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EXPERIMENTALLY INDUCED DISEASE

A Descriptive Study of Lectin Histochemistry of the Placenta in Cattle following Inoculation of *Neospora caninum*

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Summary

The aim of this study was to describe the lectin-binding pattern in the placentas of cows infected experimentally with *Neospora caninum*. Four cows were inoculated intravenously with 1×10^8 tachyzoites of the NC-1 strain of *N. caninum* at 150 ± 7 days of pregnancy. Two control cows were administered a placebo. An indirect fluorescence antibody test (IFAT) was performed on serum samples obtained before and after the inoculation. The cows were killed at 30 and 37 days post inoculation. Samples of placenta were taken for histopathology and lectin histochemistry. Fetal tissues and fluids were collected for histopathology and IFAT, respectively. All infected cows had high antibody titres. All fetuses had characteristic histopathological lesions, including non-suppurative meningoencephalitis, myocarditis, hepatitis and myositis, suggesting *N. caninum* infection. Only two infected fetuses developed specific antibodies. Mild non-suppurative inflammatory infiltrates were recorded in the placentae. Differences in the lectin-binding pattern were observed between infected animals and controls in the glycocalyx (CON-A and WGA) and apical cytoplasm (RCA-I and CON-A) of the trophoblastic cells; giant trophoblastic cells (CON-A and DBA); glycocalyx (PNA, WGA) and apical cytoplasm (CON-A, WGA, PNA, DBA and RCA-I) of endometrial cells; trophoblast of the interplacentomal region (WGA); endothelium (CON-A, SBA, RCA-1 and WGA); and finally, mesenchyme (CON-A, RCA-1, SBA, PNA and DBA). These findings indicate that there is a distinctive pattern of lectin binding in the placenta of cattle infected with *N. caninum*. The direct effect of the presence of the protozoa as well as the altered expression of cytokines could explain these changes in the maternofetal interface.

Keywords: cattle; lectin histochemistry; *Neospora caninum*; placenta

Introduction

Neospora caninum is an obligate intracellular apicomplexan protozoon that infects domestic and wild animals (Dubey *et al.*, 2002, 2007; McAllister, 2016). Despite a wide host range, neosporosis affects mainly cattle and dogs, the latter acting as the definitive host (McAllister *et al.*, 1998; Dubey *et al.*, 2007). In cattle, abortion and stillbirths are the most common clinical features (Dubey *et al.*, 2006; Benavides *et al.*, 2014). Although post-natal infection is possible, vertical transmission is considered to be the most significant route of transmission, because it allows the persistence of *N. caninum* in the herd (Dubey *et al.*, 2006; Almería and López-Gatius, 2015).

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Lectins are plant-derived proteins that bind specifically but non-immunologically to certain oligosaccharides (Munson *et al.*, 1989; Gimeno and Barbeito, 2004). Physiological and pathological changes might cause alterations in the localization and concentration of carbohydrates that constitute the glycoproteins and glycolipids of cells and tissues (Walker, 1989; Gabius *et al.*, 2004). Therefore, lectins are indirect markers of these alterations and as such, they possess great value in the study of pathogenesis of diseases (Walker, 1989; Woudwyk *et al.*, 2013). In cattle, several studies have described the expression of glycoproteins in the uterine and oviduct epithelium during infection by *Tritrichomonas foetus* and *Campylobacter fetus venerealis* (Cipolla *et al.*, 1998; Cobo *et al.*, 2004). More recently, changes in lectin patterns in fetal bovine tissues infected by *C. fetus* and *Brucella abortus* have been studied (Morrell *et al.*, 2011; Fiorentino *et al.*, 2018). There have, however, been no published studies of lectin binding in tissues infected with *N. caninum*. Therefore, the aim of this study was to describe the pattern of oligosaccharide distribution in placentas from pregnant cows infected experimentally with *N. caninum*.

Materials and Methods

Animals and Experimental Design

The experiment involved six Angus cows aged 3–7 years, all being seronegative to *N. caninum* and *B. abortus*. All cows had low antibody titres to bovine viral diarrhoea virus (BVDV) by an in-house serum neutralization test (Rossi and Kiesel, 1971).

Cattle were synchronized for oestrus followed by natural breeding using two bulls, which were negative for *Trichomonas* spp., *Campylobacter* spp. and *B. abortus*. Pregnancy was confirmed by ultrasound and transrectal palpation at 30 and 45 days after mating, respectively. In addition, fetal viability was monitored monthly.

Cows at 150 ± 7 days of pregnancy were divided into two groups: four of them were inoculated with live *N. caninum* tachyzoites and the remaining two were given phosphate buffered saline (PBS). The animals were kept in the same paddock, feeding natural pasture under standard management conditions. All animals used in this study were handled according to good practices and conditions defined by the Animal Ethics Committee at the National Institute of Agricultural Technology, Argentina. For logistical reasons, cows were killed 1 week apart: one control and two infected animals at 30 days post inoculation (dpi), and the rest at 37 dpi.

Culture of Tachyzoites

To prepare the inoculum, tachyzoites of the NC-1 strain of *N. caninum* were cultured in bovine monocyte (BM) cell cultures in RPMI-1640 medium (Sigma; St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N′ 2 ethanol sulphonate (HEPES), L-glutamine (10 μg/ml), penicillin–streptomycin (10 μg/ml) and amphotericin B (20 μg/ml). The tachyzoites were harvested during their intracellular phase when 80% of the monolayer cells were infected, using a sterile cell scraper. Live tachyzoites were counted and their concentration was adjusted for inoculation.

Parasite Inoculum

The concentration used was 1×10^8 protozoa in 5 ml of PBS administered intravenously. A control inoculum comprising 4.5×10^6 BMs in 5 ml of PBS was used to challenge the control group. Two hours after inoculation, the strain was subcultivated in BM cell culture to confirm the viability of the parasite.

Clinical Monitoring, Necropsy Procedures and Samples

Cows were monitored daily until slaughter. Rectal temperature was taken for 8 days following inoculation. Animals with temperatures >39.5°C were considered febrile. Blood samples for indirect fluorescence antibody test (IFAT) were collected monthly for 5 months prior to inoculation. Once the cows were inoculated, blood samples were taken weekly until slaughter. The buffered plate antigen (BPA) test was also performed for all of the cows before and after inoculation, as previously described (Angus and Barton, 1984).

After slaughter, samples of placenta were taken and fixed in 10% neutral-buffered formalin. The fetuses were recovered for normal processing (Campero *et al.*, 2003). Briefly, fetal fluids from thoracic–abdominal cavities were collected for IFAT. Sterile samples of lung and abomasal content were obtained for aerobic and microaerophilic bacterial culture. Direct immunofluorescence (DIF) test and culture for *C. fetus* were performed on abomasal content. The abomasal content was cultured in liver infusion medium for the diagnosis of *T. foetus*. The spleen was removed and processed, and a 10% tissue homogenate was inoculated onto cultures of Madin–Darby bovine kidney (MDBK) cells for isolation of BVDV and bovine herpes virus (BHV).

Histopathological Examination

After fixation, tissues were processed routinely and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE) as previously described (Campero *et al.*, 2003).

Lectin Histochemistry

Lectin histochemistry was performed as described previously (Cobo *et al.*, 2004; Fernández *et al.*, 2014; Díaz *et al.*, 2017). Sections were dewaxed and treated with H_2O_2 0.3% in methanol (30 min) at room temperature, rinsed several times in 0.01 M phosphate buffered saline (PBS) (pH 7.2) and immersed in PBS containing 0.1% bovine serum albumin for 15 min. The sections were then incubated for 1 h at room temperature with biotinylated lectins. Seven lectins with different specificity were used (Table 1). Optimal lectin concentration was 30 mg/ml in PBS for all lectins, except for PNA, which was applied at a concentration of 10 mg/ml. The slides were incubated with an avidin–biotin–peroxidase complex (ABC) (Vector Laboratories Inc., Burlingame, California, USA) for 45 min. The horseradish peroxidase was activated by incubation for 1–2 min with 3, 3′ diaminobenzidine chromogen in a kit (Dako, Carpinteria, California, USA). Slides were rinsed in distilled water, dehydrated with graded ethanol solutions, cleared in xylene and mounted under Permount® (Fisher Scientific International, Hampton, New Hampshire, USA). Controls for lectin labelling included: exposure to horseradish peroxidase and substrate medium without lectin; and blocking by incubation with the appropriate blocking sugars (0.1–0.2 M in PBS) for 1 h at room temperature before applying lectins to the sections. The intensity of lectin binding was scored subjectively as follows: 0, no binding; 1, weakly positive; 2, moderately positive; and 3, strongly positive, as described previously (Cobo *et al.*, 2004; Morrell *et al.*, 2011; Fiorentino *et al.*, 2018). Based on these scores, we calculated the means for each group and area observed.

The structures observed in the placentomes were trophoblast, uterine epithelium, mesenchyme and endothelium. The characterization of trophoblast, mesenchyme and endothelium was performed in the interplacentomal areas.

Indirect Fluorescence Antibody Test

Serum samples from the cows and thoracic–abdominal fluids from the fetuses were analysed by IFAT for the detection of IgG antibodies to *N. caninum*, as previously described (Dubey *et al.*, 1988; Wouda *et al.*, 1997a). Serological titres of ≥200 and ≥25 were considered positive for cows and their fetuses, respectively. In all cases positive and negative controls were used.

Results

All of the infected cows became pyrexic at 2 (mean 40.4°C, range 40.3°C–40.5°C) and 7 dpi (mean 39.6°C, range 39.5°C–39.8°C). The control group had no signs of illness. All cows developed specific antibodies to *N. caninum* (antibody titres from 3,200 to 12,800) and which had decreased by around 21 dpi. Over the following weeks, titres remained relatively stable, reaching values between 1,600 and 4,000. Control animals remained seronegative during the whole trial.

Gross lesions were absent in the placenta of the slaughtered infected cows and in their respective fetuses, except for one fetus that had generalized congestion and multifocal haemorrhages in the epicardium.

Placentas from infected cows had a multifocal, mild to moderate lymphohistiocytic infiltrate with generalized congestion and multifocal haemorrhages (Fig. 1). Evidence of vertical transmission is shown in Table 2, which summarizes the microscopical lesions and antibody titres of the fetuses. Fetal samples tested negative for bacteria, viruses and *T. foetus*.

Table 1
Lectins used in the histochemical studies, their binding specificities and blocking sugars

Lectin	*Acronym*	*Binding specificity*	*Blocking sugar*
Dolichos biflorus	DBA	α-D-GalNAc	N-acetylgalactosamine
Glycine max	SBA	α-D-GalNAc; α and β-Gal	N-acetylgalactosamine
Triticum vulgaris	WGA	α-D-GalNAC >>NeuNac	N-acetylglucosamine
Concanavalina ensiformis	CON-A	α-D-Man >α-D-Gluc	α-D-methylmannose
Ulex europaeus-I	UEA-1	α-L-Fuc	M α-L-fucose
Arachis hypogaea	PNA	β-D-Gal >(1–3)GalNAc	M D-galactose
Ricinus communis	RCA-1	β-D-Gal >α-D-Gal	M D-galactose

Adapted from Goldstein and Hayes (1978).

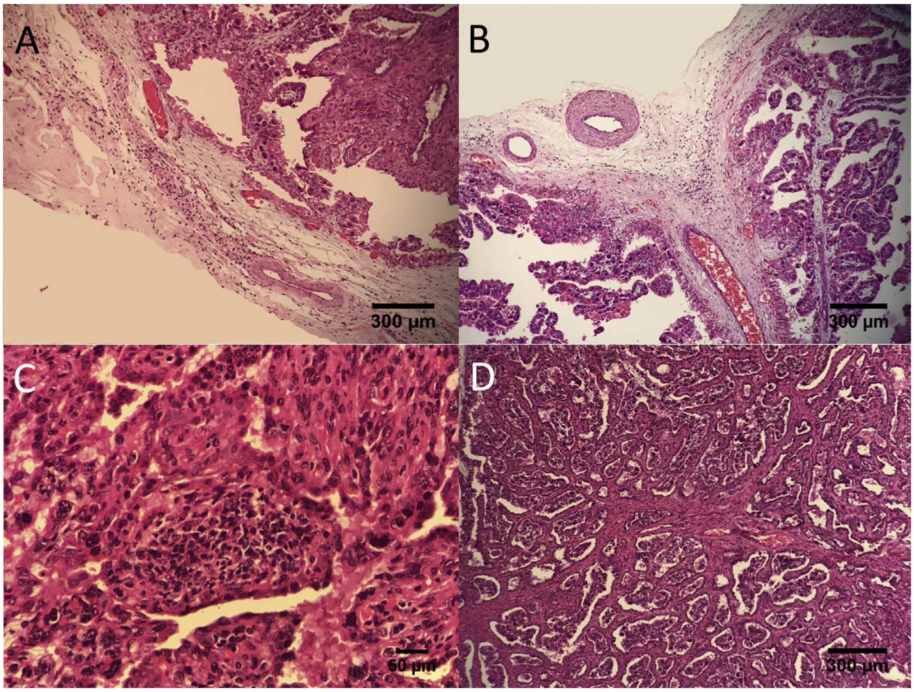

Fig. 1. Placental tissues from infected and non-infected cows. (A, B) Mild lymphohistiocytic inflammatory infiltrate and congestion in placental stroma from two infected cows. HE. (C) Focal lymphohistiocytic inflammatory infiltrate in the cotyledonal area of an infected cow. HE. (D) Absence of lesions in the placental tissue from a non-infected cow. HE.

Table 3 summarizes the lectin-binding pattern for the seven lectins. The glycocalyx of trophoblastic cells showed differences in intensity for CON-A and WGA between infected and control tissues. Moderate RCA-1 and CON-A labelling was seen in the apical cytoplasm of these cells in infected tissues. The trophoblastic giant cells (TGCs) from the infected group had stronger labelling for CON-A and DBA in comparison with the control group (Fig. 2). Differences between groups were observed for PNA and WGA in the surface of the uterine epithelium. Regarding the apical cytoplasm, the intensity of the lectin labelling showed differences between infected and control tissues for CON-A, WGA, PNA, DBA and RCA-1.

With the exception of UEA-1, most of the lectins exhibited strong labelling of the surface of the interplacentomal trophoblast (Fig. 3). WGA binding was clearly greater in the glycocalyx of the infected animals.

CON-A, SBA, RCA-1 and WGA binding of the endothelial cells differed between infected and control fetuses. Mesenchyme also showed differences for CON-A, RCA-1, SBA, PNA and DBA.

Finally, UEA-1 did not show affinity for any of the examined structures.

Table 2
Microscopical lesions and serological titres of the fetuses

Fetus ID	*Age (days)*	*Sex*	*Histopathological lesions*					*Serological titre*
			CNS	*Heart*	*Skeletal muscle*	*Liver*	*Lung*	
Infected fetuses								
335	180 ± 7	M	+	+	–	+	+	–
139	187 ± 7	F	–	+	–	+	+	25
313	180 ± 7	M	–	–	+	–	–	–
301	187 ± 7	M	+	+	+	+	–	100
Control fetuses								
5018	180 ± 7	F	–	–	–	–	–	–
350	187 ± 7	M	–	–	–	–	–	–

ID, identification number; F, female; M, male; CNS, central nervous system; +, presence of lesions; –, absence of lesions.

Discussion

Since *N. caninum* was transmitted vertically in cattle challenged at 150 ± 7 days of gestation, the altered oligosaccharide pattern in these placentas provides novel knowledge for understanding the pathogenesis of the infection at the maternofetal interface. The placenta is usually one of the most severely affected tissues, together with the fetal CNS (Dubey *et al.*, 2006). Inflammatory microscopical lesions consisting of a non-suppurative inflammatory reaction in placenta have been described (Barr *et al.*, 1990; Macaldowie *et al.*, 2004; Dubey *et al.*, 2006; Cantón *et al.*, 2014); nevertheless, alterations in the localization and concentration of the carbohydrates

Table 3
Lectin-binding pattern in the different regions of the placentas from infected and non-infected cows

Sites	*UEA-1*		*CON-A*		*RCA-1*		*SBA*		*WGA*		*PNA*		*DBA*	
	Inf	*C*	*Inf*	*C*	*Inf*	*C*	*Inf*	*C*	*Inf*	*C*	*Inf*	*C*	*Inf*	*C*
Placentomal area														
Trophoblast														
Glycocalyx	0	0	**3**	**2**	3	3	3	3	**2**	**3**	3	3	3	3
Apical cytoplasm	0	0	**2**	**1**	**2**	**1**	2	2	1	1	2	2	2	2
Mesenchyme	0	0	2	2	1	1	**0**	**1**	0	0	**1**	**0**	**0**	**2**
Endothelium	0	0	**2**	**1**	2	2	**0**	**1**	0	0	0	0	0	0
Trophoblastic giant cells	0	0	**2**	**1**	1	1	3	3	0	0	1	1	**3**	**2**
Uterine epithelium														
Glycocalyx	0	0	2	2	3	3	2	2	**1**	**3**	**3**	**2**	2	2
Apical cytoplasm	0	0	**2**	**1**	**2**	**1**	2	2	**0**	**1**	**1**	**2**	**1**	**2**
Interplacentomal area														
Trophoblast														
Glycocalyx	0	0	3	3	3	3	3	3	**3**	**2**	3	3	3	3
Apical cytoplasm	0	0	2	2	2	2	2	2	2	2	2	2	3	3
Mesenchyme	0	0	**3**	**2**	**2**	**1**	0	0	1	1	0	0	0	0
Endothelium	0	0	3	3	**3**	**2**	0	0	**0**	**2**	0	0	0	0

Four categories of staining intensity: 0, none; 1, weakly positive; 2, moderately positive; and 3, strongly positive. Mean values for each group are shown. Bold figures indicate differences in the lectin labelling between infected (Inf) and control (C) tissues.

that constitute the glycoproteins and glycolipids of placental cells are described here for the first time.

As regards the lectin-binding patterns, the uterine epithelium showed a high concentration of Gal and β-D-GalNAC (evidenced by the PNA and RCA-1 binding). These carbohydrates are involved in the junction of the chorion and the endometrium (Munson *et al*., 1989). The expression of α-D-GalNAc and possibly NeuNac, differed between infected and control animals, being higher in the latter group. NeuNac may play a significant role in the adhesion of the parasite, since Vonlaufen *et al.* (2004) showed

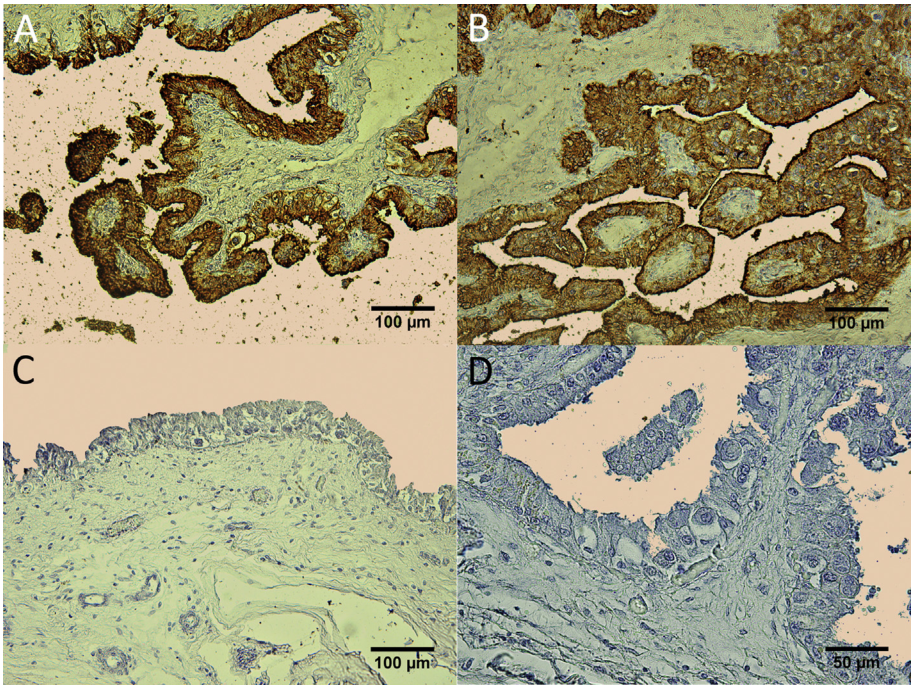

Fig. 2. Placentomes of an experimentally infected cow. (A) DBA binding in the surface and cytoplasm of trophoblastic giant cells. (B) SBA binding in trophoblastic cells. (C, D) Placentome negative for UEA-1 binding.

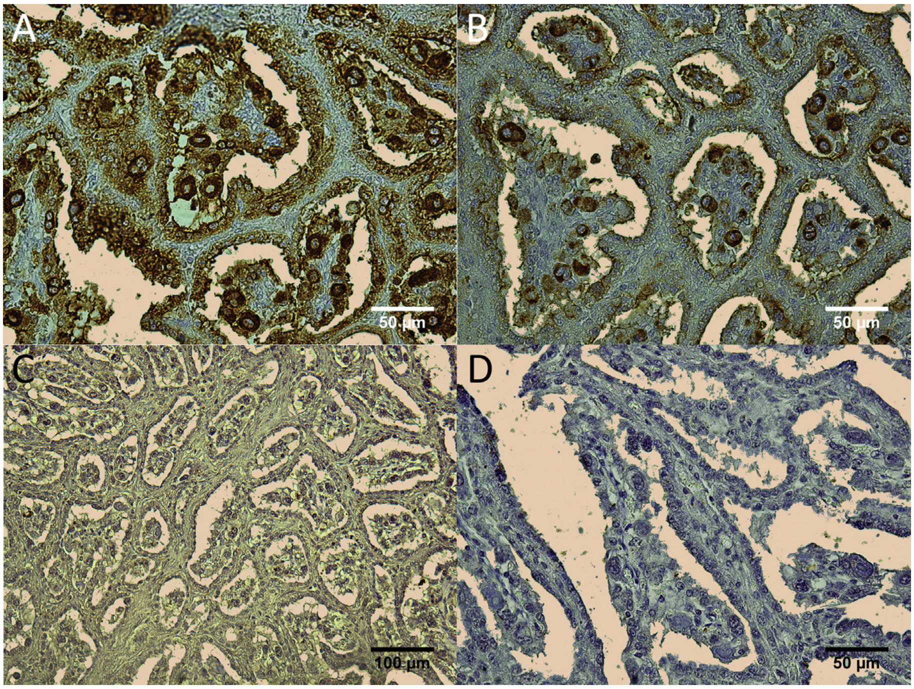

Fig. 3. Interplacentomal areas of an experimentally infected cow. (A, B) Intense SBA labelling in the glycocalyx and cytoplasm of the trophoblastic cells. (C, D) Interplacentomal area negative for UEA-1 binding.

that bradyzoite adhesion improved when NeuNac was removed from the surface of Vero cells. Nevertheless, tachyzoites did not show any changes in their capacity for invasion (Vonlaufen *et al.*, 2004).

The trophoblast of both regions (placental and interplacentomal) expressed high concentrations of α-D-GalNAc, α-D-Man, β-D-Gal, α-D-Gluc and NeuNac, particularly in the glycocalyx and, to a lesser extent, the cytoplasm. Such carbohydrates are likely to be involved in the functioning and anchorage of the chorion (Munson *et al.*, 1989). Recent findings indicate that trophoblastic cells are more susceptible, while the maternal placenta is capable of limiting multiplication and adhesion of the parasite (Regidor-Cerrillo *et al.*, 2014; Jiménez-Pelayo *et al.*, 2017). Nonetheless, during tachyzoite multiplication, both fetal and maternal tissues suffer focal lesions. We hypothesize that the differences found are due to early invasion of the parasite into the maternofetal interface from 10 to 15 dpi, in order to reach the bloodstream, as reported before (Barr *et al.*, 1994; Buxton *et al.*, 2002; Maley *et al.*, 2003; Macaldowie *et al.*, 2004). Horcajo *et al.* (2017) demonstrated that the presence of *N caninum* is able to modulate the transcriptome of mononuclear trophoblast cells, changing their glycosylation pattern.

Alterations in the epithelium of gravid uteruses caused by protozoa, such as *T. foetus*, are described in heifers and mice (Cobo *et al.*, 2004; Woudwyk *et al.*, 2013). The changes we found in the uterine epithelium might be associated with modification of cytokine expression induced by *N. caninum*, as described by Almería *et al.* (2011) and Regidor-Cerrillo *et al.* (2014).

The TGCs, which are a characteristic feature of the bovine placenta, compose the epithelial lining of the cotyledons (trophoblast) together with uninucleate trophoblast cells (Klisch *et al.*, 2010; Peter, 2013; Santos *et al.*, 2017). These cells modify the uterine epithelium, allowing maternofetal union (Wooding and Wathes, 1980). We found that TGCs expressed high concentrations of α-D-GalNAC (both on the surface and the cytoplasm), which is in agreement with other studies (Klisch *et al.*, 2010; Jones *et al.*, 2015), even in other ruminant species of similar gestational stage. Nevertheless, concentrations of other carbohydrates such as β-D-Gal (PNA), α-D-Man (CON-A) and α-L-Fuc (UEA-1) were lower, both in the infected and control groups, unlike in other reports (Munson *et al.*, 1989; Fiorentino *et al.*, 2018). TGCs expressed higher concentrations of α-D-GalNAc (DBA) and α-D-Man (CON-A) in the infected animals. Machado *et al.* (2007) concluded that TGCs had in-vitro phagocytic activity and, therefore, they might play an important role in the pathogenesis of vertical transmission of *N. caninum*. However, most research indicates that hormone production is the main function of TGCs (McNaughton and Murray, 2009). In the absence of phagocytic activity, the altered expression of cytokines could indirectly change the lectin-binding pattern, similar to that proposed for the uterine epithelium. This hypothesis is based on the results of experimental models with trophoblast cultures in which pro-inflammatory cytokines induced modifications in gene expression (Noyola-Martínez *et al*., 2014).

TGCs have granules containing pregnancy-associated glycoproteins (PAGs), which bind specifically to DBA (Munson et al., 1989; Klisch and Leiser, 2003). The concentration of PAGs and its terminal N-acetyl-galactosamine varies throughout the different stages of pregnancy (Klisch *et al*., 2006). Furthermore, *N. caninum* infection modifies the plasma concentrations of PAGs (López-Gatius *et al*., 2007). Therefore, alterations in the concentration of PAGs or its terminal N-acetyl-galactosamine could explain the differential DBA labelling in the TGCs observed herein.

Interplacentomal areas showed minor alterations in the lectin-binding pattern, probably because they become infected after lesions in the placental area have already spread (Dubey *et al*., 2006).

The endothelium of the placental vessels expressed large amounts of α-D-Man and β-D-Gal (CON-A and RCA-1), partially opposed to the findings of previous reports (Alroy *et al*., 1987). The differences observed between infected and controls animals, although mild, might be due to the capacity of *N. caninum* to multiply in endothelial cells (Wouda *et al*., 1997b; Buxton *et al*., 2002). This is a critical step for the immunopathogenesis of the infection, because endothelial cells react very rapidly by increasing the adhesion of neutrophils (Taubert *et al*., 2006).

In summary, this paper describes the alterations in the lectin-binding pattern of the placentas of cattle infected experimentally with *N. caninum*. Lectin histochemistry represents a valuable technique in the study of the pathogenesis of bovine neosporosis, although further research is needed in order to achieve a better understanding of this disease.

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Conflict of Interest Statement

The authors declare no conflict of interest with respect to the publication of this manuscript.

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