Accepted Manuscript

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PII: S0021-9975(13)00383-6

DOI: 10.1016/j.jcpa.2013.12.003

Reference: YJCPA 1704

To appear in: *Journal of Comparative Pathology*

Received Date: 3 May 2013

Revised Date: 10 October 2013

Accepted Date: 7 December 2013

Please cite this article as: Cantón, G.J., Konrad, J.L., Moore, D.P., Caspe, S.G., Palarea-Albaladejo, J., Campero, C.M., Chianini, F., Characterization of Immune Cell Infiltration in the Placentome of Water Buffaloes (*Bubalus bubalis*) Infected with *Neospora caninum* During Pregnancy, *Journal of Comparative Pathology* (2014), doi: 10.1016/j.jcpa.2013.12.003.

EXPERIMENTALLY INDUCED DISEASE

Short Title: *Neospora caninum* Infection of Water Buffaloes

Characterization of Immune Cell Infiltration in the Placentome of Water Buffaloes (*Bubalus bubalis*) Infected with *Neospora caninum* During Pregnancy

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Summary

Neospora caninum infection in cattle stimulates host immune responses, which may be responsible for placental damage leading to abortion. Susceptibility of water buffaloes (*Bubalus bubalis*) to neosporosis is not well understood, although vertical transmission and fetal death have been documented. The aim of this study was to characterize the immune response in the placentome of water buffalo following experimental infection in early gestation with the Nc-1 strain of *N. caninum.* Placentomes were examined by immunohistochemistry using antibodies specific for T-cell subsets, natural killer cells and $CD79_{\alpha cy}$ cells. Placental inflammation was characterized by the infiltration of $CD3^+$ and $CD4^+$ T cells and T cells expressing the γδ T-cell receptor. The distribution of these cellular subsets in buffalo placentomes was similar to that previously described in cattle infected with *N. caninum* in early gestation, but the lesions were milder, which may explain the lower number of abortions observed in this species after infection.

Keywords: water buffalo; *Neospora caninum*; placentome; immunity

Neospora caninum is a pathogenic protozoan parasite for which a wide range of warm-blooded animals act as intermediate hosts (Dubey *et al.*, 2007), but the organism causes disease only in cattle and dogs (Buxton *et al.*, 2002). Evidence of *N. caninum* infection in water buffalo (*Bubalus bubalis*) has been reported (Huong *et al.*, 1998; Rodrigues *et al.*, 2004).

After experimental infection of pregnant water buffaloes in early gestation with *N. caninum*, vertical transmission was confirmed and lesions were observed in

placentomes and fetuses. This demonstrated the potential of *N. caninum* as an abortifacient in water buffaloes (Chryssafidis *et al.*, 2011;Konrad *et al.*, 2012).

N. caninum produces fetal and placental lesions severe enough to cause mortality (Barr *et al.*, 1990; Maley *et al.*, 2003). Additionally, *N. caninum* stimulates a T helper (Th) 1 immune response, which limits multiplication of the organism (Innes *et al.*, 1995); however, this response may also cause placental damage leading to abortion (Innes *et al.*, 2002; Maley *et al.*, 2006).

The aim of the present study was to characterize the inflammatory cell infiltrate in placentomes collected from pregnant water buffalo infected experimentally with *N. caninum* in early gestation (Konrad *et al.*, 2012). Twelve Mediterranean adult pregnant *Neospora*-seronegative water buffaloes were divided into four groups. Three animals in group A were infected at 70 days of gestation (dg) and culled at 28 days post infection (dpi). Three animals in group B were infected at 90 dg and culled at 28 dpi. Four animals in group C were infected at 90 dg and culled at 42 dpi. Two control animals in group D received uninfected Vero cells at 70 or 90 dg and both were killed at 28 dpi. Challenged animals each received 1×10^8 tachyzoites of the Nc-1 strain of *N. caninum* (Dubey *et al.*, 1998) intravenously.

After infection, no clinical signs were observed; however, one fetus from one dam infected at 70 dg was found to have died before the dam was killed. Non-suppurative inflammation was frequent in placentomes and fetal tissues of infected animals. No lesions were observed in control fetuses. *N. caninum* was identified by immunohistochemistry (IHC) or polymerase chain reaction (PCR) in placentomes and fetuses from the infected animals. Placentome and fetal tissues from the two control animals were negative by PCR and IHC (Konrad *et al.*, 2012).

During necropsy examination randomly-selected placentomes were collected and fixed in zinc salts fixative (ZSF; pH 7.0–7.4; González *et al*., 2001) for IHC and in 10% neutral buffered formalin for in-situ hybridization (ISH). Phenotypic characterization of the cellular infiltrates was performed using the IHC technique described by Maley *et al.* (2006) and the IHC was scored as described by Cantón *et al.* (2013b). Sections were incubated overnight with monoclonal antibodies (mAbs) specific for the T-cell marker CD3 (MM1A; VMRD, Pullman, Washington USA), the Th cell marker CD4 (IL-A11; VMRD), the cytotoxic T cell marker CD8 (CC58; AbD Serotec, Kidlington, Oxfordshire, UK), T cells expressing the γδ form of the T-cell receptor (γδ T cells; IL-A29; VMRD), natural killer cells (NKp46; CD335; AbD Serotec) and B cells expressing $CD79_{\alpha cy}$ (HM57; Dako, Glostrup, Denmark). Sections of ZSF-fixed water buffalo lymph nodes were used as positive control tissues.

The scores from individual placentomes were averaged into a single score for each animal. Given the limited sample sizes, the potential effects of time of infection or culling were not considered. Non-parametric two-tailed Mann-Whitney tests allowing for ties were conducted on the pooled data in order to investigate differences in the distribution of scores between infected and control animals for each cell type. Statistical significance was assessed at the 5% level.

A mild to severe $CD3^+$ T cell infiltrate surrounded necrotic foci in the caruncle or within necrotic fetal villi (FV) in placentomes from dams of group A (Fig. 1). Higher scores were obtained in the placentome from the animal carrying a non-viable fetus, compared with those from dams carrying viable fetuses. Group B and **C** placentomes were also infiltrated with T cells, but to a lesser extent than for those of group A. In caruncles from Group D animals, there was sparse to mild infiltration of T cells.

A sparse to mild infiltration of $CD4^+$ T cells surrounded necrotic areas in the caruncle in all of the placentomes from group A dams and these scores were higher in the dam carrying the non-viable fetus. In group B animals there was sparse to mild infiltration in most of the samples, with a few placentomes that had marked infiltration. Infiltrates in group C animals were sparse and restricted to the caruncles, as were infiltrates in animals of group D.

Infiltrates of $CD8^+$ T cells in samples from all animals in groups A, B and C were sparse to mild. The group A dam carrying the dead fetus had a slightly higher $CD8^+$ T cell infiltration score when compared with the other two animals in the group. The $CD8^+$ T-cell scores in group D animals were low.

In group A animals there was sparse to moderate infiltration of γδ T cells surrounding areas of necrosis in the caruncle. The score was higher in the dam with the dead fetus compared with the scores for the other two dams in the group. Similarly, in animals of group B, a sparse to moderate infiltration of γδ T cells was observed. In group C animals, some placentomes had a sparse infiltration of γδ T cells surrounding necrotic foci in the caruncle. Scores from group D animals were similar to those for animals in group C.

NK cell infiltration was sparse to mild in samples collected from animals in group A, with no detectable differences between the dams carrying live or dead fetuses. Similar results were seen in group B animals. In group C and D animals there was sparse NK infiltration of the placentomes.

Cells expressing $CD79_{\alpha cy}$ morphologically and histologically resembled trophoblast cells rather than B cells. Mononuclear cuboidal cells and occasional binucleate cells were also labelled. Placentomes in group A animals contained $CD79_{\alpha cy}^+$

cells morphologically similar to trophoblast cells in the caruncle and FV, and these were not associated with pathological changes. In animals of groups B and C, $CD79_{\alpha cy}^{+}$ cells had a similar distribution to that described for group A. In the two group D animals $CD79_{\alpha cy}$ labelling was scored as sparse to mild.

There were no significant differences in infiltration scores between infected and negative control dams for any of the cell subsets (P >0.1611). Means and standard errors of the means (SEMs) of different immune cell infiltration scores in placentomes from the different groups are presented in Table 1 and Fig. 2.

ISH was performed as described by Anderson *et al.* (2001) in 10 placentomes selected from different animals in each group, using digoxigenin-labelled riboprobes (both sense and antisense) to detect cells expressing mRNA encoding interleukin (IL)-12p40 (Cantón *et al.*, 2013a) and interferon (IFN)-γ. Cytokine cDNA-transfected Chinese hamster ovary cells (CHO cells) expressing ovine IL-12 and IFN-γ mRNA were used as positive controls for the ISH. Sections treated with the sense RNA probe were used as specificity controls.

Sparse infiltration of IL-12p40- and IFN-γ-expressing cells was observed in placentomes from animals in groups A, B and C, but there was no detectable difference between groups. IL-12p40 and IFN-γ mRNA was located in the cytoplasm of mononuclear cells scattered in the caruncle and in some cases associated with necrotic foci (Fig. 3). Some individual cytokine-expressing cells were observed in the base of the caruncle in samples from animals in groups A, B, C and D, and these were usually associated with blood vessels.

Phenotypic analysis of the immune cell infiltrates in these animals showed a trend for higher infiltration scores for the infected dams, and in particular the one

carrying a dead fetus, but these differences were not significant, probably due to the low number of animals in each experimental group. The most common infiltrating cells were $CD3^+$ Th cells and γδ T cells, while $CD4^+$ and $CD8^+$ T cells and NK cells were less numerous. A similar cellular distribution was observed in the placentomes from cattle infected experimentally in early gestation (Maley *et al.*, 2006), although in that study infiltration by $CD4^+$ T cells was more marked. In the present study, the placental infiltration by $CD3^+$ and $CD4^+$ T cells and γδ T cells was less severe when compared with cattle inoculated during early gestation (with the exception of the dam carrying the dead fetus) (Maley *et al.*, 2006). *Neospora* is largely controlled by $CD4^+$ T lymphocytes with the production of Th1 cytokines (Innes *et al.*, 1995; Williams *et al.*, 2000). The lower numbers of $CD4^+$ T cells present in infiltrates in this study, compared with previous studies of bovine placentomes of similar gestational age (Maley *et al.*, 2006), may explain the less severe clinical outcome observed in buffalos.

Although the role of γδ T cells in combating *N. caninum* infection is unknown, marked infiltration of placental tissues were shown in the present work. Some studies have demonstrated that γδ T cells are the first line of defence against pathogens in ruminants (Entrican, 2002), and are able to produce pro-inflammatory cytokines (Raghupathy, 1997). However, other authors have associated γδ T cells in the placentomes of cattle infected in early gestation with *N. caninum* with the occurrence of fetal death (Maley *et al.*, 2006).

Maley *et al.* (2006) also described a higher number of NK cells in the placentomes from *N. caninum*-infected cows carrying dead fetuses, suggesting a possible role for these cells in the immunopathogenesis of neosporosis. NK cells are able to direct the adaptive immune response towards a Th1 response (Klevar *et al.*,

2007); however, in the animals of the present study the number of NK cells was low and no differences were observed between dams carrying viable and non-viable fetuses.

Doubts about the identity of $CD79_{\alpha cy}^{+}$ cells in ruminant placentomes have been raised by Cantón *et al.* (2013b). In the present work, morphological and histological similarities were observed between $CD79_{\alpha cy}^{+}$ cells in water buffalo placentomes and those previously observed in cattle. Further studies are needed to identify and characterize B lymphocyte infiltration and the role of these labelled trophoblast cells.

Low Th1 cytokine gene expression was demonstrated in the placentomes collected from infected water buffalos. These Th1 type cytokines have an essential role in protecting against infection with *N. caninum* (Innes *et al.*, 1995; Khan *et al.*, 1997; Bartley *et al.*, 2004); however, it has also been hypothesized that if this Th1 response is exacerbated it may jeopardize pregnancy (Raghupathy, 1997; Quinn *et al.*, 2002). Placental samples used in this study were generated from an experiment that showed *N. caninum* to be an abortifacient in water buffaloes (Konrad *et al.*, 2012). Similar studies have been carried out in cattle, but with different doses (Macaldowie *et al.*, 2004) that may explain the observed differences in clinical outcome (Collantes-Fernández *et al.*, 2004). Nevertheless, lower doses of *N. caninum* tachyzoites than used by Konrad *et al.* (2012) have been shown to cause abortion in other ruminants (Dubey and Lindsay, 1990). Therefore, we cannot rule out the possibility that the lower abortion rates in water buffalos, when compared with cattle, were due in part to the doses used. When compared with cattle, however, it is tempting to suggest that in water buffalo the mild inflammatory response and the low numbers of Th1 cytokine-expressing cells in the placentome may have been insufficient to prevent transplacental transmission, but were,

at the same time, mild enough not to precipitate a pro-inflammatory response sufficient to cause abortion in all but one of the animals.

Acknowledgments

The authors acknowledge the Scottish Government Rural and Environment Science and Analytical Services Division (UK), Instituto Nacional de Tecnología Agropecuaria (AESA 203971) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 2412) (Argentina) for funding this study. T. Lischinsky is thanked for sampling the animals and Dr. D. Buxton for useful and constructive discussion.

Conflict of Interest Statement

The authors of this paper have no financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

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Received, May 3rd, 2013

Accepted, December 7th, 2013

Figure Legends

Fig. 1. Severe infiltration of $CD3^+$ T cells in the caruncle surrounding and within a necrotic fetal villus (fv) in a placentome collected from the group A water buffalo, in which the fetus was non-viable. IHC.

Fig. 2. Mean of the scores of the different phenotypes of inflammatory cells in placentomes. Letters on the horizontal axis represent groups. Error bars indicate standard error of the mean (SEM).

Fig. 3. Positively labelled IL-12p40-expressing cells (in black) in a placentome collected from water buffalo in group C. ISH.

Table 1

Mean ± SEM of immune cell infiltration scores in placentomes of water buffaloes

Cell type	*Group A*			*Group B*	*Group C*	*Group D*
	Overall	*Non-viable*	*Viable*			
CD3	2.78 ±0.64	4.00 ±0.00	2.17 ±0.30	1.73 ±0.33	2.09 ±0.32	1.65 ±0.15
CD4	1.66 ±0.47	2.57 ±0.20	1.25 ±0.16	1.20 ±0.12	1.15 ±0.05	1.08 ±0.08
CD8	1.27 ±0.15	1.60 ±0.09	1.12 ±0.06	1.11 ±0.05	1.12 ±0.05	1.12 ±0.03
γδTCR	1.83 ±0.41	2.67 ±0.11	1.41 ±0.11	1.62 ±0.16	1.44 ±0.12	1.37 ±0.22
NKp46	1.05 ±0.20	1.00 ±0.21	1.16 ±0.30	0.73 ±0.11	0.73 ±0.20	0.43 ±0.23
$CD79_{\alpha cy}$	1.77 ±0.40	1.14 ±0.14	2.08 ±0.23	1.13 ±0.13	1.15 ±0.09	1.28 ±0.12