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Peripheral and placental immune responses in goats after primoinfection with *Neospora caninum* at early, mid and late gestation

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ABSTRACT

Neospora caninum can cause reproductive failure in goats. However, the pathogenesis of neosporosis in this domestic species remains largely unknown. We recently demonstrated that the outcome of experimental infection by *N. caninum* in pregnant goats is highly dependent on the time of gestation, during which infection occurs. In the present study, we examined the peripheral and placental immune responses in these groups of goats infected with 10^6 tachyzoites of the Nc-Spain7 isolate at early (G1, at day 40 of gestation, dg), mid (G2, 90 dg) and late (G3, 120 dg) gestation, together with a group of non-infected goats as a control group (G4). Seroconversion was observed as early as day 10 post-infection (pi) in all goats from G1 that aborted earlier (10–11 pi). The remaining infected goats had seroconverted by day 14 pi. Similar IFN-γ kinetic profiles were found in sera from goats in G1 and G2 with a significant increase in the IFN-γ levels on days 7 and 10 pi. This increase was not observed in G3. A similar pattern of placental cytokine expression was found in all infected groups. IFN-γ and IL-4 showed the highest increase, followed by a weaker up-regulation in TNF-α and IL-10. The lowest up-regulation was observed for IL-12 expression. In summary, this study provides information regarding the dynamics of immune responses and their relationship with the outcome of *N. caninum* infection in goats during gestation.

1. Introduction

Neosporosis, which is caused by the apicomplexan protozoan parasite *Neospora caninum,* is considered one of the main infectious causes of abortion in cattle worldwide (Dubey et al., 2007). Protozoan abortion is traditionally associated with *Toxoplasma gondii* in small ruminants; however, recent studies consider *N. caninum* to be an important abortifacient in goats and sheep (Moreno et al., 2012; González-Warleta et al., 2014; Gharekhani et al., 2016). Interestingly, there has been an increase in the number of serological studies reporting a broad range of prevalence rates for *N. caninum* in goats, which can reach 25% in specific geographical areas (Dubey and Schares, 2011; Andrade et al., 2013; Liu et al., 2015; Zhou et al., 2015; Gazzonis et al., 2016).

The pathogenesis of neosporosis in goats remains largely unknown. According to studies performed in cattle and sheep, the maternal immune response regulation and development of the foetal immune system throughout gestation may play a pivotal role in the outcome of *N. caninum* infection (Dubey et al., 2006; Arranz-Solís et al., 2016; Horcajo et al., 2016). By contrast, few studies have been performed in goats. Therefore, in a recent study, we conducted intravenous experimental infections using the Nc-Spain7 isolate, that has been shown to be highly virulent for cattle (Caspe et al., 2012; Regidor-Cerrillo et al., 2014) and sheep (Arranz-Solís et al., 2015), in pregnant goats at three different time points of gestation (Porto et al., 2016). The results showed that, similar to cattle and sheep, the clinical consequences of *N. caninum* infection in pregnant goats are dependent on the time of gestation. Experimental infection during the first term of gestation resulted in abortion, mainly through parasite proliferation and damage to foetal tissues; however, one early abortion, at 10–11 days post

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infection, similar to that described in ovine toxoplasmosis (Owen et al., 1998; Castaño et al., 2014) and without parasite detection in placenta and foetuses, was also observed. Infection during the second term of gestation resulted in abortions and the birth of apparently healthy kids. Infections during the third term of gestation can lead to premature parturition and the birth of weak kids. To gain further insight into the role that the immune response plays in *N. caninum*-infected pregnant goats, the present study investigated the development of both peripheral and placental immune responses in goats that were experimentally infected with *N. caninum* at early (40 day of gestation, dg: day of gestation), mid (90 dg) and late (120 dg) gestation. The results will allow us to better understand the pathogenesis of caprine neosporosis. In addition, we performed this study under analogous experimental conditions to those used in studies on sheep for a proper comparison (Arranz-Solís et al., 2015).

2. Material and methods

2.1. Experimental design

A full description of the goats, inocula and experimental design was previously reported by Porto et al. (2016). Briefly, 21 primiparous goats were randomly distributed into three groups (G1, G2 and G3; n = 7 for each) and inoculated in the jugular vein with 10^6 tachyzoites of the *N. caninum* Nc-Spain7 isolate at 40 (G1), 90 (G2) and 120 (G3) dg. Another nine goats were placed in a control group (G4; n = 9), and three goats were inoculated with phosphate-buffered saline (PBS) at each time point of tachyzoite inoculation for groups G1-G3. Three animals from G4 were culled at the average time points when abortions or births occurred in the G1, G2 and G3 groups, thus providing a negative control for further analyses.

The procedures of this experiment were approved by the Ethics Committee for Animal Use (CEUA) of the Universidade Federal de Alagoas under protocol number 59/2013 and are in accordance with the current legislation of the Brazilian College of Animal Experimentation (COBEA)

2.2. Clinical examination and collection of biological samples

Transabdominal ultrasonography was used to determine foetal viability once weekly for the first 2 weeks post-infection (wpi) and then twice weekly until the detection of foetal death. Blood was collected from the jugular vein in 10 ml tubes, without anticoagulant on days −2 and 0 of the inoculation and then twice weekly until foetal death or parturition. Serum was obtained by centrifugation and stored at −80 °C until serological analyses. When foetal death was detected or immediately after parturition dams and kids, as well as the corresponding control animals, were euthanized by an IV overdose of sodium thiopental, followed by potassium chloride. Immediately after euthanasia, three placentomes from goats that presented foetal death and three caruncles from goats that gave birth were collected. These samples either showed macroscopic lesions or were randomly selected in the absence of lesions, were transversely cut into 2- to 3-mm-thick slices and preserved in RNAlater solution (Qiagen, Hilden, Germany) at −80 °C for the subsequent cytokine mRNA expression analysis using real-time PCR (qPCR).

2.3. Serological analysis: IgG responses

The levels of IgG antibodies against *N. caninum* were measured using indirect ELISA as described previously (Arranz-Solís et al., 2016). Briefly, *N. caninum* soluble extract (Álvarez-García et al., 2003) was used as antigen to coat 96-well microtiter plates. Serum samples were diluted 1:100 in 3% bovine serum albumin diluted in PBS containing 0.05% Tween 20 (PBS-T) and incubated for 1 h at 37 °C. After four washes in PBS-T, horseradish peroxidase conjugate protein G (Biorad, Hercules, USA) diluted 1:5000 in PBS-T was added and incubated for 1 h at 37 °C. After four washes, 100 µl per well of ABTS substrate (Roche Diagnostics, Mannheim, Germany) was added. The reaction was stopped after 15 min at RT by the addition of 100 µl per well of a solution of 0.3 M oxalic acid, and the optical density (OD) was read at 405 nm (OD405). In each plate, positive and negative control sera were included. Sera from a *N. caninum* naturally infected goat with OD = 1.2 and from a non-infected goat with OD = 0.1 were used as positive and negative control, respectively. For each plate, the OD values at 405 nm were converted into a relative index percent (RIPC) using the following formula RIPC = (OD 405 sample − OD 405 negative control)/(OD 405 positive control − OD 405 negative control) × 100. A RIPC value ≥ 10 indicates a positive result.

2.4. IFN-γ response

IFN-γ levels in sera were measured using the Bovine IFN-γ ELISA development kit (Mabtech AB, Nacka strand, Sweden), following the manufacturer's instructions. The colour reaction was developed with the addition of 3,3′,5,5′-tetramethylbenzidine substrate (TMB, Sigma-Aldrich, Madrid, Spain) and incubated for 5–10 min in the dark. Reactions were stopped by adding 2N H_2SO_4 (100 µl/well). The plates were read at 450 nm. The cytokine concentrations (pg/mL) in serum samples were quantified by referencing a standard curve generated with recombinant cytokines provided with the kits.

2.5. RNA extraction and reverse transcription

The total RNA for cytokine expression analysis was extracted from approximately 10 mg of three placentomes or caruncles (depending on whether foetal death was detected or the animal gave birth, respectively) per goat using the Maxwell® 16 LEV simplyRNA Purification Kit (Promega, Wisconsin, USA) following the manufacturer's instructions. The RNA concentration and purity was assessed spectrophotometrically using the NanoPhotometer® Classic (Implen, München, Germany), and RNA integrity was assessed by electrophoresis on a 1% agarose gel. Reverse transcription was performed using the master mix SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Paisley, UK) in a 20 µl reaction containing 2.5 µg total RNA. The resulting cDNA was diluted 1/10 and used in real-time quantitative PCR (qPCR) to analyse cytokine mRNA expression.

2.6. Cytokine real-time quantitative PCR

Cytokine mRNA expression analysis was performed as previously described by Arranz-Solís et al. (2016) for ovine samples. Briefly, the primers used for IFN-γ, IL-4, IL-10, IL-12p40, TNF-α and the housekeeping gene β-actin mRNA expression analysis were those described for ovine samples (Arranz-Solís et al., 2016) after confirming their compatibility for amplifying caprine mRNA sequences by comparing their sequences with caprine mRNA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). Real-time PCR reactions were performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For each target gene, a seven-point standard curve, prepared as previously described by Arranz-Solís et al. (2016), was included in each batch of amplifications. The minimal coefficient of regression value for all standard curves used in this study was > 0.995, and the slope values were between −3.20 and −3.50. The relative quantification of cytokine mRNA expression levels (x-fold change in expression) was performed using the comparative $2^{-\Delta\Delta Ct}$ method, as previously described by Schmittgen and Livak (2008). For data analysis, results of placentomes from goats that aborted (G1: n = 7; G2: n = 4) and caruncles from goats that gave birth (G2: n = 3;