

# Treatment with Conditioned Medium from Tenocytes Primary Cell Cultures Accelerates Histological and Functional Recovery of Achilles Tendon in Tenotomized Mice

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## DOI:

10.32098/mltj.01.2024.01

## LEVEL OF EVIDENCE: 5

## SUMMARY

**Background.** Tendon injuries are responsible for a crescent number of people retirement around the world. Recently, cell-based therapies has been shown to be effective for the treatment of tendon injures.

**Objective.** We aimed to evaluate the effect of local treatment with conditioned medium produced by tenocytes cell cultures on the histological and functional recovery in tenotomized mice.

**Methods.** The tendon cells were cultured for conditioning culture medium that will be used as treatment. The animals were subjected to right Aquilles tenotomy and treated with saline solution (SAL), DMEM (DMEM) and DMEM conditioned in tenocytes cultures (CM) and compared to the control group (CTRL). For histological analysis, tissues were stained with HE. Tendon functionality was measured using the Achilles Functional Index (AFI) and mechanical sensitivity through the paw withdrawal threshold (PWT) using the Von Frey test. Analyzes were performed at 7-, 14- and 21-days post-injury (dpi). Statistics were performed by ANOVA-2 followed by Tukey's post-test,  $p < 0.01$ .

**Results.** In the histological analysis, the CM group showed better tissue organization when compared to SAL and DMEM groups. The CM group showed improvement in the PWT at 7° and 14° dpi ( $2.24 \pm 1.15$ ;  $2.66 \pm 1.06$ ) compared to the DMEM ( $0.15 \pm 0.07$ ;  $0.45 \pm 0.76$   $p < 0.01$ ) and SAL ( $0.13 \pm 1.15$ ;  $0.77 \pm 0.95$   $p < 0.01$ ). The CM group showed functional improvement at 7° and 14° dpi ( $-40.4 \pm 12.6$ ;  $-36.6 \pm 10.4$ ) compared to the DMEM ( $-76.5 \pm 11.7$ ;  $-71, 6 \pm 7.9$ ,  $p < 0.01$ ) and SAL ( $-88.8 \pm 15$ ;  $-71.4 \pm 12.6$   $p < 0.01$ ).

**Conclusions.** We conclude that treatment with tenocytes conditioned medium accelerates tendon recovery, promoting improvement in mechanical sensitivity, functionality and tissue organization in the proposed injury model.

## KEY WORDS

*Cell therapy; tendinopathy; tenocytes; tendon; recovery.*

## INTRODUCTION

Tendon injuries are responsible for a crescent number of people retirement around the world (1). It is widely described in literature that tendon injuries represent more than fifty percent of all lesions in athletes, as well as that chronic pain and loss of muscle physiology represents very common symptoms observed in patients developing tendinopathies (2, 3).

Histological reports describe tendon as a connective tissue with fibrous-elastic texture constituted by a large and dense collagen network (3, 4). Tendon tissue is formed by specialized fibroblast cells denominated tenocytes which are responsible by collagen synthesis and maintenance of biochemical components of tendons (4, 5). Although several studies demonstrated that tenocytes are able to produce different components for preservation of tendon tissue integrity, there are few studies demonstrating the potential effect of these cells on the recovery of injured tissue (6, 7)

Rupture of tendon triggers histological events initiating with an inflammatory period which is characterized by leucocytes migration to injured tissue and intense collagen degradation (8). This phase is followed by proliferation of tenocyte with consequent elevation of collagen synthesis. The last period of tissue repair is characterized by intense metabolism of extracellular matrix as well as by remodeling of collagen fibers into the injured tissue (9-11). The duration of these periods is determinant to assure the time of tendon recovery and to reestablishment of tendon functionality.

It is already evidenced that tenocytes present significant participation on tendon repair after tissue injury. In fact, tenocytes are responsible for maintenance of tissue repair during proliferation phase of tendon recovery (12-14). Previous reports also demonstrated that cellular therapy utilizing local insertion of tenocytes into injured tendons promotes significant acceleration in the tendon recovery (15, 16). However, there are no pre-clinical studies describing the effect of compounds released from tenocytes on the tendon Achilles in tenotomized subjects. In this way, the current work aimed to evaluate the effect of local treatment with conditioned medium produced by tenocytes cell cultures on the histological and functional recovery of Achilles tendon in tenotomized mice.

## MATERIALS AND METHODS

### Animals

Swiss mice were provided by animal facilities of Federal University of Pará (UFPa). Primary cell cultures of tenocytes were produced utilizing tendon tissue isolated from 2 days post-natal mice (PN) while adult mice (30-35 g) were utilized in experiments of Achilles tendon rupture. These animals were kept in polypropylene cages at 25 °C

in controlled dark/light cycle (12:12) with food and water *ad libitum*. All experimental procedures were previously approved by the ethical committee for care and use of laboratory animals from UFPa (Protocol number: 4512080418 – Date of approval: June 21, 2018).

### Tenocyte primary cell cultures and preparation of conditioned medium

Primary cell cultures of tenocytes were performed as previously described (17). Briefly, mice were deeply anesthetized with ketamine/xylazine solution (80 mg/kg and 12 mg/kg), quickly decapitated and their Achilles tendons were dissected. Tendon tissues were then transferred to Petri's plate containing cold calcium media free (CMF) solution. After that, tendons were minced in 3mm pieces and placed in cell culture dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Tenocytes cultures were maintained in CO<sub>2</sub> stove at 37 °C until reach total cellular confluence.

As there are currently no protocols utilizing conditioned medium from tendon cells, the authors chose to use confluent cultures to produce the conditioned medium based on established protocols from previous tendon cell culture studies, that cultivated those cells until confluence (15, 18, 19). Besides that, previous analyses described that muscle and tendon-derived cells achieved higher cell numbers and full confluence more rapidly (20). This confluent could enhance the secretion of bioactive factors, contributing to a more robust conditioned medium. The conditioned medium of tenocyte cell cultures was based on adaptation of previously described protocols (21). After reaching confluence, the culture medium was removed, and then tenocyte cells were washed with a calcium-free medium. The decision to wash the cells with CMF before conditioning the medium aimed to eliminate any detached cells, ensuring the purity of the conditioned medium. This approach, rooted in well-established cell culture practices, enhances the reliability of experimental results by minimizing potential influences from floating cells on the composition of the conditioned medium (22). Afterward, cell cultures were incubated with 6 ml of DMEM without any supplementation was added for conditioning by the tenocyte cells themselves over a 6-hour period in CO<sub>2</sub> stove to production of conditioned medium. After this period, conditioned medium was collected from tenocytes cell cultures and centrifuged to ensure the absence of cells. It was centrifuged at 1,500 rpm for five minutes. This approach was based on previous protocols for centrifugation of tendons cells cultures (23, 24), to enhance the reliability of the conditioned medium by minimizing the presence of cells. Supernatant was carefully collected and maintained at -80 °C until experimental utilization.

### Achilles tendon tenotomy protocol

Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg)/xylazine (12 mg/kg) solution. Tibia region of the right paw was tricotomized and a longitudinal skin incision was performed to access Achilles. After that, the right tendon was transected in axial fashion at 0.5 cm from calcaneal insertion followed by Kessler suture (25, 26). Afterwards, the tendon and skin were sutured with non-absorbable monofilament polyamide thread number 4.0 from TechFio® and subjected to local asepsis.

After surgical treatment the animals were kept in cages without movement restriction or legs immobilization. The post-anesthetic recovery of the animals was monitored for 2h. Animals were subdivided in control group (CTRL, n = 9); saline group (SAL, n = 9), vehicle DMEM group (DMEM, n = 9) which were submitted to tendon rupture followed by surgical suture and post treated *in loco* with saline or DMEM, respectively and conditioned medium group (CM, n = 9) which was submitted to tendon rupture, surgical suture and posteriorly treated *in loco* with conditioned medium from primary cultures of tenocytes. All treatments were administered into the peritendinous region with a 26-gauge needle once every two days after injury until day 21.

### Histological analysis

Achilles tendons of control and experimental groups were evaluated at 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> days post injury (dpi). Tendon tissues were quickly dissected and fixed in 4% paraformaldehyde during 12h as described by Moraes *et al.* (27). Briefly, tendons were washed three times with 0.1M phosphate buffer pH 7.4 and cryoprotected by sequential immersion in gradient of sucrose solution (10%, 20% and 30%). Longitudinal sections (20 µm) of tendon tissues were stained with hematoxylin-eosin (HE) and coverslipped using Permount® (Fisher Scientific, New Jersey/U.S.A). The images were visualized and recorded utilizing a light microscopy (Nikon, Eclipse E800 Yokohama, Japan) and analyzed by ImageJ® software.

### Von Frey behavioral test (Mechanical sensitivity)

The hind paw withdrawal threshold was determined using von Frey filaments (SORRI-BAURU, Brazil) ranging from 0.02 g to 10 g. Experimental procedures were realized as a blind test as previously described (28). The tests for control and ruptured groups began after 5 minutes of habituation. The series of von Frey filaments were applied from below customized platform as following. Ipsilateral hind paw of control or ruptured mice were pressed with one of a series of filaments gradually increasing stiffness (0.02-10 g) applied to the plantar surface for 5-6 s for each filament. Ipsilateral hind paw of control or ruptured mice were pressed with one of a series of filaments gradually increasing stiffness (0.02-10

g) applied to the plantar surface for 5-6 s for each filament. Each filament was applied 10 times and the minimum value that caused at least 3 responses which were recorded as paw withdrawal thresholds (PWT). Acute withdrawal, bite, licking or shaking the ipsilateral posterior limb and vocalization were considered positive signs of withdrawal. The average of these values was used for data analysis. The withdrawal threshold was determined to each animal before surgery at 0, 3-, 7-, 14- and 21-days post injury in independent groups.

### Achilles functional index

Tendon functionality was measured utilizing Achilles functional index (AFI) as proposed by Murrell *et al.* (29). Control and experimental animals had their hind paws painted with nontoxic blue ink and then the animals were placed in the walkway apparatus (10 × 60 cm) leaving their footprints on a white paper. The animal footprints were recorded, digitalized and evaluated using the ImageJ software. The values of footprint length (FL), foot spreading (FS) and the intermediary test factor (ITF) were applied in the followed equation of Achilles functional index  $AFI = 74 (FL) + 161(FS) + 48(ITF) - 5$ .

### Statistical analysis

All results were expressed as mean and standard derivation. The comparison among groups was evaluated using analysis of variance (ANOVA-2) followed by Tukey post-test. P-value < 0.01 was considered as significant and all statistical tests were performed using BioEstat 5.2 Software.

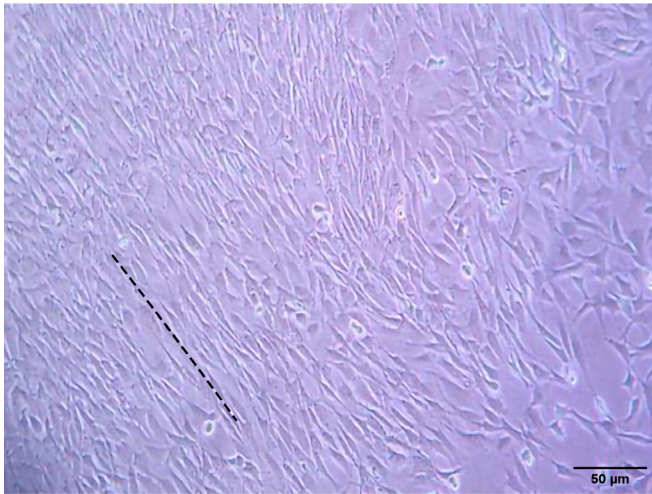
## RESULTS

Data presented in **figure 1** show primary cell cultures of tenocytes at 10 days of development. As observed in the photomicrographs, tenocyte *in vitro* present elongated morphology and a pattern of organization with rows of cells next to each other.

We also have studied the histological alterations in tendon tissue of tenotomized animals. Our data have shown intense tissue disarrangement in tenotomized animals treated with saline or DMEM in the 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> days post injury (**figure 2**). However, tenotomized animals which received local injection of conditioned medium from primary cell cultures of tenocytes presented better tissue organization when compared with tenotomized groups (**figure 2**).

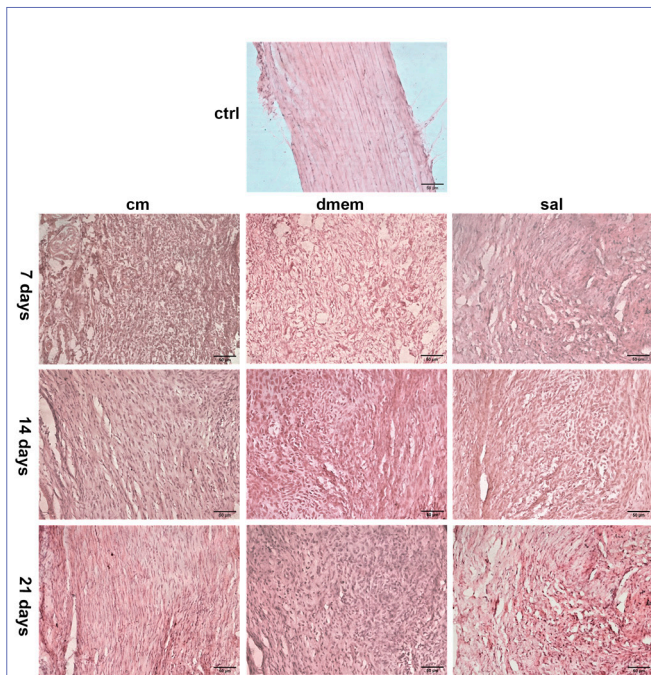
Nociceptive evaluation demonstrated no differences in paw withdrawal thresholds (PWT) among the non tenotomized animals (**figure 3**). However, tendon ruptured animals presented significant decrease in the PWT values for ipsilateral paw at 7 (SAL =  $0.13 \pm 1.15$ ; DMEM =  $0.15 \pm 0.07$ ; CM =  $2.24 \pm 1.15$ ), 14 (SAL =  $0.77 \pm 0.95$ ; DMEM =  $0.45 \pm 0.76$ ; CM =  $2.66 \pm 1.06$ ) and 21 (SAL =  $1.40 \pm 1.03$ ;





**Figure 1.** Microscopy images of primary cell cultures from tenocytes obtained from the Achilles tendon of 1- or 2-day old mice in ten days of cultivation.

Line show pattern of organization and cell proliferation to spaces not yet filled. 20x objective, 50 µm scale bar.

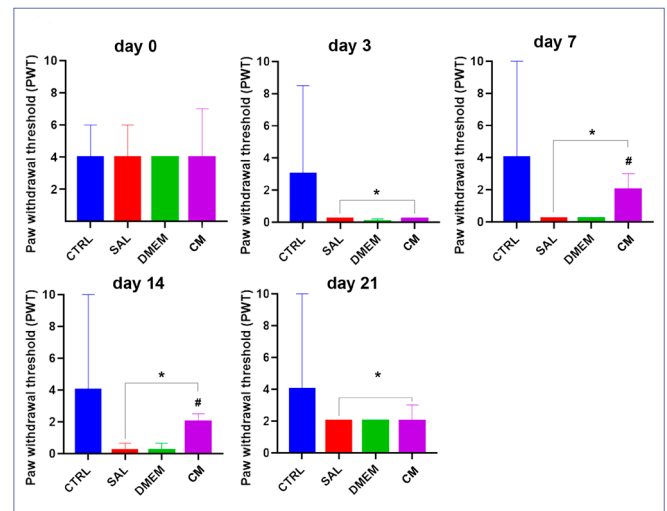


**Figure 2.** Effects of treatment with conditioned medium from tenocytes primary cell cultures on tissue organization at 7-, 14- and 21-days post-injury.

Tenotomized groups treated with saline (SAL), DMEM and Conditioned Medium from tenocytes (CM). HE marking. 20x objective, 50 µm scale bar, n = 9.

DMEM =  $1.40 \pm 1.03$ ; CM =  $2.06 \pm 1.90$ ) days post lesion when compared with control at 7 ( $6.0 \pm 3.46$ ) and 14 ( $5.33 \pm$

$4.16$ ) days post lesion ( $p < 0.01$ ). Our data also showed that DMEM treatment was not able to prevent the decrease in PWT induced by tendon rupture. On the other side, treatment with conditioned medium from primary tenocytes cultures prevented the decrease in PWT induced by tendon rupture at 7 (CM =  $2.24 \pm 1.15$ ) and 14 ( $2.66 \pm 1.06$ ) days post lesion when compared with ruptured groups and at 7 (SAL =  $0.13 \pm 1.15$ ,  $p < 0.01$ ; DMEM =  $0.15 \pm 0.07$ ,  $p < 0,01$ ) and 14 (SAL =  $0.77 \pm 0.95$ ,  $p < 0.01$ ; DMEM =  $0.45 \pm 0.76$ ,  $p < 0.01$ ) days post lesion.

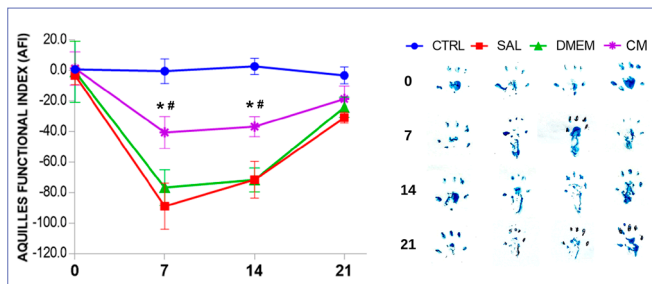


**Figure 3.** Effects of treatment with conditioned medium from tenocytes primary cell cultures on the mechanical sensitivity of mice.

CTRL: Control group, SAL: Saline group, DMEM group and Conditioned Medium from tenocytes (CM). Analyses performed at 0 before surgery, 3-, 7-, 14- and 21-days post injury. Data are presented as mean  $\pm$  SD. \*Significant difference ( $p < 0.01$ ) vs CTRL and # ( $p < 0.01$ ) vs DMEM, n = 9.

In concern of functional evaluation, our data have shown no statistical differences among the days in the Achilles functional index of non tenotomized control group. On the other way, tenotomized animals treated with saline presented significant reduction of Achilles functional index at 7 (CTRL =  $0.33 \pm 8.19$  vs SAL =  $-88.82 \pm 15$ ,  $p < 0.01$ ); 14 (CTRL =  $2.86 \pm 5.40$  vs SAL =  $-71.43 \pm 12.66$ ,  $p < 0.01$ ) and 21 (CTRL =  $-3.02 \pm 5.46$  vs SAL =  $-33.69 \pm 6.49$ ,  $p < 0.01$ ) days post injury. Similar results were observed in tenotomized animals treated only with DMEM at 7 (CTRL =  $0.33 \pm 8.19$  vs DMEM =  $-76.55 \pm 11.72$ ,  $p < 0.01$ ); 14 (CTRL =  $2.86 \pm 5.40$  vs DMEM =  $-71.62 \pm 7.91$ ,  $p < 0.01$ ) and 21 (CTRL =  $-3.02 \pm 5.46$  vs DMEM =  $-37.16 \pm 10.40$ ,  $p < 0.01$ ). However, animals tenotomized which were treated with conditioned medium of primary tenocytes cell cultures presented elevated values of Achilles functional index when

compared with tenotomized animals treated with saline at 7 (SAL =  $-88.82 \pm 15$  vs CM =  $-40.46 \pm 12.60$ ,  $p < 0.01$ ); 14 (SAL =  $71.43 \pm 12.66$  vs CM =  $-36.66 \pm 10.43$ ,  $p < 0.01$ ) or DMEM at 7 (DMEM =  $-76.55 \pm 11.72$  vs CM =  $-40.46 \pm 12.60$ ,  $p < 0.01$ ) and 14 (DMEM =  $-71.62 \pm 7.91$  vs CM =  $-40.46 \pm 12.60$   $p < 0.01$ ) days post lesion.



**Figure 4.** Effects of treatment with conditioned medium from tenocytes primary cell cultures on the functional recovery in Achilles Functional Index (AFI) and conformational gait pattern of the animals.

Analyzes were performed at 7, 14 and 21 days after injury. CTRL: Control group, SAL: Saline group, DMEM group and Conditioned Medium from tenocytes (CM). Values are expressed as mean  $\pm$  SD. \*Significant difference ( $p < 0.01$ ) vs CTRL and # ( $p < 0.01$ ) vs DMEM,  $n = 9$ .

## DISCUSSION

The histological and functional recovery of tendon tissue is a slow and painful process in patients that suffer total rupture of Achilles tendon (30, 31). In this way, several studies have suggested therapeutic strategies to promote a fast and efficient process of tendon recovery after surgical intervention (32, 33).

In current study we demonstrated that treatment with conditioned medium from tenocytes primary cell cultures is able to accelerate the histological and functional recovery of tendon in tenotomized mice. Utilization of cell-based therapies for treatment of tendinopathies already has been described in literature (34-36). However, our study represents the first one to demonstrate that conditioned medium from tenocytes maintained *in vitro* is able to exert beneficial effect on the tendon recovery. Studies performed by Chen *et al.* demonstrated that injections of tenocytes directly into the injured tendon tissue increase the expression of type I collagen as well as accelerate tissue organization (15). These finds support our hypothesis that compounds produced by tenocytes exert beneficial effect to improvement of tendon during it recuperation after mechanical injuries. In fact, all of these observations are in agreement with our histological results demonstrating that treatment with conditioned medium accelerates tissue organization in tenotomized animals.

Other relevant find in the present work was the anti-nociceptive effect exerted by treatment with conditioned medium from tenocytes in culture. As widely demonstrated in literature an important symptom of tendon rupture is the intense local pain (12, 37) which makes very hard the physiotherapeutic intervention in the patients. The molecular mechanism involved in the maintenance of prolonged pain in the injured tendon remains unclear, but increased inflammatory response, intense process of innervations, excessive production of nociceptive agents such as glutamate and substance P are associated with this phenomenon (38, 39). Our data showed that treatment with conditioned medium have significantly attenuated the paw sensibility in tenotomized animals being this effect observed since the first days after tendon rupture. Although posterior experiments need to be performed to clarify this phenomenon, the capacity of tenocytes to produce and release anti-inflammatory cytokines, grown factors and substances able to inhibit substance P activity could explain the effect observed in animals treated with conditioned medium from tenocyte (7, 16, 40).

## CONCLUSIONS

The beneficial effect exerted by the treatment with conditioned medium from tenocyte primary cell cultures on the paw sensibility of tenotomized animals was followed by significant attenuation of dysfunction in the march pattern induced by tendon rupture. Previous studies already demonstrated that better in the Achilles tendon functional index represents an important indicator of efficient treatments (41). In this context, the present pre-clinical study opens perspective to utilization of conditioned medium from tenocytes for treatment of different tendinopathies.

## FUNDINGS

This work was supported by Fundação Amazônia de Amparo a Estudos e Pesquisas (FAPESPA) and Pró-Reitoria de Pesquisa e Pós Graduação da Universidade Federal do Pará (PROPESP/UFPA).

This work was supported by grants from Brazilian funding agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq 306172/2014-3 (AMH), #483414/2012-3- CAPES / Pro-Amazônia #3288/2013 (AMH and KRMO). AMH is CNPq research fellow.

## DATA AVAILABILITY

Data are available under reasonable request to the corresponding author.

## CONTRIBUTIONS

AM: experiments, data analysis and interpretation, writing - original draft. MLM: data analysis and interpretation. DRDP; MDF, LKRL: experiments, data analysis and interpretation. SASdM, RdSB, EdJOB, ACFP, KRHMO: data analysis and interpretation, resources, writing - original

draft. AMH: conceptualization and design, experiments, data analysis and interpretation, resources, writing - original draft, writing - review & editing.

## CONFLICT OF INTERESTS

The author declare that they have no conflict of interests.

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