Histol Histopathol (2016) 31: 515-522

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Radiofrequency preserves histoarchitecture and enhances collagen synthesis in experimental tendon injury

Flavia Emi Akamatsu[1], Samir Omar Saleh[2], Flávio Hojaij[3], Carlos Augusto Real Martinez[4], Mauro Andrade[1], Walcy Rosolia Teodoro[5] and Alfredo Luiz Jacomo[1]

[1]Department of Surgery, Laboratory of Medical Research-Division of Human Structural Topography, Faculty of Medicine of the University of São Paulo (FMUSP), [2]Surgical Clinic of Faculty of Medicine of the University of São Paulo (FMUSP), [3]Department of Surgery Medicine, Laboratory of Medical Research, Faculty of Medicine of the University of São Paulo (FMUSP), [4]Post-Graduate Program in Health Sciences of the São Francisco University Medical School (USF), Bragança Paulista-SP and [5]Division of Rheumatology, Faculty of Medicine of the University of São Paulo (FMUSP), Brazil

Summary. We investigated the action of radiofrequency (RF) on the healing process after inducing experimental lesions of the Achilles tendon in rats. *Wistar* rats were surgically subjected to bilateral partial transverse sectioning of the Achilles tendon. The right tendon was treated with radiofrequency (RFT), whereas the left tendon served as a control (CT). On the third postoperative day, the rats were divided into three experimental groups consisting of ten rats each, which were treated with monopolar radiofrequency (Tonederm™) until they were sacrificed on the 7th, 14th or 28th days. The histological specimens were studied for inflammatory cell content, collagen types I and III, immunostaining and morphometry. Total collagen were biochemically analyzed and to evalute fibroblast and myofibroblast proliferation by vimentin and α-actin smooth muscle immunohistochemistry methods. Statistical analysis was performed using the Student's t-test, the sign test and the Kruskal-Wallis test to compare tendons treated with radiofrequency with the non-treated tendons (α=5%; α=10%). Larger amounts of collagen I with hydroxyproline content and myofibroblast cells were clearly evident within 7 days ($p<0.05$). No difference was observed in the inflammatory cell content between the groups. We found better collagen arrangement with RF administration across the entire time studied. Radiofrequency administration preserves histoarchitecture and enhances collagen synthesis during the initial phases of cicatrization, suggesting that the treatment can provide improved stiffness during the most vulnerable phases of tendon healing. Clinical studies may include RF among the therapeutic tools in tendinous lesion management.

Key words: Tendon, Collagen, Healing, Radiofrequency

Offprint requests to: Dra. Walcy Rosolia Teodoro, Faculdade de Medicina da Universidade de São Paulo, Disciplina de Reumatologia, Av. Dr. Arnaldo 455, sala 3124, CEP 01296-903, São Paulo, SP, Brasil. e-mail: matrix@lim17.fm.usp.br

DOI: 10.14670/HH-11-660

Introduction

Tendon injuries are common in adults, necessitating over 300,000 surgical tendon repairs each year in the United States (Pennisi, 2002). These injuries are not only responsible for large health care costs but also result in lost work time and individual morbidity (Dunning et al., 2010). Unfortunately, tendons heal poorly due to their limited regenerative potential, and many repairs require revision (Sharma and Maffulli, 2005). Moreover, recovery following tendon repair can be protracted, ranging from months to years, and at best, healed tendons retain 60% of their initial mechanical properties. Tendon injury may not only result in the deprivation of mobility or abnormal joint kinematics but may also cause damage to tissues adjacent to the joint, such as cartilage, which may eventually lead to morbidity, pain and osteoarthritis. For example, injury-related swelling and increased local pressure on a tendon may prevent

blood flow in the adjacent area. Lacerations and ruptures are two common acute tendon injuries, and both often occur in athletic settings. Once injured, a tendon undergoes a slow, spontaneous healing process, and surgical intervention is sometimes required to facilitate this process. However, tendon healing often results in the formation of scar tissue or disorganized matrix consisting largely of dense collagenous fibers, and hence, repaired tendons have inferior mechanical properties compared with intact tendons. Consequently, repaired tendons are at risk of re-injury at the repair site. Another common negative consequence of tendon healing is the formation of adhesions (Wong et al., 2009). Tendons are composed of connective tissue consisting of a parallel array of collagen fibers and elongated fibroblasts or fibrocytes that form a highly ordered extracellular matrix. Collagen molecules assemble into filamentous collagen fibrils, consisting of multiple microfibrils (Ottani et al., 2001), which aggregate to form collagen fibers. All of the hierarchical structural levels of these fibers, the main structural components, are aligned parallel to the long axis of the tendon, making the tendon ideal for carrying and transmitting large tensile mechanical loads (Provenzano and Vanderby, 2006). Degenerative changes have been held responsible for the total rupture of the human Achilles tendon, as supported by tendon histology studies in which abnormal orientation of the collagen fibers, changes in collagen composition and mucoid tissues, and hypoxic injuries have been implicated (Kannus and Józsa, 1991; Kannus et al., 1998). Tendon healing occurs in three overlapping phases. In the initial, inflammatory phase, erythrocytes and inflammatory cells, particularly neutrophils, enter the site of injury. In the first 24 hours, monocytes and macrophages predominate, and phagocytosis of the necrotic material occurs. Vasoactive and chemotactic factors are released with increased vascular permeability, the initiation of angiogenesis, the stimulation of tenocyte proliferation, and the recruitment of more inflammatory cells (Murphy et al., 1994) as well as the initiation of type III collagen synthesis (Ippolito et al., 1980; Lin et al., 2004). Within approximately 6 weeks, the remodeling stage commences. During this stage, the healing tissue is resized and reshaped. A corresponding decrease in cellularity, collagen and glycosaminoglycan synthesis occurs (Murphy et al., 1994; Samuel, 2009). Recent tissue regeneration strategies aim to improve the outcome of tendon repair. In this study, we chose to use the model of Achilles tendon injury in rabbits reported by Tibor et al. (2012) which was modified for rats, in which the intensity of the lesion is expressive, allowing the evaluation of different therapeutic approaches with homogeneity of results.

Radiofrequency (RF) administration produces heat due to molecular friction caused by polarity changes, and its therapeutic use aims at the reduction of a local inflammatory response (Tasto et al., 2003). This therapeutic approach has been used during surgery on the patellar tendons of rabbits (Hecht et al., 1999; Tibor et al., 2012) to shrink redundant collagenous tissue through the effects of thermal energy and to increase joint stability. The influence of shrinkage on the biomechanical properties of collagenous tissue has been evaluated in this model (Hecht et al., 1999; Pötzl et al., 2004).

The RF effect has been observed in another modality of application (coblation and ablation) via angiogenesis to regulate a variety of growth factors, such as vascular endothelial growth factor (VEGF) and proteins of the cellular membranes; however, this effect has not been observed in non-ablative modalities (Tasto JP et al., 2003). RF ablation enhances angiogenesis in the early stages of healing and yields a quicker return to mechanical integrity of rabbits' Achilles tendons (Tibor et al., 2012). This observed mechanical integrity could be related to the effect of RF administration on the arrangement and orientation of the collagen fibers as well as the synthesis and quantity of different types of collagen. We aim to evaluate the effect of non-ablative RF administration on collagen synthesis and to measure the remodeling of collagen I and collagen III after 7, 14 and 28 days following the experimental injury. These data will contribute to the use of RF administration as a therapy for Achilles tendon lesions.

Materials and methods

Animals and experimental protocol

This study was approved by the Ethics Committee for the Analysis of Research Projects Protocol 164/10. All animals received humane care in compliance with the experimental protocols of the Ethical Principles in Animal Experiments adopted by the Brazilian Association of Animal Testing.

Sixty adult male *Rattus norvegicus albinus Wistar* individuals weighing 250 to 300 g were housed in the São Paulo University School of Medicine's facilities. Five animals were housed per cage and were given food and purified tap water ad libitum. The animals were operated upon in the CEPEC (Center for Study and Research in Surgery, Department of Urology of the Faculty of Medicine). For the experiment, the right Achilles tendon was selected; the left Achilles tendons of the same animals were used as controls. Rats were placed under general anesthesia with 4% isoflurane and maintained anesthetized by mask using a 1.5-3% inspired fraction of isoflurane. After anesthesia, the distal portions of the right and left legs of the animal were subjected to disinfection with 10 mg/l chlorhexidine gluconate solution and shaved.

The animals were placed in a sterile field on a heated surgery table and covered with a sterile surgical drape. The skin was cut longitudinally 0.5 cm from the calcaneal insertion lateral to the tendon; both the peritendons and tendons were exposed. The animals were surgically subjected to bilateral partial transverse

sectioning of the Achilles tendon. The injury in this animal model was realized by dissection and transverse hemisection using a no. 15 scalpel blade perpendicular to the collagen fibers, 2.5 mm from the Achilles insertion on the lateral side of the Achilles tendon. We took care to separate the flexor hallucis longus, which is medially adjacent to the Achilles tendon. After transection, the skin was closed by a continuous suture with Prolene 0-6 (Ethicon). After surgery, the rats were kept warm until they recovered consciousness. The rats were administered analgesics (200 mg/kg paracetamol) orally every 24 hours for three days. The rats were left without cast immobilization. During the time they were kept housed, they were acclimatized to 12 h light-dark cycles. On the third postoperative day, the rats were divided into three experimental groups and treated with monopolar RF (Tonederm™) adjusted to 650 kHz and 2 W for two minutes twice weekly until they were sacrificed on the 7th, 14th or 28th days. The contact area for RF administration was circular with an area of 0.78 cm^2 and a diameter of 10 mm to contact the tendon of the animal. After treatment, the animals were euthanized using CO_2 chamber. The animals were placed in chambers previously filled with pure gas, or preferably, gas with 30% added oxygen to reduce stress caused by hypoxia. Because CO_2 is twice as heavy as air, it sits at the bottom of the chamber, and the chamber opening was oriented to the top. CO_2 lethality stems from central nervous system depression. The animals were kept in the chamber for 10 minutes until their deaths were confirmed (Nolen, 2009).

Specimen preparation

Achilles tendons were collected through dissection, with the animal placed in the prone position. The specimens were collected for histological and immunofluorescence analysis and quantification of collagen. To study the effects of RF administration on the matrix organization, the Achilles tendons were fixed by immersion in 10% neutral buffered formalin. After decalcification for 1 to 4 days with 7% nitric acid, the tissue was neutralized by soaking it in a sodium sulfate solution for one day and then washed for 10 hours under running water. The tendon samples were cut from a longitudinal position. After being embedded in paraffin, the tissue was sliced using a microtome. From each tendon, 6 slices were obtained at 4-5 μm thickness spaced at 50 μm. Thin sections were stained according to the hematoxylin and eosin (HE) double staining method. Picrosirius staining was performed using 0.2% Sirius red (Direct Red 80, C. I. 35780, Aldrich, Milwaukee, WI) (Dayan et al., 1989) diluted in a saturated picric acid solution, which was applied to observe the structure of the collagen fibers. Additional subserial sections from all of the paraffin blocks were used for immunohistochemistry and immunofluorescence (Junqueira et al., 1982).

Histomorphometry of the inflammatory infiltrate

To quantify the stained cells, a point-counting stereologic method (Gundersen et al., 1988) was employed using a reticulum formed by 100 points and 50 lines, each measuring 25 μm in length, adapted to a conventional microscope. At 400× magnification, the Achilles tendons covered in each field were calculated according to the number of points hitting connective tissue as a proportion of the total grid area. Then, we counted the number of total cells within the tendon. The inflammatory cells were quantified as the number of cells present in each field divided by the tissue area. The final results were then transformed to a cells/mm^2 metric by adjusting the units.

Immunofluorescence and immunohistochemistry

Antigen retrieval was carried out by enzymatic treatment of the Achilles tendons with 4 mg/ml bovine pepsin (10,000 U/ml) (Sigma Diagnostics, St. Louis, USA) in 0.5 N acetic acid followed by incubation for 45 minutes at 37°C. Then, the slips were incubated overnight with a rabbit polyclonal antihuman Col I and III antibody [1:50 Sigma Chemical Co., Saint Louis, MO, USA] produced in our laboratory according to the previous instructions of Miller and colleagues (Miller and Rhodes, 1982). For negative controls, sections were incubated with fetal bovine serum instead of the primary antibody. The same tissue treatment was used for the immunofluorescence detection. The sections were incubated with the same primary antibody, diluted with PBS plus 1% bovine serum albumin overnight at 4°C in a humid atmosphere. The sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Sigma Chemical Co.; dilution 1:50) as a secondary antibody and mounted in an aqueous mounting medium. The specificity of the antibodies was validated by Western blot. The Col I and III fibers were quantified in the Achilles tendon control and experimental groups by image analysis. Briefly, two assessors blinded to animal group employed an image analysis system, which consisted of an Olympus camera mounted on an Olympus microscope which sent the images to an LG monitor using a computer-controlled (Pentium 1330 Mhz) digitizing system (Oculus TCX, Coreco Inc., St. Laurent, Quebec, Canada). The images were processed by *Image-Pro Plus 6.0* software (Media Cybernetics, Inc., Bethesda, MD, USA). For each tendon, sixteen samples each from the control group and the experimental group were analyzed at a magnification of 400x. The collagen fiber densities in this compartment were expressed as the number of fibers divided by the total area studied. The final results were expressed as the number of collagen fibers per total area (Samuel, 2009).

To evaluate vimentin in the Achilles tendons, 4 μm paraffin sections were immuno-stained with goat

polyclonal vimentin (1:4000) (C-20 Santa Cruz Biotechnology Inc.; sc-7557) and monoclonal anti-α smooth muscle actin (1:5000) (Clone 1 a 4 Dako; A 2547) diluted in BSA. Streptavidin was used in combination with biotinylated horseradish peroxidase (Dako Corp.; dilution 1:1000) and diaminobenzidine tetrahydrochloride in the context of Harris Hematoxylin cytoplasmic counterstaining to detect evidence of antigen expression by the cells.

Collagen quantification using 4-hydroxyproline

The collagen content was determined by measuring the 4-hydroxyproline content according Bergman and Loxley (1972). The dry weights of lyophilized samples (n=40 for each time point) were measured, and the samples were transferred to 13x100 mm culture tubes (Corning,NY14831) containing 5 ml of 6 N HCL. In separate tubes, 0 (blank), 4, 6, 8, 10 and 12 μg amounts of the 4-hydroxyproline standard (Sigma) were prepared to establish a standard curve for each experiment. The samples were hydrolyzed at 100°C for 22 hours and then oxidized by adding chloramine-T reagent and incubating them at room temperature for 25 min. After oxidation, a chromophore was developed by adding Ehrlich's reagents to each sample and incubating them at 65°C for 20 min. The absorbance of each standard and sample was measured at 560 nm by spectrometry. The collagen content was calculated by multiplying the 4-hydroxyproline content by 7.0, as has been previously described (Samuel, 2009). The collagen content for each sample was determined by dividing the total collagen content by the total weight of each sample.

Statistical analysis

For quantitative variables, the means ± standard errors of the mean results from replicated samples, or from combined independent experiments when between-experiment variation allowed the reliable combination of raw data, were compared. Comparison of multiple groups was performed using analysis of variance (ANOVA) followed by Bonferroni post hoc tests, or a Student's t-test, sign test and Kruskal-Wallis test to compare tendons treated with RF with non-treated and normal tendons. The statistical procedures were performed with SPSS statistical software, version 17.0 (SPSS Inc., Chicago, Ill., USA).

Results

Effect of RF administration on histological features of healing tendons

Histopathologic analysis showed that the groups treated with monopolar RF (RFT) after 7 days displayed normal architecture, characterized by parallel or linear orientation of collagen bundles, a low degree of vascularization and fibroblast proliferation (Fig. 1D). In contrast, the CT specimens displayed remarkable architecture distortion, which was modified by large pale areas with the disruption of the normal linear orientation of collagen bundles and characterized by a wavy pattern of fibrils (Fig. 1A). No significant difference in the inflammatory infiltrate was observed between the CT and RFT groups at any time studied (Fig. 1B,D,F,H,J,L). These results were supported by the analysis of collagen fibers in the RFT group stained with picrosirius and observed under polarized light after 7 days, which showed a more linear pattern during recovery (Fig.1C) compared with the CT group (Fig. 1A). Nevertheless, there was no difference in the morphologic evaluation between the RFT and CT groups at 14 or 28 days.

Through the quantitative analysis using 4-hydroxyproline, the total collagen content in the tendon samples was demonstrated to be significantly higher for the RFT group after 7 days compared with the CT group ($P<0.05$); no differences between CT and the other groups were found (Fig. 3B).

Analysis of Col I, Col III, vimentin and α-Actin smooth muscle expression in tendons

The immunofluorescence analysis of collagen I showed a significant increase in the expression of this protein in the RFT group after 7 days of treatment (Fig. 2B) compared with the CT group (Fig. 2A), corroborating the data obtained through the HE and picrosírius staining analyses (Fig. 1A,C). The immunohistochemistry for vimentin and α-actin smooth muscle showed higher expression of vimentin in the RFT group after 7 days compared with the CT group (Fig. 2C,D), corroborating the data obtained through the HE analysis that identified the fibroblasts in the damaged area (Fig. 1A,C). The α-actin smooth muscle presented similar features in the RFT and CT groups at this time.

Additionally, the histomorphometric analysis of the tendons in the damaged area also showed a significant increase in Col I ($p<0.10$) (Fig. 3A), which is in agreement with our results pertaining to biochemical total collagen (Fig. 3B). However, no difference was observed between the CT and RFT groups for Col I at 14 or 28 days, and Col III did not show significant

Table 1. Total 4-Hidroxyproline (μg/mg) quantification in experimental tendon injury in rats treated with radiofrequency and controls.

Group/Time	7 days	14 days	28 days
Control (CT)	53.53±8.73	74.21±28.37	73.13±15.76
Radiofrequency (RFT)	*60.01±7.84	62.24±11.19	69.65±21.67

Date are expressed on Means ± SD from triplicate determinations; *$p<0.05$.

differences at any time studied.

Discussion

This study shows that RF preserves the structure of the fibers and stimulates the synthesis of collagen type I in the initial, most vulnerable stage of the tendon healing process. We found better collagen arrangement upon RF administration at all the times studied, suggesting closer regeneration to a normal tendon, which is the goal of collagen arrangement during tendon healing. Seven days after surgery, new collagen fibers grew extensively in the repair site in a random arrangement; after fourteen days, the collagen fibers began to form an axial morphological aspect (Sasaki et al., 2012). These results were similar to those found by Sheffler et al. (2005a,b), who observed intense collagen remodeling after RF treatment. The increase in collagen I matrix protein expression in experimental tendons seven days after surgery indicates that the RFTs were stiffer than the CTs at the repair site, which may indicate an increased rate of repair over the first week, therefore leading to less susceptibility to rupture. The tissue predisposition to rupture has been related to the production of fine fibers, such as collagen type III (Maffulli et al., 2000; Satomi et al., 2008).

The amount of collagen I was higher after seven days; thus, RF administration appears to preserve collagen I in tissue at the beginning of the tendon healing process, thus giving better structure to the collagenous matrix in this vulnerable phase and suggesting an improvement in the viscoelasticity of the tissue. The viscoelasticity of tendons results from collagenous proteins, water, and the interactions between collagens and proteoglycans (Purslow et al., 1998; Elliott et al., 2003). Conversely, at high strain rates, tendons become stiffer and more effective in transmitting large muscular loads to bone (Wang, 2006).

Type I collagen increased after RF treatment

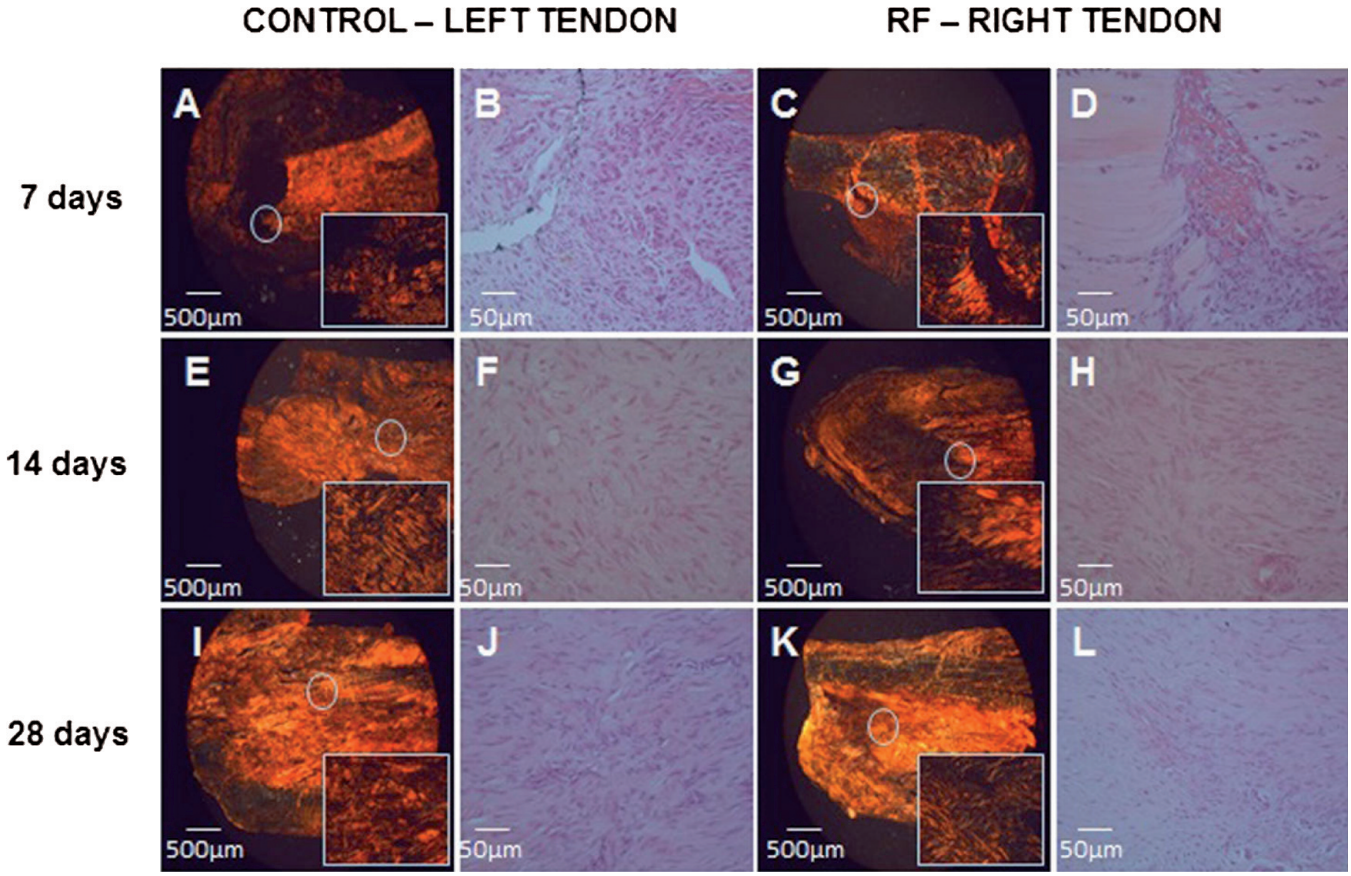

Fig. 1. Tendon samples obtained from CT and RFT therapy group - rats stained with picrosirius **(A, C, E, G, I, and K)** and hematoxylin-eosin **(B, D, F, H, J, and L)**. Note the linear and parallel orientations of the collagen bundles after 7 days of treatment (**C**, detail), indicated by an increase in reddish-orange birefringence and by the inflammation in the lesion region **(D)** when compared with architecture distortions in their collagen bundles (**A**, detail) and diffuse inflammatory cells **(B)** in the CT group. After 14 **(E and G)** and 28 **(I and K)** days, the orientations of the collagen bundles and inflammatory cells of the 14- **(F and H)** and 28-day **(J and L)** groups were similar to those of the CT group.

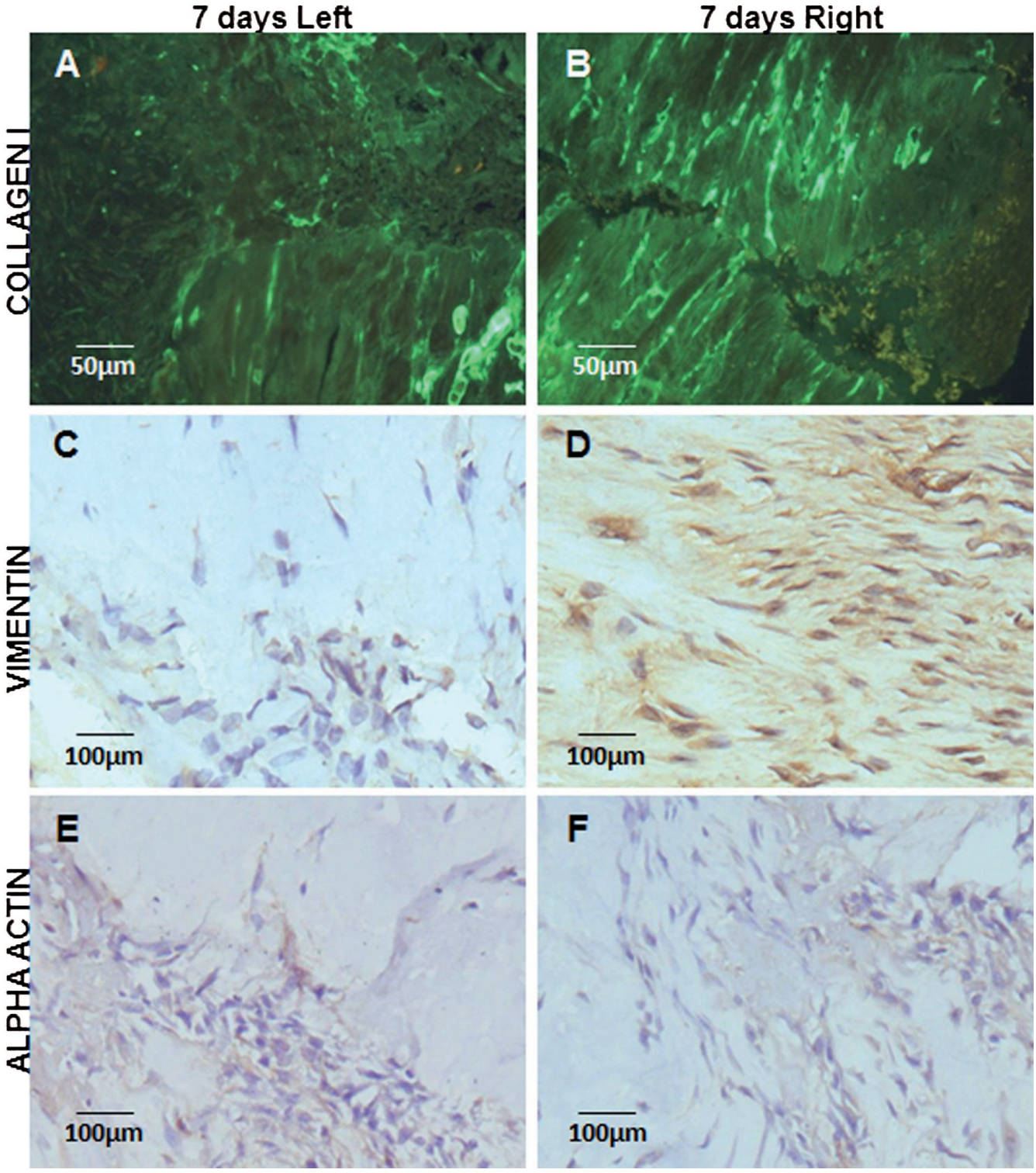

Fig. 2. Immunofluorescence of collagen I and immunohistochemistry for vimentin and α-actin smooth muscle of tendon samples obtained from the control (CT) and RF therapy (RFT) groups of rats. The RFT group shows intense collagen I expression **(B)** characterized by an increase in thick fibers in the injured area compared with the CT group **(A)**. Note the increase in vimentin expression in the injured area **(D)** in the RFT group compared with the CT group **(C)**. In contrast, actin smooth muscle expression was similar for the RFT **(E)** and CT **(F)** groups for this treatment period.

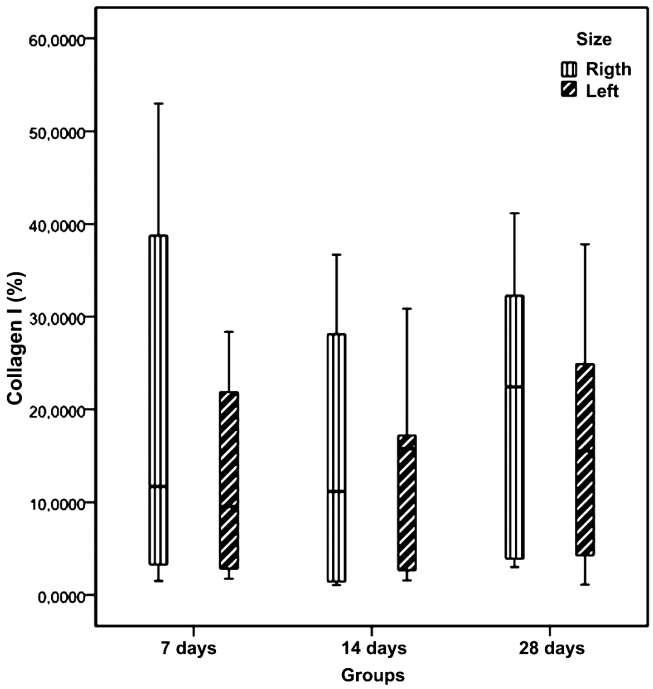

Fig. 3. Morphometric analysis of type I collagen fibers in the tendons submitted to radiofrequency therapy RFT (right) CT (left). The green fluorescence areas for type I collagen were quantified with significant increase in collagen I after 7 days of RFT administration; *p<0.10.

observed in ours histomorphometric results is of great importance to the recovery of the tendon, because this type of collagen is the main constituent of thick fibers, and it promotes the resistance of structures such as tendons and ligaments.

Additionally, we observed increased expression of vimentin in the lesion after 7 days of treatment with RF, showing that fibroblast proliferation was stimulated in the evaluated region, which once again may explain the increased expression of collagen I observed in our study. Moreover, α-actin smooth muscle in the same region was equivalent between groups, suggesting that RF administration may stimulate collagen synthesis without changing the phenotypes of the cells. These results optimize the use of RF as an initial therapy in the tissue recovery process because a change in the amount of α-actin smooth muscle would be expected following injury should fibrosis occur.

We did not detect the involvement of RF in the inflammatory processes of the studied cells. Observations of this phenomenon have been inconsistent in the literature; previously, when lasers have been used on tendons and skin, they have resulted in both decreased (Laraia et al., 2012) and increased inflammation (Choi et al., 2013). Such discrepant findings can be explained by the differences between stimuli and application protocols, which are likely to yield wide variation. Despite the existence of few conclusive studies using RF therapy for tendon recovery, we suggest another therapeutic method for recovering injured tendons, especially in the early stages of the healing process, which is the most vulnerable phase of regeneration. RF administration remains very speculative due to controversial and varied results and especially due to the lack of an application protocol.

References

Choi S., Shin J.H., Nam S.W., Jang H., Tao T., Kwak H.W., Jin K.H., Lee G.I. and Park H.K. (2013). Mid-Long term effect of non-ablative high radiofrequency therapy on the rabbit dermal extracellular matrix. Conf. Proc. IEEE Eng. Med. Biol. Soc. 2013, 3761-3764.

Dayan D., Hiss Y., Hirshberg A., Bubis J.J. and Wolman M. (1989). Are the polarization colors of picrosirius red-stained collagen determined only by the diameter of the fibers? Histochemistry 93, 27-29.

Dunning K.K., Davis K.G., Cook C., Kotowski S.E., Hamrick C., Jewell G. and Lockey J. (2010). Costs by industry and diagnosis among musculoskeletal claims in a state workers compensation system: 1999-2004. Am. J. Ind. Med. 53, 276-284.

Elliott D.M., Robinson P.S., Gimbel J.A., Sarver J.J., Abboud J.A., Iozzo R.V. and Soslowsky L.J. (2003) Effect of altered matrix proteins on quasilinear viscoelastic properties in transgenic mouse tail tendons. Ann. Biomed. Eng. 31, 599-605.

Gundersen H.J., Bagger P., Bendtsen T.F., Evans S.M., Korbo L., Marcussen N., Møller A., Nielsen K., Nyengaard J.R. and Pakkenberg B. (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96, 857-881.

Hecht P., Hayashi K., Lu Y., Fanton G.S., Thabit G. 3rd, Vanderby R. Jr and Markel M.D. (1999). Monopolar radiofrequency energy effects on joint capsular tissue: potential treatment for joint instability. An in vivo mechanical, morphological, and biochemical study using an ovine model. Am. J. Sports Med. 27, 761-771.

Ippolito E., Natali P.G., Postacchini F., Accinni L. and De Martino C. (1980). Morphological, immunochemical, and biochemical study of rabbit Achilles tendon at various ages. J. Bone Joint Surg. Am. 62, 583-998.

Junqueira L.C., Montes G.S. and Sanchez E.M. (1982). The influence of tissue section thickness on the study of collagen by the Picrosirius-polarization method. Histochemistry 74, 153-156.

Kannus P. and Józsa L. (1991). Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. J. Bone Joint Surg. Am. 73, 1507-1525.

Kannus P., Jozsa L.,Jarvinen T.A., Jarvinen T.L., Kvist M., Natri A. and Jarvinen M. (1998). Location and distribution of non-collagenous matrix proteins in musculoskeletal tissues of rat. Histochem. J. 30, 799-810.

Laraia E.M., Silva I.S., Pereira D.M., dos Reis F.A., Albertini R., de Almeida P., Leal Junior E.C., de Tarso C. and de Carvalho C. (2012). Effect of low-level laser therapy (660 nm) on acute inflammation induced by tenotomy of achilles tendon in rats. Photochem. Photobiol. 88, 1546-1550.

Lin T.W.,Cardenas L. and Soslowsky L.J. (2004). Biomechanics of tendon injury and repair. J. Biomech. 37, 865-877.

Maffulli N., Ewen S.W., Waterston S.W., Reaper J. and Barrass V. (2000). Tenocytes from ruptured and tendinopathicachilles tendons produce greater quantities of type III collagen than tenocytes from

normal achilles tendons. An in vitro model of human tendon healing. Am. J. Sports Med. 28, 499-505.

Miller E.J. and Rhodes R. K. (1982). Preparation and characterization of the different types of collagen, Methods Enzymol. 82 (Pt A), 33-64.

Murphy P.G., Loitz B.J., Frank C.B. and Hart D.A. (1994). Influence of exogenous growth factors on the synthesis and secretion of collagen types I and III by explants of normal and healing rabbit ligaments. Biochem. Cell Biol. 72, 403-409.

Nolen R.S. (2009) Revision process begins for AVMA euthanasia guidelines. J. Am. Vet. Med. Assoc. 235, 246-247.

Ottani V., Raspanti M. and Ruggeri A. (2001). Collagen structure and functional implications. Micron 32, 251-260.

Pennisi E. (2002). Tending tender tendons. Science 295, 1011.

Pötzl W., Heusner T., Kümpers P., Marquardt B. and Steinbeck J. (2004) Does immobilization after radiofrequency-induced shrinkage influences the biomechanical properties of collagenous tissue? An in vivo rabbit study. Am. J. Sports Med. 32, 681-687.

Purslow P.P., Wess T.J. and Hukins D.W. (1998). Collagen orientation and molecular spacing during creep and stress-relaxation in soft connective tissues. J. Exp. Biol. 201(Pt 1), 135-142.

Provenzano P.P. and Vanderby R. Jr (2006). Collagen fibril morphology and organization: implications for force transmission in ligament and tendon. Matrix Biol. 25, 71-84.

Samuel C.S. (2009). Determination of collagen content, concentration, and sub-types in kidney tissue. Methods Mol. Biol. 466, 223-225.

Sasaki K., Yamamoto N., Kiyosawa T. and Sekido M. (2012). The role of collagen arrangement change during tendon healing demonstrated by scanning electron microscopy. J. Electron Microsc. (Tokyo) 61, 327-334.

Satomi E., Teodoro W.R., Parra E.R., Fernandes T.D., Velosa A.P., Capelozzi V.L. and Yoshinari N.H. (2008). Changes in histoanatomical distribution of types I, III and V collagen promote adaptative remodeling in posterior tibial tendon rupture. Clinics (São Paulo) 63, 9-14.

Sharma P. and Maffulli N. (2005). Tendon injury and tendinopathy: healing and repair. J. Bone Joint. Surg. Am. 87, 187-202.

Scheffler S., Chwastek H., Schönfelder V., Unterhauser F., Hunt P. and Weiler A. (2005a). The impact of radiofrequency shrinkage on the mechanical and histologic properties of the elongated anterior cruciate ligament in a sheep model. Arthroscopy 21, 923-33.

Scheffler S., Chwastek H., Schönfelder V., Hunt P. and Weiler A. (2005b). The impact of radiofrequency shrinkage on the mechanical and histologic properties of the elongated anterior cruciate ligament in a sheep model arthroscopy. J. Arthroscopy Rel. Surg. 21, 923-933.

Tasto J.P., Cummings J., Medlock V., Harwood F., Hardesty R. and Amiel D. (2003). The tendon treatment center: new horizons in the treatment of tendinosis. Arthroscopy 19 (suppl 1), 213-223.

Tibor L.M., Leek B.T., Chase D.C., Healey R.M., Linn M.S., Tasto J.P. and Amiel D. (2012). A biomechanical assessment of tendon repair after radiofrequency treatment. Am. J. Orthop. (Belle Mead NJ) 4, E115-121.

Wang J.H. (2006). Mechanobiology of tendon. J. Biomech. 39, 1563-1582.

Wong J.K., Lui Y.H., Kapacee Z., Kadler K.E., Ferguson M.W. and McGrouther D.A. (2009). The cellular biology of flexor tendon adhesion formation: an old problem in a new paradigm. Am. J. Pathol. 175, 1938-1951.

Accepted September 4, 2015