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Andrological, pathologic, morphometric, and ultrasonographic findings in rams experimentally infected with *Brucella ovis*

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ABSTRACT

Brucella ovis is considered the most important infectious cause of reproductive disorders in sheep. The disease is characterized by epididymitis, subfertility and infertility in rams. *B. ovis* occasionally results in abortion in ewes, as well. The aim of this study was to evaluate kinetic changes in the reproductive organs of rams experimentally infected with *B. ovis*. Nine rams were experimentally inoculated intrapreputially with 2 mL of a suspension containing 1.2×10^9 CFU (colony-forming units)/mL of *B. ovis* (strain ATCC25840). In addition, 50 μL of a suspension containing 1.2×10^{10} CFU/mL of the same *B. ovis* strain was inoculated into each conjunctival sac, resulting in 3.6×10^9 CFU total per ram. Six of nine infected rams had developed clinical changes in the tail of the epididymis at 30 days post-infection (dpi), but these changes regressed in 50% of these rams. Ultrasound demonstrated an increase in the area of the tail of the epididymis ($P < 0.001$), reduction in the area of the testes ($P < 0.001$), and an increased length and width of the seminal vesicles ($P < 0.001$) during the course of infection. A sperm granuloma was diagnosed on the basis of ultrasonography findings. Microscopically, there was epididymitis, testicular degeneration, and seminal vesiculitis. Inflammatory cells were detected in the semen even before the development of epididymitis. Moreover, inflammatory cells were also found in the semen of asymptomatic rams, indicating that the presence of leukocytes in the ejaculate is a valuable method for screening potential carriers of infections in the genital tract.

1. Introduction

Epididymitis, caused by *Brucella ovis* infection in rams, is the most important reproductive infectious disease of sheep worldwide (Burgess et al., 1982). The disease is caused by a gram-negative bacterium of the genus *Brucella*, which is facultative intracellular coccobacilli that preferentially infects sheep (Enright, 1990). *B. ovis* infection is associated with chronic epididymitis and impaired fertility in rams (Biberstein et al., 1964; Searson, 1987). Occasionally, abortions in ewes and increased perinatal mortality rates are also observed (Ficapal et al., 1998; Molello et al., 1963; Osburn and Kennedy, 1966). Lesions caused by *B. ovis* are restricted to the genital tract of rams (Buckrell et al., 1985), and they are primarily located in the epididymis, although they eventually affect the testis and vesicular gland as well (Biberstein et al., 1964; Ficapal et al., 1998; Walker et al., 1986). Therefore, *B. ovis* infection

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causes significant economic losses for the sheep industry due to reproductive failure and culling of breeding animals (Carpenter et al., 1987).

A definitive diagnosis of *B. ovis* infection requires laboratory tests, including serology, bacteriology, and PCR (Xavier et al., 2009). Clinical signs may support a presumptive diagnosis, but considering the limitations of clinical examination for identifying rams infected with *B. ovis*, ultrasound may be a useful tool to improve clinical assessment in these cases because this method has been used in small ruminants in other circumstances (Ahmad and Noakes, 1995; Gouletsou et al., 2003). However, there are major obstacles for widespread use of ultrasonography in andrological diagnosis, including the scarcity of information about normal parameters and the lack of correlational studies between ultrasonographic findings and the actual stage and nature of the injury (Griffin and Ginther, 1992).

The aim of this study was to perform clinical, andrological, ultrasonographic and histopathological evaluations of reproductive organs in rams that were experimentally infected with *B. ovis*, as well as to correlate seminal parameters with ultrasonographic changes.

2. Material and methods

2.1. Experimental rams and inoculation

This experiment was approved by the Institutional Ethics Committee on Animal Experimentation (CETEA-UFMG, Protocol 02/2007). Nine crossbred rams aged 1–3-year-old were used in this study. Rams were fed hay and commercial ration throughout the experiment, which took place in Belo Horizonte, Brazil (19.52°S, 43.57°W) from April 28, to October 24, 2008.

Estrus was induced in two crossbred ewes that were used for semen sampling. They were treated for induction of estrus by applying 2 mg of estradiol cypionate (ECP – Pfizer, Brazil) intramuscularly 48 h before collection of semen. This protocol was repeated throughout the experiment whenever necessary.

The rams were experimentally inoculated intrapreputially with a 2 mL suspension containing 1.2×10^9 CFU (colony forming units)/mL of *B. ovis* (strain ATCC25840). In addition, 50 μL of a suspension containing 1.2×10^{10} CFU of the same strain was inoculated into each conjunctival sac, resulting in 3.6×10^9 CFU total per ram (Blasco, 1990). Prior to inoculation, all rams were considered to be free of *B. ovis* infection based on negative serological test results and the absence of *Brucella* isolates from blood, semen, urine, and preputial wash samples. Infection was confirmed by PCR (Xavier et al., 2010), bacteriology and serological tests, including agar-gel immunodiffusion (AGID) and complement fixation (CF) (Alton et al., 1988).

Rams were clinically monitored throughout the experiment. Length and width of the tail of the epididymis and testes were measured with a caliper to correlate with clinical and ultrasonographic findings. The length of the testis was measured dorsal to ventral, making sure to isolate the head and tail of the epididymis. The width of the testis was measured laterally mid-testicle. The length of the tail of the epididymis was measured dorsal to ventral, making sure to isolate the testis. The width of the tail of the epididymis was measured laterally mid-tail. Additionally, testis consistency was evaluated and scored from 1 (flaccid) to 4 (normal).

2.2. Semen collection and evaluation

Semen samples were obtained immediately before inoculation and every 30 days post-infection (dpi) for 180 days. All samples were collected using an artificial vagina under aseptic conditions. Semen sampling and ultrasonographic examination, for any given time point, were always performed on the same day.

Semen samples were placed in a water bath at 37 °C immediately after collection. Volume and concentration were measured. Motility, spermatic vigor and turbulence were subjectively assessed. Sperm concentration was determined by diluting semen samples (1:400) in a formalin-saline solution (Hancock's solution) using a Neubauer chamber with light microscopy (400×). For sperm morphology, fresh semen samples were fixed in buffered saline formalin at 37 °C and evaluated by phase contrast microscopy (1X 1000-Olympus BX-41). The percentage of abnormalities in spermatozoa was calculated based on 100 cells per sample. To identify inflammatory cells in the ejaculate, semen smears were stained with Quick Panoptic and examined under light microscopy.

2.3. Ultrasonographic examination

Ultrasound images of the testes and epididymis were obtained with a Chisone 500 Vet (Veterinary Ultrasound, Chisone Medical Imaging. Ltd., China) using a 5 MHz linear transducer. Rams were physically restrained by two assistants while the testes were immobilized without pressure. Initially, the left testicle was scanned with the probe in a transverse orientation. The probe was then placed on the testicular surface and moved in a dorsal–ventral direction for evaluation of the parenchyma and mediastinum testis. The height and depth through the plane corresponding to the largest circumference of scrotum was also measured. The probe was then placed in the same position on the tail, body and head of the left epididymis. The same process was conducted with the right testis and epididymis.

Images of the vesicular glands were obtained transrectally. Height and length of the vesicular glands were measured with a 5 MHz linear transducer coupled to a flexible, plastic tube.

2.4. Histopathology and morphometry

At 180 dpi, all rams were treated with 2 mg of xylazine intramuscularly, and then euthanized by electrocution. Samples from the tail, body and head of each epididymis, testis, ampulla of vas deferens, seminal vesicle, and bulbourethral gland were collected at 192 dpi. Lymph node (inguinal and internal iliac), spleen, liver, kidney and urinary bladder samples were also collected at 192 dpi. Tissues were fixed by immersion in 10% buffered formalin or, in the case of the testes, in Bouin's solution, and the tissues were processed for paraffin embedding and hematoxylin and eosin (HE) staining.

Morphologic evaluation and differential cell count of the seminiferous epithelium were performed as previously described (Santos et al., 1999). For reference, tissue samples collected at a slaughterhouse from nine 3-year-old rams aged 1–3 years were used. These reference animals were seronegative for *B. ovis* and did not have lesions.

Staging of the seminiferous epithelium was based on shape, position of the nuclei of spermatids and the presence of meiotic divisions. Cell populations in the seminiferous epithelium were estimated by counting the nuclei of different cell types, including germ cells and Sertoli cells. Twenty cross sections of seminiferous tubules from each ram were counted. Only circular cross sections were included. The following cell types were identified and counted: spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, round spermatids, and Sertoli cells, as previously described (Santos et al., 1999).

Tubular diameter was measured in 20 randomly selected circular cross sections of seminiferous tubules in stage 1 of the seminiferous epithelium cycle. The same approach was used to measure the height of the seminiferous epithelium in two distinct points of the same tubule (results were expressed as an average of these two measurements). Measurements were obtained with the aid of a micrometer adjusted to the ocular.

Volumetric proportions of testicular structural components were evaluated using an ocular grid with 25 points (KPL Zeiss, Germany) under a 10× objective. The grid was superimposed on 40 randomly selected microscopic fields totaling 1000 points per section. Volumetric proportions of interstitium, tubules, seminiferous epithelium and tubular lumen were also evaluated.

2.5. Statistical analysis

Quantitative data with normal distribution were analyzed by ANOVA followed by Student–Newman–Kews (SNK) test. Volumetric proportion data were subjected to angular transformation prior to ANOVA. Correlative analysis of data with normal distribution was performed by Pearson's method. Non-parametric data were subjected to Kruskal–Wallis test. Correlations between data without normal distribution were assessed

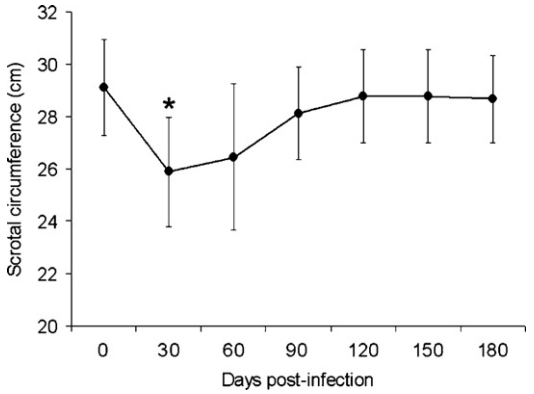

Fig. 1. Scrotal circumference of rams experimentally infected with *Brucella ovis* during the course of infection. Data points represent mean and standard deviation for nine rams. Asterisk indicates a statistically significant difference when compared to pre-infection (time 0) values (*$P < 0.05$).

with Spearman's test. Statistical analyses were performed using GraphPad InStat software version 3.05 (GraphPad InsStat Software, Inc., USA).

3. Results

3.1. Clinical findings in rams experimentally infected with B. ovis

Prior to inoculation, none of the rams had any clinical or ultrasonographical changes in the testis and epididymis. Furthermore, all rams were negative for *B. ovis* infection by serological methods (AGID and CF), bacteriology and PCR of semen, urine and preputial wash samples prior to inoculation (data not shown).

At 30 dpi, all rams were serologically positive for *B. ovis*. Specifically, 8 of 9 rams were serologically positive for *B. ovis* by AGID, and all rams were positive by CF. In addition, all rams were positive by PCR or bacteriology for at least one time point during the time course of infection (data not shown).

Clinical changes were observed in the testis and epididymis after infection with *B. ovis*. Scrotal circumferences decreased at 30 dpi, but returned to pre-infection values by 60 dpi (Fig. 1).

Clinically, 66.7% (6/9) rams developed palpable changes characterized by increased volume and consistency of the epididymis at 30 dpi. Five of the six affected rams had unilateral changes. Four affected rams displayed increased sensitivity to palpation. Nodules were palpable in two rams, one in the tail of the right epididymis and the other in the tail of the left epididymis. At 60 dpi, these changes remained in two of the six affected rams. In addition to increased volume of the epididymis, a palpable nodule was detected in one of these rams (ram 3). One ram had increased volume and consistency of the right tail of the epididymis only at 60 dpi, but that ram did not exhibit any other changes after this time point.

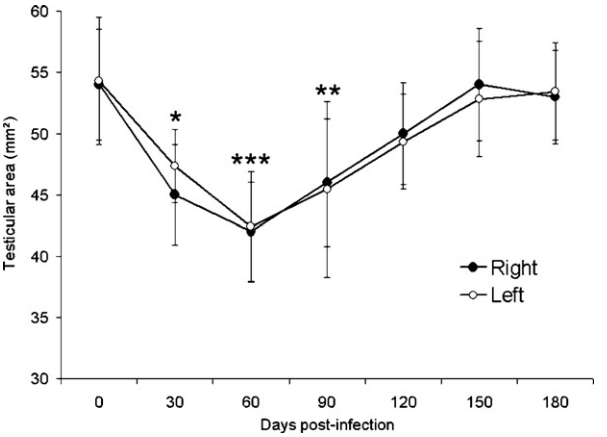

Fig. 2. Testicular area (mm^2) of rams experimentally infected with *Brucella ovis* during the course of infection. Data points represent mean and standard deviation of nine rams. Asterisks indicate a statistically significant difference when compared to pre-infection (time 0) values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

3.2. Ultrasonographic changes in rams experimentally infected with B. ovis

Testicular parenchyma was ultrasonographically homogeneous with moderate to high echogenicity. Six rams had hyperechoic spots in the testicular parenchyma before inoculation (bilateral in four rams and unilateral in two), which remained detectable throughout the time course of the experiment. These spots were interpreted as focal areas of fibrosis (Barth et al., 2008). The mediastinum testis was observed in all testes in the center of the testicular parenchyma. A decrease in the area of both testes was detected between 30 and 90 dpi (Fig. 2).

Ultrasonographic measurements of the tails of the epididymides indicated bilateral enlargement at 150 dpi; however, on average, only the right epididymis remained significantly increased until the end of the experimental period (Fig. 3).

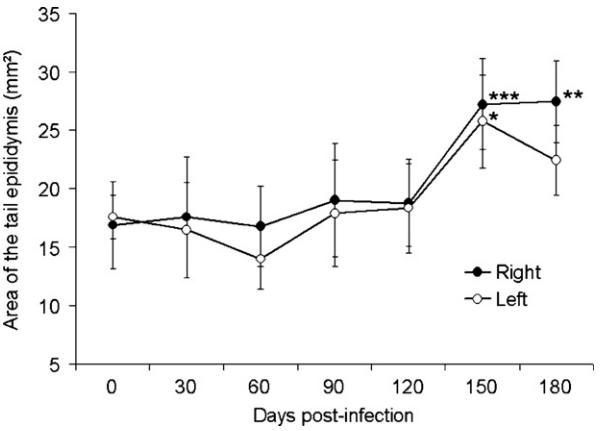

Fig. 3. Area of the tail of the epididymis (mm^2) of rams experimentally infected with *Brucella ovis* during the course of infection. Data points represent mean and standard deviation for nine rams. Asterisks indicate a statistically significant difference when compared to pre-infection (time 0) values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

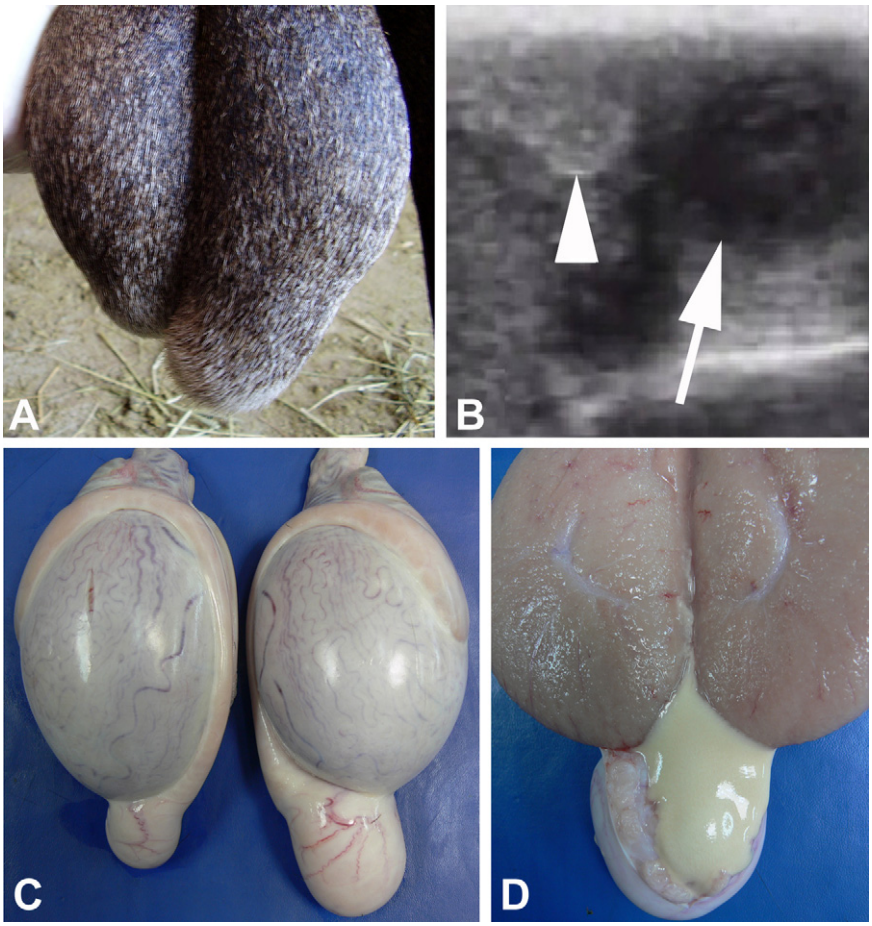

Fig. 4. Clinical, ultrasonographic and macroscopic findings at 180 days post-infection for a case of sperm granuloma in the tail of the epididymis in a ram experimentally infected with *Brucella ovis* at 180 days post-infection (ram 3). (A) Markedly increased volume of the tail of the right epididymis. (B) Ultrasonographic image of nodules in the tail of the right epididymis of ram 3, one hyperechoic nodule (arrowhead) and another anechoic nodule (arrow). Image obtained with a Chisone Vet Ultrasound 500 and a 5 MHz linear probe. (C) Asymmetric tails of the epididymides with a marked enlargement of the tail of the right epididymis. (D) Cut surface of the tail of the right epididymis draining a creamy, yellowish content from the anechoic nodule indicated in (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Changes in echogenicity of the epididymis were observed in three rams (rams 2, 3 and 5), which consisted of mildly increased echogenicity in the tail of the epididymis. At 60 dpi, an anechoic nodule measuring 3 mm^2 was noted in one ram (ram 2), though the nodule did not change the volume of the tail of the epididymis. Another ram (ram 5) had bilateral anechoic nodules, both measuring 2 mm^2.

The most significant change in the tail of epididymis was observed in ram 3 at 30 dpi. A unilateral (right side), anechoic nodule measuring 112 mm^2 with well-defined margins was found in the tail of the epididymis. By 60 dpi, the nodule measured 78 mm^2, and it was reduced to 25 mm^2 by 90 dpi. At 90 dpi, a palpable nodule in the right tail of the epididymis was detected but not differentiated from surrounding tissues by ultrasound. At 120 dpi, this palpable nodule measured 35 mm^2, had increased echogenicity, and could be differentiated from the surrounding tissues. Another nodule with an anechoic area of 78 mm^2 was observed at 120 dpi, as well. At 150 dpi, the nodules had a hyperechoic area of 136 mm^2 and an anechoic nodule of 105 mm^2. At 180 dpi, the nodules had a hyperechoic and anechoic area of 128 mm^2 and 234 mm^2, respectively (Fig. 4). On postmortem examination, the anechoic nodule observed by ultrasound had a creamy, yellow appearance (Fig. 4), whereas the hyperechoic nodule corresponded to a sperm granuloma.

There was an increase in the length and height of the seminal vesicles at 90 dpi that remained until the end of the experiment (Fig. 5). However, there were no changes in the echogenicity of these glands.

3.3. Andrological findings in rams experimentally infected with B. ovis

Ejaculates from all rams collected before inoculation had seminal parameters that were within the normal reference values (CRBA, 1998).

Changes in sperm morphology were initially observed at 30 dpi (Table 1), with a significant increase in the percentage of spermatozoa with droplet coiled tail. At 60 dpi,

Table 1
Sperm defects in rams ($n = 9$) experimentally infected with *Brucella ovis* at different time points after infection.

Days post-infection	Percentage of different types of defects						
	Detached head	Coiled tail	Coiled tail with droplet	Dag defect	Midpiece defect	Distal droplet	Proximal droplet
0	2.00 ± 0.00^{a}	3.67 ± 4.18^{a}	0.00 ± 0.00^{a}	5.75 ± 3.69^{a}	1.00 ± 0.00^{a}	6.67 ± 9.14^{a}	4.50 ± 3.54^{a}
30	1.00 ± 0.00^{a}	15.00 ± 9.73^{b***}	5.17 ± 4.54^{b*}	6.63 ± 7.33^{a}	0.00 ± 0.00^{a}	4.38 ± 5.83^{a}	3.00 ± 0.00^{a}
60	2.13 ± 1.36^{b**}	8.50 ± 4.76^{a}	1.83 ± 1.17^{ab}	4.71 ± 4.54^{a}	5.50 ± 6.36^{a}	8.33 ± 9.24^{ac}	0.00 ± 0.00^{a}
90	2.50 ± 2.12^{a}	12.44 ± 5.15^{b***}	2.00 ± 0.00^{a}	2.67 ± 1.66^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{ac}	0.00 ± 0.00^{a}
120	0.00 ± 0.00^{a}	17.22 ± 10.01^{b***}	1.33 ± 0.58^{a}	11.67 ± 12.50^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{bc*}	1.00 ± 0.00^{a}
150	3.33 ± 2.73^{b*}	14.78 ± 7.60^{b***}	1.00 ± 0.00^{a}	6.67 ± 4.64^{a}	0.00 ± 0.00^{a}	2.33 ± 1.53^{ac}	0.00 ± 0.00^{a}
180	2.00 ± 0.00^{a}	16.78 ± 5.09^{b***}	2.00 ± 0.00^{a}	4.00 ± 2.69^{a}	6.00 ± 0.00^{a}	0.00 ± 0.00^{bc*}	2.00 ± 1.00^{a}

Different letters in same column indicate significant differences by SNK test.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

morphological parameters were statistically similar to pre-infection values, but the percentage of spermatozoa with a coiled tail after infection otherwise remained increased. Spermatozoa with a dag defect, middle piece defect, proximal droplet or distal droplet had no significant variations during the experimental period. The total number of sperm with defects increased at 30 dpi and remained increased up to 180 dpi (Fig. 6).

Inflammatory cells were completely absent in all ejaculates collected before inoculation (Table 2). However, at 30 dpi, 44.44% (4/9) of rams had inflammatory cells in their ejaculates, including neutrophils, macrophages and lymphocytes. At 180 dpi, 100% (9/9) of the rams had inflammatory cells in their ejaculates. The severity of inflammatory cells in the ejaculates ranged from mild to severe. Fig. 7 illustrates a severe accumulation of inflammatory cells in the ejaculate. Shedding of inflammatory cells was intermittent in two of nine rams.

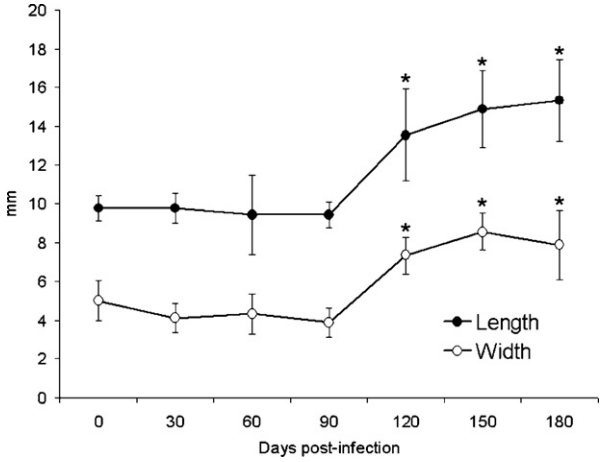

Fig. 5. Length and width values for seminal vesicles of rams experimentally infected with *Brucella ovis* during the course of infection. Data points represent mean and standard deviation for nine rams. Asterisk indicates statistically a significant difference when compared to pre-infection (time 0) values ($^{*}P < 0.01$).

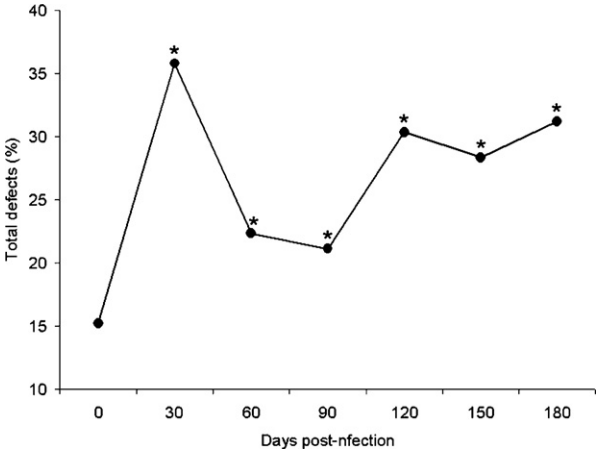

Fig. 6. Percentage of total defects in the ejaculate of rams experimentally infected with *Brucella ovis* during the course of infection. Data points represent the average for nine rams. Asterisk indicates a statistically significant difference when compared to pre-infection (time 0) values ($^{*}P < 0.05$).

Table 2
Inflammatory cells in ejaculate from rams experimentally infected with *Brucella ovis* at different time points after infection.

Animal	Days post-infection						
	0	30	60	90	120	150	180
1	–	–	–	+	+	+	+
2	–	–	+	+	–	+	+
3	–	+++	++	+++	+++	+++	+++
4	–	+	+	+	+	+	+
5	–	–	–	–	+	+	+
6	–	+	+	+	+	+	+
7	–	–	+	–	+	+	+
8	–	–	+	+	+	+	+
9	–	++	++	++	+	+	++

Severity of inflammatory cells in the ejaculate: mild (+), moderate (++) and severe (+++).

3.4. Pathologic changes in rams experimentally infected with B. ovis

Two rams (rams 3 and 9) had inflammatory infiltrates in the spermatic cord characterized by mild, interstitial, focal

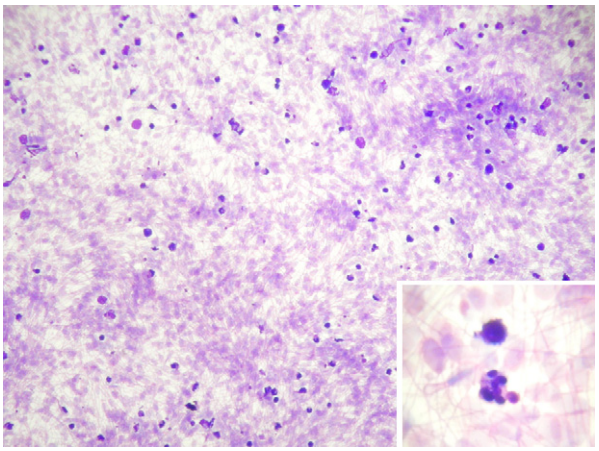

Fig. 7. Semen sample smear from a ram experimentally infected with *Brucella ovis* that developed epididymitis and seminal vesiculitis (ram 3). Inset: predominance of neutrophils. Quick Panoptic staining.

accumulation of histiocytes, lymphocytes and plasma cells. One ram (ram 3) had a similar lesion in the ampulla of the vas deferens. All rams (9/9) had inflammatory changes in the seminal vesicles, which were characterized by interstitial, multifocal infiltration of lymphocytes and plasma cells (Fig. 8) that ranged from mild (5/9), to moderate (2/9), to severe (2/9). These lesions were bilateral in six rams. Four rams (rams 3, 5, 9, and 10) had neutrophils in the lumen of the acini.

In tail of the epididymis, all rams developed interstitial lympho-histio-plasmacytic inflammatory infiltrates. These infiltrates were bilateral in six rams. The distribution of infiltrates was focal in six rams, multifocal in two rams, and diffuse in one ram (ram 3). Severity of the infiltrates was mild in two rams, moderate in seven rams, and severe with sperm granuloma formation, vacuolization of the epithelium, and accumulation of giant cells in one ram (ram 3) (Fig. 8). Interstitial, mild, focal, lympho-histio-plasmacytic infiltrates were also observed in the head of the epididymis in all rams, and the infiltrates were bilateral in six rams. Eight rams had similar lesions in the body of the epididymis, and the lesions were bilateral in three rams.

All rams had histological changes compatible with testicular degeneration, which were characterized by vacuolation and desquamation of the seminiferous epithelium and associated with dilation of the seminiferous tubule lumens. Some germ cells had an increased cytoplasmic eosinophilia and nuclear condensation. These findings were bilateral in five rams and unilateral in four rams. Intensity of testicular degeneration ranged from mild (3/9) to moderate (6/9).

No histological changes were observed in the glans penis, prepuce, urinary bladder, kidneys, spleen, liver, or the iliac and inguinal lymph nodes at the end of the experimental period.

Morphometrically, the testes from experimentally infected rams had higher volumetric proportions of interstitium and lumen when compared to those of uninfected rams. The infected rams also had lower volumetric proportions of the seminiferous epithelium. Furthermore, infected

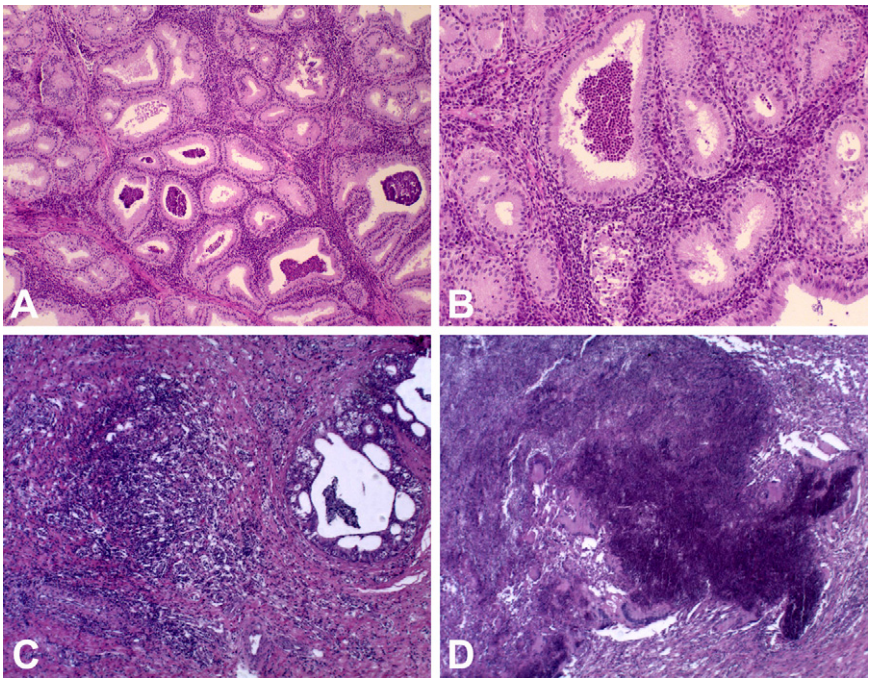

Fig. 8. Genital lesions in a ram experimentally infected with *Brucella ovis* (ram 3). (A and B) Seminal vesiculitis with diffuse lympho-histio-plasmacytic interstitial infiltrates and intraluminal accumulation of neutrophils. (C) Epididymitis in the tail of the epididymis with diffuse interstitial accumulation of inflammatory cells and hyperplasia and intra-epithelial vacuolation in the epididymal duct. (D) Sperm granuloma with interstitial accumulation of spermatozoa surrounded by inflammatory cells with several giant multinucleated cells. Hematoxylin and eosin staining.

Table 3
Testicular histomorphometry and differential cell counts in the seminiferous epithelium of rams experimentally infected with *Brucella ovis* (infected) or healthy, uninfected rams (reference).

Morphometric parameters	Groups	
	Infected ($n=9$)	Reference ($n=9$)
Tubule diameter (μm)	93.44 ± 5.69[a]	88.02 ± 6.50[b*]
Epithelium height (μm)	27.11 ± 0.60[a]	27.89 ± 2.33[a]
Number of round spermatids	123.29 ± 17.62[a]	117.18 ± 16.13[a]
Number of paquitone	41.08 ± 12.65[a]	40.13 ± 14.75[a]
Number of preleptotene	21.64 ± 3.69[a]	21.90 ± 11.87[a]
Number of spermatogonia A	1.96 ± 0.33[a]	1.76 ± 0.38[a]
Number of Sertoli cells	5.61 ± 1.26[a]	8.43 ± 1.90[a]
Total cells	193.57 ± 31.56[a]	189.39 ± 39.69[a]
Volumetric proportion of interstitium (%)	21.42 ± 2.71[a]	17.74 ± 2.60[b*]
Volumetric proportion of lumen (%)	18.26 ± 2.12[a]	14.09 ± 1.93[b**]
Volumetric proportion of epithelium (%)	60.33 ± 1.73[a]	68.18 ± 3.87[b***]

Different letters in same line indicate significant differences by SNK test.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

rams had seminiferous tubules with larger diameters in comparison to those of the uninfected reference group (Table 3).

3.5. Clinical and ultrasonographic finding correlated with seminal changes in rams experimentally infected with B. ovis

As expected, there was a significant, positive correlation between ultrasonographic and biometric measurements obtained with calipers. Interestingly, there was a negative correlation between the area of the tail of the epididymis and sperm concentration. Ultrasonographic measurements of length and depth for the seminal vesicles were highly correlated with the area of the tail of the epididymis. Sperm concentration was positively correlated with total motile sperm per ejaculate and total motility, whereas it was negatively correlated with total defects. Total motility was positively correlated with total motile sperm in the ejaculate and negatively correlated with total defects. Testicular consistency was positively correlated with vigor ($r=0.20$, $P<0.05$) and negatively correlated with inflammatory cells in the ejaculate ($r=-0.44$, $P<0.0002$). Vigor had a positive correlation with turbulence ($r=0.52$, $P<0.0001$). Correlation analysis is summarized in Table 4.

4. Discussion

This study provided a thorough, andrological evaluation of rams experimentally infected with *B. ovis*. To the best of our knowledge, this is the first report of using ultrasound to evaluate the kinetics of changes induced by *B. ovis*, although ultrasonography has been previously used for studying experimental *Arcanobacterium pyogenes*-induced orchitis (Gouletsou et al., 2004). In addition, we demonstrated that rams experimentally infected with *B. ovis* often develop clinical changes. However, in most of the cases, these clinical signs are highly non-specific. Therefore, this study suggests that, while ultrasonography improves clinical assessment of infected rams, a conclusive diagnosis under field conditions requires laboratory diagnosis (Xavier et al., 2009). Our results from experimental infection parallel clinical manifestations during natural infections because some of the experimental rams would have been considered asymptomatic in the field. Thus, ultrasonography increases the sensitivity of clinical assessment because it may help identify early changes during infection.

Importantly, microscopic examination of semen smears is a suitable screening method for the diagnosis of *B. ovis* infection, although other bacteria (i.e. *Actinobacillus seminis* or *Histophilus somni*) as well as infections of the reproductive and/or urinary tract can cause the same change. All experimentally infected rams in this study shed neutrophils and other inflammatory cells in the semen. Assessment of inflammatory cells in the semen has proven to be a suitable approach under other conditions as well (Bagley et al., 1984; Jansen, 1980). It is noteworthy that *B. ovis*-infected rams often shed the organism in the semen (Xavier et al., 2010), which is an important source of organisms for transmission. In cases of artificial insemination, the use of appropriate antibiotics in the extender inactivates the organism (Moustacas et al., 2010).

Lesions were evident in the tail of the epididymis and the seminal vesicles, and the pattern of inflammatory infiltrates observed in this study is similar to previous reports (Buckrell et al., 1985). According to Jansen (1980), histopathological lesions in naturally infected rams are more frequent in the seminal vesicles than in the epididymis, which is in concordance with our findings. The inflammatory process in the seminal vesicles may be a major source for inflammatory cells in the ejaculate. Considering that seminal vesicles cannot be assessed by traditional biometric methods, ultrasound has the advantage of allowing for noninvasive measurement of the seminal vesicles, which in this study provided clear demonstration of seminal vesicle involvement during *B. ovis* infection.

This study also helps establish normal and pathological parameters for ultrasonographic evaluation of the geni-

Table 4
Correlation between clinical, ultrasonographic and spermatic parameters from rams experimentally infected with *Brucella ovis*.

	TD	TM	TMSE	SC	EV	EC	LSV	DSV	LTERC	WTELC	LTTERC	WTERC	ATEL	ATER	LLTC	WLTC	RTLC	RTWC	ALT
TM	$r=-0.13$ $P<0.18$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TMSE	$r=-0.23$ $P<0.03$	$r=0.38$ $P<0.0006$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SC	$r=-0.25$ $P<0.02$	$r=0.21$ $P<0.04$	$r=0.84$ $P<0.0001$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EV	$r=0.02$ $P<0.40$	$r=0.05$ $P<0.32$	$r=0.40$ $P<0.0002$	$r=0.03$ $P<0.41$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EC	$r=-0.8$ $P<0.25$	$r=0.18$ $P<0.07$	$r=0.09$ $P<0.22$	$r=-0.01$ $P<0.45$	$r=0.13$ $P<0.14$	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LSV	$r=0.27$ $P<0.01$	$r=-0.15$ $P<0.11$	$r=-0.27$ $P<0.01$	$r=-0.39$ $P<0.0005$	$r=0.08$ $P<0.27$	$r=0.28$ $P<0.01$	–	–	–	–	–	–	–	–	–	–	–	–	–
DSV	$r=0.24$ $P<0.02$	$r=-0.11$ $P<0.18$	$r=-0.28$ $P<0.01$	$r=-0.35$ $P<0.002$	$r=0.03$ $P<0.41$	$r=0.23$ $P<0.03$	$r=0.85$ $P<0.0001$	–	–	–	–	–	–	–	–	–	–	–	–
LTERC	$r=0.17$ $P<0.07$	$r=-0.21$ $P<0.04$	$r=-0.17$ $P<0.07$	$r=-0.39$ $P<0.0004$	$r=0.34$ $P<0.002$	$r=0.38$ $P<0.001$	$r=0.62$ $P<0.0001$	$r=0.51$ $P<0.0001$	–	–	–	–	–	–	–	–	–	–	–
WTELC	$r=-0.02$ $P<0.41$	$r=-0.03$ $P<0.39$	$r=-0.13$ $P<0.13$	$r=-0.26$ $P<0.01$	$r=0.12$ $P<0.15$	$r=0.42$ $P<0.0001$	$r=0.23$ $P<0.03$	$r=0.23$ $P<0.03$	$r=0.37$ $P<0.001$	–	–	–	–	–	–	–	–	–	–
LTTERC	$r=0.16$ $P<0.10$	$r=0.02$ $P<0.43$	$r=-0.18$ $P<0.07$	$r=-0.39$ $P<0.0004$	$r=0.26$ $P<0.01$	$r=0.36$ $P<0.001$	$r=0.55$ $P<0.0001$	$r=0.40$ $P<0.0003$	$r=0.64$ $P<0.0001$	$r=0.37$ $P<0.001$	–	–	–	–	–	–	–	–	–
WTERC	$r=0.16$ $P<0.09$	$r=0.12$ $P<0.15$	$r=0.03$ $P<0.38$	$r=-0.11$ $P<0.2$	$r=0.24$ $P<0.02$	$r=0.36$ $P<0.001$	$r=0.16$ $P<0.1$	$r=-0.006$ $P<0.48$	$r=0.26$ $P<0.01$	$r=0.47$ $P<0.0001$	$r=0.44$ $P<0.0001$	–	–	–	–	–	–	–	–
ATEL	$r=0.03$ $P<0.40$	$r=0.02$ $P<0.44$	$r=-0.03$ $P<0.41$	$r=-0.6$ $P<0.31$	$r=-0.01$ $P<0.44$	$r=0.55$ $P<0.0001$	$r=0.51$ $P<0.0001$	$r=0.50$ $P<0.0001$	$r=0.37$ $P<0.001$	$r=0.30$ $P<0.005$	$r=0.40$ $P<0.0002$	$r=0.22$ $P<0.03$	–	–	–	–	–	–	–
ATER	$r=0.02$ $P<0.42$	$r=0.07$ $P<0.26$	$r=-0.07$ $P<0.29$	$r=-0.22$ $P<0.03$	$r=0.16$ $P<0.09$	$r=0.73$ $P<0.0001$	$r=0.59$ $P<0.0001$	$r=0.49$ $P<0.0001$	$r=0.50$ $P<0.0001$	$r=0.49$ $P<0.0001$	$r=0.64$ $P<0.0001$	$r=0.38$ $P<0.001$	$r=0.76$ $P<0.0001$	–	–	–	–	–	–
LLTC	$r=0.10$ $P<0.21$	$r=0.24$ $P<0.02$	$r=0.10$ $P<0.19$	$r=-0.10$ $P<0.19$	$r=0.21$ $P<0.03$	$r=0.61$ $P<0.0001$	$r=0.34$ $P<0.002$	$r=0.30$ $P<0.01$	$r=0.42$ $P<0.001$	$r=0.42$ $P<0.0001$	$r=0.34$ $P<0.002$	$r=0.34$ $P<0.002$	$r=0.57$ $P<0.0001$	$r=0.55$ $P<0.0001$	–	–	–	–	–
WLTC	$r=0.02$ $P<0.41$	$r=0.26$ $P<0.01$	$r=0.15$ $P<0.11$	$r=0.13$ $P<0.14$	$r=0.02$ $P<0.41$	$r=0.61$ $P<0.0001$	$r=0.10$ $P<0.20$	$r=0.10$ $P<0.2$	$r=0.42$ $P<0.0001$	$r=0.34$ $P<0.002$	$r=0.34$ $P<0.002$	$r=0.34$ $P<0.002$	$r=0.50$ $P<0.0001$	$r=0.38$ $P<0.001$	$r=0.50$ $P<0.0001$	–	–	–	–
RTLC	$r=0.02$ $P<0.43$	$r=0.23$ $P<0.03$	$r=0.12$ $P<0.17$	$r=-0.39$ $P<0.0004$	$r=0.26$ $P<0.01$	$r=0.67$ $P<0.001$	$r=0.35$ $P<0.001$	$r=0.37$ $P<0.001$	$r=0.81$ $P<0.0001$	$r=0.63$ $P<0.0001$	$r=0.35$ $P<0.001$	$r=0.28$ $P<0.01$	$r=0.60$ $P<0.0001$	$r=0.56$ $P<0.0001$	$r=0.81$ $P<0.0001$	$r=0.63$ $P<0.0001$	–	–	–
RTWC	$r=0.11$ $P<0.17$	$r=0.07$ $P<0.29$	$r=0.03$ $P<0.40$	$r=0.06$ $P<0.30$	$r=-0.03$ $P<0.39$	$r=0.52$ $P<0.0001$	$r=0.08$ $P<0.25$	$r=0.06$ $P<0.3$	$r=0.16$ $P<0.1$	$r=0.29$ $P<0.01$	$r=0.19$ $P<0.06$	$r=0.23$ $P<0.03$	$r=0.50$ $P<0.0001$	$r=0.41$ $P<0.0002$	$r=0.39$ $P<0.001$	$r=0.76$ $P<0.0001$	$r=0.55$ $P<0.0001$	–	–
ALT	$r=0.03$ $P<0.39$	$r=0.23$ $P<0.03$	$r=0.07$ $P<0.27$	$r=0.02$ $P<0.44$	$r=-0.3$ $P<0.40$	$r=0.73$ $P<0.0001$	$r=0.32$ $P<0.004$	$r=0.37$ $P<0.001$	$r=0.21$ $P<0.04$	$r=0.35$ $P<0.001$	$r=0.27$ $P<0.01$	$r=0.22$ $P<0.03$	$r=0.74$ $P<0.0001$	$r=0.61$ $P<0.0001$	$r=0.61$ $P<0.0001$	$r=0.60$ $P<0.0001$	$r=0.60$ $P<0.0001$	$r=0.57$ $P<0.0001$	–
ART	$r=-0.02$ $P<0.44$	$r=0.13$ $P<0.14$	$r=0.01$ $P<0.46$	$r=-0.71$ $P<0.24$	$r=0.06$ $P<0.32$	$r=0.73$ $P<0.0001$	$r=0.47$ $P<0.0001$	$r=0.48$ $P<0.0001$	$r=0.37$ $P<0.001$	$r=0.41$ $P<0.0002$	$r=0.47$ $P<0.0001$	$r=0.28$ $P<0.01$	$r=0.76$ $P<0.0001$	$r=0.70$ $P<0.0001$	$r=0.65$ $P<0.0001$	$r=0.48$ $P<0.0001$	$r=0.70$ $P<0.0001$	$r=0.48$ $P<0.0001$	$r=0.85$ $P<0.0001$

Abbreviations: ART = area of right testicle; ALT = area of the left testicle; RTWC = right testis width – caliper; RTLC = right testicle length – caliper; WLTC = width of the left testicle – caliper; LLTC = length of the left testicle – caliper; ATER = tail area of the right epididymis; ATEL = tail area of the left epididymis; WTERC = width of the tail of the right epididymis – caliper; LTTERC = length of the tail of the right epididymis – caliper; WTELC = width of the tail of the left epididymis – caliper; LTERC = length of the tail of the left epididymis – caliper; DSV = depth seminal vesicle; LSV = length seminal vesicle; EC = scrotal circumference; TMSE = total motile sperm in ejaculate; TM = total motile; TD = total defects; SC = sperm concentration; EV = ejaculate volume.

tal system in rams. Reference values for ultrasonographic measurements, as well as a clear description of the normal morphology, are essential for studying pathological processes of the male genital system (Chandolia et al., 1997; Eilts et al., 1993; Griffin and Ginther, 1992; Ülker et al., 2005). In this study, we were able to perform ultrasonographic volume measurements of the testis, epididymis, and seminal vesicles, which were then correlated with other andrologic parameters. For example, the reduction in testes area values after infection with *B. ovis*, which was precisely detected by ultrasonography, is likely due to the intimate anatomical relationship of the testes and epididymides. This relationship may compromise testicular thermoregulation because of the inflammatory process in the epididymis (Gouletsou et al., 2004). Changes in echogenicity and increased volume were observed in the tail of the epididymis in response to infection. Furthermore, some of the ultrasonographic findings observed in this study (particularly in ram 3) are compatible with previously described ultrasonographic findings in rams with sperm granulomas (Karaca et al., 1999).

Pathological changes observed in the seminiferous epithelium, which was reflected in morphometric parameters, are consistent with a diagnosis of testicular degeneration (Costa et al., 2007; Gouletsou et al., 2004). Testicular degeneration has been described in experimental infection with *Arcanobacterium pyogenes*, which results in degeneration mostly during the acute phase of infection (Gouletsou et al., 2004).

Sperm quality decreased in response to infection. Moreover, a negative correlation was noted between the area of the tail of the epididymis as measured by ultrasonography and sperm concentration. Increased area of the tail of the epididymis, in this case, reflects the acute phase of infection and inflammation. In addition, some of the sperm defects observed in this study may be attributed to inflammation in the tail of the epididymis (Barth and Oko, 1989; Cupp and Briggs, 1965). Moreover, inflammatory cells in the ejaculate may also induce sperm tail defects (Aziz et al., 2010). Leukocytes in the semen increase sperm abnormalities due to oxidative stress on phospholipids of the sperm membrane affecting permeability of the membrane (Lackner et al., 2010; Zalata et al., 1998).

Andrological findings observed in this study parallel those described in naturally infected rams. *B. ovis* often causes asymptomatic infections, but it is interesting that symptomatic infections often result in unilateral changes (Bagley et al., 1985; Hughes and Claxton, 1968; Kennedy et al., 1956). A sperm granuloma was also diagnosed in this study, and it has been clinically associated with *B. ovis* infection in the field (Karaca et al., 1999; Xavier et al., 2009). In this study, some of the andrological changes were transient and more prominent during the initial stages of infection, which is in agreement with previous reports that rams infected with *B. ovis* can become asymptomatic a few weeks post-infection (Cameron and Laurner, 1976).

In conclusion, we report a thorough, andrologic and ultrasonographic evaluation of the kinetics of changes during *B. ovis* infection in rams. These results demonstrate that *B. ovis* has a detrimental effect on sperm quality even in the absence of significant clinical changes. These results also demonstrate the need for laboratory confirmation to obtain a conclusive diagnosis under field conditions.

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