Neural plasticity, healing and functionality after traumatic or chemical Achilles tendon injury

Running title: Neuroplasticity and tendon injuries

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Plasticidade neural, cicatrização e funcionalidade após lesão traumática ou química do tendão de Aquiles

Introdução: As tendinopatias tem caráter multifatorial e os mecanismos subjacentes a lesão e complicações ainda são incertos. Hipóteses degenerativas e inflamatórias sobre esses mecanismos são discutidas, porém a detecção tardia da lesão pode deixar dúvidas sobre os processos envolvidos. Objetivo: No presente estudo, buscamos comparar as alterações estruturais e funcionais tardias no tendão de Aquiles após lesão por degeneração ou inflamação. Materiais e Métodos: Ratos *Wistar* foram divididos em três grupos: controle, ruptura e colagenase. Para a avaliação funcional da locomoção, usamos o índice funcional de Aquiles em 0, 2, 7, 14 e 21 dias após a lesão. O tecido foi analisado por H&E, escala de Bonar e DAPI a fim de avaliar a organização, vascularização e densidade celular. Para avaliar a plasticidade neural, o tecido foi imunomarcado com o anticorpo neurofilamento 200 e analisado por microscopia de fluorescência. Resultados: A organização tecidual, morfologia celular, número de células e vascularização foram mais comprometidos no grupo ruptura. Por outro lado, o comprometimento funcional e a presença de ramos nervosos no tecido foram identificados em ambos os grupos, colagenase e ruptura. Conclusão: A lesão degenerativa e inflamatória apresentam características estruturais bem distintas na fase tardia, embora ambas tenham implicações funcionais e neuroplásticas semelhantes.

Palavras-chave: Lesão tendínea, colagenase 1, ruptura, tendão de Aquiles, regeneração, plasticidade neural.

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Introduction: Tendinopathies have multifactorial causes and the mechanisms underlying the injury and complications are still uncertain. Degenerative and inflammatory hypotheses on these mechanisms are discussed, but the late detection of the lesion may leave doubts about the processes involved. Aim: In the present study, we intended to compare the late structural and functional changes in the Achilles tendon after injury due to degeneration or inflammation. Materials and Methods: Wistar rats were divided into three groups: control, rupture and collagenase. To assess function, we measured the Achilles functional index at 0, 2, 7, 14 and 21 days post-injury. The tissue was analyzed by H&E, Bonar scale and DAPI in order to evaluate organization, vascularization and cell density. To evaluate neural plasticity, the tissue was immunolabeled with neurofilament 200 antibody and analyzed by fluorescence microscopy. Results: Tissue organization, cell morphology, number of cells and vascularization were more compromised in the rupture group. On the other hand, functional impairment and presence of nerve branches in the tissue were identified in both groups, collagenase and rupture. Conclusion: Degenerative and inflammatory lesions have very distinct structural characteristics in the late phase, although both have similar functional and neuroplastic implications.

Keywords: Tendon injuries, collagenase 1, rupture, Achilles tendon, regeneration, neuronal plasticity.

Introduction

Achilles tendon injuries are a lower limb musculoskeletal impair frequently encountered, with an incidence around 31 per 100,000 per year[13, 29]. These injuries are multifactorial and their pathogenesis and repair process are not fully understood. Athletes and middle-age people are commonly affected and lose their functionality and occupational activities [13, 29, 16, 31]. Its clinical presentation is characterized by pain, edema and decreased biomechanical, which negatively impact on functional capacity and quality of life[29, 20].

Despite its clinical relevance, progress in understanding tendinopathy is as recent as the understanding that tendon injuries are related to inflammatory (tendinitis) and/or degenerative (tendinosis) processes[16, 20, 32]. In addition, it has already been shown that inflammation is not one of the main causes of this disorder, with a predominant role only in the acute phase[28]. The failure in the healing response is the responsible for progressive tendon degeneration, characterized by collagen fiber rupture, increased cell density, hypervascularization and deposition of fundamental substance[36].

To advance the understanding of the processes involved in tendon repair, experimental models of tendinopathy and their late impact are a relevant discussion. Several experimental models for Achilles tendon injury have been proposed, such as those by chemical induction (injections of collagenase, cytokines, prostaglandins and fluoroquinolone) and by mechanical induction (repetitive effort and total or partial tendon rupture)[21, 24, 35, 19]. In highlight, the models of chemical induction by collagenase and mechanical induction by tendon rupture have the advantage of less time required for the experiment and easier reproducibility, in addition to distinct characteristics, since the former simulates the degenerative characteristics while the latter the inflammatory ones[15].

Advantages in using the murine model are also noticeable due to the possibility of functional analysis. The impact of the injury on the limitation of functional capacity is of great relevance to assess the therapeutic efficacy of different interventions. Therefore, the use of functional tests such as gait analysis has become increasingly common in rodent models because it is not invasive and is able to investigate painful responses associated with tendinopathy[7, 12, 22].

There is evidence that the painful condition manifested in tendinopathy is not only associated with structural changes in the tendon, as it has already been shown that local cells and peripheral neural fibers respond differently in this condition, contributing to nociceptive behavior[9]. It is known that healthy tendon is practically devoid of nervous supply[1]. However, after tendon injury, there is intense nerve growth, with synthesis of neurotrophic and chemical factors[14], in addition to other substances responsible for various changes such as increased cellularity, hypervascularization and hyperalgesia[1, 2].

Although there are several models of Achilles tendinopathy, there are no studies that compare experimentally these lesion models, as well as their respective developments and late histological and functional implications. Thus, we seek to compare the impact of two different models of Achilles tendon injury, one traumatic and other degenerative, on the histological, functional and neuroplastic aspects on the remodeling phase.

Materials and methods

Animals

Fifteen adult male Wistar rats (200 to 250 g) were housed in polyacrylic cages with controlled temperature and lighting (21 ± 2 °C; 12 h/12 h light-dark cycle). The access to

food and water was *ad libitum*. Rats were previously acclimated one week before experiments and all experimental procedures were performed in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and approved by the institutional Animal Research Ethics Committee (CEUA-UFPA nº 4243280416).

Experimental Groups

Animals were randomly divided into three experimental groups: control (CTRL, 5 animals); rupture (RUP, 5 animals) and collagenase (COL, 5 animals). CTRL rats did not receive any procedure, only realized functional tests. RUP and COL were intraperitoneally anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). RUP rats were submitted to Achilles tenotomy followed by skin sutures as described by Moraes and colleagues[26]. COL rats received 30 µL of collagenase I (10 mg/mL) (Sigma® C5138) with a 26-gauge needle into the paratendinous region. The rats were supervised daily and weighed before tenotomy at 7, 14 and 21 days post-injury (dpi). All animals were killed by intraperitoneal injection with the aforementioned anaesthetics followed by decapitation at 21 dpi. Achilles tendons were dissected and fixed overnight in 4% paraformaldehyde and cryoprotected by 30% sucrose. Longitudinal sections of 20 µm of tendon tissues were cut on a Leica cryostat (model CM3050 S) at -24 °C and collected on glass slides.

Morphology

The tendon sections were stained with hematoxylin-eosin (HE) for further analysis. The images were visualized and recorded under a light microscope (Nikon Eclipse 50i, Japan) and analyzed using the ImageJ v1.47 software (NIH, USA). For the semiquantitative

analysis, the Bonar scale was used to assess the degree of tissue imparment[6]. Parameters such as tenocyte morphology, fundamental substance, collagen and vascularization are considered for analysis and scores vary from 0 to 3. For this study, only the tenocyte morphology and vascularization were considered and two slides from each animal were evaluated by blinded examinator.

Number of cells

The total number of cells in tendons was measured by a direct count of DAPI-stained nuclei. Sections of the tissue were washed with distilled water, permeabilized using 0.1% Triton X-100, incubated with DAPI solution (1:10.000) and mounted on glass slides with N-propyl-gallate. Cell nuclei were analysed by fluorescence microscopy and the number of cells was determined using ImageJ v1.47 by automatic counting tool.

Immunofluorescence

The specimens were washed in PBS and sequentially treated with 50 mM ammonium chloride, 0.25% triton X-100 and 1% BSA. After washing with PBS, sections were incubated with a polyclonal anti-Neurofilament 200 antibody (1:200, Sigma-Aldrich). After overnight incubation with the primary antibody, samples were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000; Molecular Probes, USA) for 2 h. Nuclei were stained with 1:10.000 DAPI (Sigma, USA) and mounted on glass slides. Negative controls were made by omission of the primary antibodies during incubation and resulted in absence of immunoreactivity. The sections were analyzed on a fluorescence microscope (Nikon Eclipse 50i, Japan).

Achilles functional index (IFA)

Tendon functionality was measured utilizing Achilles functional index (AFI) as proposed by Murrell et al.[27]. All animals had their hindpaws painted with nontoxic blue ink and then were placed in the walkway apparatus (10 x 60 cm) leaving their footprints on a white paper previously placed on the floor of the apparatus. Functional assessments were performed before and after the rupture surgery on the 2^{nd}, 7^{th}, 14^{th} and 21^{st} dpi. The animal footprints were recorded, digitalized and evaluated using the ImageJ v1.47 software. The values of footprint length (FL), foot spreading (FS) and the intermediary test factor (ITF) were applied in the following equation of Achilles functional index: AFI = 74 (FL) + 161 (FS) + 48 (ITF) – 5. The evaluators were blinded to the identity of the animals.

Statistical analysis

Data are reported as mean ± standard deviation (SD) or median and interquartile range. Multiple comparisons were made using Kruskal-Wallis or one-way ANOVA test, and p values less than 0.05 were considered statistically significant. All statistical analyses were performed using the software Prism 6 (GraphPad, USA).

Results

Traumatic and degenerative model have differences in the pattern of tissue organization, tenocyte morphology and vascularization

Chemical or mechanical Achilles tendon injury did not imply on body mass gain in any time of experiments (data not showed). There was an expressive difference in general tissue organization between RUP and COL when compared to CTRL. In RUP, disorganization of the matrix and cell alignment, increase in cell density as well as

rounded nuclear morphology were found in the tissue. In COL, areas of disorganization could be noticed, but the predominance was of elongated and oriented cells surrounded by a more organized matrix, more similar to the histological characteristics of CTRL (Figure 1). These characteristics are confirmed by the significant increase in the Bonar score for tenocyte morphology ($p = 0.0002$) and vascularization ($p < 0.0001$) only in RUP in relation to CTRL and COL (Figure 2A, 2B).

Traumatic model induces increase in the number of cells

The number of cells was quantified after labeling the tissue with the nuclear marker DAPI. Representative photomicrographs of the tissue in each experimental condition are shown in Figure 3A. We observed that RUP showed an increase in the number of cells when compared to CTRL and COL ($p < 0.0001$) (Figure 3B).

Traumatic and degenerative models demonstrate decline in functional performance

In view of the histological changes found, we sought to identify whether functional changes also occurred. By calculating the AFI, we found that COL and RUP showed significant worsening in the function of the Achilles tendon in the 2^{nd} ($p = 0.005$), 7^{th} ($p = 0.009$), 14^{th} ($p = 0.032$) and 21^{st} ($p = 0.008$) dpi, suggesting that the worsening in the functional performance of the calcaneus tendon occurs in both types of injury induction, when compared to CTRL (Figure 4).

Traumatic and degenerative models present neural plasticity

As the functional results pointed to a compromise in both groups, we sought to identify whether this functional impairment was related to implications for the nervous system. We performed immunostaining for NF200 in order to check the presence of axons of neurons in the tendon. We observed immunoreactivity in both RUP and COL, although with more expressive marking in RUP (Figure 5). In both groups, the marking was located in the paratendon. This penetration of nerve branches was not observed in CTRL.

Discussion

The use of animal models to induce tendinopathy is important to understand the repair mechanisms and to test possible appropriate therapies. We evaluated the effects of chemical and mechanical induction on the morphofunctional and neuroplastic aspects of the Achilles tendon. We show that, after chemical or mechanical induction of tendon injury, different morphological aspects and similar functional and neuroplastic aspects are observed. The presence of nerve branches between the collagen mesh and the functional worsening suggest that there is structural and functional plasticity in both models[18].

Mechanical induction resulted in greater tissue disorganization and greater cell density. This was expected, as it has been previously reported that experimental tendon rupture leads to changes such as modification of morphology, excessive proliferation of tenocytes, rupture of collagen fiber and neovascularization[20, 25, 34], consistent with our findings regarding mechanical induction. Interestingly, the chemical induction group showed milder changes, with small areas of disorganization. The study by De Cesar Netto et al. showed that the serial application of a low dose of collagenase compared to a single high dose injection, induces more pronounced and persistent histological and biomechanical changes[8]. Another study used two collagenase injections, each

administered on consecutive days, and obtained histological scores that indicated tissue damage, also suggesting that the severity of the injury may be associated with the amount and frequency of injected collagenase[17]. This finding is consistent with our results, showing that a single injection of collagenase I in high concentration is able to induce changes in the tissue, however such changes are less severe than those provided by mechanical induction, in the same repair phase, as we observed in our study.

We showed that hypervascularization and increased cellularity occurred only in animals that had the tendon sectioned. Studies report that vascular function and angiogenesis are regulated by vascular endothelial growth factor (VEGF), which is poorly expressed in the healthy adult tendon, but is increased in several animal models of acute injury or mechanical overload[33, 5]. On the other hand, in chemical induction, Orfei and collaborators showed neoangiogenesis and an increase in the number of cells between days 3 and 15 after chemical induction by collagenase I injection, followed by a decrease in these parameters from day 15 to day 45[30]. This suggests that, after 15 days of chemical injury induction, hypervascularization and increased cell density are no longer prevalent phenomena, corroborating our findings on the 21st day after induction of collagenase injury.

Functional decline was observed in both experimental groups in our study. This finding is in line with other studies that suggest damage to the biomechanical properties resulting from changes in the injured tendon microarchitecture[26, 4]. However, the functional worsening in the group treated with collagenase I is inconsistent with the histological results we obtained for this group. Studies indicate that, after tendon injuries, there is the synthesis of neurotrophic and chemical factors[14], such as nervous growth factor (NGF)[1], substance P[3] and glutamate[10], among others responsible for changes such as hyperalgesia, which can lead to functional limitation[23, 11]. The production of these

substances would explain the deficit in the functional performance of COL, whose changes in the microarchitecture of the tendon were mild. In addition, the penetration of nerve branches in the tendon of both experimental groups was detected, reinforcing the hypothesis of the presence of substances and factors that may contribute to the functional worsening.

Considering our findings on the neural plasticity of the tendon, in mechanical induction models, it is known that there is extensive neural plasticity, with the appearance of nerve fibers in the tendon during the repair process[14]. Our work is the first to show the presence of axons of neurons between the collagenous mesh after chemical induction in the Achilles tendon. On this model, previous studies have reported the occurrence of the expression of sensory neuropeptides and this seems to be associated with heal failure and pain. In other studies, with an animal model of musculoskeletal degenerative diseases, the occurrence of axonal growth as a possible mechanism for generating pain and functional impairment has also been demonstrated[1, 11].

Our study is the first to compare two injury-inducing models, one mechanical and the other chemical, in the late repair phase. Despite our findings, the study had some limitations described below. Histological results were examined three weeks after the injury started, while chronic tendinopathy in humans develops over a longer period. We suggest that in future studies, repeated weekly injections of collagenase and a longer duration of follow-up may better simulate the condition of chronic tendinopathy. Another limitation is the identification of the presence of nerve fibers in the tendon after inducing the injury, whose quantification of the marking intensity could not be performed, which prevents us from stating that there was a greater occurrence in RUP, according to the previous qualitative analysis.

Finally, this study plays a fundamental role in understanding the damage caused to tendons in chemical and mechanical models. With this, it is possible to establish new more efficient therapeutic strategies for better tissue regeneration and functional recovery, taking into account the specificities of the lesions. As tendinopathy is a progressive, multifactorial disorder and may or may not present inflammation, it is unrealistic to expect a single experimental model to ensure understanding of all aspects of tendinopathy, making it necessary that the therapeutic approaches developed are appropriately directed to each profile of impairment.

Declaration of interest

The authors report that they have no conflicts of interest.

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Figure legends

Figure 1. Effect of the type of induction to injury in the pattern of tissue organization. Representative histology images of longitudinal tendon Achilles section stained with hematoxylin-eosin (HE). Tendon histology slides were collected from animals 21 days post-injury. The arrow indicates the presence of disarrangement among the collagen mesh in RUP. Inset with digital zoom factor 2.0 demonstrates tenocyte morphology. Magnification 200×, scale bars = 50 μm.

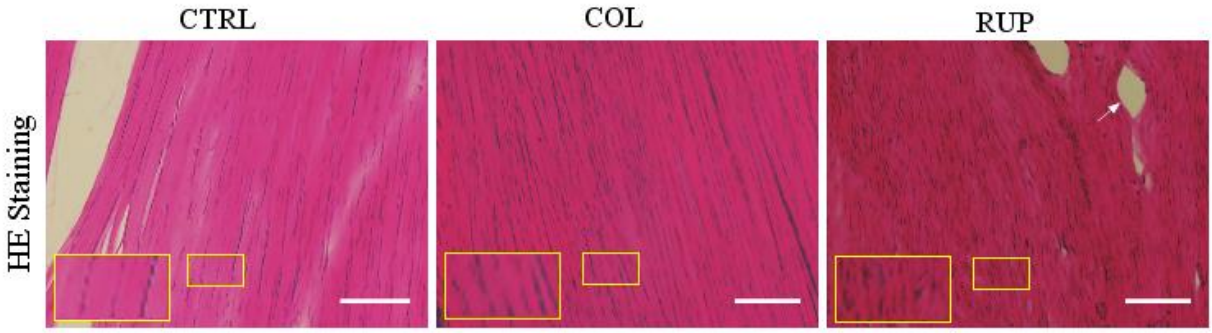


Figure 2. Effect of the type of injury induction on tenocyte morphology (A) and vascularization (B). Semi-quantitative analysis in the Achilles tendon according to the Bonar scale after 21 days of injury. Histological sections were stained with HE and analyzed by scale. Data are presented as median ± interquartile range. N = 5 animals/group. Statistical analysis: Kruskal-Wallis test; (A) *p = 0.0002 for RUP vs. CTRL and COL; (B) *p < 0.0001 for RUP vs. CTRL and COL.

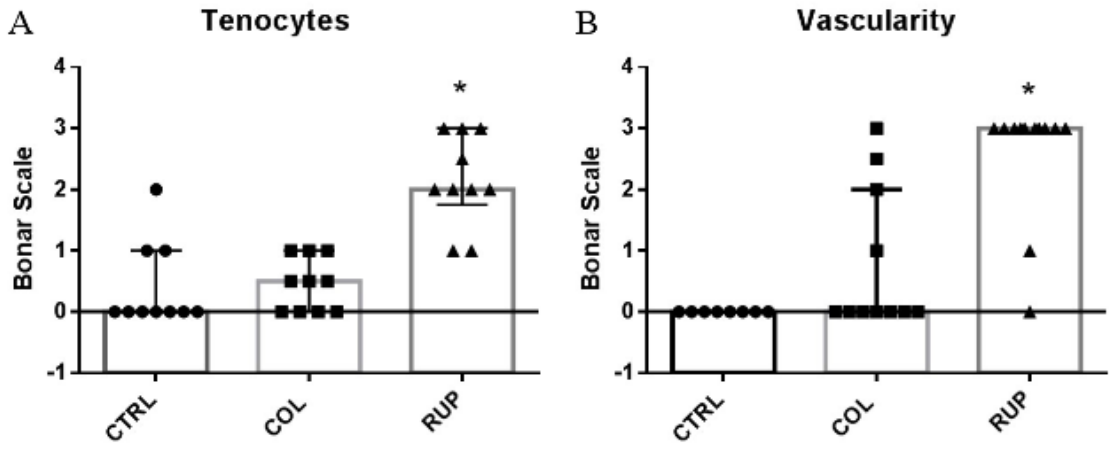


Figure 3. Effect of injury induction on the number of cells in the Achilles tendon. Photomicrographs of DAPI staining in tendon tissue in each experimental condition (A). Quantification of the number of cells (B). Data are reported as mean ± SD. N = 5 animals/groups. Statistical analysis: ANOVA-Tukey; *p < 0.0001 for RUP vs. CTRL and COL. Magnification 200×, scale bars = 50 μm.

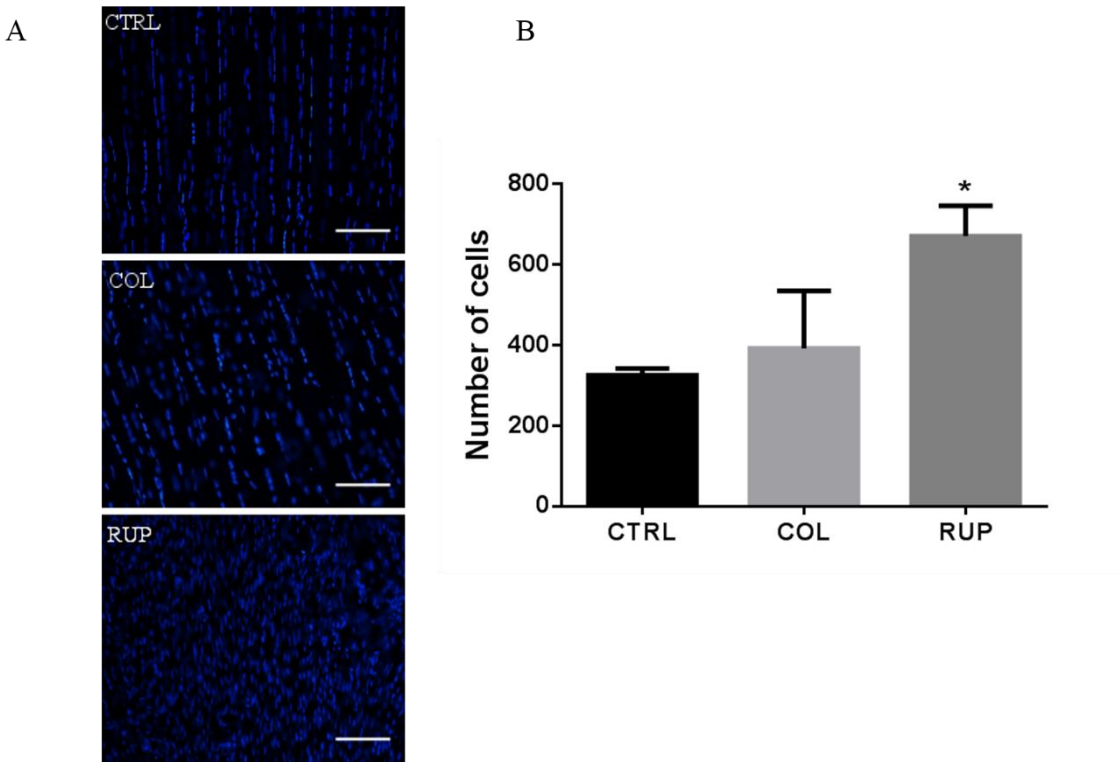


Figure 4. Effect of injury induction on AFI. Rats subjected to surgical division of the Achilles tendon (RUP) and collagenase injection (COL) were assayed for tendon function by the AFI on the days 0, 2, 7, 14 and 21 post-injury. A control group of animals (CTRL) was not subjected to tendon injury. Data are reported as mean ± SD for n = 5 animals/group. Statistical analysis: ANOVA–Tukey; *p < 0.05 for RUP vs. CTRL and COL.

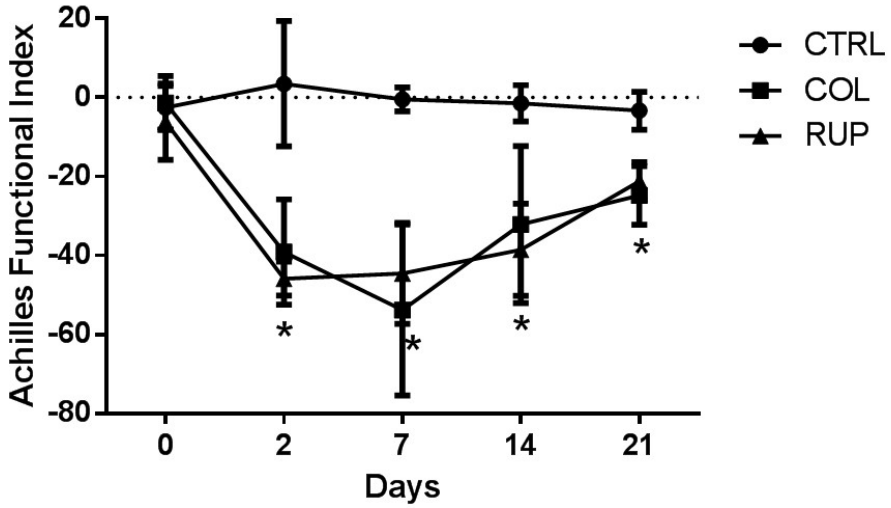
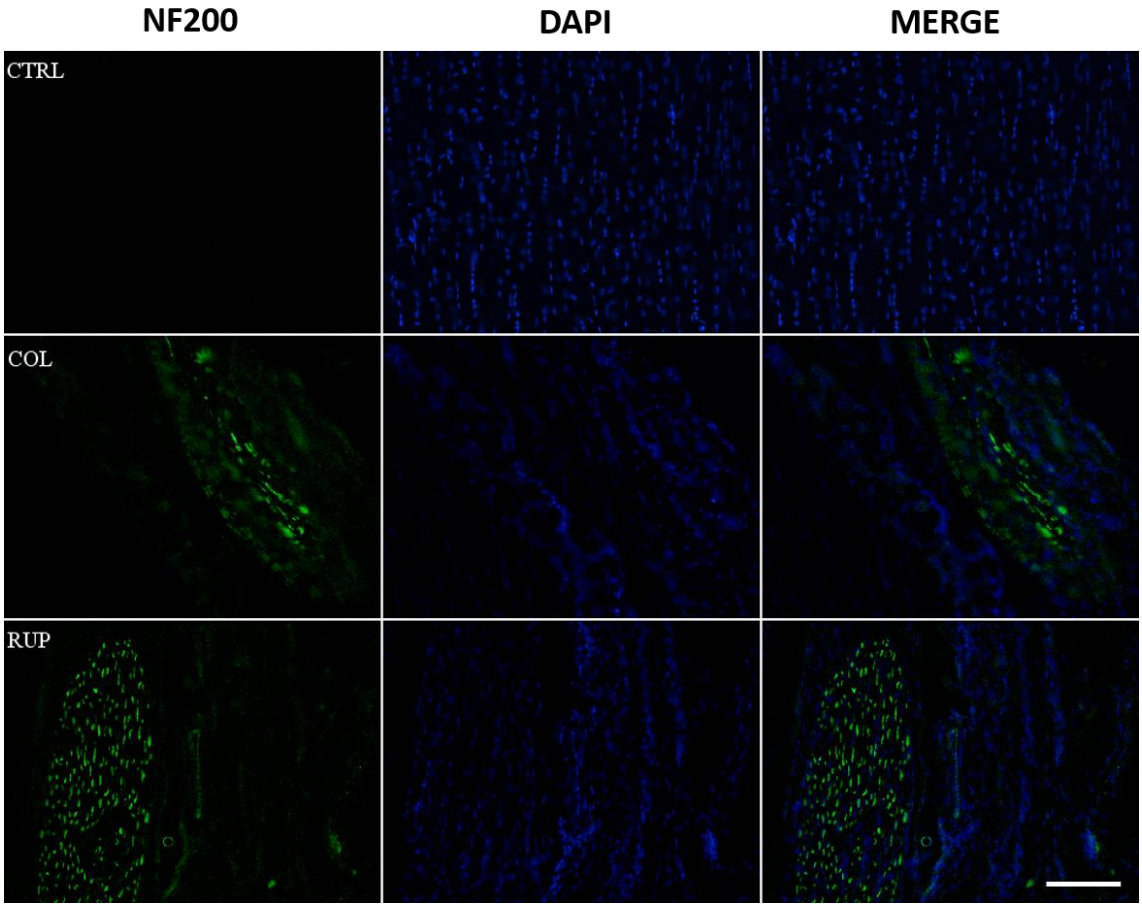


Figure 5. Effect of injury induction type on neural plasticity of the Achilles tendon in rats. Immunofluorescence for NF200 and DAPI in longitudinal tendon cuts showing that both groups present penetration of nerve branches between the collagen mesh, which is not observed in the CTRL group. Magnification 200×, scale bars = 50 μm.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