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Apoptosis and cell proliferation in the mouse model of embryonic death induced by *Tritrichomonas foetus* infection

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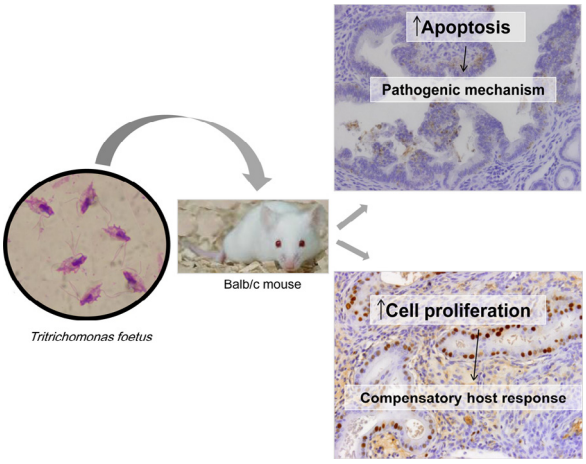
HIGHLIGHTS

- The cytopathic effect induced by *Tritrichomonas foetus* alters uterine epithelial kinetic.
- Uterine cell death index is higher in infected mice that lost their *conceptus*.
- Uterine cell proliferation rate is increased in *T. foetus*-infected animals.
- Apoptosis participates in the pathogeny of the abortion during tritrichomonosis.
- The increase in the cell proliferation may act as compensatory host response.

GRAPHICAL ABSTRACT



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ABSTRACT

Bovine tritrichomonosis is a sexually transmitted disease caused by the protozoon *Tritrichomonas foetus* and characterised by embryonic-death and abortion. During pregnancy, the processes of cell proliferation and death play a crucial role for blastocyst implantation and the subsequent maintenance of early pregnancy, and their misbalance may lead to the abortion. In this study, we aimed to investigate whether cell proliferation and death may be altered during tritrichomonosis. For this purpose, we used pregnant BALB/c mice as an alternative experimental animal model that has successfully reproduced the infection. We analysed the immunohistochemical expression of active caspase-3 and proliferating cell nuclear (PCNA) antigens in the endometrium of infected mice. We found an increase in the number of caspase-3 positive cells in infected mice that were not pregnant at the necropsy. Besides, the number of positive proliferating cells increased in the uterine luminal epithelium of infected animals killed at 5–7 days post

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coitum (dpc). Pregnant infected mice killed at 8–11 dpc showed higher proliferation than control animals. We suggest that the cytopathic effect induced by *T. foetus* in the uteri of infected mice may induce the apoptosis of the epithelial cells and, as a result, promote a compensatory proliferative response. The information described here will be helpful to further study the pathogenesis of the bovine tritrichomonosis.

1. Introduction

Bovine tritrichomonosis is a sexually transmitted disease caused by the flagellated protozoon *Tritrichomonas foetus.* This parasite colonises the reproductive tract of cows and causes temporary infertility, early embryonic-death and, in some cases, abortions (BonDurant, 2005). In countries in which cattle are extensively raised with natural service, it is an endemic disease that causes important economic losses (Rae, 1989).

The complexity of handling bovine experimental systems has led to the development of alternative experimental animal models (Hook et al., 1997; St Claire et al., 1994; Van Andel et al., 1996). To date, the BALB/c is the most sensitive strain to *T. foetus*, in which a persistent infection with lesions similar to those described in bovine natural infection can be well-reproduced (Hook et al., 1997; Van Andel et al., 1996). In addition, it has been shown that mice treated with oestrogen were infected at a higher percentage than those untreated (St Claire et al., 1994). We have satisfactorily reproduced the disease using low-dose oestrogens to synchronise oestrus in non-pregnant BALB/c mice (Soto et al., 2005). Shortly later, we have reproduced bovine tritrichomonosis in pregnant BALB/c mice treated with a low dose of estrogens (Barbeito et al., 2008). Furthermore, Agnew et al. (2008) also characterised a pregnant mouse model to study the pathogenesis of the disease. Lesions found in uteri of non-pregnant (Monteavaro et al., 2008) and pregnant (Barbeito et al., 2008; Woudwyk et al., 2013) mice are very similar to those found in experimentally infected heifers (Cobo et al., 2004). Therefore, these findings further support the use of the murine model to study the present disease.

In the mouse, the uterine implantation of the embryos occurs at 4.5 dpc. This process involves sequential processes of proliferation, differentiation and regression of the endometrium in different regions and at different times (Bell, 1983; Parr et al., 1987). During the implantation, apoptosis of uterine epithelial cells begins with the adhesion of the blastocyst to the nearest epithelium, and it is induced by the trophoblast (Schlafke and Enders, 1975). After implantation, an appropriate balance of apoptosis and cell proliferation is also essential for the successful maintenance of pregnancy (Demir et al., 2002). In this work, we aimed to determine whether changes in cell proliferation and cell death in the endometrium of BALB/c mice infected with *T. foetus* may be involved in the pathogenesis of embryonic-death during tritrichomonosis.

2. Materials and methods

2.1. Animals

Seventy-six 6–8-week-old female BALB/c mice, bred in our facility, were housed according to the regulations of the National Administration of Medicine, Food and Medical Technology (ANMAT, 1996) with controlled temperature and airflow. Food and water were available *ad libitum*. Lighting was provided on a 12 h light/dark cycle.

2.2. Synchronisation of oestrous cycle

In order to synchronise oestrus, animals were intramuscularly injected with 5 μg of β-estradiol 3-benzoate (EB) suspended in 0.1 ml of sterile sesame oil. Vaginal cytology was performed 24 and 48 h later to test the evolution of the oestrous cycle. Vaginal mucus obtained by aspiration with a micropipette and diluted with 5 ml of phosphate buffered saline (PBS; pH 7.2, 0.01 M) was microscopically examined to define the stage of the oestrous cycle (Allen, 1922).

2.3. Experimental design

Two days after cycle synchronisation, animals were divided into two experimental groups: A) control group (n = 36) and B) infected group (n = 40). Females of the infected group were inoculated with the pathogenic strain of *T. foetus*, whereas mice of control group were inoculated with PBS. Vaginal samples of both groups were analysed after estradiol inoculation by cytology from day 12.

Mice in oestrus were paired with healthy BALB/c males, and those that showed vaginal plug, considered as day 0 of pregnancy, were included in the study.

Then, infected and control mice were killed from 5 to 7 dpc, when decidual reaction occurs after blastocyst implantation, and from 8 to 11 dpc, a period that corresponds to the placentation phase. Mice were also subdivided in pregnant (with presence of implantation sites at the necropsy) and non-pregnant subgroups (no implantation sites, but presence of inflammatory changes) (Table 1). Uterine horns were removed and samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax and sectioned at 5 μm for immunohistochemistry. All animals listed in Table 1 were processed for immunohistochemistry and analysed by microscopy.

2.4. *Intravaginal* T. foetus *inoculation*

A pathogenic *T. foetus* strain isolated from bovine preputial washes (Soto et al., 2005) was cultured in trypticase-yeast-extract-maltose medium (TYM) (Diamond, 1987) and then the sample was centrifugated at 900 *g* three times with phosphate buffered solution (PBS, pH 7.2) for 10 min. Each member of the infected group was intravaginally inoculated with 10 μl of a suspension containing 9×10^7 *T. foetus*/ml, whereas the control group was intravaginally inoculated with PBS. Infected animals were sampled weekly by vaginal washes for *T. foetus* culture to confirm the vaginal infection through protozoa isolation and also to verify protozoa viability. Vaginal cytology was used to confirm the presence of *T. foetus* and to determine the oestrous cycle phase. The presence of mobile protozoa with typical *T. foetus* morphology in mucus samples of inoculated mice confirmed the infection (Soto et al., 2005).

2.5. Immunohistochemistry

Paraffin-embedded sections were dewaxed with xylene and hydrated through a decreasing graded alcohol scale. Then, the specimens were incubated with hydrogen peroxide 0.03% in methanol for 30 min at room temperature to inhibit endogenous

Table 1
Groups of animals.

Days post coitum	Control group		Infected group	
	Pregnant	Non-pregnant	Pregnant	Non-pregnant
5–7	11	5	6	11
8–11	15	5	5	18
Total	26	10	11	29

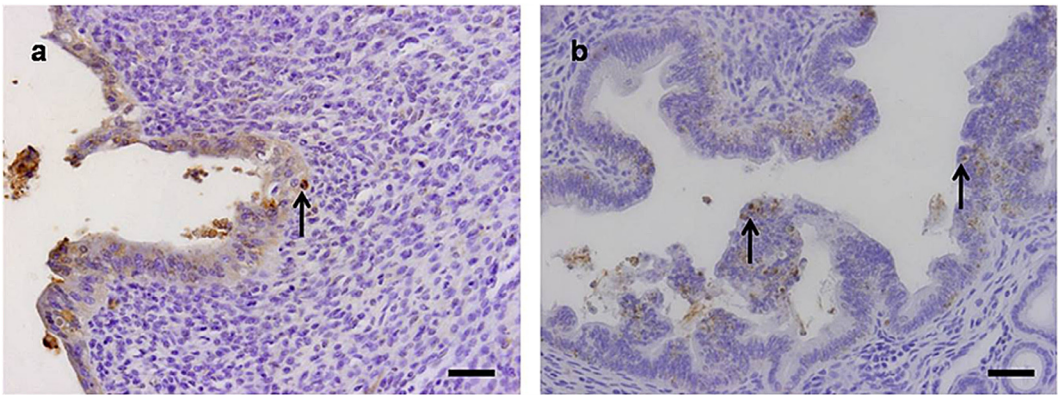

Fig. 1. Immunostaining for active caspase-3 in the uterine horns of BALB/c mice. a) Control group, 9 dpc. b) Infected-group with *T. foetus*, 11 dpc. Scale bar: 20 μm. dpc: days post coitum. Arrows: apoptotic positive cells.

peroxidase activity. Antigen retrieval was performed using microwave 750 watts for 5 min twice in citrate buffer (pH 6.0). Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) or 2% BSA + 5% skim milk for PCNA antigens or active caspase-3 antigen, respectively, for 30 min in a humid chamber at room temperature (for PCNA antigens) or 4 °C (for active caspase-3 antigen), followed by the primary antibody incubation (see below). Liquid 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen (DakoCytomation, Glostrup, Denmark), and Hill's hematoxylin was used for counterstaining. Control negative sections were prepared by omitting primary antibody. Positive controls were sections of mouse lung used in a previous work (Eöry et al., 2013).

2.5.1. Proliferation assessment

Mouse monoclonal anti-PCNA was used as a primary antibody (clone PC 10, ascites fluid, Sigma Chemical Co., St. Louis, MO, EE.UU, 1/3000 dilution). The primary antibody was pretreated with Dako ARK (Animal Research Kit) Peroxidase System (code K3954) to reduce the possible reactivity of the detection system with endogenous immunoglobulin. Non-specific binding sites of endogenous biotin were blocked with an avidin-biotin blocking kit (Cat.#HK102-5K, Biogenex, Fremont, CA). The antibody-Dako ARK polylinker complex was then incubated for 1 h at 37 °C, rinsed in PBS 3 times for 5 min each, revealed, and counterstained.

2.5.2. Apoptosis assessment

The sections were incubated with rabbit anti-caspase-3 (active form) polyclonal antibody (1/800 dilution in 0.1% BSA) (Cat. #AB3623 Chemicon International, Inc., Temecula, CA) overnight at 4 °C. Anti-rabbit EnVision detection system + HRP (DakoCytomation) was then used for 30 min at room temperature. Sections were then rinsed in PBS twice for 5 min, treated with DAB, and counterstained.

2.5.3. Cell proliferation and cell death quantification

PCNA and caspase-3 immunostaining were used to calculate cell proliferation and death percentages (CP%, CD%, respectively). PCNA positive cells showed brown nuclear staining, whereas in caspase-3 positive cells the staining was in their cytoplasm. To calculate the CD% and CP%, the following formula was used:

$$\mathrm{CP\%/CD\%} = \frac{\text{PCNA/caspase positive cells}}{\text{Total number of epithelial cells per section}}$$

Ten images per slide were captured for each animal analysed, and a minimum of 150 cells within the uterine epithelium were counted at ×1000 magnification by two independent observers.

2.6. Analysis of data

The data were analysed by one-way ANOVA followed by Fisher's LSD post-hoc test. Significant difference was considered when $p < 0.05$. Data were expressed as mean ± standard deviation (SD).

3. Results

3.1. Cell death %

In non-pregnant infected mice killed at 5–7 dpc, caspase-3 positive cells were observed within the uterine epithelium, in the endometrial glands exudate and lamina propria. At 9 dpc, the caspase-3 positive cells were found only within the luminal epithelium (Fig. 1). The CD% of each infected pregnant group did not differ regarding control groups at the corresponding dpc studied. Caspase-3 positive cells were found in the luminal and glandular epithelia at 5–7 dpc, but not at 8–11 dpc. Those infected-mice of non-pregnant group showed a significant increase in the CD% in the uterine epithelium with respect to the remaining groups (Fig. 2).

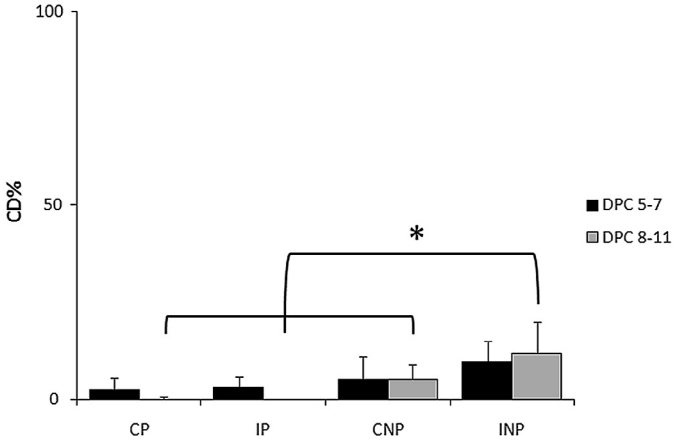

Fig. 2. Cell death percentage (CD%). Uterine epithelium. Days 5–7 pc: A significant increase in the apoptosis was observed in non-pregnant infected mice with respect to the rest of the groups (*) ($p < 0.01$). Days 8–11 pc: Significant differences were observed between non-pregnant infected mice and the rest of the groups (*) ($p < 0.05$). The results are expressed as bars showing the means and their standard deviations. The data were analysed by ANOVA test followed by Tukey's post-hoc test. CP: control pregnant; IP: infected pregnant; CNP: control non-pregnant; INP: infected non-pregnant.

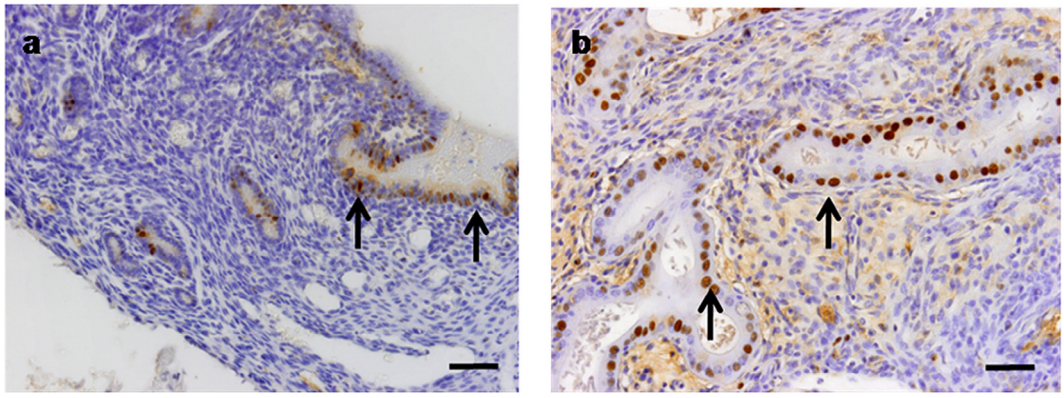

Fig. 3. Immunostaining for PCNA in the uterine horns of BALB/c mice. a) Control group, 8 dpc. b) Infected-group with *T. foetus*, 8 dpc. Scale bar: 20 μm. dpc: days post coitum. Arrows: PCNA positive cells.

3.2. Cell proliferation %

PCNA positive cells were found in the uterine epithelium of infected pregnant and non-pregnant mice, killed at 5–7 dpc when compared to controls. Infected pregnant mice killed at 8–11 dpc showed an increase in the cell proliferation of the uterine epithelium (Figs 3 and 4).

4. Discussion

The pathogenic mechanisms of *T. foetus* have been the aim of study of several authors. Singh *et al.* showed that *T. foetus* induces apoptosis of bovine vaginal (Singh et al., 2004) and uterine epithelial cells (Singh et al., 2005) by the action of a cysteine-protease known as CP30. In addition, it has been shown that *T. foetus* induces the apoptosis of bovine oviductal epithelial cells (Benchimol et al., 2006; Midlej et al., 2009) and oocytes *in vitro* (Benchimol et al., 2007). These results are just some examples that support the apoptosis as a critical process on the pathogenesis of bovine tritrichomonosis.

In this paper, we showed changes in cell proliferation and death in the uteri of infected mice. The percentage of caspase-3 positive cells in the uterine epithelium of infected and control pregnant mice killed at 5–7 dpc showed no significant difference, which is in accordance with the natural implantation processes (Correia-da-Silva et al., 2004). In addition, infected and control pregnant mice showed low apoptotic percentages at 8–11 dpc at the end of the implantation and the beginning of the placentation. However, infected non-pregnant group showed the highest percentage of apoptosis. Therefore, we suggest that apoptosis in infected animals may be triggered by the cytotoxic effect of *T. foetus* and it may be one of the mechanisms responsible for the failure of the implantation and the early embryonic losses that occur during infection. Besides, the increase in the apoptosis of uterine epithelium from 5 to 7 dpc may alter the normal implantation process as well as the secretion of the uterine milk necessary for the embryo nourishment.

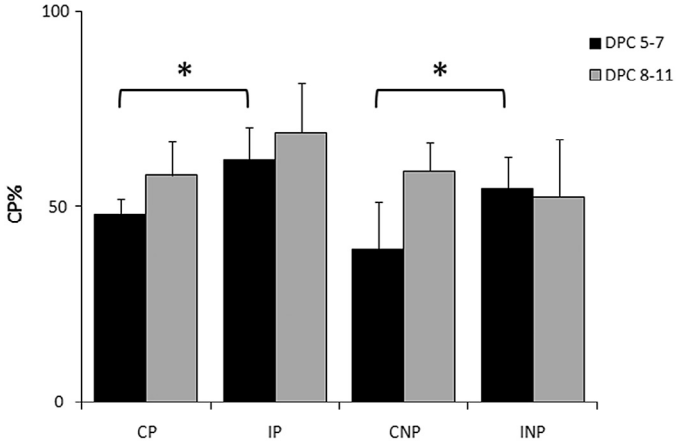

Fig. 4. Cell proliferation percentage (CP%). Uterine epithelium. Days 5–7 pc: A significant increase in cell proliferation was observed in non-pregnant infected mice with respect to control non-pregnant (*) ($p < 0.001$). Days 8–11 pc: The increase in cell proliferation was significant in the infected pregnant mice with respect to control pregnant (*) ($p < 0.05$). The results are expressed as bars showing the means and their standard deviations. Data were analysed by ANOVA test followed by Tukey's post-hoc test. CP: control pregnant; IP: infected pregnant; CNP: control non-pregnant; INP: infected non-pregnant.

Alterations of cell death and proliferation may be involved in the loss of conceptus during early pregnancy (Correia-da-Silva et al., 2004). Here, pregnant and non-pregnant infected mice killed between 5 and 7 dpc and those infected still pregnant females killed between 8 and 11 dpc showed an increase in cell proliferation in the uterine epithelium when compared to controls. However, we suggest that this higher proliferation is just a general response to the cytotoxic effects and mechanical irritation induced by the protozoan.

In previous studies of *T. foetus* infection during early pregnancy, we have found changes in the local immune response at the maternal-fetal interface (Woudwyk et al., 2012), as well as in the expression of uterine epithelial carbohydrates (Woudwyk et al., 2013). These changes and the here reported allow us to support the hypothesis of the abortion as a multifactorial process. We conclude that in the present murine model of bovine tritrichomonosis, induction of the apoptosis is an additional mechanism involved in the pathogenesis of the early embryonic-death that occurs during *T. foetus* infection, being the increase in cell proliferation as just a compensatory host response.

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