Reprod Dom Anim doi: 10.1111/rda.12265
ISSN 0936–6768

Intermediate Filament Proteins Expression and Carbohydrate Moieties in Trophoblast and Decidual Cells of Mature Cat Placenta

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Contents

The aim of this study was to characterize cytoskeletal intermediate filament proteins and glycoconjugates of syncytiotrophoblast, cytotrophoblast and decidual cells of feline endotheliochorial placenta. Samples from 12 normal pregnant female cats, after 45 ± 5 days of gestation, were obtained removing the uterine horns by hysterectomy. Sections were processed for routine observation and for immunohistochemistry using anticytokeratin, antivimentin and antidesmin antibodies. In addition, lectin histochemistry was performed using a panel of several biotinylated lectins to characterize glycosides expression profile. Cytotrophoblast and syncytiotrophoblast showed immunoreactivity only with acidic and basic cytokeratins. Decidual cells were only positive to vimentin, consistent with their origin from endometrial fibroblasts. Trophoblast expressed a broad population of glycans, highly exposing terminal *N*-acetyl glucosamine residues and non-sialylated galactose and *N*-acetyl galactosamine oligomers. Oligosaccharides bound by *Phaseolus vulgaris* erythroagglutinin were the only highly branched N-linked residues evidenced in cats, and they were restricted to the syncytium. Unlike results reported on humans, mice and rats on lectin affinity of decidual cells, sialid acids and complex N-linked oligosaccharides were not demonstrated in cats. Glycosylation of proteins determines many of their final properties, thus becoming essential for the embryo-maternal dialogue during implantation and placentation. Changes in glycosylation pattern have been related to pathological pregnancies in other species. Hence, the knowledge about glycosylation profile of the normal cat placenta may lead to a better understanding of both normal and pathological reproductive events.

Introduction

The chorioallantoic placenta is a complex and transitory organ in mammals displaying wide structural differences among species. In domestic cats (*Felis catus*), it is classified as a girdle, lamellar and endotheliochorial (Wooding and Burton 2008). The placental girdle is located between two cupular paraplacental poles. In the girdle of mature placentae, the following areas can be defined: (i) the chorioallantoic membrane; (ii) the lamellar zone, where the chorionic villi contact maternal vessels, (iii) the junctional zone and (iv) the glandular layer. In the lamellar zone, two types of structures of foetal origin can be particularly determined in addition to blood vessel cells: the cytotrophoblast (CT) and the syncytiotrophoblast (ST) (Wislocki and Dempsey 1946; Dempsey and Wislocki 1956; Bjorkman 1973; Malassinéé 1974; Leiser and Enders 1980; Leiser and Koob 1993; Jones et al. 2005; Miglino et al. 2006).

The presence of decidual giant cells, like those found in human and rodent haemochorial placenta, is not constant at all in carnivores. In cats, decidual cells (DC) are the unique permanent cells of labyrinthine maternal connective tissue. These cells have been morphologically described using optical and electron microscope in this species (Wislocki and Dempsey 1946; Dempsey and Wislocki 1956; Bjorkman 1973; Malassiné 1974; Leiser and Koob 1993; Jones et al. 2005) but dismissed in ferrets (Lawn and Chiquoine 1965), dogs (Fernández et al. 2000) and minks (Winther et al. 1999).

Intermediate filament (IF) proteins have been used as cellular lineage markers due to their high specificity (Fernández Alonso et al. 1984). In normal carnivore placenta, IF distribution was established for dogs (Fernández et al. 2000), minks (Winther et al. 1999) and partially in cats placental girdle (Barbeito et al. 2004; Walter and Schonkypl 2006). In a previous work, we established the trophoblastic origin of the cells present in the subinvoluted placental sites of the dog using intermediate filament markers (Fernández et al. 1998). The expression of IF vimentin as well as the absence of IF cytokeratin was reported for DC in the cat placenta (Barbeito et al. 2004; Walter and Schonkypl 2006). However, IF desmin immunostaining pattern has not been described yet for these cells in cats. In Wislocki and Dempsey's (1946) pioneer work, it is mentioned that some DC are binucleated; however, this observation was not confirmed in later publications (Malassiné 1974; Leiser and Koob 1993; Walter and Schonkypl 2006).

Trophoblast (TB) and endometrium cell membrane glycoconjugates play a critical role involving the maternal and embryonic cellular adhesion during the implantation process and further placentation (Carson 2002; Jones and Aplin 2009). Glycoconjugates of animal tissues in normal and pathological conditions have been studied in the last decades using lectins proteins, which specifically bind carbohydrates (Gimeno et al. 1995; Gabius et al. 2002; Diessler et al. 2003; Sant'Ana et al. 2009; Woudwyk et al. 2013). Glycosylation pattern of several species was published in many articles (Arkwright et al. 1991; Bulmer and Peel 1996; Jones et al. 1996, 1997, 1999, 2000, 2002, 2004a,b, 2007, 2008; Fernández et al. 2000; Sandoval et al. 2001; Enders et al. 2006; Tatsuzuki et al. 2009; Klisch et al. 2010). However, to our knowledge, glycosylation profile studies of the placenta in any member of Feliformian suborder apart from that of Alroy et al. (1987) have never been described.

The aim of this study was to provide information about the expression of IF proteins and the distribution of glycoconjugates in CT, ST and DC for further understanding the cat chorioallantoic girdle placenta.

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Materials and Methods

Tissue samples

Twelve normal female cats' uterine horns removed by hysterectomy were examined after 45 ± 5 days of pregnancy. Procedures were entirely carried out according to the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). Tissues were fixed in 10% neutral formaldehyde, dehydrated through increasing grades of alcohol and embedded in paraffin wax. Sections were stained with haematoxylin and eosin for histological observation. Paraffin-embedded sections mounted on slides coated with poly-l-lysine (Sigma Diagnostics, St. Louis, MO, USA) were deparaffinized with xylene, hydrated in decreasing grades of alcohol, incubated with 0.03% H_2O_2 for 30 min at room temperature to inhibit endogenous peroxidase activity and rinsed in deionized water and PBS.

Immunohistochemical techniques

The following commercial antibodies were used: AE1 and AE3 monoclonal anti-mouse acid and basic pancytokeratins (1 : 100) (Zymed, St. Louis, MO, USA), monoclonal anti-swine vimentin, clone V9 (pre-diluted) (Dako, Carpinteria, CA, USA) and monoclonal anti-swine desmin, clone DE R 11 (1 : 100; Vector, Burlingame, CA, USA). The ABC method was applied according to the manufacturer's instructions (Vector). The slides were incubated with horseradish peroxidase for 4–10 min in a buffered Tris–HCl solution (0.05 M, pH 7.6) containing 0.02% 3,3′ diaminobenzidine tetrahydrochloride (DAB) and 0.05% H_2O_2. Previously tested human ileum, colon, non-pregnant uterus and prostatic gland were used as positive controls. At least one section was used as a negative technique control by omitting the primary antibody and incubated with 1% bovine serum albumin (BSA). Positively stained cells were identified by the dark golden brown DAB H_2O_2 reaction product.

Counting of cells

For counting binucleated DC, histological images were observed using a 40× magnification objective of a microscope (Olympus BX50; Olympus, Tokyo, Japan) and captured using a digital video camera (DP71; Olympus). From each animal, total amount of DC in 10 randomly selected fields were also counted. Percentage of binucleated DC was then calculated.

Lectin histochemical techniques

Lectin histochemistry was performed as described elsewhere (Gimeno et al. 1995). Briefly, sections were incubated for 30 min with BSA 1% to block unspecific proteins binding and were incubated for 60 min with 30 μg/ml biotinylated lectins listed in Table 1 (except for PNA lectin which was used in a 10 μg/ml PBS solution), except for the negative controls that were incubated with 1% BSA. Table 1 also lists their acronyms and major sugar specificities (Jones et al. 1997, 2000, 2004b).

Table 1. Lectins used in this study and their major specificities

Acronym	Source	Major specificity
BSA-1B	*Bandeiraea (Griffonia) simplicifolia*	Galα1,3Gal-; Galα1,4Gal
BS-II	*Bandairaea (Griffonia) simplicifolia*	α,β-GlcNAc
CON-A	*Concanavalina ensiformis*	α-D-Man > α-D-Glc
DBA	*Dolichos biflorus* (horse gram)	GalNAcα1,3(LFucα1,2) Gal-β1,3/4GlcNAcβ1-
DSA	*Datura stramonium* (Jimson weed)	β1,4GlcNAc, *N*-acetyl-lactosamine > chitotriose
PHA-E	*Phaseolus vulgaris* erythroagglutinin (Kidney bean)	Bisected complex N-linked sequences
PHA-L	*Phaseolus vulgaris* leukoagglutinin (Kidney bean)	β1-6-linked GlcNAc in tri/tetra-antennary complex N-linked sequences
PNA	*Arachis hypogaea* (peanut)	Galβ1,3GlcNAcβ1- >Galβ1,4GlcNAcβ1-
RCA-1	*Ricinus communis*	β-D-Gal
SBA	*Glycine max* (soybean)	Terminal GalNAcα1- >Galα1
SJA	*Sophora japonica*	α and β GalNac > α and β Gal
sWGA	Succinyl-WGA	(β1-4-D- GlcNac)
UEA-1	*Ulex europaeus-1* (gorse)	L-Fucα1,2Galβ1,4-GlcNAcβ1-
WGA	*Triticum vulgaris* (wheat germ)	(D-GlcNAc)*n*, NeuNAcα2,3

Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; Man, mannose; Glc, glucose; GalNAc, *N*-acetyl-galactosamine; Fuc, fucose; NeuNAc, neuraminic acid.

Samples were then rinsed three times in PBS and incubated with the avidin–biotin complex (ABC; Vector). DAB was used as chromogen, and samples were counterstained with Mayer's haematoxylin.

Results

Immunohistochemistry

At the junctional and labyrinthine zone, both CT and ST showed immunoreactivity for acidic and basic cytokeratins, which was stronger in CT (Fig. 1a). Gland epithelial cells were also positive for cytokeratins. Decidual cells were positive for vimentin but negative for the remaining antibodies (Fig. 1b). A 19.4% of them were binucleated.

Endothelial cells were positive for vimentin while desmin immunoreactivity was only found in vessels and muscle cells.

Lectin histochemistry

Trophoblast and DC lectin-staining patterns were examined. Results are summarized in Table 2. Trophoblast of feline placenta expressed a broad population of glycans, highly exposing terminal *N*-acetyl-glucosamine (GlcNAc) residues. Positive reaction with RCA, SJA, SBA, DBA and PNA lectins revealed abundant non-sialylated Gal and GalNAc oligomers.

Concerning DC, quite heterogeneous results for GlcNAc residues were obtained among specific lectins. Most of the Gal/GalNAC binding group lectins were

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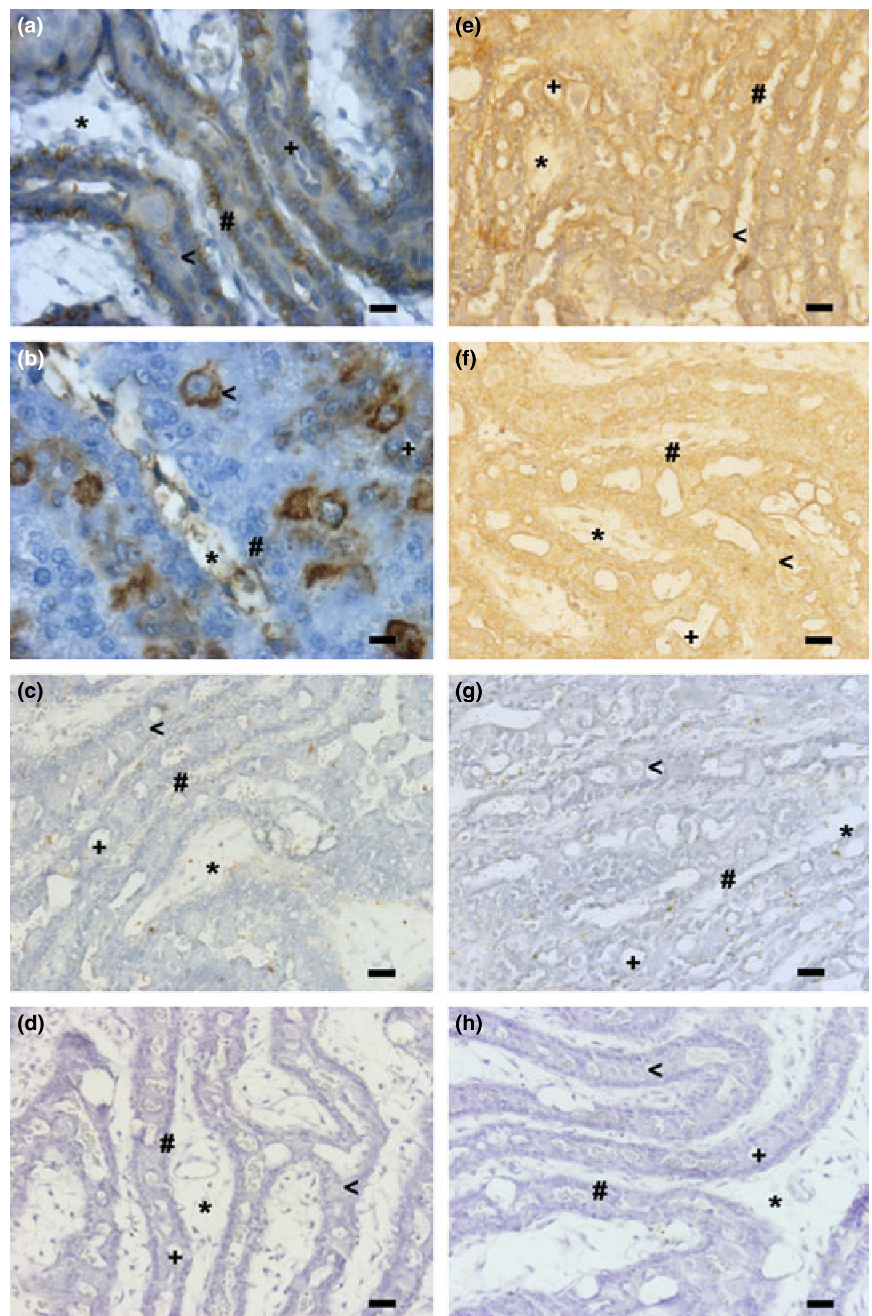

Fig. 1. Immunohistochemistry (a–d) and lectinhistochemistry (e–h) in lamellar zone of mature cat placenta. (a) Antipancytokeratins antibodies. Positivity is strong in cytotrophoblast (CT) and weak in syncytiotrophoblast (ST). (b) Antivimentin antibody. The decidual cells show strong reactivity. (c) Antidesmin antibody. All the lamellar zone structures are negative. (d) Negative control for immunohistochemistry. (e) WGA. The affinity is moderate in CT and weak in ST and decidual cells. (f) DBA. The affinity is weak in all the structures analysed. (g) UEA-1. The lamellar zone is negative. (h) Negative control for lectin histochemistry. Scale bar: 20 μm (b), 40 μm (a, c–h). #, cytotrophoblast; *, mesenchyma; +, maternal vessel surrounded by syncytiotrophoblast; <, decidual cell

reactive to DC. α-Mannose (recognized by Con-A) was also evidenced. Neither complex N-linked carbohydrates nor terminal fucosyl (recognized by UEA-I) residues could be found; however, fucosylated oligomers were demonstrated by DBA. Differences between non-decidual and decidual stromal cells were only found with BS-II, DBA and Con-A lectins. Decidual cells were reactive to those lectins whereas non-DC were not.

Discussion

Intermediate filament immunostaining pattern observed in the cat placenta resembles that found in dogs (Fernández et al. 2000), rats (Glasser and Julian 1986), mice (Oliveira et al. 2000), short-tailed fruit bat (*Carollia perspicillata*) (Rasweiler et al. 2000) and humans (Can et al. 1995). Immunoreactivity to vimentin and the absence of reaction to cytokeratins and desmin of DC are consistent with their proposed origin from endometrial fibroblasts, thus these cells are likely to originate from maternal connective tissue. Similar results were found in previous works (Barbeito et al. 2004; Walter and Schonkypl 2006). Nevertheless, no data regarding desmin immunoreactivity were reported in those works. The absence of desmin immunostaining in DC of cats reported here differs from staining in the rat (Glasser and Julian 1986), mouse (Oliveira et al. 2000) and the bat *C. perspicillata* DC (Rasweiler et al. 2000). In humans, the expression of desmin decreases with advancing gestation (Can et al. 1995). Taking into

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Table 2. Lectin affinity for trophoblastic and decidual cells of the feline placenta

	TB		
Lectin	CT	ST	DC
BSA-1B	0	1	1
BS-II	1	2	1
CON-A	1	2	1
DBA	1	1	1
DSA	1	2	1
PHA-E	0	1	0
PHA-L	0	0	0
PNA	1	1	1
RCA-I	1	2	1
SBA	1	1	1
SJA	1	1	0
sWGA	0	0	0
UEA-I	0	0	0
WGA	2	1	0

TB, trophoblast; CT, cytotrophoblast; ST, syncytiotrophoblast; DC, decidual cells; 0, negative; 1, weak; 2, moderate staining.

account our results and previous findings, we conclude that there are differences in DC desmin expression among species.

Vimentin immunodetection allowed us to identify DC in cat placentae, opposite to the negative results obtained in the dog placentae (Fernández et al. 2000). Cats do not develop the puerperal disorder known as subinvolution of placental sites (SIPS), a disease characterized by excessive invasion of TB cells into uterine tissues. It is thought that DC may prevent trophoblastic invasion (Knöfler et al. 2008); therefore, the presence of DC in cats might prevent the development of SIPS.

The morphological characteristics of DC in cats have been described by different authors (Wooding and Burton 2008); nevertheless, only Wislocki and Dempsey (1946) described the existence of binucleation or multinucleation in these cells. In our work, we did not find trinucleated or multinucleated cells, but the percentage of binucleated cells was nearly 20%. The presence of abundant binucleated DC was described in the bat *C. perspicillata* (Rasweiler et al. 2000) but not in other species.

Glycosylation pattern has not been studied in cats, but it was studied in other carnivores such as hyenas, minks and dogs. While dogs and minks, as well as cats, develop endotheliochorial placentae, hyenas develop a haemochorial placenta. The foetal structure that takes part in feto–maternal interface is syncytial in cats, dogs and hyenas, and cellular in minks (Wooding and Burton 2008). Common lectin histochemical findings in TB facing maternal tissues include exposure of α-glucosyl, α-mannosyl, *N*-acetyl-lactosamine and sialyl residues, and absence of fucosyl termini, except for haemophagous zones in hyenas. Similar to cat profile, bisected complex N-linked sequences were demonstrated in hyenas; hyenas and minks also expose non-bisected ones. Cat placentae expressed fucosylated oligomers recognized by DBA and *N*-acetyl-galactosamine residues bound by SBA and BSA1B. This saccharides profile is similar to what was described in dogs (Fernández et al. 1998; Fernández et al. 2000; Sandoval et al. 2001) but not in hyenas and minks (Jones et al. 1997, 2007; Enders et al. 2006). Data about binding of other lectins presented here are not available for dogs, hyenas and minks.

The finding of GlcNAc and non-sialylated Gal and GalNAc residues in feline TB is consistent with that of most groups of animals, regardless of the type of placenta they develop. Heterogeneous staining patterns among lectins specific for GlcNAc residues were also seen. Binding of WGA, DSA, BS-II, in addition to sWGA negative results, suggests that those glycans could have been masked by sialylation of their non-reducing termini. Results from Con-A labelling can be attributed to the presence of either α-mannose or α-glucose oligomers. Although terminal fucosyl residues were not detected, DBA binding suggested the presence of α1-2 fucosylated Gal sequences similar to the blood group A family. Lack of simple fucosyl termini in cats was also in agreement with results reported for the remaining species except for camelids (Jones et al. 2000, 2002; Jones and Aplin 2009). Differences concerning α1-2 fucosylated oligomers are striking even among species with the same placental barrier or type of trophoblast facing maternal tissues (Jones et al. 2000, 2002, 2004a,b; Jones and Aplin 2009; Klisch et al. 2010; Aplin and Jones 2012). Enzymes, which catalyse fucose α1-2 linking, are more diverse among eutherians than those that catalyse α1-6 fucosylation, an evolutionarily ancient modification (Aplin and Jones 2012). However, recent research indicated that DBA lectin also binds a sialylated structure lacking fucose; therefore, DBA staining data must be carefully interpreted (Klisch et al. 2008; Aplin and Jones 2012).

Complex *N*-glycans bisected by a GlcNAc residue were detected only in the ST, whereas non-bisected sequences were not found in any TB population. Except for camelids, peccaries and pigs, as well as cats, the remaining species co-expressed bisected and non-bisected complex *N*-glycans (Jones et al. 2000, 2002, 2004a,b, 2007; Tatsuzuki et al. 2009; Klisch et al. 2010).

Based on the variety of these results, it seems difficult to find a common evolutive pattern, as there are common aspects between cats and phylogenetically distant species and, on the other hand, there are wide differences among members of closer groups.

Syncytiotrophoblast lectin affinity was somewhat different from that of CT. Those differences might reflect diversity in the biological properties of the cells. Restriction of binding of PHA-E to ST strongly suggests the presence of complex N-linked sequences bisected by a GlcNAc in this syncytium (Green and Baezinger 1987). As bisecting GlcNAc modification results in the suppression of further branching and elongation, it is surmised that cat placenta exhibits lesser branching of glycans in ST than in CT (Takahashi et al. 2009). Among other consequences, low branching inferred here does not allow galectin–glycoprotein lattices formation. Those galectin–glycoproteins lattices are known to modulate cell growth, differentiation, migration and survival by regulating receptor turnover (Rabinovich et al. 2007). For instance, it has been reported that the interaction of galectin 3 with high-branched glycans from heavily glycosylated TK receptors enhances agonistic signalling, leading to cell proliferation and

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migration (Rabinovich et al. 2007; Lalancette-Hebert et al. 2012). Both galectin 3 and IGFR, a high-branching glycosylated receptor (Masnikosa et al. 2006), were detected by our group in the endotheliochorial placentae of dogs and cats, especially in early proliferative and invasive CTs (Diessler et al. 2013, in press). However, studies regarding the functional relationship between those molecules and placental glycans have not been performed to the present.

It should be noted that most detailed reports on functional aspects of ST glycosides concern haemochorial placentae (Getsios and MacCalman 2003; Iijima et al. 2006). It is tempting to speculate that ST which do not directly contact maternal blood, but instead are involved in cell–cell interactions with the basal aspect of endothelium, might expose glycosides of different kinds, quantities or distribution.

Lectin affinity profile of DC was not comprehensively studied in other species. Some sialid acids and complex N-linked oligosaccharides were demonstrated in human, mice and rats DC (Bulmer and Peel 1996; Jones et al. 1996). Alroy et al. (1987) also reported WGA positivity in feline DC from control cats, as a part of α mannosidosis diagnosis. Results presented here are not in agreement with those.

This work describes the glycoconjugates moieties and intermediate filament composition in trophoblast and DC of the feline placenta. As carbohydrates provide adhesion forces and specificity for cellular recognition, it is not surprising that their modifications result in pregnancy loss (Jeschke et al. 2010). In this regard, it has been reported that mucin-associated endometrial glycans are reduced in women suffering recurrent miscarriage (Aplin et al. 1996). Concerning the TB, glycosylation pattern of glycoproteins such as syncytin is modified in pre-eclamptic women placentae (Chen et al. 2006). In pregnant women suffering altered glycaemia, major changes in placental affinity for SBA, PNA and DBA were reported (Sgambati et al. 2007). Also, qualitative changes in glycosylation pattern of specific placental glycoproteins between normal and missed abortion placentae were reported in humans (Serman et al. 2004). Changes in glycoconjugates of endometrium are also involved in the pathogeny of diseases causing early embryo loss in domestic animals, namely bovine tritrichomonosis (Cobo et al. 2004; Monteavaro et al. 2008; Woudwyk et al. 2013), but the relationship between placental glycosylation and pathologic pregnancies has not been investigated. In this regard, the knowledge of the normal pattern of the species is of great value for future research.

Acknowledgements

The authors gratefully thank Ht Rubén Mario for his technical support.

Conflict of interest

The authors have no conflict of interest.

Author contributions

CGB, EJG, PEF and ELP designed the experiment. PEF, CGB, AP and EJG performed the experiment. PEF, HHO, AP, CGB and EJG carried out the lectin histochemical and immunohistochemical techniques. MED, PEF, HHO, ELP, CGB and EJG observed the slides and determinated the results. ELP and CGB performed the cell counting. ELP, MED and CGB built the tables and figures. All the authors discussed the results. MED, PEF and CGB carried out bibliographic searching. CGB, MED, HHO, ELP and PEF wrote the first version. MED, CGB, PEF, ELP and EJG revised the first version and wrote the reply to referees.

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Submitted: 22 Jul 2013; Accepted: 10 Nov 2013

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