Transosseous Application of Low-Intensity Ultrasound at the Tendon-Bone Interface Affects the Healing Rate and Up-regulates Simultaneously the Expression of Collagen Type I and $tRNA^{Gly}$

Loukia K. Papatheodorou, Katerina Grafanaki, Stamatina Giannouli, Dimitrios I. Fotiadis, Senior *Member, IEEE,* Constantinos Stathopoulos, and Konstantinos N. Malizos

***Abstract*— The present study investigates the effect of transosseous low-intensity pulsed ultrasound (LiUS) during lingamentization process on the healing at tendon graft-bone interface in rabbits. Analysis of the RT-PCR products showed statistically significant up-regulation of genes encoding collagen type I and $tRNA^{Gly}$ in the study group compared to the control group. Histological examination indicated a faster healing rate and a more efficient lingamentization process after ultrasound treatment. Our results suggest that transosseous application of LiUS enhances the healing rate of the tendon graft-bone interface, possibly by affecting the expression levels of significant genes.**

I. Introduction

THE process of ligamentization includes the histological and structural remodeling of the tendon graft to ligamentous tissue [1]-[3]. There is little information documenting the mechanism of healing process at the tendon graft interface. Joint ligament reconstruction is usually performed by the implantation of a tendon graft in to a bone tunnel.

In vitro studies have demonstrated that exposure to ultrasonic energy stimulates proliferation of fibroblasts. Possible mechanisms involve increase of their metabolic activity, affecting primarily the synthesis of collagen [4], [5]. Animal studies have shown that transcutaneous application of low intensity pulsed ultrasound (LiUS) accelerate the fracture healing and tendon and ligament healing process [6]-[11]. Recently, experimental studies have proven the efficacy of the transosseous application of LiUS for both enhancement and monitoring of the bone healing process applying the most modern smart implant technologies [12]-[15]. Despite such well-documented studies, the mechanisms through which LiUS interact with living tissue remain elusive in molecular level.

Experimental studies have shown that many key-molecules such as growth factors or extracellular matrix proteins play important role in the remodelling process of the tendon graft [16], [17]. Collagen undergoes changes in concentration and biochemical properties during ligamentization process and its synthesis seems to be stimulated by additional growth factors like TGF-β1 and controlled mechanical stretching [18]. There are three dominant types of collagen, type I, II and III. Type I collagen is the most abundant structural protein in bones, tendons and cruciate ligaments. It has been observed that glycine represents more than 30% of the amino acids in the collagen triple helix. There are four codons specifying glycine and analysis of collagen type I genes have shown that 89% of glycine codons are GGU/C. Previous studies have demonstrated that tissues synthesizing type I collagen are associated with increased level of $tRNA^{Gly}$ and therefore is suggested that up-regulation of collagen synthesis also stimulates protein synthesis rates for rapid incorporation of glycine residues [19].

The purpose of this study was to investigate the effect of transosseous LiUS on molecular level during the healing of a tendon graft-to-bone interface in rabbits, by examining any possible effects on the expression levels of type I collagen, and its association with the expression of $tRNA^{Gly}$ genes using semi-quantitive RT-PCR. 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 after LiUS transosseous application.

II. Materials and Methods

A. Study Model and Animal

Forty-four male New Zealand White rabbits of 3 months old were used in this study. All rabbits underwent a bilateral surgical procedure, which has been based on the technique developed by Wang *et al.* [20]. The anterior cruciate ligament (ACL) was excised and replaced with the long

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L. K. Papatheodorou is with the Department of Orthopaedic Surgery & Musculoskeletal Trauma, University of Thessaly, GR 41110 Larissa, Greece (tel: +302410682722; fax: +302410670107; e-mail: lpapath@med.uth.gr).

K. Grafanaki is with the Department of Biochemistry & Biotechnology, University of Thessaly, GR 41222 Larissa, Greece.

S. Giannouli is with the Department of Biochemistry & Biotechnology, University of Thessaly, GR 41222 Larissa, Greece.

D. I. Fotiadis is with the Unit of Medical Technology and Intelligent Information Systems, Computer Science Department, University of Ioannina, GR 45110 Ioannina, Greece.

C. Stathopoulos is with Department of Biochemistry & Biotechnology, University of Thessaly, GR 41222 Larissa, Greece.

K. N. Malizos is with the Department of Orthopaedic Surgery & Musculoskeletal Trauma, University of Thessaly, GR 41110 Larissa, Greece and with Center for Research & Technology – Thessaly (CERETETH), Institute of Biomedical Research & Technology (BIOMED), GR 41222 Larissa, Greece.

digital extensor in both knees. 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 bone through sutures anchored on a 10mm screw implanted at the metaphyseal-diaphyseal junction, with the knee at 30° of flexion. A custom-made ultrasound transducer was implanted onto the bone along the surface adjacent to the bone tunnel [Fig. 1]. The surgical wound was irrigated and closed in layers with absorbable sutures. Postoperatively all animals received antibiotic prophylaxis for five days and analgesic medication for one day. The general activities of the animals and the local wound condition were inspected daily. 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 E.U. guidelines for "The care and use of animals in research".

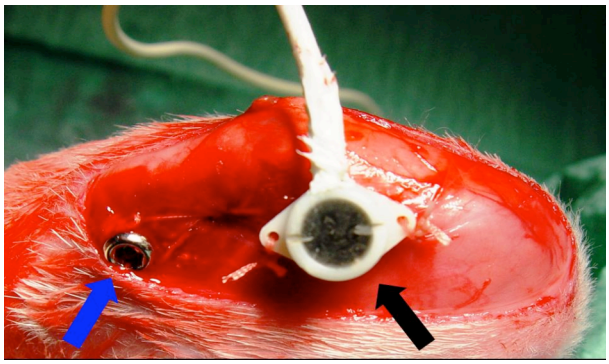

Fig. 1. Ultrasound transducer implantation. Anchoring of distal end of the tendon graft on a screw implanted distally to the bone tunnel (blue arrow). The implanted ultrasound transducer onto the bone is indicated by the black arrow.

B. *Transosseous Application of LiUS*

The right knees of all animals were exposed to LiUS for 20min/day, starting from the first day post-operatively until sacrifice, and were regarded as the study group. The ultrasound device responsible for generating the LiUS treatment has been previously described [13], [14]. The ultrasound transducer (contact-type, acoustic impedance 6 MRayls, longitudinal, central frequency 1 MHz, custom-made, ValpeyFisher Corp., Hopkinton, MA, USA) was encapsulated in a plastic case, 6mm in thickness and 8mm in diameter. The ultrasound signal consisted of 200μs bursts of 1 MHz sine waves, with a pulse repetition rate of 1 kHz and 30mW/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.

C. *RNA Quantification and Primer Development*

Twenty-eight animals were sacrificed at different time intervals at 1,2,3,5,6,7,8,9,12,14,17 and 21 days. Total RNA was extracted according to the manufacturer's instructions using the Total RNA isolation kit-Nucleospin RNA II (Macherey-Nagel) from 30 mg of each tissue sample after homogenization in a micro-dismembrator (Ultra Turax, IKA-Werke). 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 and 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.

D. *Semi-quantitative RT-PCR*

RT and simultaneous PCR reactions were undertaken with 1 μg total RNA using the Robus T II RT-PCR kit from Finnzymes (Finland) according to the manufacturer's instructions. 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). All reported experiments were in the linear range of the PCR reaction.

The amplified products were separated on 2% agarose gels, imaged using a Vilber-Lourmat TP-001-FDC image analyzer (Vilber Lourmat, Germany GmbH) and integrated density values calculated using ImageQUANT TL 2005 software (Amersham Biosciences). Data from individual

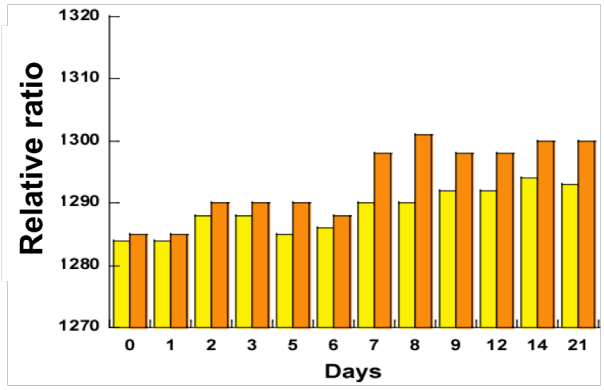

Fig. 2. Changes in mRNA levels of collagen type I. Average mRNA normalized ratios (relative ratios) from three different samples of the control group (yellow bars) and the study group (orange bars) are presented.

transcript levels were normalized to the 28S rRNA that was determined not to vary during preliminary experiments (data not shown) in order to permit semi-quantitative comparisons in mRNA expression.

E. Histological Examination

Analysis was carried out on tissue samples obtained from 16 animals after euthanasia at 1,2 and 3 weeks. The samples were fixed for 48 hours in phosphate buffered formalin before decalcification in 10% formic acid. Tibial bone tunnels were cut into 5mm 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, 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.

F. Statistical Analysis

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

III. Results

The total expression levels of collagen type I mRNA by semi-quantitative RT-PCR in both study and control group are shown in Fig. 2. It was observed that collagen type I mRNA levels increased progressively in both groups. However it became evident that when we compared the

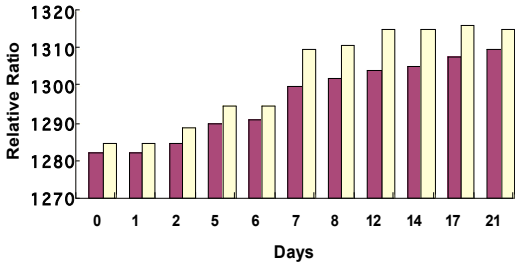

Fig. 3. Changes in levels of $tRNA^{Gly}$ from all samples of the control group (red bars) and the study group (yellow bars).

transcription levels for collagen type I in the study group we found them significantly higher compared to the control group (~20%, P=0.001).

Similar to collagen type I, levels of $tRNA^{Gly}$ are gradually increased in both groups [Fig. 3]. However, significantly elevated levels (30%) were demonstrated for $tRNA^{Gly}$ in the study group compared to the control group, especially between days 5 and 14. Such elevated levels of $tRNA^{Gly}$ verified the fact that tissues which over-produce collagen utilize higher concentrations of $tRNA^{Gly}$ [Fig. 4].

Our histological analysis revealed remarkable changes during time, at the tendon-bone interface. Although vascularity has been observed from the first week (data not shown) it was increased in samples from the study group compared with samples from the control group at the end of the treatment. The tendon graft, at 3 weeks 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. 5]. This could be observed as early as the third week. Similar findings were not observed in the control animals at this time. The tendon graft showed foci of increased cellularity. There was not morphologically detectable degeneration of the tendon grafts.

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.

IV. Discussion

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 mediated by a

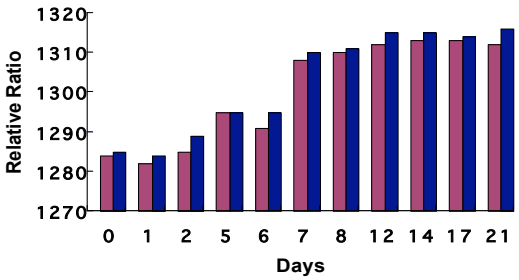

Fig. 4. Comparison of the expression levels showing simultaneously up-regulation of collagen type I (blue bars) and $tRNA^{Gly}$ (red bars) genes in the study group.

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 collagen type I and $tRNA^{Gly}$.

As it has been previously reported, the three types of collagen in conjunction with other components of extracellular matrix are the major determinants of tissue

structure and function. In our study we observed that expression of collagen type I is significantly increased in the study group compared to the control group. Although we observed an expected slight increase of collagen type I expression also in the control group, it is obvious that the application of LiUS on the study group significantly affected the expression levels of collagen type I as a response to this external signal. Therefore we conclude that LiUS treatment may promote through collagen type I a more efficient production of collagen fibers at tendon graft-bone interface. Subsequently this rearrangement facilitates the early graft incorporation within the tibial-bone tunnel.

As it has been previously reported, tissues synthesizing type I collagen are associated with increased level of tRNA molecules, especially $tRNA^{Gly}$. In our study we verified that up-regulated expression of $tRNA^{Gly}$ is in accordance with significantly increased collagen levels [19]. Our results support earlier observations and suggest that there might be two essential effects of LiUS application. One could include

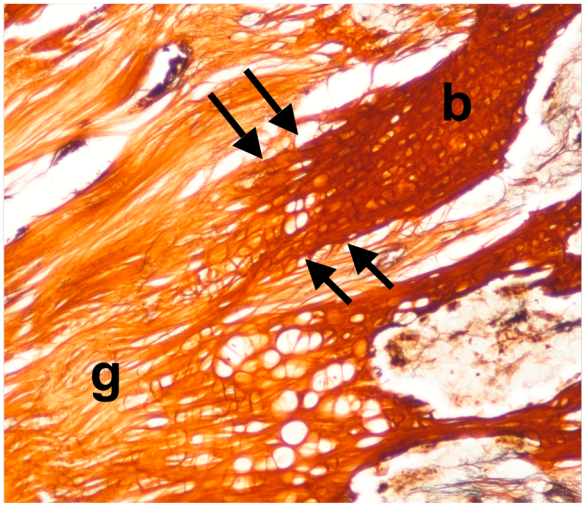

Fig. 5. Tendon graft-bone interface, at the end of the third week (LiUS treated animal). Note that in the intervening connective tissue there are collagen fibers that clearly connect the graft and the surrounding bone (black arrows) (original magnification X10, Gordon-Sweet stain). b: bone, g: graft.

direct effect on the gene expression and the other one could include the indirect up-regulation of protein synthesis rate. Therefore, the fact that collagen and $tRNA^{Gly}$ up-regulation are observed simultaneously could explain either pathways.

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. 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. 5].

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 – 8 MRayls) [21]. 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 three weeks that revealed stable graft incorporation within the tibial bone tunnel in the study group compared to 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 enhances the healing rate of the tendon graft-bone interface in rabbits. This effect is more likely mediated by the up-regulation of collagen synthesis and by altering the protein synthesis rate through the production of higher levels of specific tRNA molecules. 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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