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Differential susceptibility of human trophoblastic (BeWo) and uterine cervical (HeLa) cells to *Neospora caninum* infection

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ABSTRACT

Neospora caninum is an apicomplexan parasite, closely related to *Toxoplasma gondii,* and causes abortion and congenital neosporosis in cattle worldwide. Trophoblast cells act in mechanisms of innate immune defense at the fetal-maternal interface and no data are available about the interaction of *Neospora* with human trophoblasts. Thus, this study aimed to verify the susceptibility of human trophoblastic (BeWo) compared with uterine cervical (HeLa) cell lines to *N. caninum*. BeWo and HeLa cells were infected with different parasite:cell ratios of *N. caninum* tachyzoites and analyzed at different times after infection for cell viability using thiazolyl blue tetrazole and lactate dehydrogenase assays. Both cell lines were also evaluated for cytokine production and parasite infection/replication assays when pre-treated or not with *Neospora* lysate antigen (NLA) or human recombinant IFN-γ. Cell viability was increased up to 48 h of infection in both types of cells, suggesting that infection could inhibit early cell death and/or induce cell proliferation. *Neospora* infection induced up-regulation of the macrophage migration inhibitory factor (MIF), mainly in HeLa cells, which was enhanced by cell pre-treatment by NLA or IFN-γ. Conversely, parasite infection induced down-regulation of the transforming growth factor (TGF-β), mostly in BeWo cells, which was decreased with NLA or IFN-γ pre-treatment. HeLa cells were more susceptible to *Neospora* infection than BeWo cells and IFN-γ pre-treatment resulted in reduced infection indices in both cell lines. Control of parasite growth was mediated by IFN-γ through an indoleamine-2,3-dioxygenase-dependent mechanism in HeLa cells alone. Based on these results, we concluded that BeWo and HeLa cells are readily infected by *N. caninum*, although presenting differences in susceptibility to infection, cytokine production and cell viability. Thus, these host cells can be considered in comparative approaches to understand strategies used by *N. caninum* to survive at the maternal-fetal interface.

1. Introduction

Neospora caninum is an obligate intracellular apicomplexan parasite, closely related to *Toxoplasma gondii*, and successfully infects a wide variety of host cell types in vitro (Dubey et al., 2007). Attachment to and invasion of host cells are crucial steps in the establishment of infection and subsequent survival and proliferation of the parasite (Lei et al., 2005). *Neospora caninum* was first identified in dogs, causing neuromuscular disease (Dubey et al., 1988), and currently is recognised as a major cause of abortion in cattle worldwide, resulting in great economic losses in livestock (Dubey et al., 2007).

Natural and experimental infections have been demonstrated in a broad range of intermediate hosts including domestic, wildlife, and laboratory animal species (Björkas et al., 1984; Björkas and Presthus, 1989; Dubey et al., 2002). In non-human primates, fetal infection was evidenced after experimental *N. caninum* inoculation into pregnant females, with lesions similar to those found in congenital toxoplasmosis (Barr et al., 1994). Therefore, humans could be probable hosts of the parasite, raising the question of its zoonotic potential (Dubey et al., 2007). Although there are no conclusive reports of infection in humans (McCann et al., 2008), serological evidence of exposure to the parasite was demonstrated in different human populations (Tranas et al., 1999), including our previous study that showed higher seropositivity to *N. caninum* in immunocompromised patients compared with healthy subjects (Lobato et al., 2006).

Neospora caninum can be horizontally transmitted by ingestion of tissues infected with tachyzoites or cysts containing bradyzoites, or by ingestion of food or drinking water contaminated by oocysts shed in the feces of canine definitive hosts. However, vertical transmission accounts for the vast majority of infections in cattle, by transplacental transmission of tachyzoites from the infected dam

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to her fetus during pregnancy. As a result abortion or congenital infection may occur, depending on the time during gestation when the fetus becomes infected (Dubey et al., 2007).

During normal pregnancy, immune responses are preferentially driven to a T helper type 2 (Th2) profile, with the production of anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and transforming growth factor (TGF)-β by both maternal and fetal cells to guarantee the immune tolerance to the fetus (Saito, 2000). In contrast, there is a reduced production of pro-inflammatory cytokines such as IL-12 and IFN-γ associated with a Th1 immune response in the uterine environmental, which are detrimental to fetal life (Clark et al., 1999).

Infections by intracellular protozoan parasites such as *N. caninum* and *T. gondii*, however, induce a typical Th1 response and alter the cytokine profile of the maternal-fetal interface (Innes et al., 2002; Filisetti and Candolfi, 2004). Previous studies have shown that trophoblasts of placenta are directly involved in the pathogenesis of congenital toxoplasmosis (Ferro et al., 1999). For neosporosis, in vivo studies showed the parasite invasion of placental trophoblasts in *N. caninum*-infected mice (Long and Baszler, 1996). Recently, a cloned and 'immortalised' ovine trophoblast cell line was used for studying the pathogenesis of neosporosis (Haldorson et al., 2006), and bovine trophoblastic binucleated cells were evaluated as phagocytic cells in bovine transplacental infection of *N. caninum* (Machado et al., 2007). However, to date there are no available studies analyzing the interaction of *N. caninum* and human trophoblastic cells.

The role of trophoblast cells in the immunology of pregnancy, especially in the presence of infections by intracellular parasites, has been studied using well-established cell lines, such as human BeWo choriocarcinoma cells (Oliveira et al., 2006; Barbosa et al., 2008). These cells have morphological properties common to normal trophoblasts, including the secretion of cytokines as IL-4, IL-6, IL-8, IL-10, TNF-α and macrophage migration inhibitory factor (MIF) (Bennett et al., 1997; Fujisawa et al., 2000; Arcuri et al., 2001).

Considering the major transplacental transmission of *N. caninum* and the lack of data about the interaction of this parasite with human trophoblasts, this study aimed to verify the susceptibility of BeWo trophoblastic cells to *N. caninum* infection, in comparison with human uterine cervical (HeLa) cells, by analyzing the cell viability, cytokine profiles and rates of infection and parasite replication in both cell lines, since they are human transformant cells that may be cultured under identical in vitro conditions.

2. Materials and methods

2.1. Cell culture

BeWo and HeLa cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (GIBCO, Paisley, UK), supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all reagents from Sigma Chemical Co., St Louis, MO, USA) and 10% heat-inactivated FBS (Cultilab, Campinas, Brazil) – complete medium – in a humidified incubator at 37 °C and 5% CO_2.

2.2. Parasite and antigen

Neospora caninum tachyzoites (Nc-1 isolate) (Dubey et al., 1988) were maintained by serial low-passage in BeWo or HeLa cell cultures in supplemented RPMI medium without adding FBS (incomplete medium) and parasite suspensions were obtained as previously described (Silva et al., 2007). Briefly, tachyzoites were harvested by scraping off the cell monolayer after 2–3 days of infection, passed through a 26-gauge needle to lyse any remaining intact host cells, and centrifuged at low speed (45*g*) for 1 min at 4 °C in order to remove host cell debris. The supernatant containing parasite suspension was collected, washed twice (720 *g*, 10 min, 4 °C) in PBS (pH 7.2) and the resulting pellet was resuspended in PBS. Parasites were counted in an hemocytometric chamber under 0.4% Trypan blue staining for further infection experiments and antigen preparation.

Neospora caninum lysate antigen (NLA) was prepared as previously described (Silva et al., 2007), with minor modifications. Parasite suspension was lysed by 10 freeze–thaw cycles and the presence of probable live parasites was monitored by light microscopy under Trypan blue staining. After centrifugation (10,000*g*, 30 min, 4 °C), the supernatant was collected, filtered on a 0.22 μm membrane and its protein concentration determined (Lowry et al., 1951). Different batches of NLA were prepared and pooled together to obtain the required protein concentration. NLA aliquots were stored at −20 °C until use in cell treatment experiments.

2.3. Neospora caninum infection

Non-infected BeWo and HeLa cells were cultured in 96-well plates (2×10^4 cells/200 μL/ well) in complete medium for 24 h at 37 °C and 5% CO_2. Cells were then washed with medium and infected with *N. caninum* tachyzoites at different ratios of parasite:cell (2:1, 5:1 or 10:1) in incomplete medium. As a control, cells were incubated with medium alone. After 24, 48 or 72 h of incubation at 37 °C and 5% CO_2, plates were centrifuged (400*g*, 10 min), the supernatant was collected and stored at −70 °C for lactate dehydrogenase (LDH) activity assays. Cells were analyzed for viability using the thiazolyl blue tetrazole (MTT) assay. Two independent experiments were performed in triplicate for each condition.

2.4. LDH and MTT assays

Cell lysis was evaluated by the measurement of LDH released in culture supernatants as described elsewhere (Lucisano-Valim et al., 2002; Alves et al., 2010). The activity of LDH was measured using a commercial diagnostic kit according to the manufacturer's instructions (LDH Liquiform, Labtest Diagnóstica S.A., Lagoa Santa, Brazil) and expressed in U/mL. As a positive control, total cell lysis was obtained with 0.2% Triton X-100.

Cell viability was evaluated by colorimetric assays using MTT (Sigma Chemical Co.) as previously described (Mosmann, 1983). After different time points of infection, BeWo and HeLa cells were pulsed with MTT at 0.5 mg/mL 4 h prior to the end of the culture. Formazan particles were solubilised in 10% SDS and 50% N, N-dimethyl formamide. The O.D. was read at 570 nm in a plate reader (Titertek Multiskan Plus, Flow Laboratories, Mc Lean, USA). Results were expressed as percentages of viable cells in relation to controls (100% of cell viability).

2.5. Pre-treatment of BeWo and HeLa cells and N. caninum infection

Non-infected BeWo and HeLa cells were cultured in 96-well plates (5×10^4 cells/200 μL/ well) in complete medium for 24 h at 37 °C and 5% CO_2. Cells were washed with medium and treated with human recombinant IFN-γ (R&D Systems, Minneapolis, MN, USA) at 10 ng/mL, NLA at 10 μg/mL or non-treated (NT, complete medium alone) for an additional 24 h at 37 °C and 5% CO_2. Next, cells were again washed with medium and infected with *N. caninum* tachyzoites at a ratio of 5:1 (parasite:cell) in incomplete medium. As controls, treated or untreated cells were kept with medium