Vet. Res. 38 (2007) 399–408
© INRA, EDP Sciences, 2007
DOI: 10.1051/vetres:2007004

Original article

Tritrichomonas foetus damages bovine oocytes in vitro

Marlene BENCHIMOL[a]*, Reginaldo da SILVA FONTES[b], Ângelo José BURLA DIAS[b]

[a] Laboratório de Ultraestrutura Celular-Universidade Santa Ursula, Rua Jornalista Orlando Dantas 59, Botafogo, Rio de Janeiro, CEP 22231010, Brazil
[b] Setor de Fisiologia e Biotecnologia da Reprodução de Ruminantes/Laboratório de Reprodução e Melhoramento Genético Animal/ Centro de Ciências e Tecnologias Agropecuárias-Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil

(Received 27 February 2006; accepted 1 December 2006)

Abstract – *Tritrichomonas foetus* is an extracellular parasite of the urogenital tract in cattle. It causes infertility and abortion, but there is no documented information on the susceptibility of bovine oocytes to the parasite, except by one article that claimed no effects of *T. foetus* on oocytes or embryos. The aim of the present study was to study the effects provoked by *T. foetus* when in interaction with bovine oocytes. Oocytes were obtained from cow ovaries and divided into two groups: (1) one group contained cumulus cells, whereas (2) a second group was denuded from these cells. Light microscopy, video microscopy and scanning electron microscopy (SEM) revealed that exposure of oocytes to *T. foetus* caused rapid adhesion of the trichomonads to cumulus cells and to the zona pellucida (ZP). Motile parasites were observed for 12 h. The ZP was completely damaged, and the parasites were able to infiltrate beneath the ZP and reached the oocytes directly when the oocytes were denuded of the cumulus cells. Both the oocytes and the cumulus cells exhibited morphological characteristics compatible with apoptosis after interaction with *T. foetus,* such as chromatin condensation, the presence of several cytoplasmic vacuoles, with intact cellular membranes and organelles. The results from this study demonstrate that when a large number of *T. foetus* interacts with oocytes in vitro damage and apoptosis are provoked in the cow's reproductive cells. The behavior of this parasite as one of the causes of cattle infertility is discussed.

***Tritrichomonas foetus* / oocytes / cow / ultrastructure / infertility**

1. INTRODUCTION

Tritrichomonas foetus is the causative agent of cattle trichomonosis, one of the most prevalent sexually transmitted diseases in cattle. The infection varies in cows from a mild vaginitis or cervicitis, to endometritis, transient or permanent infertility, and abortion, causing significant economic losses. The mechanisms by which *T. foetus* causes infertility and abortion are not defined.

The molecular mechanism by which *T. foetus* colonizes mucosal surfaces is not well defined nor is the mechanism(s) of tissue damage understood. There is a need for a deeper understanding of the pathogenic mechanisms of this parasite because this will delineate the host-parasite relationship more clearly.

T. foetus initially adheres to and infects the vagina, causing vaginitis, and then

* Corresponding author:
marleneben@uol.com.br

moves to the uterus and oviduct [11]. An early investigation [15] demonstrated that the parasites are also able to invade the placenta, resulting in placentitis.

Although the effects of *T. foetus* on cell lines [4, 17–19], on scraped bovine cells [5], and directly in cows [1, 13] are well studied, the only information available in the literature of the direct effect of this parasite on oocytes [3] is distinct from our findings. These authors did not find detrimental effects of *T. foetus* on oocytes or on in vitro-fertilized embryos. Thus, only limited information is available describing in detail the changes on oocytes associated with *T. foetus* infection.

It is well known that infection by *T. foetus* may result in transient or permanent infertility in cows. *T. foetus* has been reported to attach itself and provoke damage to bovine primary vaginal and uterine epithelial cell cultures [19], inhibit their division [16], cause inflammation of the uterus [13], and uterine tubes [1], invade bovine placental tissue [15], and produce enzymes capable of attacking host tissues [4]. However, only limited information is available describing the effects of the interaction between *T. foetus* and reproductive organs.

It has been suggested that apparent infertility due to embryonic death is the most common result of *T. foetus* infection [15]. It is possible that active trichomonad infections ordinarily prevent fertilization or terminate pregnancies before maternal recognition of pregnancy so that many abortions usually pass unnoticed.

The purpose of the study reported here was to investigate the behavior of *T. foetus* in the presence of fresh oocytes obtained from bovine ovaries, and to search for possible damage provoked by parasites to this reproductive cell. We took advantage of video microscopy and scanning electron microscopy, demonstrating that *T. foetus* avidly binds to oocytes, provoking injury to this reproductive cell. The possible role of this infection in cattle infertility is discussed.

2. MATERIALS AND METHODS

2.1. Microorganisms

The K strain of *T. foetus* was isolated by Dr H. Guida (Embrapa, Rio de Janeiro, Brazil) from the urogenital tract of a bull. Cultures have been maintained in TYM Diamond medium [8]. The cells were grown for 24 h at 36.5 °C, which corresponds to the logarithmic growth phase.

2.2. Oocytes

Bovine ovaries were collected in the slaughterhouse, immersed in sterile phosphate balanced salt solution (PBS) containing 0.05 g/L gentamicine sulfate, and transported to the laboratory within 2 h. Cumulus-oocyte complexes were from ovarian follicles, using an 18-gauge needle. Oocytes surrounded by a multilayer of compact follicular cells were washed twice in PBS and transferred to a 199 culture medium (M-5017, Sigma, MO, USA) supplemented with 10% fetal cow serum. One experimental group (30 oocytes) had the cumulus cells mechanically removed by micropipetting whereas in another group (30 oocytes) the cumulus was maintained. Both groups, 25 oocytes each, were coincubated with *T. foetus.* Ten oocytes were used as the control, without *T. foetus* interaction. Viability tests were based on visual morphology under phase contrast microscopy, as used for in vitro fertilization, and thus allowed us to select the best oocytes.

2.3. Coincubation and attachment assay

For interaction analysis, the isolated oocytes were exposed to *T. foetus* K, a

prolonged culture, in a cell ratio of 10:1, 100:1, 1 000:1 and 10 000:1 parasites-oocytes, for 30 min to 12 h at 37 °C. Only oocytes exhibiting healthy morphology were used, following the criteria of De Loss et al. [7].

The cells were equilibrated in incubation medium containing two parts of complete DEMEM (pH 7.2), and one part of Diamond medium (W/D 2:1) for 15 min at 37 °C (5% CO_2) prior to the addition of parasites. In control experiments, parasites were omitted.

2.4. Light microscopy, video microscopy

During parasite interaction, the cells were observed under phase-contrast light Axiophot 2 microscopy (Zeiss, Oberkoken, Germany) and photos were obtained. Video microscopy was performed using Nikon equipment.

2.5. Scanning electron microscopy

After interaction, oocytes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Afterwards, the samples were washed in PBS, post-fixed in 1% OsO_4, dehydrated in ethanol, critical point dried with CO_2, and sputter-coated with gold-palladium. The samples were examined in a JEOL 5800 scanning electron microscope.

3. RESULTS

3.1. Obtaining fresh oocytes

In order to investigate the effects of *T. foetus* when in contact with reproductive cells, oocytes were mechanically isolated from fresh bovine ovaries, and only those oocytes exhibiting high quality appearance were used. This method provided sufficient amounts of isolated oocytes. Sixty high-quality oocytes were used in every experiment, and 25 oocytes were artificially denuded. Isolated oocytes were morphologically controlled before, during and after interaction with *T. foetus.* Viability tests were based on visual morphology, as used for in vitro fertilization, such as the presence of a refractive halo when observed by DIC or phase contrast microscopy, intact zona pellucida (ZP), observation of the nucleus in relief, absence of cytoplasm retraction, a homogenous shape and size. Only those oocyte-cumulus complexes with tightly compacted cumulus and evenly granulated cells were used, thus allowing us the selection of the best oocytes.

3.2. *T. foetus* changes

The interaction assays were performed with different concentrations of parasites-per oocyte, with a time ranging from 30 min to 12 h in culture medium (Figs. 1–5). Light and video microscopy proved the intense movement of *T. foetus* towards the oocytes, which resulted in adhesion within 1 h (Fig. 1), and caused total viability loss of the oocyte in 4–6 h (Figs. 1–3). Parasites in low concentrations such as 10:1 and 100:1 or short times (1–2 h) did not show evident damage to the oocytes when the cumulus cells were intact (Fig. 4). Higher concentrations such as 1 000 and 10 000 parasites per oocyte exhibited intense cell damage, which was very high after 6 h interaction with *T. foetus* (Figs. 1–3, 5). The damage was even higher when the cumulus cells had been removed (Fig. 3).

T. foetus adhered by its posterior end, where the axostyle is located. The flagella were maintained externalized, and the parasites presented their pear-shape during the adhesion process (Figs. 2, 3). Trichomonad aggregates were formed and completely covered the oocyte surface, as observed by light (Fig. 1) or scanning electron microscopy (Figs. 2, 3).

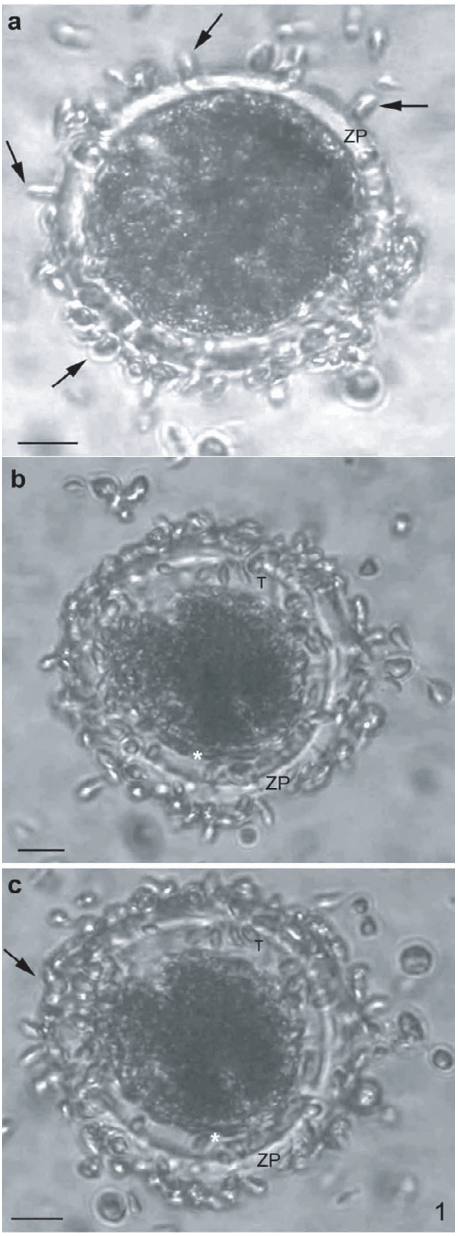

Figure 1. *T. foetus* on the surface of bovine oocyte and its gradual invasion by light microscopy. Parasites were co-incubated with fresh oocytes in a cell ratio of 1 000:1 and observed by phase contrast video microscopy for one hour. Note that parasites (arrows) adhered to the surface of the zona pellucida (ZP) after one hour. In Figure 1a, it is possible to observe that the oocyte maintains its integrity and healthy-appearance. In Figures 1b, 1c, the oocyte presents an unhealthy-appearance, with retracted cytoplasm, and *T. foetus* cells are seen invading the zona pellucida. Note that *T. foetus* (T) in Figure 1b is just invading, and has succeeded in (c). Asterisks point to trichomonads attached to the oocyte plasma membrane. Bars = 20 μm.

3.3. Damage evidence

A first morphological sign of a cytophathic effect in the oocyte was a gradual displacement of the ZP which presented severe injury (Figs. 1b, 1c, 5). Large areas denuded of cumulus cells were seen on the oocyte surface where abundant trichomonads were adhered (Fig. 1). Extensive destruction of the oocyte was seen (Figs. 1, 2). The parasites were able to cross the ZP, and after 3–4 h they were

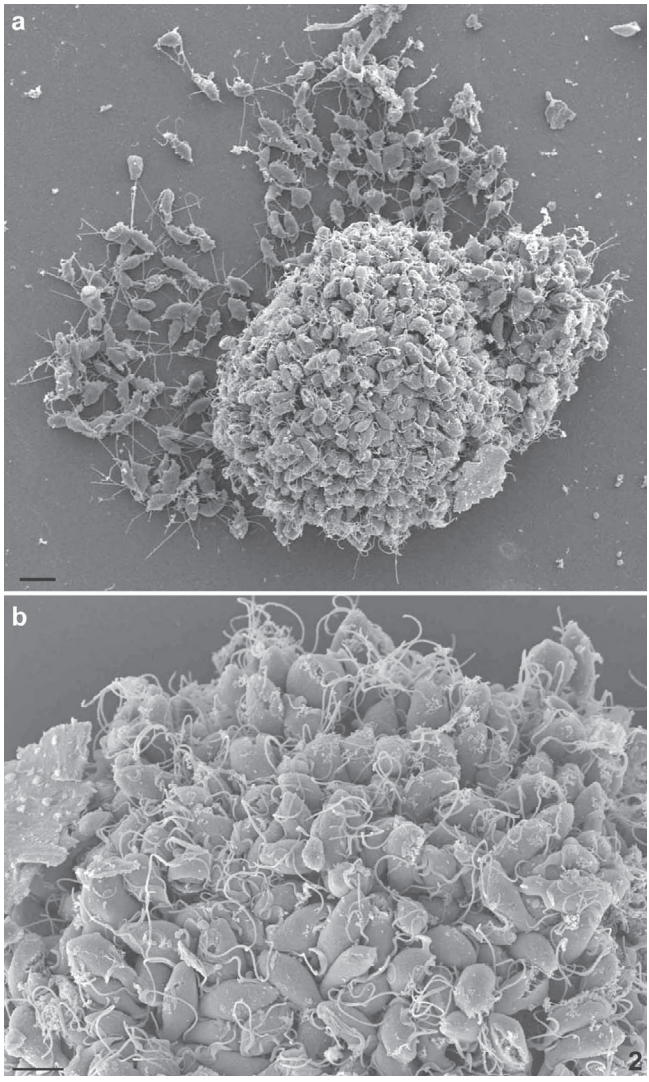

Figure 2. SEM of interaction of *T. foetus* (T) with the cow's oocyte for 2 h in a cell ratio of 1 000:1. Several *T. foetus* cells are seen adhered to the oocyte, and completely cover the gamete. Note that *T. foetus* does not modify its original morphology and displays externalized flagella and pear-shape. Figure 2b is a higher magnification of Figure 2a region. Bars = 20 μm.

found beneath the ZP, which was disrupted (Figs. 1, 3). Wide spaces were formed between the cumulus cells covering the ZP within three hours after co-incubation (Figs. 1a–1c).

Morphological changes of the cumulus cells when observed by transmission electron microscopy were compatible with apoptosis, such as chromatin condensation, and intense vacuolization, which were dif-

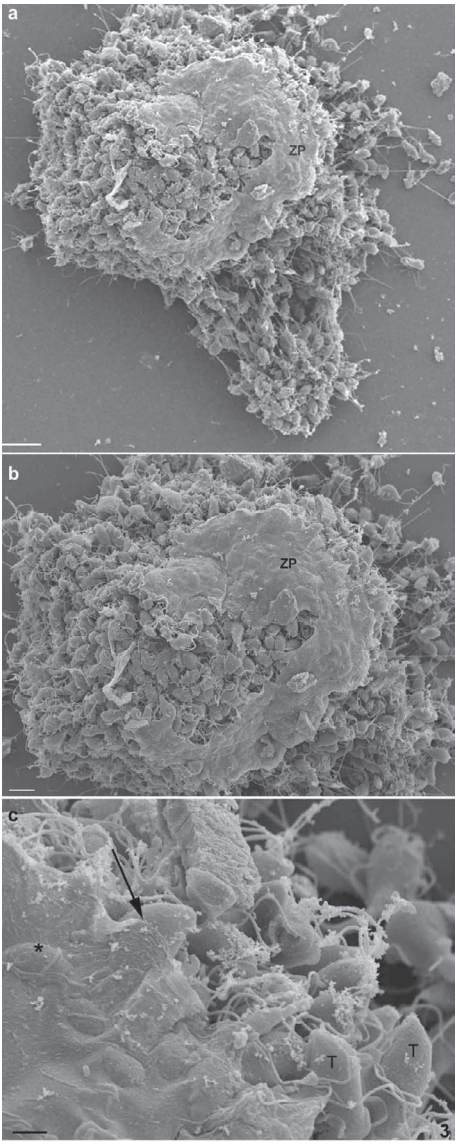

Figure 3. SEM of interaction of *T. foetus* (T) with cow's oocyte for 4 h, after initial cell concentration of 1 000 parasites per cell. Several *T. foetus* cells are seen adhered to the oocyte already denuded of cumulus cells. Note that *T. foetus* completely covered the gamete and provoked a detrimental effect in the zona pellucida. Progressive injury of the zona pellucida (ZP) and invasion (arrow) of *T. foetus* (T) in the cow's oocyte is seen after 4 h interaction. Note the rupture of the zona pellucida (ZP) and trichomonads (T) can be seen below the zona pellucida (asterisks). Bars: a = 50 μm; b = 15 μm; c = 10 μm.

ferent from the control (Fig. 5a). These morphological features were considered as morphological signs of cell death by apoptosis (Fig. 5). However this only occurred when *T. foetus* were added in high concentrations such as 1 000:1.

4. DISCUSSION

It is well known that infection by *T. foetus* may result in transient or permanent infertility in cows. *T. foetus* has been reported to attach and provoke damage to bovine

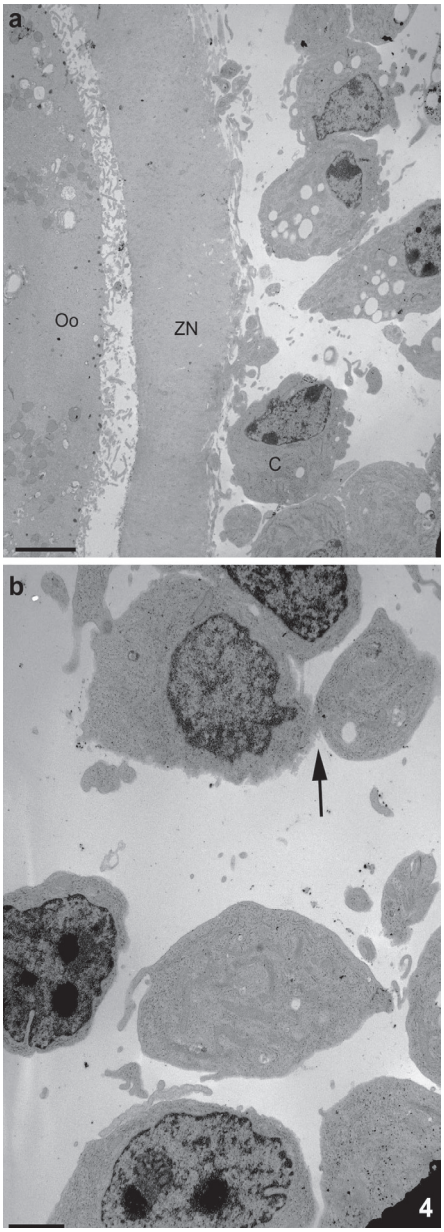

Figure 4. Transmission electron microscopy of the oocyte (Oo) presenting cumulus cells (C) after the first hour of interaction with *T. foetus* in a cell ratio of 100:1 oocyte. Note that the zona pellucida (ZN) is still intact (a) and trichomonads are already interacting with the cumulus cells (arrow). Bars: a = 10 μm; b = 2 μm.

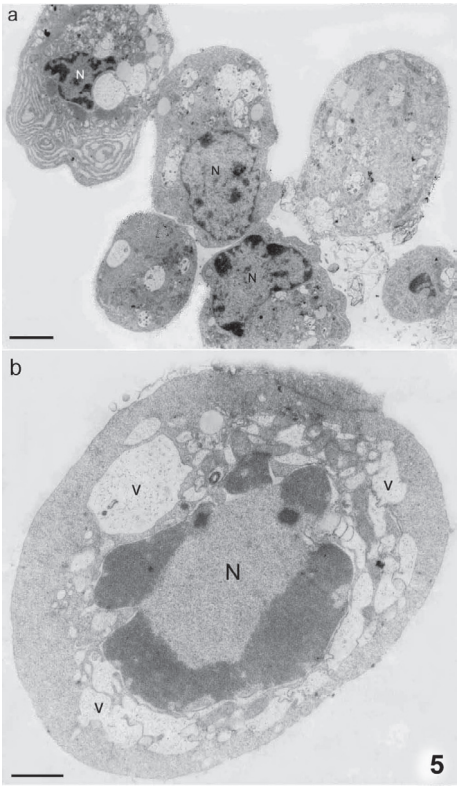

Figure 5. Transmission electron microscopy of cumulus cells before (a) and after (b) *T. foetus* interaction in a cell ratio of 1 000:1 for 6 h. Note that morphological changes of the cumulus cells are compatible with apoptosis, such as chromatin condensation, and intense vacuolization (V), which are different from the control (a). Bars: a = 3 μm; b = 1 μm.

primary vaginal and uterine epithelial cell cultures [18, 19], inhibit host-cell division [16], cause inflammation of the uterus and uterine tubes [1], invade bovine placental tissue [15], and produce enzymes capable of attacking host tissues [4]. However, only limited information is available describing the effects of interaction between *T. foetus* and reproductive cells.

The mammalian egg is surrounded by an extracellular coat, the ZP, which plays

an important role during fertilization. The ZP binds the spermatozoon in a species-specific manner, induces acrosome reaction, and prevents penetration of more than one spermatozoon. *T. foetus* is a parasitic protozoan that causes infertility and abortion. However, there is not documented information on the mechanisms that could lead to infertility. To determine the effect of *T. foetus* on fertilization, we added parasites to fresh bovine oocytes. Light and scanning electron microscopy (SEM) revealed that exposure of oocytes to *T. foetus* caused rapid adhesion of the trichomonads to the intact ZP, or to the cumulus cells. Video microscopy allowed us to follow the intense movement of the parasites towards the oocytes, and the invasion of *T. foetus* into the reproductive cell. In addition, on the contrary to *T. vaginalis* that assume an amoeboid shape when adhering to epithelial cells [9], *T. foetus* maintained its shape and the externalized flagella, suggesting a different behavior of these two species.

When examined by SEM, the oocytes exhibited significant damage in the ZP, not visible in the controls (with no trichomonad contact) or when low amounts of *T. foetus* were added, such as 10:1, or 100:1. When added in higher concentrations, such as 1 000:1 *T. foetus* was able to invade the cell compartment and provoked signs of cell death.

The fact that there is little damage until 1 000 *T. foetus* per oocyte is in accordance with the work of Bielanski et al. [3], who did not detect a detrimental effect on the fertilization and development of embryos caused by *T. foetus.* It is important to note that this latter group used *T. foetus* in a concentration of 500:1 cells. Thus, we suggest that a large number of parasites are necessary to damage this reproductive cell. Whether this occurs in vivo, we do not know, but if a large number of trichomonads is infecting a cow, and if the observations presented here are real in vivo, reproductive failure may occur.

T. foetus multiplies very rapidly and, on the contrary to sperm cells, is able to divide during the interaction process with target cells. It is well known by recent publications that *T. foetus* cells are able to survive and divide under adverse conditions, since they form pseudocysts [10]. It was shown that pseudocysts present internalized flagella and are able to adhere to host-cells [12, 14].

In the present work, we observed that cumulus cells display morphological characteristics of apoptosis, with intense chromatin condensation, and cytoplasmic vacuolization, differently from the controls. The oocyte became retracted, presenting evident signs of cell lesion. Recently, it has been demonstrated that *T. foetus* induces apoptotic cell death in bovine vaginal epithelial cells [18] and in cultured bovine uterine epithelial cells [19]. In addition, Sommer et al. [21] showed that *T. vaginalis* cystein proteases can induce apoptosis in human epithelial cells. Programmed cell death (PCD) is a genetically regulated physiological process of cell demise that is central to the development and homeostasis of multicellular organisms [20]. The nuclear changes observed after *T. foetus* interaction could be due to the activation of different proteases (endonucleases) during the process of death. An intense vacuolization was also detected. These features have also been found during the apoptotic and autophagic degeneration process of several cell types such as *T. thermophila* induced by staurosporine [6].

Barttlet [2] observed that when coitus with an infected bull occurred during the period of infection, no pregnancy was initiated. This finding clearly indicated the highly deleterious effect of typical initial infections upon the early phases of reproduction of the bovine female. This author also stated that *T. foetus* characteristically terminates pregnancies so early in

gestation (e.g. day 1 to day 70) that a recognizable conceptus is not aborted, or if aborted, is not observed. Thus, active trichomonad infections ordinarily terminate pregnancies early, and many abortions usually pass unnoticed. Parsonson et al. [13] did a very thorough study of infection on the outcome of pregnancy and found that pregnancies were not usually terminated as early as in the Bartlett paper [2], since pregnant infected cows were killed after 15–100 days in the Parsonson work [13]. In the Bartlett paper [2], it was not clear whether the infected bulls had been very fertile before they were infected. Therefore, it is not clear whether the end of pregnancy was entirely due to *T. foetus* or to other factors.

Thus, it appears that *T. foetus* is a parasite that functions as a death inducer in cells of the reproductive tract. Although it is well recognized and documented that *T. foetus* causes infertility, its direct effects are not available in the literature.

In the present report, we suggest that *T. foetus* provokes damage to the reproductive cell, not only injuring the ZP, but also provoking cell death. We reasoned that if *T. foetus* could cause cytotoxicity in vitro to oocytes, damage could occur in vivo as well, and this would be an important fact in determining pregnancy outcome in cows infected by this parasite. It is important to take in mind that the study reported here was conducted in vitro. Further studies are necessary to find out whether it happens in vivo. We should remember that the in vivo environment includes innate and acquired immune systems that could blunt the effect of the trichomonads on oocytes or embryos.

Anyway, answers to questions about the possible penetration and adherence of microorganisms to the oocyte-cumulus complexes and to the ZP of oocytes and embryos in different steps of the in vitro fertilization procedure are of great concern.

Thus, we hope that this in vitro work may shed some light on future possibilities concerning cattle sterility involving trichomonads.

ACKNOWLEDGEMENTS

This work was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), PRONEX (Programa de Núcleo de Excelência, Brazil), FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro, Brazil), FENORTE (Fundação do Norte Fluminense, Brazil) and AUSU (Associação Universitária Santa Úrsula, Brazil).

REFERENCES

[1] Anderson M.L., BonDurant R.H., Corbeil R.R., Corbeil L.B., Immune and inflammatory responses to reproductive tract infection with *Tritrichomonas foetus* in immunized and control heifers, J. Parasitol. (1996) 82:594–600.

[2] Bartlett D.E., *Tritrichomonas foetus* infection and bovine reproduction, Am. J. Vet. Res. (1947) 8:343–353.

[3] Bielanski A., Ghazi D.F., Phipps-Toodd B., Observations on the fertilization and development of preimplantation bovine embryos in vitro in the presence of *Tritrichomonas foetus*, Theriogenology (2004) 61:821–829.

[4] Burguess D.E., Knoblock K.F., Daugherty T., Robertson N.P., Cytotoxic and hemolytic effects of *Tritrichomonas foetus* on mammalian cells, Infect. Immun. (1990) 58:3627–3632.

[5] Corbeil L.B., Hodgson J.L., Jones D.W., Corbeil R.R., Widders P.R., Stephens L.R., Adherence of *Tritrichomonas foetus* to bovine vaginal epithelial cells, Infect. Immun. (1989) 57:2158–2165.

[6] Christensen S.T., Chemnitz J., Straarup E.M., Kristiansen K., Wheatley D.N., Rasmussen L., Staurosporine-induced cell death in *Tetrahymena thermophila* has mixed characteristics of both apoptotic and autophagic degeneration, Cell Biol. Int. (1998) 22:591–598.

[7] De Loss de F., Van Vliet C., Van Maurik P., Kruip T.A.M., Morphology of immature

bovine oocytes, Gamete Res. (1989) 24:197–204.

[8] Diamond L.S., The establishment of various trichomonads of animals and man in axenic cultures, J. Parasitol. (1957) 43:488–490.

[9] González-Robles A., Lazaro-Haller M., Espinosa-Castellano M., Anaya-Velazquez F.A., Martinez-Palomo A., *Trichomonas vaginalis:* ultrastructural bases of the cytophatic effect, J. Eukaryot. Microbiol. (1995) 42:641–651.

[10] Granger B.L., Warwood S.J., Benchimol M., De Souza W., Transient invagination of flagella by *Tritrichomonas foetus*, Parasitol. Res. (2000) 86:699–709.

[11] Honigberg B.M., Trichomonads of veterinary importance, in: Kreier J.P. (Ed.), Parasitic Protozoa, vol. II, Academic Press, London, 1978, pp. 207–242.

[12] Mariante R.M., Coutinho L.L., Benchimol M., Pseudocysts adhere in a contact dependent manner, Parasitol. Res. (2004) 92:303–312.

[13] Parsonson I.M., Clark B.L., Dufty J.H., Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers, J. Comp. Pathol. (1976) 86:59–66.

[14] Pereira-Neves A.N., Ribeiro K.C., Benchimol M., Pseudocysts in trichomonads- new insights, Protist (2003) 154:313–327.

[15] Rhyan J.C., Wilson K.L., Burguess D.E., Fetal and placental lesions in bovine abortion due *Tritrichomonas foetus*, Vet. Pathol. (1988) 25:350–355.

[16] Silva-Filho F.C., De Souza W., The interaction of Trichomonas vaginalis and Tritrichomonas foetus with epithelial cells in vitro, Cell Struct. Funct. (1988) 13:301–310.

[17] Singh B.N., Lucas J.J., Beach D.H., Shin S.T., Gilbert R.O., Adhesion of Tritrichomonas foetus to bovine vaginal epithelial cells, Infect. Immun. (1999) 67:3847–3854.

[18] Singh B.N., Lucas J.J., Hayes G.R., Kumar I., Beach D.H., Frajblat M., Gilbert R.O., Sommer U., Costello C.E., *Tritrichomonas foetus* induces apoptotic cell death in bovine vaginal epithelial cells, Infect. Immun. (2004) 72:4151–4158.

[19] Singh B.N., Hayes G.R., Lucas J.J., Beach D.H., Gilbert R.O., In vitro cytophatic effects of a cysteine protease of *Tritrichomonas foetus* on cultured bovine uterine epithelial cells, Am. J. Vet. Res. (2005) 66:1181–1186.

[20] Steller H., Mechanisms and genes of cellular suicide, Science (1995) 267:1445–1449.

[21] Sommer U., Costello, C.E., Hayes G.R., Beach D.H., Gilbert R.O., Lucas J.J., Singh B.N., Identification of *Trichomonas vaginalis* cysteine proteases that induce apoptosis in human epithelial cells, J. Biol. Chem. (2005) 280:23853–23860.

To access this journal online:
www.edpsciences.org/vetres