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Toxoplasma gondii transmission by artificial insemination in sheep with experimentally contaminated frozen semen

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ABSTRACT

Toxoplasma gondii is a parasite considered one of the major causes of reproductive problems in sheep. Furthermore, the presence of the agent in ram semen urges the possibility of sexual transmission in this species. The aim of this study was to evaluate if ram's frozen semen spiked with *T. gondii* tachyzoites would be able to cause infection in sheep by laparoscopic artificial insemination (AI). Nine ewes tested seronegative to anti-*T. gondii* antibodies by the modified agglutination test (MAT) were superovulated and inseminated to collect embryos. Animals were divided into two groups: G1 (n = 5), ewes inseminated with semen containing 4×10^7 tachyzoites; and G2 (n = 4), ewes inseminated with tachyzoite-free semen (control group). To confirm infection, ewe's blood samples were collected on days −14, −7, 0, 7, 14, 21, 28, 35, 49 and 57 after AI for analysis by MAT and PCR. Tissue samples of these ewes were also collected for histopathology and immunohistochemistry (IHC). Seven days after AI, all ewes of group G1 had specific antibodies to *T. gondii*, while those of G2 were negative. *Toxoplasma gondii* DNA was detected in the blood of one ewe and parasites were observed in tissues of all five animals inseminated with contaminated semen, indicating that semen freezing protocol does not affect *T. gondii* transmission by artificial insemination in sheep.

1. Introduction

Toxoplasma gondii is a parasite of warm blooded animals, including humans, and felids are the definitive hosts [1–3]. Sheep toxoplasmosis is an important cause of economic losses due to reproductive disorders [4,5] and horizontal transmission occurs by ingestion of oocysts shed by cats in the environment [6]. Vertical transmission is particularly important for pregnant ewes [7], since it can cause serious damage to the offspring [8].

Toxoplama gondii DNA has been detected in semen of various animal species [9–12] and venereal transmission of the parasite has also been tested in dogs, sheep and goats [13–16].

In sheep, transmission was proved experimentally by natural mating [14] and infection also reported by artificial insemination (AI) with the use of fresh semen experimentally contaminated with tachyzoites [15]. However, there is no information on the literature regarding the transmission of this parasite by AI of ram semen after freezing. The aim of this study was to evaluate the *T. gondii* transmission in sheep via laparoscopic AI after freezing of semen experimentally contaminated with tachyzoites.

2. Materials and methods

The protocols used in this study were approved by the Ethics Committee of Animal Use (CEUA) from Universidade Federal Fluminense (UFF), Rio de Janeiro, Brazil under protocol number 260. Animals' management followed the ethical principles for the use of research animals established by the National Committee for Animal Experimentation Control [17,18].

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2.1. Animals

Nine Santa Inês ewes were serologically tested negative for toxoplasmosis and other reproductive infectious diseases and those seronegative were selected for this study. An adult Santa Inês ram, also seronegative for toxoplasmosis, was used as a semen donor for artificial insemination. Before the start of the study all animals were vaccinated to clostridiosis.

2.2. Semen collection and analysis

Semen was collected using an artificial vagina (at 42 °C), immediately transferred to the laboratory and analyzed according to Brazilian College of Animal Reproduction criteria [19]. The volume of ejaculate was recorded directly from the graduated collection tube. The sperm progressive motility was determined by phase-contrast microscopy at a magnification of ×200, on a warm stage at 37 °C. The percentage of motile spermatozoa was assessed with a scale of 0–100% and sperm concentration determined using improved Neubauer counting chamber. A drop of thoroughly mixed 200 fold diluted semen (with 0.9% NaCl) was placed on the chamber. The grid was observed by a phase contrast microscope at a magnification of ×200 and sperm concentration was determined as sperm cells/ml. The total volume of diluent to be used was calculated to give a concentration of 25×10^6 viable spermatozoa per straw (0.25 ml).

2.3. Cultivation and concentration of T. gondii tachyzoites

Toxoplasma gondii tachyzoites of the RH strain were cultivated by intraperitoneal passages in Swiss Webster mice. For recovery of the parasites from peritoneal exudates, sick mice were killed, bled out, and skinned to expose the abdomen. Tachyzoites were aspirated from mice peritoneal cavities by injecting 5 ml of phosphate buffered saline (PBS) with a 21 gauge needle and a 5 ml syringe [2], centrifuged at 1.500×*g* for 10 min and counted in Neubauer chamber using a phase contrast microscope at magnification of ×200.

2.4. Semen contamination and cryopreservation

The semen dilution was performed in two stages using a commercial semen extender (BotuBov®, Botupharma, Brazil). In the first step, the fraction A of the extender (without cryoprotectant) was added at room temperature and diluted semen was separated into two parts of equal volume. Before freezing, one portion of the diluted semen was experimentally contaminated with *T. gondii* to give a concentration of 10^7 taquizoytes/0.25 ml; the other portion of the semen was not contaminated and therefore destined to inseminate ewes of the control group.

After dilution, semen was maintained in a water bath for 10 min at room temperature for stabilization. The contaminated and control semen were subjected to the same cooling curve in ice-water bath from 25 °C to 5 °C in approximately 2 h. Then the fraction B of extender containing cryoprotectant (glycerol), previously chilled to 5 °C, was added to the two dilutions (contaminated and control semen). After homogenization, the samples were packaged in 0.25 ml straws (25 million viable sperm per straw). The straws were placed in an isothermal box 15 cm above liquid nitrogen level for 15 min and after this period placed in liquid nitrogen.

2.5. Sperm viability and bioassay in mice

To assess sperm viability after cryopreservation, one straw with contaminated and another with control semen were thawed in water bath at a temperature of 37 °C for 30 s and evaluated in microscope for strength and sperm motility. To detect *T. gondii* presence and infectious potential, a PCR [20,21] and a bioassay [22] were performed.

In the bioassay, two straws (0.5 ml) of each semen sample were also thawed and inoculated intraperitoneally (i.p.) for each of four adult Swiss Webster mice and a control group was inoculated with the same volume of PBS. Mice were observed for six weeks and dead animals were assessed for *T. gondii* tachyzoites in fresh peritoneal exudates under 400× magnification in a phase contrast microscope. Serology (MAT) and brain cyst searches were performed in the surviving animals. Tissue samples of kidney, lung, heart, liver, spleen and stomach of all mice used in the bioassay were collected for histopathological analysis.

2.6. Hormonal treatment, artificial insemination and infection dose

Initially, on a random day of the estrous cycle, all ewes received intravaginal sponges (60 mg of medroxyprogesterone acetate; Progespon, 60 mg MAP; Progespon®, Syntex, Buenos Aires, Argentina) (Day 0). On Day 9 the implants were substituted for new implants. Ewes received a total dose of 130 mg pFSH (Folltropin®, Bioniche, Canada) given as six decreasing doses (30, 30, 20, 20, 15 and 15 mg) at 12 h interval beginning at 07:00 a.m. on Day 11. On Day 13 implants were removed and females received 0.0375 mg of d-cloprostenol im injections (Prolise®, Tecnopec, São Paulo, Brazil), and 500 I.U. eCG im (Folligon®, Schering Plough, São Paulo, Brazil).

Intrauterine insemination procedures of both groups were performed with deposition of a 0.25 ml dose of semen in each uterine horn 40 and 56 h after sponge removal (Day 15 and Day 16), under laparoscopic visualization (total of four doses per ewe). Each 0.25 ml dose contained 25×10^6 sperm cells (total of 100×10^6 sperm cells per sheep). Contaminated semen contained additional 10^7 *T. gondii* tachyzoites per 0.25 ml dose (total of 4×10^7 tachyzoites per sheep), according to Moraes et al. [15]. Ewes were divided into two groups: G1 (n = 5), animals inseminated with contaminated semen and G2 (n = 4), inseminated with semen free of tachyzoites (control group).

Six days after AI, ewes were submitted to endoscopic visualization of the corpora lutea and oocytes and embryos were surgically recovered via longitudinal ventral laparotomy according to the techniques described by Gonçalves et al. [23]. The structures were classified in accordance with the criteria established by the International Embryo Transfer Society - IETS [24].

2.7. Assessment of Toxoplasma gondii infection

2.7.1. Clinical examination

All ewes were submitted to anamnesis and clinical examination on days −14, −7, 0 before the AI and daily until 15 days after AI. The parameters evaluated were: heart rate, respiratory rate, rectal temperature and body weight using a scale.

2.7.2. Serology

Blood was collected from ewes of both groups on days −14, −7, 0, before and 14, 21, 28, 35, 49, 57 after AI. Samples were collected through jugular vein puncture, centrifuged and sera stored at −20 °C until tested by the modified agglutination test (MAT) according to protocols established by Dubey and Desmonts [25]. In short, 25 μL of formalin-fixed whole tachyzoites antigen solution were added to 25 μL of the previously diluted sera in 96-well polystyrene plates; positive and negative controls were added. The plate was incubated at 37 °C overnight and results were based on the sedimentation profile of the tachyzoites suspension, where the formation of a web indicated the presence of specific IgG

antibodies anti-*T. gondii* and the formation of a blue dot the absence of antibodies.

2.7.3. Molecular analysis

DNA was extracted from the semen and buffy coats of all sheep using DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. Buffy coats were collected from the blood parts previously stored with EDTA in 5 ml tubes after centrifugation at 500×*g* for 10 min.

After extraction, all samples and controls were subjected to Nested PCR using primers Tox-5, Tox-8, Tox-9, Tox-11 for amplification of the 529 bp repeat element present in *T. gondii* DNA [20,21]. Negative and positive controls were added to each set of PCRs. After the reaction, the final sequence of 162 bp was detected by electrophoresis in 2% agarose gel, stained with GelRed ™ (Biotium) and visualized under ultraviolet light.

2.7.4. Histopathological analysis

At the end of the experimental period, all ewes were euthanized, necropsied and tissue specimens from brain, liver, kidney, heart, lungs, skeletal muscle, lymph nodes, spleen, uterus and ovary were analyzed macro- and microscopically. Tissues of the mice used in the bioassay of contaminated semen tachyzoites were also collected and analyzed as such. All specimens were fixed in neutral-buffered, 10% formalin and processed in paraffin by the usual histological techniques for hematoxylin-eosin (H&E). Immunohistochemical staining was performed only for the samples collected from sheep.

2.7.5. Immunohistochemistry (IHQ)

Toxoplasma gondii was investigated in sections of the brain, liver, kidney, heart, lung, skeletal muscle, uterus and ovary of sheep from groups G1 and G2 by IHC using the technique described by Silva et al. [26]. The sections were deparaffinized and hydrated, and the endogenous peroxidase was blocked with a 3% hydrogen peroxide solution. Then, sections were incubated in a Target Retrieval solution, pH 9 (DAKO Corp. Carpinteria, CA, USA) at 96 °C water bath for 30 min for antigen recovery. A solution of milk and 10% bovine serum albumin for 30 min was used to block nonspecific binding. The sections were incubated for 30 min with primary rabbit polyclonal anti-*T. gondii* antibody (Neomarkers, Fremont, CA, USA) diluted 1:200. The sections were treated with streptavidin-biotin-peroxidase (LSAB, DAKO Corp. Carpinteria, CA, USA) as recommended by the manufacturer. The chromogen Diaminobenzidine (Chromogen System, code K3468, DAKO Corporation, Carpinteria, CA, USA) was used to reveal the life cycle stages of the parasite, and all samples were counterstained with Harris hematoxylin.

Histological sections of sheep brain positive for *T. gondii* were used as positive controls. A negative control of the IHC reaction was designed for each sample by omition of the primary antibody, allowing the discrimination of possible false positive reactions. The animals were considered positive by IHC when tachyzoites or cysts were stained in brown by DAB in at least one of the examined organs.

3. Results

3.1. Sperm viability and bioassay in mice

The analysis of fresh ram semen presented the following sperm characteristics: whirling 5, vigor 4, motility 95% and sperm concentration of 3×10^9 sperm cells/mL. After freezing and thawing of the control and contaminated semen samples, the motility was 35% and vigor 4.

Mice submitted to the bioassay with contaminated semen became sick and died within two weeks. Infection with *T. gondii* was confirmed by observation of tachyzoites in peritoneal exudates. Mice inoculated with the control semen did not die, were seronegative by MAT and tissue cysts were not found in their brains.

3.2. Hormonal treatment and embryo recovery

The mean number of structures and recovery rates of superestimulated ewes artificially inseminated with contaminated semen (G1) or non-contaminated semen (G2) are shown in Table 1.

In group G1 (ewes inseminated with contaminated semen) the mean number of 5.6 CL was observed at the moment of embryo recovery by endoscopic evaluation of ovaries, while in the control group (G2) it was observed a mean number of 2.2 CL. Three of the five ewes from G1 (60%) responded to the superovulation protocol (presented two or more CL), while only one of the four ewes responded to the superestimulated protocol in the G2 (25%). The recovery rate (number of collected structures/CL visualized) was high in both groups (G1 = 96.4%, G2 = 77.8%), indicating an efficient embryos collection method. The percentage of transferable embryos (grades 1, 2, 3/total collected structures) was 33.3% in the contaminated group and 100% to the control group.

3.3. Assessment of Toxoplasma gondii infection

3.3.1. Clinical examination

At clinical examination, only one of the five animals of group G1 showed apathy, lethargy, head pressing, anorexia, hypothermia and died seven days after insemination with the contaminated cryopreserved semen. No clinical changes were observed in the four animals of group G2 (control group).

3.3.2. Serology

Seroconversion occurred in all ewes of group G1 seven days after insemination with contaminated semen. Titers were 1:25 in two ewes, including the one that died seven days after insemination (DAI), and 1:100 in three ewes. All four ewes from group G2 (control group) were seronegative for anti-*T. gondii* antibodies.

3.3.3. Molecular analysis

Toxoplasma gondii DNA was present in contaminated semen and no trace of the parasite DNA was detected in control semen. The parasite DNA was also detected in the buffy coat of one ewe of group G1 (contaminated semen group) 35 DAI.

3.3.4. Histopathological analysis

Toxoplasma gondii tachyzoites were observed in the liver and intestine samples of mice inoculated with contaminated semen. The lungs presented severe congestion and focal mononuclear inflammatory infiltrate, as well as severe and diffuse areas of edema whereas in mice inoculated with control semen no histopathological change was observed.

At the necropsy of group G1 ewes, the animal which died seven days after AI had macroscopic lesions characterized by reddish areas in the cerebral and cerebellar grooves, edema and presence of evident vascularization with engorged vessels. Lungs presented multiple and extensive, not sizzling, dense and reddish areas. No macroscopic changes were observed in the examination of tissues of other ewes of both groups.

The histopathological findings of the group G1 ewe that died after the AI included severe congestion, gliosis and mild lymphocytic inflammatory infiltrate in the brain. Severe congestion and mild diffuse lymphocytic inflammatory infiltrate was present in the kidney and liver. Lungs contained accentuated and diffuse areas of

Table 1
Mean numbers of structures and recovery rates of superestimulated Santa Inês ewes artificially inseminated after freezing of semen experimentally contaminated with *Toxoplasma gondii* tachyzoites (G1) or of non-contaminated semen (G2).

	Contaminated semen G1 (n = 5)	Non-contaminated semen G2 (n = 4)
Number of CL at embryo recovery	5.6	2.2
Structures collected (ova and embryos)	5.4	1.8
Transferable embryos (Grade 1)	0	1.8
Transferable embryos (Grade 2)	0	0
Transferable embryos (Grade 3)	1.8	0
Transferable embryos (Grades 1, 2, 3)	1.8	1.8
Unfertilized ova	3.4	0
Degenerated embryos	0.2	0
Recovery rate %	27/28 (96.4%)	7/9 (77.8%)
Transferable embryo rate %	9/27 (33.3%)	7/7 (100%)
Superovulated ewes %[a]	3/5 (60%)	1/4 (25%)

[a] Superovulation protocol response was considered when animals presented two or more corpora lutea at embryo recovery.

lymphoplasmocytic inflammatory infiltrate affecting bronchi, bronchioles and alveoli featuring bronchopneumonia, severe congestion and areas of atelectasis. Slight and diffuse neutrophilic inflammatory infiltrate were observed in lymph nodes, along with splenic hypoplasia. Ovary and uterus presented severe congestion. Changes of the other four ewes of group G1 were characterized by slight congestion and lymphoplasmocytic inflammatory infiltrate in the heart, kidney, lungs and liver. The uterus, ovary and brain had slight congestion; the brain had also gliosis.

In the control group G2, no pathological changes were observed at macroscopic examination. This group did not exhibit relevant histopathological changes, only characterized by slight congestion in skeletal muscle and ovary, mild congestion in uterus, slight congestion and slight lymphoplasmocytic inflammatory infiltrate in the heart, liver, lungs and kidney.

Toxoplasma gondii cysts were not observed at histopathological examination of tissues from ewes from both groups. However, round-shape cysts with variable sizes, characteristic of *Sarcocystis* spp., were observed in the skeletal muscle and heart of all ewes.

3.3.5. Immunohistochemistry (IHC)

Immunoreactivity to polyclonal anti-*T. gondii* antibodies were observed in all females from group G1 (contaminated semen group) in at least one tissue tested. *Toxoplasma gondii* immunostained cysts with variable sizes and tachyzoites inside macrophages were observed in the heart (2/5), brain (2/5), liver (1/5), skeletal muscle (1/5), uterus (3/5) and ovary (2/5). The ewe that died seven days after infection presented immunostained small and round *T. gondii* cysts in the brain, skeletal muscle, liver, heart, and ovary. Fig. 1 shows immunostained round cysts containing bradyzoites, observed in the different fields of uterus tissue from one sheep belonging to group G1. Control group ewes (G2) were negative for anti-*T. gondii* antibodies in IHC.

Big round-shaped parasitic cysts observed at histopathology, characteristic of *Sarcocystis* spp., did not show immunoreactivity to anti-*T. gondii* antibodies, confirming the efficacy of the primary rabbit antibody for *T. gondii*.

Table 2 summarizes the results from the present study, correlating diagnosis and detection of *T. gondii* in the tissue and blood of ewes inseminated with contaminated frozen semen (G1) and sheep inseminated with control semen (G2).

4. Discussion

The present study investigated the transmission of *T. gondii* in sheep through AI after freezing with experimentally contaminated semen. Different methods had been used so far to assess the possibility of sexual transmission of this parasite in sheep. One previous study proved transmission via AI of experimentally contaminated fresh semen [15] and another via natural mating [14]. But the present research demonstrated, for the first time, that contaminated semen remains infectious to ewes even after submitted to the freezing protocol. Although the high parasite load used in this study might not express what happens in natural infections, it establishes the necessity to monitor *T. gondii* contamination in frozen semen, since the possibility of transmission to inseminated ewes is now proven.

It became, therefore, necessary to investigate whether *T. gondii* would keep its viability under specific protocols used for semen cryopreservation. The preservation of *T. gondii* tachyzoites in nitrogen was proposed using a freezing curve of -1 °C to −70 °C [2]. In this work, a different protocol was performed, consisting of cryopreservation at −196 °C employing the semen extender containing glycerol as cryoprotectant. Only DNA of the parasite had been detected in frozen semen of naturally infected ram [9], but its potential to infect and cause clinical disease in ewes after artificial insemination with frozen semen had never been tested.

Antibodies anti-*T. gondii* were detectable by MAT in all five ewes from group G1 seven days after insemination. This result corroborates the findings of other researches [14,15] where experimental infection with *T. gondii* induced a rapid immune response, with early detection of IgG five to seven days after infection. Also, one of the five sheep inseminated with contaminated semen presented clinical signs of apathy, lethargy, head pressing, anorexia and hypothermia, dying one week after insemination. Lopes et al. [14] also observed hypothermia and anorexia in two experimentally infected sheep with *T. gondii* from the 3rd to the 11th days post-infection.

Macroscopic examination of the tissues of this referred dead sheep showed presence of reddish areas with evident vascularization, edema and engorged vessels in the cerebral and cerebellar grooves. These brain lesions were similar to those found by Da Motta et al. [27] in an aborted fetus. Histopathological examination of the brain demonstrated severe congestion, gliosis and lymphocytic inflammatory infiltrate. These changes are often observed in sheep [2,27,28] and goats [16,29] with *T. gondii* infection. Analysis of the lungs showed bronchopneumonia, the main pathological finding and probably a secondary complication following *T. gondii* infection and which led to the death of the animal. In addition, IHC test revealed cysts in the brain, skeletal muscle, liver, heart and ovary, confirming the infection by *T. gondii* via AI with experimentally contaminated frozen semen.

According to Dubey [2], tissue cysts of *T. gondii* might be observed in histological analysis of various organs, especially in the brain, heart and skeletal muscle. However, besides the described injuries, we did not observe cysts of this parasite in any of the analyzed tissue samples by histopathology. Other authors [26,30] also did not observe *T. gondii* cysts by conventional histopathological analysis H&E. According to Esteban-Redondo & Innes [31]

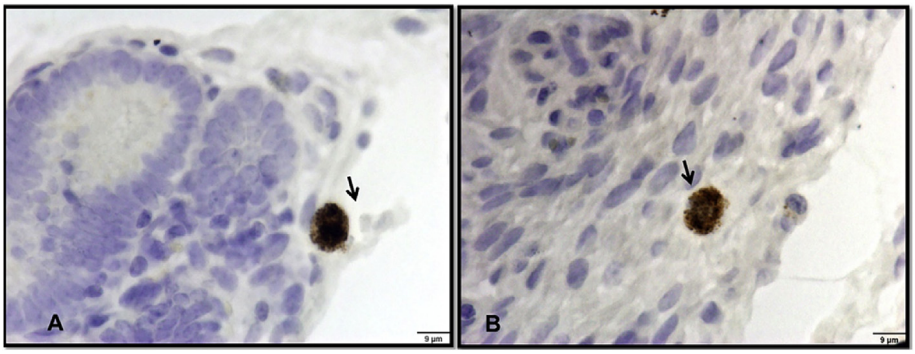

Fig. 1. (A–B) Immunostained sections of different fields of uterine tissues from one sheep inseminated after freezing of semen experimentally contaminated with *Toxoplasma gondii* tachyzoites. (A–B) Uterus – Round cysts stained with anti-toxoplasma antiserum (arrows), Scale bar: 9 μm.

Table 2
Toxoplasma gondii in tissue and blood of sheep inseminated after freezing of semen experimentally contaminated with *Toxoplasma gondii* tachyzoites (G1) or of non-contaminated semen (G2).

Sheep	MAT titers	Immunohistochemistry								PCR
		Heart	Brain	Liver	Muscle	Lung	Kidney	Ovary	Uterus	Blood
1	1:100	–	–	–	–	–	–	–	±	±
2	1:25	±	–	–	–	–	–	–	–	–
3	1:25	±	±	±	±	–	–	±	–	–
4	1:100	–	–	–	–	–	–	–	±	–
5	1:100	–	±	-	-	–	–	±	±	–
6	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–

+: positive –: negative.

the detection of *T. gondii* in large animals tissue is difficult because the parasite might be present in an animal tissue fragment that was not examined.

Confirmation of sheep infection by *T. gondii* via AI with frozen semen was also performed by IHC and PCR. Immunohistochemical analysis is an important definitive diagnosis which allows confirmation of *T. gondii* infection [26,27,32]. Immunorreactivity to anti-*T. gondii* antibodies was observed in all sheep from G1 in at least one sample of tissue: heart, brain, skeletal muscle, liver, uterus and ovary. The immunostained structures consisted of *T. gondii* cysts and tachyzoites inside macrophages. This is in agreement with other researches, in which immunostained tachyzoites and cysts were observed in the brain, heart and liver of sheep [26,27,33].

At histopathological analysis of both skeletal and cardiac muscles we observed large parasitic cysts with oval shape, with characteristics and morphology of *Sarcocystis* sp. These structures were negative in the IHC, what demonstrated the specificity of the rabbit primary anti-*T. gondii* antibody. This was also observed by Silva et al. [26].

Molecular analysis showed the presence of *T. gondii* DNA in the blood of one of the five sheep (20%) inseminated with contaminated semen 35 DAI. However, Moraes et al. [15] identified 14 of 15 positive sheep by the presence of *T. gondii* DNA in blood samples (93.3%) between seven and 123 DAI with fresh contaminated semen. This disparity in results can be explained by the difference of evaluation period in each research, which in this study represented 14 days before the AI and up to 57 days after infection.

Toxoplasmosis in sheep can lead to pregnancy loss, early embryonic death and resorption, fetal death, mummification, abortion, stillbirth and neonatal death [34]. Our results suggest that *T. gondii* infection with frozen semen at the used dose affected fertilization and/or embryonic development due high number of unfertilized oocytes and poor quality embryos. However it was not possible to perform association between groups due to the low number of collected embryos in both groups. According to numerous studies associating *T. gondii* to reproductive disorders, more researches are needed to correlate it to pre-implantational embryo development in sheep.

Although toxoplasmosis is not mentioned by OIE [35] or the Brazilian legislation [36] among the risk diseases for the commercialization of goat and sheep semen, in view of the present results we suggest increased control of importing or freezing ram semen, since toxoplasmosis is an endemic disease and is not being controlled.

5. Conclusion

The cryopreservation of ram semen did not inhibit the infectivity and pathogenicity of *T. gondii* tachyzoites to ewes. These findings open a route of investigation to assess the risk of *T. gondii* transmission through AI with naturally contaminated frozen semen.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2016.12.004.

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