIPUS-treated sample compared with the control sample

VEGF: vascular endothelial growth factor; RUNX2: runt-related transcription factor 2; BMD: bone mineral density; pQCT: peripheral quantitative computed tomography systems

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