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Original article

Morphological analysis of testicles in cats with disorders of sex development

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Abstract

Disorders of sex development (DSD) are rare in cats. They can be caused by chromosomal aberrations, gene mutations or other undefined factors. The aim of the present study was to compare the histological structure and immunohistochemical reactivity of testes in cats with DSD and in healthy cats. The research material consisted of the gonads of four cats – phenotypic males with an incorrect structure of the reproductive system. The control group consisted of the testes of four healthy cats – routinely castrated phenotypical males. The material was fixed with formalin and embedded in paraffin; the sections were stained with hematoxylin and eosin. The immunohistochemical investigation were performed using monoclonal and polyclonal antibodies directed against desmin, vimentin, actin of smooth muscles, S100 protein and MCM3 protein. The results obtained allow concluding that the testes of cats with DSD differed in certain respects, mainly in the number of blood vessels, from the normal testes. Moreover, the results of immunohistochemical examination indicate that in the testes of cats with DSD the number of supporting cells is lower, the amount of interstitial cells is comparable and spermatogenesis is correct es compared to those determined in the control gonads. The number of blood vessels in cats with DSD is reduced by about 30%. It confirms the recommendations for castration of these animals in order to eliminate the potential inheritance of sex development disorders.

Key words: cat, disorders of sex development, histopathology, immunohistochemistry

Introduction

Sex in mammals is determined genetically. That is why we refer to genetic sex (it is determined by the presence of proper chromosomes in the genome set XX or XY) of the gonadal type (dependent on differentiation of bipotential gonad into the ovary or testicle) and of the somatic type (conditioned by the total sexual characteristics dependent on hormones produced in the gonads). Disorders of sex determination and differentiation are usually diagnosed based on hermaphroditic genitals or signs of feminization of males and masculinization of females. More difficult to diagnose is a complete reversal of gender, i.e. the lack of phenotypic bisexual traits accompanied by disorders of reproductive function (infertility) (Christensen 2012, Meyers-Wallen 2012).

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Disorders of sex development (DSD) can be caused by chromosomal aberrations, gene mutations or other undefined factors (Meyers-Wallen 2006). Among chromosomal aberrations altering sex development the most common are sex chromosome aneuploidies (mainly monosomy X and trisomy XXY). Also translocation of the Y chromosome fragment carrying the *Sry* gene into the X chromosome or autosome causes disorders of sex development in XX males (Poth et al. 2010, Meyers-Wallen 2012).

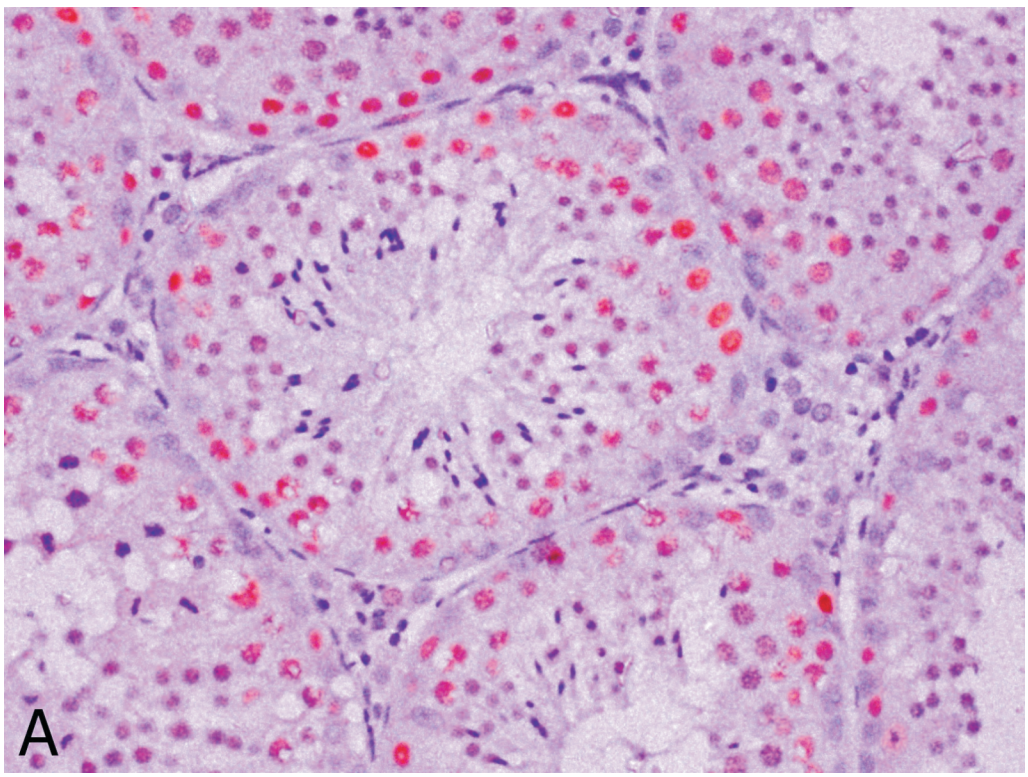
Such disorders of sex development, unlike in other species, are quite rare in cats. The most frequently reported were isolated cases equivalent to the Klinefelter's syndrome in humans (47XXY) (Leaman et al. 1999), monosomy X (Johnston et al. 1983, Dybdahl Thomsen et al. 1987, Szczerbal et al. 2015), isolated hypospadias (King and Johnson 2000), true hermaphroditism (Schlafer et al. 2011), abnormal phenotypic sex with the correct set of XY chromosomes (Nowacka-Woszuk et al. 2014) or in the case of a translocation between chromosome X and Y (Szczerbal et al. 2015). Other types of sex development disorders (XY DSD and XX DSD) or their accompanying changes in cats are described less often. Schulman and Levine (1989) reported pyometra in a male uterus *(uterus masculinus)* in a cat. Reynolds et al. (2014) described a structure disorder of the final section of the digestive tract and urogenital system in a cat (38XY, SRY positive) with a sex disorder consisting in the presence of recto-vaginal fistula and incorrect structure of penis and scrotum (PURSM – partial urorectal septum malformation). There have been reported only isolated cases of feminization of males and cryptorchidism, which definitely is more common in dogs and often co-occurs with other disorders of sex development (Meyers-Wallen et al. 1989, Meyers-Wallen 2012). Millis et al. (1992) determined the frequency of this disorder at 1.7% in the group of 1345 cats hospitalized in a ten-year period, and Yates et al. (2003) reported 1.3% cases of cryptorchidism during a 4.5-year period. While the reversed sex syndrome (78, XX with the occurrence of testis or ovotestis) has been described in many breeds of dogs, such a pathology has not been observed in cats so far (Switonski et al. 2011, Christensen 2012,). It is suspected that the basis of this anomaly, as in humans or dogs, is a mutation that has not been clearly identified. Dysgenetic gonadal neoplasia, especially of testis, has been reported in dogs, as in humans (Dzimira et al. 2015).

The aim of the present study was to compare the histological structure of testes in cats suffering from the disorder of sex development against healthy cats through routine hematoxylin and eosin staining and with the use of immunohistochemical reaction. Antibodies against desmin and actin of smooth muscles were used to demonstrate the presence of mioid cells in the wall of the seminiferous tubules and smooth myocytes in the wall of epididymal and blood vessels. Antibodies against vimentin were used to demonstrate the cells of mesenchymal origin in the testes. The S100 protein was used due to its prevalence in the cells of the body, including interstitial cells and supporting cells. The MCM3 cell proliferation marker was used to determine cells in the phase of mitotic divisions in the seminiferous tubules. The preparations were viewed under the Olympus CX-41 light microscope coupled with a DP-20 photographic camera.

Materials and Methods

The research material consisted of the gonads of four cats, hybrids, phenotypic males aged between 6 and 18 months, surgically removed during routine neutering and sent for histopathological examination in years 2012-2015 from among 4,000 cats – patients of the Department of Reproduction. During clinical examination of the cats, prior to the surgical procedure, the following developmental anomalies were identified: bisexual genitalia (not fully developed, blind vagina, penis hypoplasia with residual penile spines) with normal scrotum with testicles or with scrotum deprived of its septum. These were classified as evident features of sex development disorders. Three of those cats had earlier been diagnosed in the course of molecular and cytogenicity tests as testicular DSD cats with a male karyotype (38,XY) (Nowacka-Woszuk et al. 2014). The control tissues included of testes of four healthy cats, phenotypic males aged from 7 to 12 months collected during a routine castration surgery.

The material was fixed in 10% buffered formalin, dehydrated in alcohol, dipped in xylene, embedded in paraffin and sliced into 5 µm sections, which were then stained with hematoxylin and eosin. The immunohistochemical analyses were conducted on 4 µm-thick paraffin sections mounted on silanized slides. For dewaxing and hydration, the preparations were carried through a series of alcohols. The slides were heated in citrate buffer (pH 6.0, 97°C, 20 min) to retrieve the antigens. Then the slides were double washed with TBS, block in 10% normal serum with 1% BSA in TBS. Afterwards, monoclonal and polyclonal antibodies from Dako (Gdynia, Poland) which cross-react with cat tissues were applied; i.e. vimentin (M7020 code, clone Vim 384, 1: 100), desmin (code M7060, clone D33, 1: 100), smooth muscle actin (code M0851, clone 1A4, 1: 100), protein S-100 (code



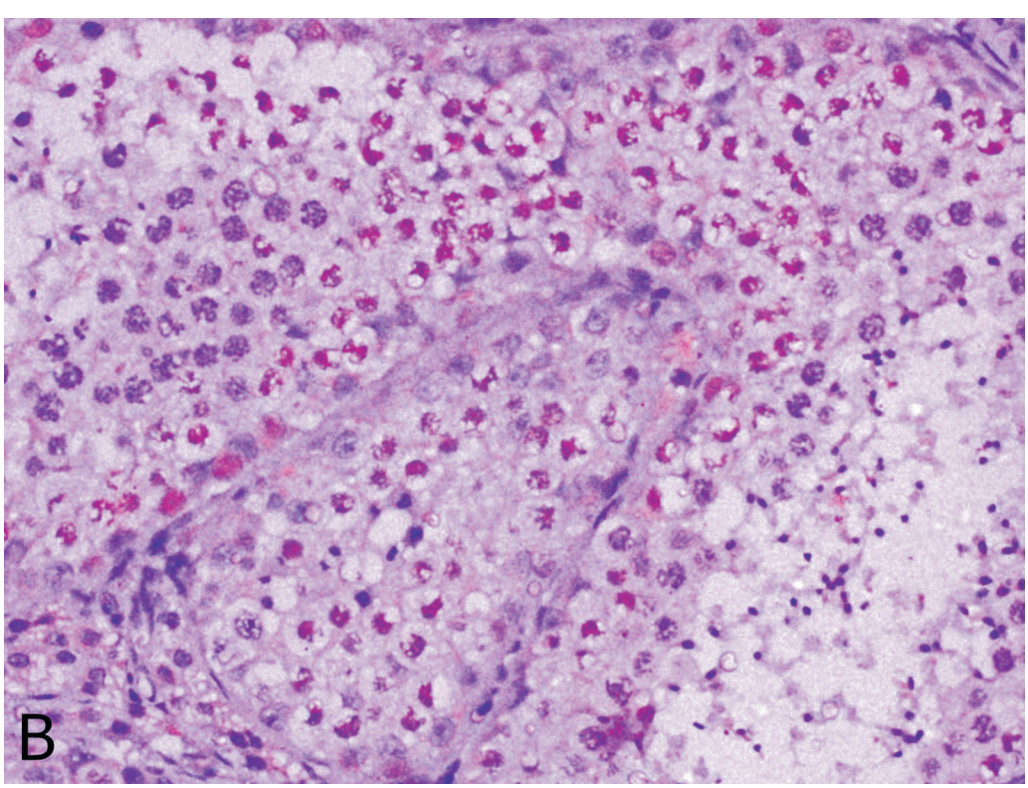


Fig. 1. A, B

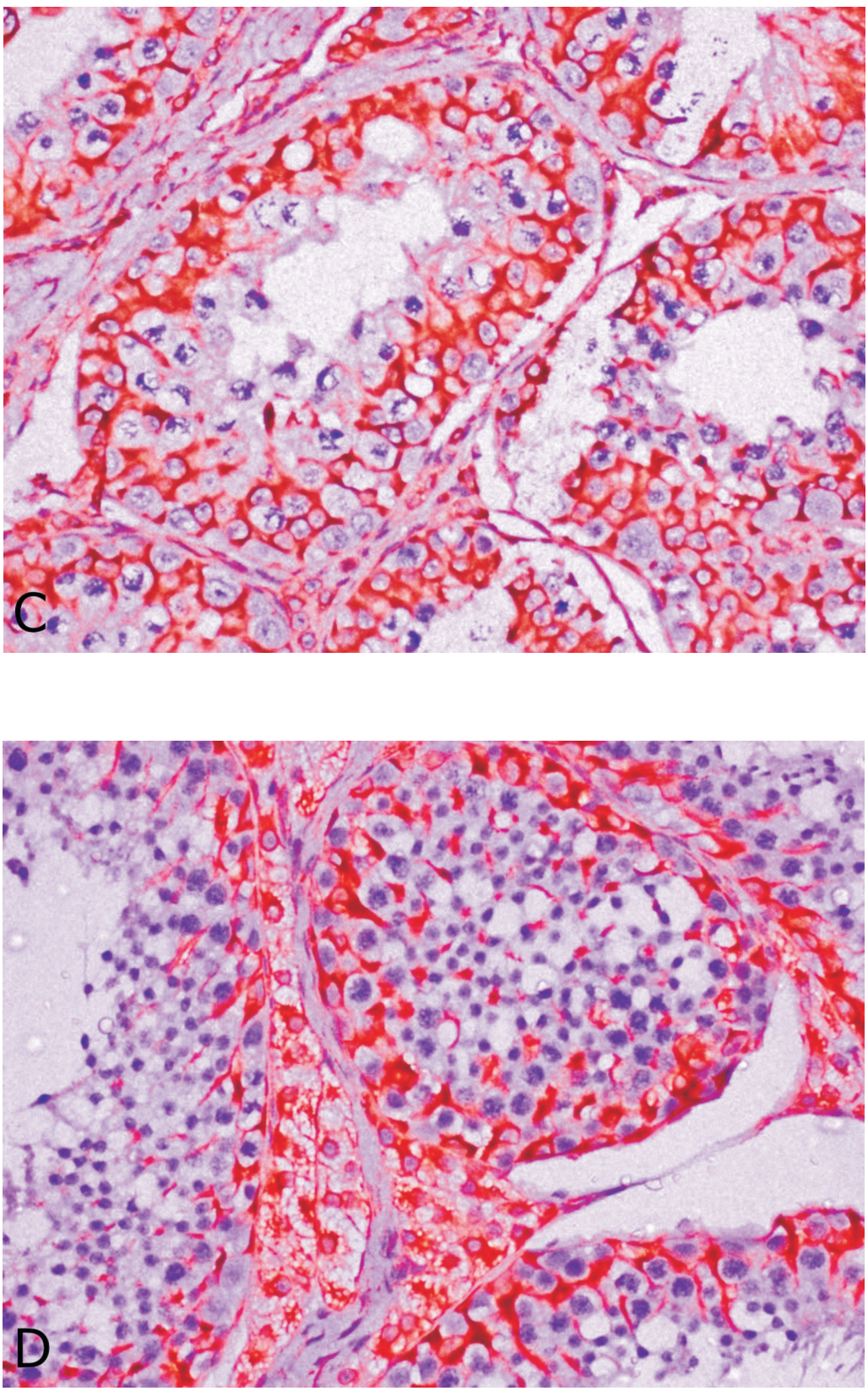


Fig. 1. The immunohistochemical reaction: with anti MCM 3 antibody directed against dividing cells: A – control group – positive reaction in the nuclei of most dividing cells (+++), B – cat with DSD, positive reaction in much less nuclei (+/++), 400x; with antibody directed against vimentin: C – control group – a strong (+++) reaction of interstitial and supporting cells, D – cat with DSD, the strong reaction of interstitial cells, supporting weaker, x400

Table 1. Summary of immunohistochemical examination results of testes in healthy cats and in cats with DSD.

No.	Phenotypic sex, age	Form of external genital organs	Histopathol. examination	Desmin	Vimentin	SMA	S100	MCM3	Cytogenetic test	Reference
1.	Control 1 Male, 8 months	correct	Testicles with spermatozoa	+++	+++	+++	+++	+++, >80%	not tested	–
2.	Control 2 Male 7 months	correct	Testicles with spermatozoa	++	+++	++	+++	+++, >70%	not tested	–
3.	Control 3 Male, 10 months	correct	Testicles with spermatozoa	+++	+++	+++	+++	+++, >80%	not tested	–
4.	Control 4 Male, 12 months	correct	Testicles with spermatozoa	+++	+++	+++	+++	+++, >80%	not tested	–
5.	Case 1 Male, 10 months	Vestigial penis, hypospadias – urethral opening (meatus) on the head of the penis; preserved frenulum, missing septum in scrotum	Testicles with spermatozoa	from + to ++	++	++	+++	++, 40-60%	38 XY, *SRY* pos.	Nowacka-Woszuk et al., 2014
6.	Case 2 Male, 10 months	Penis with penal spine, urethral opening (meatus) in normal position; preserved frenulum, blind vagina (1.5 cm long)	Testicles with spermatozoa	from + to ++	++	++	+++	++, 40-60%	38 XY, *SRY* pos.	Nowacka-Woszuk et al., 2014
7.	Case 3 Male, 6 months	Vestigial penis, missing scrotum, gonads under the skin near groin	Testicles without spermatozoa	from + to ++	++	++	+++	+, <30%	38 XY, *SRY* pos.	Nowacka-Woszuk et al., 2014
8.	Case 4 Male, 18 months	Crotch hypospadias. Gonads in scrotum consisting of two separate parts	Testicles with spermatozoa	from + to ++	++	++	+++	++, >60%	not tested	

Z0311, polyclonal, 1: 600) and MCM3 (code M263, clone 101, 1:30); the slides were incubated at room temperature for 20 min. Subsequently, the slides were washed in TBS. After rinsing, endogenous peroxidase was blocked in 3% solution of hydrogen peroxide for 10 minutes. The immunohistochemical reactions were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB). Finally, the slides were counterstained with hematoxylin. Either a positive or a negative control was performed for each marker studied. The specificity of the immunolabelling was verified by incubation with PBS instead of the specific primary antibody, and the sections of the canine skin were used as positive controls.

The preparations were evaluated on the basis of the positively stained cells, and the intensity of the color reaction. The strength of the reaction was assessed based on the percentage of positively reacting cells (0-5%) – no reaction (-), 6-25% – weak reaction (+), 26-50% – moderate reaction (++), over 50% – strong reaction (+++).

Results

Histopathological structure of the gonads of the cats with disorder of sex development did not differ from the structure of the testes of healthy, properly developed animals. A normal histological structure was observed, i.e. interstitial cells in the sublayer of the connective tissue, the correct seminiferous tubules with supportive cells on the basilar membrane and with germ cells. In three cases the presence of sperm was revealed in the lumen of the tubules. In one case in a 6-month old cat with DSD there were no spermatozoa in the seminiferous tubules. There were supporting cells and spermatocytes visible in the tubules and interstitial cells in the sublayer of the connective tissue. Testicles of cats in the control group have shown a proper structure of the seminiferous tubules, with supporting cells and numerous spermatogonia. Interstitial cells were visible in the sublayer.

Immunohistochemical reactions gave positive results with the individual components of the cats' testes. The resulting color reactions were clear, moderate (++) to intense (+++). Antibodies directed against vimentin gave positive reactions, from moderate to intense, in the interstitial cells and supporting cells. Desmin gave positive reactions in all cases where there was smooth muscle tissue or even single mioidal cells (membrane of the seminiferous tubules). An antibody directed against dividing cells (MCM3) gave a positive reaction with the nuclei of such cells within seminiferous tubules, i.e. cells in spermatogonia form. The reaction of brown or dark brown color was positive in over 50% of the cells in the control group (+++) and 26 -50% of the cells (++) in the group of cats with DSD. The results of immunohistochemical tests are summarized in Table 1 and shown in Fig. 1. Positive reactions with antibodies directed against desmin and actin of smooth muscles allowed showing blood vessels in the interstitial tissue of testes. These vessels were counted in five randomly selected fields at 400X magnification. The counting results are presented in Table 2 and Fig. 2.

Table 2. The number of blood vessels in testes of healthy cats and in cats with DSD.

Case	Number of vessels (average from 5 view fields at 400X magnification)
Control 1	6 ± 1
Control 2	5 ± 2
Control 3	6 ± 2
Control 4	7 ± 1
Case 1	4 ± 2
Case 2	5 ± 2
Case 3	4 ± 1
Case 4	4 ± 1

Discussion

Testes of cats with disorder of sex development did not differ morphologically from the testes of healthy cats. This observation was also reported by other authors. Bredal et al. (1997) described the lack or only single spermatozoa in the light of the tubules in the presence of supporting and interstitial cells in Himalayan cats with pseudo hermaphroditism. King and Johnson (2000) found correctly formed testes with numerous spermatozoa in a cat with hypospadias. Similarly to the results of examination of the testes in healthy cats of various ages obtained by Siemieniuch and Wocławek-Potocka (2007) in their own study, quite significant morphological differentiation of the seminiferous epithelium cells was found in cats with DSD aged 7-8 months. No such differentiation was observed in a six-month old animal with DSD. However, it is difficult to clearly determine whether it was the result of disorder or failure to reach sexual maturity.

The reaction of vimentin was observed in supporting and interstitial cells, which had been reported in the literature in the testes of males of other domestic animals. For example, in the testes of healthy adult dogs the positive response of interstitial and supporting cells was shown by Banco et al. (2010), which is associated with a mesenchymal origin of these cells. In the testes of healthy cats, the reaction was positive in numerous supporting cells, with intense dark brown color. In testes of cats with DSD the response of these cells was less intense, but still clear, with brown color. Such a reaction leads to the conclusion that in the testes of cats with sex disorder there were fewer supporting cells observed as compared with the testes of healthy cats. *Sry* and *Sox9* genes, which are responsible for the development of male gonads, initiate the creation of supporting cells as the first cells specific for male gonads. The final number of supporting cells in the testes of an adult male determines the number of stem cells for which they can provide support and therefore regulates the level of sperm production and fertility of the male (Wilhelm et al. 2013). This leads to a presumption of lower fertility of cats with DSD as compared to the healthy males.

The positive reaction of interstitial cells with vimentin in testes of healthy cats as well as those with sex disorder was not as clear and intense as of supporting cells. The cells in both groups of the examined testes reacted intensively with the antibody directed against the S100 protein. It was a cytoplasmatic reaction, which is consistent with the literature data (Haimoto et al. 1987). These cells of slightly foamy cytoplasm were observed in quantities from a few to several in the interstitial tissue between the seminiferous tubules. The origin of these cells is debatable. It is believed that they are of a mesenchymal origin however epithelial origin also

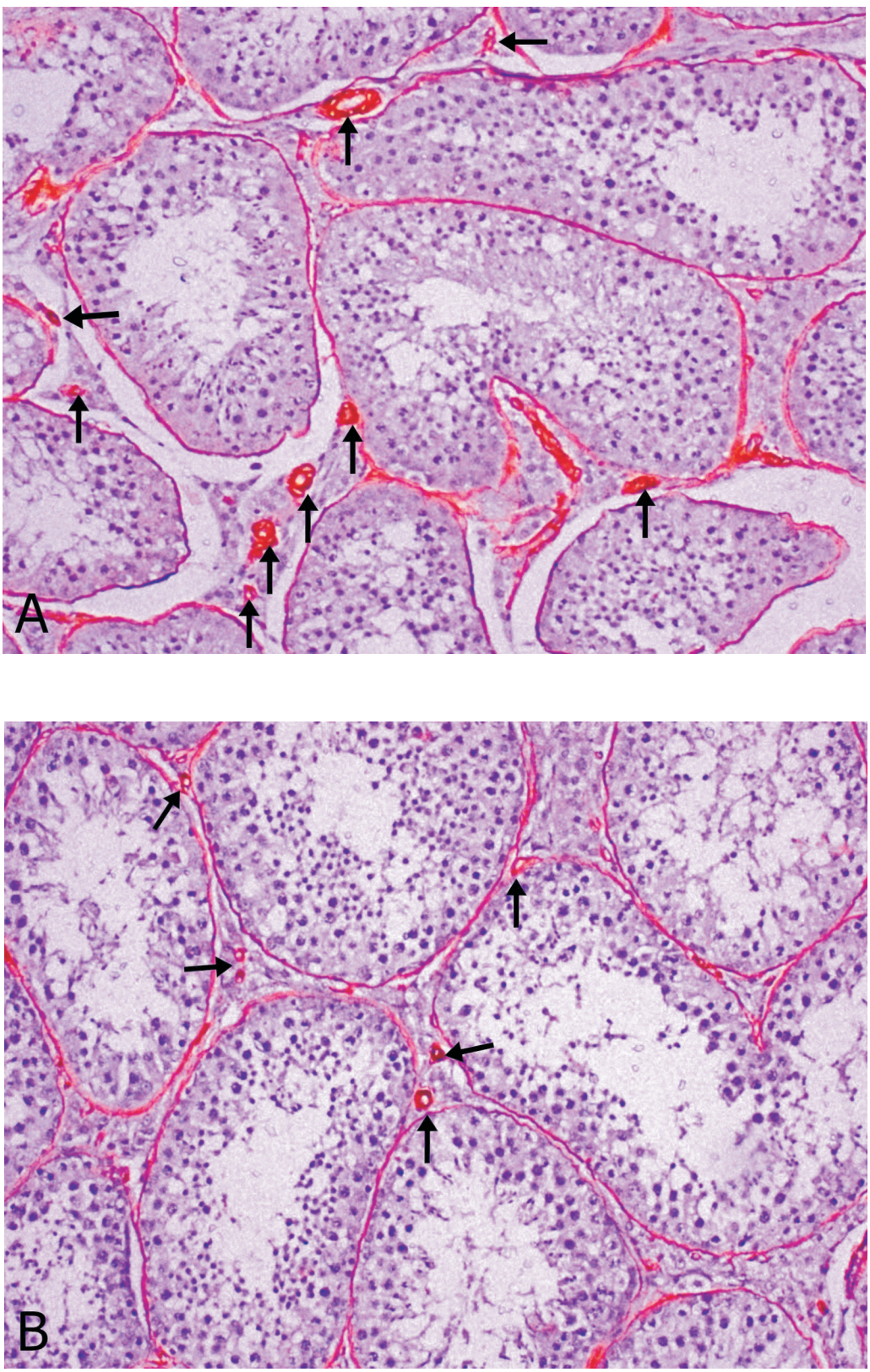


Fig. 2. Number of blood vessels in the testis (arrows), Fig. A. control group (cat No. 2) and Fig B cat with DSD (case No. 2). Immunohistochemical staining anti SMA, x200

has its supporters. The amount of these cells is continuously increasing during the development and it is suggested that the new cells are recruited from existing precursor cells and do not originate from cell division (Wilhelm et al. 2013). The presence of these cells in the testes of cats with DSD in similar quantities as in the testes of healthy cats suggests that they are the source of testosterone responsible for the development of secondary and tertiary sex characteristics in the animals under study.

In all mioidal cells desmin immunolabelling was disclosed. A positive reaction of the highest intensity was observed around many sections of the epididymal duct and vessels, and the lowest intensity around the seminiferous tubules where only single mioidal cells reacted. Smooth muscle actin reacted intensely with the muscle layer of blood vessels and muscle layer of the epididymal duct. The reaction of both antibodies marking smooth myocytes showed a significantly thicker, more pronounced lamina muscularis around the epididymides in healthy cats compared to those with DSD. In healthy cats 3-5 layers of myocytes were observed, whereas in cats with DSD – just 2-3 layers.

Determining desmin and actin of smooth muscles allowed showing the location and amount of blood vessels in the testes of the examined cats. The results obtained give grounds to conclude that the testes of cats with sex disorder contain approximately 1/3 less blood vessels and in the vast majority they are smaller.

Prognostic significance of proliferating cells was determined by the MCM3 antibody. In an animal with DSD, in which there were no spermatozoa observed in the light of seminiferous tubules, the positive response was weak with less than 25% of the positively responding cells.

The results obtained allow concluding that despite sex development disorders in cats with male karyotype 38 XY, *Sry* positive, the histological structure of the developed testicles is not significantly different from the structure of testicles in healthy cats. In the testes of cats with DSD there can be observed a smaller number of supporting cells, a normal but reduced spermatogenesis, the presence of interstitial cells and the number of blood vessels reduced by approximately 30%. This research did not determine the quality of the semen and sperm but their presence may indicate the male reproductive capabilities provided their physical ability to mate (construction of the penis). This confirms the recommendations for castration of these animals in order to eliminate the potential inheritance of sex development disorders.

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Author contribution

SD and WN participated in the design of the study. SD performed the experiments, analyzed data and drafted the manuscript. WN examined the animals. JAM participated in the discussion and corrected the manuscript. All authors read and approved the final version of the manuscript.

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