DOI: 10.2478/s11686-011-0052-8
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Acta Parasitologica, 2011, 56(3), 245–254; ISSN 1230-2821

VERSITA

Pathological and immunological findings in placentas from pregnant BALB/c mice infected with *Neospora caninum* at early and late stages of gestation

Inmaculada C. López-Pérez[1], Esther Collantes-Fernández[1], Silvia Rojo-Montejo[1], Vanesa Navarro-Lozano[1], Verónica Risco-Castillo[1], Valentín Pérez-Pérez[2], Juana Pereira-Bueno[2] and Luis Miguel Ortega-Mora[1]*

[1]Saluvet Group, Animal Health Department, Veterinary Faculty, Complutense University of Madrid, Avenida Puerta de Hierro, s/n, 28040 Madrid, Spain. [2]Animal Health Department, Veterinary Faculty, University of León, 24071 León, Spain

Abstract

Neospora caninum is transmitted from a cow to its foetus by vertical transmission and the timing of infection in gestation is an important factor in determining the disease outcome. Few studies have explored the role of the placenta in the outcome of *N. caninum* infection during pregnancy. Here, we described the *N. caninum* presence, parasite load, local immune response, and histopathological lesions at the materno-foetal interface after infection of BALB/c mice at early and late stages of gestation. In mice infected at early gestation, *N. caninum* DNA was detected in foetoplacentary units 7 days post-infection (PI) and in the placenta, but not in viable foetuses on day 14 PI, indicating that the parasite was multiplying primarily in the placental tissues without reaching the foetus. Moreover, parasite DNA was detected in resorptions, suggesting that foetal death could be a consequence of infection. An increase in IFN-γ, TNF-α and IL-10 expression was observed in *N. caninum* PCR-positive placentas, which could favour *N. caninum* foetal transmission and be harmful to both the placenta and the foetus. Histopathological analysis revealed necrosis affecting both the maternal and foetal sides of the placenta. At late gestation, transmission occurred rapidly following infection (day 3 PI), but parasite were rarely found. In addition, an increase in cytokine expression was observed in spleen and placental tissues from infected animals, while a downregulation in IL-4 expression was only observed in the spleen. Finally, necrosis in the placenta was limited to the maternal side, suggesting that the parasite is mainly multiplying in the placental tissue at this stage. Thus, the results of the present study indicate that the placenta may be actively involved in *N. caninum* pathogenesis.

Keywords

Neospora caninum, gestation, parasite load, cytokine expression

Introduction

Neospora caninum is a protozoan parasite that causes abortion in cattle worldwide (Dubey and Lindsay 1996). The parasite can be transmitted from a cow to its foetus by either endogenous or exogenous transplacental infection (Trees and Williams 2005). In addition, vertical transmission contributes to the persistence of the parasite in the herd by propagating the infection to successive generations (Dubey *et al*. 2006).

Foetal immunocompetence develops gradually through gestation, and the timing of infection in gestation is an important factor in determining the disease outcome (Maley *et al*. 2006, Rosbottom *et al*. 2008). In cattle, inoculation with tachyzoites during early gestation induces foetal death (Dubey *et al*. 1992; Barr *et al*. 1994; Williams *et al*. 2000, 2003; Macaldowie *et al*. 2004), whereas infections occurring later in pregnancy usually result in the birth of congenitally infected but clinically normal calves (Barr *et al*. 1994, Williams *et al*. 2000, Innes *et al*. 2001, Maley *et al*. 2003). The placenta may play a key role in the pathogenesis of neosporosis. During pregnancy, the immune system is modulated, and the expression of Th2-type cytokines predominates (Wegmann *et al*. 1993, Raghupathy 1997). This bias is evident locally at the foeto-maternal interface (Lin *et al*. 1993). *N. caninum* multiplication during pregnancy may increase Th1-type cytokines, which could contribute to foetal death due to immune-mediated pathological alterations (Innes *et al*. 2002, Quinn *et al*. 2002a); however, few studies have explored this hypothesis. In

*Corresponding author: luis.ortega@vet.ucm.es

cattle, *N. caninum* infection in early gestation, which results in foetal death, has been found to be associated with a much greater increase in placental Th1- and Th2-type cytokine expression, placental necrosis, and inflammatory cell infiltration compared to cattle infected later in gestation, when the foetus survives (Rosbottom *et al*. 2008).

In-depth studies to understand the immunological and pathological mechanisms involved in *N. caninum* infection in cattle are difficult due to practical constraints. Thus, pregnant mouse models have been developed to study the effect of *N. caninum* infection during pregnancy. The day of infection during pregnancy seems to be a determining factor in foetal losses and in the rate of transmission to the offspring. Mice inoculated at early gestation show an increase in resorptions and a high vertical transmission rate; in contrast, no foetal losses and low transmission rates are detected when dams are infected late in gestation (Long and Baszler 1996; Liddell *et al*. 1999; Quinn *et al*. 2002b; López-Pérez *et al*. 2006, 2008). We previously developed a pregnant mouse model to further explore the pathogenesis of *N. caninum* infection during gestation (López-Pérez *et al*. 2006, 2010). In this model, we detected an increase in IL-4 expression in placentas from mice infected at mid-gestation, which might favour the multiplication of the parasite and facilitate its transmission to the foetus across the placenta. Furthermore, during the initial infection, when the parasite is mainly multiplying in the placenta, we observed an increase in TNF-α expression in *N. caninum* PCR-positive placentas, which could be associated with embryotoxic effects (López-Pérez *et al*. 2010).

The aim of this work was to evaluate the role of the placenta in the outcome of pregnancy after infection with *N. caninum* at early and late gestation. Thus, we investigated the parasite presence, parasite load, local and systemic immune response, and the lesion severity at the materno-foetal interface.

Materials and methods

Mice

Seven-week-old female BALB/c mice were obtained from a commercial supplier (Harlan Interfauna Ibérica, Barcelona, Spain). These mice were free of common viral, parasitic, and bacterial pathogens, according to the results of routine screening procedures performed by the manufacturer. Mice were fed *ad libitum* in a controlled environment with light/dark cycles of twelve hours. At eight weeks of age and after using the Whitten effect (Whitten 1957), females were mated for one night. Day 0 of gestation was determined by the presence of the vaginal mucoid plug.

Parasite and preparation of inoculum

Neospora caninum Nc-1 tachyzoites were maintained *in vitro* by continuous passage in MARC-145 cells, as described previously (Pérez-Zaballos *et al*. 2005). Cultures were scraped and parasites were passed through a 25-gauge needle to break up cells and release tachyzoites. The parasites were then centrifuged for 15 minutes at 1,350 *g* and re-suspended in sterile phosphate-buffered saline (PBS, pH 7.4). Viability was determined by Trypan blue exclusion, followed by counting with a Neubauer chamber. Parasites were re-suspended in sterile PBS at the required dose in a final volume of 200 µl/mouse and used immediately to infect mice.

Experimental design and samples

Pregnant BALB/c mice were infected subcutaneously with 2×10^6 Nc-1 tachyzoites on days 0 (early gestation, group 1) and 14 (late gestation, group 2) of gestation. A group of pregnant mice was inoculated with PBS on days 0 and 14 of gestation and kept as an uninfected control group. Eight infected dams and four control animals were sacrificed with CO_2 gas, at random, at each time point. Animals from group 1 were sacrificed on days 7 and 14 post-infection (PI). Mice from group 2 were sacrificed on day 17 of gestation. Different samples were taken depending on the day of sacrifice. In group 1, the complete foetoplacentary unit was collected on day 7 of gestation (7 days PI), while on day 14 of gestation (14 days PI), the foetuses, resorptions, placentas, and maternal spleen from each dam were recovered. In group 2, brain and lung from foetuses were collected separately on day 17 of gestation (3 days PI) together with placentas and maternal spleen from each dam. In group 1, foetal mortality (resorptions) was determined on day 14 of gestation (day 14 PI) on the basis of small foetal size (≤ 3 mm) or lack of discernible foetal tissue at an implant site containing placenta tissue (Long and Baszler 1996). The percent of resorption per dam was calculated as $R/(R + V) \times 100$ in every dam, where R is the number of resorbed foetuses and V is the number of viable foetuses per animal (Krishnan *et al*. 1996). Resorptions observed on day 17 of gestation (3 days PI) in group 2 were not considered for PCR analysis, as these could have occurred prior to experimental infection. Foetoplacentary units, foetuses, and resorptions were individually processed for PCR analysis. All of the collected placentas were divided into two portions: one-half used for PCR analysis, and the other half was processed for either cytokine expression or histological analysis. Finally, spleens from dams were collected for cytokine expression analysis. Placentas containing live foetuses were subjected to local immune response and histological analyses, whereas resorptions were only used for parasite DNA detection due to their small foetal size.

Detection and quantification of N. caninum DNA

Genomic DNA was extracted using the commercial kit Real Pure DNA Extracción ADN genómico (Durviz, Valencia, Spain) according to the manufacturer's instructions. For parasite DNA detection, a nested PCR reaction to amplify the in-

ternal transcribed spacer 1 (ITS1) region of *N. caninum* was performed as previously described (Buxton *et al*. 1998). Parasite DNA in PCR-positive tissues was quantified by real-time PCR (Collantes-Fernández *et al*. 2002) using an ABI PRISM™ 7300 Sequence Detector Machine (PE Applied Biosystems, Foster City, California, USA) and the commercial kit Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Paisley, United Kingdom). We used primer pairs from the *N. caninum* Nc-5 sequence to quantify parasites and primers from the 28S rRNA gene to quantify host DNA. Samples were run in duplicate in separate tubes. *N. caninum* organisms were quantified by interpolation of cycle threshold (the fractional cycle number reflecting a positive PCR result) values on a standard curve of DNA equivalent to 1×10^{-1}–1×10^{4} tachyzoites. The amount of DNA per sample was normalised by quantification of the 28S rRNA gene, and a standard curve was generated with five-fold serial dilutions of mouse brain DNA quantified by UV spectrophotometry. Data were analysed with Sequence Detection System Software v.1.6 (PE Applied Biosystems) and parasite load was expressed as the number of tachyzoites/µg host DNA. The detection limit for the assay was 1×10^{-1} tachyzoites, as described previously (Collantes-Fernández *et al*. 2002).

Total RNA extraction and real-time RT-PCR

Cytokine expression was evaluated by real-time RT-PCR. Spleen and foetal placentas were placed in TRI REAGENT (Sigma, St. Louis, Missouri, USA) and disrupted using a Polytron PT1600E homogeniser (Kinematica, AG, Lucerne, Switzerland). Total RNA was extracted according to the manufacturer's instructions, and RNA integrity was checked via agarose gel electrophoresis. Synthesis of cDNA was performed with SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations. The primer sequences used in this study for cDNA amplification of IFN-γ, IL-10, IL-4, and β-actin were previously published (Varona *et al*. 2005, López-Pérez *et al*. 2010). β-actin was employed as an endogenous reference for each corresponding sample. Placentas from the control group were analysed simultaneously with placentas from the infected group. The amount of cDNA was determined by real-time PCR on an ABI PRISM™ 7300 Sequence Detector Machine (PE Applied Biosystems) with the commercial kit Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Each determination was performed in triplicate, and the cycle threshold (Ct) value was obtained using Sequence Detection System Software v.1.6 (PE Applied Biosystems). For relative quantification of gene expression, the comparative threshold cycle method was used. The relative n-fold changes in expression of each target cytokine was normalised to the endogenous reference (β-actin) and calculated relative to the control group, producing the -ΔΔCt value (Livak and Schmittgen 2001). The local immune response was analysed exclusively in foetal placentas containing live foetuses; resorptions were only recovered for parasite DNA detection due to their small foetal size.

Histopathological studies

Foetal placentas were processed by routine histological methods. Tissues were fixed in 10% neutral buffered formalin and dehydrated with graded alcohols prior to being embedded in paraffin wax, and stained with haematoxylin and eosin (Pereira-Bueno *et al*. 2003). Analysis was based on the identification of characteristic *N. caninum* lesions in the placenta, as previously described (Long and Baszler 1996, Dubey *et al*. 2006).

Analysis of data

The Chi-square and Fisher *F* tests were used to compare foetal mortality and rates of parasite detection. The Mann-Whitney *U*-test was employed to analyse differences in the percent of resorption between infected and uninfected dams. Differences in parasite burden were analysed by a nonparametric Kruskal-Wallis test. When statistical differences were found, a Dunn's multiple comparison test was employed to examine all possible pairwise comparisons. When statistically significant differences were obtained using the Kruskal-Wallis test, but the multiple comparison test did not yield significance, the results obtained by the Kruskal-Wallis test were preferred, as suggested by Morrison (Morrison 2002). The Student's *t*-test was used to compare the differences in cytokine expression between infected and uninfected dams. All of the statistical analyses were performed using GraphPad Prism v.5.02 (San Diego, California, USA) software.

Results

Foetal mortality

The consequences of the infection in the outcome of gestation were evaluated by investigating the foetal mortality rate (Table I). In group 1, resorptions were observed in 5 out of 8 infected dams and in 3 out of 4 of uninfected animals that were sacrificed on day 14 of gestation (day 14 PI). No differences in foetal mortality ($P = 0.7464$, $\chi^2 = 0.10$) or percent of resorption per mouse ($P = 0.730$, $U = 13.50$, Mann-Whitney *U*-test) were found between infected and uninfected dams.

N. caninum DNA presence and parasite load

To analyse the pattern of parasite transmission from dams to the foetus, *N. caninum* DNA presence (Table II) and parasite load (Fig. 1) were evaluated in foetoplacentary units, foetuses, and placentas. In group 1, *N. caninum* DNA was present in foetoplacentary units at day 7 of gestation (7 PI), when the

Table I. Foetal mortality in dams infected on day 0 (group 1) of gestation and sacrificed on day 14 of gestation

		Foetal mortality	
Group 1	infected dams	7/75 (9.3%)*	9.4 (0.0–20.8)†
	control dams	4/43 (9.3%)	11.0 (2.3–31.8)

*Number of resorptions/total number of viable foetuses (percentage). †Median of resorptions per litter (lower and upper quartiles).

Table II. Detection of *N. caninum* DNA by nested-PCR in foetoplacentary units, foetuses, resorptions and placentas from mice infected on days 0 (group 1) and 14 (group 2) of gestation with 2×10^6 Nc-1 tachyzoites. Dams from group 1 were sacrificed on days 7 and 14 of gestation and dams from group 2 were sacrificed on day 17 of gestation

	Samples collected	Nested-PCR	
		no. samples*	per litter†
Group 1	foetoplacentary units[a]	11/83 (13.3%)	10.6 (2.5–21.7)
	viable foetus[b]	0/68 (0.0%)	0.0 (0.0–0.0)
	resorptions[b]	4/7 (57.1%)	0.0 (0.0–87.5)
	placentas[b]	24/68 (35.3%)	31.0 (11.2–56.2)
Group 2	viable foetus[c]	1/55 (1.8%)	0.0 (0.0–0.0)
	placentas[c]	11/64 (17.2%)	5.0 (0.0–33.6)

*Number of positive samples/total number of samples analyzed (percentage); †Median of positive samples per litter (minimum and maximum values); [a]foetoplacentary units collected on day 7 of gestation (7 PI); [b]samples collected on day 14 of gestation (14 PI); [c]samples collected on day 17 of gestation (3 PI).

foetus and the placenta were recovered together. On day 14 PI (day 14 of gestation), when the two samples were collected separately, parasite DNA was only detected in placentas and resorptions, but not in viable foetuses. *N. caninum* presence was more often detected in resorptions than in foetoplacentary units ($P = 0.0135$, Fisher *F*-test). Parasite burden was significantly higher in placenta and resorptions ($P = 0.0066$, $\chi^2 = 14.24$, Dunn's multiple comparison test) compared to foetoplacentary units. In group 2, parasite DNA was found in placentas and in one foetus at 3 days PI (day 17 of gestation). Parasite DNA was more often detected ($P < 0.014$, $\chi^2 = 6.10$) and a higher parasite burden was observed in the placentas compared the foetuses ($P < 0.0001$, $U = 20.0$, Mann-Whitney *U*-test).

Cytokine expression

We evaluated patterns of cytokine expression in the spleen and placenta to determine whether the local and systemic immune responses differed (Fig. 2). In group 1, we observed a significant increase in the expression of IFN-γ, TNF-α, IL-10 and IL-4 in spleens from infected dams compared to the control group ($P < 0.004$–0.0001, $t = 3.7$–11.5, Student *t*-test). In placentas, we found that only IFN-γ and IL-10 expression was significantly upregulated in the infected group ($P < 0.002$–0.006, $t = 2.8$–3.3, Student *t*-test). In group 2, an upregulation of IFN-γ and IL-10 expression was observed in the spleens of infected dams ($P < 0.01$–0.0001, $t = 4.0$–6.8, Student *t*-test), while TNF-α and IL-4 were significantly downregulated ($P < 0.02$–0.0004, $t = 2.8$–4.7, Student *t*-test). In foetal placenta, an increase in the expression of IFN-γ, TNF-α, IL-10, and IL-4 was observed in infected dams compared to the uninfected animals ($P < 0.02$–0.0002, $t = 2.5$–4.4, Student *t*-test).

We also compared the patterns of cytokine expression in the PCR-positive and -negative placentas from infected mice (Fig. 3). In group 1, we observed significantly higher levels of IFN-γ, IL-10 and TNF-α expression in PCR-positive compared to PCR-negative placentas ($P = 0.0346$–0.003, $t = 2.2$–3.1, Student *t*-test); however, no differences were found in IL-4 mRNA levels ($P = 0.34$, $t = 0.853$, Student *t*-test). As placentas were randomly assigned to either cytokine expression analysis or histopathological analysis, only one nested PCR-positive placenta was assessed for cytokine expression in group 2. Thus, we did not analyse the differences between PCR-positive and -negative placentas for this group.

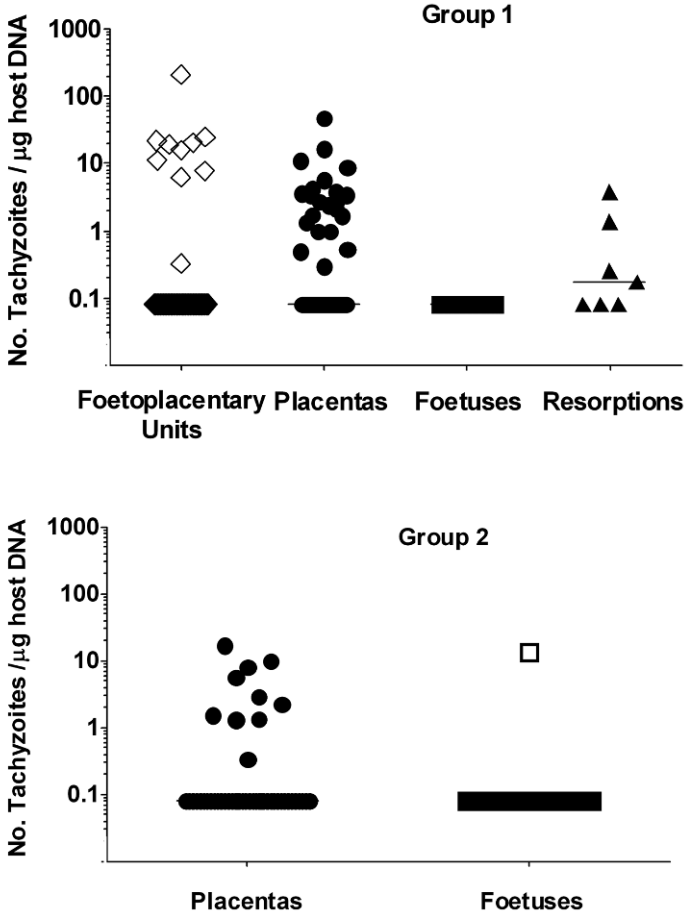

Fig. 1. Parasite load in foetoplacentary units, placentas, foetuses, and resorptions following infection of dams with 2×10^6 Nc-1 tachyzoites on day 0 (group 1) or day 14 of gestation (group 2). Dams from group 1 were sacrificed on days 7 and 14 of gestation, and dams from group 2 were sacrificed on day 17 of gestation. Each point represents the parasite load of a single sample, and the points were horizontally spread out for ease of visualizing overlapping values. Horizontal lines correspond to mean value for each type of sample. Because the *N.caninum* detection limit by real-time PCR is 10^{-1} parasites (Collantes-Fernández *et al*. 2002), all positive samples had ≥ 0.1 parasites, while negative samples (0 parasites) were represented on the log scale as having < 0.1 parasites

Histopathological lesions in foetal placentas

The main lesions found were necrosis accompanied by a slight inflammatory infiltrate, which was diffusely distributed in relation to necrosis (Fig. 4). In group 1, these multifocal areas of necrosis affected both the maternal and foetal sides of the placenta, while in group 2, necrosis was mainly found on the maternal side of the placenta and was surrounded by a slight mononuclear inflammatory infiltrate. Necrosis was found in 59% (13/22) of placentas from group 1 and 75% (12/16) of placentas from group 2; inflammatory infiltration was observed in 18% (4/22) of placentas from group 1 and 50% of placentas (8/16) from group 2.

Discussion

To date, most experimental mouse models of *N. caninum* infection have been developed to study vertical transmission, foetal mortality and the systemic immune response (Cole *et al*. 1995; Long and Baszler 1996; Liddell *et al*. 1999; Quinn *et al*. 2002b, 2004; Omata *et al*. 2004; Kano *et al*. 2005; Lopez-Pérez *et al*. 2006). Despite the importance of transplacental transmission, few studies in mice have addressed the possible role of the placenta in the outcome of infection. Previously, a high parasite burden was observed in the placentas from dams infected at mid-gestation, suggesting that *N. caninum* primarily multiplies in the placenta during the initial stages of infection. Moreover, IL-4 was upregulated in placentas which may enhance *N. caninum* susceptibility at the materno-foetal interface and favour transmission to the progeny (López-Pérez *et al*. 2010). In addition, we also previously reported that infection at the three pregnancy periods produced different foetal and neonatal mortality and transmission rates to the offspring (López-Pérez *et al*. 2006, 2008). Thus, in the present study, we investigated the presence of *N. caninum*, the parasite load, the local immune response and histopathological lesions at the foeto-maternal

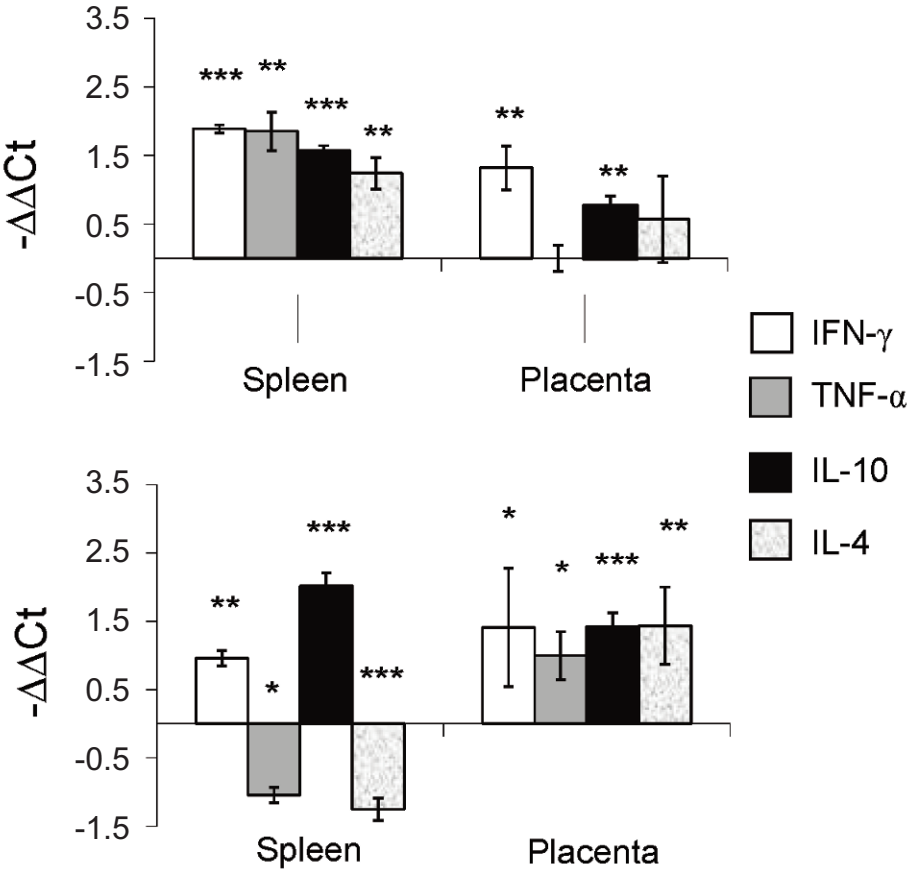

Fig. 2. Cytokine expression in spleens and foetal placentas from infected and uninfected dams. β-actin was employed as an endogenous reference for each corresponding sample, and each determination was performed in triplicate. The relative n-fold change in expression of each target cytokine was normalized to the endogenous reference (β-actin) and calculated relative to the control group to produce the -ΔΔCt value (Livak and Schmittgen 2001). The -ΔΔCt value for control group was zero. Data were horizontally spread out for ease of visualizing overlapping values. The bars represent the mean values of cytokine expression and the standard error. The asterisks represent significant differences between infected and uninfected dams by the Student *t*-test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001)

interface in BALB/c mice infected at early and late stages of gestation.

In mice infected at early gestation, *N. caninum* DNA was detected in foetoplacentary units on day 7 of gestation. The presence of parasite in foetal placentas, but not in foetuses, on day 14 of gestation could indicate that the parasites were mainly multiplying in the placenta without reaching the foetus. As gestation progresses, the parasite may reach the tissues of viable foetuses without causing death, leading to a moderate vertical transmission at birth (López-Pérez *et al*. 2006). Similar findings have been reported in studies of other intracellular pathogens, such as *Chlamydophila abortus* (Buendía *et al*. 1998) and *Toxoplasma gondii* (Ferro *et al*. 2002), in which the organisms probably reached the foetus from day 16 of pregnancy onwards. Furthermore, parasite DNA was detected in resorptions on day 14 of gestation, suggesting that the foetoplacentary units invaded by *N. caninum* may have been resorbed as a consequence of infection.

An increase in IFN-γ, TNF-α and IL-10 expression was observed in *N. caninum* infected placentas, which could be harmful to both the placenta and the foetus. Both IFN-γ and TNF-α have been shown to have potentially embryotoxic effects, affecting the development of murine foetuses and causing foetal resorption (Chaouat *et al*. 1990). In *N. caninum* infection in cattle, IFN-γ mRNA was detected by *in situ* hybridisation in infiltrating cells from placentas containing dead foetuses (Maley *et al*. 2006). In addition, in malaria, high mRNA and protein levels of TNF-α in placenta were associated with low birth weight (Moormann *et al*. 1999). Previously, we observed a high neonatal mortality after the infection of dams in the first term of gestation (López-Pérez *et al*. 2008), which might be associated with placental damage due to IFN-γ and TNF-α. Indeed, histopathological analysis revealed necrosis affecting both the maternal and foetal sides of the placenta. Thus, the placental necrosis observed which is likely exacerbated by the local immune response due to the presence of the parasite, might be responsible for the high neonatal mortality rate reported previously (López-Pérez *et al*. 2008).

The precise mechanisms that compromise pregnancy or permit the vertical transmission of *N. caninum* remain to be

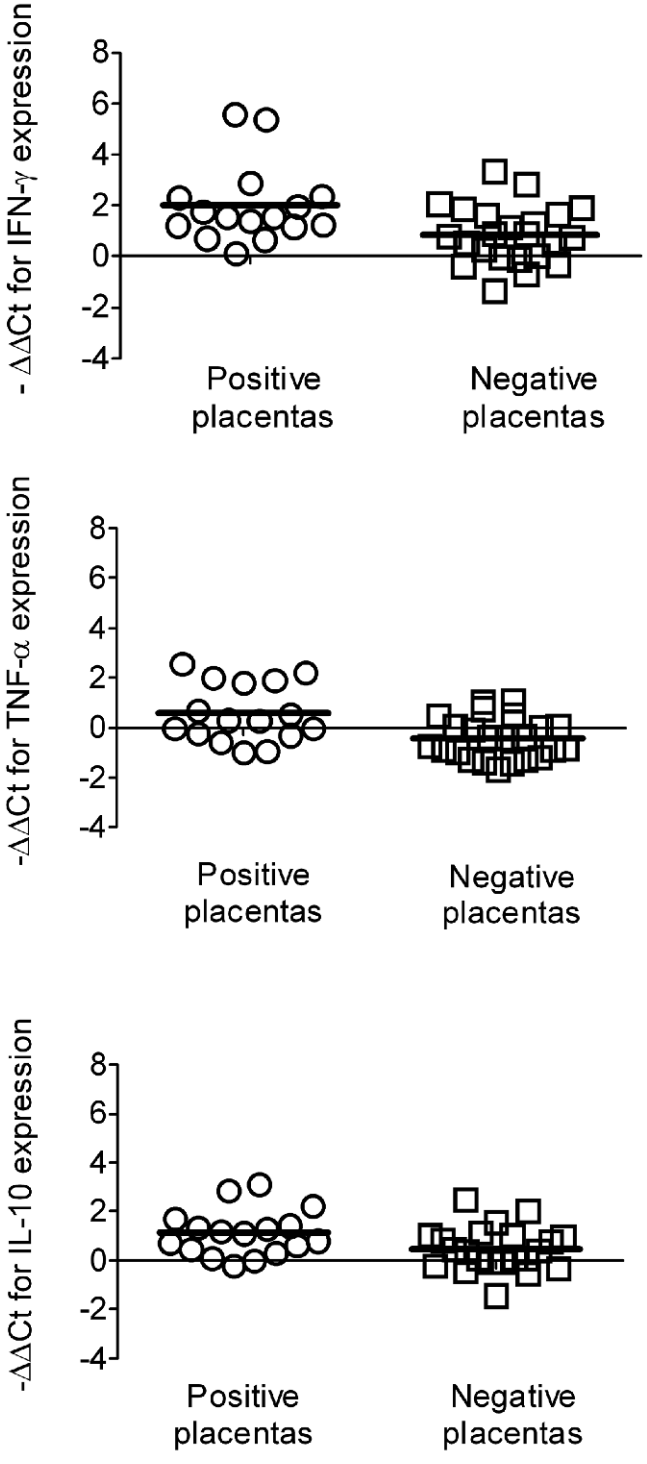

Fig. 3. IFN-γ, IL-10 and TNF-α expression in PCR-positive and -negative placentas from group 1. The data are represented as individual points in each sample, and the horizontal line corresponds to the median value

elucidated. It is possible that TNF-α could induce maternal natural killer (NK) cells to produce IFN-γ, which may increase the phagocytic properties of trophoblast cells (Amarante-Paffaro *et al*. 2004), facilitating parasite internalisation without neutralisation (Abou-Bacar *et al*. 2004). Moreover, studies on the human BeWo trophoblast cell line suggest that IFN-γ is necessary for adhesion of *T. gondii*-infected monocytes, thereby increasing materno-foetal transmission (Pfaff *et al*. 2005). Then, trophoblast cells are not able to limit *T. gondii* multiplication when stimulated by IFN-γ, in contrast to other

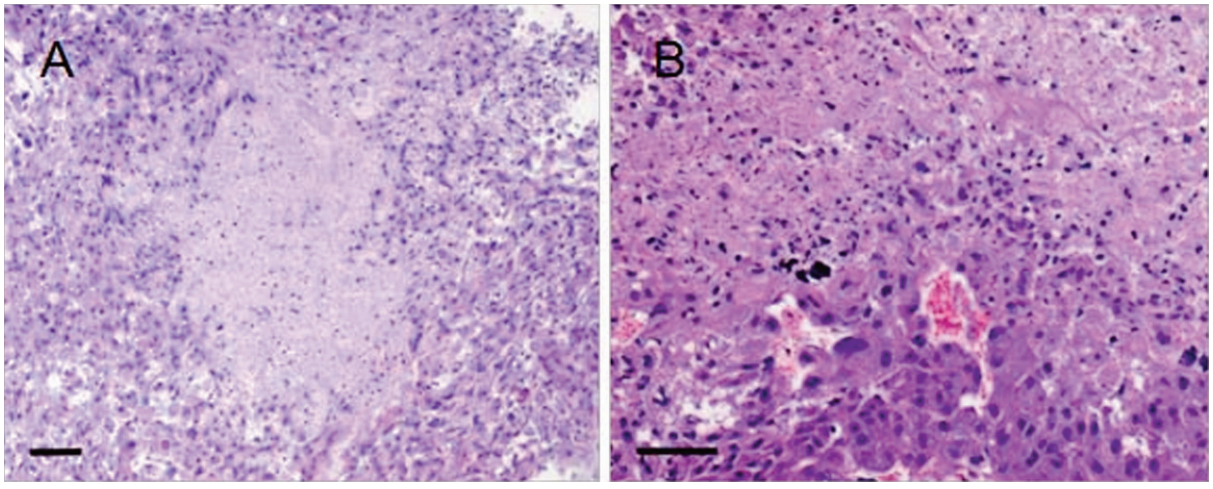

Fig. 4. Placental lesions associated with *N. caninum* infection. Zones of necrosis were observed in both foetal and maternal placental tissues in group 1 (panel A), whereas necrotic areas, surrounded by a slight mononuclear inflammatory infiltrate, were only found in maternal placental tissue from group 2 (panel B). Scale bar = 10 μm

cell types (Pfaff *et al*. 2005). In addition, it is known that IL-10 is a regulatory cytokine that can control both the IFN-γ and the TNF-α response and block immunopathology (Eperon *et al*. 1999, Quinn *et al*. 2004). However, IL-10 might also enhance *N. caninum* invasion rates and intracellular replication, as this cytokine has been implicated in increasing the susceptibility of trophoblast cells to *T. gondii* infection (Barbosa *et al*. 2008). Therefore, the increase in the expression of IFN-γ and IL-10 detected in infected placentas could favour foetal transmission of *N. caninum*. However, more studies are needed to determine the roles of these cytokines on *N. caninum* infection during pregnancy.

At late gestation, transmission occurs rapidly following infection, but parasite was rarely found. However, the infection of a greater number of foetuses is likely to occur because we previously detected the successful transmission of parasite to 49% of pups at day 60 post-partum (López-Pérez *et al*. 2008). This discrepancy could be due to the minimal amount of time between *N. caninum* infection at late gestation and sample recovery, as an increase in time between infection and necropsy permits the detection of a higher vertical transmission rate (Liddell *et al*. 1999, López-Pérez *et al*. 2008). In this group, necrosis was limited to the maternal side of the placenta, suggesting that the parasite mainly replicates in the placental tissue at this stage.

The decrease in the IL-4 mRNA levels in spleen detected at late gestation may be able to further limit *N. caninum* multiplication in maternal tissues (Long and Baszler 2000), reducing both the number of parasite that reach the placenta and the vertical transmission to progeny, as previously observed (López-Pérez *et al*. 2006, 2008). IL-4 and IFN-γ compete for the binding of IFN-γ activation sequence motifs in macrophages, interacting in a functional manner (Ohmori and Hamilton 1997). Thus, in the presence of constant levels of IFN-γ, decreasing IL-4 levels may have the same effect on macrophage activation as increasing IFN-γ levels (Long and Baszler 2000). In addition, a clear predominance of either a type 1 or a type 2 immune response was not evident in the placenta. Perhaps the increase in type 1 cytokines as a consequence of the infection during late gestation may have occurred too late to affect an existing well-established type 2 immune response at the materno-foetal interface (Williams *et al*. 2000).

Comparison between the bovine and the murine experimental models is difficult due to the limited information available on the expression of cytokines and the parasite load at the materno-foetal interface in pregnant cattle. Only Rosbottom *et al*. (2011) described how *N. caninum* experimental infection in early gestation resulted in foetal death, which was associated with a much greater increase in placental cytokine expression, placental necrosis, and inflammatory cell infiltration, compared to cattle infected later in gestation, when the foetus survived (Rosbottom *et al*. 2011). In persistently infected cattle sacrificed at 20 and 33 weeks of gestation, an increase in both type 1 and type 2 cytokines were also detected in maternal and foetal epithelial and stromal fibroblastoid cells (Rosbottom *et al*. 2008). Moreover, a higher number of PCR-positive tissue samples were observed in naturally infected foetuses corresponding to the first and second pregnancy periods compared to the last trimester (Collantes-Fernández *et al*. 2006). Thus, despite the obvious differences between the bovine and murine placenta (epitheliochorial and haemochorial, respectively), the mouse model can provide a valuable tool for *N. caninum* studies.

In the present work, comparisons between groups are inappropriate because the groups were not matched for time post-infection or for pregnancy stages. However, the results show that the degree of development of the placenta and the local immune response seem to influence the pattern of parasite transmission to the foetus. Indeed, the higher number of PCR-positive placentas observed at mid-gestation (López-

Pérez *et al*. 2010) could explain why infection at this stage provoked greater foetus damage than infection in early or late gestation, leading to the highest mortality and vertical transmission rates along time (López-Pérez *et al*. 2006, 2008).

In conclusion, these preliminary data suggest that the placenta may be actively involved in *N. caninum* pathogenesis. However, further studies are necessary to clarify the relationship between the immune response and parasite transmission at the materno-foetal interface.

Acknowledgements. We would like to thank Raquel Expósito for their excellent technical assistance, and Ricardo García Mata for statistical analyses. We express our gratitude to Dr. Ángel Zaballos and Dra. Rosa Varona for their helpful comments to the manuscript. This work has been supported by a grant from the Spanish Government (AGL2001-1362). Inmaculada C. López-Pérez has been financed by Spanish Ministry of Education and Science. This experiment complied with the current local laws of animal protection in the European Union.

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(Accepted May 17, 2011)