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Observations on the fertilization and development of preimplantation bovine embryos in vitro in the presence of *Tritrichomonas foetus*

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Abstract

Tritrichomonas foetus, a world-wide distributed parasitic protozoan is a cause of infertility and abortion. There is no documented information on the susceptibility of bovine embryos to the parasite. To determine the effect of *T. foetus* on fertilization and embryonic development of preimplantation bovine embryos, we added approximately 10^4/ml or 10^6/ml *T. foetus* (Belfast strain) to sperm cells and oocytes prior to in vitro fertilization (IVF) or to presumptive zygotes 24 h post-fertilization. Light and scanning electron microscopy (SEM) revealed that exposure of oocytes or embryos at any stage of development to *T. foetus* caused rapid adhesion of the trichomonads to the embryonic intact zona pellucida (ZP) and to trophoblastic cells of hatched blastocysts. Treatment of contaminated embryos with 0.25% trypsin for 3 min did not render them free from *T. foetus*. Motile parasites were not observed after 18 h incubation in IVF medium, or after 72 h in synthetic oviductal fluid (SOF) embryo culture medium. The percentages of cleaved zygotes, blastocysts and hatched embryos resulting from culture of experimental and uninfected control groups of embryos were not different ($P > 0.05$). *Tritrichomonas foetus* was not detected in embryonic cells of ZP-intact or hatched embryos when examined by transmission electron microscopy (TEM). In conclusion, *T. foetus* has no detrimental effect on the fertilization and development of IVF embryos and the potential risk of transmission of trichomonosis is unlikely, due to the limited survival of the parasite in IVF culture conditions.

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Keywords: *Tritrichomonas foetus*; Embryo; Semen; Fertilization; Zona pellucida

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1. Introduction

Bovine trichomonosis is caused by a world-wide distributed protozoal parasite, *Tritrichomonas foetus*, initially recognized over a century ago [1–3]. Due to its substantial reproductive pathogenicity and economic losses, trichomonosis is a reportable disease in most countries and has been included in List B of infectious diseases by the Office International des Epizooties (O.I.E.) [4]. *Tritrichomonas foetus* is transmitted mainly by the venereal route from infected bulls to cows, with subsequent confinement of the trichomonads to the epithelial cells of the reproductive tract causing a relatively mild inflammatory reaction. Salpingitis, endometritis, repeated breeding, abortion and pyometra are noted in affected cows [5,6]. It has been shown that the *T. foetus* can survive in fresh and cryopreserved semen; artificial insemination (AI) has been considered as a potential route of spreading the infection [7,8]. Although it is well recognized and documented that *T. foetus* causes infertility, there is no information available in the literature on its direct effects on the fertilization and development of preimplantation bovine embryos. Thus, to better understand the role of *T. foetus* in the pathogenesis of trichomonosis, the effect of *T. foetus* on fertilization and early embryonic development, and the transmission of *T. foetus* to embryos in an in vitro culture system were investigated.

2. Materials and methods

2.1. In vitro fertilization and embryo culture

Unless otherwise noted, all chemical reagents used during IVF and embryo culture were supplied by Sigma (St. Louis, MO, USA).

Bovine ovaries, collected immediately after slaughter, were rinsed and washed twice in fresh phosphate-buffered saline (PBS) containing 100 μg/ml streptomycin, 100 IU/ml penicillin and 25 μg/ml fungizone. Cumulus–oocyte complexes (COC) were aspirated from ovarian follicles 3–6 mm in diameter, using an 18-gauge needle and vacuum pressure pump. Oocytes surrounded by multi-layers of compact follicular cells were washed twice in PBS and then once in the maturation medium, composed of tissue culture medium (TCM-199) with Earle's salts supplemented with 10% fetal cow serum (FCS), 1 μg/ml estradiol, 35 μg/ml FSH-P (Folltropin, Bioniche Animal Health, Belleville, Ont., Canada), 10 IU/ml hCG (Ayerst), 0.4 mM glutamine, 0.2 mM sodium-pyruvate and 50 μg/ml gentamycin. Oocytes were matured in 400 μl of the maturation medium overlaid with silicone oil in culture dishes (Nunclon, Cat. No. 176740, 4000 Roskilde, Denmark) at 38.5 °C and in 5% CO_2 in air with 100% humidity for 24 h. After maturation, oocytes were washed three times in modified Tyrode's solution (TALP-HEPES) and once in fertilization medium (TALP-F) before being transferred to fertilization droplets. The 50 μl drops of IVF-TALP contained hypotaurine (10 mM), penicillamine (20 mM), epinephrine (1.0 mM), heparin (10 μg/ml) and 0.6% bovine serum albumin [9].

A swim-up method was used to prepare spermatozoa for IVF [10]. Semen from six bulls (free of *T. foetus*) was collected, pooled and frozen at a commercial AI center, and then was used for the experiments. Briefly, 150 μl of thawed sperm was pipetted under 1 ml of sperm medium (S-TALP) and incubated for 60 min at 38 °C, followed by aspiration of the top

800 μl of medium, twice centrifugation of sperm supernatant, and dilution of the sperm pellet to the required concentration for IVF. Oocytes were fertilized in droplets (50 μl) of IVF-TALP, with approximately 1×10^6/ml of motile sperm cells and then incubated at 38.5 °C in 5% CO_2 in air.

Eighteen hours post-insemination, the cumulus cells were removed from the oocytes by vortexing for 2 min in 1 ml PBS. Subsequently, presumptive zygotes were washed in PBS medium before being transferred to 500 μl of modified synthetic oviductal fluid (SOF) and incubated for 7 days under silicone oil at 38.5 °C in 5% CO_2, 5% oxygen and 90% nitrogen [11]. At the end of this incubation period, the number of embryos that had cleaved and developed to the morula or blastocyst stage was determined. The proportions of fertilized ova that had developed to each of these stages were evaluated by the Chi-square test. Differences for which the probability of occurrence by chance was <0.05 were considered significant.

2.2. *Tritrichomonas foetus* cultivation

Frozen *T. foetus* (Belfast strain, Cat. No. 30166) in 5% DMSO was obtained from the American Type Culture Collection, (Rockville, MD, USA). Upon thawing, *T. foetus* was cultivated in Winters medium [12] (pH 7.2), with 0.25% of FCS at 37 °C in a humidified atmosphere of 5% CO_2. One liter medium contained 3 g beef extract, 10 g Bacto-Peptone, 10 g dextrose, 0.9 g agar and 1 g NaCl. Stock cultures were routinely passaged at 48-h intervals in the same medium. For experimentation, the trichomonads were harvested in log phase (24 h) and centrifuged at $2000 \times g$ for 15 min. Pellets were washed in a small volume of medium, and trichomonads were counted using a Neubauer chamber and then re-suspended at required concentrations in the sperm and IVF media. The presence and motility of parasites in culture media and in association with embryos in all experiments were assessed at 400× magnification.

2.3. *Experimental design*

In a preliminary experiment, frozen–thawed semen was spiked with *T. foetus* and used for swim-up procedure [10]. This resulted in poor survival of sperm and low sperm count, and did not allow it to be used for IVF (results are not shown). Therefore, to determine the effect of the parasite on fertilization, *T. foetus*, at a concentration of 1×10^4/ml (approximately 500 trichomonads) or 1×10^6/ml (approximately 50,000 trichomonads) in 2 μl was added directly to the fertilization-drop medium containing non-contaminated spermatozoa resulting from swim-up.

In other experiments, to determine the direct effect of the *T. foetus* on embryonic development, non-contaminated sperm was used for IVF and trichomonads were then added either to presumptive zygotes 24 h post-fertilization or to unhatched expanded and hatched, blastocyst-stage embryos cultured in SOF medium.

2.4. *Trypsin treatment of embryos*

Zona pellucida-intact embryos at morula and blastocyst stages with an intact zona pellucida (ZP) were exposed to *T. foetus* (1×10^4 or 1×10^6/ml) in SOF medium for 3 h.

Subsequently, embryos were washed five times by transfer through multi-well plates, sequentially containing 2 ml PBS with 1% BSA and then two wells with 0.25% trypsin solution (Gibco BRL, Burlington, Ont., Canada) for 3 min, followed by five washes in PBS containing 10% FCS. Washed embryos were examined using light microscopy to assess motility and presence of trichomonads adhered with ZP of embryos. Some embryos were fixed and processed for scanning electron microscopy (SEM) [13].

2.5. Scanning electron microscopy

Groups of ZP-intact and hatched embryos exposed to *T. foetus* after washing were fixed in 3% glutaraldehyde and processed for SEM and transmission electron microscopy (TEM) [12].

3. Results

Exposure of oocytes, zygotes and embryos to *T. foetus* resulted in adherence of multiple trichomonads to the ZP (Fig. 1). Movement of affected embryos by beating flagella was easily visible using light microscopy. Scanning electron microscopy revealed multiple trichomonads attached to the surface of the ZP (Fig. 2A). Some trichomonads were attached to the ZP by their flagella, while others were embedded in craterous fenestrations of the ZP (Fig. 2B).

Direct contamination of IVF medium or exposure of zygotes to *T. foetus* in SOF medium had no effect on fertilization and subsequent embryonic development (Table 1). There were no motile spermatozoa or *T. foetus* movement observed after 18 h post-insemination. In contrast, motile spermatozoa were present in IVF control cultures

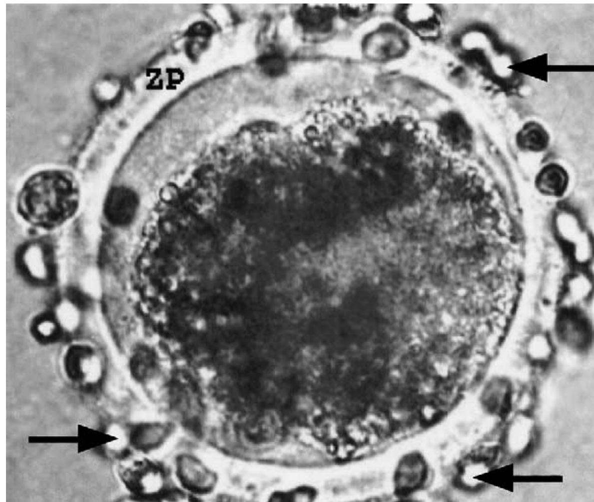

Fig. 1. *Tritrichochomonas foetus* (arrows) adhered to the surface of the zona pellucida (ZP) (400×).

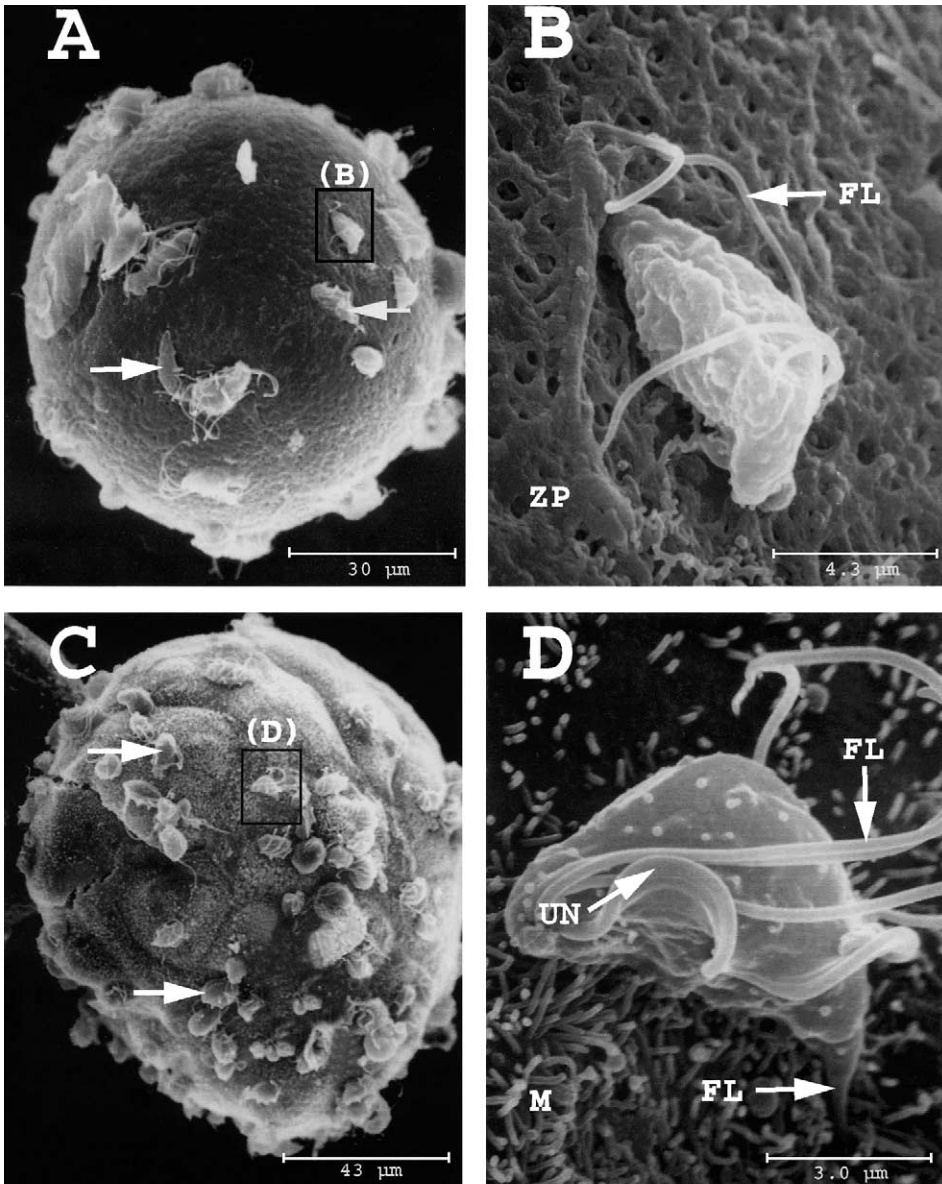

Fig. 2. (A) Scanning electron micrographs of bovine blastocyst showing *Tritrichomonas foetus* (arrows) adhering to ZP (1100×). (B) Higher magnification from (A), ZP: zona pellucida; Fl: flagella (7700×). The parasite's posterior end is embedded in the ZP. (C) Multiple parasites (arrows) attached to the surface of hatched embryo (770×). (D) Higher magnification from (B), M: trophoblastic microvilli (11,000×). The three anterior flagella, flagellum undulating membrane (UN) and the posterior flagellum buried in microvilli are clearly visible.

Table 1
Result of embryonic development in the presence of *T. foetus* (10^4/ml and 10^6/ml) in TALP-F medium and SOF culture medium

Experimental group/medium	Embryos			
	Number of ova inseminated	Cleaved number (%)	Blastocyst number (%)	Hatched blastocyst number (%)
TALP-F control	151	91 (60)	29 (19)	11 (7)
TALP-F + *T. foetus* (10^4/ml)	140	80 (57)	28 (20)	10 (7)
TALP-F + *T. foetus* (10^6/ml)	137	80 (58)	25 (18)	9 (7)
SOF + *T. foetus* (10^4/ml)	121	76 (63)	26 (21)	10 (8)
SOF + *T. foetus* (10^6/ml)	125	74 (49)	22 (18)	7 (7)
SOF control	196	120 (61)	43 (22)	15 (8)

Within each column, there are no significant differences among treatments. SOF: synthetic oviductal fluid, TALP-F: in vitro fertilization medium.

containing non-contaminated sperm cells and embryos. Survival of the trichomonads in SOF cultures was limited to 72 h.

Direct exposure of expanded blastocysts to *T. foetus* resulted in hatching rates not different from that observed in control embryos not exposed to the parasite (38% versus 40%, $P > 0.05$; Table 1). *Tritrichomonas foetus* was attached to the microvilli of trophoblastic cells of hatched embryos (Fig. 2C and D). An attempt to locate the *T. foetus* in embryonic cells by TEM was not successful.

Exposing contaminated embryos ($n = 55$) to trypsin solution did not render them free from the parasite. It was noted that trichomonads retained their motility in trypsin washes for at least 2 h.

4. Discussion

The present study demonstrated for the first time that *T. foetus* apparently has no effect on fertilization and preimplantation embryonic development, even at concentrations that likely exceeded those present during infection in the reproductive tract [14,15]. These findings may be strengthened somewhat by observations in which breeding bulls infected with *T. foetus* resulted in a 68% pregnancy rate [6]. However, there is no detailed information on the relationship between timing of exposure to *T. foetus* and breeding time. Because the parasites do not replicate in the vagina until after an incubation period of several days, there can be no barrier to fertilization at this time [16]. Otherwise, it can be assumed that the reduction in fertility is due to the prevention of implantation by an unfavorable uterine environment or from interference with placental circulation, resulting in early embryonic mortality and abortion [6,17]. In our in vitro experiments, *T. foetus* was added to embryo cultures both pre- and post-fertilization to mimic the hypothetical possibility of natural infection of oocytes and embryos from zygotes to hatched blastocyst stages which take place during the passage of embryos through the reproductive tract. From the practical aspect of the safety of embryo transfer (ET), it is conceivable that healthy embryos could be associated with *T. foetus* when they are collected from the oviduct and

uterus at the early stages of infection when the reproductive epithelium and its secretory properties are not yet affected by the parasite. Likewise, it could be assumed that in later stages of pregnancy, endometritis will prevent the collection of viable embryos and thus diminish the risk of disease transmission by ET. The possibility of production of contaminated embryos by aspiration of oocytes for IVF from ovarian follicles seems to be unlikely, as the presence of parasites was not confirmed in blood or other body fluids [2].

Since it was reported that *T. foetus* can survive in frozen semen [18,19], it should be considered as a potential means of introducing the parasite to the IVF system. It was interesting to note the inhibitory effect of *T. foetus* on sperm motility during swim-up and IVF. To our knowledge, despite numerous reports dealing with infection and transmission of *T. foetus* by bulls or semen, there is no relevant information on the interaction of this parasite with spermatozoa. This requires further investigation.

From the present observations, it appeared that *T. foetus* interacted with embryos by adherence to the ZP and embryonic cell membranes through their flagella and the cell body in a manner similar to bovine vaginal epithelial cells and Madin-Darby canine epithelial cells [20–22]. However, under the conditions used in the present study, the parasite had no ability to penetrate the ZP or affect embryonic survival by release of enzymatic components such as proteases, glycosidases and neuraminidase, or other cytotoxins that were reported to affect tissues [3,21–24].

Further, it has been shown that even extended treatment of contaminated embryos with trypsin, commonly used in embryo washing media, has no immediate effect on the release of trichomonads from ZP or the parasite survival. This corroborates the findings of other authors that the *T. foetus* is not affected by trypsin [21]. Nevertheless, the present study demonstrated that, using IVF, it was possible to produce transferable embryos from contaminated semen and embryos. However, it is important to recognize the limitations of in vitro experimental findings in the determination of embryo–pathogen interactions under natural conditions [25,26].

In general, the cultivation of *T. foetus* in vitro requires specific culture conditions and composite media. Despite that, we observed that the parasite has the ability to survive for a considerable period of time in the simple ET/IVF culture media. From a sanitary point of view, this may be important when uterine stage embryos are collected from infected cows and transferred to recipients without an extended period of culture or when they are cryopreserved. In this context, production of embryos by IVF, that requires relatively longer periods of embryo culture, appears to be more appropriate to prevent the possibility of disease transmission by ET.

Tritrichomonas foetus, like *Neospora caninum* is a parasitic aborifatient. The mechanism of association of *N. caninum* with early embryonic mortality and the potential for its transmission by preimplantation embryos has recently been investigated. *Neospora caninum*, in contrast to *T. foetus*, does not attach firmly to the intact ZP, but has the ability to invade ZP-free, blastocyst-stage embryos and cause their degeneration [27]. It has been demonstrated that *N. caninum* is not transmitted by ET [28]. It is not known whether *T. foetus* can be transmitted by ET. However, in a case of embryo production from cows infected with *T. foetus*, it could be advisable to collect oocytes for IVF rather than embryos from a superovulated and inseminated cow to avoid the potential of recovering contaminated embryos from the uterus.

In conclusion, it appears that *T. foetus* has no detrimental effect on the fertilization and development of IVF embryos, and the potential risk of transmission of trichomonosis by IVF embryos seems to be unlikely due to the limited survival of the parasite in IVF culture conditions. Furthermore, detailed studies on the transmission of trichomonosis by ET are warranted.

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