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Immunohistochemical analysis of smooth muscle cells and volumetric density of the elastic system fibers of wild boar (*Sus scrofa*) penis

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Abstract

The purpose of the present study was to verify the smooth muscle cell distribution and elastic system fibers volumetric density (V_v) in the corpus spongiosum and corpus cavernosum of the wild boar penis. Adult wild boars (n = 13) were used. The penile mid shaft fragments were fixed with 4% phosphate buffered formalin solution and/or Bouin's liquid during 24–48 h, and processed using standard histological techniques. The sections were stained with Weigert's Resorcin-Fucsin with previous oxidation. The elastic system fibers V_v was determined in 25 random fields of each fragment using M42 test system. For immunohistochemical analysis, monoclonal anti-α actin smooth muscle was used. The histochemical methods detected elastic system fibers in both corpus spongiosum and corpus cavernosum of all animals. The elastic fibers V_v average was 36.6% ± 0.9 for corpus spongiosum and 11.7% ± 0.5 for corpus cavernosum. Through immunocitochemistry, a small quantity of smooth muscle cells was visualized in intimate relationship with blood vessels wall. The great amount

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of elastic fibers and the smooth muscle cell distribution beneath the endothelium suggest that these fibers may have an important role in penile erection process in the penis of wild boars.

Keywords: Penis; Wild boar; Elastic fibers; Stereology; Immunohistochemistry; Smooth muscle cells

1. Introduction

The general understanding of the morphological changes and physiology of penile erection is obtained through several studies considering different animal models (Pinheiro et al., 2000; Yesilli et al., 2001; Hellstrom, 2001; Burnett, 2001) such as rats (Pinheiro et al., 2000; Giuliano, 2000; Lee et al., 2002), domestic animals, primates (Paick et al., 1992; Hellstrom et al., 1994; Hellstrom, 2001; Bischoff, 2001), and rabbits (Qiu et al., 2000; Yesilli et al., 2001).

Morphological and quantitative data concerning wild boar are still scarce, and there is need for more information, mainly because these animals are used as biological models and commercially as potential protein sources (Swindle et al., 1988).

The wild boar (controlled by Environment Brazilian Institute) is a representative ancestor of domestic swine, being considered the same species *Sus scrofa* (Nickel et al., 1979; Nowak and Paradiso, 1983) because mating of domestic swine female with a wild boar produces fertile descendents. In China, since 4900 B.C. wild boars were maintained as domestic animals and after succeeding generations, domestication of swine occurred (Nickel et al., 1979).

Although the gross anatomy of wild and domestic swine is different, histological structural elements are similar in mammals, but with special characteristics for each species (Banks, 1992).

Elastic and collagen fibers are important penile constituents and maintain the penile structure during erection, and allow adequate resistance during the return to the non-erect state (Hsu et al., 1994; Sattar et al., 1994; Da Silva and Sampaio, 2002; Bastos et al., 2004).

The scientific literature, in general, focuses on the general morphology of the wild boar penis (Nickel et al., 1979). There is, however, no information about qualitative or quantitative connective tissue elements. The purpose of the present study is to gain a greater understanding of the wild board penis using immunohistochemical methods and morphometrical analysis of the elastic fibers in the corpus spongiosum and corpus cavernosum.

2. Material and methods

The ethical committee of the State University of Rio de Janeiro approved the research protocol. Adult wild boars (n = 13, Javali in Brazil) were obtained from a commercial farm (Profauna Limited), and slaughtered when weighing from 40 to 50 kg. After sacrificed, the penis was removed and immediately fixed in 4% phosphate buffered formalin solution and/or Bouin's liquid for 24–48 h. Afterwards, penile mid shaft segments were processed according to the standard histological techniques for paraffin embedding.

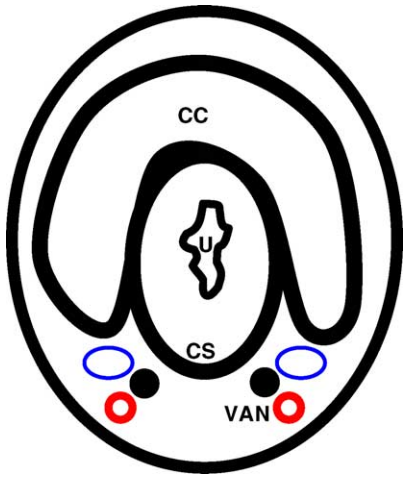

Fig. 1. Anatomical schematic drawing (transversal section) of the wild boar penis U = urethra, CC = corpus cavernosum, CS = corpus spongiosum, VAN = vein, artery, nerve.

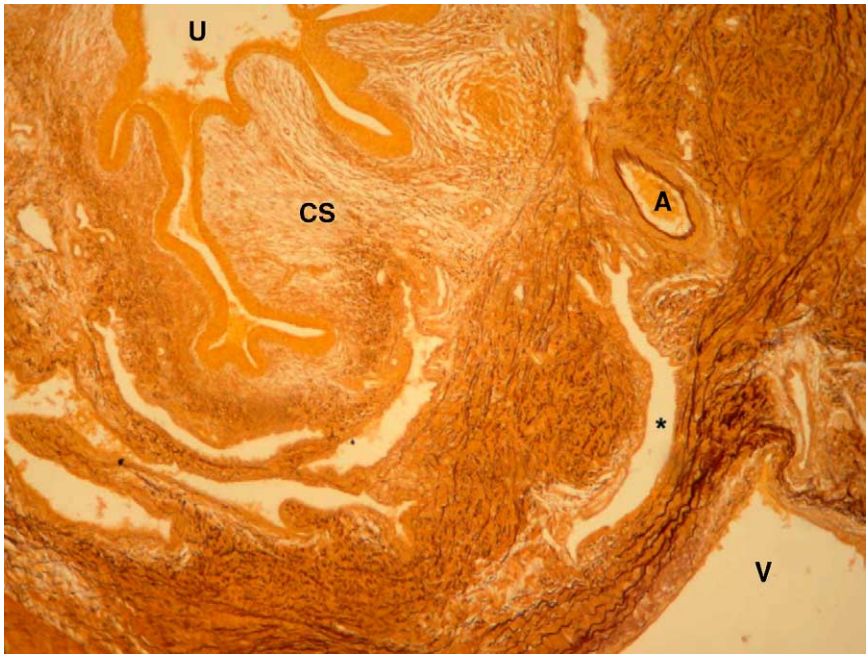

Fig. 2. Light micrograph of the corpus spongiosum (CS) showing the ventral urethra (U). Elastic fibers are abundant in stroma. Artery (A); vein (V); cavernous sinus (*). Weigert's Resorcin-fucsin, ×100.

2.1. Quantitative analysis

From each penis, five different sections were selected from five fragments. Then, five random fields were evaluated from each section. There were, therefore, 25 test areas from each penis. For the stereological analysis, 5 μm sections were stained with Weigert's Resorcin-Fucsin stain to detect the elastic system fibers (Bradbury and Era, 1996). The data were expressed as volumetric densities (%). The analyzed fields were digitized with ×200 final magnification using a video camera coupled to a light microscope. The selected histological areas were then quantified using M42 test-grid system on the digitized fields on a color monitor screen. From stereological principles in isotropic tissue, the distribution area of a specific structure, as determined on a two-dimensional section of a structure, is proportional to the volume distribution of the structure (Pinheiro et al., 2000; Chagas et al., 2002; Mandarim-de-Lacerda, 2003). The volume density of the histological components was calculated by $V_v = P_p/P_t$, where V_v is the volume density, p is the tissue component under consideration (elastic fibers), P_p is the number of test points associated with p, and P_t is the number of points of the test system. Line and point probes are obtained in a field by superimposing a grid consisting of an arrangement of lines and points (Weibel et al., 1966; Cruz-Orive and Weibel, 1990; Mandarim-de-Lacerda, 2003). The M42 multipurpose test-system was used where only well-preserved structures not crossing the test-system forbidden line were considered. The short line length (d) was used to calibrate the test-system,

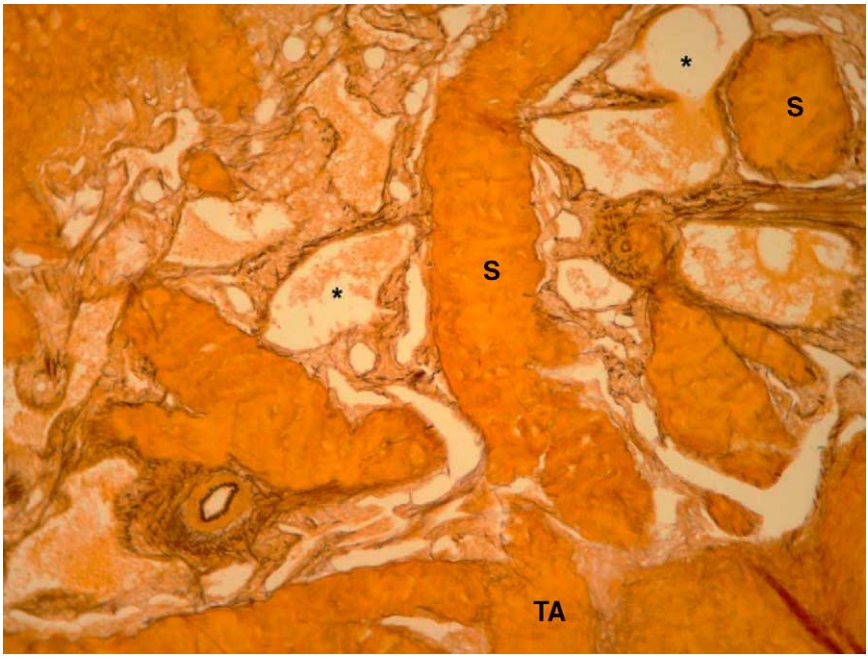

Fig. 3. Cross-section of the wild boar penis. Note the tunica albuginea (TA), intracavernosal septum (S) and cavernous sinus (*). Weigert's Resorcin-Fucsin stain, ×200.

the line length LT is 21*d*, the test-area AT is 36.36*d*2, and it has 42 test-points (PP). The stereological methods were described in detail elsewhere (Weibel et al., 1966; Gundersen et al., 1988; Cruz-Orive and Weibel, 1990; Mandarim-de-Lacerda, 2003).

2.2. Statistical analysis

The data were analyzed in the software Graphpad Instat (Graphpad) to verify the Gaussian distribution. To compare the quantitative data of corpus spongiosum and corpus cavernosum, the Student's *t*-test was used ($p < 0.05$ was considered significant).

2.3. Immunohistochemistry

The standard avidin biotin conjugate (ABC) immunostaining procedures, with appropriate positive and negative controls, were used to detect smooth muscle cells and elastin. Briefly, sections from formalin-fixed and paraffin-embedded samples were de-waxed, hydrated in a graded series of ethanol solutions of decreasing concentrations until the solution was all water and then washed in phosphate buffered saline (PBS) for 5 min. The sections were treated for 30 min with 3% hydrogen peroxide solution in methanol to block endogenous peroxidase activity. The sections were washed in three drops of PBS, incubated in a humid chamber at 37 °C for 30 min with 1% goat serum and then incubated at

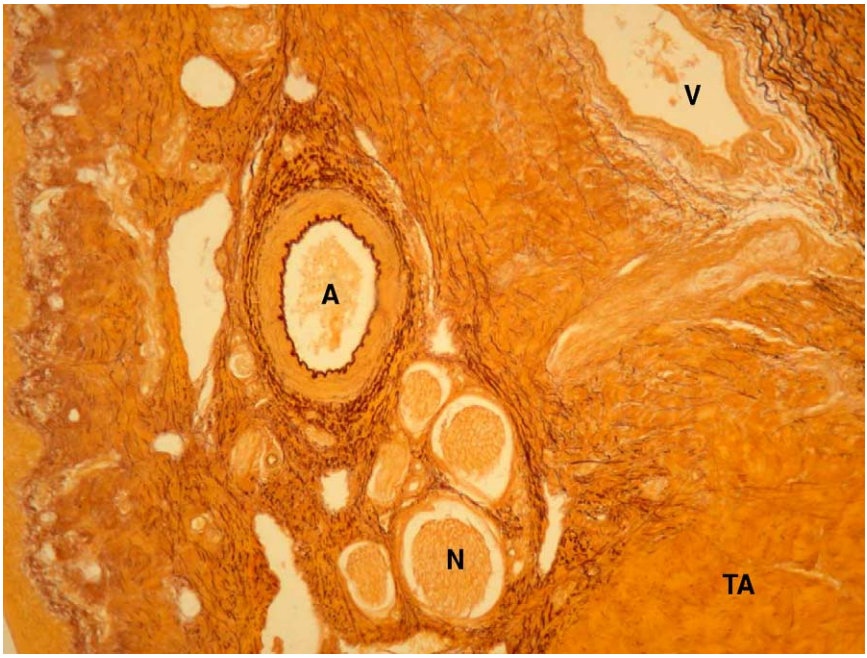

Fig. 4. Elastic fibers surrounding nerves and vessels underneath and laterally to spongy body. Tunica albuginea (TA); vein (V); nerve (N); artery (A). Weigert's Resorcin-Fucsin stain, ×200.

4 °C in a humid chamber with monoclonal anti-smooth muscle α actin (Dako) diluted to 1:600 for 12–14 h. Subsequently, the sections were washed in three drops PBS and incubated at room temperature in a humid chamber with the biotinylated secondary antibody En Vision (Dako) (1:600) for 30 min, washed in three drops PBS and incubated at room temperature in a chamber with ABC complex (extravidin 1:100) for 30 min. The sections were washed in three drops PBS and revealed by treating with a 3′,3-diaminobenzidine tetrahydrochloride solution containing 0.1% hydrogen peroxide (v/v), washed in distilled water, dehydrated in a increasing concentration series of ethanol solutions and mounted with rapid mounting media for microscopy. The negative controls were processed by replacing the anti-smooth muscle α actin antibody with PBS and no indication of staining was observed.

3. Results

The wild boar has a fibroelastic penis with a corkscrew shape that contains two erectile structures: the supero-lateral corpus cavernosum and the ventral corpus spongiosum that surrounds the penile urethra. Both structures were covered by a dense connective tissue capsule—the tunica albuginea (Figs. 1 and 2). The tunica albuginea projects intracavernosal pillars or septa, mainly in the corpus cavernosum (Fig. 3).

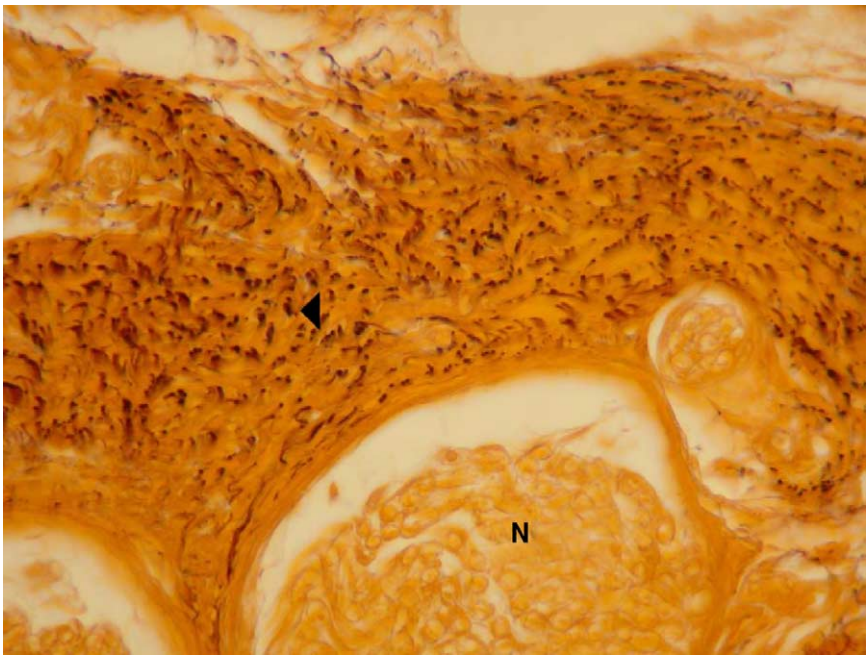

Fig. 5. High magnification of a nerve showing the great density of elastic fibers in longitudinal pattern. Nerve (N), elastic fibers (arrow head). Weigert's Resorcin-Fucsin stain, ×400.

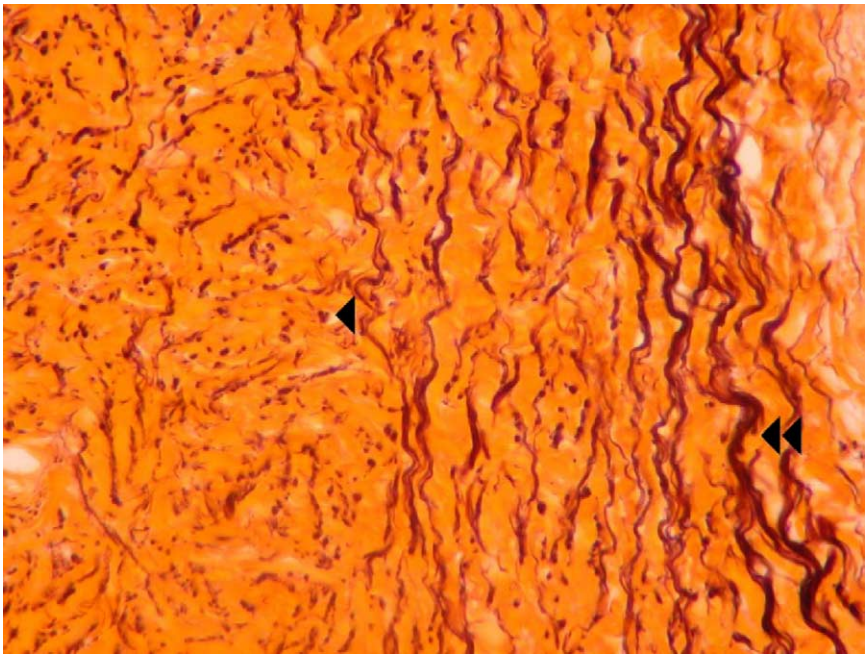

Fig. 6. Elastic fiber system at the corpus spongiosum outer periphery. Elastic fibers are interspersed among collagen bundles, forming inner and outer layers when they are longitudinally (arrow head) and transversely (double arrow head) sectioned. Weigert's Resorcin-Fucsin stain, ×400.

An irregular elastic fiber network was distributed throughout the penis. Underneath and lateral to the corpus spongiosum neurovascular bundles were bilaterally observed (Fig. 1 and Fig. 4). Connective tissue elements, mainly elastic system fibers, were abundant (Fig. 5 and Fig. 6). In the corpus spongiosum, the amount of elastic fiber was greater (36.6%) as compared with the corpus cavernosum (11.7%). These fibers often had a tortuous profile and surrounded sinusoids in the corpus spongiosum (Figs. 5 and 6, Fig. 7). The quantitative data on the elastic system fibers are summarized in the Table 1.

The immunohistochemistry revealed smooth muscle cells only in blood vessel walls (Fig. 8) and beneath the endothelium from communicating blood spaces or sinusoids found in the corpus spongiosum and cavernosum (Fig. 9). There were no single smooth muscle cells in the connective tissue.

Table 1
Volumetric density (penis distribution) in corpus spongiosum and corpus cavernosum

Parameter	Corpus spongiosum	Corpus cavernosum
Mean	36.6 (%)	11.7(%)
S.D. (±)	0.9	0.5
Minimum	35.2	11.1
Maximum	38.1	13.2

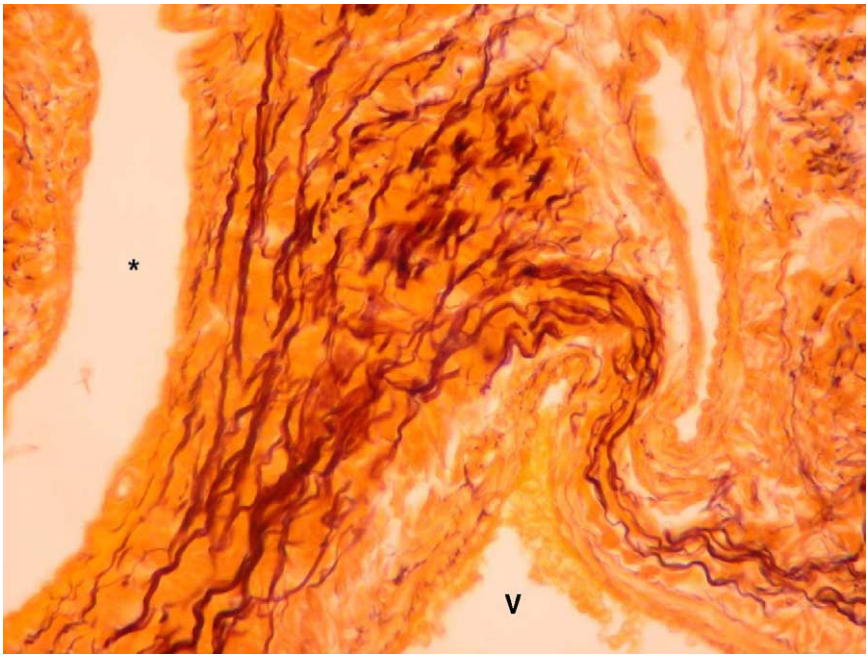

Fig. 7. High magnification of elastic fibers lamellae interspersed through the cavernous sinus (*) and vein (V). Weigert's Resorcin-Fucsin stain, ×400.

4. Discussion

Weigert's Resorcin-Fuchsin staining was used after oxidation to detect the oxitalan, elaunin and elastic fibers (Fullmer, 1958; Cotta-Pereira et al., 1976; Pinheiro et al., 2000) so as to be able to quantify these elastic system components. The stereological method has been used to quantify and especially to determine the amount (percentage) of the elastic fiber system in rat penile (Pinheiro et al., 2000) and other extracellular matrix elements of the human prostate (Chagas et al., 2002). The stereological method has been recommended by several authors (Cruz-Orive and Weibel, 1990; Pinheiro et al., 2000; Chagas et al., 2002; Bastos et al., 2004; Mandarim-de-Lacerda, 2003) to avoid the bias that frequently occurs during automatic or semiautomatic computerized image analyses that could overestimate or underestimate the analyzed structures (Sattar et al., 1994).

Through the M42 grid test system, the elastic fiber systems were abundant in the corpus spongiosum of the wild boar, demonstrating a greater volumetric density in contrast to the corpus cavernosum. These data revealed a typical fibroelastic penis.

Normal tissue development and several tissue or organ support structures depend on the intercellular and cell–matrix interactions (Zhang et al., 1995; Kierzenbaum, 2002). According to previous data (Cotta-Pereira et al., 1976; Kreis and Vale, 1993), extracellular matrix elastic system fibers are characterized by great extension qualities and elastic recoil. Tissues that are constantly submitted to tensile strength are rich in elastic fibers (Kreis

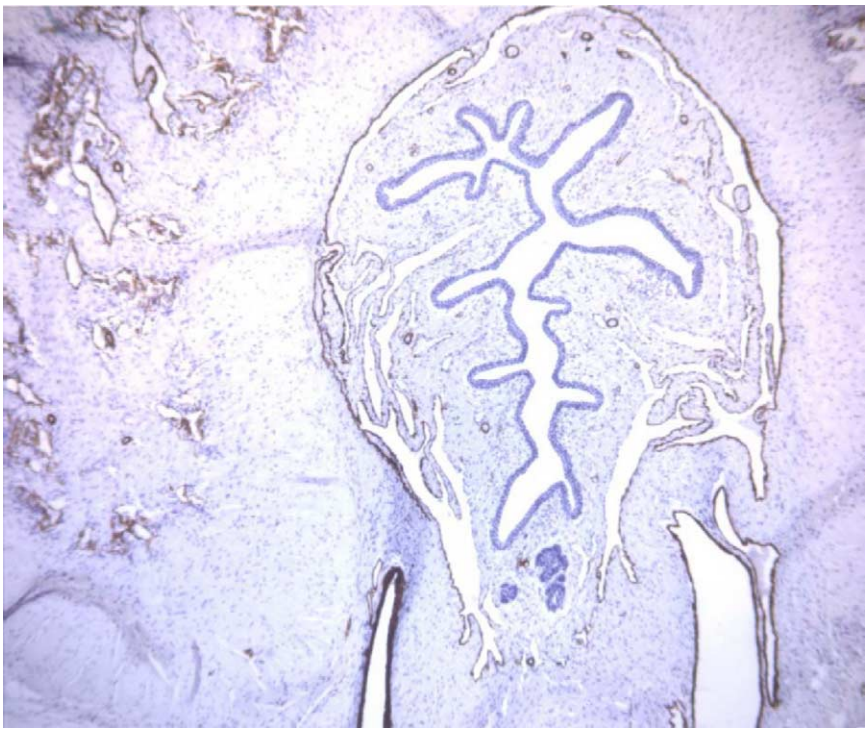

Fig. 8. Light micrograph of smooth muscle cells around the vascular sinus in corpus spongiousum and vascular endothelium. ABC immunostaining anti-smooth muscle α actin, ×50.

and Vale, 1993; Haralson and Hanssel, 1995; Bastos et al., 1999). The fiber locations and arrangement are related to their different functionality which reflect local tissue mechanical properties (Cotta-Pereira et al., 1976; Hay, 1991; Haralson and Hanssel, 1995).

Intumescence and detumescence processes require a complex structure. These processes are closely related to fibrous connective tissue element distributions, mainly the elastic fibers, and collagen bundles that also have a stretching function during erection (Sattar et al., 1994). The penile function also depends on the elastic fiber system.

In mammals, the classification of different penis types is based on erectile tissue or the penile connective tissue (Nickel et al., 1979; Delmann and Brown, 1982; Banks, 1992). In animals, with a vascular penis, erection is a consequence of size increase and organ hardening (Swenson, 1996). In animals with a fibroelastic penis, the erection is essentially a result of length increasing, where the penis emerges from the prepuce due to sigmoid flexure straightening (Nickel et al., 1979; Delmann and Brown, 1982; Banks, 1992; Swenson, 1996).

Although elastic fibers in the corpus spongiosum and the corpus cavernosum provide elasticity and reliance critical properties, few quantification data on the fiber distributions are available for the mammalian penis.

Elastic fibers are the major extracellular matrix components of the human fetal urethra (Bastos et al., 1999). Elastic fiber volumetric density in the corpus spongiosum increases in

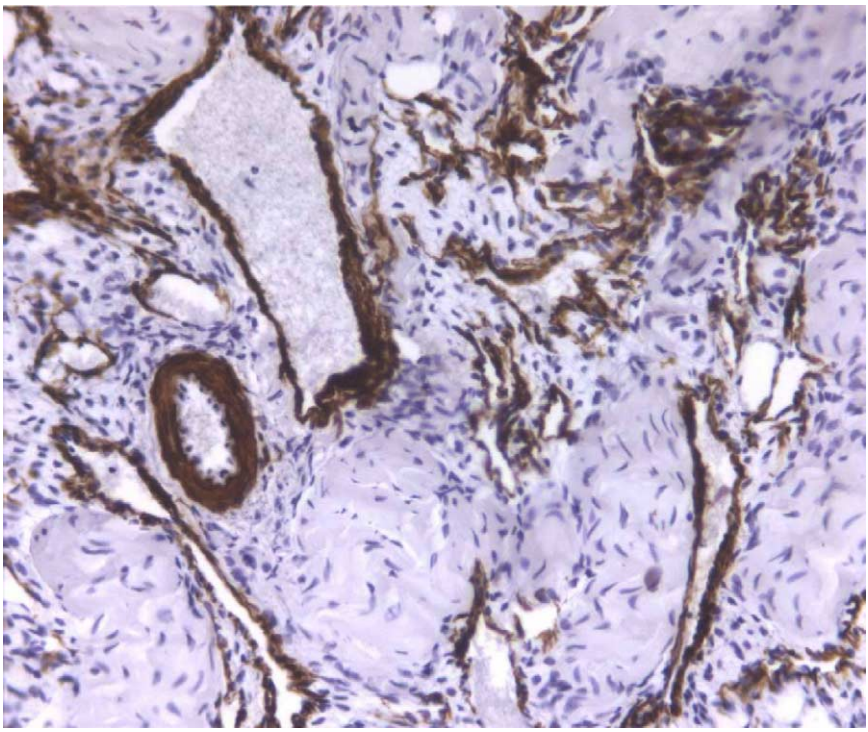

Fig. 9. Smooth muscle cells are associated with blood vessel walls in corpus cavernosum, but absent in connective tissue of the corpus spongiosum and corpus cavernosum. ABC immunostaining anti-smooth muscle α actin. ×200.

a linear fashion during fetal development (from 5.2% at 14th week to 14.8% at 16th week). In a young male, the elastic fiber volumetric density is about 18% (Bastos et al., 2004, in press).

In rat corpus cavernosum, Pinheiro et al. (2000) found 9% of elastic fibers, using the stereologic method. Sattar et al. (1994) found normal human penis elastic fibers to represent 9% of the total fibrous connective elements. In the previous study, the light intensity and color contrasting observation on computerized images (semiautomatic method) was used.

The present results showed that the wild boar penis is a fibroelastic organ with prominent elastic fibers in the corpus spongiosum (V_v = 36%) and corpus cavernosum (V_v = 11%). The elastic fibers system displayed a more tortuous pattern in the corpus spongiosum as compared with the corpus cavernosum. As in other mammals, these fibers may have an important role in corpus spongiosum expansion and reliance during voiding urine, erection and ejaculation (Swenson, 1996; Da Silva and Sampaio, 2002; Bastos et al., 2004).

The fibroelastic penis is typical for domestic pigs and cattle, but without intumescence, i.e., there is volume increase in contrast to the human penis that is a vascular type. Thus, the sigmoid flexure in these animals is important for penile straightening.

Information on elastic fiber quantification in the wild boar penis has not been previously reported. Thus, the present study is the first report considering the fibroelastic penis type in this animal species.

The present study demonstrated by stereological, histochemical, and immunohistochemical methods, that the wild boar penis is a typical fibroelastic structure important to expansion and reliance during erection. These data provide morphological variables for further comparative studies mainly with human or other mammalian species.

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