Pathological findings in genital organs of bulls naturally infected with *Besnoitia besnoiti*

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Abstract. Bulls chronically affected by bovine besnoitiosis can suffer from sterility. There is limited information about the distribution of *Besnoitia* cysts and their associated lesions within the male genital organs. This work describes the gross and histological abnormalities in the genital organs of 6 bulls chronically infected with *Besnoitia besnoiti*, including both clinically (n=4) and subclinically (n=2) affected cases. Parasitic cysts were observed in the genital organs of all the clinically affected bulls. The tissue cysts were most commonly found within the pampiniform plexus (4/4), where they were often seen within venous vascular walls and associated with vasculitis, followed by epididymis (3/4), tunica albuginea (2/4) and penis (1/4). In decreasing order of their frequency, observed abnormalities included: seminiferous tubule degeneration, testicular fibrosis, testicular necrosis, lack of/or diminished numbers of spermatozoa, testicular atrophy and Leydig cell hyperplasia. Only one of the subclinically infected bulls had few *Besnoitia*-cysts within the pampinoform plexus, which was associated to small areas of necrosis and mineralization in the ipsilateral testicle. Results indicate that *Besnoitia* cysts and genital abnormalities are frequent in bulls chronically affected by bovine besnoitiosis, while they are mild and scarce in subclinically affected ones. Moreover, present data show that *Besnotia*-associated testicular lesions can occur without the presence of cysts within the testicular parenchyma. *B. besnoiti* cysts seem to have a tropism for the vascular structures of the spermatic chord, which may cause testicular abnormalities via vascular damage, reduced blood flow and/or impaired thermoregulation and subsequently lead to the observed testicular lesions.

Keywords: *Besnoitia besnoiti*; bovine besnoitiosis; Leydig cell hyperplasia; testicular necrosis; testicular atrophy.

Introduction

Bovine besnoitiosis is caused by *Besnoitia besnoiti,* an obligate intracellular protozoan parasite, which belongs to the phylum Apicomplexa (Cortes et al. 2014). The disease is widely distributed in Africa, Asia and Europe (Álvarez-García et al. 2013), and it is considered to be an emerging disease in Europe (https://www.efsa.europa.eu/en/efsajournal/pub/1499).

B. besnoiti is suspected to have a heteroxenous life cycle, with both domestic (cattle) and wild bovids (antelopes) acting as intermediate hosts. The definitive host has not been identified yet, but a wild carnivore is suspected (Cortes et al. 2014). Bovine besnoitiosis has 2 distinct clinical stages: an acute stage which lasts approximately 1-2 weeks, followed by a chronic stage, which is lifelong (Cortes et al. 2014). The acute phase is associated with proliferation of tachyzoites and is characterized by increased body temperature, weakness and generalized edema. Besides, the chronic phase is characterized by scleroderma and alopecia, which are associated with formation of tissue cysts (Cortes et al. 2014). *Besnoitia* cysts are frequent within mucous membranes, sclera, skin and subcutaneous tissue, but can be present in many other tissues throughout the body, including the reproductive organs (Cortes et al. 2014). Despite reports that besnoitiosis can be associated with sterility in bulls (Álvarez-García et al. 2013; Cortes et al. 2014; Esteban-Gil et al. 2016), its pathogenesis is mostly unknown. As far as we are aware, there are no reports describing the lesions in genital organs of bulls subclinically infected by the *B. besnoiti*. There are only few works describing the lesions in genital organs of clinically affected bulls and some of the information is discrepant (Kumi-Diaka et al., 1981; Sekoni et al., 1992; Cortes et al. 2005; Fernández-García et al., 2010; Dubey et al., 2013; Nieto-Rodríguez et al., 2016). The latter is likely due to the fact that most of the reported pathological data is based on the examination of single cases (Cortes et al. 2005; Sekoni et al., 1992; Fernández-García et al., 2010; Dubey et al., 2013; Nieto-Rodríguez et al., 2016). While some works indicate the presence of *Besnoitia* cysts within the testes (Kumi-Diaka et al., 1981; Sekoni et al., 1992; Dubey et al., 2013) others report lack of intratesticular cysts (Nieto-Rodríguez et al., 2016). Furthermore, the term orchitis is often used when referring to bovine besnoitiosis (Kumi-Diaka et al., 1981; Álvarez-García et al. 2013, Cortes et al. 2014), but it is not clear whether the encountered testicular lesions are necessarily primarily inflammatory or, alternatively, they may be secondary to for example vascular damage.

The aim of the present work is to describe the gross and histological abnormalities as well as the tissue distribution of *Besnoitia* cysts in the genital organs of bulls clinically and subclinically infected by *B. besnoiti* in order to get further insights into the pathogenesis of these lesions.

Materials and methods

Animals

In May 2010, seven bulls (No. 1 to 7) and ten cows coming from a beef cattle herd located in Aragon, in the North-east of Spain, were slaughtered for sanitary reasons. The herd was known to be chronically affected by bovine besnoitiosis based on previous examinations observing skin abnormalities, parasitic cysts within the conjunctiva as well as on serological analysis (Table 1). Serological determinations were carried out using an indirect fluorescent antibody test (IFAT) (Fernández-García et al., 2009) as well an Enzyme-linked immunosorbent assay (ELISA) (Fernández-García et al., 2010). The bulls were between 1 and 6 years old and belonged to 2 different bovine breeds: Parda Alpina and Pirenaica. Bulls No. 1 to 6 were euthanized for sanitary reasons, as they were seropositive for *B. besnoiti*. Based on a clinical examination performed in April 2010, bulls No. 2 to 5 were clinically affected, having scleroderma, hyperkeratosis, multifocal alopecia and/or parasitic cysts within the conjunctiva. The remaining two bulls (No. 1 and 6) had no macroscipical abnormalities nor clinical signs and were therefore considered to be subclinically infected. Bull No. 7 was euthanized due to a leg fracture, had no gross lesions suggestive of besnoitiosis and was seronegative for *B. besnoiti*. Therefore, bull No. 7 was used as negative control for this study.

Sample collection, macroscopic examination and histopathology

A large number of samples were collected in the slaughterhouse including genital and non-genital organs. Sampled genital organs included the entire testes, epididymides, spermatic cords, penis and skin samples of the external genitalia area. The non-genital organs sampled were: eyelid, third eyelid, the entire eye globe, pharynx, trachea, lung, tongue, esophagus, liver and skin from 2 additional locations (perineum and neck).

Upon gross examination of the testes, complete longitudinal sections and subsequent serial cross-sections were performed. When multifocal testicular gross lesions were observed, samples including affected and non-affected areas were taken. For the remaining testes, samples consisted of 1 cm-thick cross-sections of the testicular parenchyma at the midlevel, including the tunica albuginea. Epididymis and spermatic cord were sampled in all animals at the level of the tail and approximately 2 cm distance from the testicle, respectively. All collected tissues were placed in 10% formalin, routinely processed for histology and stained with hematoxylin and eosin (H&E). Histopathological assessment was performed by 2 veterinary pathologists (LGR and JL), which were case-control blinded. To further characterize the cells where the tissue cysts were located,

immunohistochemistry (IHC) for von Willebrand factor (polyclonal rabbit antibody,[a] dilution: 1:250) and Vimentin (monoclonal mouse antibody[b] dilution 1:100) was performed.

Results

Macroscopic testicular lesions were observed only in two of the bulls (Nos. 2 and 3) clinically affected by bovine besnoitiosis. Bull No. 2 had moderate unilateral testicular atrophy (Fig. 1). Bull No. 3 had multifocal to coalescing white and irregular areas of up to 2 x 1 cm in size within one testis (Fig. 2). No gross abnormalities were observed in the testes from the other examined bulls. Epididymis, spermatic cord, penis and skin from the external genitalia from all bulls were macroscopically unremarkable.

The main histological findings are detailed in Table 2. *Besnoitia* cysts were observed in the genital organs of all clinically affected bulls (Nos. 2 to 5) and of one of the subclinically affected ones (No. 6), with a bilateral and unilateral distribution, respectively. No *Besnoitia* cysts were observed in any of the studied tissues (genital and non-genital) from bulls No. 1 and 7. The tissue cysts observed in H&E-stained sections were typical mature multilayered *Besnotia* cysts of between approximately 150 and 400 µm. While some of the tissue cysts had no associated inflammatory reaction, others were surrounded by variable numbers of macrophages, lymphocytes, plasma cells and eosinophils. Within the genital organs, the cysts and its associated inflammation were more frequently observed within the pampiniform venous plexus (five out of six bulls). The tissue cysts were located within the intervascular connective tissue and within the vein walls of the plexus, often bulging into their lumen (Fig. 3). No tissue cysts were observed in the studied cross-sections of the testicular artery. *Besnoitia* cysts were observed within the epididymis of three of the four clinically affected bulls, which were always bilaterally distributed and located mostly within the interstitium (buls 3 to 5). Two of the clinically affected bulls (No. 4 and 5) had low numbers of *Besnoitia* cysts within the skin collected from the external genitalia. Dermal cysts were associated with orthokeratotic hyperkeratosis of variable intensity. One of the bulls (No. 4) had cysts within the connective tissue of the *corpus spongiosum* of the penis. No cysts were observed within the testicular parenchyma of any of the bulls, with few cysts being observed within the tunica albuginea of the testes from 2 clinically affected bulls (No. 2 and 3). Histologically, one clinically affected bull (No. 3) and a subclinically affected one (No. 6) had multifocal areas of necrosis within the testicular parenchyma, being severe and bilateral in the former and mild and unilateral in the latter one. The necrosis was accompanied with multifocal areas of dystrophic mineralization, and there was no significant inflammatory cellular infiltration associated to them (Fig. 4). The three testicles from the 2 bulls with areas of necrosis had the concomitant presence of parasitic

cysts within the ipsilateral pampiniform venous plexus. All the clinically affected bulls and one of the subclinically affected ones (bull No. 1) had variable degree of atrophy of testicular germinal epithelium of the seminiferous tubules, which was often accompanied with variable degree of interstitial fibrosis (bulls Nos. 2, 3 and 5). Unilateral absence of spermatozoa was observed within the epididymis and seminiferous tubules from two of the clinically affected bulls (Nos. 2 and 3). Furthermore, in bull No. 3, the number of spermatozoa observed within the contralateral epididymis and testis were very scant. In addition, the atrophic testis from bull No. 2 had a diffuse and moderate increased number of well-differentiated interstitial cells compatible with Leydig cell hyperplasia, which accompanied a marked seminiferous tubule atrophy and absence of spermatozoa (Fig. 5). Regarding the other studied organs, *Besnoitia* cysts were observed in decreasing order of frequency in skin (tissue cysts observed in 4 out of the 6 bulls), tongue (2 of 6) and nasal mucosa (2 of 6), and conjunctiva (1 of 6) and trachea (1 of 6). No parasitic cysts were observed in liver, lung, third eyelid and esophagus. The cysts were typically located within the interstitium and often surrounded by a chronic granulomatous inflammatory reaction. No other relevant lesions were observed in the studied tissues. IHC showed that the cysts-containing cells within the vein walls of the pampiniform venous plexus were positive for Vimentin but negative for von Willebrand Factor VIII (Fig. 6).

Discussion

Although genital abnormalities are often listed within the lesions caused by *B. besnoti* in bulls, accurate pathological descriptions are lacking. The most comprehensive pathological description so far dates from 1981 (Kumi-Diaka et al., 1981), which provides with a brief description of the main gross and histopathological findings in animals from a cattle herd suffering from an outbreak of besnoitiosis in Nigeria. Moreover, as far as we are aware, there are no reports describing the lesions of bulls subclinically infected by *B. besnoiti*.

In the present work, the frequent finding of testicular lesions in bulls chronically affected by besnoitiosis together with the lack of lesions in the testes of the negative control suggests that the observed lesions were consequence of the disease. Moreover, the lower number of tissue cysts and milder associated lesions in subclinically infected bulls compared to the ones in clinically affected ones further supports this idea. The latter two animals were also the ones with lowest serological titres for *B. besnoiti*.

The most common lesion was the seminiferous tubules atrophy, which was present in all clinically infected and one of the subclinically infected bulls. In clinically affected bulls this lesion was often associated with fibrosis and lack of/or diminished numbers of spermatozoa. One bull from each group had areas of testicular necrosis,

being much more prominent, even grossly visible, in the clinically affected bull than in the subclinically infected one. Regarding the distribution of the parasitic cysts, the pampiniform venous plexus was the most frequent location, adding up to previous descriptions of the parasite within this tissue (Kumi-Diaka et al., 1981; Dubey et al., 2013). The second most common location was the epididymis, where they had also been reported before (Kumi-Diaka et al., 1981; Dubey et al., 2013). One bull had *Besnotia* cysts within the *corpus spongiosum* of the penis, a finding only reported once before (Nieto-Rodríguez et al., 2016), confirming for this organ to be an uncommon location for the *Besnoitia* cysts. Interestingly, no cysts were observed within the testicular parenchyma, which is in contrast with some of the previous works (Kumi-Diaka et al., 1981; Sekoni et al., 1992; Dubey et al., 2013) but similar to another one (Nieto-Rodríguez et al., 2016). Given the lack of inflammatory cells observed within the testicular parenchyma, the observed necrosis within the testes should be regarded as 'testicular necrosis' rather than as 'orchitis' (Nieto-Rodríguez et al., 2016). This however does not preclude the possibility of orchitis to occur, for instance if *B. besnoiti* cysts happen to be present within the testicular parenchyma (Kumi-Diaka et al., 1981; Dubey et al., 2013). In any case, the observations in this study suggest that testicular necrosis may be common in bulls clinically and chronically affected by bovine besnoitiosis and, most importantly, that the development of these lesions can occur without the presence of the cysts within the testicular parenchyma.

The *Besnoitia* cysts observed within the pampiniform venous plexus were often located within the vascular walls, some without associated inflammatory cells but others had vasculitis with abundant macrophages, lymphocytes, plasma cells and eosinophils. The cysts were located within cells, which stained negative with IHC for Von Willebrand factor and strongly positive with IHC for Vimentin within their cytoplasm. These results indicate that the host cells are not endothelial cells. Indeed, previous works using transmission electron microscopy have suggested that the cyst-containing cells are of myofibroblast origin (Dubey et al., 2013). Current and some previous observations suggest that *B. besnoiti* cysts may have a high tropism for the pampiniform plexus (Kumi-Diaka et al., 1981).

Kumi-Diaka (1981) considered three main mechanisms to explain the *Besnoitia*-associated testicular lesions and subsequent sterility: i) direct effect of the tissue cysts and its associate inflammatory reaction present within the testicular interstitium and seminiferous tubules; ii) the presence of cysts within the testicular vessels and its associated inflammation may lead to a reduced blood supply which may cause the testicular lesions; iii) Poor heat exchange through thickened and heavily parasitized scrotum. Although these mechanisms are not necessarily mutually exclusive, current results suggest that a direct effect of the *Besnoitia* cysts do not seem to

have played a role in the development of the observed testicular abnormalities and therefore that the first hypothesis is not essential for the development of these lesions. Given that the vessels of the spermatic chord was the most common location of the cysts in both clinically and subclinically infected bulls, it seems reasonable to consider this location as important for the pathogenesis of the lesions. A reduced blood supply due to tissue cysts and inflammation within the pampiniform plexus may lead to reduced blood flow and/or impaired thermoregulation (Sekoni et al., 1992), which may explain the observed testicular atrophy, degeneration of seminiferous tubules, lack or reduced numbers of spermatozoa and potential infertility. Although vascular-associated testicular damage may potentially also be secondary to damage in the testicular artery (i.e. via thrombosis and ischemia), no parasitic cysts or lesions were observed within the studied sections of this artery. Finally, although cysts present within the tunica albuginea and external genitalia may also have contributed to a reduced testicular thermoregulation capacity, in the present study this may only have occurred to a limited extent, as they were observed in low frequency and numbers in these locations.

In addition to the above-discussed lesions, one of the clinically affected bulls had Leydig cell hyperplasia. As far as the authors are aware, Leydig cell hyperplasia has not been reported before in cases of besnoitiosis in any species. It is known that conditions causing cessation of spermatogenic activity and tubular atrophy can lead to Leydig cell hyperplasia (Naughton et al., 1998). Therefore, we speculate that the observed Leydig cell hyperplasia may have been secondary to the observed concomitant testicular atrophy and degeneration of seminiferous tubules.

Conclusions

In conclusion, this study provides a detailed description of male genital abnormalities and *Besnoitia*-cyst distribution in clinically and subclinically infected bulls. The study indicates that both *Besnoitia*-cysts and testicular abnormalities are frequent in bulls chronically affected by bovine besnoitiosis, while they are mild and scarce in subclinically affected animals. Results also show that the testicular lesions can occur without the presence of cysts within the testicular parenchyma. Moreover, *B. besnoiti* cysts seem to have a high tropism for the pampiniform plexus, which may cause the testicular abnormalities via reduced blood flow and/or impaired thermoregulation.

Sources and manufacturers

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Conflicts of interest/Competing interests

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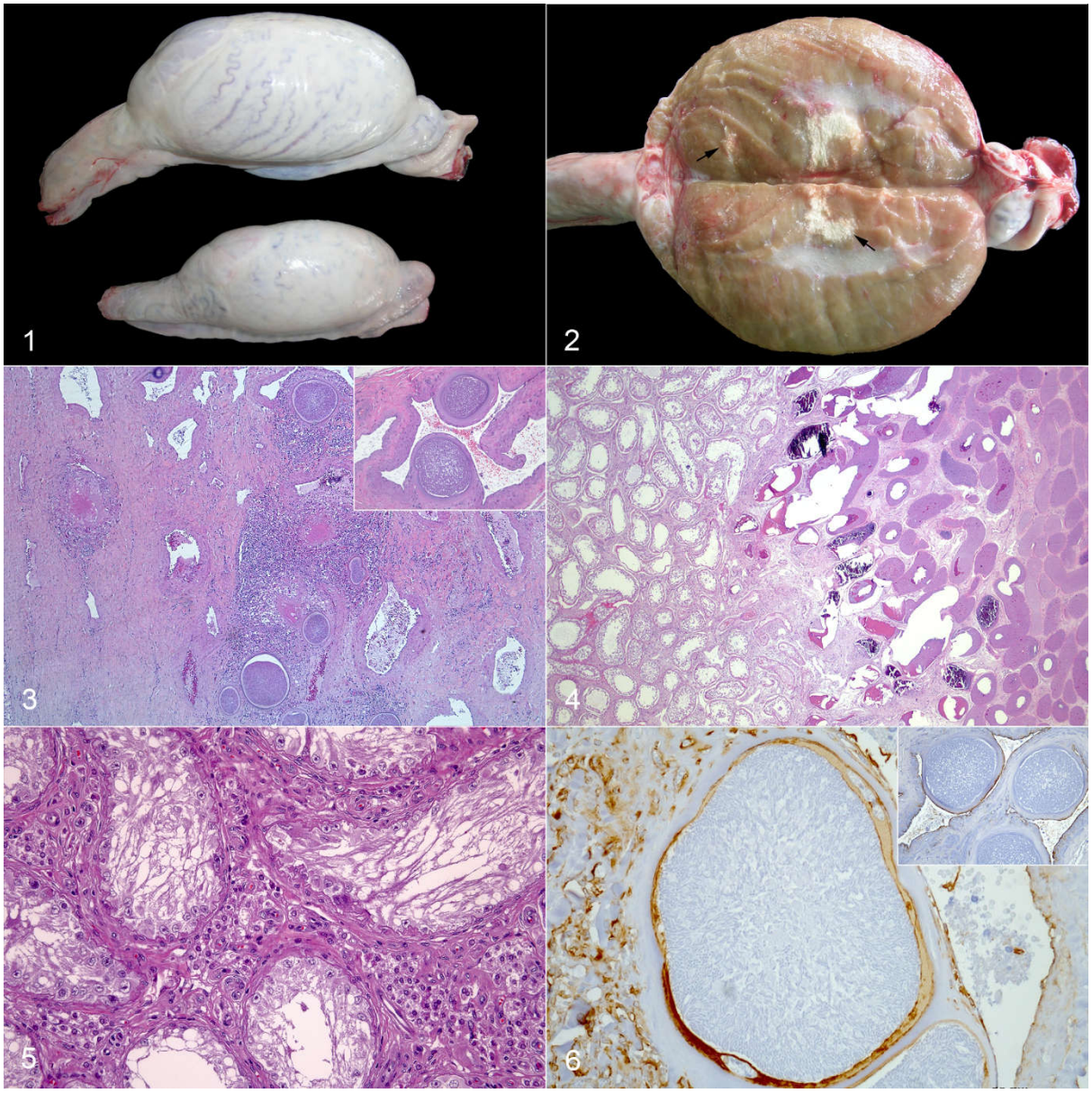
Figure Legends

Figures 1-6 Pathological findings in genital organs of bulls affected by bovine besnoitiosis **Figure 1** Unilateral testicular atrophy, Testes, Bull No. 2. The testis below is moderately reduced in sized compared to the other one **Figure 2** Testis, Bull No. 3. Testicular parenchyma has multifocal white and irregular areas of necrosis and mineralization (arrows) **Figure 3** Pampiniform venous plexus, Bull No. 3. Numerous degenerated and non-degenerated *Besnoitia* cysts are present in the connective tissue and often within the vascular walls. Most of the tissue cysts are associated with a moderate granulomatous and eosinophilic inflammatory reaction. H&E. Inset: Two *Besnoitia* cyts are present within vein wall and markedly bulge into its lumen. No inflammatory cells are present. H&E **Figure 4** Testis, Bull No. 4. There is a well-demarcated and focally extensive area of necrosis which contains multifocal areas of dystrophic mineralization. Remaining seminiferous tubules have marked degeneration of their germinal epithelium. H&E **Figure 5** Testis, Bull No. 2. There is a moderate and diffuse Leydig cell hyperplasia within the interstitium of the testicular parenchyma. Germinal epithelium contains only basal Sertoli cells and there is a diffuse loss of the spermatogenic cells. H&E **Figure 6** Pampiniform plexus, Bull No. 2. *Besnoitia*-containing cells within the vascular walls have a diffuse intracytoplasmic brown staining. IHC for Vimentin. Inset: Endothelial cells have a diffuse brown cytoplasmic staining. Cells containing *Besnoitia* cysts are negative. IHC for von Willebrand Factor.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318 **Table 1**. Breed, age, clinical and serological data from the 7 studied bulls. IFAT: indirect fluorescent antibody
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Bull No.	Breed	Age (years)	IFAT for *B. besnoiti* (antibody titer)		Clinically affected
			January 2010	May 2010	
1	Parda alpina	1,6	1:200	1:200	No
2	Pirenaica	3,6	1:400	1:400	Yes
3	Parda alpina	6	1:1600	1:800	Yes
4	Parda alpina	4,7	1:3200	1:1600	Yes
5	Parda alpina	4,6	1:3200	1:800	Yes
6	Parda alpina	1,5	1:400	negative	No
7	Pirenaica	1,5	negative	negative	No

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338 **Table 2.** Cyst distribution and histopathological lesions in the genital organs of the studied bulls. Results from
339 each bull are presented as 'testicle 1 / testicle 2'. Lesions and presence of cysts were semiquantified as follows,
340 respectively: -: absence; +: mild/low; ++: moderate; +++: severe/abundant.

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Bull No.	Presence of cysts and associated granulomatous and eosinophilic inflammation			Testicular necrosis and mineralization	Seminiferous tubules atrophy	Interstitial testicular fibrosis
	Testicular parenchyma	Epididymis	Pampiniform plexus			
1	- / -	- / -	- / -	- / -	+ / -	-/-
2	- / -	- / -	+ / +	- / -	+++ / +	++/-
3	- / -	+ / +	+ / ++	+++ / +	+++ / +	++/+
4	- / -	++ / ++	++ / +++	- / -	+ / -	-/-
5	- / -	+ / +	+ / ++	- / -	+ / -	+/-
6	- / -	- / -	+ / -	+ / -	- / -	-/-
7	- / -	- / -	- / -	- / -	- / -	-/-
Total	0	3	5	2	5	3

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