Manuscript Title:

The effect of anabolic androgenic steroids on the biomechanical properties of the Achilles tendon: experimental study.

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Abstract

Background

The effect of anabolic androgenic steroids on tendons has not yet been fully elucidated. Aim of the present study was the evaluation of the impact of anabolic androgenic steroids on the biomechanical and histological characteristics of Achilles tendons.

Methods

Twenty-four male Wistar rats were randomized into four groups with exercise and anabolic steroids (nandrolone decanoate) serving as variables. Protocol duration was 12 weeks. Following euthanasia, tendons' biomechanical properties were tested with the use of a modified clamping configuration. Histological examination with light and electron microscopy were also performed.

Results

In the group of anabolic steroids and exercise the lowest fracture stress values were observed, while in the exercise group the highest ones. Histological examination by light and electron microscopy revealed areas of collagen dysplasia and an increased epitendon in the groups receiving anabolic steroids and exercise.

Conclusions

These findings suggest that anabolic androgenic steroids reverse the beneficial effect of exercise, thus resulting in inferior maximal stress values.

Keywords

- Anabolic androgenic steroids;
- Achilles tendon;
- Rupture;
- Biomechanics

1. Introduction

Anabolic Androgenic Steroids (AAS) are synthetic derivatives of testosterone. Since the discovery of the molecule of testosterone in 1935 [1], the use of androgenic compounds as ergogenic aids has attracted the attention not only of professional athletes but also of the greater masses [2], [3] and [4]. This extensive AAS consumption has raised a lot of interest regarding their impact on the organism. Under this scope, many of the aspects of the systemic deleterious actions of AAS have been already elucidated [5] and [6]. As far as the impact of AAS on tendons is concerned, the first evidence on a relationship between AAS and tendon injuries came from case reports [7], [8], [9], [10] and [11]. The studies that followed could be characterized to a certain extent contradicting, mainly due to methodological differences. The extrapolation of reliable data from biomechanical testing of small laboratory animals' tendons, without affecting their biomechanical characteristics is technically challenging. In order to overcome this difficulty the modified cryo-jaw technique for biomechanical testing has been proposed in the literature [12] and [13]. The aim of the present study was the determination of the effect of the AAS use on the biomechanical and histological parameters of the Achilles tendon (AT) in Wistar rats.

2. Materials and methods

2.1. Laboratory animals

Twenty-four male 12-week-old Wistar rats were used for the needs of the present study (200–250 g). The animals were housed under conditions of controlled temperature (23 ± 2 °C) and humidity (60%). There was a 12 h light/dark circle and access to food and water was *ad libitum*. A positive vote was granted by the Ethics Committee of the local Veterinary Directorate and all procedures were conducted in accordance with ethical recommendation of the European Communities Council Directive of November 24, 1986 (86/609/EEC). Prior to study inclusion, all animals were kept for a week in the laboratory premises in order to minimize stress. The animals were randomized into four equal groups with AAS treatment and exercise serving as variables: (1) Control Group (n = 6): No

intervention; (2) AAS group (n = 6): AAS administration/no exercise; (3) Exercise Group (n = 6): Exercise/no AAS; (4) AAS and Exercise Group (n = 6): Combination of AAS administration and exercise. The four groups did not differ in terms of size and weight ($p > 0.05$). At the end of the protocol statistically significant differences were seen among the groups ($p = 0.006$). The control group showed the highest weight (mean weight: 384 g, SD 37), followed by the exercise group (mean weight: 370 g, SD 32), the anabolic group (mean weight: 320 g, SD 43) and anabolic and exercise group: 314 g, SD 31).

2.2. AAS compound and administration protocol

Nandrolone Decanoate was administered intramuscularly (i.m.) at the gastrocnemius twice a week at a dosage of 5 mg/kg for a total period of 12 weeks. This is a mega-dose, equivalent to that taken by professional athletes and bodybuilders [2]. In the exercise and in the control group, the vehicle of Nandrolone Decanoate (sterilized sesame oil) was administered at the same site and at the same time points, as placebo. Injections were made by turns at both legs in order to minimize soft-tissue irritation.

2.3. Exercise protocol

The exercise protocol that also lasted for 12 weeks consisted of training in a custom-made motorized running wheel. A period of one week preceded the start of the exercise protocol, in order to ensure that the rats would get acquainted with the exercise procedure. The animals were exercised for 30 min each day, five days per week at a speed of 0.5 m/s. The constant speed did not permit a more intense exercise for anyone of the groups. The level of activity of the animals when not exercising was not quantified; however, no behavioral abnormalities were observed during the protocol between the different groups.

2.4. Biomechanical analysis

All animals were euthanized at 12 weeks under ether anesthesia. The ATs along with the gastrocnemius muscles and foot from both legs were harvested, as previously described [12]. An alternative clamping technique employing rapid freezing was developed for the biomechanical study of rat bone-Achilles tendon-muscle units. The clamping device consisted of two separate parts (Fig. 1). The first was a pincers-like clamp for bone fixation, while the second a modified cryo-jaw. The modified cryo-jaw comprised of a liquid nitrogen cup mounted on the inferior load frame and a structure that formed a cavity with adjustable dimensions. The latter was placed in the medial axis of the device at a higher level with respect to the liquid nitrogen cup. The muscle was placed and fixed inside the cavity, ensuring that the musculotendinous junction was a few millimeters distant to the cryo-jaw. Subsequently, the bone was fixed to the pincers-like clamp and mounted on the upper part of the load frame. For the present study a MTS MiniBionix 858 (MTS System Corp., Eden Prairie, MN, USA) load frame was utilized. Liquid nitrogen was poured inside the cup in order to achieve rapid freezing of the muscle. Afterwards, the mechanical testing was initiated with a displacement rate of 1 mm/min. The axial force exerted on the specimen was measured using a 500 N Instron Tensile Load Cell (Instron, Canton, MA, USA). The required liquid nitrogen volume was accurately determined in a series of pre-tests by placing a T-type thermocouple probe inside the tendon tissue at the musculotendinous transition area, providing real-time temperature measurements. Use of 75 cm^3 of liquid nitrogen resulted in a total temperature drop of the tendon of about 10 °C, while the muscle was frozen to a satisfactory degree in order to withstand loads until tendon failure, without any noticeable slippage. During the biomechanical testing, room temperature remained constant at 25 ± 2 °C.

2.5. Histological examination

ATs were subjected to light and electron microscopy examination. For the needs of light microscopy the tendons were fixed in 10% formalin at room temperature. Subsequently, the tissues were

142 embedded in paraffin, sectioned and mounted on glass microscope slides. Specimens were stained
143 with hematoxylin–eosin and examined under light microscopy at different magnifications.

144 For the needs of electron microscopy, the ATs were fixed with 2.5% glutaraldehyde in 0.1 M
145 phosphate buffer (pH 7.2) for 2 h, postfixed with 1% osmium tetroxide for 2 h, dehydrated in a graded
146 series of alcohol followed by propylenoxide, and embedded in Spurr resin. Ultrathin sections were cut
147 and stained with uranyl acetate, and specimens were examined and photographed with a Zeiss 9S
148 transmission electron microscope (Carl Zeiss, Germany). Sections from the biomechanically tested
149 specimens were stained with toluidine blue for the determination of the cross sectional area for the
150 calculation of fracture stress. After the fixation, 10 consecutive cross-sections of each Achilles
151 tendon, 10 μm thick, were obtained at the site of the rupture. The sections were absolutely transverse
152 and the whole cross-sectional area of the tendon appeared in each section. The cross-sectional area of
153 each one sections was measured using MatLab (MathWorks, Natick, MA, USA) and their mean value
154 was assessed for each specimen. The mean cross-sectional area of each tendon was used for the
155 assessment of the maximal sustained stress of the tendon, based on the fact that, when the tendon is
156 subjected to quasi-static tension, its mechanical behavior is not affected by its internal fluid pressure.
157 Additionally, the tensile bearing capacity of the liquid phase of the extracellular matrix of the tendon
158 is obviously negligible and therefore it can be excluded from the stress assessment procedure. The
159 value of the rupture stress for each tendon was then calculated through the division of the maximal
160 load to the measured effective cross-sectional area.

161

162 ## 2.6. Statistical analysis

163 Data are expressed as mean ± 1 standard deviation (SD) for continuous variables. The Kolmogorov–
164 Smirnov test was used in order to assess the normality of the distributions. Analysis of Variance
165 (ANOVA) was used for multiple between group comparisons. Significance levels were adjusted by
166 applying the Bonferroni–Holmes correction for multiple comparisons, in order to maintain a family-
167 wise $\alpha = 0.05$. Differences were considered as statistically significant if the null hypothesis could be
168 rejected with >95% confidence ($p < 0.05$).

3. Results

3.1. Biomechanical testing

A characteristic load–displacement curve from the present series of experiments is depicted in Fig. 2. The three portions, typical for soft tissues under tension, are clearly distinguished: an initial non-linear region, followed by one almost linear, leading eventually to a sudden drop due to tendon failure. The results indicate that by rapid freezing only, the muscle provides the necessary fixation force capable to sustain significant tension loads without slippage and without affecting the mechanical behavior of the "gage-length" of the tendon. The rupture occurred in the mid-substance of the tendons. Differences in terms of load were observed among the groups; however, they were not statistically significant (Table 1). Statistically significant differences were observed between maximal stresses of the four groups (Table 1). The AAS-exercise group exhibited the lowest maximal stresses among the four groups, while the exercise-group the highest (Table 1).

3.2. Histological analysis

Light microscopy in the control group (Fig. 3A) revealed normal alignment of the collagen fibers. In the exercise group, anticipated micro-damages were observed, which were typical for an exercise protocol (Fig. 3B). In the AAS group collagen fiber alignment remained relatively normal, when compared to the control group (Fig. 3C). Finally, in the AAS-exercise group, the specimens provided a more dramatic picture with collagen fiber derangement, increased vascularization and increased cellularity (Fig. 3D). Furthermore, in both AAS groups, a thicker epitendon was evident (Fig. 4). The examination of the tendons with the use of electron microscopy revealed areas of collagen dysplasia especially in the AAS-exercise group with derangement of the alignment of the collagen fibers and micro-damages (Fig. 5).

4. Discussion

The results of the present study suggest that the beneficial effect of exercise on the biomechanical behavior of tendons is reversed by AAS administration. Our study is supported by biomechanical data derived from a specific configuration, which enables tendons to remain relatively unaffected by the deep freezing procedure. This fact was confirmed by the use of pinch thermometers at the musculotendinous junction, while no signs of specimen slippage were observed.

Maximal rupture stress sustained by the tendons differed significantly between the study groups. The fact that the exercise group was able to sustain the greatest stresses can be attributed to the exercise protocol [14] and [15]; the ATs in the exercise group achieved 66% higher fracture stress values compared to the control group. On the other hand, the combination of AAS and exercise resulted in the lowest stress values among the four groups. The existing literature on the effect of AAS on tendon biomechanics has up to now provided contradicting results. In the study of Wood et al. [16] the modulus of elasticity was examined by testing bundles of three or four pre-conditioned fascicles from flexor digitorum superficialis rat tendons, using a modified Wingfield fiber tensometer. This study failed to show any statistically significant differences between the four groups. However, this methodology is not very safe, as only a part of the tendon is examined.

The biomechanical studies that followed were those of Miles et al. and Inhofe et al. [17] and [18] in ATs from rats that were trained and treated with AAS. The authors of the former concluded that AAS resulted in a stiffer tendon that failed with less elongation. In the study of Inhofe et al. [18] these alterations were reversible after discontinuation of AAS treatment for 12 weeks. An inherent limitation of those studies was that a part of the tendon was frozen and held tightly with the additional use of a serrated collet, a fact that in a structure of less than 4 mm length, could have affected the biomechanical properties of the tendon in its entity.

In the most recent biomechanical study of Marqueti et al. [19] the biomechanical behavior of Achilles tendons under the influence of AAS treatment and exercise (jumps in water with concurrent increasing load) was examined. Maximum stress did not differ significantly among the four groups, whereas significant differences in the modulus of elasticity were observed. However, it should be

noted that the cross-sectional area was measured prior to the biomechanical testing with the use of metal calipers, thus potentially affecting the extrapolated stress values.

The observed histological alterations of collagen dysplasia, increased vascularization and cellularity, micro-damages of collagen fibers and increased synovial layer are in accordance with previous experimental studies. In a series of articles Michna demonstrated alterations in the morphology and ultrastructure under electron microscopy in flexor digitorum longus tendons of female exercised mice treated with AAS on a short-term (one week) and long-term (10 weeks) basis [20], [21] and [22]. Dysplastic, as well as ruptured and dissociated collagen fibrils in the hormone-treated animals were observed, the occurrence of which seemed to be time-dependent [16] and [17]. In the histological studies of Marqueti et al. [23] a thick fibrosis layer covering the tendon was observed in the groups that received AAS. Additionally, AAS treatment decreased both concentration and active form of MMP-2, thus suggesting a blocking of tendon (collagen) remodeling. Whether the decreased maximal stress observed in our study, could be associated with such an increased and prolonged anabolic state that does not permit tendon remodeling, remains to be further evaluated. The first study to examine the effect of AAS on human tendons was that of Evans et al. [24]. Specimens of four human ruptured tendons (longitudinal strips of 5 mm × 1 mm) were examined by light and electron microscopy. Two of the tendons came from individuals that confessed being AAS users, while the other two served as controls. Areas of dysplasia were observed in all four specimens. However, the area of the tendon, from which the specimens were obtained, was adjacent to the rupture end. This fact alone could explain the ultrastructure similarities seen, as it could be anticipated that in that area collagen architecture would be *per se* deteriorated.

The present study presents also certain limitations. The relatively low number of animals per group does not permit undisputable conclusions as far as the absence of statistical significance between the groups in terms of rupture force and cross-sectional area are concerned. Additionally, the present biomechanical methodology does not permit the extrapolation of absolute values of the biomechanical parameters of the tendons and is appropriate for the conduction of comparative studies between

groups, mainly because of the observed difference in the temperature and the measured cross-sectional area at the moment of rupture.

Conclusively, the present study suggests that AAS treatment reverses the beneficial effect of exercise on the biomechanical behavior of the Achilles tendon, as it significantly decreased the maximal sustainable tendon stress.

Conflict of interest

The authors declare that no conflict of interest exists.

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Tables

Table 1: The values of fracture forces, cross-sectional area of the tendons and fracture stress among the four different groups. There were statistical significant differences in the fracture stress between the control group (C) and the exercise group (E) and between the exercise group (E) and the exercise and anabolics group (AE).

Parameter	Controls (C)	Exercise (E)	Anabolics (A)	Anabolics & Exercise (AE)	Significant Differences
Fracture Force [N]	41.7 ± 7.1	46.6 ± 10.9	45.3 ± 10.1	53.4 ± 14.5	None
Area (μm^2)	23,000 ± 8750	17,800 ± 1600	21,000 ± 7,600	26,000 ± 5,700	None
Fracture Stress (MPa)	15.7 ± 2.6	26.1 ± 7.2	19.1 ± 2.5	15.0 ± 3.4	$C\text{-}E^{2(2)}$, $E\text{-}A^{1(4)}$, $E\text{-}AE^{3(2)}$

1:p<0.05, 2:p<0.01, 3:p<0.001, 4:p=0.06, (): significance levels after normalization for t_f weights (it also stands for t0 weights, but the indices might change a bit)

Figures

Figure 1: The biomechanical testing device setting with the two separate parts of the clamping device are depicted.

376 Figure 2: Characteristic force–displacement curve of the present study.

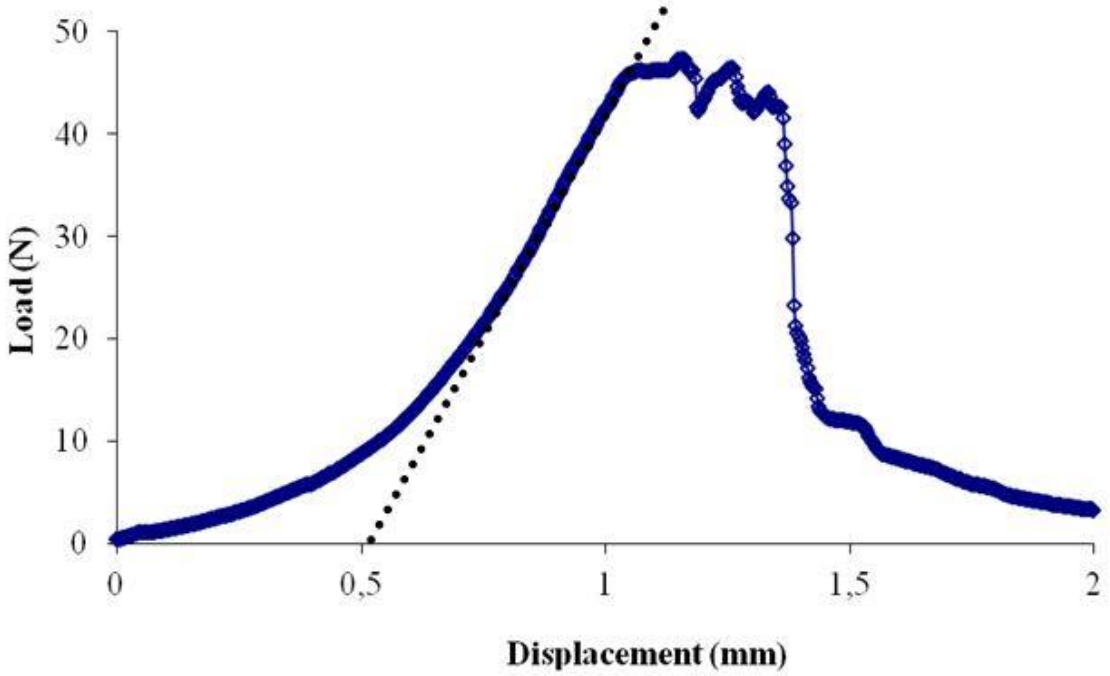

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Figure 3: Histological findings of the Achilles tendons of the different groups. The control group (A) shows normal collagen fiber alignment. Microdamages are seen in the exercise group (B). No major differences are observed in the anabolic groups (C) compared to the control group. Microdamages, increased cellularity and vascularity, as well as derangement of collagen fibers are seen in the anabolics and exercise group (D).

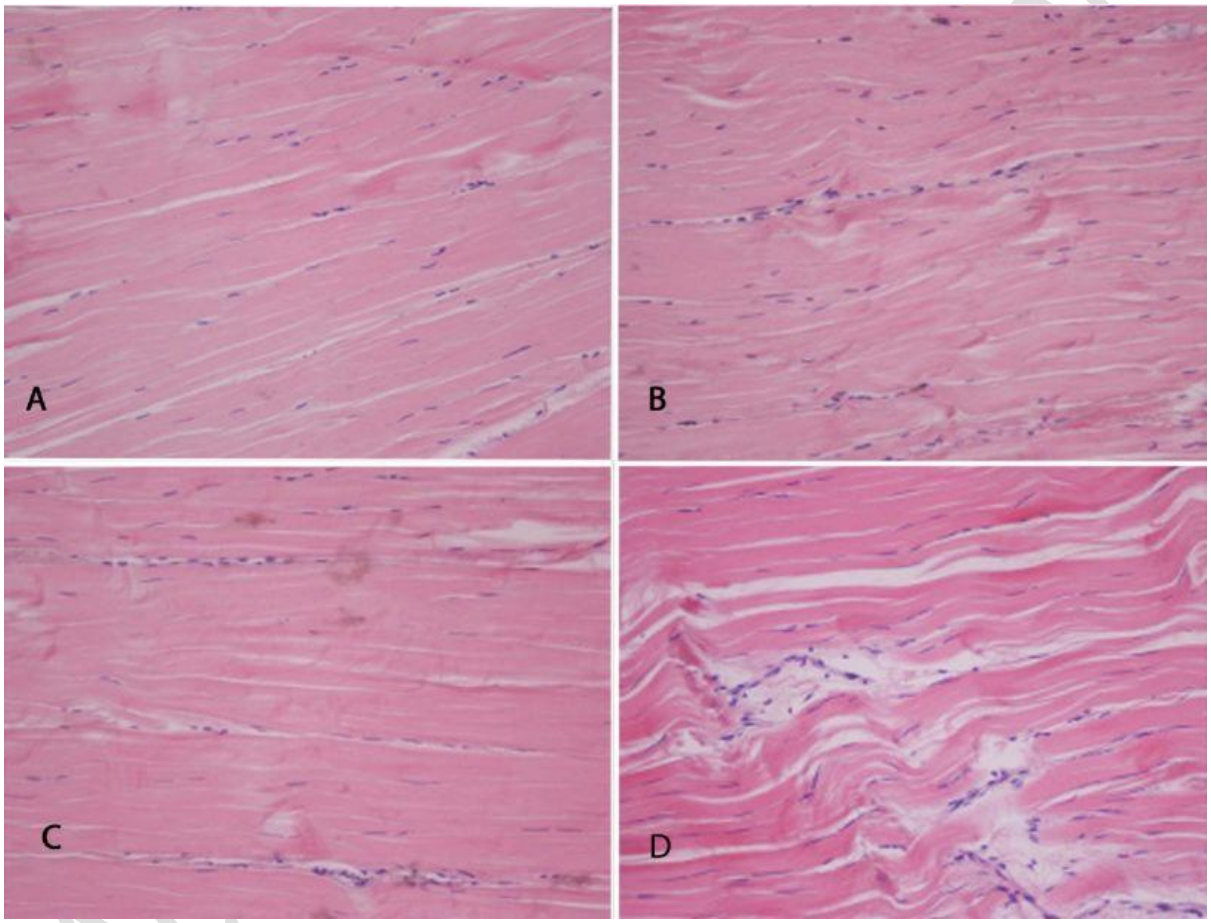

Figure 4: Histological findings of the Achilles tendons of the different groups. The control group (A) shows normal collagen fiber alignment. Microdamages are seen in the exercise group (B). No major differences are observed in the anabolic groups (C) compared to the control group. Microdamages, increased cellularity and vascularity, as well as derangement of collagen fibers are seen in the anabolics and exercise group (D).

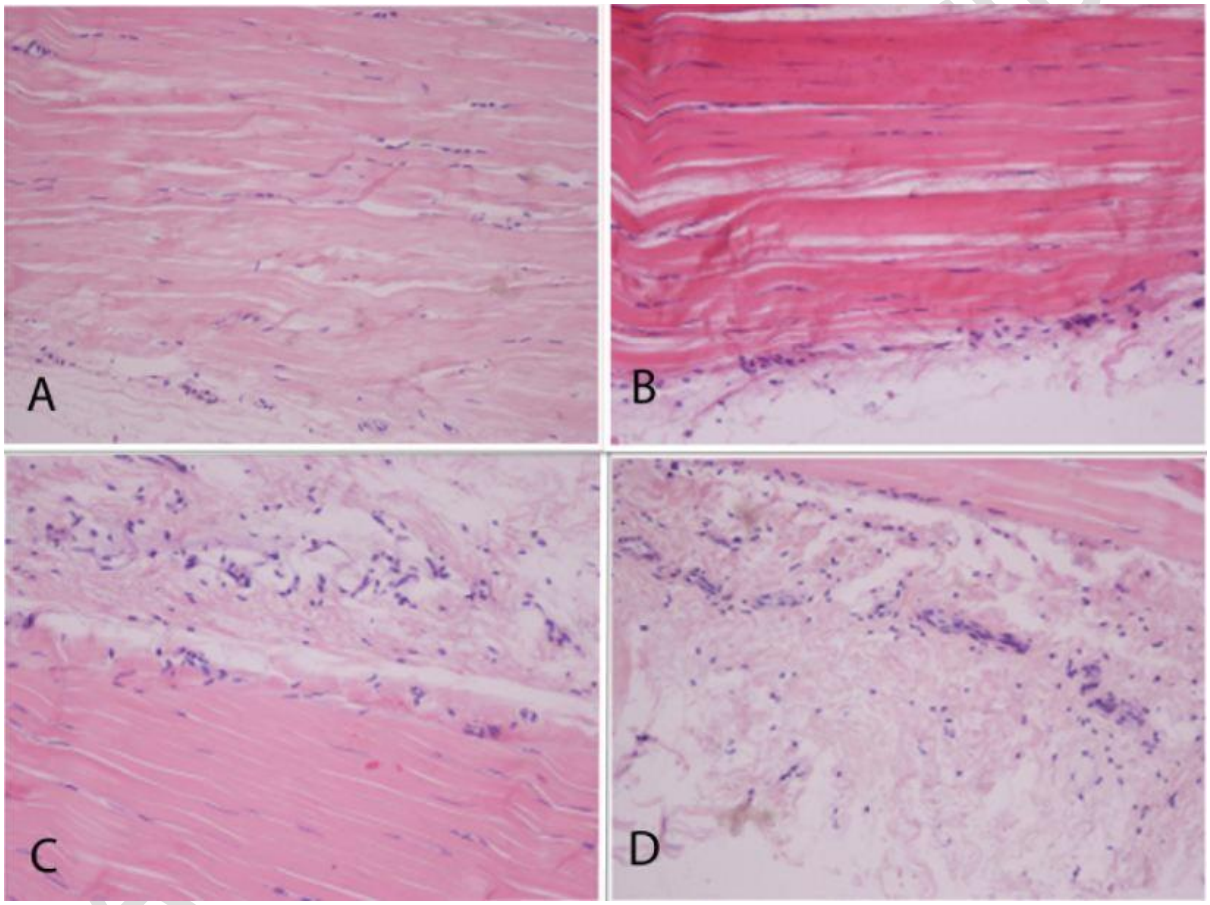

Figure 5: Electron microscopy examination. On the left side a typical tendon of a rat from the control group is seen. The alignment of the fibers is normal, without signs of collagen dysplasia or micro-rupture. On the right side a typical tendon from a rat from the AAS and exercise group is depicted. Collagen dysplasia and micro-ruptures are present.