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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.

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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

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