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● *Original Contribution*

EFFECT OF TRANSOSSEOUS APPLICATION OF LOW-INTENSITY ULTRASOUND AT THE TENDON GRAFT-BONE INTERFACE HEALING: GENE EXPRESSION AND HISTOLOGICAL ANALYSIS IN RABBITS

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Abstract—The present study investigates the effect of transosseous low-intensity pulsed ultrasound (LiUS) on the healing at tendon graft-bone interface, in molecular and histological level. The anterior cruciate ligament (ACL) in both knees of 52 New Zealand White rabbits was excised and replaced with the long digital extensor. A custom-made ultrasound transducer was implanted onto the medial tibial condyle, adjacent to the surface of the bone tunnel at both knees of the rabbits. The LiUS-treated right knees received 200-μs bursts of 1 MHz sine waves at a pulse repetition rate of 1 kHz and with 30 mW/cm^2 spatial-average temporal-average intensity for 20 min daily (study group), while the left knee received no LiUS (control group). Thirty-six rabbits were used to perform semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis from both study and control groups for transforming growth factor-β1 (TGF-β1), biglycan and collagen I. RT-PCR products showed statistically significant upregulation of biglycan and collagen I gene expression in the study group, while TGF-β1 gene expression exhibited a bimodal profile. Histological examination performed in 16 rabbits from both groups supported the findings of the molecular analysis, indicating a faster healing rate and a more efficient ligamentization process after ultrasound treatment. These findings suggest that transosseous application of LiUS enhances the healing rate of the tendon graft-bone interface, possibly by affecting the expression levels of genes significant for the tendon to bone healing process. (E-mail: malizos@med.uth.gr) **© 2009 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Low-intensity ultrasound (LiUS), Tendon graft-bone interface healing, TGF-β1, Biglycan, Collagen type I, Anterior cruciate ligament (ACL).**

INTRODUCTION

The process of ligamentization (transformation of a tendinous graft into a ligament) includes the histological and structural remodelling of the tendon graft during healing at the tendon-bone interface and thereafter (Amiel et al. 1986; Goradia et al. 2000; Marumo et al. 2005). Joint ligament reconstruction with a tendon graft involves the implantation of the graft ends into a bone tunnel. There is little information documenting the mechanism of healing at the tendon graft-bone interface at molecular level. A number of essential genes are involved in this process and their expression can be regulated through complex biochemical pathways. The biochemical processes leading to healing of a tendon graft to bone remain unclear.

Experimental studies have shown that many key molecules such as growth factors or extracellular matrix proteins play an important role in the remodelling pro-

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cess of the tendon graft (Marui et al. 1997; Lo et al. 1998; Yasuda et al. 2004). Transforming growth factor-β (TGF-β) is a prominent regulator of tendon healing affecting cellular functions and gene expression through the activation of the Smad and non-Smad signalling pathways (Molloy et al. 2003; Moustakas and Heldin 2005; Tsubone et al. 2006). However, the precise role of TGF-β in tendon graft-bone interface healing is unclear due to complex interactions and the presence of multiple cytokines involved in the process. A number of *in vitro* studies have demonstrated that transforming growth factor-β1 (TGF-β1) stimulates fibroblast proliferation and increases proteoglycan and collagen synthesis (DesRosiers et al. 1996; Sakai et al. 2002). Biglycan is among the proteoglycans upregulated by TGF-β *in vitro* (Kahari et al. 1991; Hildebrand et al. 1994). It is a small leucine-rich proteoglycan that is involved in matrix organization and in cell-matrix interactions, together with other proteoglycans. It is considered as an important mediator of growth-factor binding and modulates the biological activities of a variety of growth factors through interaction via its glycosaminoglycan moieties as well as its core protein (Ruoslahti and Yamaguchi 1991; Hildebrand et al. 1994). It has been shown that the expression levels of mRNA of biglycan are increased earlier in ligament healing (Lo et al. 1998). In addition, experimental studies have shown that exogenous administration of TGF-β1 to the tendon graft-bone interface increases mRNA expression of collagen type I (Murphy et al. 1994; Yamazaki et al. 2005). It has been observed that collagen undergoes changes in concentration and biochemical properties during ligamentization process and its synthesis seems to be stimulated by growth factors like TGF-β1 and controlled mechanical stretching (Kim et al. 2002).

Previous *in vitro* studies have demonstrated that exposure to ultrasonic energy stimulates proliferation of fibroblasts and increases their metabolic activity, affecting primarily the synthesis of collagen (Webster et al. 1980; Ramirez et al. 1987). Animal studies and subsequent clinical trials have shown that transcutaneous application of low intensity pulsed ultrasound (LiUS) accelerate fracture healing (Duarte 1983; Heckman et al. 1994; Kristiansen et al. 1997; Rubin et al. 2001). Transcutaneous application of LiUS has also been shown to have a positive effect on the healing process of other tissues, including tendon, ligament and bone-tendon junction healing (Takakura et al. 2002; Ng et al. 2003; Sparrow et al. 2005; Leung et al. 2006; Lu et al. 2006; Qin et al. 2006; Yeung et al. 2006; Walsh et al. 2007). Recently, studies in animal models have proven the efficacy of the transosseous application of LiUS on the enhancement of the bone healing process by utilizing modern implant technologies (Hantes et al. 2004; Protopappas et al. 2005; Malizos et al. 2006). The ultrasound induced mechanical stimulation on the tissues has been demonstrated to have positive effects at all stages of bone healing, through enhancement of angiogenesis, increased fibroblast proliferation and increased collagen, interleukin and vascular endothelial growth factor production. However, the exact mechanisms and the biochemical pathways through which LiUS interact with living tissue remain elusive. In the present study, we probed the hypothesis, that transosseous application of LiUS may enhance the healing rate of tendon graft-bone interface, by affecting the expression levels of important genes that contribute in many metabolic processes. The purpose of this study was to investigate the effect of transosseous LiUS on molecular level during ligamentization process in rabbits, using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). We selected to study the expression level of TGF-β1, biglycan and collagen type I genes because previous reports were supportive of the notion that these factors mediate important biochemical pathways in the complex process of tissue healing (DesRosiers et al. 1996; Sakai et al. 2002; Molloy et al. 2003; Yasuda et al. 2004; Yamazaki et al. 2005). Finally, we performed histological analysis in order to examine any possible correlation between the alteration of gene expression levels at tendon graft-bone interface and earlier tissue reconstruction observed after LiUS application.

MATERIALS AND METHODS

Study model and animal

Fifty-two male New Zealand White rabbits of 3 months old and weighing 3.0 to 3.5 Kg were used in this study. The experimental protocol was approved by the Institutional Review Board of the University Hospital (Animal Care and Use Committee, Faculty of Medicine, University of Thessaly) and the study was performed under the European Union guidelines for the care and use of animals in research. All rabbits underwent a bilateral surgical procedure, which has been based on the technique developed by Wang and colleagues (2005). Animals were anesthetized before surgery via intramuscular injection using a combination of ketamine, midazolam and atropine. Both lower limbs were shaved, scrubbed and prepared with sterile technique. Through a lateral parapatellar incision, the knee joint was opened. The long digital extensor tendon was dissected off distally at the musculotendinous junction while the proximal femoral attachment was left intact. The anterior cruciate ligament (ACL) was excised. The tibial tunnel was created with a graft size-matched drill through the ACL footprint and exited on the anteromedial aspect of the proximal tibia. The distal end of the graft was re-routed intra-articularly, pulled into the tibial tunnel and fixed to

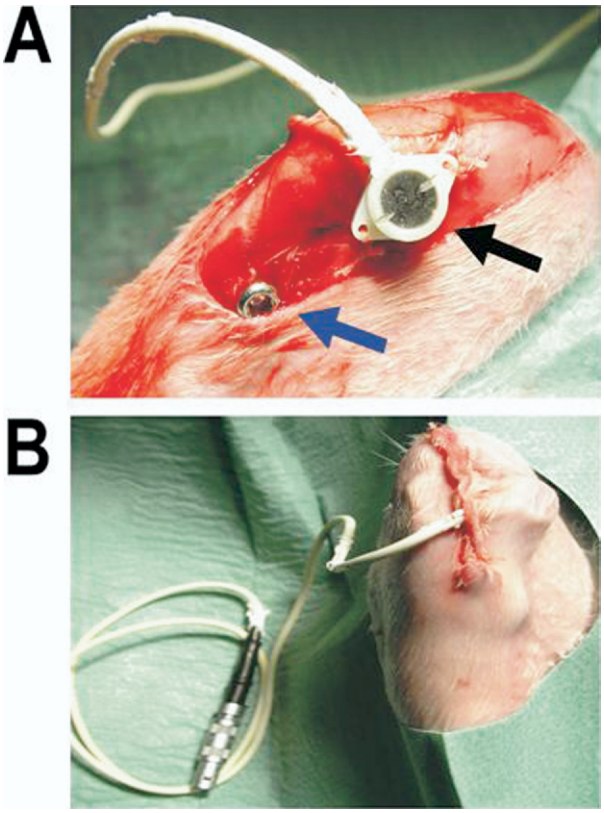

Fig. 1. Ultrasound transducer implantation. (A) Anchoring of distal end of the tendon graft on a screw implanted distally to the bone tunnel (blue arrow). The implanted ultrasound transducer onto the bone is indicated by the black arrow. (B) The external overextension of the transducer cable through the surgical wound, which allows ultrasound propagation.

bone through Ethibond sutures (Ethicon, Inc., Somerville, NJ, USA) anchored on a 10 mm screw implanted at the metaphyseal-diaphyseal junction, with the knee at 30° of flexion. An ultrasound transducer was implanted onto the bone, at the anteromedial surface of the medial tibial condyle adjacent to the bone tunnel, with the use of an absorbable suture (Fig. 1A). The transducer is custom-made disk-shaped and encapsulated in a plastic case (acrylonitrile butadiene styrene-ABS), 6 mm in thickness and 8 mm in diameter (Lead Metaniobate piezoelectric material, custom product, Valpey Fisher Corp., Hopkinton, MA, USA). The surgical wound was irrigated and closed in layers with absorbable sutures (Fig. 1B). Postoperatively, all animals received 0.2 ml enrofloxacin (Baytril, Bayer, Athens, Greece) for 5 d as an antibiotic prophylaxis and analgesic medication for 1 d. Throughout the study, each rabbit was housed individually in a standard rabbit cage and received food and water ad libitum. The general activities of the animals and the local wound condition were inspected daily.

Transosseous application of LiUS (LiUS treatment)

The right knees were exposed to LiUS for 20 min/d, starting from the first day postoperatively until sacrifice and were regarded as the study group. The ultrasound device responsible for generating the LiUS treatment has been previously described (Protopappas et al. 2005; Malizos et al. 2006). The ultrasound transducer (contact-type, acoustic impedance 6 MRayls, longitudinal, central frequency 1 MHz, custom-made, ValpeyFisher, Hopkinton, MA, USA) was encapsulated in a plastic case. The ultrasound signal consisted of 200 μs bursts of 1 MHz sine waves, with a pulse repetition rate of 1 kHz and 30 mW/cm^2 spatial average and temporal average intensity (70 mW/cm^2 spatial-peak temporal-average intensity). The left knees of all rabbits received sham treatment, (transducer transplantation but no application of LiUS) and served as the control group. Immediately after LiUS application, the right knee was examined for local complication. There were no wound infections, cable track infection or other postoperative local or systemic complications.

RNA quantification and primer development

Thirty-six animals were sacrificed with an overdose of sodium pentobarbital at different time intervals with four rabbits in each subset at 1, 2, 5, 7, 9, 12, 14 and 21 d. Tissue samples including tendon grafts in bone tunnel from both knees were immediately dissected and stored at −80°C. Total RNA was extracted according to the manufacturer's instructions using the total RNA isolation kit-Nucleospin RNA II (Macherey-Nagel GmbH & Co. KG, Duren, Germany) from 30 mg of each tissue sample after homogenization in a micro-dismembrator (Ultra Turax, IKA-Werke GmbH & Co. KG, Staufen, Germany). The samples were incubated in the presence of DNase I to avoid any possible DNA contamination and the RNA was eluted in 50 μl of RNase-free water. All the samples were checked for any possible degradation or any detectable DNA contamination on agarose/formaldehyde gels (1.2%) in the presence of MOPS and ethidium bromide. Subsequently, the samples were quantified in a UV spectrophotometer at 260 nm and they were stored at −80°C.

Primer design for the RT-PCR experiments was based on the partial cDNA sequences of the corresponding genes from Oryctolagus cuniculus that were available through GenBank. As an internal control, we used the corresponding sequence for the 28S rRNA gene. All the primer sets, annealing temperatures, number of PCR cycles and expected product size are summarized in Table 1.

Table 1. Primer sequences used for semiquantitative RT-PCR analysis

Gene	Annealing temperature (°C)	Product size (bp)	Primer sequences (5' to 3')	GenBank accession number
TGF-β1	55	268	F-CGGCAGCTGTACATTGACTT R-AGCGCACGATCATGTTGGAC	AF020217
Biglycan	61	406	F-CGGCAGCTGTACATTGACTT R-GGTTGTTGAAGAGGCTGATGCCG	AF020290
Collagen type I	60	480	F-GACTTCTACAGGGCTGACCAGCCTC R-GCAATGCTGTTCTTGCAGTGGTAGG	D49399
28S rRNA	61	510	F-CATTGTGAAGCAGAATTCACCAAGC R-GCTCTGCTACGTACGAAACCCCG	AF460236

Semiquantitative RT-PCR

RT and simultaneous PCR reactions were undertaken with 1 μg total RNA using the Robus T II RT-PCR kit from Finnzymes (Espoo, Finland) according to the manufacturer's instructions. The one step RT-PCR kit reactions were including first an M-MuLV reverse transcriptase cDNA synthesis for 1 h at 48°C, followed by its inactivation for 5 min at 94°C. Subsequently, the DNA polymerase and the set of specific primers that were included in the reaction mixture could amplify the cDNA sequence that is initially produced (30 cycles). Cycle optimization was performed for each primer set and for all reported experiments amplification levels were compared in the linear range of the PCR reaction. All samples underwent the one step RT-PCR at the same time to avoid potential variations in experimental conditions. All reported experiments were in the linear range of the PCR reaction.

The amplified products were separated on 2% agarose gels, stained with ethidium bromide, imaged using a Vilber-Lourmat TP-001-FDC image analyzer (Vilber Lourmat, Eberharzell, Germany GmbH) and integrated density values calculated using ImageQUANT TL 2005 software (Amersham Biosciences, Piscataway, NJ, USA). All reported experiments were in the linear range of the image analysis system. Data from individual transcript levels were normalized to the 28S rRNA that was determined not to vary during preliminary experiments (data not shown) in order to permit semiquantitative comparisons in mRNA expression.

Histological examination

Analysis was carried out on tissue samples obtained from 16 animals after euthanasia at 1, 2 and 3 wk. The samples were fixed for 48 h in phosphate buffered formalin before decalcification in 10% formic acid. Tibial bone tunnels were cut into 5 mm sections for paraffin embedding. This resulted in preparations of serial blocks from precisely defined areas at the tibial tendon-bone tunnel interface. Three micron sections were cut with a Leica Microtome (Leica Microsystems GmbH, Wetzlar, Germany). Serial sections from the tibial tunnels stained with hematoxylin and eosin, with Masson trichrome and with Gordon Sweet reticulin stain. Features assessed were the degree of bone-tendon incorporation, vascularity at the interfaces, orientation of the fibers and overall tendon quality within the tunnel. The histologic samples were initially examined blindly regarding the group of origin (either study or control group) and timing of sampling.

Statistical analysis

After normalization of all RT-PCR data to facilitate equal loading of gels for quantitative comparisons of

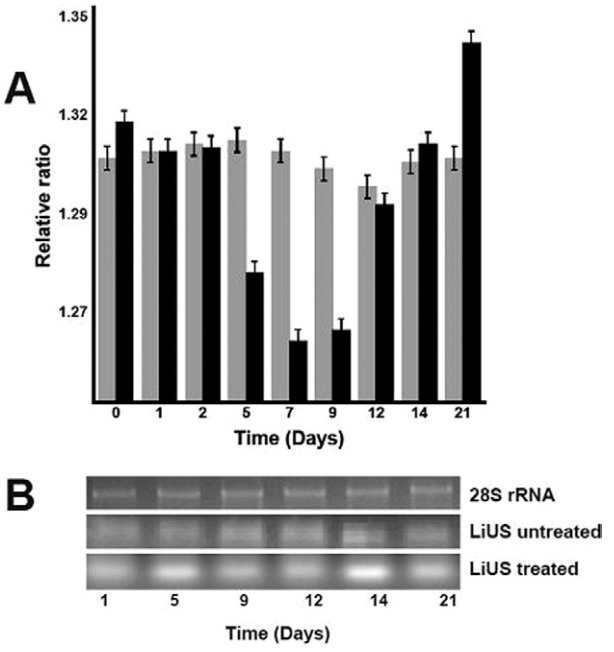

Fig. 2. Changes in mRNA levels of TGF-β1 during the period of treatment (21 days). (A) Average mRNA normalized ratios (relative ratios) from three different samples of the control group (grey bars) and the study group (black bars) are presented. Values are expressed as mean ± standard deviation. (B) Representative results for TGF-β1 (control group and study group) and 28S rRNA using RT-PCR and 1 mg total RNA extracted from each sample. RT-PCR and analysis was performed as described in Materials and Methods.

amplified PCR products, we calculated the gene expression relative ratios between the control group and the study group. Data comparison was evaluated using the paired-samples t-test. Statistical significance was accepted for $p < 0.05$ after statistical analysis that was performed using the SPSS v13.0 package.

RESULTS

The total expression levels of TGF-β1 mRNA by semiquantitative RT-PCR in both study and control group are shown in Fig. 2. All transcript levels were normalized to the constantly expressed 28S rRNA, described in the materials and method section. All the results are presented as the ratio of the integrated density values that were analyzed with the ImageQUANT TL 2005 software of all transcription values between the control group and the study group that received the application of LiUS. From the results presented in Fig. 2, it is evident that TGF-β1 expression levels at the initial phase of the treatment were slightly higher in the study group compared with the control group (~10%). Such elevated levels of TGF-β1 have also been reported before under ultrasound application, especially during the first hours of treatment (Lai and Pittelkow 2007; Harle et al. 2005). After the initial phase of treatment the TGF-β1 levels decline slightly and until day 2 are comparable to those of the control group and they did not vary significantly. Although our initial observations where expected according to what has been previously reported, interestingly, we recorded a significant decrease of TGF-β1 levels after day 5 until day 12. Especially between days 5 and 9, only 40% of the control group expression level can be observed in the study group (days 0-9, $p = 0.001$). After day 12, the initial expression level is restored and increased gradually after day 14. Significantly elevated levels of mRNA (30%) were demonstrated for TGF-β1 in the study group compared with the control group, from day 14 until day 21 (days 12-21, $p = 0.003$). During all the experimental analyses, the samples derived from the control group showed no essential alteration of expression levels for TGF-β1 and they remained relatively stable during the 21-d period.

Figure 3 illustrates the total expression levels of biglycan mRNA in both groups (study and control). It was observed that biglycan mRNA levels increased progressively in both groups. However, it became evident that when we compared the transcription levels for biglycan in the study group, we found them significantly higher compared with the control group (~50% at day 21, $p = 0.003$).

When we compared the mRNA levels in both groups for biglycan and collagen type I, we noticed that the expression levels of both genes follow the same

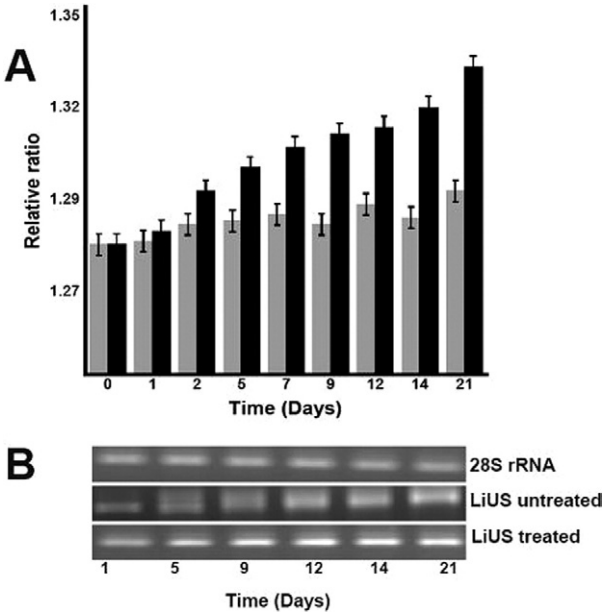

Fig. 3. Changes in mRNA levels of biglycan during the period of treatment (21 days). (A) Average mRNA normalized ratios (relative ratios) from three different samples of the control group (grey bars) and the study group (black bars) are presented. Values are expressed as mean ± standard deviation. (B) Representative results for biglycan (control group and study group) and 28S rRNA using RT-PCR and 1 mg total RNA extracted from each sample. RT-PCR and analysis was performed as described in Materials and Methods.

pattern. Similar to biglycan, collagen type I mRNA levels are gradually increased in both groups (Fig. 4). However, the difference in the levels of collagen type I expression between the control group and the study group was not as wide as for biglycan (20%, $p = 0.001$).

Our histological analysis revealed remarkable changes during time, at the tendon-bone interface. Although vascularity has been observed from the first week (Fig. 5), it was increased in samples from the study group compared with samples from the control group at the end of the treatment (21 d, Fig. 6). This increased vascularity was most evident at the tendon-bone interface and it was seen in the tibial sections. The tendon graft, at 3 wk was surrounded by a cellular "reactive" connective tissue. This tissue formed a "bridge" between the tendon graft and the surrounding woven trabeculae. Using Masson trichrome and reticulin (Gordon-Sweet) stains, we observed thick collagen fibers passing through this newly formed connective tissue and connecting the graft and the bone (Fig. 6). This could be observed as early as the third week. Similar findings were not observed in the control animals at this time (Fig. 7). The tendon graft showed foci of increased cellularity. There was not morphologically detectable degeneration of the tendon grafts.

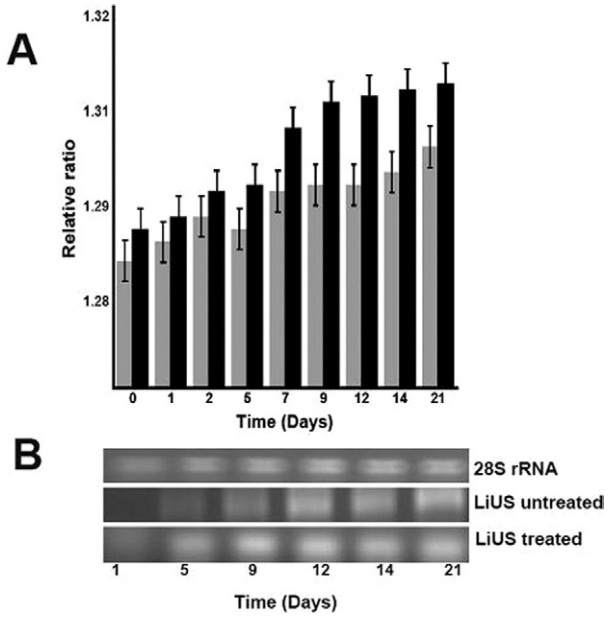

Fig. 4. Changes in mRNA levels of collagen type I during the period of treatment (21 days). (A) Average mRNA normalized ratios (relative ratios) from three different samples of the control group (grey bars) and the study group (black bars) are presented. Values are expressed as mean ± standard deviation. (B) Representative results for collagen type I (control group and study group) and 28S rRNA using RT-PCR and 1 mg total RNA extracted from each sample. RT-PCR and analysis was performed as described in Materials and Methods.

Combined evidence deriving from both molecular and histological analyses described above, indicate that LiUS treatment is the proximal cause of altered gene expression in the study group. Moreover, our results strongly indicate that LiUS treatment is responsible for faster healing at the tendon graft-bone interface. These changes are reflected in a more general reprogramming of positive biological effects that lead, at the end of the treatment, to a histological profile that supports a faster healing rate and indicates strongly the success of the procedure.

DISCUSSION AND SUMMARY

Ligamentization is a process that occurs during and after the healing at tendon graft-bone interface and throughout the entire length of the tendon graft. This complex process is affected by various biomechanical factors and a number of essential genes. However, it is still not known whether the expression levels of specific genes could affect ligamentization and healing process. The present study demonstrates that transosseous LiUS treatment affect healing at tendon graft-bone interface in rabbits through alteration of the expression levels of specific genes like TGF-β1, biglycan and collagen type I.

TGF-β1 is known to be a multifunctional cytokine, present in many biological tissues, that induces new matrix synthesis and production of various growth factors. In the present study, TGF-β1 expression levels exhibit an unexpected bimodal profile in the study group, while the expression levels of the same gene in the control group remains unaltered. This peculiar effect seems to be connected with the application of LiUS in the study group. In the beginning of the treatment, we observed that TGF-β1 levels in the study group were about 10% higher than those of the control group. As has been previously reported in studies on cell cultures, such initial elevated levels of TGF-β1 seem to represent an immediate response to ultrasound application. However, it has been reported that TGF-β1 levels decline gradually after few hours something that we also observed until day 2 (Lai and Pittelkow 2007; Harle et al. 2005). Interestingly, this gradual attenuation of TGF-β1 levels was consistent and more obvious after day 5 and until day 9. Although this is an unexpected observation, it might

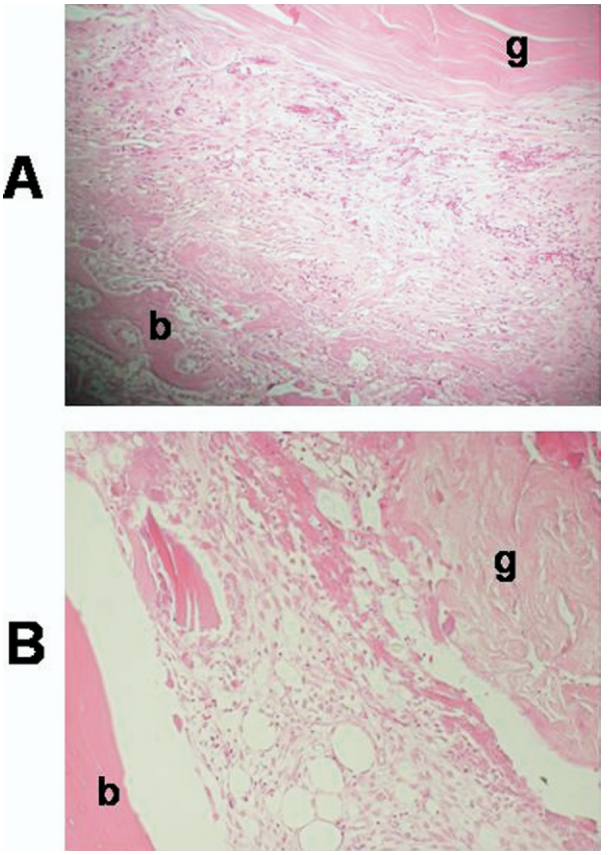

Fig. 5. (A) LiUS treatment at first week showed an active interface with plump cells and blood vessels between graft and bone (hematoxylin and eosin stain original magnification ×20). (B) Control at first week. Interface showed looser connective tissue with few blood vessels (hematoxylin and eosin stain original magnification ×40). b = bone; g = graft.

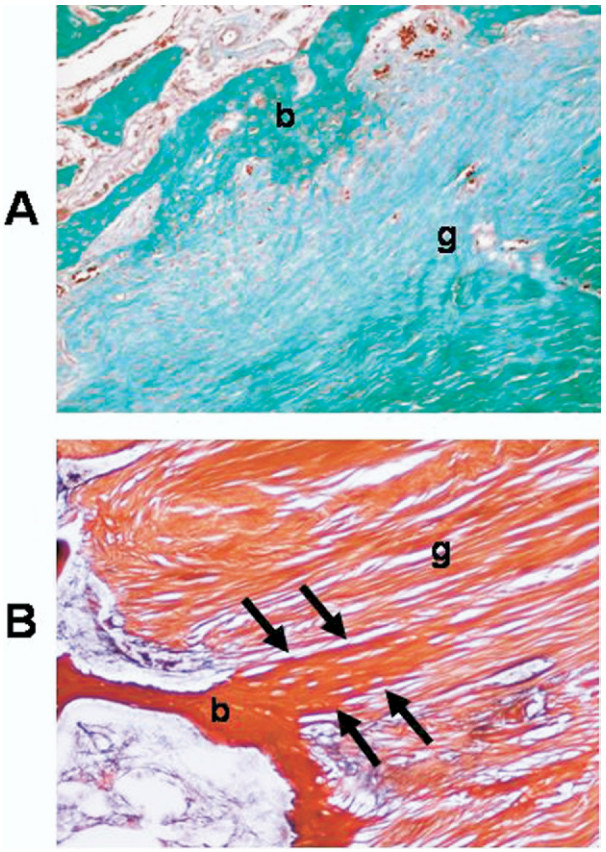

Fig. 6. (A) Tendon graft-bone interface, at the end of the third week (LiUS treated animal). Note that in the interposed connective tissue, there were collagen fibers connecting the bone with the graft. (original magnification ×100, Masson-trichrome stain). (B) Tendon graft-bone interface, at the end of the third week (LiUS treated animal). Note that in the intervening connective tissue there are collagen fibers that clearly connect the graft and the surrounding bone (arrows) (original magnification ×100, Gordon-Sweet stain). b = bone; g = graft.

indicate a reply signal for a slower cell differentiation process to assist tissue reconstruction during the tendon graft-bone interface healing. It is possible that this signal that is related to the decreased TGF-β1 expression levels can change the cell's dynamics towards biochemical pathways that are necessary for an accelerated healing process at tendon graft-bone interface. Such a biochemical pathway could involve proteins of the non-Smad pathway that may provide quantitative regulation (Moustakas and Heldin 2005; Tsubone et al. 2006). During ligamentization process and as neo-angiogenesis is progressive (Rougraff et al. 1993), the levels of TGF-β1 are normalized and at the end (day 21), they appear increasing compared with the control group. Our results are in accordance with previous reports. The same pivotal regulation of TGF-β1 by ultrasound has been observed in human osteoblast cell lines (Harle et al. 2005). Moreover, it has been demonstrated in an animal study that ultrasound treatment in a high-dose application enhances ligament repair by upregulating expression of TGF-β1 (Leung et al. 2006). The same study reports that stimulation of TGF-β1 was extended only in the presence of ultrasound application, thus, concluding that long-term treatment with this therapy could obtain further improvement. Therefore, we assume that this TGF-β1 bimodal profile could be attributed to the LiUS application and, moreover, this effect possibly accelerates the remodelling process of the graft at the tendon graft-bone healing interface. However, the biochemical pathways that are affected by the bimodal regulation of TGF-β1 should be further investigated.

As it has been previously reported, biglycan is among the proteoglycans that play important role in the organization and the regulation of extracellular matrix. Previous studies have shown that biglycan is up-regulated by TGF-β *in vitro* and in addition it has been demonstrated that biglycan's expression levels of mRNA are increased earlier in ligament healing (Kahari et al. 1991; Hildebrand et al. 1994; Lo et al. 1998). In our study, we observed that expression of biglycan is significantly increased in the study group compared with the control group. This gradually elevated expression of biglycan may facilitate a rapid rearrangement of the extracellular matrix in the study group. Although we observed an expected slight increase of biglycan expression also in the control group, it is obvious that the application of LiUS on the study group significantly affected the expression levels of biglycan as a response to this external signal. Therefore, we conclude that LiUS treatment may promote through biglycan a more efficient

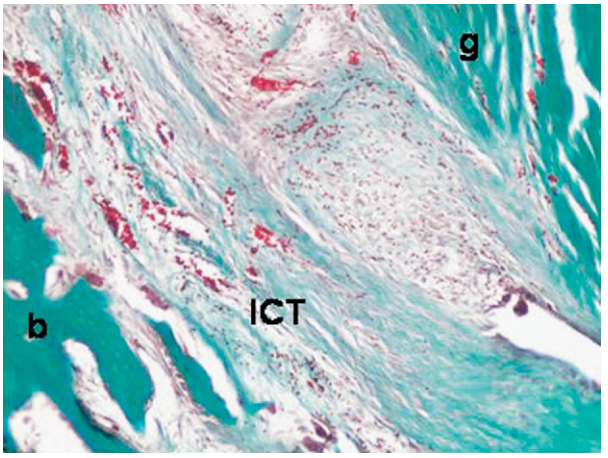

Fig. 7. Tendon graft-bone interface, at the end of the third week (control animal). In the intervening connective tissue (ICT) there are blood vessels and few random thin collagen fibers (original magnification ×100, Masson-trichrome stain). b = bone; g = graft.

rearrangement of the extracellular matrix. Subsequently, this rearrangement facilitates the early graft incorporation within the tibial-bone tunnel. It is interesting to notice that although we measured reduced expression levels of TGF-β1 between days 5 to 9 in the study group, the levels of biglycan were continuously increased. This observation can be explained by the fact that the LiUS application may stimulate other factors that probably affect the expression of biglycan and it seems that biglycan expression in our study is not solely dependent on the levels of TGF-β1.

These findings concerning the pattern of mRNA levels observed for biglycan are in good agreement with previous reports suggesting that biglycan might directly affect matrix assembly and biomechanical strength in healing through its association with collagen fibrillogenesis (Boykiw et al. 1998). In addition, it has been reported that exogenous administration of TGF-β1 to the tendon graft-bone interface increase mRNA expression of collagen type I (Murphy et al. 1994; Yamazaki et al. 2005). Therefore, it was intriguing to investigate the effect of LiUS in collagen type I expression on the healing process at tendon graft-bone interface. Our findings showed that LiUS application leads to collagen type I mRNA increased levels in the study group. However, collagen-encoding gene displays a rather moderate increase in the study group compared with the control group and compared with the biglycan pattern. Although the observed difference in the collagen type I expression levels between the study group and control group is measurable albeit lower than biglycan, we noticed that between day 5 and day 9, the levels of collagen type I increase gradually despite the decreased TGF-β1 expression levels in the study group. This observation, however, can be explained by the fact that the effect of LiUS on these specific genes seems to be different and possibly the amount of TGF-β1 that is produced between days 5 and 9 is sufficient to support stimulation of both biglycan and collagen expression.

In a next step, and in order to verify the observations concerning the effects of LiUS on the expression level of significant genes that have been reported to mediate the ligamentization process, we performed histological analysis of samples from 16 rabbits representing both groups. Histological evidence further confirms hypothesis, that LiUS treatment is indeed an applicable method to achieve a faster rehabilitation of injuries and to further study specific mechanisms of this effect in molecular level in order to have a fast and reliable method of predicting the course of the treatment.

In previous studies, analysis of the morphological findings indicated that the tendon graft undergoes a process of faster ligamentization in LiUS-treated animals (Lu et al. 2006; Walsh et al. 2007). The findings from our molecular analysis were indeed supported by our histological observations. We have demonstrated that incipient ligamentization can be visible as early as the end of the third week. Moreover, the formation of collagen fibers connecting the graft with newly formed trabeculae of woven bone, eventually establish anchoring of the graft into the surrounding lamellar bone of the hosting tunnel. The "bridging" collagen fibers are best depicted by the application of a particular reticulin stain (Fig. 6B).

These findings demonstrate that transosseous application of LiUS in daily dosages of 20 min affects the expression levels of significant genes like TGF-β1, biglycan and collagen type I and support the hypothesis that they enhance tendon graft healing to bone through effects on the molecular level. The transosseous application through the use of an implanted transducer overcomes the interference of the overlaying soft tissues. As opposed to conventional transcutaneous treatment regimens, the LiUS energy is neither attenuated within the soft tissues nor significantly reflected back at the bone surface, but rather most of the energy is transmitted into the bone and propagates efficiently into it. This can be justified by the fact that the transducer is in direct contact with the bone surface and is also acoustically matched to the bone (the transducer's impedance is 6 MRayls, while typical values for the bone's acoustic impedance range from 4 to 8 MRayls) (Raum et al. 2005). In this respect, transosseous LiUS facilitates the direct exposure of the tendon graft-bone interface to the LiUS energy. The characterization of the genes under study is in agreement with the histological findings at 3 wk that revealed stable graft incorporation within the tibial bone tunnel in the study group compared with the control group. Furthermore, in the histological analysis, it was verified that the study group exhibited earlier healing indicating a more efficient ligamentization process.

In conclusion, our study suggests that direct application of LiUS on the bone surface adjacent to the tendon graft-bone tunnel affects the expression of specific genes that encode essential molecules involved in the healing and the ligamentization of the tendon graft. This effect possibly enhances the healing rate of the tendon graft-bone interface in rabbits. Therefore, these findings indicate that after joint ligament reconstruction with tendon grafts, ultrasound treatment may facilitate earlier resumption of full activity and function, reducing the socioeconomic burden on the patient.

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