Collagen Fibers in the Healing Process of Rat Achilles Tendon Rupture Using Different Times of Ultrasound Therapy

Thiago Saikali Farcic,^{1,2,*} Cristiano Schiavinato Baldan,^{1,2}
Aline Fernanda Perez Machado,^{2,3} Leonardo Affonso Massabki Caffaro,¹
Igor Fagioli Bordello Masson,² and Raquel Aparecida Casarotto¹

¹Department of Physical Therapy, Faculdade de Medicina, Universidade de São Paulo (FMUSP), São Paulo, Brazil.

²Department of Physical Therapy, Universidade Paulista (UNIP), São Paulo, Brazil.

³Master's and Doctoral Programs in Physical Therapy, Universidade Cidade de São Paulo (UNICID), São Paulo, Brazil.

Thiago Saikali Farcic, PhD

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*Correspondence: Department of Physical Therapy, Universidade Paulista (UNIP), São Paulo-SP, Brazil. Yojiro Takaoka Avenue, 3500, Santana de Parnaíba 06500-000, SP, Brazil, (e-mail: thiagofarcic@hotmail.com)

Objective: The aim of this study was to evaluate the organization of collagen fibers in the healing process of rat Achilles tendon rupture using different times of ultrasound therapy (TUS).

Approach: Forty Wistar rats were selected. Among these, 32 were submitted to total *tenotomy of the calcaneus tendon* and divided into 5 groups: control group (CG, $n=8$), without tenotomy or any treatment; tenotomy group ($n=8$), with tenotomy and without treatment; TUS groups—TUS3 ($n=8$), TUS4 ($n=8$), and TUS5 ($n=8$)—submitted to tenotomy and treated with TUS for 3, 4, and 5 min per effective radiating area (ERA), respectively. The animals were sacrificed on the 12th post-operative day. The tendons were surgically removed for analysis of the collagen fiber organization using the birefringence technique (OR, optical retardation).

Results: The collagen fibers exhibited better aggregation and organization in the UST3, TUS4, and TUS5 groups compared with CG ($p < 0.05$). The TUS5 group had *better response rates* in intergroup comparison.

Innovation: The dose response of therapeutic TUS is influenced by many variables. The scientific evidence to support the dosimetry is insufficient. The application time is an important variable to be considered in TUS. In this study, the longer the application time, the better for organization and aggregation of collagen fibers in the rat tendon.

Conclusion: TUS applied for 5 min per ERA presented higher dose response to the organization of collagen fibers in the healing process of rat Achilles tendon rupture.

Keywords: birefringence, dosimetry, wound healing, Achilles tendon, ultrasonic therapy, tendon injuries

INTRODUCTION

ACHILLES TENDON RUPTURE is a serious and common injury. It is higher prevalence among athletes who practice high-impact sports, which involve jumping and running.¹ It can also occur spontaneously in patients with tendon degeneration with increased amount of collagen type III, reduced amount, and aggregation stage of type I.²

The postsurgery rehabilitation of Achilles tendon could take up to 18 weeks to enable the patient to return to work.³ During the recovery period, the injured area is immobilized to prevent functional complications. However, a longer immobilization period may lead to morphological changes in tendon.⁴ Tendon healing can be divided into three phases: inflamma-

tory, proliferative, and remodeling.⁵ Both proliferative and remodeling phases are responsible for important phenomena in tendon healing. During these two phases, there is intense proliferation of fibroblasts, which are responsible for the synthesis of collagen and the alignment of these fibers in the longitudinal axis of the tendon⁶ ensuring adequate tissue repair biomechanical proprieties similar to noninjured tissue.

In recent years, researchers have studied the effects of therapeutic treatments with ultrasonic therapy to modulate and accelerate the tendon repair process.^{7–9} The times of ultrasound therapy (TUS) therapeutic effects are associated with the anticipation of events related to the inflammatory response: mast cell degranulation; histamine, leukocytes, and macrophase release; increased cellular metabolism and synthesis of type I and III collagen; better alignment and arrangement of collagen fibers, promoting the acceleration of the healing process in tendons; and reduced edema.^{10–12}

There are many dosimetric parameters that have influenced the dose response of therapeutic TUS, such as coupling agents,¹³ duty cycle,¹⁴ intensities,¹⁵ emission modes,¹⁶ application time,⁹ and frequencies.¹⁷ Therefore, the use of TUS in tendon repair is difficult, because there are no guidelines or systematic reviews for its parameters.¹⁸ Studies, *in vitro* and *in vivo*, demonstrate positive effects of ultrasonic therapy in tendon healing¹⁹; there are many studies about some parameters as frequency and intensity^{17,20,21}; however, the application time is still poorly studied.

Robertson¹⁸ conducted a review on dose–response relationship of TUS performed in randomized clinical trials. The author suggests that there is an insufficient amount of clinical trials to identify a dose–response relationship to answer questions about TUS dosage and therefore further studies should be conducted to justify the dosimetry of TUS.

CLINICAL PROBLEM ADDRESSED

Among the adjustable TUS variables, the application time influences the amount of energy applied to the tissue, which is calculated by the following formula: Total energy per treatment (Joules) = spatial average temporal average (SATA) (Watts/cm²) × effective radiating area (ERA) (cm²) × time (seconds).²² The treatment time is not often the topic of studies on TUS. In our previous study,⁹ we evaluated the effects of 1, 2, and 3 min of TUS treatment per transducer area on the organization of collagen fibers in rat tendons. The results obtained showed that TUS applied for 3 min per transducer area

presented higher dose response to the organization of the collagen fibers on the healing process of rat tendons compared with tenotomized nontreated group and to the application time of 1 and 2 min per transducer area.

In this study, we hypothesized that longer TUS application times could produce better organization of collagen fibers. Therefore, the purpose of this study was to evaluate the effects of 3, 4, and 5-min therapeutic ultrasound treatment per transducer area on the organization of collagen fibers in rat tendons.

MATERIALS AND METHODS

Design

This study is a randomized controlled prospective study. The research was approved by the Research Ethics Committee of the Medical School of the Universidade de São Paulo (USP), São Paulo, SP, Brazil (authorization no. 065/11). All animals received humane care in strict compliance with the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation. The animals were submitted to euthanasia on the 12th postoperative day (13th day of study).

Animals

The sample consisted of 40 male, adult rats (*Rattus norvegicus albinus*) of the Wistar-EPM 1, with a mean age of 90 days and weighing between 250 and 350 g. The animals were randomly classified into 5 groups of 8 rats each. The number of animals needed to achieve statistical significance was based on Eckelman *et al.*'s.²³ study. The animals were housed in propylene cages and fed standard rat chow and water *ad libitum*, in a controlled environment, under controlled temperature (22 ± 25°C) light–dark cycle (12/12 h).

Surgical technique

The animals were weighed and anesthetized with veterinarian tiletamine hydrochloride and zolazepam hydrochloride (Zoletil 50; VIRBAC®) at a dose of 25 mg/kg of body mass through intraperitoneal injections. The posterior areas of the right tibia, corresponding to the location of the Achilles tendon, were epilated. Afterward, asepsis was achieved with 70% alcohol. The skin and the panniculus carnosus were cut longitudinally over the site corresponding to the middle third of the Achilles tendon, exposing the tendon to obtain a cross-section (Fig. 1A). After sectioning the tendon, the skin incision was completely sutured in the central area using 4.0 nylon thread (Fig. 1B). All the aforementioned procedures were carried out by a trained researcher. The model for the total

Figure 1. (A) Longitudinal incision and exposure of the Achilles tendon on the rat's posterior hindlimb; (B) Surgical incision suture with 4.0 nylon thread. (C) Irradiation of the Achilles tendon on the rat's posterior hindlimb.

tenotomy of the middle portion of the Achilles tendon (transverse incision of the dissected tendon) was based upon experiments performed by da Cunha *et al.*,²⁴ Koeke *et al.*,²⁵ and Carrinho *et al.*²⁶ Postoperatively, the animals received a single intramuscular dose of Ceftriaxone in the contralateral hindlimb for antibiotic prophylaxis and were returned to sterilized cages without immobilization.⁹

Treatment protocol

The equipment used in this experiment was Sonacel BIOSSET[®] TUS emitter (São Paulo, SP, Brazil). Its transducer design was modified to fit the posterior portion of the rat's hindlimb and the size of the lesion. The equipment was calibrated with an acoustic scale (GT210 Glutymax[®]) and an oscilloscope (Intermetro[®]), before the beginning of the experiment. The ultrasonic irradiation was applied by a trained researcher using the following parameters: 1 MHz frequency, pulsed mode with 20% of the duty cycle (2 ms emission/8 ms interval), 100 Hz repetition frequency, 0.5 W/cm² of intensity (SATA), and 0.5 cm² (ERA). The treated area was 1 cm² in size. The animals were divided into the following groups: control group, (CG, $n=8$) without surgery and TUS; tenotomy group, (TG, $n=8$) with total tenotomy of the Achilles tendon and without TUS; TUS3 ($n=8$), TUS4 ($n=8$), and TUS5 ($n=8$) groups submitted to tenotomy and treated with TUS for 3, 4, and 5 min per transducer area, totaling 6, 8, and 10 min of treatment, respectively.

During the TUS application, the transducer was placed perpendicular to the treated area using the dynamic technique. Water-soluble gel was used as a coupling agent to better conduct the waves and facilitate movement over the rat's skin.²⁷ During the procedure, the animals were also stabilized with a standard retainer (Fig. 1C).²⁴

The animals were submitted to irradiation once a day, during 10 days, with 2 days of interval after the fifth treatment day to simulate the clinical practice.^{24,25} All the animals were sacrificed on the 12th postoperative day.

Histological analysis

The animals were sacrificed using a CO₂ chamber at 40% of carbon dioxide concentration with prior induction of anesthesia with veterinarian tiletamine hydrochloride and zolazepam hydrochloride at a dose of 25 mg/kg of body mass through intraperitoneal injections and the damaged tendon areas were surgically removed. For the quantitative assessment of tendon repairs, the removed tendons were placed in a 10% formalin solution for 24 h and were subsequently dehydrated with four alcohol baths of 1 h each at dilutions of 50%, 70%, 80%, and 100%. The samples were then washed in xylene for one hour and embedded in paraffin at 58°C. The blocks were longitudinally cut into serial sections using a microtome (LAICA[®]) with a standard thickness of 7 μ m. The sections were mounted on glass slides without staining and identified by a blinded rater.

The uneven penetration of light through an object is a measure of birefringence. The birefringence measures the numeric difference between maximum and minimum values of refractive indices of collagen fibers. This measure assesses the organization, the aggregation states, and the alignment of the tendon collagen fibers.^{24,25} For the birefringence analyses, the slides for each group were immersed in distilled water (refractive index $n=1.333$) for 30 min. The optical retardation (OR) was measured using a polarized light microscope (LEICA[®]) with a 10 $\times$ /0.22 objective, pol. 0.9 condenser, Senarmont $\lambda/4$ compensator, and monochromatic light ($\lambda=546$ nm) obtained by an interference filter (LEICA) of the LAMAV (Laboratório de Materiais Vítreos do Departamento de Engenharia de Materiais da São Carlos Federal University). These types of analyses have been used to quantitatively measure the degrees of organization of the collagen fibers in several studies. The resulting measures, in degrees, were converted into nanometers (nm) by multiplying the values by 3.03.²⁴ The total birefringence of the

collagen fibers was measured after soaking the tendons in distilled water. To perform the measurements along the axis of the tendon, the longitudinal axis of the collagen fibers was oriented 45° from the direction of the propagation of the transmission light. In this position, the collagen fibers have the highest OR.^{24,25} Measurements were taken at five different points on the central areas of the tendons, which corresponded to the injured area. The birefringence data were collected by two previously trained, independent, and blinded raters.⁹

Statistical analysis

To check whether data presented normal distribution, the Kolmogorov–Smirnov test was used. For the evaluation of the birefringence analysis, unpaired Kruskal–Wallis test was used, followed by Dunn's *post-hoc* test. The significance level adopted for the statistical tests was 5% ($p < 0.05$) and the values were presented as mean and standard deviation.

RESULTS

The tests revealed statistically significant differences between the TG, TUS3, TUS4, and TUS5 and CGs ($p < 0.05$). The analysis among groups has demonstrated statistically significant differences

when comparing the OR data between the following groups: TUS3 $\times$ TG, TUS3 $\times$ CG, TUS3 $\times$ TUS5, TUS4 $\times$ TG, TUS4 $\times$ CG, TUS4 $\times$ TUS5, TUS5 $\times$ TG, and TG $\times$ CG (Fig. 2A).

A qualitative histological analysis using polarized light microscopy allows the definition of normality standards regarding the organization of the collagen fibers (Fig. 2B). When comparing the images in Fig. 2B, it is possible to observe that the animals of the TUS3, TUS4, TUS5, and CGs demonstrated better organization of the collagen fibers than TG. For intergroup comparison, no significant differences were observed when compared with CG.

DISCUSSION

The comparison between the studied groups showed that the TUS3, TUS4, and TUS5 groups presented better organization and aggregation of collagen fibers than TG. The findings showed that the TUS5 group presented better organization and aggregation of collagen fibers with values similar to those of the CG in the birefringence analysis, with no statistically significant differences between the groups. In addition, the OR values tend to increase with longer application times in the treated groups, corroborating the findings of Farcic *et al.*⁹

Figure 2. (A) OR values between CG, TG, TUS3, TUS4, and TUS5. TUS3-TUS3 group; TUS4-TUS4 group; TUS5-TUS5 group; *significant difference. (B) Images related to the qualitative observations of the birefringence analysis of the rat tenotomized tendons. Specimens were positioned with the longitudinal axis of the collagen fibers at 45° . In the TG, TUS3, and TUS4 images, deficiencies in the organization of the collagen fibers can be observed when compared with TUS5 and CGs. CG, control group; OR, optical retardation; TG, tenotomy group; TUS, times of ultrasound therapy.

Collagen is the major protein in tendons, which accounts for 70% to 86% of the dry weight.^{2,24,27} Rats are the most commonly employed *models* in the evaluation of birefringence in tendon collagen fiber healing.^{9,24,25} The tissue injury tends to decrease the OR value, corroborating the data found in the present study, where TG showed the lowest OR value, followed by TUS3, TUS4, TUS5, and CGs. During the process of tissue repair, the OR values tend to increase.

The findings of Farcic *et al.*,⁹ suggested that 3 min of TUS application time per transducer area provided a better collagen fiber organization in rat tendons during the tissue repair process, showing that longer application times led to a better collagen fiber organization. During the tendon excise procedure, it was possible to observe that the animals submitted to longer TUS application times had better tissue quality considering adherence and anatomy of the dissected tendon.

The amount of time that a tendon is exposed to tensile force will change its mechanical proprieties. The tendon viscoelastic properties will depend on the amount of time that this tissue is exposed to deformations or strains.^{9,28} Therefore, 5 min of TUS application have sufficient biomechanical effects (compression and/or tension) to promote the mechanisms of cellular and matrix extracellular signaling for the realignment of the collagen fibers similar to that of the tendon with no injury.

The time application is not only dependent upon the size area of the injury, but also the amount of energy applied on the tissue as Alexander *et al.*²² demonstrated in a systematic review about the use of TUS in soft tissue shoulder pathology. The treatment time depends on the area of the lesion. The time recommended by Oakley²⁹ to treat the transducer area is 1 or 2 min per transducer area. According to this author, subsequent treatment times should be increased by 30 s. Hoogland³⁰ recommends a maximum treatment time of 15 min and at least 1 min in each treated area. Watson¹¹ assumes 1 min per transducer area. The Draper's article¹⁷ provides a basis on the time required for application as a function of the thermal effects required on muscle tissue. When it comes to the mechanical effect, the 5-min time appears frequently.^{7,27,31,32}

To our knowledge, the only experimental study that discusses the time of application is Farcic *et al.*,⁹ which indicates that times greater than 1 min per treatment area are more effective in aligning the collagen fibers of injured tendons. The results of the present study point out that application times greater than those proposed by Farcic

*et al.*⁹ provides a better tendon tissue healing, showing that the time of 5 min per transducer area promoted an alignment and aggregation state of the collagen fibers equal to the noninjured tendon.

It is worth mentioning that the size of the treated area should be considered in studies to allow future comparisons. The research of da Cunha *et al.*²⁴ and Koeke *et al.*²⁵ did not describe the size of the area to be treated, but the description of the remaining parameters provided the necessary information for the calculation of the treated area such as application time per transducer area.

One hypothesis to explain the differences between the results of this and previous studies is that the control of the treated areas may not have been accurately measured, making it impossible to compare findings.^{7,8,31,32}

da Cunha *et al.*²⁴ studied the effects of continuous and pulsed TUS on Achilles tendon injury in an animal model. The authors treated an experimental group with the same parameters of the present study, which includes the total application time of 5 min per section. The authors conclude that these groups had a better tendon healing process with a superior alignment of collagen fibers and a significant collagen deposition when compared with continuous and mock TUS groups. Our study showed that it is necessary to apply 5 min per transducer area, not only per section. As the treated area is twice transducer area, it is necessary to double the application time, totaling 10 min.

The dose standards employed in the present study were those most often used in TUS studies, except the treatment time, which is the variable studied. The frequency (1 MHz), intensity (0.5 W/cm²), and duty cycle (20%) were based on the research of Koeke *et al.*,²⁵ Farcic *et al.*,⁹ and Aiyegbusi *et al.*³²

The application time determines the amount of energy applied to the treated tissue. Alexander *et al.*²² showed that studies, which employed the average energy of 4.2 J per treatment session, were clinically effective, whereas those that applied an average of 2.019 J were not successful. Those energy values per treatment were obtained from the formula as follows: treatment energy (Joules) = SATA (W/cm²) × ERA (cm²) × application time (seconds), and it is important to remember that SATA intensity is calculated according the formula: SATA (W/cm²) = average intensity (W/cm²) × duty cycle (%).²² This systematic review indicates that it is necessary to increase the amount of energy applied to patients for ultrasound therapy to be effective. Data obtained in this study showed that the energy of 30 J (TUS5) presented the best result in

the organization and aggregation of collagen fibers in rat tendons.

Our results indicate that a high amount of energy with low SATA intensity and high application time promote a better modulation of the tendon tissue healing. We could use high intensity and shorter application times to obtain the same amount of energy added to the tissue, but high intensities are contraindicated in the early tissue repair process, since the thermal effects could increase the inflammatory reaction, which is not desirable at this stage of the injury.³³

Therefore, further researches should be conducted to determine the dosimetry of TUS. We recommend studies that aim at a higher efficiency of TUS with higher intensities and shorter application times and studies that investigate possible adverse effects related to long application times in animal models. Additionally, clinical trials are needed to confirm the *in vivo* and *in vitro* findings.

INNOVATION

It can be concluded that TUS applied for 5 min per transducer area provided the best results in the organization and aggregation of collagen fibers in rat tendons during the tissue repair process, producing significant changes in OR, with no significant differences when compared with CG.

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AUTHOR DISCLOSURE AND GHOSTWRITING

This article was expressly written by the authors listed. No ghostwriters were used.

ABOUT THE AUTHORS

Thiago Saikali Farcic, PT, PhD, is a professor at Universidade Paulista (UNIP), and re-

KEY FINDINGS

- The longer the application time, the better for organization and aggregation of collagen fibers in the rat tendon;
- TUS applied for 5 min per ERA provided the best results in the healing process of rat Achilles tendon rupture;
- TUS3, TUS4, TUS5, and CG demonstrated better organization of the collagen fibers than TG.

ceived his degree from Universidade de São Paulo (USP). He works at UNIP and USP, and researches the treatment of musculoskeletal injuries and physical therapy equipment in wound healing. **Cristiano Schiavinato Baldan, PT, PhD**, is a professor and Director of the Physical Therapy department at UNIP, and received his degree from USP. He works as a physiotherapist and researches physical therapy equipment in wound and tendon healing and the rehabilitation of orthopedic injuries at UNIP and USP. **Aline Fernanda Perez Machado, PT, MS**, received her degree from Universidade Federal de São Paulo. She works as a physiotherapist and is a professor at UNIP and Universidade Cidade de São Paulo, where her research interests include reconstruction and plastic surgery, aesthetic treatments, general women health and wound healing treatments, including physical therapy equipment in wound healing. **Leonardo Affonso Massabki Caffaro, PT, MS**, received his degree from USP where he works as a physiotherapist and researches physical therapy equipment in wound healing and in oncologic effects. **Igor Fagioli Bordello Masson, PT, PhD**, is a professor at UNIP, and received his degree from Universidade Federal de São Paulo. He works as a physiotherapist and researches physical therapy equipment in wound and tendon healing, and the rehabilitation of orthopedic injuries at UNIP. **Raquel Aparecida Casarotto, PT, PhD**, received her degree from Universidade Federal de São Paulo. She works as a physiotherapist and is a professor at USP, where she researches physical therapy equipment in wound and tendon healing. Her other research interests include wound healing treatments, oncological effects, and general physical therapy equipments.

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Abbreviations and Acronyms

- CG = control group
 ERA = effective radiating area
 OR = optical retardation
 SATA = spatial average temporal average
 TG = tenotomy Group
 TUS = times of ultrasound therapy