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Original Article

Testosterone replacement maintains smooth muscle content in the corpus cavernosum of orchietomized rats



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Abstract *Objective:* To evaluate the effects of testosterone (T) on the maintenance of corpus cavernosum (CC) structure and apoptosis.

Methods: Animals were divided into three groups: sham operation group ($n = 8$) underwent sham operation; Orchietomized (Orchiec)+ oily vehicle group ($n = 8$) underwent bilateral orchietomy and received a single dose of oily vehicle by intramuscular injection (i.m.) 30 days after orchietomy; and Orchiec + T group ($n = 8$) underwent bilateral orchietomy and received a single dose of T undecanoate 100 mg/kg i.m. 30 days after the surgery. Animals were euthanized 60 days after the beginning of the experiment with an anesthetic overdose of ketamine and xylazine. Blood samples and penile tissue were collected on euthanasia.

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Azan's trichrome staining was used to evaluate smooth muscle, Weigert's Fucsin-Resorcin staining was used to evaluate elastic fibers and Picrosirius red staining was used to evaluate collagen. Apoptosis was evaluated using TUNEL technique.

Results: T levels decreased in Orchiec + oily vehicle when compared to sham operation and Orchiec + T groups ($p < 0.001$). T deprivation reduced trabecular smooth muscle content and penile diameter and T replacement maintained both parameters ($p = 0.005$ and $p = 0.001$, respectively). No difference was observed in the content of sinusoidal space ($p = 0.207$), elastic fibers ($p = 0.849$), collagen ($p = 0.216$) and in apoptosis ($p = 0.095$).

Conclusion: Normal testosterone levels maintain CC smooth muscle content and do not influence elastic fibers, collagen content and apoptotic index. Further studies should be performed in order to investigate the mechanisms by which androgen mediates its effects on CC structure. © 2017 Editorial Office of Asian Journal of Urology. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Testosterone (T) is known to be an essential hormone involved in normal sexual male response, including erectile function [1,2]. Besides that, it is well established that normal erectile function requires the correct balance of hormonal factors, such as T, and corpus cavernosum (CC) histological structure [1,2]. Although there are clinical evidences demonstrating that low levels of T are associated to erectile dysfunction (ED) [3], this subject is still controversial, since some authors have not observed any association between T levels and ED [4]. In fact, the exact role of androgens in erectile function and dysfunction remains unclear [1,5,6].

Traish et al. [5,6] observed that androgen deprivation by surgical castration damages the histological structure of CC, which leads to veno-occlusive dysfunction, an important cause of organic ED [1,5,6]. Recently, Miranda et al. [7] evidenced that T deprivation decreases smooth muscle and sinusoidal space content, an effect reversed by testosterone replacement. The decrease of smooth muscle content in response to androgen deprivation is believed to be due to increased cellular apoptosis [1,8]. In fact, there are several data demonstrating increased apoptotic cells following androgen deprivation [1,8]. Besides that, T replacement prevents CC structures apoptosis, suggesting that androgen may have a role in apoptotic cascade [8].

Therefore, the aim of the study is to evaluate the effects of T on the maintenance of CC histological structures (smooth muscle, collagen, elastic fibers and sinusoidal space) and on apoptosis in order to better elucidate the interplay among androgens and CC structures.

2. Materials and methods

2.1. Animals and study design

The experimental protocol was approved by Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) Ethical Committee for Research and all efforts were made to minimize discomfort, distress and animals' suffering. All experimental procedures were carried out according to the

International Guiding Principles for Biomedical Research Involving Animals of the Council for International Organization of Medical Sciences and The International Council for Laboratory Animal Science.

Ninety-day-old male Wistar rats (~250 g), obtained from the animal facility of UFCSPA, were used. The animals were maintained under standard conditions of temperature $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a 12 h light/dark cycle (lights off at 5 p.m.). The animals were fed a standard laboratory rat chow and had water available *ad libitum*.

Animals were divided into three groups: sham operation group ($n = 8$) underwent sham operation; Orchiectomized (Orchiec) + oily vehicle group ($n = 8$) underwent bilateral orchiectomy and received a single dose of oily vehicle by intramuscular injection (i.m.) 30 days after orchiectomy; and Orchiec + T group ($n = 8$) underwent bilateral orchiectomy and received a single dose of T undecanoate (Nebido[®]; Bayer Schering Pharma, Berlin, Germany) 100 mg/kg i.m. 30 days after the surgical procedure [9]. Both oily vehicle and T undecanoate were injected into the animals' muscle biceps femoris in the right hind leg.

All surgical procedure were performed under sterile condition and ketamine and xylazine (10 mg/kg and 80 mg/kg i.p., respectively) anesthesia. Animals from Orchiec + oily vehicle and Orchiec + T groups were submitted to bilateral orchiectomy. The surgical procedure was performed with a 2 cm scrotal midline incision and both testes were removed. Sham operation group underwent the same surgical procedure and manipulation; however, testes were not removed. Ibuprofen (Buprovil[®]; Multilab, São Jerônimo, Brasil) 20 mg/kg, 8-8 h, was given by gavage during 2 consecutive days. Animals' body weight was monitored for T undecanoate dose adjustment.

All animals were euthanized 60 days after the beginning of the experiment with an anesthetic overdose of ketamine and xylazine. Immediately after euthanasia, while the animals were still alive, a cardiac puncture was performed in order to collect blood samples for total T determination. Total T levels were determined by electrochemiluminescence assay (Elecys[®]; Roche Diagnostics, Indianapolis, IN, USA). Results are expressed in ng/dL.

Penile tissue was obtained by dissecting its skin up to the crura. Sterile cotton gauze was used to gently remove

excess blood. The penis was immediately fixed in 10% buffered formalin and penile mid-shaft segments were embedded in paraplast according to standard histological techniques.

For histomorphometric analysis, a total of five 100 μm -equidistant transversal penile tissue sections (5 μm -thick) were taken from each specimen. From each section, five random fields were analyzed, totaling 25 evaluated fields per animal, per stain used [10]. Tissue sections followed routine histological staining.

Azan's trichrome staining, which highlights smooth muscle (red) and connective tissue (blue), was used to assess the mean percentage of smooth muscle in penile cavernosal tissue. The same staining was used to quantify sinusoidal space. Corpus cavernosum elastic system components were evaluated using Weigert's Fucsin-Resorcin staining, with previous oxone oxidation, in order to detect oxitalan, elauin and elastic fibers [10] and in order to quantify collagen fibers, all sections were also stained with Picosirius red and were observed under light polarization due to collagen fibers' birefringence [11].

Histological analysis was performed by the same examiner (GH), who was blind to the animal's treatment. Image acquisition and digitalization were performed with a digital camera (DP72; Olympus, Tokyo, Japan) coupled to a light microscope (BX51; Olympus). All images were obtained in a standardized method to ensure uniformity.

Penile diameter measurements were performed in triplicate in five transversal penile tissue sections ($\times 20$ final magnification), per rat, using ImageJ 1.45s (NIH, <https://imagej.nih.gov/ij/>, USA). For statistical analysis, mean values for each rat (the mean of the five sections and triplicate measurements) were calculated and used for comparisons of the three groups.

Smooth muscle, sinusoidal space ($\times 400$ final magnification) and elastic fibers content ($\times 1000$ final magnification) were quantified using a manual point-counting method, as previously described [12]. The percentage of smooth muscle, sinusoidal space and elastic fibers content were calculated by dividing the number of points counted per number of total points $\times 100$.

Collagen content was determined using semi-automated color segmentation method, performed in Image-Pro[®] Plus software (Media Cybernetics, Rockville, MD, USA), as previously described [12].

For statistical analysis, mean values for each CC (the mean of the 25 images analyzed per rat) were calculated and used for comparisons of the three groups.

In situ Terminal Deoxynucleotidyl Transferase-mediated dUTP nick-end Labeling (TUNEL) procedure was also performed in order to detect *in situ* apoptotic cells in penile formalin-fixed, paraplast-embedded 5 μm -thick sections. Click-iT[®] TUNEL Alexa Fluor 488 Imaging Assay (Invitrogen; Baltimore, MD, USA) was used according to the manufacturer's instructions. Briefly, after formalin-fixed, paraplast-embedded 5 μm -thick sections' deparaffinization and hydration, the slides were pre-treated with 2% glycine and ammonium chloride 50 nmol/L for blocking aldehyde group and were incubated with TdT and EdUTP reaction cocktail for 60 min at 37°C in a humidified chamber. Afterwards, penile sections were

incubated with Click-iT[®] reaction cocktail for 30 min at room temperature for fluorescent detection of EdUTP. Penile tissue nuclei were counterstained with Hoechst 33342 for 15 min at room temperature. The slides were, then, mounted with *SlowFade*[®] Gold Antifade Mountant (S36937; Thermo Fisher Scientific Inc., Waltham, MA, USA) and with glass coverslips. Positive control sections were prepared with DNase incubation. TdT reaction cocktail was omitted in negative control sections.

A total of two 100 μm -equidistant transversal penile tissue sections were analyzed. From each section, five random fields were evaluated, totaling 10 evaluated fields per animal.

Apoptotic cells were visualized using a fluorescent microscope (BX51; Olympus) coupled with a digital camera (DP72; Olympus). After image acquisition ($\times 600$ final magnification), the number of TUNEL-labeled nuclei and the number of Hoechst-labeled nuclei were counted in 10 random fields per animal. The mean values were obtained for each rat and these means were used to calculate apoptotic index, which was obtained by dividing the number of TUNEL-labeled nuclei by Hoechst-labeled nuclei (total number of nuclei) $\times 100$ [13]. Results are expressed in percentage (%).

2.2. Statistical analysis

All variables were tested for normality using Shapiro–Wilk test. Data were expressed as mean \pm SD or as median (quartiles). One-way Analysis of Variance followed by Tukey *post-hoc* test was used to compare means of T, body weight, penile diameter, smooth muscle, sinusoidal space, elastic fibers and collagen content among groups. Pearson correlations were used to verify the correlation between T levels and smooth muscle content. Apoptotic index was compared using Kruskal–Wallis test, due to the non-normally distributed data.

Statistical analyses were performed using SPSS software version 12.0 (SPSS, Chicago, IL, USA). Statistical significance was set at $p \leq 0.05$.

3. Results

Baseline body weight was 245.50 ± 48.80 g in sham operation group, 242.12 ± 38.50 g in Orchiec + oily vehicle group and 247.00 ± 47.42 g in Orchiec + T group ($p = 0.977$).

As expected, T levels were statistically decreased in Orchiec + oily vehicle group (7.02 ± 2.52 ng/dL; $n = 8$) when compare to sham operation (358.10 ± 178.38 ng/dL; $n = 7$) and Orchiec + T groups (237.68 ± 153.01 ng/dL; $n = 6$) ($p < 0.001$). This result indicates that T replacement was effective.

T deprivation reduced mean trabecular smooth muscle content ($p = 0.005$) as well as penile diameter ($p = 0.001$) and androgen replacement maintained normal values in both parameters (Table 1). In addition, there was a positive statistically significant correlation between T levels and mean trabecular smooth muscle content ($r = 0.613$; $p = 0.005$; Fig. 1). However, no statistical significant difference was observed in the mean content of sinusoidal

space ($p = 0.207$), elastic fibers ($p = 0.849$) and collagen ($p = 0.216$) (Table 1 and Fig. 2).

No statistical significant difference was observed in penile apoptotic index among groups ($p = 0.095$, Table 1 and Fig. 3).

4. Discussion

In the present study, we demonstrated that T replacement of castrated rats maintained trabecular smooth muscle content as well as penile diameter and did not alter the content of sinusoidal space, elastic fibers and collagen. Furthermore, T deprivation and replacement did not change CC apoptotic index.

Erectile function is a complex event that requires the synchronism of neural, vascular and hormonal factors as well as the integrity of CC tissue [1] and any imbalance in these components may result in ED [5]. Studies have highlighted the importance of androgens in erectile function, penile tissue function and structure [1,2,7]. Probably, one of the most important structures in penile CC is trabecular smooth muscle, which is responsible for penile detumescence and erection [14] and is closely related to veno-occlusive function [1,6]. As a consequence, decreased trabecular smooth muscle content may lead to ED due to poor venous outflow resistance [15].

Our data indicate that androgen replacement maintained trabecular smooth muscle content and testosterone deprivation decreased its levels. Our results are consistent with some previous studies [5–7,16]. Traish et al. [5] demonstrated that castration significantly decreased trabecular smooth muscle content. Seven days after orchietomy, T replacement was given and restored this parameter to control levels [5]. Albeit Miranda et al. [7] have replaced T after 1 month of androgen ablation; the authors have found similar results: decreased absolute values of smooth muscle in the castrated group and that T replacement restored its content.

Although some studies have demonstrated that androgens act directly in smooth muscle content, the exact mechanism by which androgen deprivation reduces smooth muscle content is not known [5]. It has been observed that androgen deprivation induces the onset of apoptotic

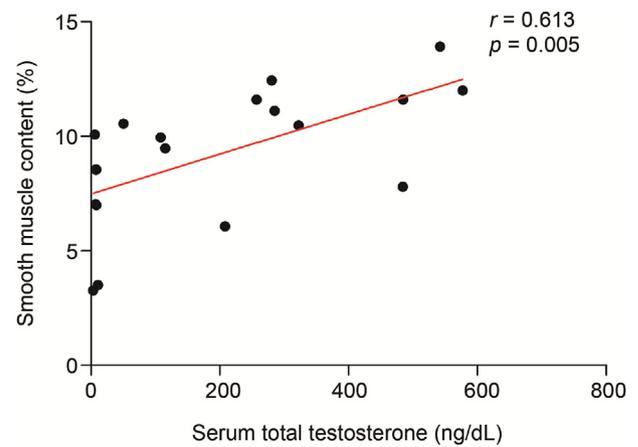


Figure 1 Correlation between testosterone levels and mean trabecular smooth muscle content. Correlation was performed using Pearson's correlation test.

cascade in penile CC [1,8,17]. Moreover, experimental studies have pointed out that T supplementation can prevent apoptosis promoted by androgen ablation [8], suggesting that androgens may be involved in the control of this process [18]. The increased apoptotic index induced by androgen ablation may explain the decrease in smooth muscle content observed after castration [1] and the decrease in penile size after penile denervation [13]. Although there are strong evidences showing the effects of T withdrawal and replacement on apoptosis regulation [1,8], our results indicate that apoptosis was not influenced by long-term androgen deprivation and replacement, which are in agreement with some studies published so far [17,19,20]. Nakazawa et al. [19] have studied the effects of castration and T administration on rat urinary bladder and found no significant effect of T on TUNEL-labeled nuclei. It is important to highlight that the authors have started T replacement after 4 weeks of castration, similar to our replacement protocol. Additionally, as far as we are concerned, this is the first study published in literature that evaluated CC apoptotic index after long-term androgen deprivation and replacement.

Table 1 Penile histomorphometric analysis and apoptosis of the evaluated groups.

Parameters	Sham operation		Orchiec + oilyvehicle		Orchiec + T		p-Value
	Value	n	Value	n	Value	n	
Penile diameter (mm)	3.40 ± 0.20	7	3.04 ± 0.32*	8	3.70 ± 0.22	6	0.001
Smooth muscle content (%)	11.14 ± 2.55	8	6.86 ± 2.59 [#]	7	10.60 ± 1.62	6	0.005
Sinusoidal space (%)	40.34 ± 6.27	8	42.9 ± 3.79	7	38.02 ± 2.96	6	0.207
Elastic fibers content (%)	6.23 ± 1.03	8	6.51 ± 1.37	7	6.20 ± 0.96	7	0.849
Collagen content (%)	44.56 ± 3.96	8	40.93 ± 4.94	8	42.17 ± 2.84	7	0.216
Apoptotic index (%)	13.70 (11.09–20.75)	6	18.46 (12.81–29.54)	8	26.32 (20.44–56.09)	5	0.095

Data are expressed as mean ± SD or as median (quartiles).

* $p = 0.001$, [#] $p = 0.005$, when compared to sham operation and Orchiec + T groups.

p values obtained by one-way ANOVA, but for apoptotic index, which was obtained by Kruskal–Wallis test.

Sham operation group, sham operation; Orchiec + oily vehicle group, underwent bilateral orchietomy and received a single dose of oily vehicle by intramuscular injection (i.m.) 30 days after orchietomy; Orchiec + T group, underwent bilateral orchietomy and received a single dose of testosterone undecanoate 100 mg/kg i.m. 30 days after the surgical procedure; Orchiec, orchietomy; T, testosterone.

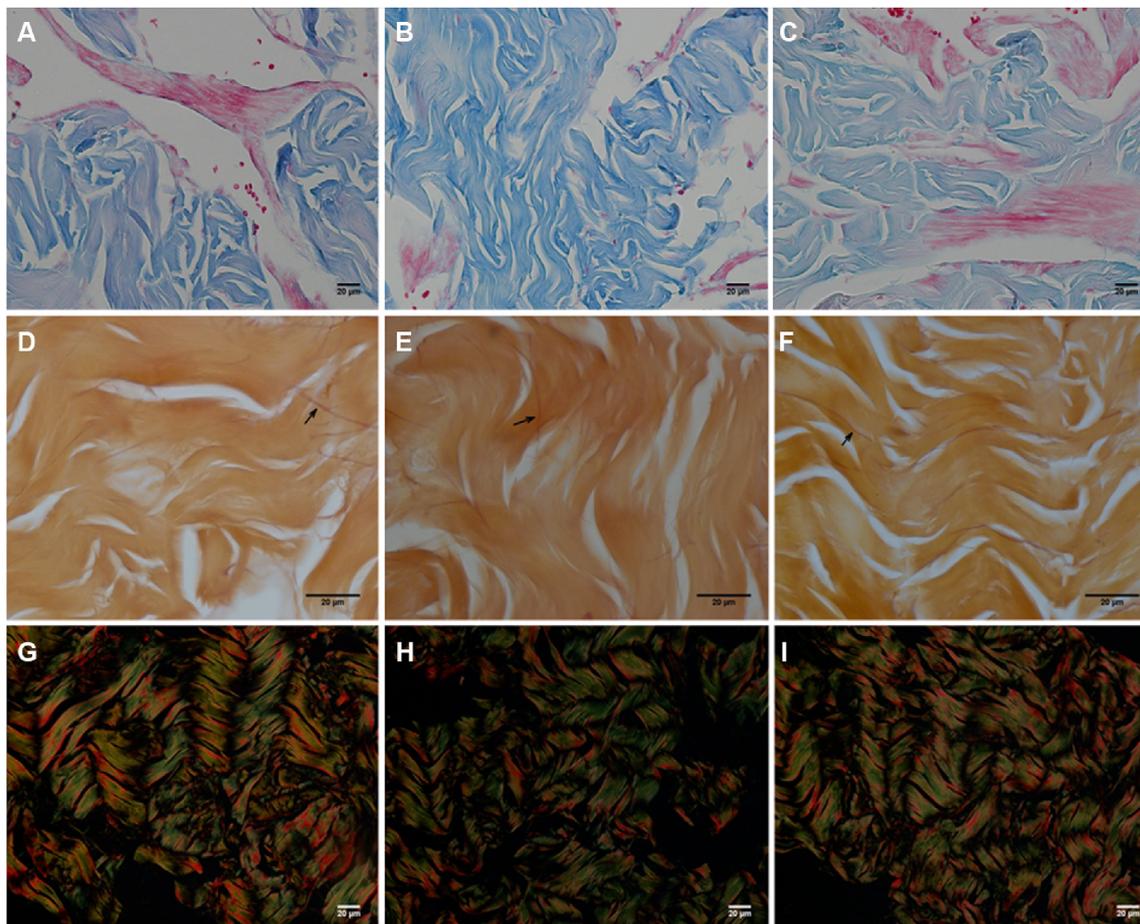


Figure 2 Photomicrographs of rat corpus cavernosum demonstrating Azan's trichrome staining (Blue: collagen and red: smooth muscle) at $\times 400$ magnification (A, B and C), Weigert's Fuchsin-Resorcin staining at $\times 1000$ magnification (arrows indicates elastic fibers) (D, E and F) and Picosirius red under polarization at $\times 400$ magnification (G, H and I). (A, D and G) Sham operation group; (B, E and H) Orchiec + oily vehicle group; (C, F and I) Orchiec + T group. All samples were collected 30 days after oily vehicle or T injections (60 days after orchietomy). Orchiec, orchietomy; T, testosterone. Scale bar = 20 μm .

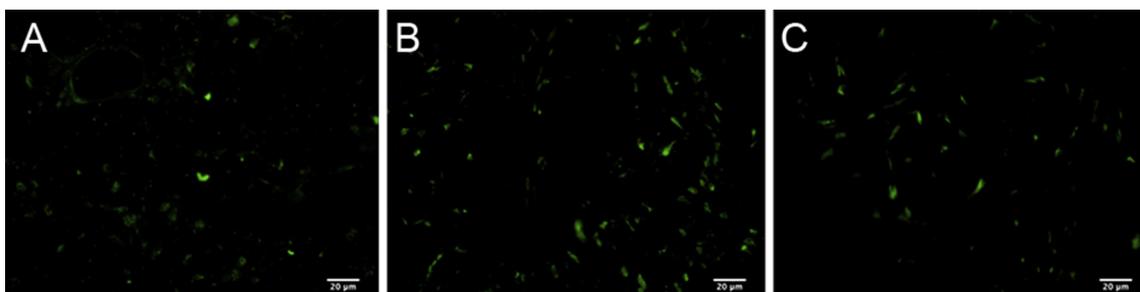


Figure 3 TUNEL-labeled nuclei (apoptotic cells) at $\times 600$ magnification. (A) Sham operation group; (B) Orchiec + oily vehicle group; (C) Orchiec + T group. All samples were collected 30 days after oily vehicle or T injections (60 days after orchietomy). Orchiec, orchietomy; T, testosterone. Scale bar = 20 μm .

Even though we have not found any statistical significant difference in the apoptotic index among groups, this does not necessarily reflect an absence of apoptosis in penile CC. Studies have indicated that the apoptotic index peaks on the 2nd day after cavernous nerve injury and returns to baseline levels 14 days after nerve damage [13]. Similarly, Yamamoto et al. [17] have observed that penile apoptotic

cells were increased after the 5th day after bilateral orchietomy and returned to baseline levels after the 14th and 20th days after the surgery. These results indicate that T withdrawal has an early effect on penile tissue and that the penis is dependent upon physiological levels of T [21]. The decrease in both smooth muscle content and penile diameter found in the castrated group could be explained

by an early apoptosis promoted by the lack of T. This apoptosis has probably returned to control levels, suggesting a timing of hormonal effect on apoptosis [18].

Although there are overwhelming evidences in literature demonstrating the importance of smooth muscle in erectile function, collagen and elastic fibers also play a pivotal role in this physiological process [22]. These histological structures are responsible for the maintenance of erection and are important to the rapid return to the non-erect state [23]. Costa et al. [23] have found that the percentage of elastic fibers in patients with severe ED was statistically decreased when compared to control group. Shen et al. [16] have observed that elastic fibers content was replaced by collagenous fibers after androgen deprivation in rats tunica albuginea. However, no study published so far has evaluated the effects of T ablation and replacement in elastic fiber content in CC. Our results indicate that androgens do not act on elastic fibers content. These data corroborate with a previous study published by Inoue et al. [24]. The authors have evaluated the biosynthesis of sex steroids in human skin and found that aromatase is closely associated with estrogen synthesis and with elastic fiber formation [24]. However, no influence of T was observed in the synthesis of elastic fibers [24].

Regarding collagen content, we have not observed any influence of T on its content. This result contradicts the ones in literature [6,16]. Different study design, animal models used and histomorphometric methods employed should explain the discrepant results observed.

Some limitations should be recognized in the current study. First, we have used an animal model to investigate the role of T on CC histology. However, the rat CC markedly differs from those in human, so extrapolation to human beings should be done carefully [22]. Second, surgical castration is not an appropriate model of late-onset hypogonadism (LOH). So every attempt to simulate LOH in animal models is not completely reliable because it does not reflect the real/clinical LOH in human beings. This raises questions regarding the model of LOH used nowadays and highlights the need for the creation of a new animal model of LOH, which would reflect more accurately what really happens in clinical practice.

5. Conclusion

In summary, physiological T levels maintain CC smooth muscle content and do not influence elastic fibers and collagen content. However, further studies should be performed in order to investigate the mechanisms and signaling pathways by which androgen mediates its effects on CC structure.

Conflict of interest

The authors declare no conflict of interest.

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