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Tendon structural adaptations to load exercise are inhibited by anabolic androgenic steroids

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The present study investigated the structural changes in the rat calcaneal tendon (CT), superficial flexor tendon (SFT), and deep flexor tendon (DFT) in response to jump exercises and anabolic androgenic steroids (AAS). Animals were divided into four groups: sedentary, trained, AAS-treated sedentary rats, and AAS-treated trained animals. Training increased the volume density (Vv%) of blood vessels in all regions of the CT and DFT, cell Vv% in the peritendinous sheath of the proximal and distal regions of the SFT and proximal region of DFT, and cell Vv% in the tendon proper of the proximal and distal regions of the SFT and DFT. The combination of AAS and load exercises showed little increased blood vessel Vv% at the proximal region of the CT, intermediate region of the SFT, and all regions of the DFT as opposed to an increase in adipose cell Vv% in the CT proximal region. The AAS reduced the levels of hydroxyproline in the proximal region of the DFT and in the distal region of the STF. In conclusion, exercise promoted benefits to the adaptation of the tendons to overload. These effects were absent when load exercise was combined with AAS. The abusive consumption of AAS contributes to tendon inertness and rigidity, and increases the potential risk of injury.

Tendons and other connective tissues contain a predominance of extracellular matrix (ECM) around the cells (Wang, 2006). The ECM of the tendons is highly organized in a hierarchical structure of collagen molecules, fibrils, fibers, fascicles, and tendon units (Kjaer, 2004; Provezano & Vanderby, 2006).

Cell–matrix interactions allow tendon cells to detect changes in the mechanical load and alter the composition of the ECM (Benjamin & Ralphs, 1998). One common adaptation is the development of a fibrocartilaginous matrix at sites to resist additional forces of compression and friction in wrap-around tendons (Benjamin & Ralphs, 1998). The arrangement adopted by collagen fibers and their association with other molecules results in a number of special properties that resist the forces of tension, compression, and friction (Benjamin & Ralphs, 1998; Shaw & Benjamin, 2007; Malheiro et al., 2009). The differences in tendon microstructures along its length reflect the different mechanical loads applied (Abrahamsson et al., 1989; Waggett et al., 1998).

Accordingly, training modifies the structural and mechanical properties (Magnusson et al., 2003), fibroblast proliferation, and collagen synthesis in human tendons (Benjamin et al., 2008). Previous studies have demonstrated that training increases proteinase activity matrix metalloproteinase-2 (MMP-2) in a rat tendon subjected to mechanical load exercise for 6 (Marqueti et al., 2006) and 7 weeks (Marqueti et al., 2008). Furthermore, different rat tendons may adapt differently to the same exercise with respect to the ability to withstand load and modulate elasticity, which are inherent characteristics of this tissue in exercise-associated movements (Marqueti et al., 2011). In addition to exercise, Malheiro et al. (2009) evaluated the rats calcaneal tendons (CTs) of control, water-adapted (3 days without overload), vertical jumping (4 sets/10 jumps, overload of 50% of the body weight for each animal over 4 consecutive days), and treadmill-running groups (30 min/day, 13 m/min on a surface inclined 5 degrees for 4 consecutive days). Tendons from the adapted and trained animals showed increased thickness, higher cellularity, and an increased blood vessel volume fraction of the peritendinous sheath.

However, the abusive consumption of anabolic androgenic steroids (AAS) leads to adverse effects on the muscle–tendon system and increases the risk of tendon ruptures in athletes (Maravelias et al., 2005). It has been suggested that the tendons are at risk because of the increased mass and strength of the muscles (Miles et al., 1992) by increasing the number of androgen receptor sites on the muscle (Casavant et al., 2007). Michna (1987) observed that administration of AAS caused an accumulation of collagen in the ECM and connective

tissue disorders in rats. Many case reports indicate that combining AAS with exercise induces deleterious alterations in the tendons and predisposes the tendons to rupture (Cope et al., 2004). These degenerative changes in the tendon occur mainly because of disorganized collagen fibers, including dysplastic fibrils with a clear disruption of the fibril interface (Michna 1986; Miles et al., 1992). AAS also increase tendon stiffness and decrease elongation and energy absorption, and these effects are potentiated by the combination of AAS with exercise, which may render the tendons weaker and predisposed to rupture (Wood et al., 1988; Miles et al., 1992; Inhofe et al., 1995). In addition, the patellar tendon presents stiffness as well as a higher tensile modulus in trained individuals using steroids (Seynnes et al., 2013). Studies from our laboratory have reported that MMP-2 activity is inhibited in different tendon regions in rats with AAS administration or association of AAS with load exercise (Marqueti et al., 2008). The AAS affected the biomechanical properties of the CT, superficial flexor tendon (SFT), and deep flexor tendon (DFT), which reduced the capacity to accommodate an initial tensional load, reduced the capacity to resist tension, and reduced deformability (Marqueti et al., 2011).

Currently, there is no evidence that shows the alterations in morphology of different tendon regions with abuse of AAS. Understanding the morphological traits is an important approach to append our previous findings in this field. In this context, we hypothesized that high doses of AAS may cause harmful effects and reduce the ability for the tendon to adapt, whereas mechanical load exercises can promote beneficial morphological changes to adapt muscle–tendon systems. The purpose of this study was to evaluate the effect of anabolic steroids and mechanical load exercises on the morphology of the peritendinous sheath and tendon proper in three different tendons.

Methods

Animals

Twenty male rats (Wistar norvegicus albinus, weighing approximately 200 ± 17 g at the beginning of the experiment) were grouped in four plastic cages at room temperature and allowed rodent chow and water *ad libitum*. All animal procedures were performed in accordance with the U.S. National Research Council's guide for the care and use of laboratory animals (National Research Council, 1996). The experimental procedures were approved by the Institutional Ethics Committee in Animal Research of the Federal University of São Carlos (protocol number 004/2006).

Experimental groups

Rats were randomly distributed into four experimental groups (five animals per group) in the following order: sedentary without AAS supplementation (S), sedentary with AAS supplementation (AAS), trained (T), and exercised with AAS supplementation (AAST). Animals in the sedentary groups were not submitted to any type of physical activity. Animals in the exercised groups were submitted to a jumping program in a plastic tube 25 cm in diameter filled with water to a height of approximately double the size of the rat standing on the back limbs. The water was held to a constant temperature of 30 ± 2 °C. After pretraining adaptation for 1 week, the animals were submitted to the experimental training protocol, which consisted of 7 weeks of using a 5 days/week training session regimen.

AAS treatment

Animals received Deca-Durabolin (nandrolone decanoate; Organon do Brasil, São Paulo, Brazil). Doses of 5 mg/kg of body mass (supraphysiologic dose) were injected subcutaneously in the back of the rat twice per week. This dosage is comparable with the dosage frequently used by athletes (Pope & Katz, 1988). The experimental groups with no AAS treatment (S and T) received the vehicle only (peanut oil plus benzyl alcohol). The treatment began on the first training week after the pretraining week and lasted for a total of 7 weeks.

Training protocol

To reduce animal stress, the animals were adapted to water in the pretraining week. This adaptation consisted of weightlifting sessions (50% body weight load) once a day for 5 days in water at 30 ± 2 °C. The overload was attached to the animal's chest using an appropriate vest that allowed for jump execution without the vest slipping off the animal's body. The number of sets (2–4) and repetitions (5–10) were adjusted daily and increased gradually. All sessions were performed after 16:00 h. After the pretraining week, the animals were submitted to the experimental training protocol, which consisted of jumps in water at 30 ± 2 °C with the overload adjusted according to the animal's body weight as previously described (Cunha et al., 2005; Marqueti et al., 2006). Briefly, the training protocol consisted of a training week where the animal executed four series of 10 jumps with a rest period of 30 s between series and the overload at 50% of the animal's body weight, and 6 training weeks with the same number of series (4), jumps (10), and resting intervals (30 s between series) but with an increased overload (5% per week) such that in the last week, the overload was at 80% of the animal's body weight. An observer was present during all training sessions. All animals were weighed three times per week.

Tissue preparation

After 7 experimental weeks, the animals were euthanized immediately after the last training session. The CT, SFT, and DFT were immediately dissected from both posterior paws (Fig. 1). The tendons were divided into their respective proximal and distal regions (for the CT) and proximal, intermediate, and distal regions (for the SFT and DFT). Tendon regions were fixed by immersion in 4% formaldehyde in phosphate buffered saline (pH 7.4) for 24 h, washed with distilled water, dehydrated in 70% ethanol, and embedded in historesin (Leica Microsystems, Heidelberg, Germany). Two-micrometer sections were obtained using glass knives, and the sections were stained with hematoxylin–eosin for tissue observation. The images were acquired using an Olympus microscope (BX51 Model, Olympus Optical Co, Tokyo, Japan) connected to an SV Micro Sound Vision digital camera (ACE, Preston South, Australia). All images were captured at ×20 magnification.

Image analysis

All regions of each tendon were analyzed, including the peritendinous sheath and tendon proper. Ten nonconsecutive

digital images per area were obtained (peritendinous and tendon proper). The images were analyzed using Photoshop software (Adobe Systems Inc., San Jose, California, USA). A planimetry system using a translucent Weibel grid (Weibel, 1969) superimposed over each image was used to determine the volume density (Vv%) of the adipose cells, blood vessels (blood vessel lumen, endothelial cells, and perivascular sheath), peritendinous sheath cells (other cells), and tendon proper cells (fibroblasts and fibrochondrocyte-like cells). The stereology was performed by the point-counting method when the structures coincided with the grid points. To estimate the volume densities, the percentage of each structure in the peritendinous sheath and tendon proper was determined by multiplying the total number of grid points that coincide with the structures of interest by 100 and dividing by the total number of grid points falling on the peritendinous sheath or tendon proper.

Hydroxyproline assay

The hydroxyproline (OH-Pro) content was determined for individual tendon regions according to the procedure of Bergman and Loxley (1970) and modified by Leite and coauthors (1995) after hydrolysis with 8 N HCl at 115 °C for 12 h. The chromophore was developed with the addition of Ehrlich's aldehyde, and the absorbance of the chromophore was measured at 550 nm. Unknown concentrations of hydroxyproline in each tissue specimen were deduced from a standard calibration curve. The results were obtained for six individual regions in each experimental group and are presented as grams of hydroxyproline per gram of wet tissue. The results are presented as mg OH-Pro per gram of wet tissue.

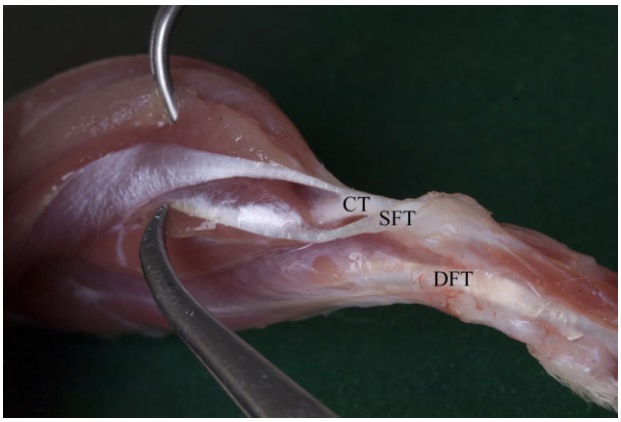


Fig. 1. Anatomical location of the calcaneal tendon (CT), superficial flexor tendon (SFT), and deep flexor tendon (DFT) of the rat hind limb, as seen from the medial aspect.

Statistical analysis

The data are presented as the mean ± standard error of the mean. The statistical analysis was initially performed by the Shapiro–Wilk test for normality and the homoscedasticity test (Bartlett criterion). All variables showed normal distribution and homoscedasticity, and one-way analysis of variance (ANOVA) followed by Tukey's multiple paired analysis was used for comparisons between tendons. Two-way ANOVA was used (taking into consideration the intervening variables T vs AAS) followed by the Bonferroni post-hoc test for multiple comparisons. In all calculations, a significance level of 5% ($P < 0.05$) was used. The Statistica 6.1 software package was used (Statsoft Inc, Tulsa, Oklahoma, USA).

Results

After 7 weeks of mechanical load exercise combined treatment with AAS, histological analysis displayed consistent differences between the groups.

CT: Comparison between the proximal and distal regions of sedentary animals

Few adipose cells were observed in the CT proximal region (Fig. 3a; Table 1). The other variables showed no significant differences between the proximal and distal regions (Table 1). A typical organization of the peritendinous sheath in the CT proximal and distal regions was observed in sedentary animals, and no visible differences between these two regions were observed (Fig. 3a,b). Compact parallel collagen bundles were observed in the tendon proper in the proximal and distal regions (Fig. 3c,d).

Table 1. Volume densities of major components of the peritendinous sheath and tendon proper in sedentary animals as determined by stereology

		Peritendinous sheath			Tendon proper
		Adipocytes	Blood vessels*	Cells†	Cells‡
CT	P	2.13 ± 0.87	2.42 ± 0.87	4.66 ± 0.62	2.16 ± 0.43
	D	–	1.31 ± 0,54	6.30 ± 1.73	2.23 ± 0.46
SFT	P	11.02 ± 3.34	2.09 ± 0.54	1.90 ± 0.45	1.54 ± 0.26
	I	–	5.00 ± 0.94[a]	8.78 ± 0.59[a]	4.14 ± 0.56[a]
	D	–	3.80 ± 0.81	3.19 ± 0.44[b]	3.00 ± 0.29[b]
DFT	P	–	1.85 ± 0.83	5.51 ± 0.67	1.93 ± 0.31
	I	–	0.94 ± 0.44	14.24 ± 1.34[a]	3.46 ± 0.31[a]
	D	–	4.84 ± 1.08[a,b]	12.17 ± 1.25[a]	1.92 ± 0.45[b]

[a]Different from proximal.

[b]Different from intermediate.

*Include endothelial and mural cells, and lumen.

†Include fibroblasts, synovial-like cells.

‡Include fibroblasts, fibrocytes, and fibrocondrocytes.

CT, calcaneal tendon; DFT, deep flexor tendon; SFT, superficial flexor tendon.

CT: Comparison between groups

Training

The load exercise increased the blood vessel Vv% in the peritendinous sheath in the proximal and distal regions of the CT (Figs 2b and 3e,f). The load exercise induced an increase of adipose cell Vv% only in the distal region of the peritendinous sheath (Figs 2a and 3f). Fibroblasts with reduced cytoplasm aligned with longitudinally arranged collagen bundles were visualized in the tendon proper in the proximal region (Fig. 3g). In the distal region, fibroblasts of the tendon proper exhibited an oval shape (Fig. 3h).

AAS

AAS treatment increased cell Vv% only in the peritendinous sheath of the distal region (Figs 2c and 3j). Curiously, aligned cells were observed in the outermost area of the distal region of the peritendinous sheath (arrowheads, Fig. 3j), which suggests the presence of synovial-like cells. The distal region of the tendon proper of the CT showed linearly arranged elongated fibroblasts (Fig. 3l).

AAS and training

Administration of AAS combined with exercise increased adipose cell Vv% in the peritendinous sheath of the CT proximal region (Figs 2a and 3m). The combination of AAS and training markedly decreased the blood vessel Vv% in the peritendinous sheath when compared with animals that were subjected only to training (Fig. 2b). Thus, load exercise seems unable to induce vasculogenesis when combined with high doses of nandrolone decanoate (Fig. 2b). No differences were observed between the experimental groups on cell Vv% in the tendon proper on the proximal and distal region of the CT (Figs 2d and 3c,d,g,h,k,l,o,p).

SFT: Comparison between proximal, intermediate, and distal regions of sedentary animals

The SFT presented interesting morphological differences between the proximal, intermediate, and distal regions of sedentary animals. Adipose cells were observed only in the proximal region of the SFT in sedentary animals (Figs 2e and 4a; Table 1). The intermediate region showed an increased blood vessel Vv% as well as cell Vv% in the peritendinous sheath and tendon proper (Fig. 4c; Table 1).

SFT: Comparison between groups

Training

Training increases the cell Vv% in the peritendinous sheath in the proximal, intermediate, and distal regions of the SFT (Figs 2g and 4g,i,k). In addition, the intermediate region of the peritendinous sheath showed aspects of fibrocartilage with typically round cells (Fig. 4i). This organization is typical of fibrocartilage and is restricted to the joint surface. Interestingly, training enhanced this phenotype. In the tendon proper, the load exercise increased the cell Vv% in the proximal and distal regions (Figs 4h,l and 5h).

AAS

Treatment with AAS showed no change in any of the variables analyzed (Figs 2e–h and 4m–r).

AAS and training

The combination of AAS and load exercise decreased the blood vessel Vv% in the peritendinous sheath of the SFT intermediate region (Figs 2f and 4u). In the proximal and distal regions, there was no difference between groups (Fig. 2f). Regarding the tendon proper, the cell Vv% increased in the proximal region and decreased in the intermediate and distal regions (Figs 2h and 4t,v). Interestingly, atypical round cells with an abundant pericellular matrix (similar to synovial cells) were observed in the peritendinous sheath of the intermediate region (Fig. 4u).

DFT: Comparison between the proximal, intermediate, and distal regions of sedentary animals

The distal region of the DFT showed a larger blood vessel Vv% than the other regions (Fig. 5e; Table 1). The intermediate and distal regions showed high cell Vv% in the peritendinous sheath (Fig. 5c,e; Table 1). Fibroblasts showed an oval shape in the tendon proper of the intermediate region (Fig. 5d), whereas in the proximal and distal regions, the cells were elongated and spindle shaped (Fig. 5b,f). Accordingly, the tendon proper in the intermediate region exhibited a greater cell Vv% (Table 1).

DFT: Comparisons between groups

Training

Exercise load increased the blood vessel Vv% in the peritendinous sheath of the proximal, distal, and intermediate regions (Figs 2j and 5g,i,k). In addition, an increase of the cell Vv% in the peritendinous sheath of the proximal region (Figs 2k and 5i) was observed, whereas other regions showed no effect of training (Fig. 2k). Synovial-like cells were observed at the edge of the peritendinous sheath in the proximal and intermediate regions (Fig. 5g,i). In the tendon proper, the load exercise promoted an increase of cell Vv% in the proximal and distal regions (Figs 2l and 5h,l). Fibroblasts of

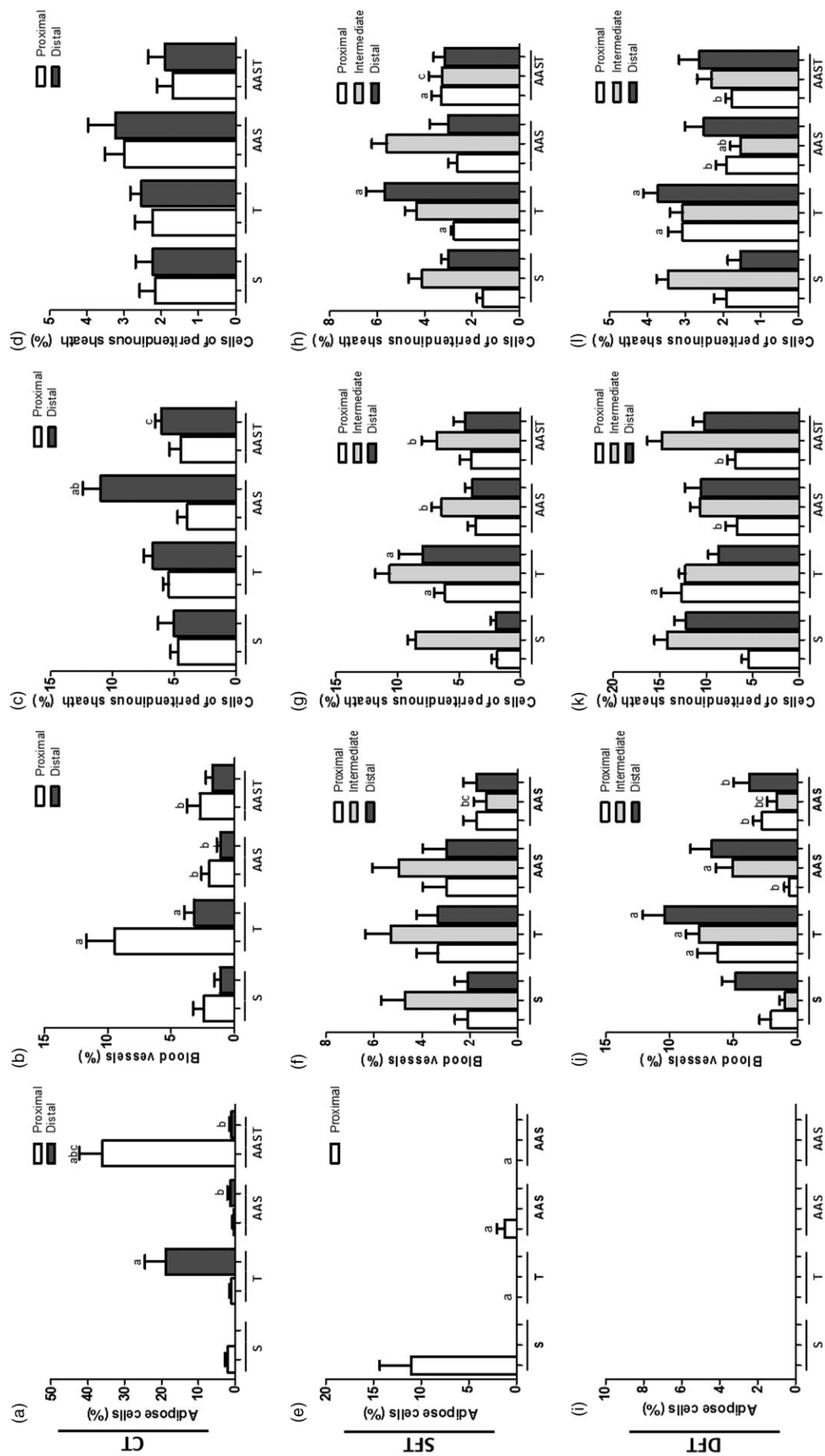


Fig. 2. Volume density variation of structural elements found in the proximal and distal region of calcaneal tendon (CT), in the proximal, intermediate, and distal region of superficial flexor tendon (SFT) and deep flexor tendon (DFT) in each experimental groups. Values were expressed as means ± standard error of the mean ($P < 0.05$). a, significant difference vs S group; b, significant difference vs T group; c, significant difference vs AAS group. The comparison was made between groups of the same region.

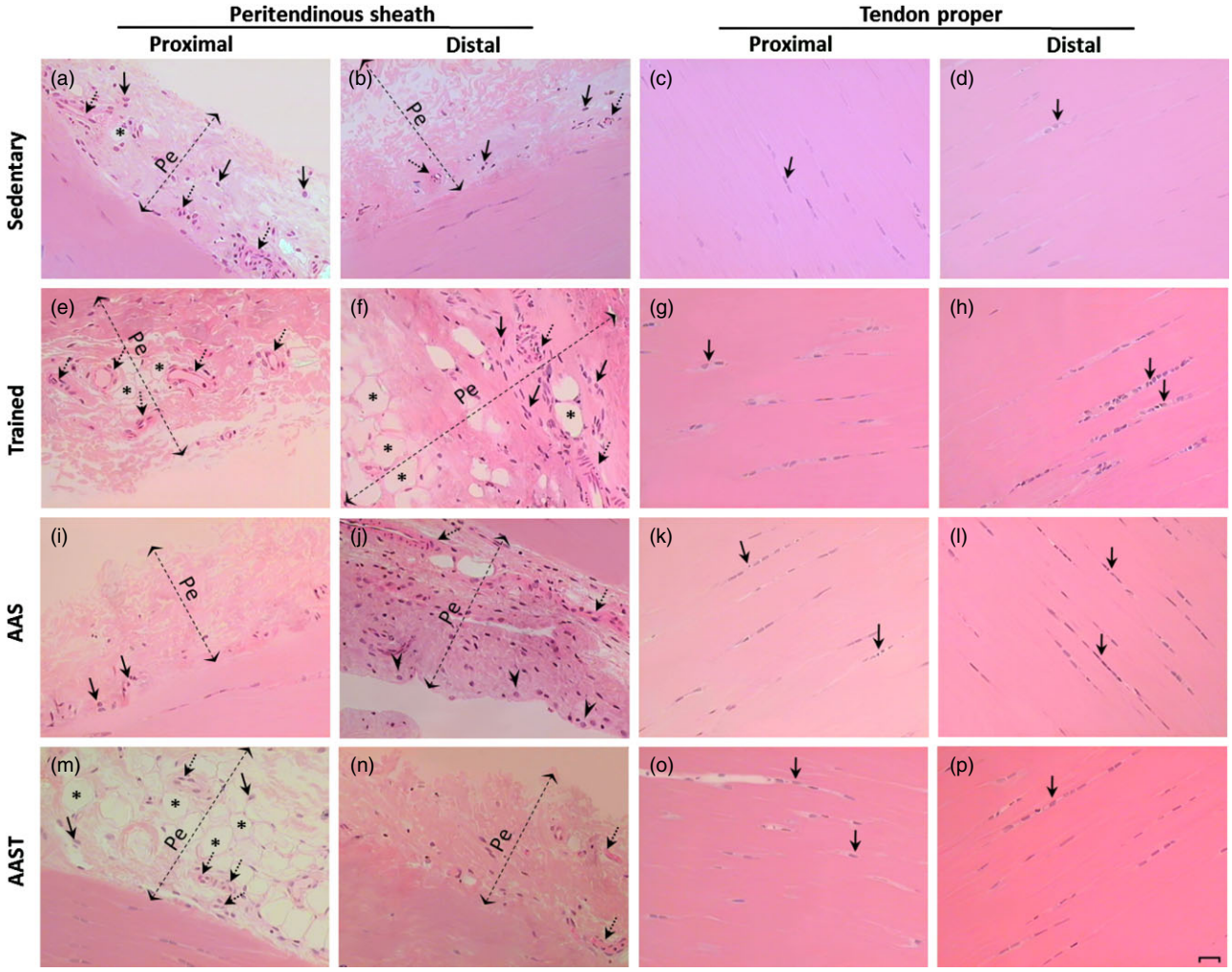
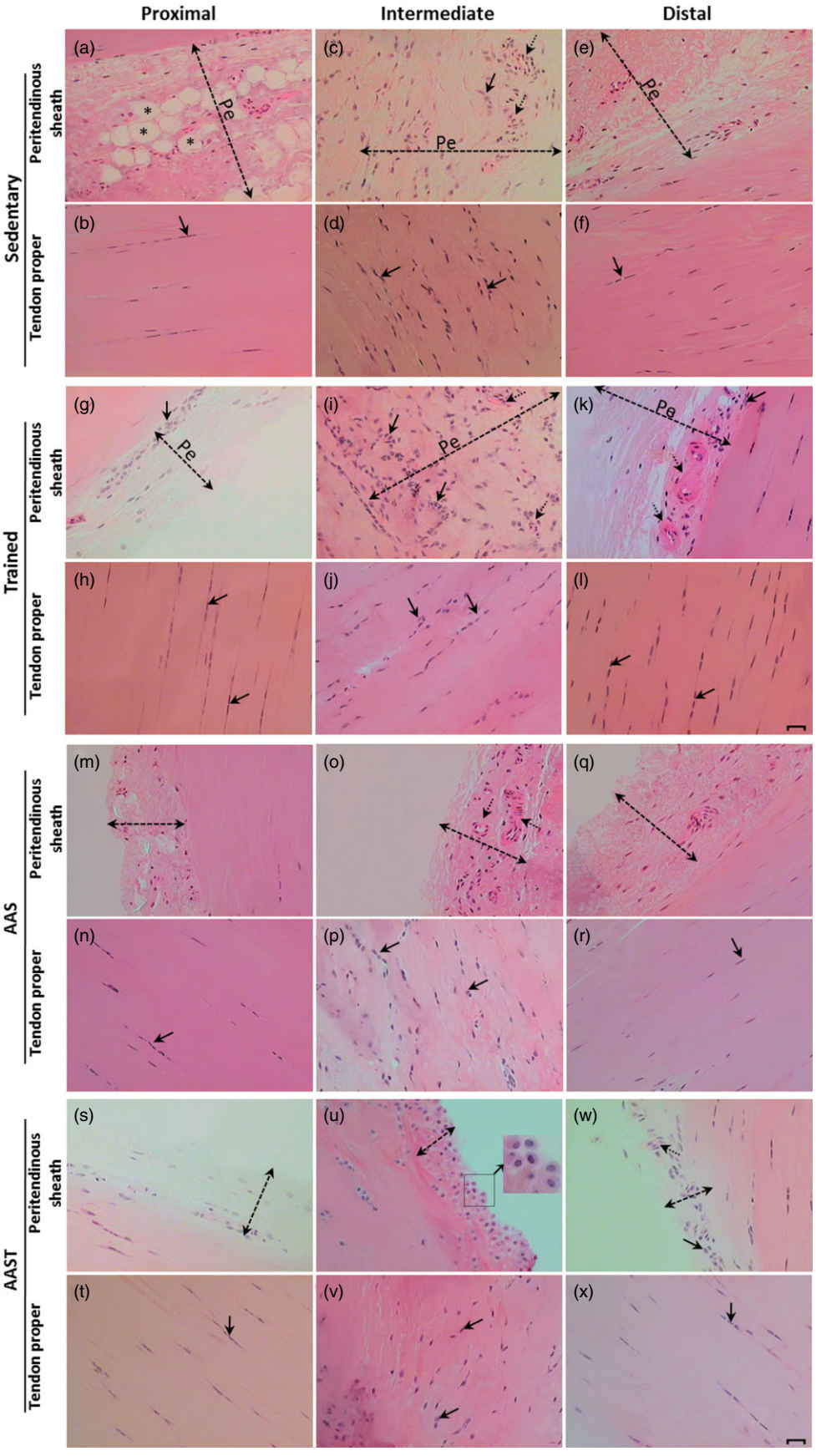


Fig. 3. Longitudinal sections of the proximal and distal regions of the calcaneal tendon stained with hematoxylin–eosin. Sedentary group: in the peritendinous sheath (Pe – dashed double-headed arrow), the proximal region shows adipose cells (asterisk) (a) and the distal region (b) shows the blood vessels (dashed arrow) and cells (arrow). In the tendon proper, the proximal (c) and distal (d) region shows the organization of collagen fibers and cells (arrows). Trained group: in the Pe, the proximal (e) and distal (f) region shows the blood vessels (dashed arrow), cells (arrow), and adipose cells (asterisk). In the tendon proper, the proximal region (g) shows cells (arrow), and the distal region (h) indicates fibrocondrocytes (arrow) in fibrocartilage site. AAS group: the proximal region (i) indicates cells (arrow), and the distal region (j) demonstrates synovial-like cells around the Pe (arrowhead), blood vessels (dashed arrow) and cells within the Pe (arrow). In the tendon proper, the proximal (k) and distal region (l) shows elongated fibroblasts (arrow). AAST group: in the Pe, the proximal region (m) shows adipose cells (asterisk), cells (arrow), and blood vessels (dashed arrow). The distal region (n) indicates some blood vessels (dashed arrow). In the tendon proper, the proximal region (o) indicates fibroblasts less aligned (arrow) than distal region (p), which shows oval and aligned fibroblasts. Barr = 30 μm.

Fig. 4. Longitudinal sections of the proximal, intermediate, and distal regions of the superficial flexor tendon stained with hematoxylin–eosin. Sedentary group: in the peritendinous sheath (Pe – dashed double-headed arrow), the proximal region (a) shows adipose cells (asterisk); the intermediate region (c) shows the blood vessels (dashed arrow) and cells (arrow), and the peritendinous sheath of distal region (e). In the tendon proper, the intermediate region (d) shows typically round fibroblasts (arrow), whereas the proximal (b) and distal region (f) shows aligned and elongated fibroblasts (arrow). Trained group: in the Pe, the proximal (g) shows cells (arrow); the intermediate region (i) indicates fibrocondrocytes (arrow) and blood vessels (dashed arrow); the distal region (k) shows cells (arrow) and blood vessels (dashed arrow). In the tendon proper, the proximal (h) and distal region (l) shows aligned fibroblasts (arrow), and the intermediate region (j) indicates wave-like collagen fibers (arrow). AAS group: Pe of the proximal (m) and distal region (q); and the intermediate region (o) indicates cells (arrow) and blood vessels (dashed arrow). In the tendon proper, the intermediate region (p) shows less aligned fibroblasts and collagen fibers (arrow) when compared with cells of proximal (n) and distal region (r) (arrow). AAST group: Pe in the proximal region (s); the distal region (w) indicates cells (arrow) and blood vessels (dashed arrow); the intermediate region (u) indicates round cells similar to synovial-like cells (dashed square). In the tendon proper, the proximal (t) and distal region (x) indicates aligned fibroblasts (arrow); and the intermediate region (v) shows less aligned cells (arrow). Barr = 30 μm.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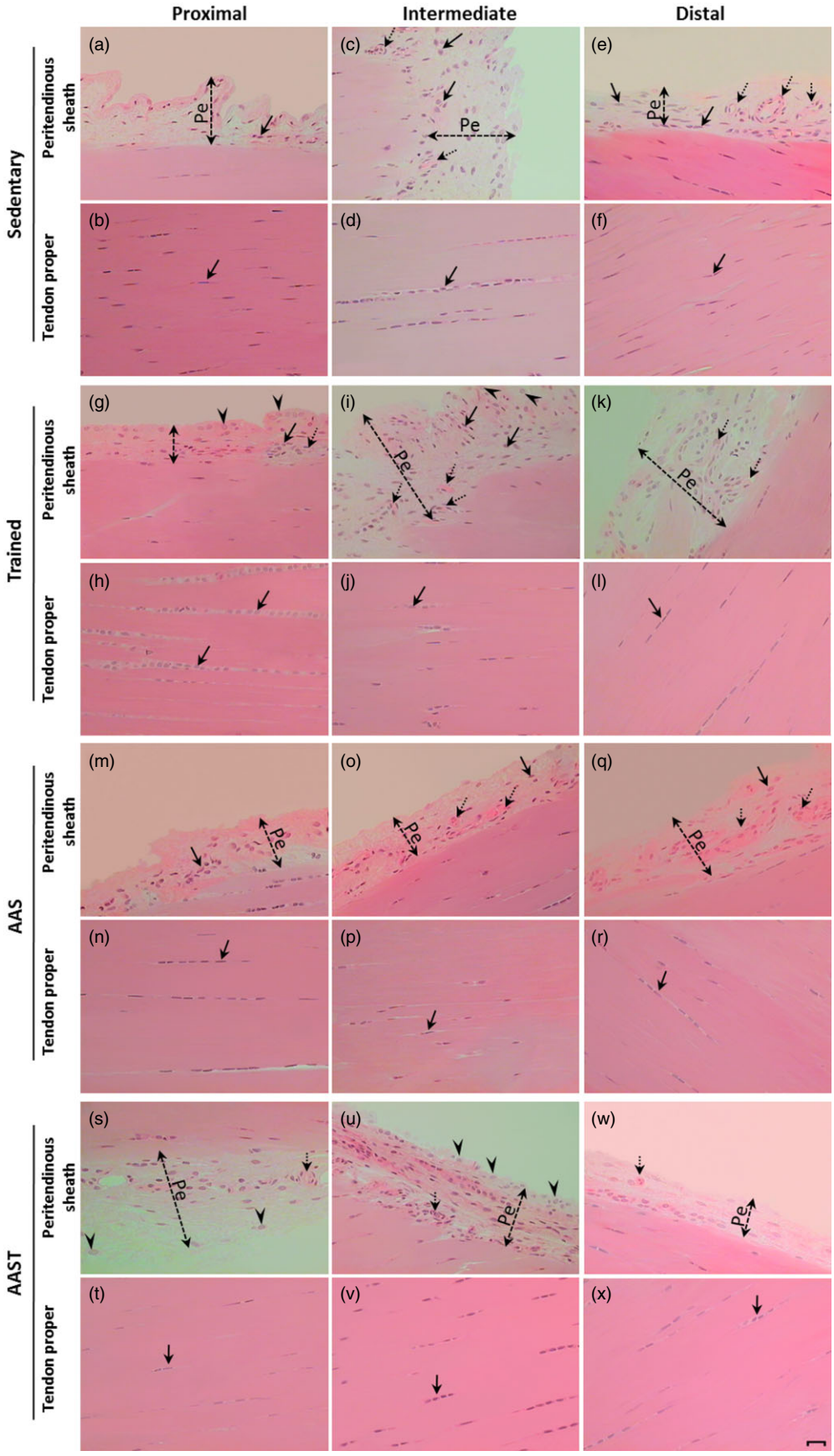
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Fig. 5. Longitudinal sections of the proximal, intermediate, and distal regions of the deep flexor tendon stained with hematoxylin–eosin. Sedentary group: in the peritendinous sheath (Pe – dashed double-headed arrow), the proximal region (a) shows cells (arrow); the intermediate region (c) and distal region (e), both, shows the blood vessels (dashed arrow) and cells (arrow). In the tendon proper, the intermediate region (d) shows round fibroblasts (arrow), whereas the proximal (b) and distal region (f) shows elongated fibroblasts (arrow). Trained group: in the Pe, the proximal (g) and intermediate region (i), both, shows cells (arrow), blood vessels (dashed arrow) and synovial-like cells (arrowhead) around the Pe. The Pe of distal region (k) indicates blood vessels (dashed arrow). In the tendon proper, the proximal region (h) shows aligned round cells surrounded by a pericellular matrix (arrow); the intermediate region (j) shows cells (arrow) and distal region (l) shows aligned and elongated fibroblasts (arrow). AAS group: in the Pe, the proximal region (m) indicates cells (arrow); the intermediate region (o) and distal region (q) shows cells (arrow) and blood vessels (dashed arrow). In the tendon proper, the proximal (n) and distal region (r) indicates elongated and aligned cells (arrow); and in the intermediate region (p), the cells exhibited an oval shape (arrow). AAST group: the Pe in the proximal region (s) and in the intermediate region (u) indicates cells (arrow), blood vessels (dashed arrow) and synovial-like cells (arrowhead) around the Pe sheath; the distal region (w) indicates blood vessels (dashed arrow). In the tendon proper, the proximal (t), intermediate (v), and distal region (x) indicates fibroblasts (arrow). Barr = 30 μm.

the proximal region showed an oval shape that was arranged in lacunae and separated by an abundant pericellular matrix (Fig. 5h). In contrast to the other groups, the fibroblasts in this region were elongated and spindle shaped (Fig. 5b,n,t).

AAS

Only in the peritendinous sheath of the intermediate region was an increase of blood vessel Vv% observed (Figs 2j and 5o). However, in the tendon proper of the intermediate region, there was a decrease of cell Vv% (Figs 2l and 5p).

AAS and training

The key finding regarding the combination of AAS and load exercise was the reduction of blood vessel Vv% in the peritendinous sheath of the proximal, intermediate, and distal regions (Figs 2j and 5s,u,x). Interestingly, simultaneous AAS administration inhibited the effects of exercise, and no increase of blood vessel Vv% was observed in any tendon region in this context. Similarly, the increase in cell Vv% observed in the tendon proper and peritendinous sheath was inhibited by AAS (Figs 2k,l and 5s,t). The intermediate and distal regions showed no difference between the groups regarding the cell Vv% in the peritendinous sheath (Fig. 2k). However, both regions showed synovial-like cells in the edge of the peritendinous sheath (Fig. 5s,u). No adipose cells were found in the DFT peritendinous sheath (Figs 2i and 5a,c,e).

Hydroxyproline (OH-Pro) concentration

Significant differences were observed when comparing the concentration of OH-Pro between the regions of sedentary animals in all tendons. The distal region of the CT (Fig. 6a) showed a higher concentration of OH-Pro. The intermediate and distal regions of the SFT (Fig. 6b) showed a higher concentration of OH-Pro than the proximal region. In the DFT (Fig. 6c), the intermediate region showed a higher concentration of OH-Pro than the proximal and distal regions.

Load exercise increased the OH-Pro concentration in the distal region of the CT (Fig. 6d) and in the intermediate and distal regions of the DFT (Fig. 6f). Administration of AAS resulted in reduced levels of OH-Pro in the proximal region of the DFT (Fig. 6f). The concentration of OH-Pro also decreased in the distal region of the TFS when load exercise was combined with AAS (Fig. 6e). In addition, this combination was not able to reverse the negative effects of AAS, as shown by the intermediate region of the DFT (Fig. 6f).

Discussion

This study evaluated the morphological aspects of different regions of the CT, SFT, and DFT. The main findings suggest that the tendons have different mechanisms of adaptation to mechanical load exercise, which vary the tissue composition according to the demand and regional function that affect the tendon proper and peritendinous sheath, including blood vessels, adipocytes, and synovial-like cells as well as fibroblasts and fibrochondrocytes. Sedentary animals: we observed an interesting morphological variation of each tendon region. The most important findings in the sedentary group are the different arrangements found in the intermediate region of the SFT and DFT combined with increased cellularity and blood vessels, which is most likely explained by the anatomy differences between the intermediate regions of these two tendons. The intermediate region of the SFT is flattened, less flexible, and firmly fixed to the calcaneal surface, which restricts the sliding of the tendon, and the intermediate region of the DFT wraps around the lateral malleolus and slides against it during contraction of the deep digital flexor muscle (Covizi et al., 2001). Fibrous regions and elongated fibroblasts appear in the proximal and distal regions of the SFT and DFT. This elongated shape and flatter fibroblast morphology have a direct relationship with the direction of the force applied (Kjaer, 2009) and represent more mature cells between collagen bundles (Oshiro et al., 2003). In addition, the material properties of the three tendons showed that the SFT and DFT were similar to each other and differed from the CT, which is consistent with the displacement at the

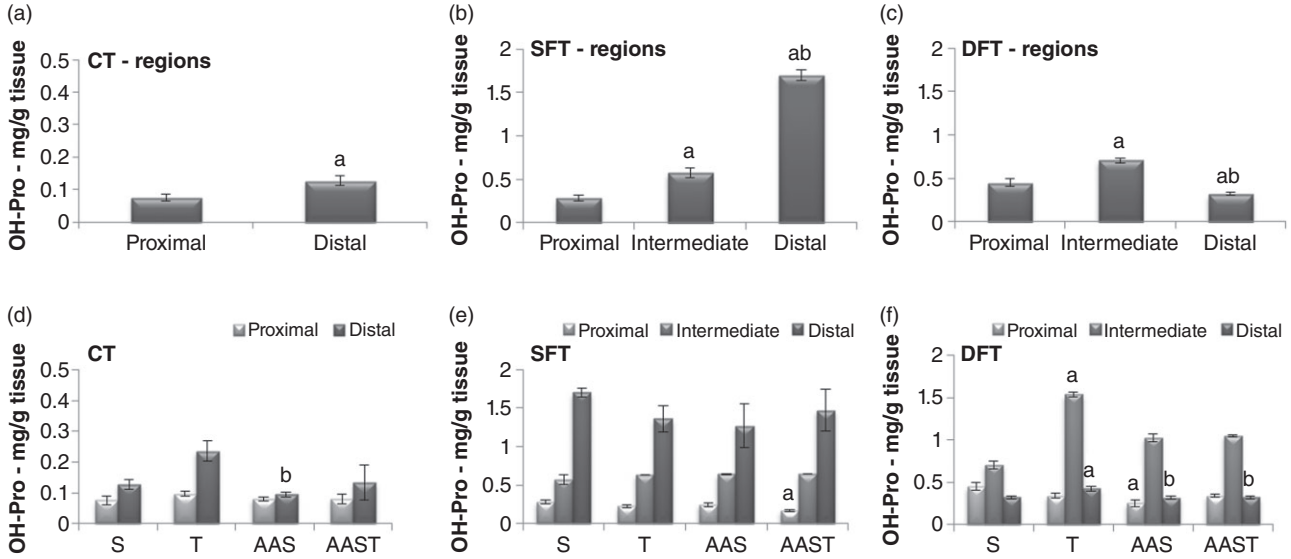

Fig. 6. The hydroxyproline (OH-Pro) content. (a–c) OH-Pro content in all regions of calcaneal tendon (CT), superficial flexor tendon (SFT), and deep flexor tendon (DFT) of sedentary animals. (d–f) OH-Pro content in all regions of CT, SFT, and DFT of experimental groups. Values were expressed as means ± standard error of the mean ($P < 0.05$). a, significant difference vs S group; b, significant difference vs T group.

maximum load, stress, strain, and elastic modulus (Marqueti et al., 2011). The CT accommodated less energy and resisted tensional load more promptly than the SFT and DFT in the sedentary group. This confirms the hypothesis that different tendons perform distinct functions in a set of movements by modulating the remodeling of the ECM and adapting to new physiological demands and morphological changes.

Effect of training

The cell Vv% of the tendon proper in the SFT and DFT (proximal and distal regions) and cell Vv% of the peritendinous sheath of the SFT (all regions) and DFT (distal region) increased significantly in response to exercise. This cellular response to exercise can be explained by an increase in local tissue demand and an increase in turnover of the connective tissue and ECM proteins because of the increased strength of muscle contraction, which promotes the mechanical stimulus for collagen synthesis (Kjaer, 2009). The mechanical load could stimulate collagen production and regulate the translation of mechanical loading to collagen synthesis in humans (Heinemeier et al., 2007). The physical connection between fibroblasts and the ECM permits the cells to sense and respond to mechanical stimuli. Similarly, the linkage between the tendon fibroblasts and tendon matrix is vital to the mechanotransduction of tendon cells and adaptation to mechanical loading demands (Chiquet et al., 2009; Heinemeier & Kjaer, 2011). Consequently, the fibroblasts respond by converting mechanical stimuli into chemical signals to modify gene expression by increasing collagen synthesis and ECM components (Chiquet, 1999; Kjaer, 2004; Chiquet et al., 2009).

The peritendinous tissue reflects many of the changes that occur within the tendon (Langberg et al., 2002). An increased cellularity and thickness of the peritendinous sheath after 1 day of vertical jump and 4 days of treadmill running was observed in the CT of rats (Malheiro et al., 2009). It was previously thought that this tendon was metabolically inactive. Currently, studies have determined that the metabolism in the human tendon is high and has the ability to adapt to changing demands, including aging, unloading, and loading (Kjaer et al., 2008). The peritendinous region is capable of increasing the metabolic activity in response to physical activity (Magnusson et al., 2003). In addition, studies using the ^{14}C bomb–pulse method indicated the limited tissue turnover in the human CT, which explains the reduced regenerative ability of this tissue (Heinemeier et al., 2013). However, other studies reported that a large degree of tendon adaptation happens in the outer region of the tendon (Babraj et al., 2005; Miller et al., 2005). Our findings show that the response to training in the peritendinous sheath indicates an increased metabolism, which is related to greater blood vessel Vv% and an adaptation following tissue remodeling during 7 weeks of training. During exercise, the blood flow can increase up to seven times in tendons compared with the normal flow at rest (Magnusson et al., 2003). An increased vascularity in the peritendinous sheath was also observed in response to vertical jumping and treadmill running (Malheiro et al., 2009).

AAS effect

The administration of AAS caused different effects in each tendon. In the CT (distal region), we observed an increase of cell Vv% in the peritendinous sheath. In the

TFS (intermediate region), the cell Vv% increased only in the tendon proper compared with the AAS group, and in the DFT (intermediate region), an increase of blood vessel Vv% in the peritendinous sheath and decreased Vv% of cells in the tendon proper was observed. There is a lack of studies involving the effects of AAS and the variables evaluated in this study in different regions and tendons. It is difficult to compare and discuss our data with other studies. However, in a previous study using the same experimental model as this work, it was shown that the combination of two types of AAS caused changes in the morphology and decreased the activity of metallopeptidase type 2 (MMP-2), thus impairing the remodeling of the CT (Marqueti et al., 2006).

Effect of exercise and AAS

AAS combined with load exercise prevented an increase of blood vessel Vv% in the CT (proximal region), SFT (intermediated region), and DFT (all regions). These findings represent an important novelty in this region. Abuse of AAS is linked to a variety of cardiovascular diseases, including cardiomyopathy, atrial fibrillation, stroke, myocardial infarction, ventricular thrombosis, systemic embolism, and acute heart failure (Hartgens & Kuipers, 2004). The effect of AAS on the blood vessels is still unclear. However, one study using the soleus muscle of rats showed that the combination of training and AAS administration inhibited the messenger RNA (mRNA) of vascular endothelial growth factor (VEGF), whereas jump training alone increased the mRNA of VEGF in relation to the sedentary and sedentary with AAS administration groups (Paschoal et al., 2009). This finding suggests a strong link between the inhibition of VEGF expression and inhibition of blood vessel Vv% on tendons in our study. Furthermore, VEGF is an important molecule for endothelial cell proliferation in the induction of angiogenesis (Prior et al., 2003).

Interestingly, the combination of load exercise and AAS caused the presence of adipose cells mainly in the proximal region of the CT. There are a variety of proposed functions for adipose cells in the tendon enthesis, which includes the facilitation of movement between the tendon fascicles, tendon, and bone, and the dissipation of stress and tension concentrated at the attachment sites (Benjamin et al., 2004). Nevertheless, Józsa and Kannus (1997) described a relationship of tendinopathy with lipids and lymphocyte infiltration in an injured human CT. Interestingly, in the AAST group, the adipose cell Vv% found in the CT was increased twofold compared with trained animals and increased fourfold compared with sedentary animals (SFT). Thus, the exacerbated adipose tissue in the AAST group can determine a not functional response of the tendon. However, further studies are required to confirm this hypothesis.

Interestingly, in our study, some synovial-like cells appeared around the peritendinous sheath in trained (SFT, proximal, and intermediate region), AAS (CT, distal region), and AAST groups (intermediate region of the SFT, and proximal and intermediate regions of the DFT). Synovial cells produce synovial fluid in tendons and joints, and this fluid helps reduce tissue friction and nutrition (Benjamin et al., 2008). The synovial sheaths form access tunnels for tendons that pass through the bone surfaces or anatomical structures that may cause friction (Józsa & Kannus, 1997; Khan, 1996). However, we found a larger amount of synovial-like cells in the AAST group in the intermediate region of the SFT, which suggests the possibility of an injury site. The proliferation and migration of synovial cells may occur in the presence of injury, which supports healing of the tendon (Chang et al., 1998). The appearance of the cluster of synovial-like cells only in the peritendinous sheath of the AAST group (intermediate region) may indicate a protective response to tendon injury; however, further investigation is required to understand the real function of these cells. In addition, biomechanical analysis showed that the combination of AAS and training led to an increased stiffness in all ruptures (Marqueti et al., 2011). Studies have suggested that long-term heavy resistance training and AAS abuse could be linked to a higher risk of human patellar tendon injury (Seynnes et al., 2013).

An interesting response to load exercise in the collagen content was observed in this study, which promoted an increase in the OH-Pro concentration in the distal region of the CT, and the intermediate and distal regions of the DFT. Load exercise increase connective tissue remodeling in the muscles and tendons, which results in the physiological adaptation of the ECM (Kjaer et al., 2005). In addition, load exercises enhance cell proliferation, which promotes protein synthesis and increased collagen levels in the ECM (Kjaer, 2004). Consequently, intense physical training is able to control collagen metabolism (Langberg et al., 1999). Studies have shown that collagen type I is synthesized and degraded in the peritendinous space of the Achilles tendon in men after 4–11 weeks of intense physical training (Langberg et al., 2001). Curiously, the OH-Pro content in the sedentary animals of this study showed a higher concentration specifically in the regions where there were more cells. Although the fibroblasts synthesize collagen, these cells occupy a considerable volume; however, even with the presence of these cells, the collagen content was higher. One possible explanation for the collagen contents is due to the prevalence of the paw position of rats inside the cages. Small cages lead to constant stress on the compression region, especially of the CT, which alters the synthesis of the ECM.

The administration of AAS or the combination of load exercise and AAS showed a decreased OH-Pro content in some tendon regions, which indicated that AAS have a negative effect on collagen metabolism. Some studies have reported that AAS may inhibit collagen synthesis in

tendons and ligaments, and induce changes in the arrangement of collagen fibrils, which leads to critical changes in tendon plasticity (Evans et al., 1998). We suggest that the reduction of the OH-Pro concentration in the AAS groups is associated with a reduction of local levels of MMP-2 in these tendons, and the total OH-Pro concentration can be from both native and degraded collagen. The AAS treatment not only caused an increase in MMP-2 but also inhibited the effect of training, which impaired tendon remodeling (Marqueti et al., 2008).

In conclusion, each region of tendons differs morphologically from each other according to the function and load applied, which changes the content and shape of the cells, cellularity of the peritendinous sheath and tendon proper, blood vessel Vv%, and collagen content. The use of AAS negatively affects these properties, mainly when the use of AAS is with training. The negative effects of AAS were characterized by a reduction of blood vessels, increased adipose cell Vv%, the presence of synovial-like cells, and a reduction of the OH-Pro content. In line with earlier studies, our current results suggest that AAS combined with exercise leads to tissue structural damage, loss of function, and increased likelihood of injury.

Perspectives

The administration of AAS resulted in minor changes of the morphology of tendons. However, the combination of AAS with load exercise failed to revert the negative morphology changes of AAS administration. These changes may cause further damage or injury by jeopardizing the tissue repair and remodeling processes as well as the morphological properties of the tendons. Aside from the structural changes observed in this study, the administration of AAS or its combination with training reduced the expression of key genes involved in tendon adaptation with collagens I and III (Marqueti et al., 2012) and loss of their mechanical properties (Marqueti et al., 2011). However, there are some questions that are still unclear: What signaling pathways are inhibited or exacerbated by AAS abuse? What is the interaction between AAS and the ECM environment? These queries need to be investigated in further research to understand how AAS influence the structural and functional tendon properties.

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