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Effects of Castration and Late Hormonal Replacement in the Structure of Rat Corpora Cavernosa

ALEXANDRE F. MIRANDA, CARLA B. M. GALLO, DIOGO B. DE SOUZA, WALDEMAR S. COSTA, AND FRANCISCO J. B. SAMPAIO

From the Urogenital Research Unit, Rio de Janeiro State University, Rio de Janeiro, Brazil.

ABSTRACT: The objective of the study was to evaluate, through quantitative methods, the structural alterations in the corpora cavernosa of rats submitted to orchiectomy as well as the role of late hormone replacement in overturning the possible structural alterations. Twenty-five male rats were assigned into 5 groups with 5 animals each and treated as follows: ORCHIEC-1 = submitted to orchiectomy and sacrificed after 1 month; C-1 = control group sacrificed after 1 month; ORCHIEC-2 = submitted to orchiectomy and sacrificed after 2 months; C-2 = control group sacrificed after 2 months; and T = submitted to orchiectomy, underwent testosterone replacement with testosterone undecanoate (100 mg/kg) after 1 month, and sacrificed after 1 month of hormonal replacement. Smooth muscle, collagen, and elastic system fibers of penile corpora cavernosa were quantified. There was a significant decrease in the absolute values of smooth muscle, sinusoidal space, and total area of corpora cavernosa after 2 months in the castrated group when compared with controls. Overall, regarding density, no significant differences were observed among the groups. The hormonal replacement with testosterone was able to reverse the alterations observed. The method used for this research allowed demonstrating that absolute values are reliable to quantify the structural alterations of corpora cavernosa structures. The results suggest that hormonal replacement, even when instituted at a late stage, is effective in reversing the corpora cavernosa's structural alterations produced by castration.

Key words: Androgen, penis, andropause, erectile dysfunction, morphology.

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Testosterone has been considered essential for maintenance of sexual function (Mills et al, 1996; Morales and Heaton, 2003). Adequate serum levels of testosterone are important not only for maintenance of erectile function, but also for cognitive processes and body mass composition. When low levels of testosterone are found in the aging male, the condition is called andropause or androgen deficiency of the aging male (ADAM; Morley and Perry, 1999; Wang et al, 2009). ADAM is estimated to be present in 6% of men at 40 years old and 12% at 69 years old (Araujo et al, 2004). Hormonal replacement has been indicated to restore the secondary alterations of hypogonadism, including sexual function (Morley and Perry, 1999; Wang et al, 2009).

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Correspondence to: Dr Francisco J. B. Sampaio, Urogenital Research Unit–UERJ, Av. 28 de Setembro, 87, Fundos, Vila Isabel, Rio de Janeiro, RJ, Brazil, CEP: 20551-030 (e-mail: sampaio@urogenitalresearch.org).

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In recent years, testosterone decrease related to aging has received special attention; nevertheless, its pathophysiological significance remains controversial (Wang et al, 2009). Studies in animals and humans have suggested that adequate levels of testosterone are necessary to preserve the integrity of penile erectile tissue. Moreover, testosterone is important for the biochemical mechanisms involved in erection, as well as being linked to erectile dysfunction (Mills et al, 1996; Granata et al, 1997; Dai et al, 1999; Shen et al, 2003; Gooren and Saad, 2006; Traish and Guay, 2006; de Souza et al, 2012). Shen et al (2003) reported that androgen deprivation caused a decrease of elastic fibers in tunica albuginea and smooth muscle in corpora cavernosa of rats, both replaced by irregularly arranged collagenous fibers. Similar findings were reported in rabbits by Traish et al (1999), who found a decrease in the trabecular smooth muscle, an alteration that was prevented with hormonal replacement. However, these experiments were performed using short-term hormone deprivation. To our knowledge, no studies have confirmed if the erectile structures are irreversibly altered after hormonal deprivation or if replacement therapy can overturn these penile changes.

Our objective was to evaluate, through quantitative methods, the structural alterations in the corpora cavernosa of rats submitted to surgical castration as

well as the role of late hormone replacement in reversing the possible structural alterations.

Materials and Methods

Animals and Experimental Design

The Animal Care and Use Committee of the State University of Rio de Janeiro approved the handling of the animals, and the study design was approved by the local ethical committee for the care and use of laboratory animals.

We used 25 male Sprague-Dawley rats of approximately 12 weeks of age from the Urogenital Research Unit biotery. The animals were assigned into 5 groups composed of 5 animals each and treated as follows: ORCHIEC-1 = group that underwent orchiectomy and were sacrificed after 1 month; C-1 = control group sacrificed after 1 month; ORCHIEC-2 = group that underwent orchiectomy and were sacrificed after 2 months; C-2 = control group sacrificed after 2 months; T = group that underwent orchiectomy, underwent testosterone replacement after 1 month, and were sacrificed after 1 month of hormonal replacement.

The first 2 groups (ORCHIEC-1 and C-1) were mainly used to determine if after 1 month of orchiectomy the erectile structures were altered and testosterone levels were low, simulating a nontreated ADAM patient.

Surgical Procedure

Under anesthesia, the scrotum was incised at the midline and the testes were exposed. In the orchiectomy groups, the testes were removed after en bloc ligature of the spermatic cord, and in the control groups, the testes were exposed, manipulated, and reinserted into the scrotum. Group T underwent testosterone replacement after 30 days of orchiectomy with a subcutaneous single dose of testosterone undecanoate (100 mg/kg; Callies et al, 2003; Gallo et al, 2012).

Testosterone Serum Levels

At the end of each experiment, the animals were anesthetized and blood was collected for testosterone serum concentrations analysis by radioimmunoassay (ICN Pharmaceuticals Inc, Costa Mesa, California).

Histological Analysis

The animals were killed by cervical fracture and the penis was harvested en bloc. The penile midshaft was fixed in 10% buffer formalin (pH 7.2) and routinely processed for paraffin embedding. From each penis, we obtained 6 transversal sections of 5-μm thickness. All samples were initially stained with hematoxylin-eosin and analyzed for tissue integrity.

Picrosirius red stain was used to analyze and quantify collagen and sinusoidal space. The picrosirius method enables sulfonic radicals in the dye to react with the amine groups of lysine, which is one of the major amino acid components of collagen, thus intensifying its birefringence. When observed under polarization, the collagen presents birefringence with varying colors, as tonalities of red, yellow, and green over a dark background.

An anti–α-actin antibody (Zymed Laboratories, Carlsbad, California) with Histostain-Plus Kit secondary antibody (Invitrogen Immunodetection, Camarillo, California) was used for characterization and quantification of smooth muscle fibers.

We had 5 animals per group in the 5 groups. In each animal, we obtained 6 images (sections). We calculated the mean of the 6 images of the same animal to obtain the animal's value. With the 5 values of 1 group, we calculated the mean of the group to compare with other groups. Thus, we had a total of 30 images per group and 150 images total.

Image Acquisition

The picrosirius red images observed under polarization were photographed with an Olympus digital camera DP70 coupled to an Olympus BX51 microscope (Tokyo, Japan) with a ×40 magnification and a resolution of 4080 × 3072 (300 pixels/in). Some images of the sections were greater than the microscope field and therefore were obtained in 2 stages and then digitally united. At the end of the image processing, they were placed on a background of the same size (4080 × 4000), and in this way, all sections remained at the same magnification and resolution, with the proportions maintained (Figure 1).

Digital Dissection

Collagen—After image standardization, we proceeded with a digital dissection of the tunica albuginea, which was completely deleted, leaving only the erectile tissue (Figure 2a). The images were transferred to Image-Pro Plus software (Media Cybernetics, Bethesda, Maryland). Using the same color-selecting tool, all dark colors in the image were selected and only the stained tissue remained; thus, everything that was not collagen was removed (Figure 2b). The next step was the confection of a mask, where the collagen became black and the rest became white (Figure 2c). By dividing the total number of black pixels by the total pixels in the image we estimated the percentage of black in the image, thus quantifying the collagen.

Sinusoidal Space—By using the images obtained previously for collagen quantification, we performed another digital treatment on which the region outside of the erectile tissue was placed completely in white (Figure 2d). In the image obtained a mask was applied, where the sinusoidal space (the black space in the image) was selected (Figure 2e). The last step was to substitute the selected (green) area with a white color and the remaining components in black (Figure 2f). By dividing the total number of white pixels by the total pixels of the image we estimated the sinusoidal space.

Smooth Muscle—After digital acquisition of the histological sections immunolabeled for smooth muscle (Figure 3a), based on the same resolutions and using the same software, we selected the smooth muscle in the corpus cavernosum that was evidenced in brown. After selection, we created a mask based on the labeled smooth muscle, where white was the muscle and black was the other structures (Figure 3b). This image was used to estimate the percentage of white in the image, by dividing the total number of white pixels by the total of pixels in the image, thus quantifying the smooth muscle.

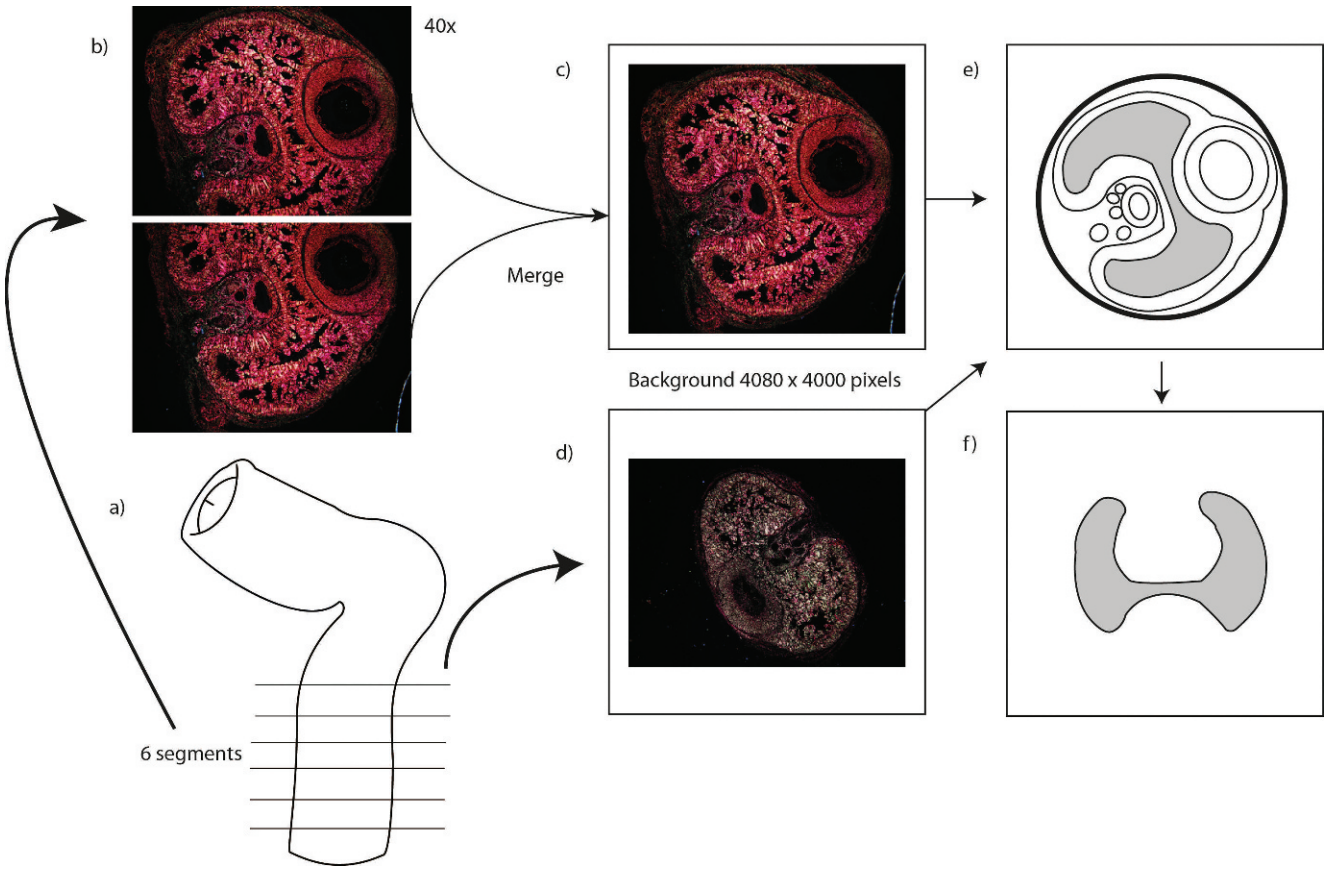

Figure 1. Anatomical and digital dissection. **(a)** Image shows the animal penis and the lines exemplify the sections acquired. **(b)** Acquisition of an image that was larger than the microscopic field (4000 × 3072 pixels). In these cases, 2 images were captured separately and merged into 1 image with 4080 × 4000 pixels **(c)**. **(d)** When the entire penis fit into the microscopic field, only 1 image was captured and adjusted to 4080 × 4000 pixels, raising the background. **(e)** All the images had the same size at the end. **(f)** The final image after the digital dissection, without any tissues around the corpus cavernosum. Color figure available online at www.androlgyjournal.org.

Corpus Cavernosum Area—By using the images obtained previously for collagen quantification, we performed another digital treatment in which the region outside of the erectile tissue was placed completely in white color. The second step was to delete the erectile tissue and substitute a black color. At the end we had an image in black and white, where the black represented the erectile tissue. Dividing the number of black pixels by the total number of pixels in the image, we obtained the percentage of the erectile tissue on the image.

We termed *density* of a structural component as the division of the result of the component analyzed by the total area of the corpora cavernosa section. In this work, for example, the collagen density corresponds to the division of the collagen area by the corpora cavernosa area in a given section.

We termed *absolute value* the percentage of a given analyzed component in relation to the total image delimited by the square.

Statistical Analysis

For the evaluation of the 2 groups sacrificed in the first month, we used Student's *t* test for comparison between the means. For the 3 groups sacrificed after 2 months, we used analysis of variance with a Bonferroni posttest.

Results

Testosterone Serum Levels

The determination of serum testosterone levels showed that orchiectomy was effective, with undetected testosterone levels in castrated rats in both the ORCHIEC-1 AND ORCHIEC-2 groups. Hormonal replacement in the T group was effective, presenting serum testosterone levels similar to those of controls after 1 month of replacement with testosterone (Figure 4).

Area of Corpora Cavernosa

The orchiectomy reduced the area of corpora cavernosa by 21% in the animals of group ORCHIEC-1 in comparison with group C-1, which shows that we may consider 1 month as a late stage for hormonal replacement therapy in our model.

Also, the area of corpora cavernosa was reduced by 22% in group ORCHIEC-2 when compared with C-1 animals. Despite these major alterations, the T group had no difference from C-2.

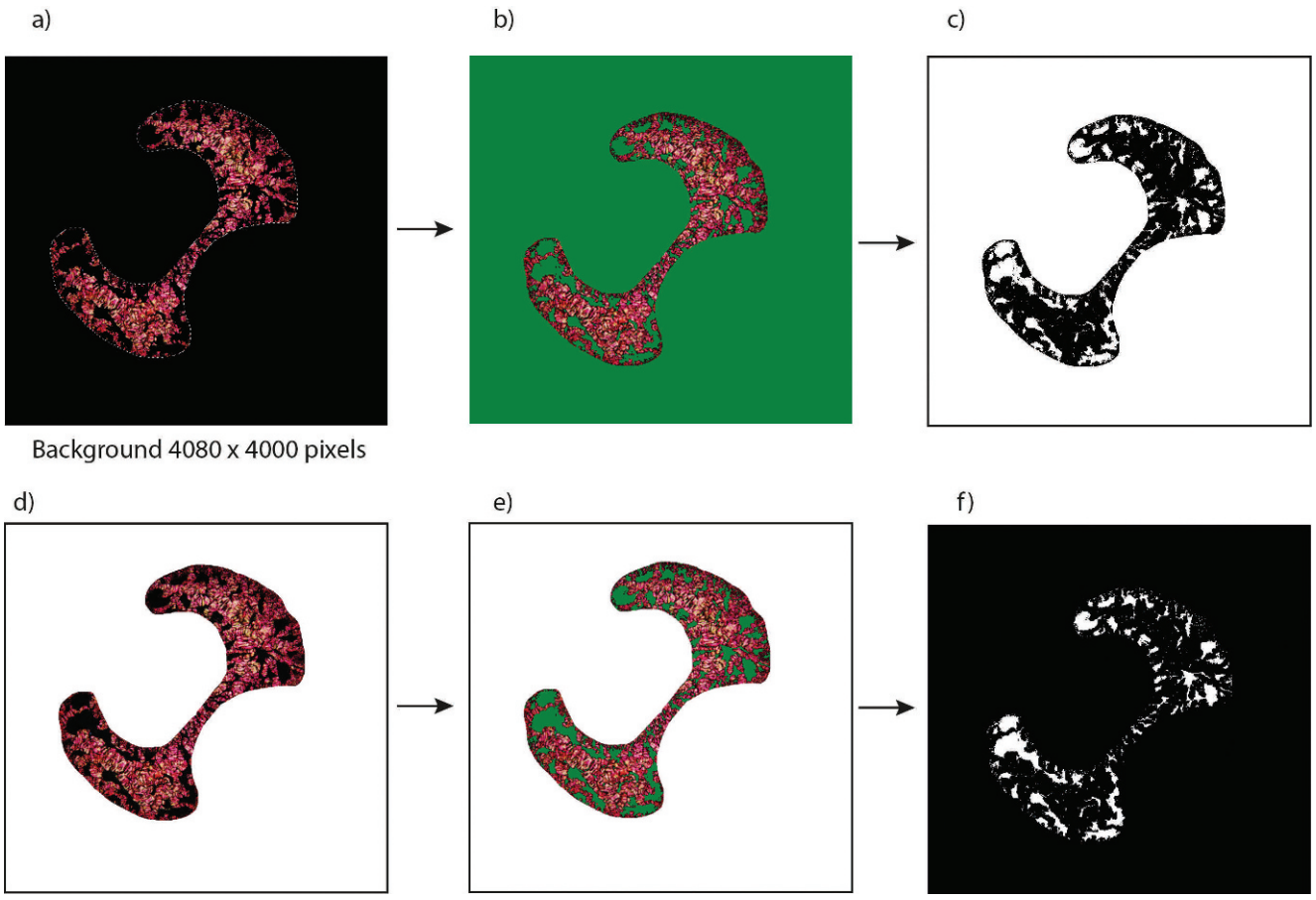

Figure 2. Digital preparation for collagen and sinusoidal space analysis. **(a)** Image after digital dissection; all external tissue including the albuginea was removed. **(b)** A mask (green) was applied in all structures, except the collagen trabeculae. **(c)** Final black and white image, where black portions represent the collagen content. **(d)** Image after digital dissection. All external tissue was removed and replaced with white. **(e)** A mask (green) was applied only in the black-colored areas, which represent the sinusoidal space. **(f)** Final black-and-white image, where the white areas represent the sinusoidal space. Color figure available online at www.andrologyjournal.org.

These findings are shown in Figure 5 and Tables 1 and 2.

Sinusoidal Space

Tissue analysis showed a significant decrease of 35% in the absolute values of sinusoidal space in corpora cavernosa after 2 months in the castrated groups when compared with controls. Again, no significant difference was observed when comparing C-2 with the hormone-replaced group (T; Tables 1 and 2).

When analyzing the sinusoidal space in relation to the corpora cavernosa area (relative values), no statistical difference was found among the groups (Tables 3 and 4).

Cavernosal Smooth Muscle

Tissue analysis showed a significant decrease (45%) in the value of cavernosal smooth muscle after 2 months in the castrated group when compared with control animals. In the testosterone-replaced group, no difference was found in relation to C-2. Also, no difference was noted comparing relative values of this structure.

Cavernosal Collagen

The absolute values of collagen content in corpora cavernosa was not statistically different between castrated and control groups after 1 month from hormonal deprivation. After 2 months, group T had an increased amount of collagen when compared with group C-2, as a consequence of the higher area of corpora cavernosa.

Discussion

The increasing aging population, which needs a satisfactory quality of life, has stimulated the search for solutions that would soften the alterations secondary to the aging process. Among the most important alter-

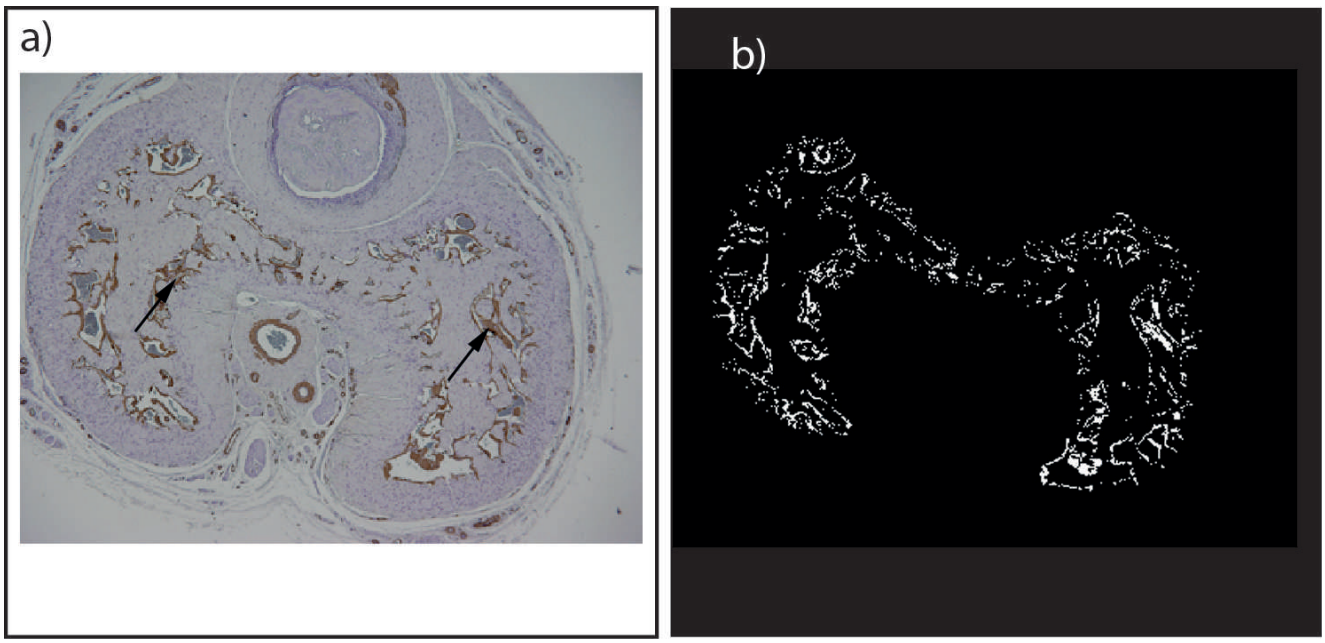

Figure 3. Digital preparation for smooth muscle analysis. **(a)** Penile transverse section immunolabeled for smooth muscle, as evidenced in brown (×40). **(b)** Black and white image created from the previous selection, where the white represents the smooth muscle. Color figure available online at www.andrologyjournal.org.

ations that affect the aging male, androgen deficiency with erectile dysfunction remains prominent.

The maintenance of an active sexual life depends on penile erectile structures, which are affected by low levels of serum testosterone (Alcorn et al, 1999; Shen et al, 2003; Gooren and Saad, 2006; de Souza et al, 2012). The analysis of testosterone levels permitted us to test the hypothesis that variations in serum testosterone will determine alterations in the rat corpora cavernosa, as was found in the present work. The remaining question was to determine if the penile structures are definitely affected by testosterone deficiency. Therefore, in the present work, we attempted to simulate, in an animal model, the more frequent clinical situation, that is, testosterone deficiency for a long period of time, because patients usually are diagnosed at a late stage. For this purpose we analyzed the structural alterations of castration (1 and 2 months), as well as the effects of late testosterone replacement, in the rat corpora cavernosa.

Commonly, the administration of a single dose of testosterone undecanoate maintains physiologic hormonal levels during 3 months in humans. However, in the rat model, a single dose is enough to maintain normal serum testosterone levels for 1 month (Callies et al, 2003). This was confirmed by our testosterone analyses, which were performed after 1 month of hormone administration and showed no difference from controls.

Several authors have reported the effects of castration on penile structures and corpora cavernosa physiology, as well as the effects of hormonal replacement on the penis of castrated animals (Mills et al, 1992; Alcorn et al, 1999; Dai et al, 1999; Traish et al, 1999). Traish et al (1999) analyzed the structures of corpora cavernosa in rabbits sacrificed 2 weeks after castration and observed a decrease in smooth muscle cells when compared with controls, whereas the group

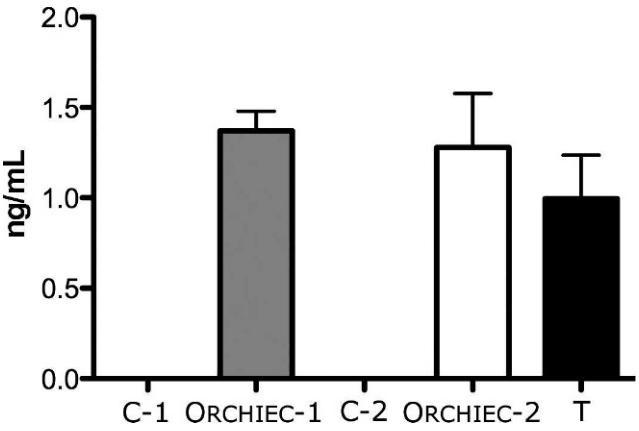

Figure 4. Serum testosterone levels (ng/mL) in the group that underwent orchiectomy and were sacrificed after 1 and 2 months (ORCHIEC-1 and ORCHIEC-2), in the control group sacrificed after 1 and 2 months (C-1 and C-2), and in the group that underwent orchiectomy and after 1 month underwent testosterone replacement with a subcutaneous single dose of testosterone undecanoate at 100 mg/kg (T).

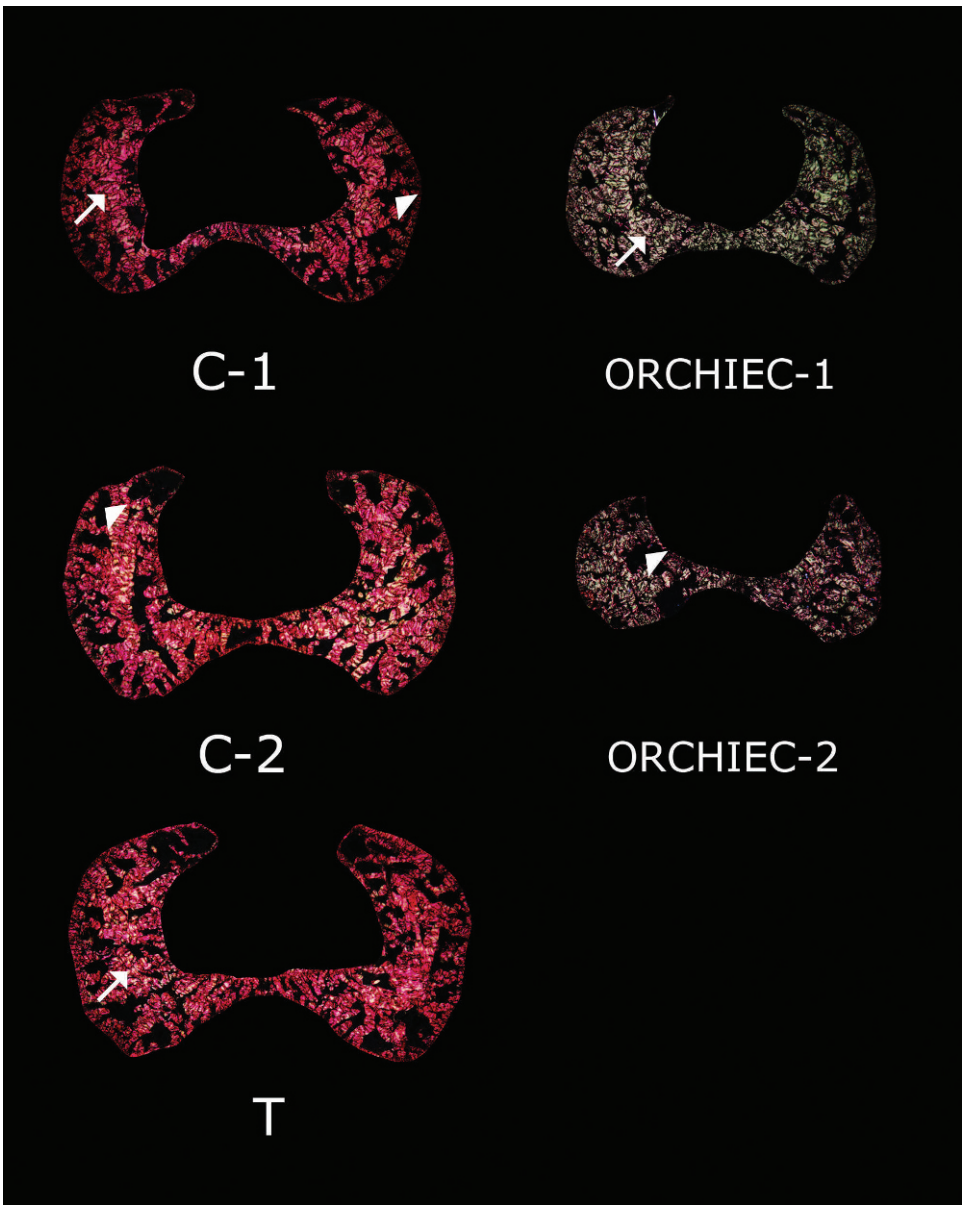

Figure 5. Corpora cavernosa after digital dissection in the different groups. Collagen structures are pointed with arrows and sinusoidal spaces are pointed with arrowheads. Picrosirius red under polarization (×40). ORCHIEC-1 = group that underwent orchiectomy and were sacrificed after 1 month; C-1 = control group sacrificed after 1 month; ORCHIEC-2 = group that underwent orchiectomy and were sacrificed after 2 months; C-2 = control group sacrificed after 2 months; T = group that underwent orchiectomy, and underwent testosterone replacement with a subcutaneous single dose of testosterone undecanoate at 100 mg/kg after 1 month, and was sacrificed after 1 month of hormonal replacement. Color figure available online at www.andrologyjournal.org.

Table 1. *Absolute values in relation to the whole image of the analyzed elements in the rat corpora cavernosa (mean ± SD; n = 5 animals per group)*

	Collagen	Smooth Muscle	Sinusoidal Space	Area of Corpora Cavernosa
ORCHIEC-1	8.5 ± 1.5	1.177 ± 2.03	3.677 ± 4.56	13.37 ± 2.1[a]
C-1	10.0 ± 3.08	1.446 ± 1.60	5.164 ± 3.26	16.81 ± 1.5[a]
P value (*t* test)	.0642	.1505	.0524	.0178[a]

Abbreviations: C-1, control group sacrificed after 1 month; ORCHIEC-1, group that underwent orchiectomy and were sacrificed after 1 month.

[a] Statistically significant difference between groups.

Table 2. *Absolute values in relation to the whole image of the analyzed elements in the rat corpora cavernosa (mean ± SD; n = 5 animals per group)*

	Collagen	Smooth Muscle	Sinusoidal Space	Area of Corpora Cavernosa
ORCHIEC-2	9.01 ± 4.83[a]	0.83 ± 1.43[a,b]	3.6 ± 4.86[a,b]	13.89 ± 2.12[a,b]
C-2	10.69 ± 6.98	1.51 ± 2.54[a]	5.58 ± 4.75[a]	17.75 ± 1.75[a]
T	12.41 ± 0.1[a]	1.44 ± 1.61[b]	6.03 ± 1.74[b]	19.77 ± 0.76[b]
P value (ANOVA)	.0087	.0044	.0004	.0004
P value (Bonferroni posttest)				
ORCHIEC-2 vs C2		<.01[a]	<.01[a]	<.01[a]
ORCHIEC-2 vs T	<.05	<.05[b]	<.001[b]	<.001[b]

Abbreviations: ANOVA, analysis of variance; C-2, control group sacrificed after 2 months; ORCHIEC-2, group that underwent orchiectomy and were sacrificed after 2 months; T, group that underwent orchiectomy, underwent testosterone replacement after 1 month with a subcutaneous single dose of testosterone undecanoate at 100 mg/kg, and were sacrificed after 1 month of hormonal replacement.

[a,b] Statistically significant difference between groups.

Table 3. *Density in percentage of the analyzed elements in the rat corpora cavernosa (mean ± SD; n = 5 animals per group)*

	Collagen	Smooth Muscle	Sinusoidal Space
ORCHIEC-1	63.67 ± 1.5[a]	8.75 ± 2.0	27 ± 4.5
C-1	59.59 ± 3.079[a]	8.91 ± 1.6	30.76 ± 3.26
P value (*t* test)	.0288[a]	.9078	.1714

Abbreviations: C-1, control group sacrificed after 1 month; ORCHIEC-1, group that underwent orchiectomy and were sacrificed after 1 month.

[a] Statistically significant difference between groups.

Table 4. *Density in percentage of the analyzed elements in the rat corpora cavernosa (mean ± SD; n = 5 animals per group)*

	Collagen	Smooth Muscle	Sinusoidal Space
ORCHIEC-2	65.41 ± 4.8	6.2 ± 1.4	26.75 ± 4.8
C-2	59.91 ± 6.97	8.59 ± 2.5	31.58 ± 4.7
T	62.72 ± 0.99	7.22 ± 1.6	30.53 ± 1.7
P value (ANOVA)	.2508	.2098	.1823

Abbreviations: ANOVA, analysis of variance; C-2, control group sacrificed after 2 months; ORCHIEC-2, group that underwent orchiectomy and were sacrificed after 2 months; T, group that underwent orchiectomy, underwent testosterone replacement after 1 month with a subcutaneous single dose of testosterone undecanoate at 100 mg/kg, and were sacrificed after 1 month of hormonal replacement.

that underwent testosterone replacement 1 week after castration presented values of smooth muscle similar to those of controls. These authors also found an increase in collagen content in the castrated group when compared with controls, whereas the group that underwent hormonal replacement presented collagen amounts similar to those of controls.

Shen et al (2003) performed a quantitative analysis by using scanning electron microscopy and found an increase in collagen content and a decrease in smooth muscle and sinusoids in rats that underwent orchiectomy and were sacrificed after 1 month. The supposed divergence between previous papers and our results could be because the authors did not take into account the absolute value, considering only the density, as final values are in fact influenced by the decrease in the total area of the penis. When we used the relative density, in fact, we found a collagen increase after 1 month of castration. On the other side, when we analyzed the absolute values we found a decrease of collagen content, which was nevertheless not statistically significant.

The increase in collagen density in the orchiectomized group (ORCHIEC-1) when compared with controls (C-1) is relative. The final value for density is based on the division between the collagen area and the transverse section area of the corpora cavernosa. Because both are reduced after orchiectomy, and the transverse section area presented greater decrease than the collagen decrease, the final value for density is apparently increased.

The evaluation of collagen density in the group of rats sacrificed after 2 months of orchiectomy showed that there was no significant difference when compared with controls, because the collagen variation was accompanied by the variation in the transverse section area of corpora cavernosa, thus maintaining similar proportions

(collagen/area) between the groups (Table 4). The absolute values showed that the orchiectomized rats who underwent testosterone replacement (group T) presented a significant increase in collagen amount when compared with the orchiectomy group without hormonal replacement (ORCHIEC-2).

Smooth muscle presented a decrease in the absolute values only in the second month after orchiectomy (Table 1). In this group, analyzed 2 months after orchiectomy, the difference was considered significant, showing a decrease in smooth muscle when compared with controls. The group that underwent orchiectomy and hormonal replacement with testosterone showed absolute values similar to controls, demonstrating that hormonal replacement was effective in reversing the process that leads to smooth muscle decrease (Table 2).

The density of sinusoidal space did not undergo significant alterations after 1 or 2 months of orchiectomy (Tables 3 and 4). Similarly to what occurred with collagen, the transverse section area of the corpora cavernosa follows the sinusoidal space variation. Nevertheless, the absolute values of sinusoidal space in the group sacrificed 2 months after orchiectomy showed significant differences when compared with control and hormonal replacement groups. The group that underwent orchiectomy (ORCHIEC-2) presented a decrease in the sinusoidal space area when compared with controls (C2), whereas the hormonal replacement group (T) showed values similar to those of controls (Table 2). In this case, the hormonal replacement was effective in restoring the area of sinusoidal spaces to normal values.

The sinusoidal space analysis suggests 2 hypotheses that would justify the erectile dysfunction in patients with low testosterone levels. In the first hypothesis, we would suppose that the decrease in the sinusoidal space is a consequence of a decrease in intracavernous pressure, secondary to a venous leak that accompanies testosterone deficiency (Dai et al, 1999). The sinusoidal filling would be incomplete because of low wall distension consequent to a low intracavernous blood pressure. In the second hypothesis, the reduction in the sinusoidal space would be a primary event, consequent to a low compliance of sinusoidal walls. In both hypotheses, the sinusoids were not able to compress the deep circumflex veins against the tunica albuginea, resulting in venous leak and low intracavernous pressure (Dai et al, 1999).

In the group that underwent orchiectomy, we found a reduction in smooth muscle and sinusoidal space. This determined a decrease in the transverse section area of the corpora cavernosa (Figure 5). The decrease of the transverse section area of the corpora cavernosa was relatively low in the first month and was more accentuated in the second month; nevertheless, the proportion of the different elements analyzed was maintained in both groups that underwent orchiectomy. Following hormonal replacement, the alterations resulting from orchiectomy were reversed, and the structures affected presented values similar to controls. The method used for this research allowed demonstrating that absolute values are reliable to quantify the structural alterations of corpora cavernosa reducing the bias caused by the tissue shrinkage.

Hormonal replacement therapy, even when instituted at a late stage, is effective in reversing the morphological changes of corpora cavernosa produced by castration in rats.

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