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Effect of the *Arrabidaea chica* extract on collagen fiber organization during healing of partially transected tendon

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Aims: After undergoing lesions, tendons have disorganized collagen fibers compared to undamaged tendons. *Arrabidaea chica* leaves have the aglycones carajurin and carajurone, components of the antocyanins, with a strong pharmacological potential due to their healing properties. Thus, the aim of this study was to investigate the effect of topical application of *A. chica* extract during tendon healing.
Main methods: The calcaneal tendon of Wistar rats was partially transected with subsequent treatment with *A. chica* extract (2.13 g/mL) followed by excision on the 7th, 14th and 21st days. Control rats received only saline treatment.
Key findings: Transmission electron microscopy analysis showed the presence of a large amount of small segments of collagen fibrils in the transected region of the tendons on the 7th day in both the control and plant-treated groups. Considering the organization of the collagen fibers, higher values of birefringence were observed under polarization microscopy in the tendons of the plant-treated group on the 14th day compared to the control group. A larger quantity of dermatan sulfate was also detected after plant treatment in the same period. However, lesser dermatan and chondroitin sulfate were detected in the plant-treated group than in the control group on the 21st day. No differences were found in the values of birefringence between these groups. Intense metachromasy was observed in both transected groups on the 21st day.
Significance: In conclusion, the use of *A. chica* extract improves collagen organization and increases the quantity of dermatan sulfate on the 14th day of the tendon healing.

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Introduction

Tendons are resistant structures that are constantly subjected to high biomechanical loads, thus becoming prone to acute and chronic injuries (Zhang and Wang, 2010). The incidence of tendon ruptures has increased dramatically due to an increase in the participation in sports by the general population (Józsa et al., 1989; Möller et al., 1996). However, other factors can cause tendon lesions, such as age (Dudhia et al., 2007), diabetes (de Oliveira et al., 2010) and hormones (Soo et al., 2011). Studies have shown that healed tendons present decreased collagen fiber organization compared to intact tendons (Park et al., 2010), with a high prevalence of re-rupture due to the formation of a fibrotic scar, restrictive adhesions, and suboptimal functionality after treatment of tendon lacerations (Józsa and Kannus, 1997).

Although several treatments for tendon lesions have been proposed in recent years, some clinical approaches remain controversial (Okamoto et al., 2010). According to Okamoto et al. (2010), surgical treatment has been described as being advantageous, including low re-rupture rates (Lea and Smith, 1972; Möller et al., 2001), good final range of motion (Cetti et al., 1998; Gigante et al., 2008), short recovery time (Möller et al., 2001; Gigante et al., 2008), and a good final outcome without muscle atrophy (Cetti et al., 1993; Gigante et al., 2008). Although, surgical repair does not consistently restore function (Shearn et al., 2011). Non-surgical treatment methods report better cosmetic appearance (Lea and Smith, 1972; Gigante et al., 2008), reduced risk of infection (Beskin et al., 1987; Carden et al., 1987) and no risk of suture granulomas (Beskin et al., 1987; Fierro and Sallis, 1995). The repair process of the calcaneal tendon is difficult because this tendon is often subjected to high demand biomechanical, becoming unable to bear elevated loads (Greca et al., 2005; Rees et al., 2006). Despite the existence of several treatments, tendon injuries are a considerable socioeconomic problem that can affect people of all ages (Dyment et al., 2012).

Tendons are formed by an abundant extracellular matrix (ECM) composed mainly of type I collagen bundles (Birk et al., 1996), proteoglycans (PGs) (Vogel and Heinegard, 1985), non-collagenous proteins (NCPs) (Aro et al., 2012a), cells and matrix metalloproteinases (MMPs), which are responsible for tissue remodeling. These components, especially collagen and PGs, are arranged to form supramolecular structures (Vidal and Mello, 1984) that confer the biomechanical properties of

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different regions of this tissue. The presence of large amounts of cross-linked collagen molecules confers tensile strength to tendons and makes them highly resistant to mechanical stress (Kjaer, 2004). These molecules have intermolecular interaction sites located at regular intervals along the collagen molecules and along the fibrils (Miller, 1985). These interactions are determined by the primary amino acid sequence, with the sites involved being rich in polar and hydrophobic residues (Hofmann et al., 1978). After tendon lesions, a mechanism called mechanotransduction is initiated wherein mechanical stress acts on the cells and induces an intracellular signaling program that promotes growth and cell survival (Vidal, 1994). Thus, the structural, biochemical and even biomechanical properties of this tissue are not completely recovered after lesions (Józsa and Kannus, 1997; Park et al., 2010). Clearly, alternative methods need to be developed to improve the efficacy of tendon repair.

For many centuries, plant extracts have been employed to treat diseases, and their active principles have great pharmacological potential. With the aim of improving tendon healing, some plants such as *Hippophae rhamnoides* (Fu et al., 2005), pineapple fruit parts (Aiyegbusi et al., 2010), safflower yellow (Liu et al., 2011), bromelain and fresh pineapple juice (Aiyegbusi et al., 2011) and *Aloe vera* (Aro et al., 2012b) have been studied. The *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. syn. *Bignonia chica* (Humb. & Bonpl.), (Bignoniaceae) is a liana widely distributed in Neotropical regions (Zorn et al., 2001). The leaves of this plant have been used in folk medicine for wound healing, the treatment of inflammation, intestinal colic, sanguine diarrhea, leucorrhoea and anemia (Zorn et al., 2001; Jorge et al., 2008). In the present study, the effect of the *A. chica* leaf extract was investigated during tendon healing. For this experiment, the crude *A. chica* leaf extract was standardized according to Jorge et al. (2008) and included the aglycones carajurin and carajurone. These compounds are anthocyanins, which are phenolic water-soluble pigments that are released after acid hydrolysis (Lima et al., 2006). Scientific interest in these pigments has recently increased due to the effective bioactivity of anthocyanins and their aglycones (Lima et al., 2006) as anti-inflammatory (Seeram et al., 2001), antioxidants (Kähkönen and Heinonen, 2003) and vaso-dilator substances (Burns et al., 2000), besides other biological properties.

According to Jorge et al. (2008), the crude extract of *A. chica* improved skin healing, with increased collagen production. These data corroborate a previous study performed in our laboratory in which a higher total collagen concentration was detected in injured tendons after treatment with the *A. chica* extract (Aro et al., in press). Antimicrobial (Höfling et al., 2010) and anti-inflammatory (Zorn et al., 2001) potentials of the leaf extract of *A. chica* have also been identified. However, there is no study about the effect of *A. chica* on collagen fiber organization. As it is known, a better organization of the collagen fibers after any kind of lesion is essential to improve the recovery of the functional properties of the tendon. Because tendon injuries are a clinical issue that requires innovative treatments, we have proposed to improve the organization of collagen fibers by the topical application of *A. chica* extract during tendon healing.

Materials and methods

Plant material and extraction

The lyophilized extract of the *A. chica* plant utilized in our study was provided by Multidisciplinary Center for Chemical, Biological and Agricultural Research, (CPQBA) — UNICAMP, after standardization of the aglycones [pigment 1 (carajurone), pigment 2 and pigment 3 (carajurin)] according to Jorge et al. (2008). These pigments were previously isolated and characterized in a study of Zorn et al. (2001) and Devia et al. (2002), and their structures can be seen in the Fig. 1. Leaves of *A. chica* (Humb & Bonpl) Verlot available in the Germplasm Bank (from access 06) were collected from the CPQBA, located in the city of Paulínia (22°45′00″ South and 47°10′21″ West). The plant material was ground with dry ice in a knife grinder, Stephen, model UM 40. The ground leaves were extracted three times with a mixture of 1:5 (v/v) ethanol/0.3% citric acid. The extracts were filtered, dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed in a vacuum, followed by lyophilization. The lyophilized extract was dissolved in saline solution (0.85%) at a concentration of 2.13 g/mL for application to the lesion site on each tendon, as described below.

Protocols for the partial transection of the calcaneal tendon and topical application of the A. chica extract

For surgical procedures, the animals were anesthetized with intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg), and the lower right paws underwent trichotomy and antisepsis with iodine alcohol. A longitudinal incision was made in the skin to expose the calcaneal tendon, and the partial transverse transection was performed in the tension region of the tendon that is located an approximate distance of 4 mm from the tendon's insertion into the calcaneus bone (Tomiosso et al., 2009). We dissolved 32 mg of the *A. chica* in 15 μL of 0.85% saline solution and applied the paste-like extract at the site of the tendon transection prior to the suture of the skin (Aro et al., in press). After application of the extract, the skin was sutured with nylon suture (Shalon 5-0) and needle (1.5 cm). From day 1 after surgery, we performed a daily topical application of the plant extract in the region of the sutured skin, gently massaging for its absorption. Due to the high penetrating power of the *A. chica* extract, this procedure was repeated until the 7th day after the surgery for a total of 7 topical applications. This same procedure was used for the transected tendons of controls that received applications of 15 μL of 0.85% saline solution. For the immobilization of the animals during the daily topical applications, a rat retainer was used (Request Privilege of Utility Model in Brazil, entitled: Retainer of rats for laser beam and biocompounds application, deposited in National Institute of Industrial Property — INPI, on 04.16.10, under n° MU9000622-4, authored by researchers Andrea Aparecida de Aro and Edson Rosa Pimentel, from Institute of Biology, UNICAMP).

Experimental groups

Sixty eight-day-old male Wistar rats ($n = 77$, weighing 250 ± 20 g), with free access to food and water, were used in the present study. The animals were separated into 7 experimental groups: the normal group (N) — rats with tendons without transection and euthanized at age eighty-two-days-old; the S7, S14 and S21 groups — rats with transected tendons treated with topical applications of saline for 7 days and euthanized on the 7th, 14th and 21st days after surgery, respectively; and the A7, A14 and A21 groups — rats with transected tendons treated with topical applications of the *A. chica* extract for 7 days and euthanized on the 7th, 14th and 21st days after surgery, respectively. This study was performed according to the Institutional Committee for Ethics in Animal Research of the State University of Campinas — Unicamp (protocol n° 1621-1).

Birefringence: image analysis and measurements

The tendons ($n = 4$) were fixed using 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH, pH 7.4) for 24 h at 4 °C. Then, the tendons were washed in water, ethanol dehydrated, diaphanized with xylene, and paraffin embedded. Longitudinal serial sections of 7 μm, obtained and after deparaffinization, were subjected to microscopy analysis. Image analyses of the tendons were evaluated to detect differences in its morphology, considering the aggregation and organization of the collagen bundles, which reflect the variation of birefringence intensity. Birefringence properties were studied using an Olympus BX53 polarizing microscope and an image analyzer (Life Science Imaging Software, Version 510_UMA_cellSens16_Han_en_00). Since birefringence appears visually as brilliance, this

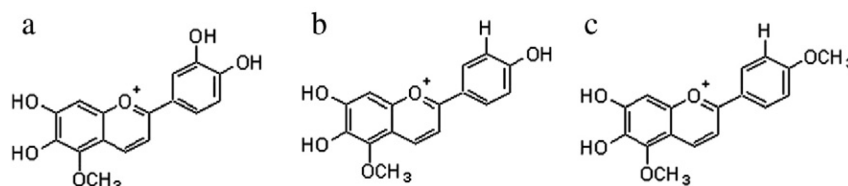

Fig. 1. Structures of the aglycones from the *A. chica* extract. a) Pigment 1 named carajurone (6,7,3′,4′-tetrahydroxy-5-methoxy-flavylium). b) Pigment 2 (6,7,4′-trihydroxy-5-methoxy-flavylium). c) Pigment 3 named carajurin (6,7-dihydroxy-5,4′-dimethoxy-flavylium).

phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits = 1 pixel). The larger tendon axis was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen bundles exhibit two kinds of birefringence: intrinsic birefringence (Bi) and form or textural birefringence (Bf) (Vidal, 1980; Vidal, 1986), total birefringence (sum of Bi and Bf) was used in this study. Sixty GA measurements of the transected regions of the tendons in each experimental group were made after immersing the sections in water, a condition in which total birefringence is highly detectable (Vidal, 1980; Vidal, 1986; Vidal and Mello, 2010). The measurements of GA were represented as arithmetic mean and standard deviation.

Transmission electron microscopy

The transected region of the calcaneal tendon of each group and the tension region of normal tendons (n = 3) were immersed in a fixative solution containing 5% glutaraldehyde and 0.25% tannic acid in Millonig's buffer (0.13 M sodium phosphate, 0.1 M sodium hydroxide, 0.03 M glucose — pH 7.4) for 24 h at room temperature. The samples were washed with Millonig's buffer, post fixed in 0.5% osmium tetroxide in the same buffer for 1 h at room temperature. After washing in water rapidly, the samples were acetone dehydrated and embedded in Epon® resin. Ultrathin sections (60–80 nm) were stained with 3% uranyl acetate for 30 min and 0.2% lead citrate in 0.1 N sodium hydroxide for 5 min and observed under an LEO 906 transmission electron microscope (Zeiss, Jena, Germany) operated at 80 kV. The images were captured with a digital imaging system for documentation.

Light microscopy analysis

After dissection, the tendons (n = 4) were fixed using a 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH — pH 7.4) for 24 h at 4 °C. The tendons were then washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal serial sections of 7 μm were obtained for microscopy analysis. For observation of PGs, the sections were deparaffinized and then were stained with 0.025% toluidine blue (TB) in McIlvaine buffer (0.03 M citric acid, 0.04 M sodium phosphate dibasic — pH 4.0) (Mello and Vidal, 2003). The sections on slides were air dried and immersed in xylene, before embedding in entellan (Merck, Rio de Janeiro, Brazil). For observation of tendon morphology, tissue sections were analyzed under an Olympus BX53 microscope.

Quantification and characterization of sulfated glycosaminoglycans

After washing in PBS (Phosphate-Buffered Saline — 5 mM phosphate buffer, 0.15 M NaCl and 50 mM EDTA), fragments from the tendons (n = 4) were immersed into acetone for 12 h and dried in a histological oven (37 °C) for 12 h. GAGs were extracted with papain solution (40 mg/g tissue) in 100 mM sodium phosphate buffer, pH 6.5, containing 40 mM EDTA and 80 mM β-mercaptoethanol for 24 h at 50 °C. After precipitation using 90% TCA (trichloroacetic acid) for 10 min at 5 °C, the samples were centrifuged and submitted to precipitation with methanol during 12 h at 5 °C. The precipitate was resuspended in water and used for the measurement of GAGs (Farndale et al., 1986). Before electrophoresis, 5 μg of each sample was treated with DNAse (10 mg/mL) in 20 mM Tris–HCl buffer, pH 7.4 for 30 min at 37 °C. Then, the sulfated glycosaminoglycans (GAGs) chondroitin (CS), dermatan (DS) and heparan sulfate (HS) were separated by electrophoresis in agarose gel (0.5%) in 0.05 M propylene diamine (Dietrich and Dietrich, 1976), at 0.1 mA for 45 min. The agarose gels were fixed in cetavlon and stained with 0.2% TB. The gels were washed with a solution containing 50% ethanol and 1% acetic acid for observation of bands. The identification of GAGs was confirmed by digestion with chondroitinases B and AC. In addition, the DS and CS were individually quantified in agarose gel by densitometry of bands using the Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA) and by comparison with a standard of 5 μg of each GAG provided by Sigma (Sao Paulo, Brazil).

Statistical analyses

For the biochemical analysis, data from different experimental groups were analyzed by *t*-test Student ($p < 0.05$) for comparisons between the treated and non-treated groups for each period, and by one-way analysis of variance followed by the Tukey test for multiple comparisons ($p < 0.05$). The Mann–Whitney test ($p < 0.05$) was used only for analysis of the birefringence measurements, also using the GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0.

Results

Analyses of images obtained from polarization microscopy (Fig. 2) showed intense brightness in normal tendons due to the high aggregation and organization of collagen fibers (Fig. 2a). The transected region (TR) of injured tendons and the proximal and distal regions (T1) that border the TR were analyzed. On the 7th day, no birefringence measurements were performed due to the low birefringence observed in the TR of groups S7 and A7, indicating complete disorganization of the ECM at this time (Fig. 2b,c).

On the 14th day after lesion, the birefringence measurements detected higher values in the TR of the A14 (85.0) compared to the S14 (62.9) groups (Table 1), indicating that *A. chica* improved the organization of the collagen fibers at this time. Observations of images of birefringence also confirmed better organization in A14 than in S14 (Fig. 2d, e). It is important to note the presence of fiber fragmentation in T1 in both S14 and A14 groups (Fig. 2d, e). There was an imbrication between TR and T1 in the collagen fibers that were not cut, and there were also newly formed fibrils throughout the region adjacent to the TR (Fig. 2f). At 21 days after injury (Fig. 2g–i), greater organization and aggregation of collagen fibers were observed compared with images of tendons 14 days after injury. At this time, measurements (Table 1) showed a gradual increase of birefringence compared to 14 days, with no differences between groups S21 (105.0) and A21 (104.8). As observed in the images (Fig. 2i), fragmentation of the collagen fibers was detected in T1 even 21 days after the lesion, with values of birefringence lower than those found in the normal tendon (Table 1).

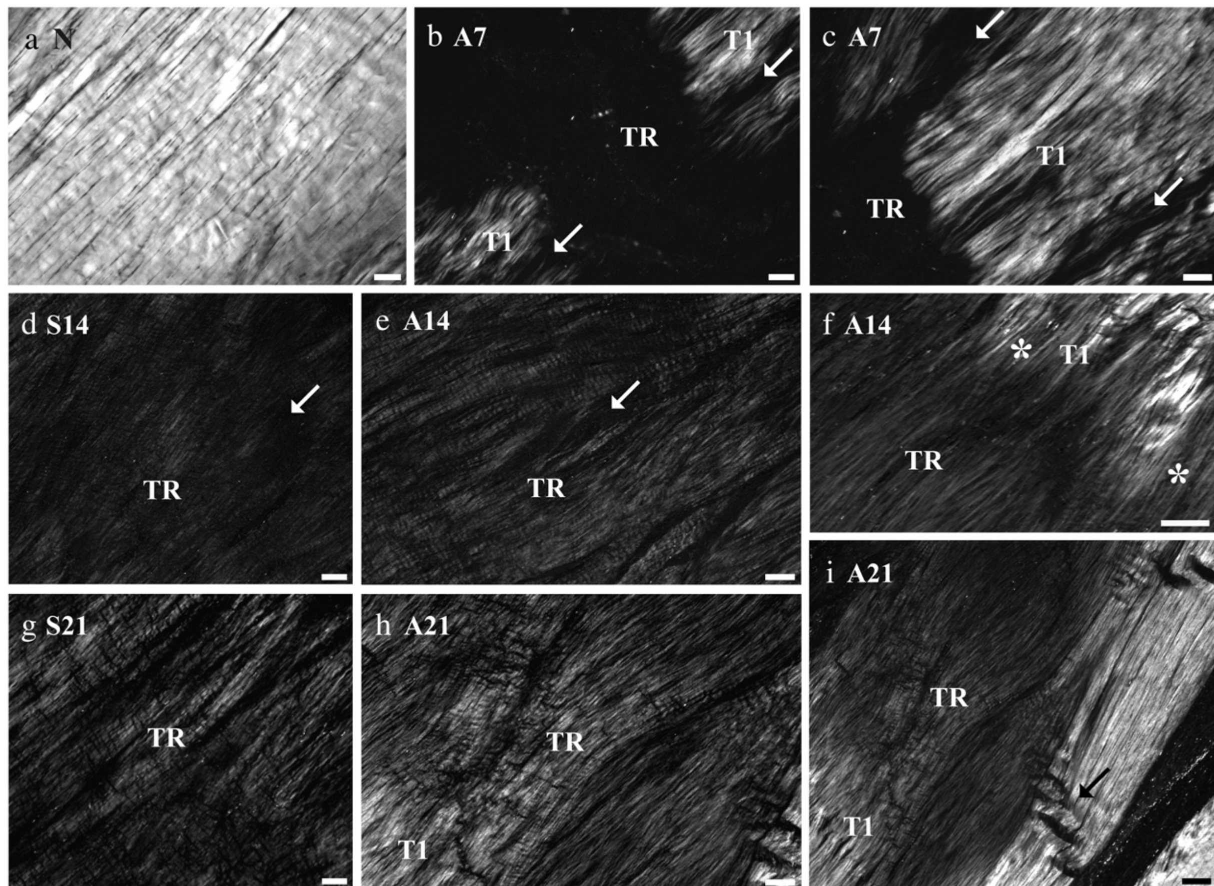

Fig. 2. Images of tendon sections using polarization microscopy. (a) group N: birefringence of the collagen fibers in the proximal region of tendon; the variation in brilliance intensity (gray levels) is due to the collagen fiber waviness. (b) group A7: TR corresponds the dark area that is due to complete disorganization of the collagen fibers. Observe the proximal and distal regions (T1), which border the TR. (c) group A7: the detail of the same image as in (b) displays the fragmentation of the collagen fibers in T1 (→). (d) group S14: In TR, the collagen fibrils are more regularly arranged along the larger tendon axis, although fibers are fragmented (→). (e) group A14: observe higher birefringence of the collagen fibers compared to the S14 and fiber fragmentation (→). (f) group A14: observe an imbrication between collagen fibers that were not cut in T1 as well as newly formed fibrils (*) throughout the region adjacent to TR. (g and h) S21 and A21 groups: the birefringence of the newly formed fibrils is more prominent in the TR compared to the earlier periods. (i) group A21: a panoramic view of the tendon showing the non-homogeneous organization of collagen fibers along the TR and T1 regions. Fragmentation of the fibers in the remaining portion of the tendon is located below the sectioned region (→). Images of the S7 group were not shown because they were similar to the A7 group. Bar = 60 μm (a, b, c, d, e, f, g, h) and bar = 120 μm (i).

Ultrastructural analysis (Fig. 3) showed highly organized longitudinal collagen fibrils in the normal group, with the presence of uniform pattern of bands (Fig. 3a). This pattern occurs because of the oriented aggregation of collagen molecules during the formation of each collagen fibril. In groups S7 and A7 on the 7th day after the injury, the collagen fibrils were completely disorganized, as shown by the fibrils arranged in various directions in TR (Fig. 3b, c). It was possible to observe the presence of small segments of collagen fibrils in the ECM of TR in both groups on the 7th day; these were likely to be the remnants of old segments (Fig. 3b, c). Regions of matrix lacking fibers were observed in S7 and A7 groups. The cells presented well-developed endoplasmic reticulum and secretory vesicles in their cytoplasm, indicating a high level of matrix component synthesis. In A7 group, an endoplasmic reticulum with enlarged cisternae was frequently observed, probably involved in the large amount of procollagen production (Fig. 4a).

The micrograph analyses on 14th day (Fig. 3d, e) revealed a greater number of newly formed collagen fibrils in TR, which were more organized in the plant-treated group than in the saline-treated group. It was still possible to observe the presence of small segments of collagen fibrils in A14 group (Fig. 3e), as well as the presence of more elongated fibroblasts and fibrils longitudinally and transversely oriented. On the 21st day after lesion (Fig. 3f, g), the presence of small segments of collagen fibrils was diminished compared to earlier phases. The longitudinally oriented collagen fibrils were more prominent, with no apparent differences between groups S21 (Fig. 3f) and A21 (Fig. 3g). Endoplasmic reticulum with enlarged cisternae was frequently detected in S21 group (Fig. 4b). In the A21 group, the plasma membrane cleft was evidenced (Fig. 4c).

The longitudinal sections of the calcaneal tendons stained with TB showed an increased number of cells and blood vessels in the TR of groups S7 and A7, with low metachromasy in both groups (Fig. 5b, c). The number of cells was also high in the 14 day groups (Fig. 5d, e),

Table 1
Birefringence measurements: GA (pixels) variability in collagen fiber organization on the TR region of the tendon. On the 14th day after lesion, a higher value in TR of the A14 group was observed compared to S14 group. On the 21st day, no difference was observed between the groups. The transected tendons had birefringence measurements lower than normal tendons.

Groups	TR (GA median)	Comparisons	Mann–Whitney test (p)
N	228.5		
S14	62.9		
A14	85.0	S14 × AI4 (TR)	(0.000)*
S21	105.0		
A21	104.8	S21 × A21 (TR)	(0.400)

GA: Gray Average. The largest axis of the tendon was positioned at 45° with respect to the crossed polarizers. The number of measurements (60) chosen at random in 12 sections from four tendons of each group.
* Significant differences between the transected groups ($p < 0.05$).

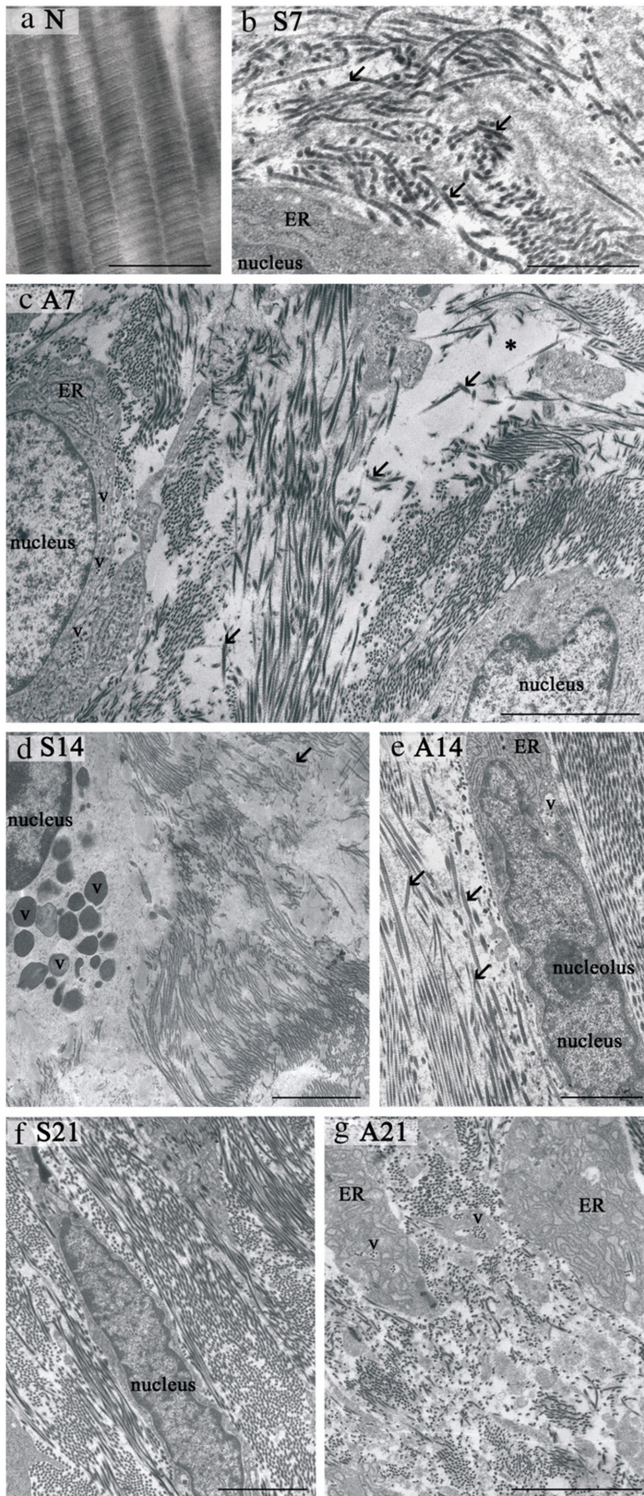

Fig. 3. Transmission electron micrographs of the tendons. A highly organized ECM may be seen in the group N, with the characteristic band pattern of collagen fibrils in longitudinal section (a). Observe in the TR of S7 and A7 groups (b, c) the collagen fibrils completely disorganized, the presence of small segments of collagen fibrils (→) in the ECM, and regions of the matrix without the presence of fibers (*). Observe on the 14th day, the higher organization of collagen fibrils in A14 (e) compared to S14 and presence of small segments of collagen fibrils (→). The fibroblasts were more elongated in S21 (f) and the collagen fibrils longitudinally and transversely oriented in S21 (f) and A21 (g) groups. Observe the presence of a well-developed endoplasmic reticulum (ER) in the transected tendons. v = secretory vesicles. Bar = 3 μm (c, f, g), Bar = 2 μm (d, e), bar = 1 μm (b) and bar = 0.5 μm (a).

with greater orientation of the matrix than at 7 days post-injury. Apparently, no differences in metachromasy were observed between the saline- and plant-treated groups on 14th day (Fig. 5d, e). The cell migration (►) from the paratenon/epitenon to the TR was evidenced in the group A14 (Fig. 5f). On the 21st day (Fig. 5g, h), metachromasy was higher in both groups compared to earlier phases, and the number of cells was still high. A panoramic view of the tendon with the high metachromasy (*) in TR of the A21 group was showed (Fig. 5i). It is important to mention that in the N group (Fig. 5a), the matrix was not stained because of the low amount of PGs. Only the nuclei were stained in the sections of normal tendons.

The total sulfated GAG quantification (mg/g tissue) showed that all groups of transected tendons had a higher concentration of these components compared to N group, but without significant differences between the saline and plant-treated groups (Table 2). Densitometry analysis of the bands (pixels) obtained from the electrophoresis (Fig. 5) showed an increase of DS (dermatan sulfate) in A14 group (679.8 ± 95.1) compared to S14 group (403.7 ± 10.4). On the 21st day, A21 group had lower amounts of DS (747.8 ± 69.3) and chondroitin sulfate (CS: 220.1 ± 18.3) compared to S21 group (SD: 958.9 ± 85.1; CS: 322.1 ± 54.6). Considering the amounts of DS and CS, with the exception of S21 group, all groups showed lower values than the N group (Fig. 6).

Discussion

Tendon healing is a slow and complex process that occurs in three phases called the inflammatory, proliferative and remodeling phases (Tomiosso et al., 2009; Dyment et al., 2012). Our purpose was to study the effect of the topical application of *A. chica* extract on collagen fiber organization during these three phases of tendon repair. On the 7th day after injury, the tendon sections showed a remarkable increase of cellularity and an increased number of blood vessels at the transected area in both the saline- and plant-treated groups. It is known that the inflammatory phase that begins immediately after injury and peaks on day 7 is characterized by an increased number of blood vessels and the migration of synovial and inflammatory cells to the site of tendon repair (Sahin et al., 2012). Fibroblasts and myofibroblasts that participate in tendon healing may arise from the tendon, epitendon, paratendon, or from a combination of these (Garner et al., 1989). This cellular migration and proliferation are due to the action of cytokines and growth factors produced from platelets and macrophages (Hoppe et al., 2013; Bedi et al., 2012). These events in the early phase of tendon healing have an important role in increasing the metabolism of cells, as shown by the presence of well-developed rough endoplasmic reticulum and of secretory vesicles observed in the micrographs of both groups on the 7th day. The increase in cellular metabolism at this phase is targeted towards the synthesis and degradation of matrix molecules to provide a temporary fragile structure at the repair site (Dyment et al., 2012).

The ultrastructural results showed the presence of small segments of collagen fibrils in the ECM of the transected region in both groups on the 7th day, although the existence of small fragments of collagen fibrils cannot be neglected. The MMPs that are more abundant in this phase (Oshiro et al., 2003; Sahin et al., 2012) can be directly related to collagen degradation. Recent work performed by our laboratory showed similar quantities of the active isoform of MMP-2 and MMP-9 in both the saline- and plant-treated groups, indicating the participation of these MMPs in the collagen degradation observed in this period (Aro et al., in press). MMP-2 and MMP-9 degrade several types of collagen, gelatin and other non-collagenous components of the ECM (Chakraborti et al., 2003) and are involved in remodeling (MMP-2) and inflammatory (MMP-9) processes (Oshiro et al., 2003; Almeida et al., 2012; Vieira et al., 2012). Therefore, concomitant with the action of MMPs, inflammatory cells and macrophages act on the removal of cell debris (Tomiosso et al., 2009) as well as fragmented and denatured collagen. Other matrix components are also removed in this early stage of the healing process,

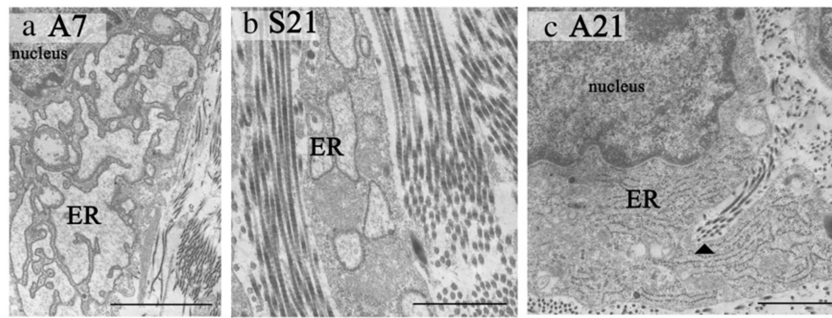

Fig. 4. Transmission electron micrographs of the tendons. Observe the presence of ER (endoplasmic reticulum) with enlarged cisternae in A7 (a) and in S21 (b). Note the presence of plasma membrane cleft (►) in A21 (c). Bar = 3 μm (a), and bar = 1 μm (b, c).

resulting in regions without the presence of matrix, as observed in the micrographs of both groups 7 days after the lesion.

The proximal region of the calcaneal tendon which is predominantly subjected to tensional forces, is especially composed of type I collagen that is highly organized into fibrils, fibers, bundles of fibers and fascicles (Józsa and Kannus, 1997; Aro et al., 2008), forming a complex supramolecular structure (Vidal and Mello, 2010). In this region, the collagen bundles are organized in a helical arrangement along the larger axis of the tendon (Vidal, 2003) and the fibers are highly oriented and aggregated, exhibiting strong birefringence (Vidal, 1980; Vidal, 1986; Vidal and Mello, 2010). On the 7th day after lesion, the low birefringence found in that region indicated the high disorganization of the ECM, justifying the absence of birefringence measurements in this period. Our ultrastructural results showed that the low birefringence is due to the presence of a disorganized network composed of newly-formed thin fibrils and to a large amount of old remnant collagen fibrils which had not yet been removed in this early phase of repair.

On the 14th day after the injury, the peak of the proliferative phase occurs (Oshiro et al., 2003; Tomiosso et al., 2009). The morphological and ultrastructural analyses showed a more compact granulation tissue in this phase, with fibroblasts more regularly arranged along the larger axis of the tendon. According to previous results performed in our

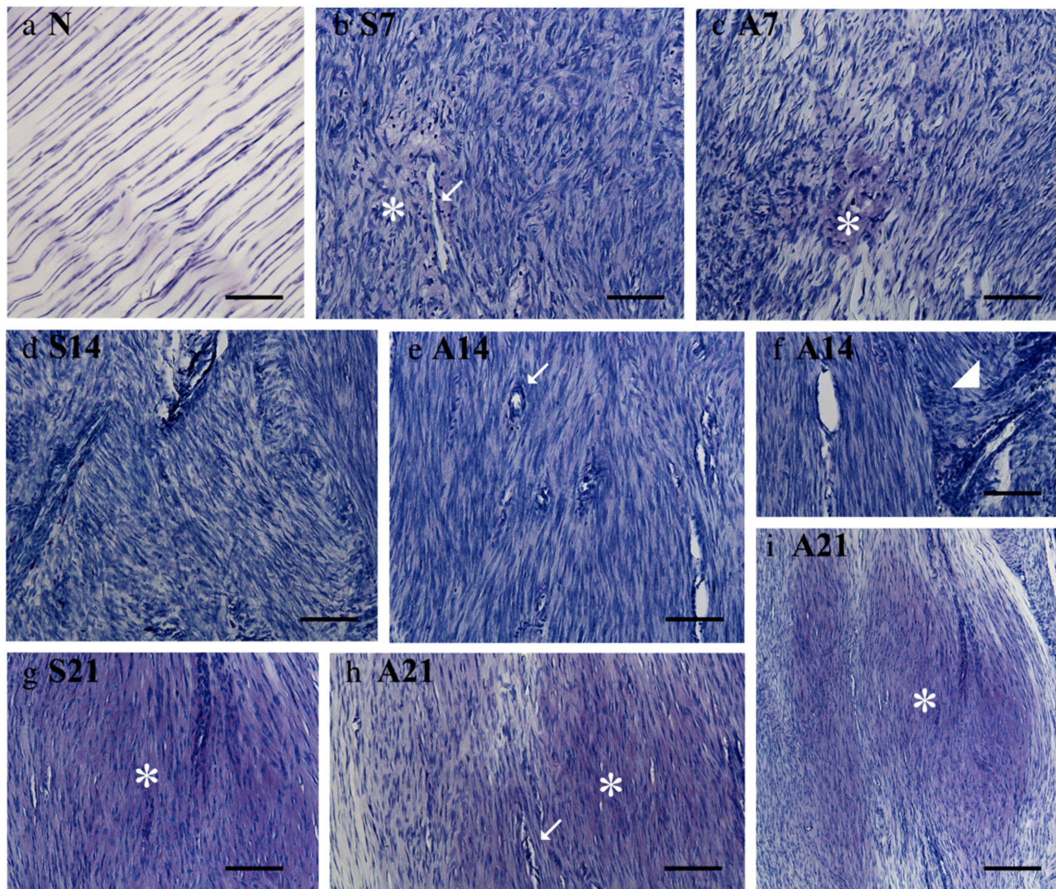

Fig. 5. Images of tendon sections stained with toluidine blue. Observe the numerous elongated fibroblasts oriented in the ECM of the proximal region of the tendon (a). In S7 and A7 groups, observe the low metachromasy in the ECM (*) of TR (b, c). In the 14 day groups, observe a better orientation of the matrix in TR (d, e), compared to the 7 day groups, with no difference in metachromasy between the saline- and plant-treated groups. Observe cell migration (►) from the paratenon/epitenon to the TR (f). On the 21st day, the metachromasy was higher in both groups compared to the earlier phases (g, h). A panoramic view of the tendon showing the high metachromasy (*) in TR of the A21 group. (→) indicates presence of blood vessels. Bar = 120 μm (a, b, c, d, e, f, g, h) and bar = 240 μm (i).

Table 2
Concentration of GAGs: S7, A7, S14, A14, B21 and A21 groups had higher values of GAGs in relation to the group N (*). Band densitometry of the electrophoresis in agarose gel: higher value of DS was observed in the A14 group compared to the S14 group ([a]); and lower values of DS ([b]) and CS ([c]) were observed in the A21 group compared to the S21 group.

Parameters	N	S7	A7	S14	A14	S21	A21
Concentration of GAGs							
GAGs	3.4	9.7	9.9	10.5	11.1	11.7	10.5
(mg/g tissue)	±1.2	±1.3*	±1.3*	±2.0*	±3.0*	±2.1*	±2.9*
Band densitometry from the electrophoresis in agarose gel							
DS	1091.0	608.6	606.9	403.7	679.8	958.9	747.8
(pixels)	±104.5	±169.8*	±166.6*	±10.4*,a	95.1*,a	85.1[b]	69.3*,b
CS	367.8	217.2	242.1	166.5	249.2	322.1	220.1
(pixels)	±37.5	±53.6*	±34.4*	±34.9*	±48.9*	±54.6[c]	±18.3*,c

([a,b,c]) Significant differences between the groups marked with the same letter, according to *t*-test Student ($p < 0.05$).
* Significant differences between the normal and the transected tendons, according to Tukey test ($p < 0.05$).

laboratory, the granulation tissue is formed by collagenous and non-collagenous proteins, MMPs, PGs, cells and new capillaries that were initially formed during the inflammatory phase, as also shown by several other studies (Garner et al., 1989; Aro et al., 2012b, in press; Dyment et al., 2012;). During the proliferative phase, the cells still have high metabolic activity, as demonstrated by the presence of the well-developed rough endoplasmic reticulum observed in the micrographs in both the saline- and plant-treated groups.

Our birefringence measurement data showed differences in the degree of ordered aggregation of the collagen fibers along the larger axis of the tendon on the 14th day after lesion. The collagen fibers in the transected region of the group treated with the *A. chica* extract showed higher birefringence compared to the saline-treated group, indicating greater organization of these fibers. The higher molecular orientation of collagen fibers observed in this group may be related to differences in the amounts of PGs. Our results showed large quantity of DS after plant extract treatment. Fibromodulin and lumican are PGs containing keratan-sulfate (KS), while decorin and biglycan are both chondroitin/dermatan sulfate PGs belonging to the ECM family of small leucine-rich PGs (Esquisatto et al., 2007; Viola et al., 2007). The small PGs are altered during the healing process (Mello et al., 1975; Mello and Vidal, 2003; Liang et al., 2008) and are intimately associated with collagen fibers to regulate fibrillogenesis in the tendons (Vogel et al., 1984; Birk and Trelstad, 1986) and restore the matrix structural organization. In addition to the small PGs that are characteristic of regions subjected to tensional forces (Vogel and Heinegard, 1985; Rees et al., 2000) as occurs in the proximal region of the calcaneal tendon where the transection was performed in the present study, the large aggregating PG aggrecan may also be found in tensional regions of tendons (Vogel et al., 1994; Rees et al., 2000). Thus, the higher amounts of DS observed in the group treated with the *A. chica* extract suggest alterations in the proportions of different PGs bearing DS.

It is important to mention that besides differences in the amounts of PGs, a previous study performed in our laboratory (Aro et al., in press) showed a possible anti-inflammatory effect of the extract of *A. chica* resulting in an improvement of the gait and reduction of MMP-9, 7 and 14 days after injury respectively. This result can indicate an acceleration of the healing process which was evidenced in the present study by a higher collagen organization of the tendons of the plant-treated group on the 14th day, compared to the saline-treated group.

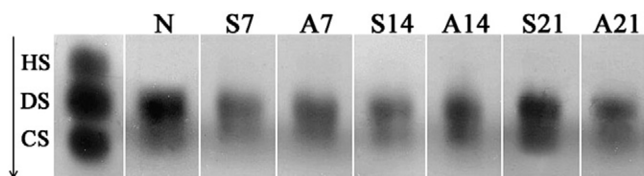

Fig. 6. Electrophoresis in agarose gel with 5 μg of GAGs in each sample. Observe different proportions of DS and CS in the groups. Band densitometry is shown in Table 2. HS, DS and CS standards are on the left.

Jorge et al. (2008) observed an acceleration of the skin healing after using the same *A. chica* leaf extract standardized in the presence of the carajurin and carajurone, corroborating our results. Zorn et al. (2001) showed an anti-inflammatory action of the lipophilic extract of *A. chica* leaves that contains the carajurin, a natural phenolic compound. The carajurin acts in synergy with other components still to be identified, that inhibit the factor NF-kB (nuclear factor-kappa B) transcription. As it is known, the factor NF-kB regulates the transcription of genes encoding several inflammatory molecules. In another study using the aqueous *A. chica* extract (Oliveira et al., 2009), substances with inhibitory activity on the inflammatory effects during the edema induction were found.

During the initial remodeling phase at 21 days after the lesion, the recess in the fibroblast plasma membranes is observed mainly in the group treated with the *A. chica* extract. It is called a plasma membrane cleft, and represents a specialized extracellular compartment (Sprague et al., 2011). Within these clefts, newly synthesized procollagen is cleaved into tropocollagen (Birk and Trelstad, 1986), which will be incorporated into the expanding collagen fibers (Birk et al., 1989). Collagen fibrillogenesis is remarkable during this period compared to the earlier phases and can be determined by the presence of regions with unidirectional organization of collagen fibers, according to birefringence observations.

In this phase, the *A. chica* extract did not influence the collagen fiber organization in the TR. No significant differences were observed in the birefringence measurements between the saline- and plant-treated groups. This result is interesting because higher birefringence in this group was expected on the 21st day, since a higher value had been observed 14 days after injury. However, this result corroborates the finding of a lesser amount of DS and CS observed in this group after plant treatment on the 21st day. The small leucine-rich PGs regulate the fibrillogenesis of type I collagen due to their association with the collagen fibers and thereby regulate the structural organization of the tissue (Vogel et al., 1984; Viola et al., 2007). Once again, differences in CS and DS quantities suggest possible alterations in the proportions of different PGs affecting the collagen organization. Further studies should be conducted to investigate whether the decrease in CS and DS after treatment with the *A. chica* extract is related to increased enzymes, which could have acted in the degradation of these components, or if there was a change in PGs turnover.

Conclusion

In the present study, we hypothesized that the topical application of *A. chica* extract could improve the organization of collagen fibers. In fact, the results reported herein demonstrate that the crude *A. chica* extract acted in the proliferative phase of tendon repair, improving the molecular organization of the collagen fibers. New strategies that improve collagen organization after tendon lesions could be interesting for clinical procedures, considering that collagen provides integrity and functionality to tendons. Thus, further studies should

be conducted to identify which molecular mechanism is involved in the better collagen reorganization, as well as the effect of the *A. chica* extract on the PG turnover.

Conflict of interest statement

The authors report no conflicts of interest.

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