

1 **Manuscript Title:**

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

4

5 **Names of the authors:**

6 Tsitsilonis Serafim, MD, M.Sc.^{1,2}, Chatzistergos E. Panayiotis, PhD³, Mitousoudis S. Athanasios,
7 PhD², Kourkoulis K. Stavros, Assoc. Professor², Vlachos S. Ioannis, M.Sc.¹, Agrogiannis George⁵,
8 MD, Fasseas Konstantinos, Professor⁴, Perrea N. Despina, Professor¹, Zoubos B. Aristides, Assoc.
9 Professor³.

10

11 **Affiliations and addresses of the authors:**

12 ¹Laboratory for Experimental Surgery and Surgical Research ``N.S. Christeas``, Athens Medical
13 School, Agiou Thoma str. 15B, 11527, Athens, Greece.

14 ²Charité – University Medicine Berlin, Center for Musculoskeletal Surgery, Augustenburger Platz 1,
15 13353, Berlin, Germany.

16 ³Unit of Biomechanics, Department of Mechanics, School of Applied Mathematical and Physical
17 Sciences, National Technical University of Athens, Zografou Campus, 15773, Athens, Greece.

18 ⁴1st Department of Pathology, University of Athens, Athens Medical School, Athens, Greece.

19 ⁵Laboratory of Electron Microscopy, Faculty of Agricultural Biotechnology, Department of Plant
20 Biology, Agricultural University of Athens, Iera Odos 75, 11855, Athens, Greece.

21 ⁶Department of Orthopaedic Surgery, University of Athens, Athens Medical School, ``Attikon``
22 University Hospital, Rimini str. 1, 12462, Athens, Greece.

23

24 **Corresponding Author:**

25 Tsitsilonis Serafim, MD, M.Sc.

26 Laboratory for Experimental Surgery and Surgical Research ``N.S. Christeas``, Athens Medical
27 School, Agiou Thoma str. 15B, 11527, Athens, Greece.

28 Telephone: 0030-2107462501

29 Fax: 0030-2107462539

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37 **Abstract**

38 **Background**

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

42 **Methods**

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

47 **Results**

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

52 **Conclusions**

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

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56 **Keywords**

- 57 • Anabolic androgenic steroids;
58 • Achilles tendon;
59 • Rupture;
60 • Biomechanics

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65 **1. Introduction**

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

81 **2. Materials and methods**

82 **2.1. Laboratory animals**

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

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

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99 2.2. AAS compound and administration protocol

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

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107 2.3. Exercise protocol

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

115

116 2.4. Biomechanical analysis

117 All animals were euthanized at 12 weeks under ether anesthesia. The ATs along with the
118 gastrocnemius muscles and foot from both legs were harvested, as previously described [12]. An
119 alternative clamping technique employing rapid freezing was developed for the biomechanical study
120 of rat bone-Achilles tendon-muscle units. The clamping device consisted of two separate parts (Fig.
121 1). The first was a pincers-like clamp for bone fixation, while the second a modified cryo-jaw. The
122 modified cryo-jaw comprised of a liquid nitrogen cup mounted on the inferior load frame and a
123 structure that formed a cavity with adjustable dimensions. The latter was placed in the medial axis of
124 the device at a higher level with respect to the liquid nitrogen cup. The muscle was placed and fixed
125 inside the cavity, ensuring that the musculotendinous junction was a few millimeters distant to the
126 cryo-jaw. Subsequently, the bone was fixed to the pincers-like clamp and mounted on the upper part
127 of the load frame. For the present study a MTS MiniBionix 858 (MTS System Corp., Eden Prairie,
128 MN, USA) load frame was utilized. Liquid nitrogen was poured inside the cup in order to achieve
129 rapid freezing of the muscle. Afterwards, the mechanical testing was initiated with a displacement rate
130 of 1 mm/min. The axial force exerted on the specimen was measured using a 500 N Instron Tensile
131 Load Cell (Instron, Canton, MA, USA). The required liquid nitrogen volume was accurately
132 determined in a series of pre-tests by placing a T-type thermocouple probe inside the tendon tissue at
133 the musculotendinous transition area, providing real-time temperature measurements. Use of 75 cm³
134 of liquid nitrogen resulted in a total temperature drop of the tendon of about 10 °C, while the muscle
135 was frozen to a satisfactory degree in order to withstand loads until tendon failure, without any
136 noticeable slippage. During the biomechanical testing, room temperature remained constant at
137 25 ± 2 °C.

138

139 2.5. Histological examination

140 ATs were subjected to light and electron microscopy examination. For the needs of light microscopy
141 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.

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162 2.6. Statistical analysis

163 Data are expressed as mean \pm 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 ($p < 0.05$).

169 **3. Results**

170 3.1. Biomechanical testing

171 A characteristic load–displacement curve from the present series of experiments is depicted in [Fig. 2](#).
172 The three portions, typical for soft tissues under tension, are clearly distinguished: an initial non-linear
173 region, followed by one almost linear, leading eventually to a sudden drop due to tendon failure. The
174 results indicate that by rapid freezing only, the muscle provides the necessary fixation force capable to
175 sustain significant tension loads without slippage and without affecting the mechanical behavior of the
176 “gage-length” of the tendon. The rupture occurred in the mid-substance of the tendons. Differences in
177 terms of load were observed among the groups; however, they were not statistically significant ([Table](#)
178 [1](#)). Statistically significant differences were observed between maximal stresses of the four groups
179 ([Table 1](#)). The AAS-exercise group exhibited the lowest maximal stresses among the four groups,
180 while the exercise-group the highest ([Table 1](#)).

181

182 3.2. Histological analysis

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

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195 4. Discussion

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

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

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

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

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

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

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

248 groups, mainly because of the observed difference in the temperature and the measured cross-
249 sectional area at the moment of rupture.
250 Conclusively, the present study suggests that AAS treatment reverses the beneficial effect of exercise
251 on the biomechanical behavior of the Achilles tendon, as it significantly decreased the maximal
252 sustainable tendon stress.

253

254 **Conflict of interest**

255 The authors declare that no conflict of interest exists.

256

257 **References**

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329 **Tables**

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331 Table 1: The values of fracture forces, cross-sectional area of the tendons and fracture stress among
 332 the four different groups. There were statistical significant differences in the fracture stress between
 333 the control group (C) and the exercise group (E) and between the exercise group (E) and the exercise
 334 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 (µm²)	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-E ²⁽²⁾ , E-A ¹⁽⁴⁾ , E-AE ³⁽²⁾

335 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
 336 also stands for t₀ weights, but the indices might change a bit)

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350 **Figures**

351

352 Figure 1: The biomechanical testing device setting with the two separate parts of the clamping device

353 are depicted.

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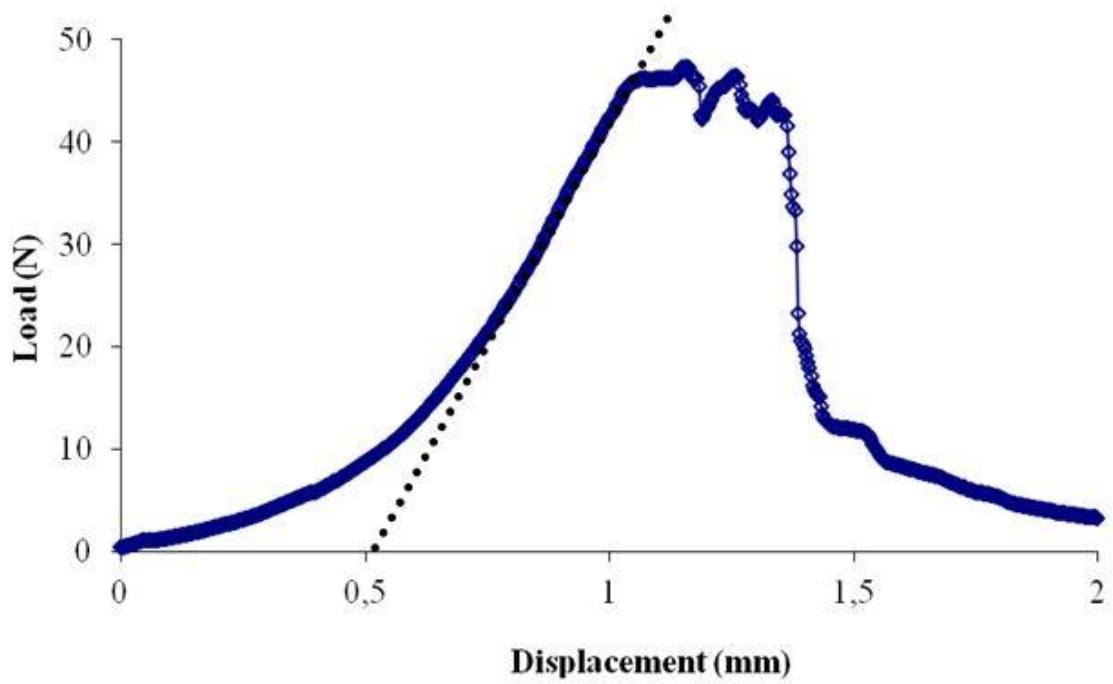
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376 Figure 2: Characteristic force–displacement curve of the present study.

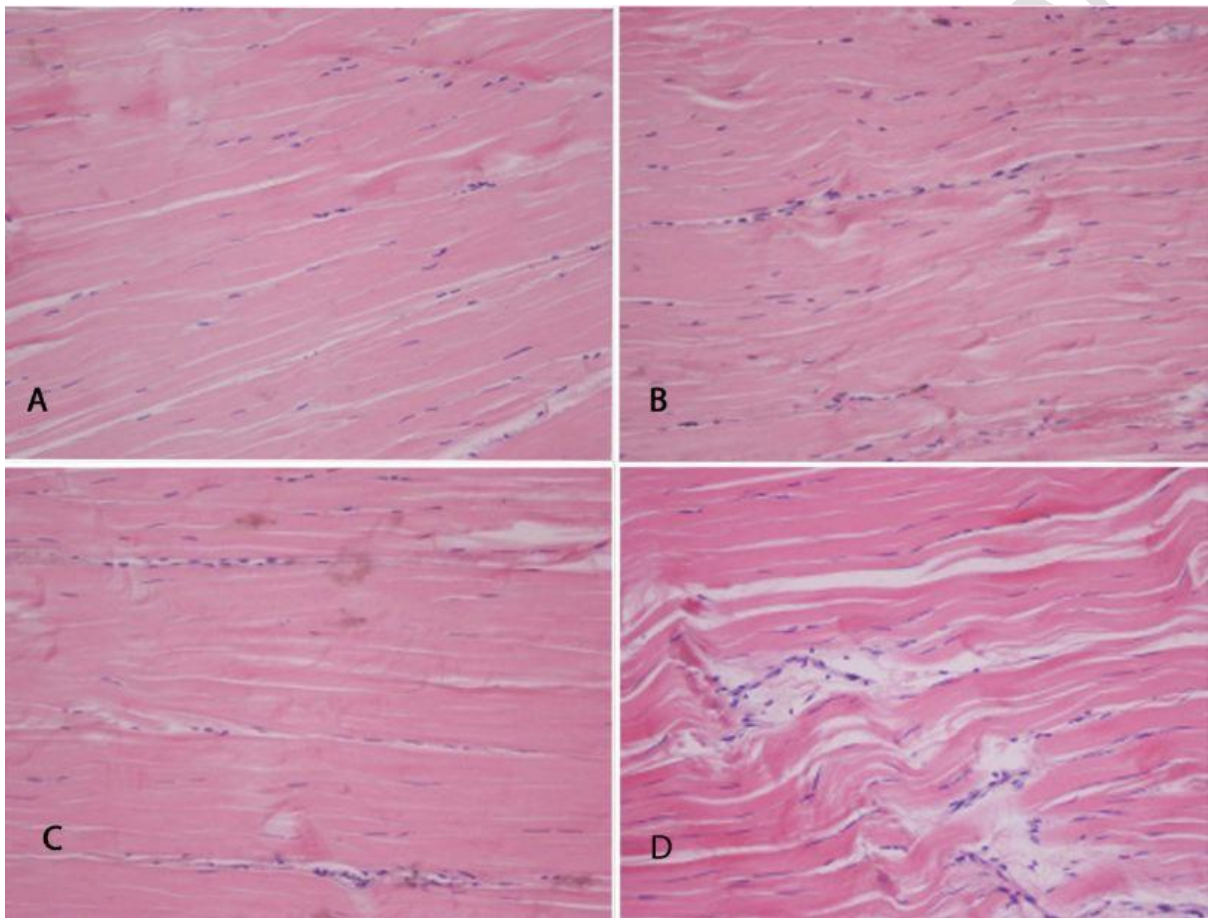


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391 Figure 3: Histological findings of the Achilles tendons of the different groups. The control group (A)
392 shows normal collagen fiber alignment. Microdamages are seen in the exercise group (B). No major
393 differences are observed in the anabolic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

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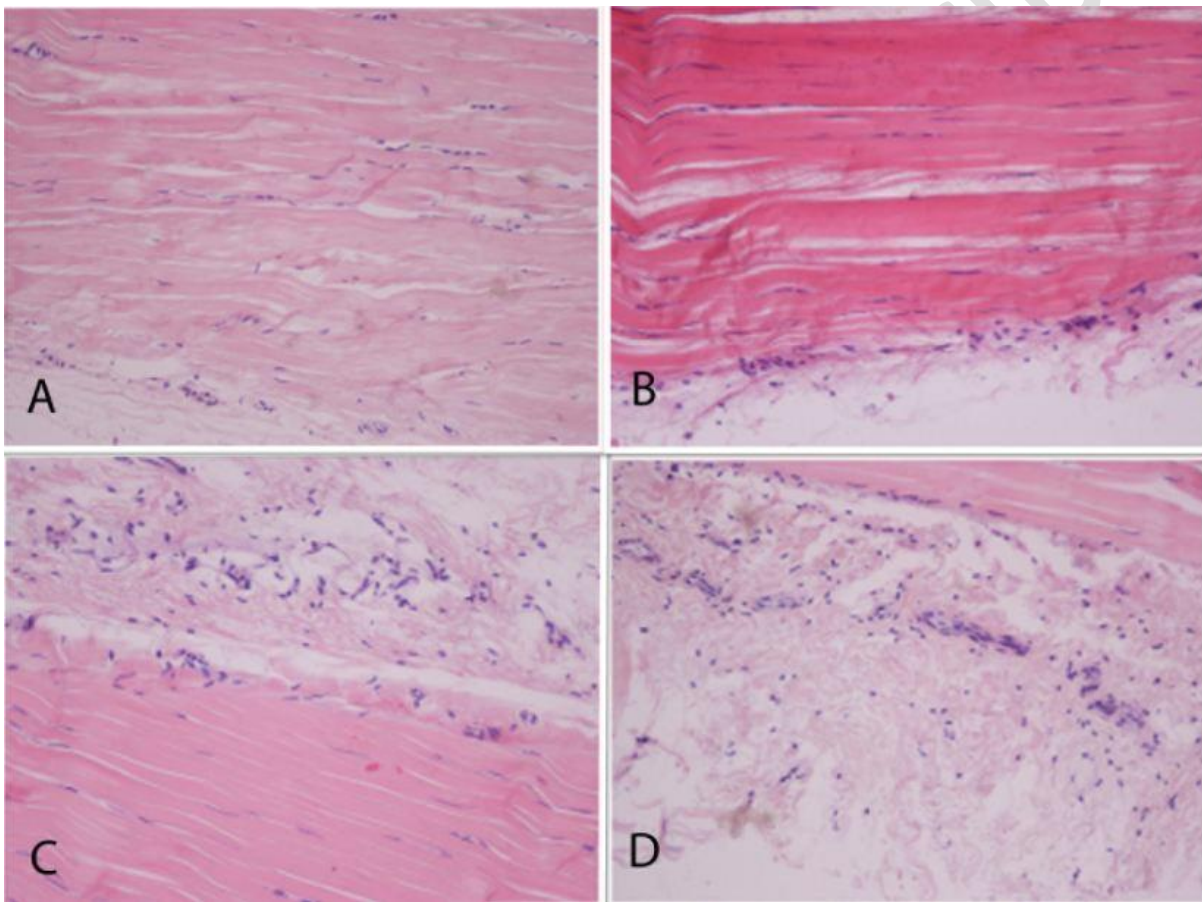
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406 Figure 4: Histological findings of the Achilles tendons of the different groups. The control group (A)
407 shows normal collagen fiber alignment. Microdamages are seen in the exercise group (B). No major
408 differences are observed in the anabolic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

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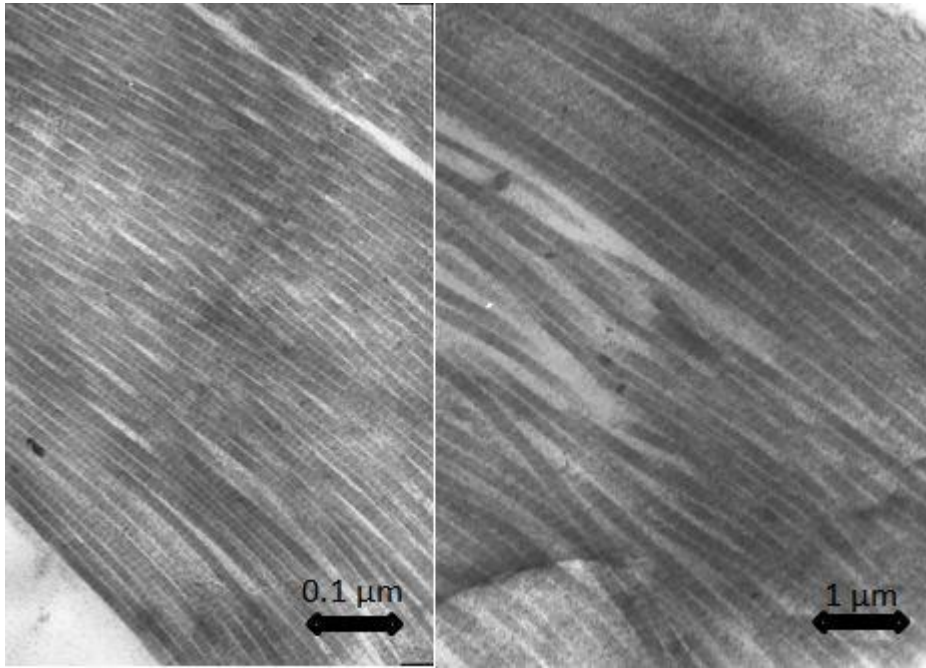
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419 Figure 5: Electron microscopy examination. On the left side a typical tendon of a rat from the control
420 group is seen. The alignment of the fibers is normal, without signs of collagen dysplasia or micro-
421 rupture. On the right side a typical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



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