

NANDROLONE INCREASES ANGIOTENSIN-I CONVERTING ENZYME ACTIVITY IN RATS TENDONS

NANDROLONA AUMENTA A ATIVIDADE DA ENZIMA CONVERSORA DE ANGIOTENSINA EM TENDÕES DE RATOS

LA NANDROLONA AUMENTA LA ACTIVIDAD DE LA ENZIMA DE CONVERSIÓN ANGIOTENSINA EN TENDONES DE RATONES



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ABSTRACT

Introduction: The renin-angiotensin system (RAS) has been associated with several biological processes of the human body, regulating, among others blood pressure and water and electrolytes balance. Moreover, RAS also regulates connective tissue growth. Recently, studies have shown that the use of nandrolone modifies the angiotensin-I converting enzyme (ACE) activity and increases collagen deposition in the heart. **Objective:** The aim of study was to evaluate the Angiotensin-I converting enzyme (ACE) activity in the superficial flexor tendon (SFT) and in serum after load exercise in combination with anabolic androgenic steroid (AAS) administration after training session and six weeks of detraining. **Methods:** Forty-eight Wistar rats were used into two groups (G1 and G2) subdivided into four subgroups: Sedentary (S); trained (T); AAS-treated (Deca-Durabolin®, 5mg/kg, twice a week) sedentary rats (AAS) and AAS-treated and trained animals (AAS-T). Trained groups performed jumps in water: four series of 10 jumps each, followed by a 30 sec interval between the series, for seven weeks. **Results:** Training increased ACE activity in the SFT compared to the control group ($p < 0.05$). Both AAS and AAS-T groups presented higher ACE activity levels ($p < 0.05$). The AAS-T increased the ACE activity only compared to the trained animals. Only the AAS-T group presented significant higher levels of ACE in the serum. In the G2 group, all experimental groups presented decreased ACE activity in the serum and in the tendon, as compared to the control group. **Conclusion:** This study indicates that AAS administration and its combination with exercise increased ACE activity of tendons. AAS abuse could compromise tendon adaptation causing maladaptive remodeling.

Keywords: steroids, renin-angiotensin system, exercise.

RESUMO

Introdução: O sistema renina-angiotensina (SRA) tem sido associado a importantes processos biológicos do corpo humano, regulando, entre outros processos, a pressão arterial e balanço hidroeletrolítico. Além disso, o SRA também regula o crescimento do tecido conjuntivo. Recentemente, foi demonstrado que a utilização de nandrolona modifica a atividade da enzima conversora de angiotensina (ECA) e aumenta a deposição de colágeno no coração. **Objetivo:** O objetivo do estudo foi avaliar a atividade de ECA no tendão flexor superficial (TFS) e no soro após exercício de força com administração de esteroides anabólicos androgênicos (EAA) durante sete semanas e após seis semanas de destreinamento. **Métodos:** Quarenta e oito ratos da linhagem Wistar foram divididos em dois grupos (G1 e G2) e subdivididos em quatro subgrupos: Sedentários (S); treinados (T); sedentários com EAA (EAAS) (Deca-Durabolin® - 5mg/kg, duas vezes por semana) e treinados com administração de EAA (EAAT). Os grupos treinados realizaram saltos na água: quatro séries de 10 saltos cada, com intervalo de 30 seg entre as séries. **Resultados:** O treinamento aumentou a atividade de ECA no TFS em comparação ao controle ($p < 0,05$). Os grupos tratados com EAA apresentaram maiores níveis de ECA ($p < 0,05$). O grupo EAA-T mostrou atividade de ECA mais elevada quando comparada ao grupo T. Além disso, o grupo EAA-T apresentou maiores níveis de ECA no soro. No grupo G2, todos os subgrupos diminuíram a atividade de ECA tanto no soro quanto no tendão. **Conclusão:** Este estudo indica que a administração de EAA e sua combinação com o exercício aumenta a atividade de ECA nos tendões. O uso abusivo de EAA pode comprometer a adaptação tendínea no qual pode provocar remodelamento mal adaptativas.

Palavras-chave: esteroides, sistema renina-angiotensina, exercício.

RESUMEN

Introducción: El sistema renina-angiotensina (RAS) ha sido asociado con varios procesos biológicos del cuerpo humano, entre ellos, regular la presión arterial y el contenido de electrolitos. Además, el RAS también regula el tejido conectivo. Recientemente, estudios han demostrado que el uso de nandrolona modifica la actividad de ACE e incrementa la deposición de colágeno en el corazón. **Objetivo:** En este modo, el objetivo del estudio fue evaluar la actividad de la enzima de conversión angiotensina (ACE) en el tendón flexor superficial (TFS) y en el suero después del ejercicio de resistencia en combinación con la administración de esteroides anabólico-androgénicos (AAS) después de la sesión de entrenamiento, y seis semanas de desentrenamiento. **Métodos:** Cuarenta y ocho ratones Wistar fueron divididos en dos grupos (G1 y G2) y subdivididos en cuatro grupos: sedentarios (S); entrenados (T); ratas sedentarias

tratadas con AAS (Deca-Durabolín® - 5 mg / kg dos veces a la semana) (AAS) y animales entrenados y tratados con AAS (AAST). Los grupos entrenados realizaron saltos en el agua: cuatro series de 10 saltos cada uno, con 30 segundos de intervalo entre las series, durante siete semanas. Resultados: El entrenamiento aumentó la actividad de ECA en TFS en comparación con el control ($p < 0,05$). Los grupos AAS y AAST mostraron mayores niveles de ACE ($p < 0,05$). El grupo AAST mostró alta actividad de ECA en comparación con el grupo T. Además, el AAST mostró niveles más altos de ACE en el suero. En G2, todos los grupos disminuyeron la actividad ACE tanto en el suero como en el tendón si comparados con el grupo control. Conclusión: Este estudio indica que la administración de AAS y su combinación con el ejercicio aumenta la actividad de ECA en los tendones. El uso abusivo de AAS puede comprometer la adaptación del tendón, lo que puede causar remodelaciones mal adaptativas.

Palabras clave: esteroides, sistema renina-angiotensina, ejercicio.

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INTRODUCTION

The renin angiotensin system (RAS) is classically the system that plays an important role in hemodynamic maintenance of human body¹. The RAS regulates blood pressure, water and electrolyte balance. Among other functions, the RAS also regulates connective tissue cell growth and the metabolic behavior of loose and dense connective tissue and sites of tissue repair².

From a pathological point of view, RAS activation results in vasoconstriction, cardiac hypertrophy and even fibrosis. In relation to the connective tissue, the RAS is involved the process of myocardial infarction and fibrosis in chronic liver disease³. Interestingly, several studies have shown that RAS is involved in bone metabolism⁴. Thus, strategies have been used to decrease the process of bone disorders through RAS inhibition⁵. However, little is known about RAS on tendon tissue remodeling.

Tendon fibrosis is associated with skeletal muscle disuse and muscle and tendon damage⁶. Angiotensin II (AngII) has been shown to induce collagen type I gene expression by activation of transforming growth factor- β 1 (TGF- β 1) signaling pathways and these effects were blocked by the AT1 receptor antagonist losartan⁷. TGF- β is widely regarded as universal mediators of fibrogenesis in diverse tissues, including tendon⁸. It is clearly demonstrated that AngII mediates atrial fibrosis by provoking mitogen activated protein kinases (MAPKs), and then stimulating endogenous synthesis of transforming growth factor- β 1 (TGF- β 1) and connective tissue growth factor (CTGF)⁹.

Also in this sense, the use of androgenic anabolic steroids (AAS) cause cardiac hypertrophy with interstitial fibrosis, which is associated with a local RAS activation¹⁰. Normally, the use of AAS has been associated with increased physical performance, wherein athletes and non-athletes use to increase the strength and muscle mass. Uncontrolled use of AAS causes changes in biomechanical properties of tendons, reducing tendon flexibility, which increases the risk of tendon rupture¹¹. Previous studies have also demonstrated that administration of AAS inhibits the activity of matrix metalloproteinases (MMPs) and collagen gene expression, possibly impairing tendon^{12,13}, and cardiac remodeling¹⁴.

Recently, were investigated¹⁵ the effects of nandrolone on the ACE activity, cardiac cytokines profile and other biomarkers. It was observed that the use of nandrolone increased the ACE activity, reduced the cytokines anti-inflammatory and increased of matrix type I collagen deposition, demonstrating that the use of AAS may cause cardiac hypertrophy associated to arterial hypertension.

Based on these previous studies, we raised the hypothesis that AAS-treatment would increase ACE activity similarly in the serum and tendon of rats and the association of exercise training and ASS-tre-

atment could enhance this effect. However, the elevated ACE levels would return to basal values after 6 weeks without AAS administration. Therefore, the aim of present study was to evaluate the ACE activity in superficial flexor tendon (SFT) and in the serum after load exercise in combination with AAS administration. We also evaluated these variables 6 weeks after the experimental groups had stopped both training and AAS treatment. This is the first study to investigate the effects of tendon ACE activity, bringing a new viewpoint for the role of this enzyme in tendons.

METHODS

Forty eight male rats (Wistar, *Rattus norvegicus albinus*; ~ 200g) were housed at room temperature with access to food and water ad libitum. All animal procedures were performed in accordance with the U.S.A. National Research Council's Guide for the Care and Use of Laboratory Animals. The experimental procedures of this study were approved by the institutional Ethics Committee in Animal Research of the University (Protocol no. 004/2006).

Rats were used into two groups (G1, and G2 and) subdivided into four subgroups (six animals/subgroup): sedentary without AAS administration (S); sedentary with AAS administration (AAS); trained (T); and trained with AAS administration (AAST).

Animals in the sedentary groups were not submitted to any type of physical activity. Animals in trained groups were exposed to jump training in a plastic tube 25 cm in diameter filled to a height of 30 cm, with water at constant temperature of $30 \pm 2^\circ\text{C}$. After a pre-training week, the animals initiated the experimental training protocol, which consisted of seven weeks of training with five training days (one session per day) per week. After seven weeks, G1 group was euthanized and G2 stopped both training and AAS treatment. G2 animals were euthanized after six weeks (S6, T6, AAS6, AAST6) of detraining.

Rats received 5 mg/kg of body mass (supraphysiological dose) of deca-durabolín (Nandrolone Decanoate; Organon do Brasil, São Paulo, Brazil) administered subcutaneously in their backs, twice a week. This dosage is comparable to the dosage frequently used by athletes. The experimental groups with no AAS treatment (S and T) received the vehicle only (Peanut oil with Benzyl alcohol). The treatment started in the first training week, and continued for seven weeks.

Training protocol

To reduce stress, the animals were adapted to water in the pre-training week. This adaptation consisted of sessions of weight lifting (50% body weight load), once a day for five days in water at $30 \pm 2^\circ\text{C}$. The jump training was induced by the instinctive reactions of rats: when animals are underwater they make an effort to jump for

breathing. The overload was attached to the animal's chest by means of a vest fitted to its body. The numbers of sets (2–4) and repetitions (5–10) were adjusted daily and gradually increased. All sessions were performed in the afternoon after 4h pm. After the pre-training week, animals were exposed to the experimental training protocol, which consisted of jumps in water, with the overload adjusted according to the animal's body weight, as previously described¹². Briefly, the training protocol consisted of a first training week, in which the animal performed four sets of 10 jumps with a rest period of 30s between sets and overload at 50% of body weight. In the next six weeks, the training protocol consisted of the same number of sets, jumps, and resting intervals, but with increased overload (5% increase per week), so that in the last week it was 80% of body weight. An observer was present during all the training sessions. All animals were weighed three times/week. The depth of the water column and the overload constituted barriers to avoid the rats to rest on their tails.

The G1 animals were euthanized immediately after the last training session. The G2 animals were euthanized after six weeks of detraining and no AAS treatment, respectively. Blood samples were obtained in ice without anticoagulant, centrifuged (10 min, 3000 x g at 4°C) and serum was stored at 20°C. The SFT tendon was immediately dissected from both posterior paws, frozen in liquid nitrogen and stored at -80°C. The prompt responses on ECM remodeling of the SFT to the demands of jump training and the treatment with AAS in our previous studies have motivated the choice of this tendon in the current study.

The samples of serum were diluted in 0.1 M Tris-HCl buffer, pH 7.0, containing 50 mM NaCl, centrifuged (10 min., 3000 x g at 4°C) and frozen at -20°C. Tendon samples were homogenized and incubated in 0.5 ml of extraction buffer (10 mM Cacodylic Acid pH 5.0; 0.15 M NaCl; 1 μM ZnCl₂, 20 mM CaCl₂, 1.5 mM NaN₃, 0.01% Triton X-100 [v/v]), at 4°C for 24 hours. After this period the solution was centrifuged (10 min, 13.000 x g at 4°C). The protein content of the samples was measured by BCA protein assay kit (Pierce, Rockford, IL), according to manufacturer's instruction, using bovine serum albumin as standard.

For the assay 5 μL serum extracts or 10 μL of homogenized tendon were incubated with the substrate Abz-FRK(Dnp)P-OH (Abz= ortho-aminobenzoic acid; Dnp = dinitrophenyl) in 0.1 M Tris-HCl buffer, pH 7.0, with 50 mM NaCl for 30 min at 37°C and centrifuged at 1000 x g for 10 min. The assays were performed at 37°C in 0.1 M Tris-HCl buffer, pH 7.0, containing 50 mM NaCl and 10 μM ZnCl₂.

The enzymatic activity was continuously monitored with a Hitachi F-2000 (Tokyo, Japan) fluorometer by measuring fluorescence at λ_{ex} = 320 nm and λ_{em} = 420 nm¹⁶. The slope was converted into μmol substrate hydrolyzed/minute on the basis of a calibration curve obtained after complete hydrolysis of each peptide. For the determination of the kinetic parameters, the enzyme concentration was chosen to hydrolyze less than 5% of the substrate present per unit time in order to obtain the initial rate. To correct for the inner filter effect we used an adjusting equation determined experimentally for 0.1 to 100 μM Abz-FR-OH, used as standard for fluorescence measurements¹⁵. The ACE activity was expressed in uF.min⁻¹.mL⁻¹.mg⁻¹ of protein.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). All variables show normal distribution and homoscedasticity (Kolmogorov-Smirnov and Levene's tests, respectively). Thus, two way ANOVA (training x AAS) followed by Tukey's multiple paired analysis was used for comparisons among treatments. Differences were considered significant when p < .05. All data were analysed by using the Statistica 7.0 software package (Stat. Soft. Tusa Inc., OK, USA).

RESULTS

Neither jumping protocol nor AAS treatment altered ACE activity in the serum (figure 1A p >0.05). Only the association of AAS and training presented significant higher levels of ACE (p=0.01). However, six weeks after experimental treatments had stopped, the levels of ACE activity decreased in the T6, AAS6 and AAST6 groups compared to the control group (figure 1B; p=0.02; p=0.01; p = .01, respectively).

Training increased the ACE activity in SFT compared to the control group (figure 2A, p=0.01). Interestingly, both AAS and AAST groups presented higher values of ACE activity levels when compared to the control (figure 2A; p = 0.01; p =0.01; respectively). Six weeks after these experimental groups had stopped both training and AAS treatment, experimental groups decreased ACE activity compared to the control (figure 2B; p=0.01).

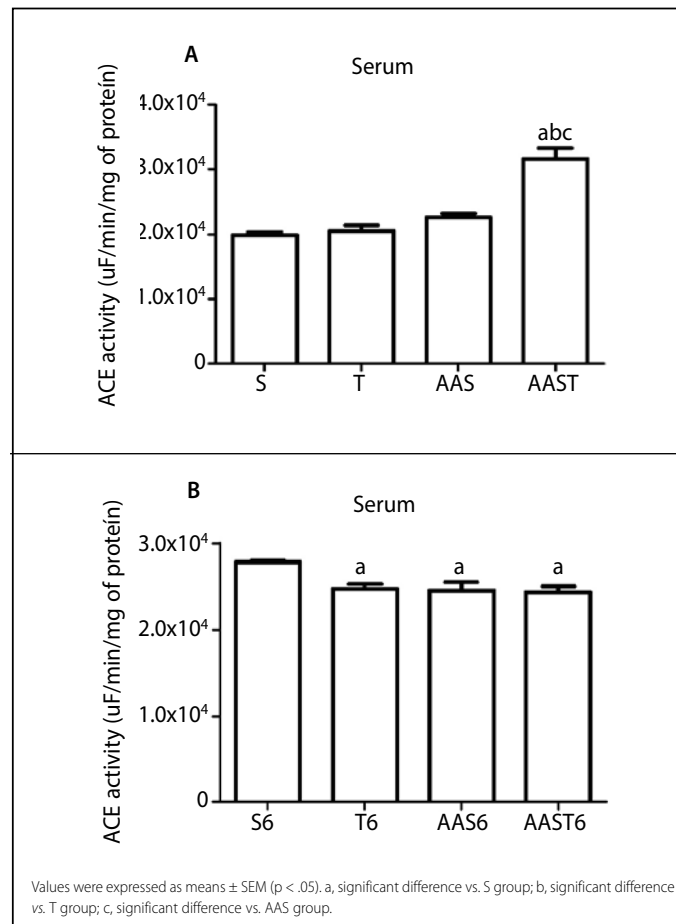


Figure 1. Angiotensin-converting enzyme (ACE) activity in the serum of sedentary animals (S), trained animals (T), sedentary with AAS administration (AAS) and trained with AAS administration (AAST). (A). ACE activity obtained immediately after the last training session. (B). ACE activity after 6 weeks of detraining and no AAS treatment.

DISCUSSION

This is the first study to investigate the effects of mechanical loading resulting from jumping exercise combined with AAS on the ACE activity of the SFT. The results demonstrated that training increased the ACE activity. Likewise, the AAS administration had increased the ACE activity in both AAS and AAST groups. After stopping exercise and AAS administration, it was observed a reduction in ACE activity in both tendon and serum compared to the control group. These findings have clinical relevance since they could be associated to harmful effect on ECM remodeling and could indicate further risk factors to tendons dysfunction in AAS users on sports field.

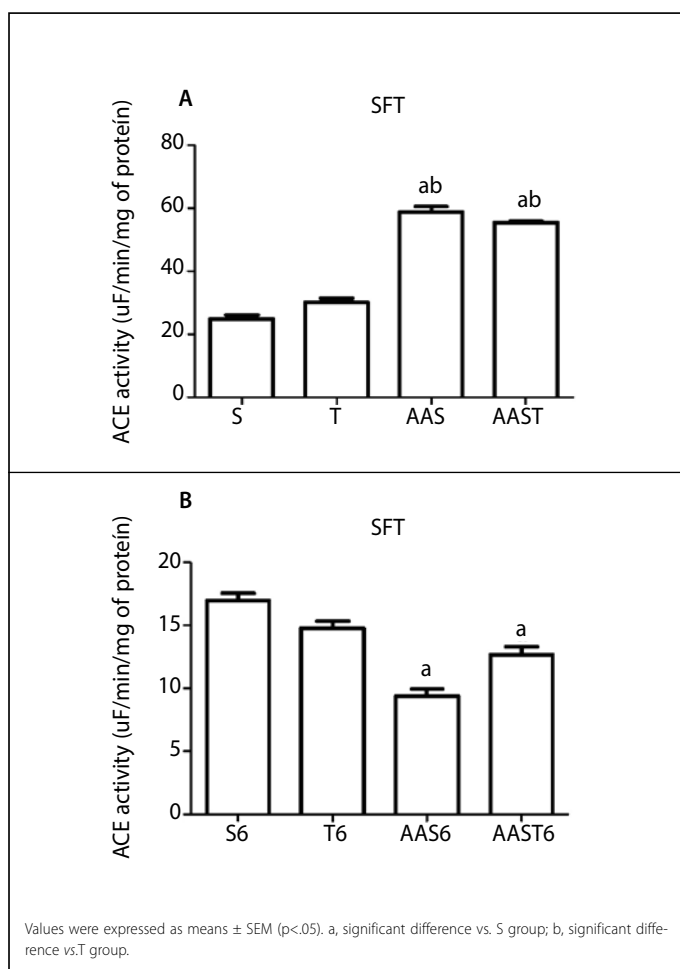


Figure 2. Angiotensin-converting enzyme (ACE) activity in SFT of sedentary animals (S), trained animals (T), sedentary with AAS administration (AAS) and trained with AAS administration (AAST). (A). ACE activity obtained immediately after the last training session. (B). ACE activity after 6 weeks of detraining and no AAS treatment.

ECM remodeling is determined by an active and dynamic process and depends on the adequate balance between synthesis and degradation of their components¹⁶. Studies showed that RAS cascade is involved in connective tissue cell growth and ECM remodeling², mainly in the heart following myocardial infarction^{17,18}. These enzymes cascade, including ACE expression has been detected in human cardiac tissues and there is a correlation between the levels of ACE and collagen type I mRNA¹⁹. Although, there is a lack of evidence that ACE activity is expressed by tendons, we demonstrated here the involvement of the local renin-angiotensin system on tendon tissue.

Interestingly, our results demonstrated that seven weeks of training increased the ACE activity, contrary to other reports^{20,21}. Probably, these discrepancies could be attributed to variations amongst the studies, such as training models and type of tissue evaluation. Concerning training models, these authors^{20,21} have used a swimming training exercise, whereas the presented study performed jumping exercise protocol. In addition, we assessed a tendon tissue differently from other studies, which analyzed cardiac muscles^{20,21}, lung and kidney²⁰. Further investigations are needed to test these discrepancies results.

It has been widely accepted that RAS cascade plays a pivotal role on the ECM remodeling, cell growth and fibrosis²². Rocha and colleagues reported²⁰ an increase in the ACE activity on the left ventricle after swimming training and AAS administration in rats. This exercise

training associated with AAS causes loss of the beneficial effects of left ventricle function induced by exercise training and maladaptive remodeling. Further deterioration of cardiac performance was detected accompanying with a local activation of the RAS consistent with the hypertrophic response. Nevertheless, there is a lack of information about the ACE activity on tendons and this relation with training and or AAS administration.

The main approach of our study was the increase of ACE activity of tendon in the AAS and AAST groups. In serum, only AAS combined with training have increased the ACE activity. These results are very intriguing comparing previous studies using the same experimental model. We have shown that AAS treatment in association with load exercise reduced total concentration of matrix metalloproteinase 2 (MMP-2: enzyme responsible for ECM remodeling and collagen degradation) and the percentage of the active mostly in SFT. The SFT responded more promptly to AAS treatment and this might be related to a higher cell density in this tendon¹². In addition, the biomechanical properties of SFT also was impaired by AAS treatment or AAS plus training, showing reduced tendon capacity to accommodate the initial tensional load, decreased capacity to resist tension and reduced deformability, contributing to the high risk of tendon rupture during training in AAS consumers¹¹. We might presume that AAS treatment in association with high load exercise induce harmful effects in SFT consider the increase of ACE activity together with abolishment of MMP-2 activity and biomechanical changes.

In line with this, our data indicate that the increase of ACE activity could be associated with ECM remodeling, once the ACE modulates the TGF- β , an important mediator of fibrous tissue formation in repairing tissue. Also, ACE increase the CTGF synthesis, which is a potent stimulator of type I collagen synthesis and fibrosis²³. It is possible to suggest that these results could be associated to tendon maladaptive remodeling, once we have previously demonstrated that AAS alters the biomechanical properties of tendons, reducing tendon flexibility, led to greater stiffness and would increase the risk of tendon rupture in AAS abusers¹¹. Recently, we also showed that AAS and ASS associated to training decreased the expression of key genes involved in tendon ECM remodeling¹³. Clinically, these findings are important in sports field, since AAS administration and their combination with exercise demonstrated harmful effects in relation to tendon ECM remodeling. The effect on tendons is likely a reflection of the delayed anabolic response of this tissue as compared to the highly vascularized and androgen-responsive associated skeletal muscle.

It is interesting to note that ACE activity has reduced in both tendon and serum after 6 weeks of detraining and upon cessation of AAS treatment in all experimental groups. The detraining induces a decrease in the synthetic activities of the tenocytes and the interruption of sudden build-up of MMPs activity²⁴, which is in agreement with the reduction of ACE activity after interruption of training. In addition, the literature has reported that many of the side effects are reversible with cessation of AAS special with reproductive/endocrine system²⁵, however there is a lack of studies to clarify whether cessation of AAS could be reversible in the tendon tissue.

Nevertheless, the reversibility of ACE activity levels after 6 weeks without AAS administration can be associated with a reversible of side effects caused by these drugs. Further researches in order to assess the MEC remodeling, such as MMPs activity and tissue morphology after six weeks of detraining and cessation AAS administration period will be performed.

CONCLUSION

In conclusion, this study indicated that AAS administration and their combination with exercise increased ACE activity, suggesting the activation of the local renin-angiotensin system on tendon tissue. This effect can be reversed with interruption of AAS treatment. Taking together these results with previous studies it is possible to speculate

that ACE activity is associated to harmful effect of AAS administration on tendon remodeling, and this fact has a clinical importance related to greater risk of tendon rupture.

All authors have declared there is not any potential conflict of interests concerning this article.

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