Experimental model of Achilles tendon injury in rats[1]

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ABSTRACT

PURPOSE: To describe an effective experimental model to study the Achilles tendon healing.

METHODS: Forty male *Rattus norvegicus albinus,* Wistar lineage adult male weighing 250 to 300g were used for this experiment and thirty were surgically submitted to bilateral partial transverse section of the Achilles tendon. The right tendon was treated with radio waves (RF) whereas the left tendon served as control. On the third postoperative day, the rats were divided into four experimental groups consisting of ten rats each which were treated with monopolar RF adjusted to 650 kHz and 2w, for two minutes twice a week and a group of normal animals without any intervention, until they were sacrificed on the 7th, 14th and 28th days, respectively. Tendons were weighed and collagen quantification was evaluated by hydroxyprolin content.

RESULTS: Significant reduction in collagen content on day 7, 14 and 28 was related to control experiment to normal tendon (7 days, $p<0.01$; 14 e 28 days, $p<0.05$).

CONCLUSION: The experimental model has been effective and available to be used to study Achilles tendon healing.

Key words: Models, Animal. Achilles Tendon. Collagen Type I. Hydroxyproline. Radio Waves. Rats.

Introduction

Injuries to tendons are among the most common injuries to the body. They include complete tendon ruptures that occur from a single overload event as well as the less dramatic but more common incomplete injuries such as tendinitis. These injuries are not only responsible for large health care costs, but they also result in lost work time and individual morbidity[1]. Although, there are medical treatments for most of these conditions, continuing efforts need to be made to improve the effectiveness of the treatments and accelerate recovery. In the past, most of these efforts have been directed at improving surgical, pharmacological and rehabilitative techniques. Despite many improvements in these techniques, there remain significant limitations in our management of these conditions.

In order to determine if a proposed animal tendon injury model is appropriate a number of conditions must be met. The correct anatomic structure must be modeled and the desired experimental condition must be simulated. Complete and incomplete injuries are modeled differently. Another important factor to determine at the outset is the outcome measure desired to assess the effectiveness of the technique being tested. Since the primary function of tendons is to transfer loads, injuries to these structures result in the interruption of load transfer and loss of function.

There are several pathologic conditions more common that occur to tendons and most appropriate to model as partial and complete tissue injuries and clinically these injuries often do not heal properly leading to significant morbidity and often require reconstructive surgery. These conditions have been simulated through partial lesion, partial tissue laceration, tendinitis, tendinosis, transecctions, hole punction, incisional wound, dropping, total transection, longitudinal lesion and hemisecction on Achilles tendon[2-12]. Techniques to prevent injury or accelerate healing after injury can be tested in these models. Overall, there are major differences in the modeling of the different types of soft tissue injuries. As a result, each type of soft tissue condition (partial injuries, complete injuries) requires different modeling approaches. Tendon repair involves a complex orchestrated series of physiological events that include protein synthesis, cell migration, and degradation of the extracellular matrix, particularly collagen[13].

An appropriate animal model should be available to study the tendon healing process. We describe a protocol for Achilles tendon injury in rats showing its effectiveness by quantifying 4-hydroxyprolina on phases of tendon regeneration and using the contralateral paw as control.

Methods

This study was approved by the Ethics Committee for the Analysis of Research Projects (CAPPesq) Protocol 164/10. The study followed the principles complied with Federal Law No. 11.794, of October 8, 2008, and Decree No. 6,689, of July 15, 2009 that regulated the Law 11.794. All animals received human care in compliance with the experimental protocols of the Ethical Principles in Animal Experiments adopted by the Brazilian Association of Animal Testing (COBEA).

Forty Adult male *Rattus norvegicus albinus,* Wistar weighing 250 to 300g were housed in the São Paulo University School of Medicine Barrier. Animals were housed five per cage were given food and purified tap water ad libitum. Ten animals were used for normal group, normal right Achilles tendon (NRT) and normal left Achilles tendon (NLT), without surgery and thirty animals were used for surgery group (S). The animals of surgery group were operated in CEPEC (Center for Study and Research in Surgery, in the Department of Urology, Faculty of Medicine, University of Sao Paulo) (Figure 1A). It was used for experiment the right Achilles tendon (SRT) and as control the left one (SLT) of the same animals of surgery group. Rats were placed under general anesthesia with 4% isoflurane and maintained anesthetized by mask with inspired fraction of 1.5-3% isoflurane (Figure 1B). After anesthesia, the distal portions of the right and left legs of the animal shall be subjected to disinfection with chlorhexidine gluconate solution 10mg and shaved.

Animals were placed in a sterile field on a heated surgery table and covered with a sterile surgical drape. The skin was cut longitudinally, lateral and 0.5cm from the calcaneal insertion lateral to the tendon, both the peritendon and tendon exposed.

The animals were surgically submitted to bilateral partial transverse section of the Achilles tendon. The injury in this animal model was represented by dissection and transverse hemisection by Nº 15 scalpel blade perpendicular to the collagen fibers, 2.5mm from Achilles insertion on the lateral side of Achilles tendon (Figure 1C-F).

We took care to separate the flexor halluces longus which is next to the Achilles tendon medially. After transection, the skin was closed by a continuous suture with Polipropilene 6-0 (Figure 1G, H). After surgery, the rats were kept warm until they recover the consciousness. Analgesics were administered (paracetamol 200mg/kg) 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 12h light-dark cycles.

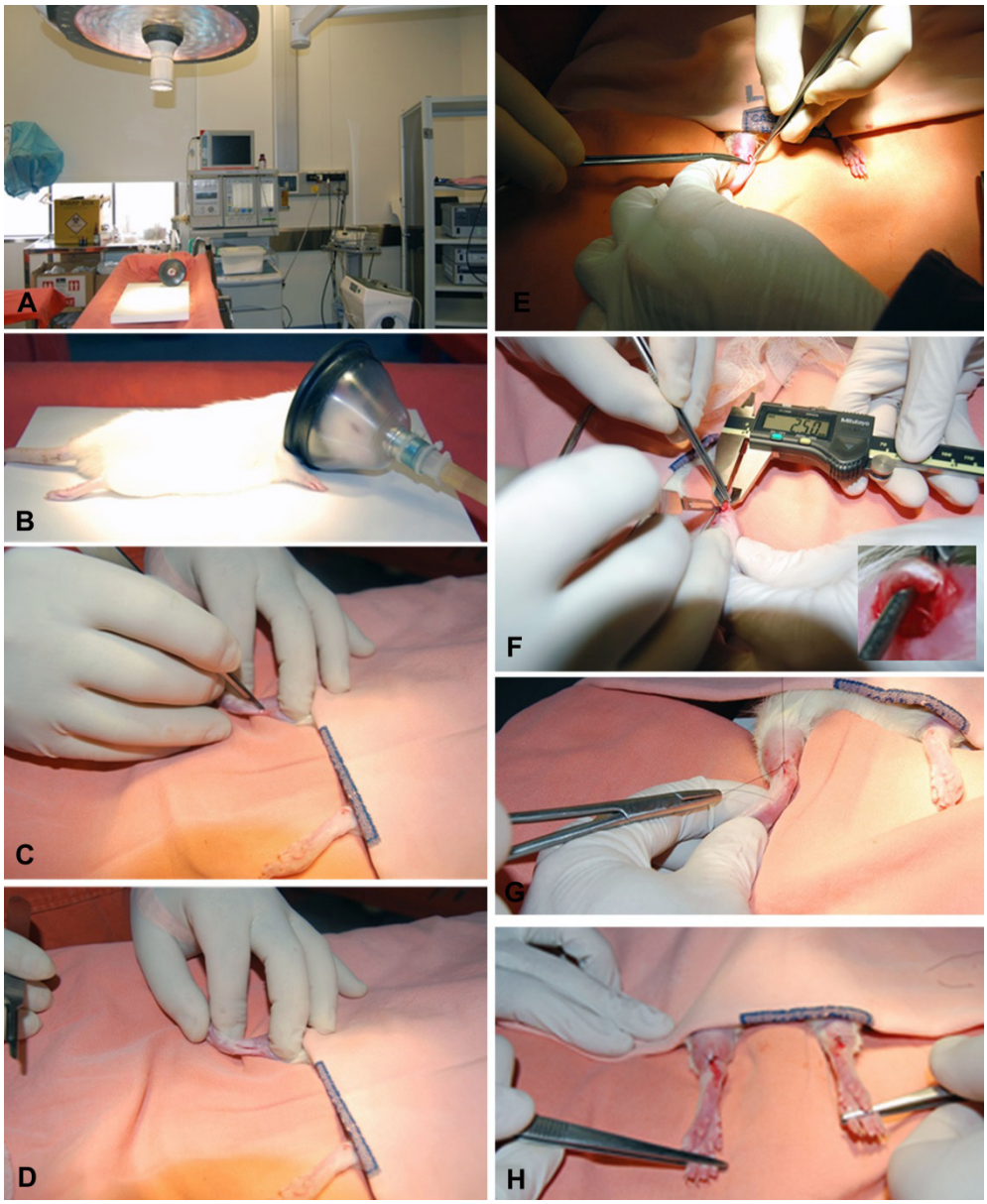

FIGURE 1 – A. Center for Teaching and Research in Surgery (CEPEC) of University of Sao Paulo Medical School. **B.** Rat before surgery. **C, D.** The skin was cut longitudinally, lateral and 0,5cm from the calcaneal insertion lateral to the tendon, both the peritendon and tendon exposed. **E.** Dissecting the tendon. **F.** The hemisection was made with a no. 15 scalpel blade perpendicular to the collagen fibers, 2.5mm from the calcaneous insertion, on lateral side of the tendon. **G, H.** The skin was closed by a continuous suture with Polipropilene 6-0.

To find out if the lesion was effective we measure the hydroxyproline content at seven, fourteen and twenty eight days and compared the control and contralateral injured tendons to a normal one. On the third postoperative day, the rats were divided into four experimental groups in which one group with normal tendon and the others three groups treated with monopolar RF (Tonederm™) adjusted to 650 kHz and 2w, for two minutes twice a week, until they were sacrificed on the 7th, 14th and 28th days.

Euthanasia

After each period of treatment the animals were euthanized using CO_2 (Carbon Dioxide). The animals were placed in chambers previously filled with pure gas, or preferably, gas added with 30% oxygen to reduce distress caused by hypoxia. As CO_2 is twice as heavy as the air it sits at the bottom of the chamber. Therefore, the chamber opening should be on the top. CO_2 is a deadly gas because it causes depression in the central neural system. The animals should be kept in the chamber for 10 minutes so that their death will be confirmed[14]. Achilles tendons were collected through their dissection with the animal in the prone position following the procedures of the methodology used. Specimens were intended for quantification of collagen.

Collagen quantification-4- hydroxyproline

The collagen content was determined by measuring the hydroxyproline content according Bergman and Loxley[15]. Dry weights of lyophilized samples (n=40 for each time point) were measured and transferred to tube,culture,13x100mm (Corning, NY14831) containing 5ml of 6N HCL. In separate tubes, 0 (blank) and 4, 6, 8, 10 and 12µg of the hydroxyproline standard (Sigma) were prepared to establish a standard curve for each experiment. Samples were hydrolyzed at 100°C for 22 hours and then oxidized by adding chloramine-T reagent and incubating at room temperature for 25 min. After oxidation, a chromophore was developed by adding Ehrlich's reagents to each sample and incubating at 65°C for 20 min. The absorbance of each standard and the samples was measured at 560 nm by spectrometry. The collagen content was calculated by multiplying the hydroxyproline content by 7.0 as previously described[16]. Collagen production for each sample was determined by dividing the total collagen content by the total weight of each sample.

Statistical analysis

Data were analyzed statistically and values are expressed by median. Normality of the variables was assessed. In order to assess whether there was any difference it was decided by nonparametric Mann-Whitney U test. Significance was accepted at $p<0.05$. Data were analyzes using the statistical package SPSS17.0 (Chicago, IL).

Results

The amount of hydroxyproline was significantly lower in injured tendons compared to normal control tendons at the study (7 days, $p<0.01$, 14 and 28 days, $p<0.05$) Tables 1 and 2.

TABLE 1 - Comparison of hydroxyproline content between Normal Left Tendon (NLT) and Surgery Left Tendon (SLT).

	NLT			SLT			
Days	Median	Minimum	Maximum	Median	Minimum	Maximum	p
7	83.07	69.04	111	56.07	34.29	70.80	0.0003**
14	83.07	69.04	111	63.62	40.88	143.65	0.0114*
28	83.07	69.04	111	73.77	61.90	81.94	0.0218*

NLT = Normal Left Tendon; SRT = Surgical Left Tendon; * = significant at 5%; ** = significant at 1%

TABLE 2 - Comparison of hydroxyproline content between Normal Right Tendon (NRT) and Surgery Right Tendon (SRT).

	NRT			SRT			
Days	Median	Minimum	Maximum	Median	Minimum	Maximum	p
7	78.50	50.85	88.73	57.66	33.13	74.32	0.0003**
14	78.50	50.85	88.73	63.56	48.07	83.73	0.0114*
28	78.50	50.85	88.73	69.83	57.25	79.43	0.0218*

NRT = Normal Right Tendon; SRT = Surgical Left Tendon; * = significant at 5%; ** = significant at 1%

Discussion

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 necrotic materials occurs. Vasoactive and chemotactic factors are released with increased vascular permeability, initiation of angiogenesis, stimulation of tenocyte proliferation, and recruitment of more inflammatory cells[17]. Tenocytes gradually migrate to the wound, and type III collagen synthesis is initiated. After a few days, the remodeling stage begins which the synthesis of type III collagen peaks during this stage, which lasts for a few weeks. In the approximately six weeks, the modeling stage commences. During this stage, the healing tissue is resized and reshaped. A corresponding decrease in cellularity, collagen and glycosaminoglycan synthesis occurs[16].

Especially at the initial inflammatory phase, the hydroxyproline content of model experiment were less than the normal tendon and this is probably because of phagocytosis of necrotic materials. Collagen typing experiments, using material from normal and ruptured Achilles tendons, demonstrated that ruptured tendons contained reduced quantities of type I Collagen as well as a significant proportion of type III collagen and this justify less amount of hydroxyproline content in our work[13,18].

The presence of type III collagen likely accounts for the decreased resistance of tendon to tensile forces, and may therefore predispose the tendon to spontaneous rupture[19].

Type I collagen is the major component of normal tendon, greatly contributing to the strength of the tissue[19,20]. As it can be observed, the content of hydroxyproline used as an indicator of collagen content with the injury was significantly lower than normal tendons at all-time points, thus confirming the effectiveness of the experimental injury. It hasn't been evaluated in this work the collagen content of tendon subjected to radiofrequency because this was for other work, so we evaluated only normal tendon compared with the control experiment, which was left tendon. Over time 28 days is suggested attempt to approach collagen recovering the injured tendon to normal.

Although the tensile strength of the healing tendon improves over time, it does not reach the levels of uninjured, normal tissue[13]. This model can be used to study tendon regeneration. It's important to state that tendon recovery is still controversial in the therapeutic resource literature.

Conclusion

The proposed experimental model has been effective and available to be used to study Achilles tendon healing.

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