ABSTRACT

The effects of the abortifacient parasite, *Neospora caninum* on bovine foetuses in early and late gestation

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Neospora caninum is an obligate intracellular protozoan parasite, which is the most frequently diagnosed abortifacient in dairy cattle in the UK and is a leading cause of abortion worldwide. *Neospora caninum* infection in early gestation is associated with foetal death whereas in late gestation, infection can result in the birth of asymptomatic, but persistently infected animals. How the parasite kills the foetus is not fully understood, but it has been suggested that more mature foetuses are better able to mount a stronger immune response to control parasite multiplication and dissemination. The ability of the bovine foetus to respond to various antigens develops in a sequential fashion during the gestation period and foetal immunocompetence starts to develop at approximately 100 days gestation age (dg), but can only fully recognise antigens during mid-gestation at around 150 dg.

Chapter 2 assessed the pathological effects of *N. caninum* on bovine foetuses in early and late gestation (70 and 210 days gestation, respectively) and also in foetuses from naturally infected dams after recrudescence of *N. caninum* in mid to late gestation. Based on results of an initial histological screen of 35 bovine foetuses and 2 new-born calves, a total of 12 foetuses and calves were selected and subjected to more detailed histological examination. Both haemolymphatic and non-haemolymphatic tissues were used. The distribution of *N. caninum* antigen, CD3-positive T cells, PAX5-positive B cells, monocytes/macrophages and neutrophils (myeloid/histiocyte antigen/calprotectin-positive), antigen presenting cells (MHCII), interferon gamma (IFN-γ) expressing cells, PCNA-positive proliferating cells and apoptotic cells (cleaved caspase 3-positive) was analysed by immunohistology. In uninfected, control foetuses in early gestation (90 days gestation), haemolymphatic tissues were moderately developed and exhibited normal morphological features with low lymphocyte turn over and no evidence of IFN-γ production. Uninfected foetuses in late gestation had fully developed haemolymphatic tissues with high lymphocyte turnover, indicative of a mature immune system. In the infected foetuses in early gestation, extensive apoptosis of lymphocytes was observed in the thymus and spleen compared to controls ($p<0.001$, student's *t*-test). No histological changes were observed in the haemolymphatic tissues of infected foetuses in late gestation. In non-haemolymphatic tissues, infected foetuses in early gestation exhibited extensive hepatocellular necrosis and apoptosis, glial cell necrosis and apoptosis in the CNS and high parasite loads in the liver, CNS and myocardium. There was no evidence of cell death in the heart despite the high parasite loads. In late gestation, histological lesions were restricted mainly to the CNS where non-suppurative inflammation and low parasite loads were observed. Other non-haemolymphatic tissues exhibited only mild mononuclear inflammatory infiltrates. The results suggest that in early gestation, tachyzoites replicate preferentially in foetal liver, brain and myocardium in the absence of an inflammatory response and cause extensive necrosis in the liver and brain. Unlike foetuses in early gestation, those in late gestation exhibited a mild to moderate mononuclear inflammatory infiltrate in various tissues dominated mainly by lymphocytes, plasma cells and smaller numbers of macrophages.

In Chapter 3, the observation that *N. caninum* appeared to induce cellular degeneration in hepatocytes but not in the myocardium was investigated in more depth. An *in vitro* tissue

culture system using the human HepG2 hepatoma cell line and the murine HL-1 cardiomyocyte cell line was used to establish the mechanism of cell death following *N. caninum* infection. The activation of the initiator and effector caspases (caspases 3, 8 and 9) was measured and the mitochondrial organisation in cells following *N. caninum* infection evaluated. Quantitative (caspase 3) and semi-quantitative (caspase 8 and 9) analyses were used to assess differences in *N. caninum*-infected and uninfected HepG2 and HL-1 cells. A significant difference was observed in the numbers of cleaved caspase 3-positive HepG2 cells at 20-36 hours post infection (p=0.029, Mann-Whitney U test) in infected cultures compared to controls. No significant difference was observed for caspase 8 and 9 expression. In HL-1 cultures, no significant difference was observed in the number of caspase 3, 8 and 9-positive cells between infected and control cultures. This suggests that *N. caninum* infection is not associated with activation of the caspase cascade in cardiomyocytes. *N. caninum* tachyzoites were detected within intact HepG2 and HL-1 cells with normal cellular morphology and which were not labelled with the caspase antibodies; whereas uninfected surrounding cells were caspase 3, 8 and 9-positive, indicating that the parasites are involved in the inhibition of the caspase pathways (intrinsic and extrinsic). The mitochondrial organisation in *N. caninum*-infected and uninfected cells was assessed in both cell lines using double immunofluorescence, which involved staining with a *N. caninum* specific polyclonal antibody and COX 1 mitochondrial marker. In the control cultures of both HepG2 and HL-1 cells, mitochondrial clumping with large aggregates of mitochondria exhibiting a punctate pattern was observed in high numbers of cells, mainly in the perinuclear region and this is suggestive of mitochondrial fragmentation, which is associated with apoptosis. Other cells within the control cultures revealed an unaltered reticular pattern of mitochondria that is consistent with the normal cellular morphology. In the infected cultures, there was mitochondrial clumping with aggregates of mitochondria detected surrounding parasitophorous vacuoles; while in neighbouring uninfected cells, large aggregates of mitochondria, exhibiting a punctate pattern were present, suggesting mitochondrial clumping and fragmentation associated with cytochrome c release and apoptosis. Other uninfected HepG2 and HL-1 cells exhibited a diffuse, homogenous distribution of mitochondria, often with an unaltered reticular pattern as was observed in the control cultures and is consistent with the normal cellular morphology. The results indicate that *N. caninum* inhibits apoptosis in infected cells and is associated with increased apoptosis in infected HepG2 cultures, while not having any effects on HL-1 cardiomyocytes.

Chapter 4 investigated the seroprevalence of *N. caninum* infection in Jamaican dairy herds. Serum samples were analysed from 499 Holstein-Friesian and Holstein Friesian crossbreed dairy cattle from three different farms in Jamaica. A seroprevalence of approximately 26% was found with the majority of seropositive animals aged 0-2 years old (25%), while the lowest seroprevalence was recorded in animals over 13 years old (13.3%). Pregnancy status was shown to influence the seroprevalence of cows, but no significant relation of seropositivity to age was found, suggesting that vertical transmission is the principal route of transmission in Jamaica.