POSSIBLE ROLE OF BOVINE TROPHOBLAST GIANT CELLS IN TRANSPLACENTAL TRANSMISSION OF *Neospora caninum* IN CATTLE

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ABSTRACT:- MACHADO, R.Z.; MINEO, T.W. P.; LANDIM JR, L.P.; CARVALHO, A.F.; SOLANGE M. GENNARI; MIGLINO, M.A.A. **Possible role of bovine trophoblast giant cells in transplacental transmission of *Neospora caninum* in cattle.** [Um possível envolvimento de células gigantes trofoblásticas na transmissão transplacentária de *Neospora caninum* em bovinos.] *Revista Brasileira de Parasitologia Veterinária*, v. 16, n. 1, p. 21-25, 2007. Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, FCAV/UNESP, Jaboticabal, SP, 14.884-900, Brasil. E-mail: zacarias@fcav.unesp.br

Neospora caninum is an aplicomplexan parasite that has brought several concerns to cattle raisers worldwide due to its relationship to fetal loss. However, the mechanism of the parasite's transplacental infection and induced abortions are not completely understood. Bovine trophoblastic binucleated cells (BNC) play a major role in the maternal-fetal interactions, migrating during the entire pregnancy from chorionic connections to uterine epithelium. This study aimed to investigate the possible role of BNC as phagocytic cells and its participation in the bovine transplacental infection of *N. caninum*. BNC was isolated by discontinuous Percoll gradient, and characterized by Hoeschst 33342 nucleus-specific staining. Isolated BNC were cultured in DMEM supplemented with 10% bovine fetal serum, and infected with 10^4 tachyzoites of *N. caninum* NC-1 strain. Parasite invasion was visualized by indirect immunofluorescence and Giemsa technique. Multiplication of parasites took place in 2-3 day cycles. Healthy cows' placenta and normal and infected cultured BNC was immunostained with monoclonal antibodies against CD-163, MAC-387 and iNOS, demonstrating their phagocyte capacity. Thus, BNC was characterized as cells with macrophagic activity, which may host *N. caninum* in vitro. Therefore, we may conclude that BNC could potentially participate in the transplacental infection of bovine neosporosis.

KEYWORDS: *Neospora caninum*, *b*ovine trophoblastic binucleated cells, immunohistochemistry, cell culture.

RESUMO

Neospora caninum é um protozoário que tem causado um grande número de problemas reprodutivos em rebanhos bovinos em todo mundo. Entretanto, os mecanismos pelos quais o parasito realiza a infecção transplacentária, não são completamente compreendidos até o momento. Células trofoblásticas binucleadas bovinas (BNC) desempenham papel importante nas interações materno-fetais, migrando durante o período de gestação das conexões coriônicas ao epitélio uterino. Este estudo objetivou a investigação sobre o papel das BNC como célula fagocitária e sua possível participação na infecção transplacentária por *N. caninum*. BNC foram isoladas por gradiente descontínuo de Percoll, caracterizadas pela coloração de Hoeschst 33342, específica ao núcleo. BNC isoladas foram cultivadas em DMEM suplementado com 10% de soro fetal bovino, sendo infectadas por 10^4 taquizoítas de *Neospora caninum*, cepa NC-1. A invasão dos parasitos foi visualizada por imunofluorescência indireta e pela coloração de Giemsa. A multiplicação dos parasitos ocorreu em ciclos de 2-3 dias. Placentas de vacas sadias, bem como BNC infectadas e sadias advindas de cultura celular, foram submetidas a imunohistoquímica com anticorpos anti-CD-163, anti-MAC-387 e anti-iNOS, demonstrando suas capacidades fagocíticas. Assim, BNC foram caracterizadas como células com atividade macrofágica, podendo albergar o *N. caninum in vitro*. Portanto, podemos concluir que as BNC tem potencial para estar participando de forma ativa na infecção transplacentária da neosporose bovina.

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PALAVRAS-CHAVE: *Neospora caninum*, células trofoblásticas binucleadas bovinas, Imunoistoquímica, cultura celular.

INTRODUCTION

Neospora caninum is a protozoan parasite recognized as one of the major causes of bovine abortion. The parasite has a worldwide distribution and antibody prevalence around 10-30% in cattle (DUBEY, 1999). The dominant route of infection in cattle is by transplacental infection, although oral infection by ingestion of oocysts shed by dogs is the key for parasite introduction in the herds (McALLISTER et al., 1998; TREES; WILLIAMS, 2005). Both acutely and chronically infected cattle with *N. caninum* may abort their foetuses or give birth to weak calves that die within the first month of life. However, the majority of infected cows give birth to clinically healthy but chronically infected calves (DUBEY, 2003).

The trophoblastic binucleated cells (BNC) play a major role in the maternal-fetal relationship. These cells are originated by mononucleated trophoblasts through cariocinesis without cytocinesis. It migrates during the entire period of pregnancy, from corionic connection to uterine epithelium, where there is a cellular fusion originating trinucleated cells, which are sent directly to the uterine epithelium. Chronologically, the BNC can be found from the 17th day until the end of pregnancy, when its numbers are reduced to about 15-20% of total trophoblastic cell population (FLOOD, 1991; WOODING, 1992). BNC are able to synthesize and release hormones such as placental lactogen, progesterone, estrogen, prostaglandins and prostacyclins, as well bovine pregnancy-associated glycoprotein (bPAG) (GROSS; WILLIAMS, 1988; MATAMOROS et al., 1994; REIMERS et al., 1985). It is also speculated that the BNC have phagocytic functions (macromolecules filter, and Fe^{+} ion storage), although this role has not yet been shown to be effective on infectious agents models.

This study aimed to investigate the possible role of BNC as fagocytic cells, and its participation in the bovine transplacental infection cycle of *N. caninum*.

MATERIAL AND METHODS

Isolation of Trophoblastic Binucleated Cells. Pregnant uterus (45 to 60 days of pregnancy) were obtained from slaughterhouses and transported in termal containers at 8ºC for primary cell culture. The uterus was sectioned and dissected until placentomes were exposed. Asepsis was assured by the use of 70º ethanol. (LANDIM JR et al., 2003). Fetal cotyledon components were separated manually, washed three times in phosphate buffer solution (PBS) and cut into small pieces to obtain a uniform cellular suspension, which was then submitted to enzimatic digestion by collagenase 0.5%. After inactivation of collagenase by washing steps, cells were ressuspended in Dulbelco's Modified Eagle Medium (DMEM – GIBCO BRL, USA) supplemented with 10% FCS, and layered on top of a Percoll discontinuous gradient (Amersham-Pharmacia Biotech, UK) at 1.043 and 1.060 g/ml, centrifuged for 20 min at 1000 x g. BNC fraction was then found at the Percoll interface described. It was washed and ressuspended in DMEM medium supplemented with 10% FCS, and cultivated on polystyrene Petri dishes, at 38.5ºC, with 5% CO_2 atmosphere and 100% of relative humidity.

Morphological Analysis. The morphologic characterization of BNC was made with HOESCHT 33342 nucleus-specific staining (ICN PHARMACEUTICALS Inc., USA). A aliquot of the cell suspension was incubated for 15 minutes, at 38ºC, with HOESCHT 33342 solution (5µg/ml). Petri dishes were observed on a epifluorescence microscope (460 to 490 nm – Olympus, Japan).

Parasite maintenance, BNC infection and slide preparation. *Neospora caninum* tachyzoites (NC-1 strain) were cultured in CV-1 cells, using RPMI medium (Sigma Inc., USA) supplemented by bovine fetal serum (BFS) at 2%. Parasites were harvested by trypsin 1% treatment of cell monolayer 3 days after infection, and passed on to a new cell bottle with confluent CV-1 cells. Each Petri dish containing BNC was inoculated with 10^4 N. caninum tachyzoites, and infection kinetics was observed by inverted-light microscope (Carl Zeiss, Germany). After total BNC disruption, the parasites were passed on to a new Petri dish for another observation period.

After 4 cycles of infection kinetics observed, a new Petri dish was infected for a 20 hour period. The infected BNC Petri dishes were then incubated at 37ºC for 5 minutes with 1% trypsin solution for cell dislocation. The content was centrifuged at 1000 x g for 10 minutes, followed by three washing cycles with PBS with 0,1% of bovine soroalbumin, using the same centrifugation protocol. Thus, cell concentration was set at 20 cells/field in 100x light microscope (Nikon, Japan). Thirty microliters triplicates of infected cell suspension was added to glass slides and let dry at room temperature. The dried samples were fixed onto the slides by the addition of 10µl of Poly-L-Lisine (Sigma)/cell spot, been followed by a new drying cycle, at room temperature. Some slides were used for Giemsa staining and others were stored in -20 ºC until immunohistochemical a direct immunofluoresce assays were realized.

Indirect immunofluorescence test. Slides with infected and normal BNC were fixed to glass slides as described above. Each antigen spot was incubated with a polyclonal mouse serum against N. caninum, at 1:1000 diluition, for 30 minutes, at 37ºC. The slides were then washed three times, for 5 minutes each, with PBS. An anti-mouse IgG whole molecule conjugated with FITC was used as secondary antibody (Sigma). The slides were again incubated at 37ºC, for 30 minutes before undergoing a new washing procedure. The slides were left to dry before being mounted with cover slip, using buffered glycerin, pH 9.0. After slide preparation, the assays were observed on a epifluorescence microscope (460 to 490 nm – Olympus).

Immunohistochemical assays. The immunohistoche-

mistry assays were performed in BNC infected slides and paraffin-embedded placentas of healthy cows. BNC slides were submitted to three different antibodies bovine polyclonal IgG anti-N. caninum, anti-iNOS (Santa Cruz Biotechnology, USA), anti-MAC-387, and anti-CD-163 (Dakocytomation, Denmark), macrophage markers. The placentas from healthy cows were tested to iNOS, MAC-387 and CD-163 expression. Immunohistochemical procedures were performed following previous description (CASTRO et al., 2004). Briefly, primary antibodies (anti-N. caninum, anti-iNOS, or anti-CD-163) were incubated at optimal dilutions. The avidin–biotin complex immunoperoxidase step was performed (DakoCytomation) and the slides stained with diaminobenzidine tetrahydrochloride (DakoCytomation). Cell spots were counterstained with Harris's hematoxylin 10% and mounted with coverslips. The reaction was read under light microscope (Nikon).

RESULTS AND DISCUSSION

There is no exact information to date about the mechanisms used by *N. caninum* to infect the bovine fetus. Experimental models of congenital toxoplasmosis have been described in the literature since early 1950s (FERRO et al., 2002). However, there is no information concerning the kinetics of events leading to understand how the parasite actually reaches the fetal tissues. In addition, there is no data describing the role played by BNC interacting with the parasites in the placenta as *in vitro* or *in vivo* experimental models.

BNCs were successfully separated from gross placental tissue by Percoll gradient, as determined by HOESCHT 33342 nucleus-specific staining. The assay revealed the presence of mono, bi, and trinucleated trophoblasts after fluorescent analysis. The cells maintained its viability for 30-40 days *in vitro*, sufficient time to allow several parasite serial passages and also other experimental protocols. After that, BNCs tend to undergo rapid apoptosis, with no new cell yield. The trophoblast, an epithelial cell of fetal origin that forms the physical barrier between mother and developing conceptus, becomes a component of the host immune system during pregnancy. BNC have been shown to play a central structural role in developmental and growth of the placenta. They originate from a mononucleated trophoblastic cells, which migrates to the uterine epithelium, where they fuse to endometrial cells, followed by hormone synthesis and release (progesterone included), before returning to the fetal side where apoptosis takes place. They compromise about 20% of the trophoblast layer at the fetal-maternal interface, where their functional properties are shown in the involvement in steroid and protein synthesis, which is necessary for pregnancy's successful outcome (WOODING, 1996). Trophoblasts also act in embryonic and fetal innate immune defense through elimination of microorganisms present at the feto-maternal interface, such as yeasts and bacterias (AMARANTE-PAFFARO et al., 2004).

After parasite challenge, BNC culture was rapidly infected by *Neospora* tachyzoites. Observation of infection kinetics demonstrated a fast parasite invasion and multiplication, with a 2-3 days average time for total cell disruption (Figure 1). In all 4 BNC challenge rounds observed, the same kinetics pattern was observed. Experiments involving infection outcome of

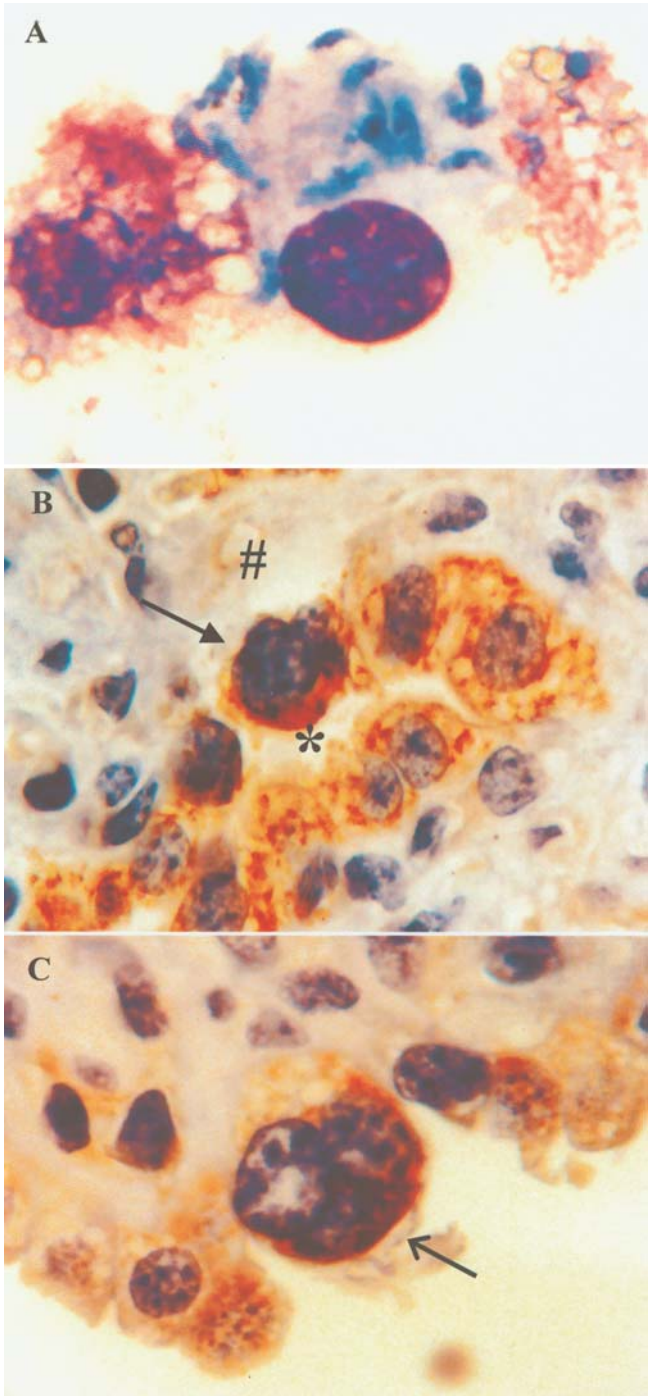

Figure 1. (A) Giemsa stained BNC infected by serial passage of NC-1 tachyzoites of *Neopora caninum in vitro*, showing multiplying parasites inside its cytoplasm, 100x; (B) Uninfected placenta showing positive immunohistochemistry for iNOS in BNC (solid arrowhead) present in the fetal (*)-maternal (#) interface, 400x; (C) Presence of CD-163 marker in BNC (open arrowhead) demonstrated by immunohistochemistry, 400x.

N. caninum in DH-82 cells, a canine histiocytoma lineage, have shown a much slower replication of the parasite, presenting full host cell lyses after an eight to ten day period (MACHADO, 2006)[a]. Indirect immunofluorescent assays revealed crescent amounts of parasites present in BNC primary cultures after serial *in vitro* passages. That increase in *N. caninum* tachyzoites was observed in relation to the number of cells infected by the parasite, as well as number of parasites inside each parasitophagous vacuole. The third and fourth passages were marked by almost total cell disruption at 48-72 h time interval. These results were also observed in slides stained by Giemsa (Figure 1A), although with smaller intensity, since direct immunofluoresce assays tends to expose a higher quantity of parasites inside the cells. However, immunohistochemistry performed with anti-*N. caninum* polyclonal antisera, used as primary antibody, revealed the same assay sensitivity as the indirect immunofluorescence, showing large amounts of tachyzoites within BNC.

Immunohistochemistry of healthy cow's placenta demonstrated positive reactivity of BNC to anti-iNOS and anti-CD163 antibodies (Figure 1B and 1C). Both markers showed higher intensity in the fetal-maternal interface. It was also possible to observe positive reactions within other trophoblastic cell lineages. When healthy and infected BNC primary culture slides were assayed with the same antibodies, positive staining was also observed. There was no difference in staining intensity between healthy and infected BNC.

In protozoan experimental models, it has been demonstrated that *Trypanosoma cruzi* uses placentary alkaline phosphatase to invade host's trophoblastic cells, rearranging actin filaments (SARTORI et al., 2003). *Toxoplasma gondii*, has the ability to invade and multiply mature and immature human trophoblastic cells *in vitro* (ABBASI et al., 2003), showing also tropism to different murine trophoplastic lineages, using a Brazilian savannah rodent - *Calomys callosus* - as the experimental model (FERRO et al., 2002). There are some evidences that viral infections affecting trophoblastic cells would lead to loss of placentary functions, followed by spontaneous abortions, poor fetal development and premature births (ARECHAVALETA-VELASCO et al., 2002). Some viral infections as HIV, adenoviruses, herpes simplex and human cytomegalovirus, use trophoblastic cell lineages to multiply and make their way into the fetus, causing irreversible damage (KOI et al., 2001; HEMMINGS; GUILBERT, 2002).

In recent years, bovine neosporosis has became a major concern for the scientific community and cattle raisers due to its ability to evade the host's defense mechanisms and cause severe fetal loss in herds worldwide (INNES et al., 2005; WOUDA et al. 1998). Infected cows present a risk of abortion up to seven times higher than uninfected counterparts (PARE et al., 1997; THURMOND; HIETALA, 1990). Abortions may take place during acute infection (exogenous transplacental transmission), or due to recrudescence of the parasite acquired in a previous contact, now entitled endogenous transplacental transmission (TREES; WILLIAMS, 2005). In this case, *N. caninum* acts like a 'biological Trojan horse', waiting for adequate conditions (immunosuppression, proper hormone environment) for a swift change from quiescent tissue cysts to rapidly dividing tachyzoites, which will promote its transmission to a new host (INNES et al., 2005). When a dam face *Neospora* infection, its immune response is caught on a major setback: infection control may cost its fetus life, since there is a physiological down regulation of pro-inflammatory cytokines to preserve gestation, although protective immunity against the parasite is dependent on those Th1 type response suppressed mechanisms (INNES et al. 2002; QUINN et al., 2002). An important trigger for this Th1-Th2 immunity shift is progesterone, abundant on early and mid gestation, affecting directly IFNγ production, which is a key resistance factor for infection control (POPE et al., 1969; KALINSKI et al., 1997; INNES et al., 2002).

BNC produces progesterone, derives from a macrophagic lineage, as shown by CD-163, MAC-387, and iNOS immunostaining, and present free passage between fetal and maternal placentomes. The use of trophoblastic cell primary cultures not only allows the observation of the parasite's virulence factors, but has also demonstrated the BNC's ability as a phagocyte, probably mediating the parasite's passage to the fetus. The major contribution of this study is the characterization of a primary BNC culture as a model for experimental infection by *N. caninum*, verifying their macrophage functions in a possible role in the transplacental infection by this parasite, being that the process of *in utero* transmission to the fetus remains unknown.

[a]MACHADO, R.Z. (2006). Personal communication.

REFERENCES

ABBASI, M.; KOWALEWSKA-GROSHOWSKA, K.; BAHAR, M.A.; KILANI, R.T.; WINKLER-LOWEN, B.; GUILBERT, L.J. Infection of placental trophoblasts by *Toxoplasma gondii*. *Journal of Infectious Diseases*, v. 188, n. 4, p. 608-16, 2003.

AMARANTE-PAFFARO, A.; QUEIROZ, G.S.; CORRÊA, S.T.; SPIRA, B.; BEVILACQUA, E. Phagocytosis as a potential mechanism for microbial defense of mouse placental trophoblast cells. *Reproduction*, v. 128, n. 2, p. 207–218, 2004.

ARECHAVALETA-VELASCO, F.; KOI, H.; STRAUSS III, J.F.; PARRY, S. Viral infection of the trophoblast: time to take a serious look at its role in abnormal implantation and placentation? *Journal of Reproductive Immunology*, v. 55, n. 1-2, p. 113-121, 2002.

CASTRO, M.B.; MACHADO, R.Z.; AQUINO, L.P.C.T.; ALESSI, A.C.; COSTA, M.T. Experimental acute canine monocytic ehrlichiosis: clinicopathological and immunopathological findings. *Veterinary Parasitology*, v. 119, n. 1, p. 73-86, 2004.

DUBEY, J.P. Recent Advances in *Neospora* and neosporosis. *Veterinary Parasitology*, v. 84, n. 3-4, p. 349-367, 1999.

DUBEY, J.P. Review of *Neospora caninum* and neosporosis in animals. *Korean Journal of Parasitology*, v. 41, n. 1, p. 1-16, 2003.

FERRO, E.A.V.; SILVA, D.A.O.; BEVILACQUA, E.; MINEO, J.R. Effect of *Toxoplasma gondii* infection kinetics on trophoblast cell population in *Calomys callosus*, a model of congenital toxoplasmosis. *Infection and Immunity*, v. 70, n. 12, p. 7089-7094, 2002.

FLOOD, P.F. The development of conceptus and its relationship to the uterus In: CUPPS, P.T. (Ed.) *Reproduction in domestic animals*. New York: Academic Press, 1991. p. 315-56.

GROSS, T.S.; WILLIAMS, W.F. Bovine placental prostaglandin synthesis: principal cell synthesis as modulated by the binucleate cell. *Biology of Reproduction*, v. 38, n. 5, p. 1027-1034, 1988.

HEMMINGS, D.G.; GUILBERT, L.J. Polarized release of human cytomegalovirus from placental trophoblasts. *Journal of Virology*, v. 76, n. 13, p. 6710-6717, 2002.

INNES, E.A.; ANDRIANARIVO, A.G.; BJÖRKMAN, C.; WILLIAMS, D.J.L.; CONRAD, P.A. Immune responses to *Neospora caninum* and prospects for vaccination. *Trends in Parasitology*, v. 18, n. 11, p. 497-504, 2002.

INNES. E.A.; WRIGHT, S.; BARTLEY, P.; MALEY, S.; MACALDOWIE, C.; ESTEBAN-REDONDO, I.; BUXTON, D. The host–parasite relationship in bovine neosporosis. *Veterinary Immunology and Immunopathology*, v. 108, n. 1-2, p. 29-36, 2005.

KALINSKI, P.; HILKENS, C.M.; SNIJDERS, A.; SNIJDEWINT, F.G.; KAPSENBERG, M.L. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naïve T helper cells. *Journal of Immunology*, v. 159, n. 1, p. 28-35, 1997.

KOI, H.; ZHANG, J.; PARRY, S. The mechanisms of placental viral infection. *Annals of the New York Academy of Sciences*, v. 943, p. 148-156, 2001.

LANDIM JÚNIOR, L.P.; MIGLINO, M.A; GARCIA, J.M. Bovine binucleate cell culture and evaluation of its secretion products in pregnancy maintenance. In: MEETING OF THE EUROPEAN PLACENTA GROUP, 10, 2003, Mainz. *Anais*… Amsterdã: Placenta, 2003. v. 24, p. A44.

MATAMOROS, R.A.; CAAMANO, L.; LAMB, S.V.; REIMERS, T.J. Estrogen production by bovine binucleate and mononucleate trophoblastic cells *in vitro*. *Biology of Reproduction*, v. 51, n. 3, p. 486-492, 1994.

McALLISTER, M.M.; DUBEY, J.P.; LINDSAY, D.S.; JOLLEY, W.R.; WILLS, R.A.; McGUIRE, A.M. Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology*, v. 28, n. 9, p. 1473-1478, 1998.

PARÉ, J.; THURMOND, M.C.; HIETALA, S.K. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. *Journal of Parasitology*, v. 83, n. 1, p. 82–87, 1997.

POPE, G.S.; GUPTA, S.K.; MUNRO, I.B. Progesterone levels in the systemic plasma of pregnant, cycling and ovariectomised cows. *Journal of Reproductive Fertility*, v. 20, p. 369–381, 1969.

QUINN, H.E.; ELLIS, J.T.; SMITH, N.C. *Neospora caninum*: a cause of immunemediated failure of pregnancy? *Trends in Parasitology*, v. 18, n. 9, p. 391-394, 2002.

REIMERS, T.J.; ULLMANN, M.B.; HANSEL, W. Progesterone and prostanoid production by bovine binucleate trophoblastic cells. *Biology of Reproduction*, v. 33, n. 5, p. 1227-1236, 1985.

SARTORI, M.J.; MEZZANO, L.; LIN, S.; MUNÕZ, S.; DE FABRO, S.P. Role of placental alkaline phosphatase in the internalization of trypomatigotes of *Trypanosoma cruzi* into HEp2 cells. *Tropical Medicine and International Health*, v. 8, n. 9, p. 832–839, 2003.

THURMOND, M.C.; PICANSO, J.P.; HIETALA, S.K. Prospective serology and analysis in diagnosis of dairy cow abortion. *Journal of Veterinary Diagnostic Investigation*, v. 2, n. 4, p. 274-282, 1990.

TREES, A.J.; WILLIAMS, D.J.L. Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. *Trends in Parasitology*, v. 21, n. 12, p. 558-561, 2005.

WOODING, F.B.P. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta*, v. 13, n. 2, p. 101-113, 1992.

WOODING, F.B.P.; MORGAN, G.; MONAGHAN, S.; HAMON, M.; HEAP, R.B. Functional specialization in the ruminant placenta: evidence of two popuplations of fetal binucleate cells of different selective synthetic capacity. *Placenta*, v. 17, n. 1, p. 75-86, 1996.

WOUDA, W.; BRINKHOF, J.; VAN MAANEN, C.; DE GEE, A.L. W.; MOEN, A.R. Serodiagnosis of neosporosis in individual cows and diary herds, a comparision of three enzyme linked immunosorbent assays. *Clinical and Diagnostic Laboratory Immunology*, v. 5, n. 5, p. 711-716, 1998.

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