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Short communication

Follicular degeneration in the ovaries of goats experimentally infected with *Trypanosoma vivax* from the Brazilian semi-arid region

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ABSTRACT

Infection by *Trypanosoma vivax* and other African trypanosomes plays an important role in reproductive disorders in male and female livestock. Outbreaks of *T. vivax* in the semi-arid region of northeastern Brazil are characterized by wasting disease in cattle, sheep and goats with hematological, cardiac and nervous compromises in addition to reproductive failures. Similar to reports from Africa, we previously observed a reduction in fertility rates and severe testicular degeneration and epididymitis in male sheep infected with *T. vivax* from this region. Although anestrus is frequently reported in goats and sheep infected with *T. vivax*, the effects of this infection on the female reproductive organs need clarification. In this study, we addressed this issue through a histopathological evaluation of ovarian follicular morphology and classification in goats experimentally infected with a *T. vivax* isolate from the Brazilian semi-arid region. The infected animals presented typical clinical signs of trypanosomosis by *T. vivax*, including anemia, hyperthermia, pallor of the mucous membranes, enlarged lymph nodes, and progressive loss of weight. All the infected goats remained anestrus throughout the experimental period and exhibited important disturbances in the ovaries, evidenced by reduced size and a smooth surface without follicles or corpora lutea, and abnormal follicular development. In addition, through PCR, we detected *T. vivax* DNA in the ovarian tissues of the infected goats. Our findings contributed to understand the female reproductive failure associated with trypanosomosis caused by *T. vivax*.

1. Introduction

The African trypanosomes *Trypanosoma brucei brucei*, *Trypanosoma congolense* and *Trypanosoma vivax* are the agents of an important livestock disease known as Nagana in Africa, where these species are cyclically transmitted by the tsetse fly. Nagana strongly compromises the productive and reproductive performance of livestock. Trypanosomosis caused by *T. vivax* can be a highly debilitating and fatal disease in domestic ruminants, mainly due to the hematological disturbances that induce severe anemia and inflammatory foci in the central nervous system (CNS), heart, liver, spleen and lymph nodes (Gardiner et al., 1989; Desquesnes, 2004; Chamond et al., 2010).

Infection by *T. vivax* plays an important role in the reproduction failures of both male and female livestock (Masake, 1980; Gardiner and Mahmoud, 1992). Reproductive disorders in males include delayed puberty, loss of libido, and severe degenerative changes of the genitalia. Testicular atrophy, degeneration and calcification have been

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documented in sheep and goats infected with *T. vivax* resulting in very poor quality semen, and may lead to a total lack of spermatogenesis. Pathological changes in *T. vivax*-infected males include epididymitis and orchitis (Isoun and Anosa, 1974; Anosa and Isoun, 1980; Sekoni et al., 1988, 1990a,b; Okech et al., 1996; Bezerra et al., 2008; Mbaya et al., 2011).

In females, trypanosomosis causes temporary or permanent anestrus, abnormal estrus cycles (Ogwu et al., 1984) and rapid decline in milk production (Batista et al., 2007). Additionally, *T. vivax* infection has induced abnormal pregnancy, dystocia, abortion, premature and low birth weights, stillbirths, transplacental fetal infection, neonatal death and other pathogenic effects on fetuses and offspring (Ogwu et al., 1986; Okech et al., 1996; Batista et al., 2012).

T. vivax was introduced into South America by cattle imported from Africa. Outside Africa, this species is only mechanically transmitted by hematophagous flies such as *Tabanus* spp. and *Stomoxys* spp. The parasite is now endemic in some regions of Brazil, Venezuela and Bolivia (Jones and Dávila, 2001; Desquesnes, 2004; Gardiner and Mahmoud, 1992; Garcia et al., 2005; Silva et al., 1999; Osório et al., 2008). In Brazil, *T. vivax* has been reported in cattle, sheep, goat and buffalo herds from the northern to the southern regions (Silva et al., 1996; Batista et al., 2007, 2009, 2012; Cuglovici et al., 2010; de Araujo Melo et al., 2011; Galiza et al., 2011). Recently, horses infected with *T. vivax* were found for the first time in Brazil (Da Silva et al., 2010).

Recent studies of outbreaks of trypanosomosis by *T. vivax* in the semi-arid region of northeastern Brazil showed very devastating and often fatal disease that creates serious economic losses in cattle, goat and sheep breeding operations due to productive and reproductive problems (Batista et al., 2007, 2009, 2012; Galiza et al., 2011). We previously evaluated the pathogenicity for sheep of one *T. vivax* isolate from the Brazilian semi-arid region. In this study, in addition to severe haematological and neurological disorders, the infected males showed severe testicular degeneration and epididymitis, and DNA of the parasite was detected in testicular and epididymal tissues using a *T. vivax*-specific PCR (Bezerra et al., 2008).

There is a paucity of information on the effects of *T. vivax* infection on the female reproductive organs of ruminants in Africa and South America. A previous study reported numerous cysts and parasites in smears from the ovaries of sheep infected with *T. vivax* in Nigeria, West Africa (Isoun and Anosa, 1974). The main goal of this study was to evaluate the effects of *T. vivax* infection on the ovaries of goats experimentally infected with a virulent isolate from the Brazilian semi-arid region.

2. Materials and methods

2.1. Composition of the experimental groups and experimental infection

In this study, we used ten female mixed breed goats, approximately 15 months of age, housed in a properly screened stall at the Veterinary Hospital of the University of the Semi-Arid (UFERSA), Mossoró, Rio Grande do Norte, Brazil. For 14 days before the inoculation of *T. vivax*, the goats were evaluated by clinical and hematological examination with approval from the local ethics committee in the use of animals of UFERSA-CEUA (process n° 23091.1901/10-98). Blood samples from all the animals were tested using a *T. vivax*-specific diagnostic PCR (Cortez et al., 2009) before and during the experimental period conducted in April and May (winter season). The goats were treated with the anthelmintic Ivermectin (Ivomec®). Healthy animals were randomly distributed into two experimental groups: one group of six goats infected with *T. vivax* (goats 1–6) and a control group composed of four goats not infected by *T. vivax* (goats 7–10). All the animals were kept under identical management conditions and were fed with Tifton hay (*Cynodon* sp.) supplemented with commercial food at 1.5% of their body weight per day, with water *ad libitum*.

The isolate of *T. vivax* used for the experimental infections was obtained from a sheep during an outbreak in São João do Rio do Peixe, Paraíba, in the Brazilian semi-arid region, where severe hematological and nervous symptoms were reported (Galiza et al., 2011). Blood samples were collected from a sheep showing very high parasitemia using 10% EDTA (ethylenediaminetetraacetic acid disodium), mixed with 8% glycerol, distributed in aliquots and frozen in liquid nitrogen. Immediately before inoculation, the cryopreserved parasites were thawed, and each animal was inoculated intravenously with 1.25×10^5 trypomastigotes of *T. vivax* as described previously (Batista et al., 2007, 2012).

2.2. Clinical exams, PCV and parasitemia assessment

Daily for 60 days post infection (dpi), the animals from both the infected and the control groups were clinically examined to assess rectal temperature and status of mucous membrane and external lymph nodes. We also performed a daily inspection of the animals for signs that indicate the occurrence of estrus, such as restlessness, sexual receptivity, edema and hyperemia of the vulva, and the presence of vaginal discharge.

Parasitemia was determined daily by microscopic determination of the number of parasites in 5 μl of peripheral blood collected from the ear and dispersed between two glass slides as standardized previously (Batista et al., 2007). At the same time, blood was collected by puncture of the jugular vein into sterile tubes containing 1.0 mg/ml EDTA for the PCV analysis and DNA preparations (Cortez et al., 2009).

2.3. Collection, macroscopic and histologic evaluation of ovaries

Surgical collection of the ovaries and macroscopic evaluation were performed 60 days after infection. Several representative pieces of ovarian cortex were fixed in Bouin solution for 48 h and preserved in 99% ethanol. The fixed tissues were embedded in paraffin, sectioned at 4.0 μm thickness and stained with hematoxylin and eosin. For qualitative assessment, 30 follicles from each ovary (right and left) per animal were morphologically classified as

normal or degenerated follicles. Follicles are classified into four classes according to the stage of follicular development: (a) primordial, formed by one layer of flattened or flattened-cuboidal granulosa cells around the oocyte, (b) primary, formed by a single layer of cuboidal granulosa cells around the oocyte, (c) secondary, constituted by an oocyte surrounded by two or more layers of cuboidal granulosa cells, and (d) tertiary, characterized by the presence of an antral cavity (Chaves et al., 2008).

The follicular morphology was evaluated taking into account the integrity of the basement membrane, the cellular density, the presence or absence of pyknotic nuclei and the integrity of the oocyte (Chaves et al., 2008). Based on these parameters, each follicle was classified as normal, type I degenerated (only the oocytes showed degeneration) or type II degenerated (the oocyte and granulosa cells were degenerated). Only follicles in which the oocyte nucleus was apparent were counted to avoid counting the same follicle twice.

2.4. Diagnosis by PCR in samples from blood and ovarian tissues

Samples of approximately 500 μL of blood and 1.0 cm^3 of ovarian tissue were collected, washed three times in PBS and preserved in ethanol for DNA preparation. The DNA samples obtained from approximately 200 μL of blood and approximately 0.3 cm^3 of tissue were subjected to the highly sensitive PCR assay (TviCatL-PCR) specific for *T. vivax*, which targets repeated gene sequences that encode the cysteine protease cathepsin L-like enzyme (Cortez et al., 2009). The DNA of *T. vivax* (from Catolé, Paraíba, Brazil) was used as the positive control for the PCR reactions, and the DNA from the blood and ovarian tissues of non-infected goats used as the negative controls.

2.5. Statistical analysis

An analysis of variance (ANOVA) was used to detect the differences between the treatments, followed by the use of the Tukey's test at a 5% significance ($p < 0.05$) level for comparison the means of parasitemia, PCV and rectal temperature for each observation time. We also compared the differences between the infected and control animals regarding morphology of the ovarian follicles.

3. Results

3.1. Clinical signs, PCV and parasitemia of infected goats

The goats infected with *T. vivax* showed hyperthermia starting on day 7 after infection with a maximum temperature of 41 °C on the 8th dpi. The average temperatures of these animals remained higher than the average values of the non-infected goats until the end of the experiment (Fig. 1). The acute phase, which persisted for approximately two weeks, was characterized by increased parasitemia and hyperthermia. The temperature of the infected animals remained high until the end of the experimental period, independent of the variations in parasitemia (Fig. 1).

Trypanosomes were found in the smears of peripheral blood from all the infected animals from the 3rd dpi. The parasitemia increased progressively, reaching a maximum between the 7th and 14th dpi, and then quickly decreased to low peaks alternating with periods lacking parasitemia detectable by direct examination of the blood smears (Fig. 2).

The infected goats, from the 15th dpi until the end of experiment, showed pallor of the mucous membranes, enlarged lymph nodes, apathy and progressive anorexia.

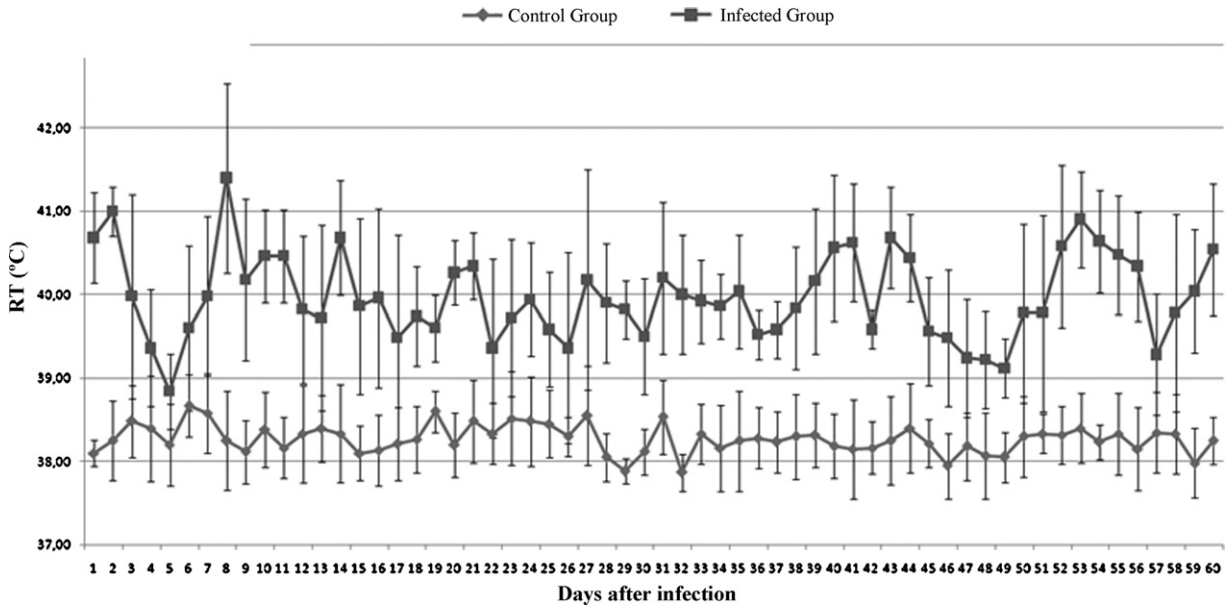

Fig. 1. Mean values of rectal temperatures (TR) °C in goats experimentally infected with *Trypanosoma vivax* and non-infected goats (control group) during the experimental period.

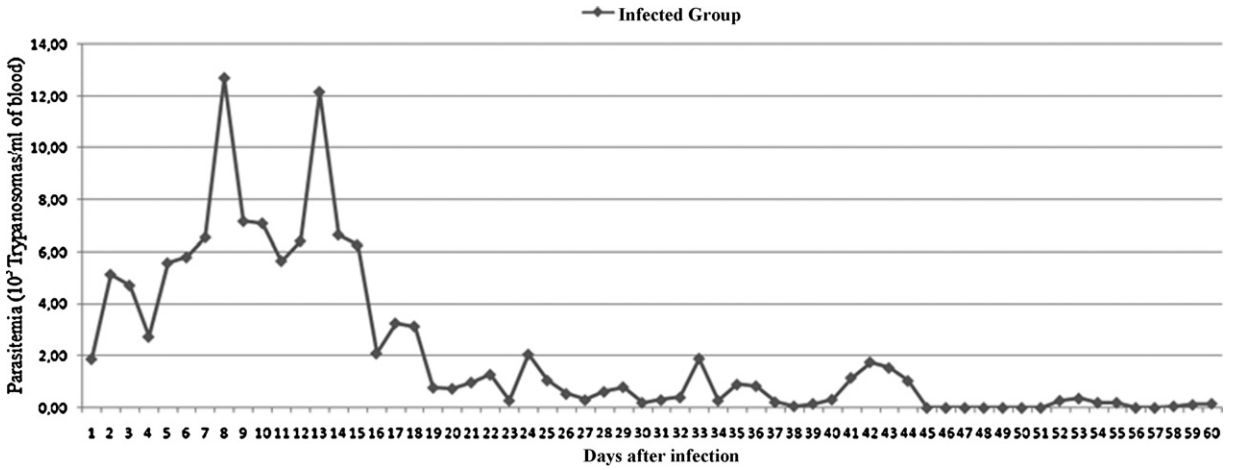

Fig. 2. Mean values of parasitemia ($\times 10^5$ trypanosomes/ml blood) of goats experimentally infected with *Trypanosoma vivax* during the experimental period.

Goat number 4 showed a worsening of clinical symptoms and, at the 9th dpi, was found dead. The chronic phase of the disease was characterized by low parasitemia, severe anemia and loss of weight. All the goats in the infected group remained anestrus throughout the experimental period, whereas the control animals showed at least one occurrence of estrus during this period.

From the 7th dpi until the end of the experiment, the five remaining infected goats showed a significant reduction ($p < 0.05$) in the mean PCV, whereas the average PCV in the control group remained within the normal range for goats. The decrease of PCV, *i.e.*, enhanced anemia, was drastic in the infected animals, reaching 11%, which corresponds to 70% below the normal value for the species examined (Fig. 3).

3.2. Macroscopic and histological evaluation of the ovaries

Macroscopic examination evidenced gross differences on the ovaries from the infected animals compared to the normal aspect of ovaries from the control goats. Ovaries from infected goats presented reduced size and a smooth surface without follicles or corpora lutea. In contrast, the animals of the control group showed ovaries with normal size, growing follicles and corpora lutea visible on the surface, as expected for healthy animals (Fig. 4).

The degrees of follicular degeneration were assessed and graded by histopathology (Table 1). Histological analysis showed that the ovaries of goats in the infected group exhibited a significant reduction ($p < 0.05$) in the number of

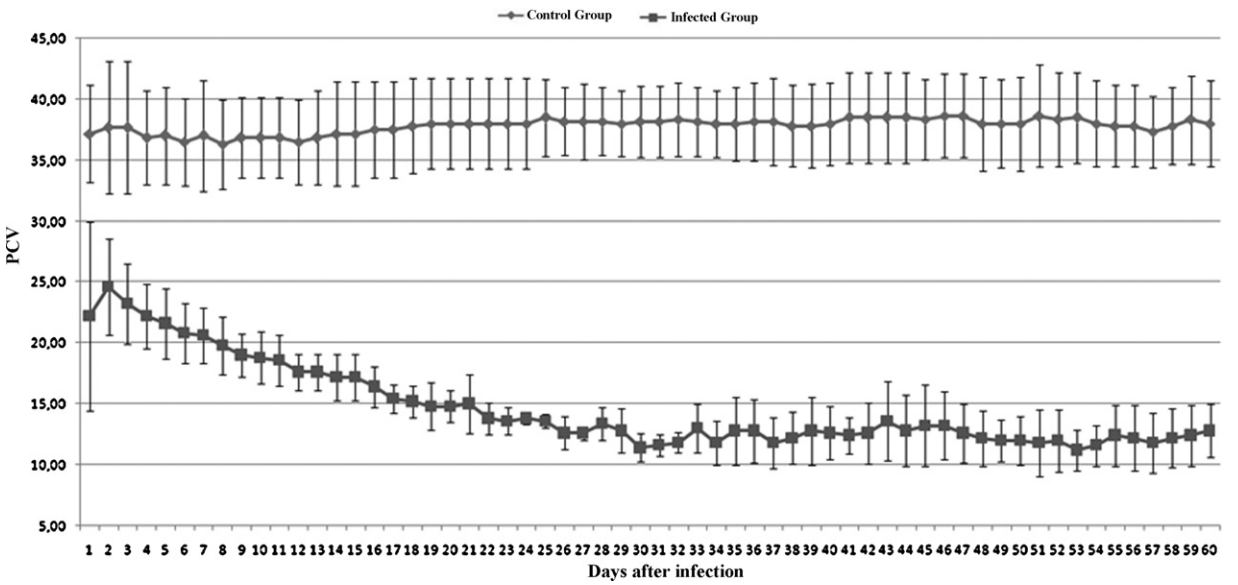

Fig. 3. Mean values of PCV in goats experimentally infected with *Trypanosoma vivax* and non-infected goats (control group) during the experimental period.

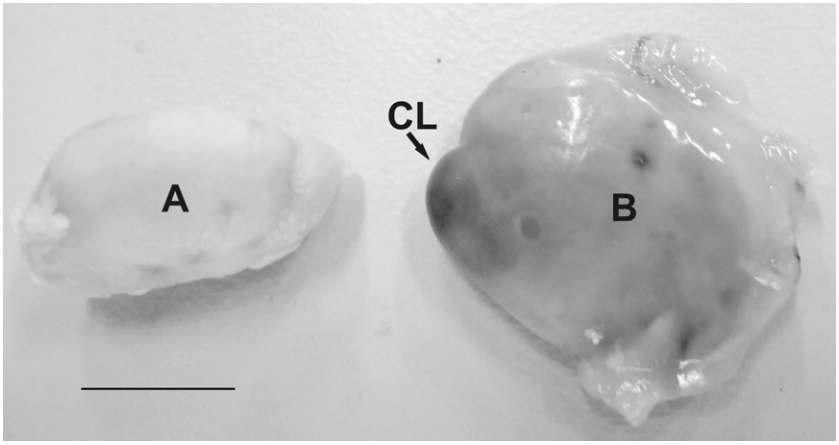

Fig. 4. (A) Macroscopic aspect from the ovary of a goat infected with *Trypanosoma vivax* evidencing reduced size and surface without follicles or corpora lutea. (B) Normal ovary from a non-infected goat showing the corpora lutea (CL). Scale bar = 1 cm.

follicles compared to those from the control group. Regarding the stage of normal follicles development, infected animals showed no significant difference between the number of primordial, primary, secondary and tertiary follicles. In contrast, in the non-infected animals, the number of primordial and primary follicles was significantly higher ($p < 0.05$) than the number of secondary and tertiary follicles.

Concerning the integrity of the follicles, no significant difference was detected in the average value of the type I degenerated follicles between the control and infected goats. The number of type II degenerated follicles was significantly higher ($p < 0.05$) in the infected group compared to the control group. Most of the follicles degenerate type II observed in the infected group was primordial and primary (Table 1). Morphologically, follicles from non-infected goats have a spherical or elliptical oocyte with a central nucleus and uniform cytoplasm, granulosa cells well organized into layers around the oocyte, and a distinguishable intact basement membrane. The degenerated follicles of type I showed a pyknotic nucleus, while those of type II showed the pyknotic nucleus with retraction of the oocyte and turgid and disorganized granulosa cells (Fig. 5).

3.3. Detection of *T. vivax* in the blood and ovarian tissue samples

Positive results for TviCatL-PCR, with the amplification of a DNA fragment of approximately 177 bp and specific for *T. vivax*, were obtained using DNA preparation from the blood samples taken from all the infected goats, even for samples collected on the 60th dpi from animals showing very low or negative parasitemia by blood smear examination. In addition, the analysis using this PCR assay for the evaluation of *T. vivax* DNA in the ovarian tissues also showed positive results for both ovaries of all the infected goats. The intensity of the amplified DNA bands was variable for both the blood and ovarian tissue samples, as well as for the samples from the right and left ovaries of the same animals (goats 1, 3 and 6). The PCR tests were negative for all the blood and tissue samples of the control animals.

4. Discussion

The goats experimentally infected with a *T. vivax* isolate from Brazilian semi-arid region in the present study showed an acute phase characterized by peaks of parasitemia between the 7th and 14th dpi, and a chronic phase characterized by waves of parasitemia alternating with undetectable parasitemia. The PCV values showed a progressive reduction, reaching a drastic reduction of 50% at the end of experimental period.

The clinical and pathological features of the experimentally infected goats corroborated the high pathogenicity and virulence of the isolate of *T. vivax* used in this study, which was obtained from a sheep with severe hematological and nervous disturbances (Galiza et al., 2011). In animals infected with *T. vivax* from this region,

Table 1
Analysis of the integrity of the oocyte and classification of ovarian follicles according to the stage of follicular development (from primordial to tertiary) from goats experimentally infected with *Trypanosoma vivax*, and comparison with results from non-infected goats (mean values and standard deviation).

Follicle category	Control group			Infected group		
	Normal	Degenerate type I	Degenerate type II	Normal	Degenerate type I	Degenerate type II
Primordial	21.33 ± 5.13 a A	1.50 ± 2.38 a B	7.00 ± 4.97 a B	3.67 ± 2.58 a B	1.33 ± 1.03 a B	18.83 ± 5.46 a A
Primary	17.35 ± 6.56 ab A	0.50 ± 1.01 a B	3.25 ± 0.50 ab B	3.67 ± 2.50 a B	1.33 ± 1.97 a B	18.17 ± 4.22 a A
Secondary	8.25 ± 6.40 bc A	0.00 ± 0.00 a B	1.75 ± 2.87 ab AB	2.83 ± 2.23 a AB	0.33 ± 0.52 a B	4.20 ± 3.35 b AB
Tertiary	2.75 ± 3.10 c A	0.00 ± 0.00 a A	0.00 ± 0.00 b A	1.50 ± 1.38 a A	0.00 ± 0.00 a A	1.83 ± 1.83 b A
Total	49.68 ± 18.20 a A	2.0 ± 0.70 b A	12.0 ± 2.97 b A	11.67 ± 1.02 a B	2.99 ± 0.68 a A	43.03 ± 8.99 b B

Means followed by same letter in column do not differ at 5% significance level. Means followed by same capital letter in line, do not differ at 5% significance level.

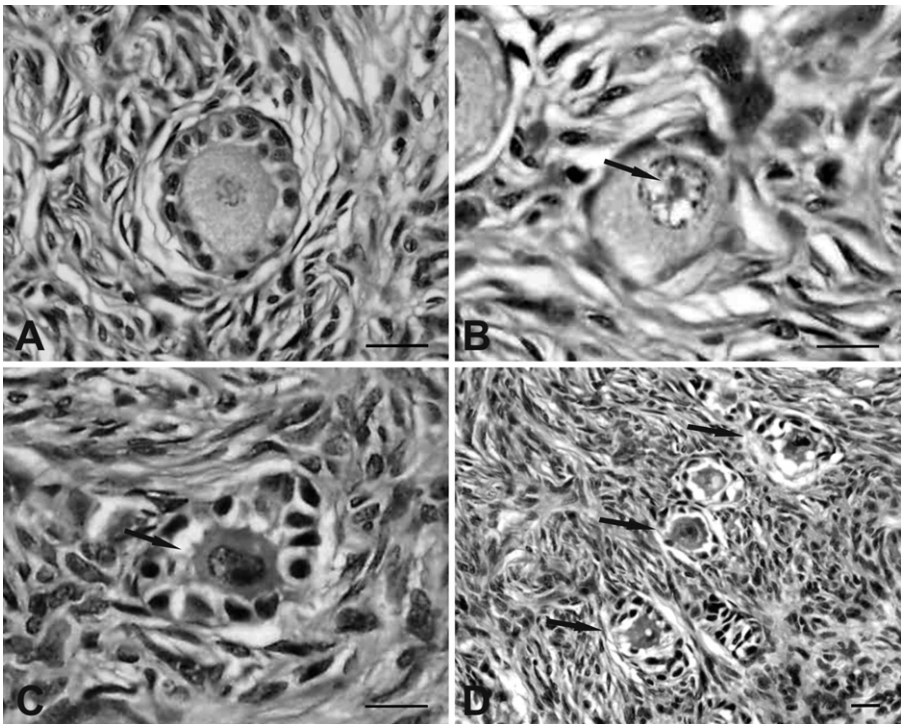

Fig. 5. Histological aspects of ovarian fragments stained with hematoxilin–eosin of goats experimentally infected with *Trypanosoma vivax*. (A) Morphologically normal ovarian follicles; (B) pyknotic nuclei (arrow) (follicle degeneration of type I); (C) follicle degenerated pyknotic nuclei and disorganization of the layers of granulosa cells (arrow) (follicle degeneration of type II); (D) follicle degeneration of type II (arrows). Scale bar = 10 μm, hematoxilin–eosin staining.

histopathological findings confirmed the parasite tropism for the CNS, as evidenced by detection of parasite in CNS tissues by PCR (Batista et al., 2007, 2011; Galiza et al., 2011). *T. vivax* was proved able to invade different organs and tissues and the pathogenesis of inflammatory and degenerative lesions has been associated with intravascular or intrasinusal trypanosomes, microtrombs of parasites and cells, immune complex and deleterious immunological responses in various organs such as the heart, spleen, CNS, lymph nodes (Masake, 1980; Kimeto et al., 1990; Batista et al., 2007, 2011; Chamond et al., 2010; Galiza et al., 2011), aqueous humor of the eye (Whitelaw et al., 1988), testicles and epididymis (Isoun and Anosa, 1974; Bezerra et al., 2008).

In this study, animals at the chronic phase of the disease caused by *T. vivax* showed clear macroscopic and histological alterations in ovarian morphology. The small sized ovaries with no follicles or apparent corpora lutea are characteristic of highly reduced or absent ovarian activity in animals showing anestrus. Histological analysis evidenced a significant reduction in the number of normal primordial and primary follicles in the infected goats while the number of follicles in the other categories remained unchanged, evidencing abnormal initial follicular development. Follicles development shown by the non-infected goats are in agreement with the estimated for healthy ruminants. In ruminants, approximately 90% of the population is represented by primordial follicles, which are the precursors of the follicles that reach the ovulatory stage (Chaves et al., 2008).

Regarding the assessment of follicular integrity, a high number of follicles of degenerated type II were found in the infected animals. The enhanced type II follicular degeneration affected the reserve pool of follicles and reduced the number of viable follicles. This finding may explain the reduction or even the interruption of cyclic ovarian activity and subsequent anestrus of goats infected with *T. vivax* in this study and in field-infected ruminants (Batista et al., 2007, 2009, 2012). Follicular degeneration and apoptosis are physiological processes responsible for normal follicular atresia or regression; a large proportion of preantral follicles normally does not ovulate and are naturally eliminated (Hussein, 2005).

The presence of *T. vivax* DNA in the ovarian tissues, detected by a species-specific TviCatL-PCR assay, was an unprecedented finding, indicating migration of the parasite through the ovaries. The absence of inflammatory response in the ovaries of infected goats in this and in previous study (Isoun and Anosa, 1974) suggested that the parasites might not migrate into parenchyma of the ovaries. However, highly positive PCR reaction using DNA preparation from ovary tissues of some infected goats at chronic phase, when animals showed very low or negative parasitemia by blood smear examination, suggested migration of the parasites to the ovaries. Using the same PCR assay, we previously had detected *T. vivax* DNA in tissue samples from the parenchyma of the testis from *T. vivax*-infected sheep showing severe testicular degeneration and epididymitis (Bezerra et al., 2008). However, any association between

the presence of *T. vivax* (parasite or parasite DNA) in testicular and ovarian tissues, intra- or extravascular localization of parasites in these organs, and the reproductive disorders reported in this and in previous studies (Isoun and Anosa, 1974; Adamu et al., 2007; Bezerra et al., 2008) require further investigations.

In agreement with our findings in the present work, a previous study (Isoun and Anosa, 1974) of sheep infected with a Nigerian isolate demonstrated the presence of *T. vivax* in smears from the ovaries exhibiting numerous cysts in the absence of any inflammatory response. The etiopathogenic mechanisms of reproductive failure in females infected with *T. vivax* are still unknown. It is believed that these changes occur by a combination of factors, including high body temperature and hematological, metabolic, hormonal and tissular disorders (Zwart et al., 1991; Van Dam et al., 1996). Infection by African trypanosomes induced degeneration of the hypothalamus, pituitary glands and gonads, with consequent disturbances in the production of the hormones necessary for normal reproductive processes (Masake, 1980).

Corroborating important ovarian disturbances in small ruminants, this study evidenced anestrus and atrophy, abnormal ovary follicular development and the presence of *T. vivax* DNA in the ovaries of goats infected with a Brazilian isolate of *T. vivax* showing anestrus. Our findings greatly contributed to the understanding of the reproductive failure of female ruminants associated with trypanosomosis by *T. vivax*.

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