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RESEARCH ARTICLE

Effect of different resistance-training protocols on the extracellular matrix of the calcaneal tendon of rats

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ABSTRACT

The calcaneal tendon extracellular matrix (ECM) is composed of collagen, non-collagenous glycoproteins and proteoglycans, and able to adapt to various biomechanical stimuli. The objective of this study was to analyze the response of different resistance-training protocols, such as hypertrophy, strength and resistance, on the organization of the calcaneal tendon after training. Wistar rats were divided into four groups: untrained (UT), resistance training (RT), hypertrophy training (HT), and strength training (ST). The protocol in a vertical climbing platform was performed thrice per week over twelve weeks. For biochemical study, the tendons of each group were minced and analyzed for gelatinases, quantification of non-collagenous proteins, sulfated glycosaminoglycans, and hydroxyproline. For morphological analysis, sections were stained with HE and toluidine blue. Non-stained sections were used for birefringence analysis under polarization microscopy. The highest hydroxyproline concentrations were found in HT (154.8 ± 14.2) and RT (173.6 ± 25.2) compared with UT (122.4 ± 27.0). A higher concentration of non-collagenous proteins was detected in the RT group (14.98 mg/g) compared with the other groups. In polarization microscopy, major birefringence was observed in HT and the lowest in ST compared with UT, indicating higher organization of collagen bundles in HT. In analysis for zymography, the presence of latent MMP-9 was more prominent in the ST group and the active MMP-9 more prominent in the HT group. For MMP-2, significant differences in the latent isoform between the HT ($184{,}867 \pm 6765$) and UT ($173{,}018 \pm 9696$) groups were found. In sections stained with toluidine blue (TB), higher metachromasia was observed in the tendon's distal region in HT and RT groups, indicating a greater amount of proteoglycans. We conclude that the different training protocols produced different responses in the ECM. The remarkable presence of MMP-2 and -9 in the hypertrophy training group may be related to the highest organization of collagen bundles and possibly a more efficient remodeling process, observed in that group, as demonstrated by images and measurements of birefringence.

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1. Introduction

The calcaneal tendon, also known as Achille's tendon, has a fibrous extracellular matrix (ECM) consisting of collagen, glycoproteins and proteoglycans (Vidal and Mello, 1984; Vidal and Mello, 2010), and it is able to adapt to different biomechanical stimuli (Aro et al., 2012). The ability of the tendons to resist various kinds of mechanical stress is related to the structural organization of their ECM (Esquisatto et al., 2003) and to the biomechanical properties of tendons, which are determined not only by the arrangement of the collagen molecules, but also association with other elements of the matrix (Benevides et al., 2004; Aro et al., 2012). These properties are based directly on the maintenance and integrity of the ECM, which involves the synthesis and degradation of components including collagen, glycoproteins, glycosaminoglycans, and proteoglycans (Bryant et al., 2008). Under normal conditions, matrix metalloproteinases (MMPs) are present in tissue remodeling (McCawley and Matrisian, 2001; Mott and Werb, 2004; Page-McCaw et al., 2007), with collagen degradation being mainly carried out by MMP-2 and MMP-9 (Kjaer, 2004).

MMPs play an important role in promoting collagen turnover in the ECM (Nagase et al., 2006), with changes in MMP levels in the tendons indicating that these enzymes may play an important

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role in tendon adaptation during overload in response to exercise (Koskinen et al., 2004; Legerlotz et al., 2007; Marqueti et al., 2012). Studies show that repetitive mechanical stress on the tendons during exercise can increase matrix remodeling, causing beneficial and/or deleterious effects on their morphology and biochemistry (Del Buono et al., 2013). The tendon undergoes remodeling in response to resistance training (Kjaer, 2004; Groth, 2004). Previous studies have shown that adequate mechanical loads are beneficial to tendons by improving the anabolic processes in tissues, particularly protein synthesis of the matrix, e.g. collagen (Zhang and Wang, 2010). In adult dogs, e.g., the expression of collagen types I and III in flexor tendons significantly decreased after the front legs were immobilized for 6 weeks (Sun et al., 2010).

Therefore deprivation of mechanical stress can produce deleterious changes in the tendon including decreased expression of several MMPs (Koskinen et al., 2004; Aro et al., 2008). Studies with tendon of rats submitted to treadmill training for three weeks showed a great amount of fibroblasts in the tissue remodeling phase due to mechanical load (Szczodry et al., 2009). In rabbits, after four weeks of exercise, significant improvement was found in tensile resistance in the peroneus brevis tendon (Viidik, 1967), while, in pigs after 12 months of training, the digital tendons had become stronger compared with control (Woo et al., 1980). Thus, a proper mechanical load can benefit the structure of the tendon matrix without causing damage during resistance exercise (Narici and Maganaris, 2006). Despite these various studies, results on biochemical and organizational aspects of the tendon's ECM in view of the different resistance exercises are still lacking. Therefore, the purpose of this study was to evaluate the effect of different types of resistance exercise on organizational and biochemical aspects of the ECM of tendons.

2. Methods

We used 60 young adult (60 days) male Wistar rats, weighing on average 250 g, The animals were divided into four groups (n = 15 for each group) and kept in standard plastic cages with wood shavings bedding, with free access to water and feed, room temperature of 23 °C, and a 12-h light–dark cycle. The animals were handled in accordance with the rules approved by the Ethics Committee on Animal Use (CEUA 096-2014-01) of Unesc. The animals were divided into the following groups: UT (untrained), RT (muscle resistance training), HT (hypertrophy training), ST (strength training).

The animals (n = 60) were randomly divided into four groups, as described above, after being familiarized with a climbing platform (Hornberger and Farrar, 2004; Scheffer et al., 2012). During the first week of training, the animals carried a load corresponding to 5% of their body mass (adjustment period). After the adaptation period, the animals started training thrice a week, with 48-h pauses, for 12 weeks. The exercise time was about 30 min. RT consisted of climbing the platform carrying a load of 10% of body weight, which was gradually increased to 20%–50%, performing 12–15 repetitions at 2-min intervals. HT consisted of climbing the platform carrying an initial load of 25% of body weight, which was progressively increased to 50%, 75% and 100%, performing 8–10 repetitions with 2-min intervals. ST consisted of climbing the platform carrying an initial load of 25% of body weight, which was gradually increased to 50%, 100%, 125% and 150%, performing 4–5 repetitions at 2-min intervals, all protocols were carried out three days per week, for 12 weeks (Scheffer et al., 2012).

2.1. Extraction of extracellular matrix components and zymography for gelatinases

The tendons were shredded and immersed in a solution containing 50 mM Tris–HCl (pH 7.4), 0.2 M NaCl, 10 mM $CaCl_2$, 0.1% Triton, and 1% protease inhibitor cocktail (Sigma) for protein extraction (100 μL of extraction buffer for each 30 μg of tissue) at 4 °C for 2 h (Marqueti et al., 2006; Silva et al., 2008). After this first extraction, the samples were incubated by adding 1/3 volume of the same solution described previously at 60 °C for five minutes. After quantification of total protein according to the method of Bradford (1976) using BSA (Sigma) a standard 20 μg of proteins were applied per sample in gel. The acrylamide-gel electrophoresis (10%) containing gelatin (1%) was performed at 4 °C, then the gel was washed with 2.5% Triton X-100 and incubated for 21 h in 50 mM Tris–HCl solution (pH 7.4), 0.1 M NaCl and 0.03% sodium azide at 37 °C. The gel was stained with Coomassie brilliant blue R-250 (Sigma) for 1 h. Then, the gel was washed with a solution containing 30% methanol and 10% acetic acid for observation of the bands corresponding to the gelatinolytic activity. In addition, 20 mM EDTA was used as positive control in the incubation buffer, which inhibited the activity of gelatinases, confirming the presence of MMPs in the gel.

2.2. Quantification of non-collagenous proteins

The total tendon extract was used to quantify protein by the method of Bradford (1976) using the Biorad kit (Biorad Protein Assay) and bovine serum albumin (BSA) as a standard at concentrations of 1, 2, 4, 8, 16 ug/uL. The reading of the samples was at 595 nm on a spectrophotometer Ultrospec, model 2100 Pro Amersham Biosciences.

2.3. Quantification of sulfated glycosaminoglycans

The tendons were chopped and dehydrated in acetone for 24 h. After dehydration, the specimens were dried for 1 h in an oven at 37 °C, weighed and sent for digestion in papain (40 mg of papain per 1 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 digestion, the sulfated glycosaminoglycans (GAGs) present in the samples were measured by the dimethylmethylene blue assay (Farndale et al., 1986).

2.4. Hydroxyproline quantification

After being washed in PBS (0.15 M NaCl and 50 mM EDTA in 5 mM phosphate buffer), the tendons were shredded and immersed in acetone for 48 h and then in chloroform:ethanol (2:1) for 48 h. The fragments were weighed and hydrolyzed in 6 N HCl (1 mL per 10 mg of tissue) for 16 h at 110 °C. The hydrolysate was neutralized with 6 N NaOH, and 20 μL of each sample was treated with a chloramine T solution, as described by Stegemann and Stalder (1967) with some adjustments. The absorbance was measured at 550 nm in a spectrophotometer. Hydroxyproline solutions in concentrations from 0.2 to 6 μg/mL were used for the standard curve.

2.5. Birefringence analysis

The tendons were fixed in 4% formaldehyde 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, dehydrated in ethanol, diaphanized with xylene and embedded in paraffin. For analysis of potential differences in birefringence, longitudinal sections of 7 μm were deparaffinized and embedded in water. The birefringence was analyzed using an Olympus BX53 polarization microscope and an image analyzer (Life Science Imaging Software Version 510_UMA_cellSens16_Han_en_00). The birefringence brightness was expressed in mean values of gray (pixels). The measurements were performed with the longest axis of the tendon

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