ALTERATIONS IN THE ACHILLES TENDON AFTER INFLAMMATION IN SURROUNDING TISSUE

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ABSTRACT

Objective: To analyze the characteristics of the Achilles tendon of rats after induction of localized inflammation in the rat paw. Methods: In our study three groups were used: inflamed group with carrageenan in rat paw (G1); saline group (G2) and control group (G3). After 4 hours the animals were euthanized and the Achilles tendon removed. Results: No significant differences were observed in the analysis of non-collagenous proteins, glycosaminoglycans and hydroxyproline in the groups but a tendency of reduction was verified in G1. As regards the organization of collagen molecules, no differences were observed between groups. With respect to MMPs activity, a stronger presence of the active isoform of MMP-2 in G1 was observed, suggesting that the remodeling was occurring. Conclusion: Thus, we conclude that the inflammatory process in rat paw may affect the remodeling of tendons located near the inflamed site. ***Level of Evidence I, Prognostic Studies – Investigating the Effect of a Patient Characteristic on the Outcome of Disease.***

Keywords: Achilles tendon/injuries. Inflammation. Carrageenan.

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INTRODUCTION

The number and the incidence of tendon injuries have increased substantially in recent decades.[1,2] It has been estimated that tendon injuries account for 30% to 50% of all sports-related injuries. Among these injuries, those that affect the Achilles tendon have been prevalent both among athletes and in the public in general. The Achilles tendon is considered the strongest in the human body,[3] but it is also the most affected by injuries, followed by the digital flexor tendon.[4]
Achilles tendon injuries generally have two different origins: (1) some symptoms are only caused by injury induced by excessive load or tendon degeneration (without any predisposing systemic disease); and (2) sometimes a systemic disease, such as rheumatoid arthritis, can manifest with symptoms in the Achilles tendon.[3]
The extracellular matrix (ECM) of the tendons is composed of collagen (mainly type I) and elastin, both comprising 65-80% and 1-2% of the tendon dry mass, respectively. Collagen and elastin are soaked in a matrix of proteoglycans, non-collagenous proteins and water.
The elements that take part in the post-injury tendon repair process, acting through the healing phases, include enzymes such as metalloproteinases (MMPs), which are involved in the remodeling of the ECM of the tendons.[5] MMP-2 (gelatinase) digests gelatin, which is a denatured form of collagen. It is reported in literature that the concentration of MMP-2 is high after injury, taking part both in the degradation and in the remodeling of collagen.[6]
Tendons and ligaments are structures that are affected by different pathologies such as strain injuries, infection and inflammation, with possible rupture. Many workers and athletes who make repetitive efforts need to temporarily suspend their daily activities when there is tendon inflammation.[7]
Inflammation can occur directly in tendons or ligaments, but in many cases the inflammation can be found in surrounding tissues. The inflammatory process in tissues close to the Achilles tendon and the alterations caused by this process in the ECM of the actual tendon is still somewhat of a mystery. Recent studies on the flexor digitorum profundus tendon[8] and on the supraspinatus muscle tendon in the shoulder[9] showed biochemical and structural changes in the ECM of these tendons when close to an inflammation site. However, there are no studies demonstrating whether there are alterations in the Achilles tendon when there is inflammation in surrounding tissues. Our analysis consists of verifying whether an inflammatory process in rat paws causes alterations in the Achilles tendon.

MATERIAL AND METHODS

Experimental Groups

The use of the animals was in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and is consistent with the ethical principles for animal experimentation adopted by the Colégio Brasileiro de Experimentação Animal (COBEA)

All the authors declare that there is no potential conflict of interest referring to this article.

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and was approved by the Committee of Ethics in Animal Experimentation of the Universidade Estadual de Campinas, SP, Brazil and filed under no. 2259-1.

Male Wistar rats weighing 140-160g were maintained with free access to feed and water during the experimental period. The animals were divided into 3 Groups: (G1) that received subcutaneous injection of 1% (0.1ml) carrageenan type IV (Sigma code 22039) dissolved in saline,[10] (G2) that received saline (0.9% NaCl) and (G3) that did not receive application in the right paw. Four hours after application the animals were euthanized by means of an isoflurane overdose and their tendons were collected.

Quantification of proteins and sulfated glycosaminoglycans

The tendon fragments were immersed in 50 volumes of 4M guanidine hydrochloride, and the supernatant was used for biochemical analyses of non-collagenous proteins by the Bradford method[11] and glycosaminoglycan dosage by the dimethylmethylene blue method.[12]

Quantification of hydroxyproline

The tendon fragments were immersed in acetone for 48 hours and chloroform-ethanol for 48 hours. Afterwards, the fragments were hydrolyzed with HCl 6N (1ml/10mg tissue) and the hydrolyzate was neutralized with NaOH 6N and treated with a solution of 1.41% chloramine T and 15% p-dimethylaminobenzaldehyde. The samples were subsequently incubated at 60°C for 15 minutes. The solution with hydroxyproline was cooled until it reached room temperature and the absorbances were measured in 550nm.

Zymography for gelatinases

The tendons were treated according to Marqueti *et al.*[13] The tendon fragments were immersed in a solution of 50mM tris-HCl (pH 7.4), 0.2M NaCl, 10mM $CaCl_2$, and 0.1% triton and a 1% protease inhibitor cocktail (Sigma P8340) for the extraction of proteins at 4°C for 2 hours. In the gels, 20μg of protein were applied per sample. The gel containing 10% acrylamide and 0.1% gelatin was kept at 4°C and after the end of the electrophoresis, the gel was washed with 2.5% triton x-100 and incubated for 21 hours in a solution of 50mM tris-HCl (pH 7.4) 0.1M NaCl and 0.03% sodium azide at 37°C. The gel was stained with coomassie brilliant blue R 250 for 1 hour. The gels were washed with a solution containing 50% methanol and 10% acetic acid, for observation of the negative bands of the proteins corresponding to the enzymes. The bands were quantified in negative image by densitometry using the Scion Image Alpha 4.03.2 software.

Analysis by light microscopy

The tendons were fixed in a solution contend 4% formaldehyde in Millonig's buffer (0.13M sodium phosphate, 0.1M NaOH - pH 7.4) for 24 hours at room temperature. Next the tendons were washed under running water for 6 hours, dehydrated in alcohol baths, submitted to diaphanization with xylol baths and embedded in paraffin (Histosec, Merck). The surgeon made a series of 7 μm lengthwise cuts. For observation of the collagen fibers, the sections were stained with 0.025% Ponceau SS and 2% acetic acid for 1.5 minutes and linear dichroism was observed under a polarization microscope.[14]

STATISTICAL ANALYSIS

The data were presented as mean ± standard deviation. The results were obtained with a number of five animals per group. After the data collection, we conducted the ANOVA statistical analysis of variance followed by Tukey's test with significance level $p<0.05$. The statistical program used was GraphPad Prism®, version 3.0.

RESULTS

In our study, we analyzed the extracellular matrix of the Achilles tendon after applying the inflammatory agent on the rat paw. In 4 hours, an edema was observed on the paw of the animals from G1 (result not shown), confirming that the inflammation induced by the carrageenan is occurring. This edema was not detected in other groups.

Our results showed that the tendon undergoes some alterations in the concentration of its structural elements, such as hydroxyproline, glycosaminoglycans and non-collagenous proteins. (Figures 1, 2 and 3) These elements showed lower concentration in G1 when compared with G2 and G3. However, these results were not significantly different ($p>0.05$), but this indicates a tendency to decrease in rats that received the inflammatory agent on the paw. As can be seen in Figures 4 and 5, the analysis of the MMP-2 bands in the zymography showed the active isoform (62 kDa) of MMP-2 with greater activity in G1 than in G2 and G3. The proMMP-2 had an increase in G1 and G2 and the activity of MMP-2 (active and intermediate isoform) increased in G1 in relation to G2 and G3. MMP-9 was not detected in the groups. As regards the linear dichroism that detects the organization of the collagen molecules, no organization was observed in the groups. (Figure 6)

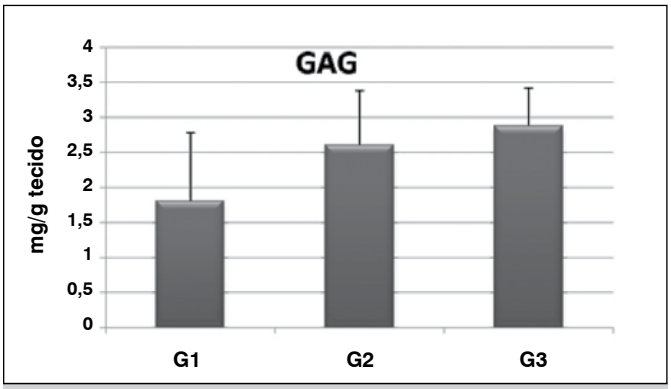

Figure 1. Concentration of *glycosaminoglycans (mg/g of tissue). A lower concentration was observed in G1, yet it is not significant when compared to G2 and G3.*

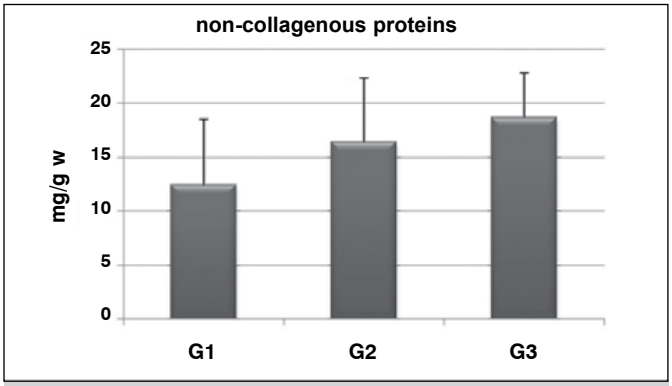

Figure 2. *Concentration of non-collagenous proteins (mg/g of tissue) A lower concentration was detected in G1, yet there was no significant difference when compared to G2 and G3.*

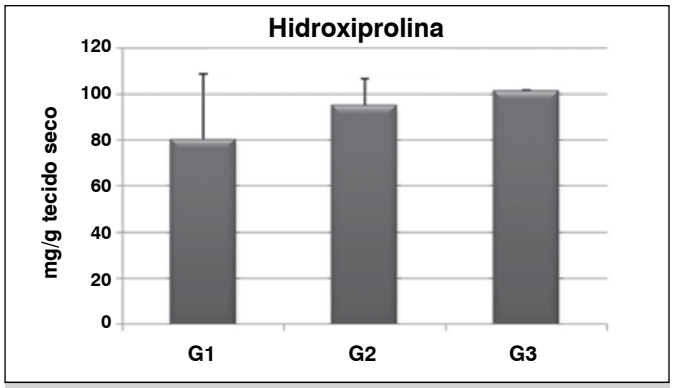

Figure 3. *Concentration of hydroxyproline (mg/g of dry tissue) from Achilles tendon. A lower concentration was detected in G1, yet there was no significant difference when compared to G2 and G3.*

DISCUSSION

Pathologies that affect the tendons, which include chronic pain and rupture, are now denominated "tendinopathies". Although the role of inflammation is still under discussion, it is known that tendinopathies are primarily degenerative conditions and that there are often no inflammatory cells around the lesion.[15]

In our analyses, we observed the effect of induced rat paw inflammation on the extracellular matrix of the Achilles tendon. Although the results of the dosages of GAG, PNC and HO-Pro in G1, G2 and G3 did not prove significantly different, we observed less concentration of these components in G1. Previous studies have demonstrated a decrease in the concentration of PNC and GAG, and structural changes in the flexor digitorum profundus tendon (FDPT) when analyzed 4 hours after rat paw inflammation induction and even cell infiltrate was evidenced in the epitenon of these tendons.[8] However, the Achilles tendon did not demonstrate structural modifications in the epitenon, or in the collagen fibers that constitute this tendon (results not shown). It is known that one of the first events to occur during an inflammatory process is the degradation of the extracellular matrix elements, for subsequent reorganization of the tissue,[16] a fact that can explain the decrease of these elements in the extracellular matrix.

The metalloproteinases (MMPs) form the main group of enzymes responsible for regulating the composition of the cellular and extracellular matrix components. The expression of the majority of MMPs is usually low in the tissues and is induced when extracellular matrix remodeling proves necessary.[17] MMPs are important regulators of extracellular matrix remodeling, and their levels change over the course of the healing and inflammation process[18] with the objective of reestablishing homeostasis through degradation. It is assumed that the greater activity of MMP-2 in G1 can explain the tendency for a decrease in HO-Pro, GAGs and PNC. The proMMP-2 increased in G1 and G2, yet in G3 it remained in a low quantity, according to the densitometry results. MMP-9 is an indicator of the inflammatory process in the tissue[19,20] yet it was not detected in our analysis, and even though our results indicated alterations in relation to gelatinases of the extracellular matrix, we can affirm that the tendon was not inflamed.

We believe that the inflammatory process had a less severe effect on the extracellular matrix of the FDPT than in previous studies[9] where an infiltration of macrophages and disorganization of the collagen fibers were observed in the supraspinatus tendon, with a decrease in the presence of fibronectin. However, no differences

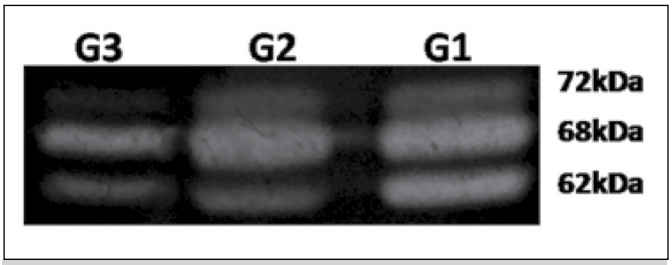

Figure 4. *Zymography analysis: the presence of active MMP-2 (62 kDa) with greater activity in G1.*

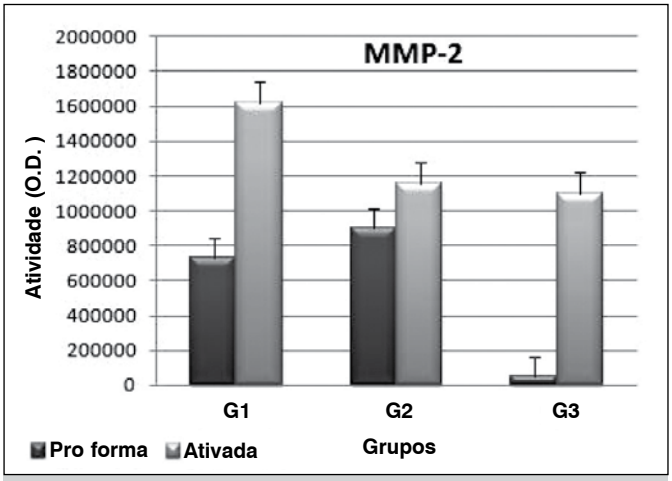

Figure 5. *Activity of MMP-2 of the tendon after inflammation installed in rat paw. The pro-MMP-2 (72kDa) and activated MMP-2 (68 and 62kDa). Observe the increase in activity of MMP-2 in G1 when compared to the same isoform in G2 and G3. The proMMP-2 presented an increase in G1 and G2.*

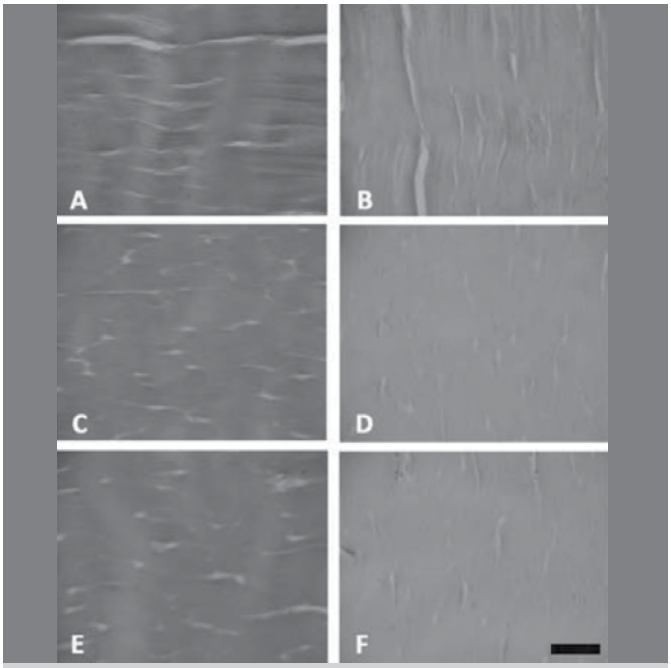

Figure 6. *Organization of collagen molecules in the Achilles tendon. In A, C and E, the sections are parallel; B, D and F, the sections are perpendicular to the plane of polarized light. A and B (G1), C and D (G2), E and F (G3). No difference was found in linear dichroism between the groups.*

were observed in the collagen molecules in our study. The Achilles tendon is located at a considerable distance from the acute inflammation induction site, which may indicate that the action of the inflammatory agents in the extracellular matrix occurred in a less intense manner. If the inflammatory process had been provoked closer to the Achilles tendon, more intense modifications of its extracellular matrix components would probably have been observed, as in previous studies.[8,9]

CONCLUSION

The results indicated that injuries located close to the tendon can give rise to modifications in the tendon tissue remodeling process, promoting an imbalance between the degradation and replacement of the extracellular matrix elements, which may produce a predisposition to future injuries and even lead to a rupture. Future studies should be conducted in order to evaluate the different levels of inflammation, as well as different sites adjacent to the tendon, to observe its influence on the composition and organization of the tendon.

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