Distribution of sialoglycoconjugates in the oviductal isthmus of the horse during anoestrus, oestrus and pregnancy: a lectin histochemistry study

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The distribution of sialic acid residues as well as other glycosidic sugars has been investigated in the horse oviductal isthmus during anoestrus, oestrus and pregnancy by means of lectin and pre-lectin methods. Ciliated cells and non-ciliated (secretory) cells exhibited different lectin binding profiles that were found to change during the investigated stages. Ciliated cells did not show any reactivity in the basal cytoplasm, while the supra-nuclear cytoplasm displayed a few of oligosaccharides with terminal and internal αmannose (Man) and/or αglucose (Glc) during oestrus and pregnancy and a moderate presence of oligosaccharides terminating in αfucose (Fuc) during oestrus; cilia exhibited a more complex glycoconjugate pattern for the presence of oligosaccharides terminating in N-acetylgalactosamine (GalNAc), GalNAcα1,3 GalNAcα1,3galactose(Gal)β1,4Galβ1,4N-acetylglucosamine(GlcNAc), Fuc, sialic acid (Neu5Ac)-αGalNAc belonging or not to the GalNAcα1,3GalNAcα1,3 Galβ1,4 Galβ1, 4GlcNAc sequence, and. αGalNAc and Neu5Acα 2,6Gal/GalNAc increased during oestrus. Cilia displayed terminal Galβ1,3 GalNAc in pregnancy, terminal αGal in anoestrus and pregnancy and terminal or internal D-GlcNAc during anoestrus and pregnancy, respectively. The whole cytoplasm of non-ciliated cells showed oligosaccharides terminating with αGalNAc, Neu5Acα2,6Gal/GalNAc, Neu5Ac GalNAcα 1,3GalNAcα1,3Galβ1,4Galβ1,4GlcNAc during the investigated stages, as well as GlcNAc in anoestrus and pregnancy. The supra-nuclear zone of non-ciliated cells exhibited oligosaccharides with terminal Galβ1,4GlcNAc and internal Man during oestrus and pregnancy as well as terminal αGal and Fuc in oestrus and Neu5Ac-Galβ1,3GalNAc in pregnancy. The luminal surface of non-ciliated cells showed glycans terminating with αGalNAc and/or Neu5Ac GalNAcα1,3 GalNAcα1,3Galβ1,4Galβ1,4GlcNAc in all specimens, oligosaccharides with terminal Galβ1,4GlcNAc and internal Man during oestrus and pregnancy, Neu5Ac α2,6Gal/GalNAc in anoestrus and oestrus, and glycans terminating with Galβ1,3GalNAc, Neu5A acα2,3 Galβ1, 4GlcNac, Neu5ac-Galβ1,3GalNAc, Neu5Ac-Galβ1,4 GlcNAc in pregnancy. These findings show the presence of sialoglycoconjugates in the oviductal isthmus of the mare as well as the existence of great modifications in the glycoconjugates linked to different physiological conditions.

Key words: histochemistry, lectins, sialic acid, glycoconjugates, oviduct, horse.

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The mammal oviduct consists of three parts: the infundibulum, the ampulla, and the isthmus. Each one of these regions is involved in specific biological events. The isthmus is considered to be a spermatozoal reservoir for several species such as cattle (Suarez *et al.*, 1990; Hunter *et al.*, 1991), hamsters (Smith and Yanagimachi 1991; Smith *et al.*, 1991), mice (Suarez, 1987), pigs (Hunter 1981; Suarez *et al.*, 1991), rabbits (Oversteet *et al.*, 1978), sheep (Hunter and Nichol, 1983), and horses (Thomas *et al.*, 1994; Dobrinski *et al.*, 1996). The isthmic region of the oviduct represents a unique biochemical milieu able to i) prevent polyspermic fertilization, ii) maintain the fertility of sperm, and iii) regulate capacitation and motility hyperactivation in order to ensure the effective condition of sperm when ovulation occurs (Suarez, 2002). Thus, the role of the isthmus appear to be of utmost importance in horses where the fertilization may occur up to 6 days after mating (Day, 1942; Burkhardt, 1949)

As in other species (Suarez, 2002), equine sperm binding to oviductal epithelium is established by interactions between oligosaccharides of cell surface-associated glycoproteins (Lefebvre *et al.*, 1995; Dobrinski *et al.*, 1996).

The epithelium of the isthmus, like that of the entire oviduct, is of the simple columnar type and consists of two types of cells: ciliated and non-ciliated (secretory) cells. Both kinds of cell have been found to be involved in sperm trapping in cattle (Hunter *et al.*, 1991; Gualtieri and Talevi, 2000) and pigs (Flechon and Hunter, 1981; Suarez *et al.*, 1991). However, non-ciliated cells are mainly involed in the synthesis and release of secretory glycoproteins that are dissolved in the oviductal fluid. Once released in the lumen, glycoproteins create an intraluminal environment able to maintain the viability and fertilizing capability of spermatozoa (Pollard *et al.*, 1991; Suarez *et al.*, 1991; Chian *et al.*, 1995) and to play a supportive role in

sperm/egg interactions (Geng *et al.*, 1997; Tulsiani *et al.*, 1997).

It is well known that the oviduct epithelium is characterised by morphological and biochemical changes in response to oestrogen and progesterone fluctuactions during the oestrus cycle. In particular, the synthesis and secretion of oviduct-specific protein have been shown to be controlled by ovarian steroids (Erickson-Lawrence *et al.*, 1989; Buhi *et al.*, 1992; DeSouza and Murray, 1995).

Glycoconjugates have been used as a mean to detect hormonal effects in the oviduct during different oestrus cycle phases in pig (Raychoudhury *et al.*, 1993) and the rabbit (Menghi *et al.*, 1995). Among the carbohydrates that constitute the oligosaccharide chains in glycoproteins, sialic acids are known to be a large family of nine-carbon carboxylated sugars that usually occupy the terminal position of oligosaccharide chains in a variety of glycoconjugates (Schauer, 1982) and to act as ligands in recognition phenomena (Varki, 1997) as well as *in vitro* sperm capacitation (Banerjee and Chowdhry, 1994; Focarelli *et al.*, 1995) and sperm-egg interaction (Geng *et al.,* 1997). In spite of their putative importance, sialoglycoconjugates have not been studied in horse. Lectins have been successfully used to demonstrate the presence of glycoconjugates in a number of studies including the *in situ* distribution of glycoconjugates in the mammalian isthmic oviduct, namely in mice (Lee *et al.*, 1983), humans (Schulte *et al.*, 1985; Wu *et al.*, 1993; Kiss *et al.*, 1998), hares (Menghi *et al.*, 1988), rats (Menghi *et al.*, 1989), pigs (Raychoudhury *et al.*, 1993; Walter and Bavdek, 1997), rabbits (Menghi *et al.*, 1995), monkeys (Jones *et al.*, 2001) and horses (Ball *et al.*, 1997).

The aim of present study was to identify and localise the oligosaccharide sequences of glycoconjugates, mainly sialoglycoconjugates, in the oviductal isthmus of horse to detect cycle-stage specific changes by means of the most commonly used lectin in glycohistochemistry. We investigated the isthmic oviduct epithelium in three very different physiological conditions (anostrus, oestrus and pregnancy) to increase our data in the equine reproduction field that is currently not as well known as other mammalian species (Squires *et al.*, 2003).

Materials and Methods

Tissue preparation

Oviducts from anoestrus (n=2, collected in November), oestrus (n=2) (with a follicle >35 mm), and pregnant (n=2) (at 6-7 months of gestation) mares were obtained from a local slaughterhouse. Immediately after collection, on the basis of gross appearance, the isthmus was separated from ampulla and fixed in Bouin's fluid for 12 h at room temperature (RT). Following fixation, the tissues were washed and dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin wax. Sections 4 μm thick were cut and, after de-waxing with xylene and hydration in an ethanol series of descending concentrations, were stained with Mayer's hematoxylin and eosin (to study the general morphology) or by means of the following histochemical methods according to Desantis *et al.* (2002).

Lectin histochemistry

The lectins used are listed in Table 1. The lectins PNA, DBA, RCA_{120}, SBA, HPA, Con A, WGA, GSA-II, UEA I, LTA were HRP-conjugated and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). SNA, MAL I and GSA I-B_4 were biotinylated lectins and were purchased from Vector Laboratories Inc.(Burlingame, CA, USA).

De-waxed and re-hydrated tissue sections were immersed in 3% H_2O_2 for 10 min to suppress the endogenous peroxidase activity, rinsed in 0.05 M Tris-HCl buffered saline (TBS) pH 7.4 and incubated in lectin solution at appropriate dilutions (Table 1) for 1 h at room temperature (RT). After 3 rinsings in TBS, peroxidase activity was visualized by incubation in a solution containing 0.05% 3,3'-diaminobenzidine (DAB) and 0.003% H_2O_2 in 0.05 M TBS (pH 7.6) for 10 min at RT before dehydration and mounting. Tissue sections incubated in biotinylated lectins (SNA, MAL I and GSA I-B_4) were rinsed 3 times with 0.05 M phosphate-buffered saline (PBS) and were incubated in streptavidin/peroxidase complex (Vector Lab. Inc.) for 30 min at RT. After washing in PBS, peroxidase was developed in a DAB-H_2O_2 solution as above.

Controls for lectin staining included: (1) substitution of the substrate medium with buffer without lectin; (2) incubation with each lectin in the presence of its hapten sugar (0.2-0.5 M in Tris buffer).

Table 1. Lectins used, their sugar specificities and inhibitory sugars used in control experiments

Lectin abbreviation	*Source of lectin*	*Concentration (μg/mL)*	*Sugar specificity*	*Inhibitory sugar*	*Reference*
SNA	*Sambucus nigra*	15	Neu5Acα2,6Gal/GalNAc	NeuNAc	Shibuya *et al*., 1987
MAL I	*Maackia amurensis*	10	Neu5Acα2,6Galβ1/GalNAc	Neu5acα2,3Galβ1,4GlcNac	Sata *et al*., 1989
PNA	*Arachis hypogea*	20	Terminal Galβ1,3GalNAc	Galactose	Lotan *et al*., 1975
DBA	*Dolichos biflorus*	15	Terminal FP>GalNAcα1,3GalNAc	GalNAc	Hammarström *et al*., 1977
RCA_{120}	*Ricinus communis*	25	Terminal Galβ1,4GlcNAc	Galactose	Baenziger & Fiete, 1979
SBA	*Glycine max*	10	Terminal α/βGalNAc	GalNAc	Hammarström *et al*.,1977
HPA	*Helix pomatia*	15	Terminal αGalNAc	GalNAc	Roth, 1984
Con A	*Canavalia ensiformis*	25	Terminal and internal αMan>αGlc	Mannose	Goldstein & Hayes, 1978
GSA I-B_4	*Bandeiraea simplicifolia*	20	Terminal αGal	Galactose	Hayes & Goldstein, 1974
WGA	*Triticum vulgaris*	25	Terminal and internal βGlcNAc>>NeuNAc	GlcNAc	Debray *et al*., 1981
GSA II	*Bandeiraea simplicifolia*	20	Terminal D-GlcNAc	GlcNAc	Shanker Iyer *et al*., 1976
UEA I	*Ulex europaeus*	25	Terminal αL-Fuc	Fucose	Sugii & Kabat, 1982
LTA	*Lotus tetragonolobus*	25	Terminal αL-Fuc	Fucose	Pereira & Kabat, 1974

Fuc, Fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; FP, Forssman pentasaccharide GalNAcα1,3GalNAcα1,3Galβ1,4Galβ1,4GlcNAc; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid.

Table 2. Summary of lectin binding to epithelium lining the isthmus oviduct of mares in different physiological stages.

Lectin	*Anostrus*		*Oestrus*		*Pregnant*	
	Ciliated cells	*Non-ciliated cells*	*Ciliated cells*	*Non-ciliated cells*	*Ciliated cells*	*Non-ciliated cells*
MAL I	–		–	–	+++ci	+++as
KOH-s-MAL I	–	–	–	–	–	–
SNA	+ci	±as/±c	++ci	++as/++c	+ci	±as/++c
KOH-s-SNA	–	–	–	–	–	–
PNA	–	–	–	–	++ci	–
KOH-s-PNA	–	–	–	–	+++ci	+++as/++sn*
DBA	+++ci	+++as/++c	+++ci	+++as/+++c	+++ci	+++as/+++c
KOH-s-DBA	++++ci	++++as/+++c	++++ci	++++as/++++c	++++ci	++++as/++++c
RCA120	–.	–	–	+as/±sn	–	+as/+sn
KOH-s-RCA_{120}	–	–	–	+as/±sn	–	++as/++sn
SBA	++ci	+++as/+c	++ci	+++as/+c	++ci	+++as/+++c
KOH-s-SBA	++++ci	++++as/++c	++++ci	++++as/++c	++++ci	++++as/++++c
HPA	++ci	+++as/++c	+++ci	+++as/++c	++ci	+++as/++c
Con A	±ci	±as	±ci/±sn	±as/+sn	±ci/±sn	±ci/±sn
KOH-s-WGA	–	–	–	–	++ci	++c*
GSA I-B_4	+ci	–	+sn	+sn	+ci	–
GSA II	++ci	+c	–	–	–	–
UEA I	±ci	–	±ci/++ap	++ap	±ci	–
LTA	±ci	-	±ci	-	±ci	–

*ap, apical region; as, apical (luminal) surface; c, whole cytoplasm; ci, cilia; s, sialidase; sn, supra-nuclear cytoplasm. *, rare positive reaction; -, negative reaction; ±, faintly visibile reaction; +,++,+++,++++, weak, moderate, strong, intense positive reactions..*

Enzymatic and chemical treatments

Before incubation in 3% H_2O_2 and staining with SNA, MAL I, PNA, DBA, RCA_{120}, WGA some sections were incubated, at 37°C for 16 h in 0.86 U/mg protein of sialidase (Type V, from Clostridium perfringens) (Sigma Chemicals Co., St. Louis, MO, USA) dissolved in 0.1 M sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$. Prior to the neuraminidase treatment, a saponification technique was performed to render the enzyme digestion effective, with 0.5% KOH in 70% ethanol for 15 min at RT (Reid *et al.,* 1978). As controls of the enzyme digestion procedure, sections were incubated in the enzyme-free buffer solution under conditions of the same duration and temperature. In control sections, cleavage of sialic acid was not evident.

Results

The isthmic segment of the mare oviduct is characterized by both a well-developed muscle layers and less extensively branched mucosal folds (Figure 1) compared to ampulla. The epithelium lining the mucosa is columnar and consists of ciliated cells and

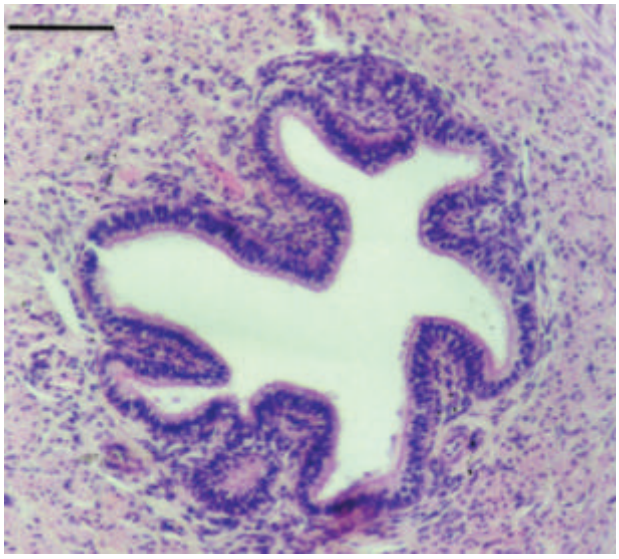

Figure 1. Cross-section of the horse oviductal isthmus. Mayer's hematoxylin-eosin staining. Bar: 100 μm.

non-ciliated (secretory) cells. The lectin-binding pattern of the epithelium in the isthmus oviduct of mares under different physiological conditions is summarized in Table 2.

MAL I was unreactive in anoestrus and oestrus (Figure 2a) while it strongly stained the cilia of ciliated cells and the apical surface of non-ciliated cells during pregnancy (Figure 2b). This reactivity was abolished after KOH treatment followed by sialidase incubation, performed to remove sialic acid residues.

SNA gave a moderate reaction for cilia during oestrus (Figure 2c) while it weakly stained cilia in anoestrus (Figure 2d) and pregnancy. In non-ciliated cells the lectin showed a faintly visible reaction of the apical surface during anoestrus and pregnancy versus moderate in oestrus, while the cytoplasm was stained moderately in oestrus and pregnancy and very weakly in anoestrus (Figures 2c, d). KOH-sialidase treatment abolished the SNA reactivity.

PNA showed a moderate reaction with the cilia during pregnancy (Figure 2e). In this physiological stage, KOH-sialidase treatment revealed cryptic binding sites in the cilia and on the luminal surface of non-ciliated cells as well as in supra-nuclear cytoplasn of some non-ciliated cells (Figure 2f).

DBA displayed a strong reactivity of the ciclia in ciliated cells and on the apical surface of the non-ciliated cells during the stages under study (Figure 2g). The cytoplasm of non-ciliated cells was stained moderately during anoestrus and strongly during oestrus and pregnancy. After KOH-sialidase, DBA showed an increased reactivity in all the above positive mentioned structures (Figure 2h).

RCA_{120} failed to stain the ciliated cells of the isthmus oviduct in each of the stages in question, whereas it weakly marked the apical surface of non-ciliated cells during oestrus and pregnancy, when the supra-nuclear cytoplasm was stained very faintly and weakly (Figure 2i). After KOH-sialidase, non-ciliated cells revealed crypitic RCA_{120} binding sites in the supra-nuclear cytoplasm as well as on the luminal surface only in pregnancy (Figure 2l).

SBA showed moderate and strong staining of cilia in ciliated cells and on the apical surface of non-ciliated cells, respectively, during the stages under study (Figure 2m). The cytoplasm of non-ciliated cells reacted weakly during anoestrus and oestrus (Figure 2m), while it was stained strongly during pregnancy. KOH-sialidase caused an increase in SBA staining in the above positive mentioned sites (Figure 2N).

HPA marked the cilia moderately during anoestrus and pregnancy (Figure 2o) and strongly in oestrus. The lectin stained the apical surface strongly and the cytoplasm of non-ciliated cells moderately in each of the stages in question (Figure 2p).

Con A gave a faintly visible reaction on the luminal surface throughout the epithelium (Figures 2 q, r). In addition, ciliated cells showed a very weak staining of their supra-nuclear cytoplasm during oestrus and pregnancy, while non-ciliated cells reacted weakly in the supra-nuclear cytoplasm (Figure 2r).

KOH-s-WGA (performed to highlight GlcNAc but not NeuNAc) was un-reactive in anoestrus and oestrus epithelium (Figure 3a), while it showed weak and moderate reactivity for the the cilia and for the whole cytoplasm of some non-ciliated cells, respectively, during pregnancy (Figure 3b).

GSA $I\text{-}B_4$ showed a weak reaction for the cilia during anoestrus and pregnancy (Figure 3c), and for the supra-nuclear cytoplasm of both ciliated cells and non-ciliated cells in oestrus (Figure 3d). GSA II stained the cilia moderately and the cytoplasm weakly in the ciliated cells of anoestrus mares (Figure 3e).

UEA I showed a faintly visible reactivity of the cilia in all specimens and a moderate staining of the apical cytoplasm in both ciliated and non-ciliated cells during oestrus (Figure 3f).

LTA displayed a faintly visible reaction of the cilia during the physiological conditions under study.

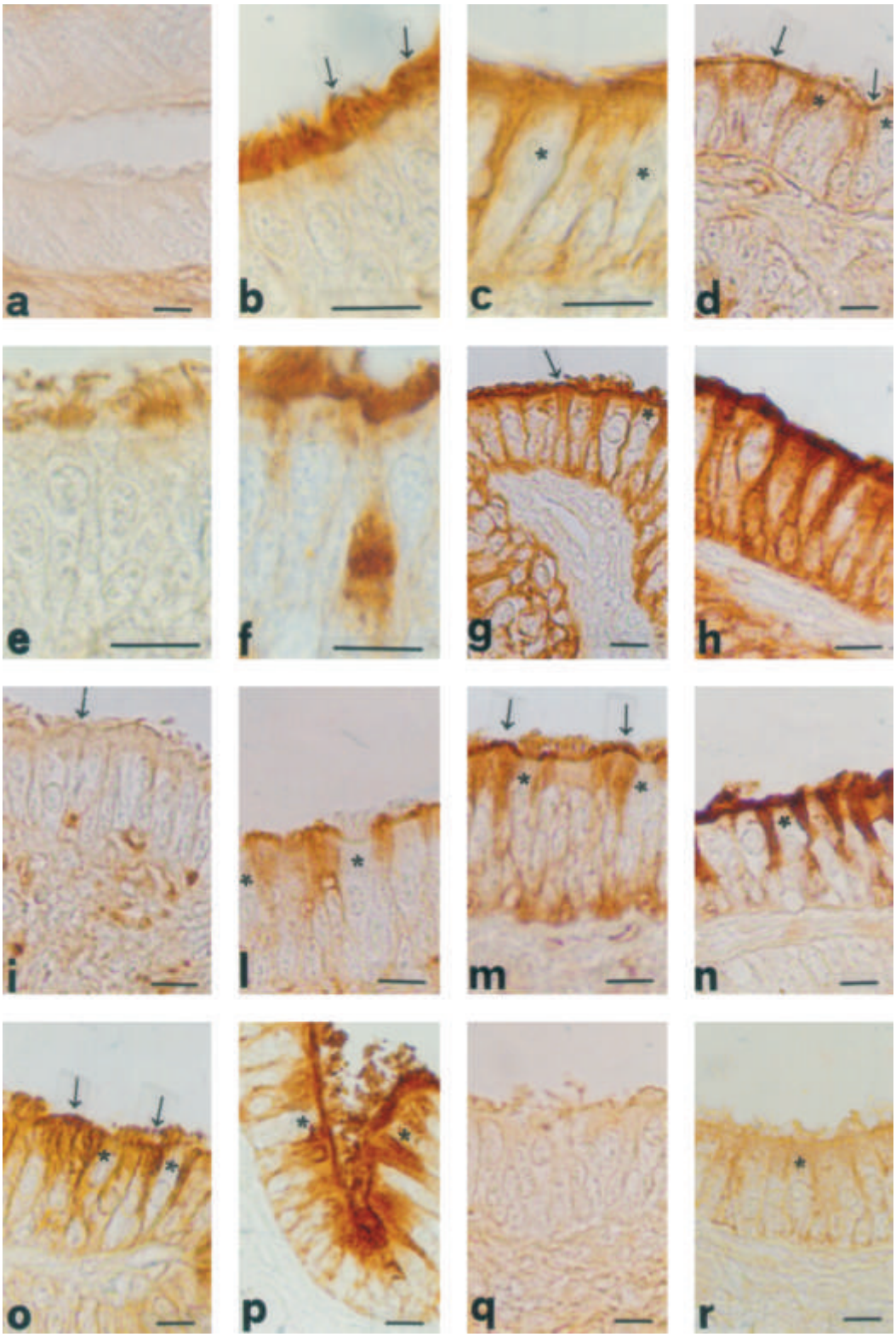

Figure 2. a. MAL I-negative staining in isthmus of oestrus mare. b. MAL I-binding sites on the epithelial surface of the isthmus during pregnancy. c. SNA-binding sites during oestrus. d. SNA staining of anoestrus isthmus showing less binding sites than estrus. e. PNA staining in pregnant mare. f. KOH-sialidase pre-treatment significantly increases the PNA binding throughout the epithelium luminal surface as well as revealed rare non-ciliated cells in pregnant mares. g. DBA-binding sites in isthmus of oestrus mare. h. After KOH-sialidase pre-treatment DBA exhibits increased reactivity in the epithelium luminal surface and in non-ciliated cells. i. RCA_{120} staining in oestrus. l. RCA_{120} staining after KOH-sialidase pre-treatment in isthmus during pregnancy. m. SBA staining during oestrus. n. KOH-sialidase pre-treatment significantly increases the SBA binding in epithelium luminal surface and non-ciliated secretory cells. o. HPA affinity for isthmus epithelium during anoestrus. p. HPA staining of isthmus in oestrus. q. Con A reactivity during anoestrus. r. Con A shows weak staining in the supra-nuclear cytoplasm of ciliated and non-ciliated cells during oestrus and pregnancy. arrows, luminal surface of non-ciliated cell. asterisk, ciliated cell Bar: 10 μm.

Discussion

In mammals, the isthmus of the oviduct is considered to be a sperm reservoir involved in maintaining sperm viable when ovulation occurs. It consists of a mucosal epithelium lining of two cell types: ciliated cells and non-ciliated (secretory) cells. In the present study, the differences in oligosaccharides and sialyloligosaccharides have been identified in the ciliated cells and the non-ciliated cells of the isthmus of the horse oviduct using lectins during various phases of the sexual-cycle.

Ciliated cells did not display any reactivity in their basal cytoplasm but they were stained in the supranuclear cytoplasm and on glycocalix cilia. The supranuclear cytoplasm reacted with GSA I-B_4 (specific for terminal α/Gal), UEA I (specific for terminal αL-Fuc) and Con A (specific for terminal and internal αMan/αGlc) during oestrus. The latter lectin stained the supra-nuclear cytoplasm also in pregnancy. Con A affinity indicates that these carbohydrate residues are contained in N-linked oligosaccharides because the lectin binds to a range of N-linked glycans from the high-Man, through the intermediate/hybrid to the small bi-antennary complex type, irrespective of bisection (Goldstein and Hayes, 1978; Debray *et al.*, 1981). The cilia glycocalix did not reveal binding sites to RCA_{120}, while it was stained by SNA (specific for Neu5Acα2,6Gal/GalNAc), DBA (specific for GalNAcα1,3GalNAcα1,3Galβ1,4Galβ1,4GlcNAc known as Forssman pentasaccharide), SBA (terminal α/βGalNAc), HPA (terminal αGalNAc), Con A, UEA I and LTA (the latter two lectins are specific for terminal αL-Fuc). After saponification with KOH and sialidase digestion, SNA staining was abolished whereas DBA and SBA reactivity increased. This indicates the presence of Neu5Acα2,6Gal/GalNAc, as well as of sialic acid residues linked to GalNAc residue of Forssman pentasaccharides. SNA and HPA binding sites were more widely expressed in oestrus than in anoestrus and pregnancy. The HPA reactivity suggests the presence of O-linked oligosaccharides (Spicer and Schulte, 1992). The weak presence of Con A binding sites on cilia indicates that the cilia glycocalix mainly consists of asialyl- and sialyl-O-linked glycoconjugates, which are more widely expressed in oestrus than anoestrus and pregnancy. Cilia showed the presence of binding sites for GSA II (specific for terminal D-GlcNAc), and for GSA I-B_4 (specific for terminal αGal) in anoestrus. Terminal αGal were also found on cilia during pregnancy when they showed a moderate presence of glycans with terminal Galβ1,3GalNAc residues (PNA staining), internal βGlcNAc (KOH-sialidase WGA procedure) as well as a strong presence of sialyloligoasaccharides ending with Neu5acα2,3Galβ1,4GlcNac (revealed by MAL I) and with sialic acid linked to

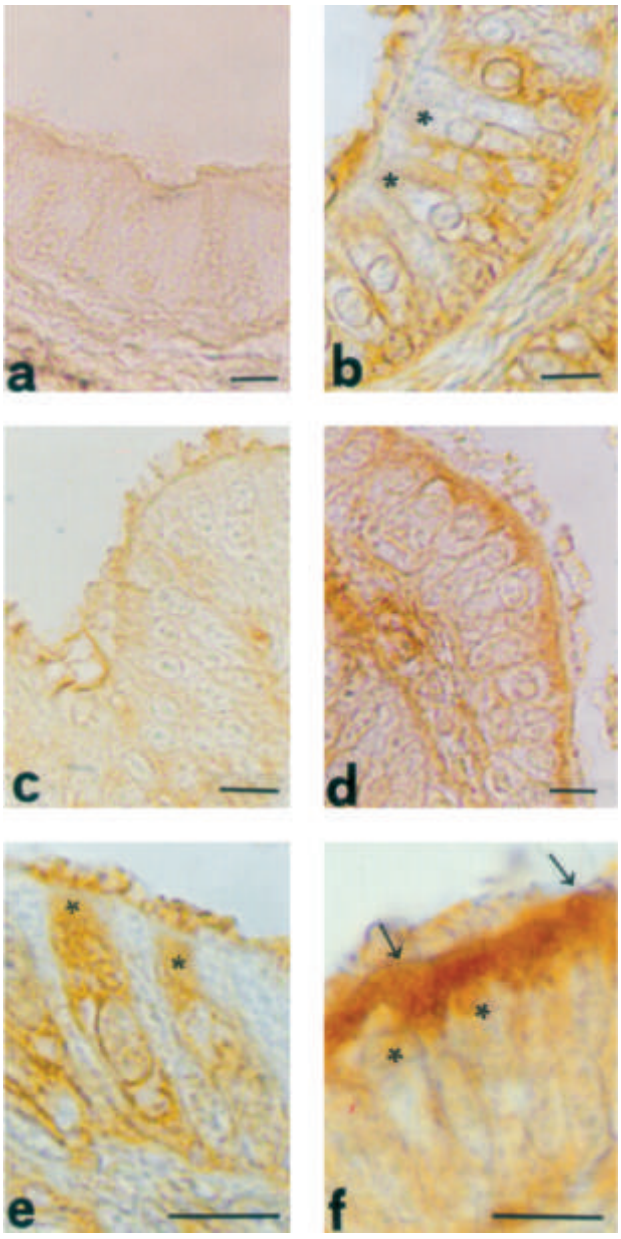

Figure 3. a. KOH-sialidase WGA staining in isthmus of oestrus mare. b. KOH-sialidase WGA reactivity for the cilia and the cytoplasm of rare non-ciliated cells in isthmus from pregnant mare. c. GSA I-B_4 labelling to isthmus during anoestrus. d. GSA I-B_4 staining of supra-nuclear cytoplasm in isthmus from oestrus mare. e. GSA II positivity of ciliated cells in isthmus during anoestrus. f. UEA-I labelling of isthmus during oestrus showing very weak reactivity of the cilia and moderate staining of apical cytoplasm in both ciliated and non-ciliated cells. arrow, non-ciliated cells. asterisk, ciliated cell. Bar: 10 μm.

Galβ1,3GalNAc (evidenced by an increase in PNA staining after KOH-sialidase treatments). Since the supra-nuclear cytoplasm of ciliated cells only exhibited reactivity to Con A, GSA I-B_4, and UEA I, it is possible to hypothesize that the many oligosaccharides found on cilia could be secreted by isthmic non-ciliated cells. Of course, it can not be excluded that they could come from the luminal fluid produced in other regions of the oviduct. The difference in the glycoconjugate pattern observed during the stages under investigation could depend on the hormonal state. Variations in the lectin binding pattern have been observed at the light microscopy level in cilia of the isthmus of cycling and hormone-treated pigs (Raychoudhury *et al.*, 1993) and rabbits (Menghi *et al.*, 1995). Sialilglycoconjugates have been found in cilia of the isthmic oviduct of hares (Menghi *et al.*, 1988), rats (Menghi *et al.*, 1989), rabbits (Menghi *et al.*, 1995), and monkeys (Jones *et al.*, 2001), as well as in humans (Schulte *et al.*, 1985). Although the role of the sialic acid residues present on cilia is not well known, they could play a role in keeping the cilia separated from one other, maintaining ciliary motility (Schulte and Spicer, 1985; Ito *et al.*, 1990), and providing the progression of capacitated spermatozoa (Hunter *et al.*, 1991).

The cytoplasm of non-ciliated cells never revealed binding sites to MAL I and LTA, whereas it reacted with SNA, DBA, SBA and HPA, during the three analysed stages. After saponification with KOH and sialidase digestion, SNA staining was abolished, SBA affinity increased in all the stages investigated, whereas DBA reactivity increased in oestrus and pregnancy. These findings reveal the constant presence of oligosaccharides terminating with Neu5Acα2,6Gal/GalNAc and, during oestrus and pregnancy, also of sialic acid residues linked to Forssman oligosaccharide. Non-ciliated cells cytoplasm also showed terminal D-GlcNAc (GSA II staining) during anoestrus, and internal βGlcNAc (KOH-sialidase WGA procedure) in rare cells during pregnancy. The present results suggest that the cytoplasm of non-ciliated cells, from the basal to apical region, contains O-linked oligosaccharides terminating with αGalNAc and/or with sialic acid linked to βGalNAc. Furthermore, in anoestrus and in pregnancy non-ciliated cells also express terminal βGlcNAc. The supra-nuclear cytoplasm of non-ciliated cells also revealed binding sites to Con A and RCA_{120} during oestrus and pregnancy, thus indicating the presence of N-linked oligosaccharides terminating or not with Galβ1,4GlcNAc in the basal cytoplasm. However, oestrus and pregnancy differed in their supra-nuclear glycoconjugates, because terminal αGal (revealed with GSA I-B_4) and sialic acid linked to Galβ1,3GalNAc (displayed with KOH-sialidase PNA procedure) were found during oestrus and pregnancy, respectively. The apical cytoplasm of non-ciliated cells was labelled with UEA I in oestrus, thus showing the presence of fucosyloligosaccharides. Changes in the non-ciliated cells lectin binding pattern related to hormonal state have been reported in mice (Lee *et al.*, 1983), pigs (Raychoudhury *et al.*, 1993), rabbits (Menghi *et al.* 1995), sheep (DeSouza and Murray, 1995) and horses (Ball *et al.*, 1997). Regarding horses, our observations are consistent with the findings of Ball *et al.* (1997) on the presence of oligosaccharides terminating in galactosides in the isthmic oviduct of mares. Sialylgalactosides have been found in the non-ciliated cells of hares (Menghi *et al.*, 1989), humans (Schulte *et al.*, 1985), monkeys (Jones *et al.*, 2001), rabbits (Menghi *et al.*, 1995), and sheep (DeSouza and Murray, 1995). Changes in the sialoglycoconjugates have been observed in rabbit (Menghi *et al.*, 1995) after hormone treatment, and an estrogen-dependent sialomucin has been found in the sheep oviduct (DeSouza and Murray, 1995). As revealed by electron microscope studies (Abe, 1996), the morphological basis of supra-nuclear lectin affinity could be the rough endoplasmic reticulum (RER), Golgi apparatus and mainly the secretory granules. The physiological role of the glycoconjugate contained in secretory granules is not well known. They could constitute the glycocolyx of the luminal plasmamembrane (Schulte *et al.*, 1985). Con A and UEA I affinity in the supra-nuclear region has been considered to be histochemical evidence of the presence of lysosome-like bodies (Schulte *et al.*, 1985). Lysosome-like vesicles have also been observed in the isthmic secretory cells of the oviduct of hamster (Abe and Oikawa, 1990a) and in rat (Abe, 1994). The large presence of O-glycans (mucyn-type glycans) observed in the supra-nuclear cytoplasm of the mare oviductal isthmus is consistent with the findings of Strous and Dekker (1992) and DeSouza and Murray (1995). These researchers found that O-glycans represents a superfamily of highly heterogeneous secretory glycoproteins constituting the majority of the glycans present in the oviductal glycoproteins. The O-linked glycans secreted by non-ciliated cells are likely to constitute the

cell coat of the luminal plasmamembrane of the epithelium (Schulte *et al.*, 1985). Glycoproteins secreted by non-ciliated cells create an intraluminal environment able to immobilise spermatozoa (Hunter, 1995), to maintain the viability and fertilizing capability of spermatozoa (Pollard *et al.*, 1991; Suarez *et al.*, 1991; Chian *et al.*, 1995) and to play a supportive role in sperm/egg interactions (Geng *et al.*, 1997; Tulsiani *et al.*, 1997). Differences in the production of oestrus-associated glycoproteins by oestrogen have been observed in the isthmus of baboon (Verhage and Fazleabas, 1988) and pig (Buhi *et al.*, 1992).

The luminal surface of the non-ciliated cells did not display binding sites to KOH-sialidase WGA, GSA I-B_4, GSA II, UEA I and LTA while it expressed a high staining intensity with DBA, SBA, and HPA during the stages under study. KOH-sialidase treatment visualized cryptic binding sites to DBA and SBA. This suggests that O-linked glycans are mainly present as oligosaccharides terminating with either αGalNAc or sialic acid linked to αGalNAc belonging or not to Forssman pentasaccharides. Furthermore, a higher presence of Neu5Acα2,6Gal/GalNAc (revealed with SNA) occurs during oestrus than oanestrus and pregnancy. During oestrus and pregnancy scarce binding sites to Con A and RCA_{120} were identified. Only in pregnant mares did the luminal surface show binding sites to MAL I, and, after KOH-sialidase treatment, cryptic binding sites to PNA and RCA_{120} were seen, thus exhibiting the presence of oligosaccharides terminanting with Neu5acα2,3 Galβ1,4GlcNac and sialic acid linked to Galβ1,3GalNAc. These present findings indicate that the luminal glycocalyx of the isthmus non-ciliated cells mainly consists of sialylgalactosyl as well as galactosyl terminating oligosaccharides which show sexual cycle-dependent changes. In horse, galactosyl residues are known to occur on the luminal surface of the oviductal epithelium (Ball *et al.*, 1997). The simultaneous presence of Con A and RCA_{120} binding sites observed on the apical surface of non-ciliated cells during oestrus and pregnancy, may depend on the presence of an asialofetuin-like molecule. Asialofetuin possesses three N-glycosylation sites with complex bi-, tri-, and tetra-antennary glycans and terminates in Galβ1,4 GlcNAc residues (Wagner *et al.*, 2002). In horse, asialofetuin blocks the binding of sperm to the oviductal epithelium by means of its terminal galactose residues (Lefebvre, 1995; Dobrinski *et al.* 1996). The sialylglycoconjugates contained on the luminal surface of non-ciliated cells seem to belong mainly to O-linked oligosaccharides (mucyn-type glycans) that are typical secretory moieties and contain more complex and heterogeneous carbohydrates than N-linked types (Fukuda, 1994). The differences in the isthmus distribution of O-linked oligosaccharides during the investigated stages may be related to the existence of a cycle-specific regulation of the spermatozoa attachment to the oviductal epithelium of horse (Thomas *et al.*, 1994). It has been proposed that, in other mammals, O-linked glycans are involved in a variety of biological phenomena including *in vitro* sperm capacitation (Banerjee and Chowdhry, 1994; Focarelli *et al.*, 1995), a selective barrier to sperm transport, modulation of sperm ascent to the site of fertilization (DeSouza and Murray, 1995) and sperm-egg interaction (Geng *et al.*, 1997; Tulsiani *et al.*, 1997). At present, the role played by sialoglycoconjugates of the epithelium lining the isthmic oviduct of horse is not known. In general, sialic acid can not only inhibit intermolecular and intercellular interactions by virtue of its negative charge but can also act as the critical ligand recognized by a variety of sialic-acid binding lectins. Thus, sialoglycoconjugates in the isthmus of the oviduct may represent a crucial component of a ligand which is recognized by endogenous lectins present on the spermatozoa and can undergo modification related to hormonal fluctuation regulating the different physiological conditions occurring in the isthmic epithelium of the oviduct during the sexual-cycle. In conclusion, the present histochemical analysis indicates differences in the lectin-binding pattern between ciliated cells and non-ciliated cells of the isthmic oviductal epithelium in horse. The presence of galactosides and sialylgalactosides on both the supra-nuclear cytoplasm of non-ciliated cells and the luminal surface epithelium could be consistent with the role played by this oviductal region in the formation of a reservoir of sperm. The variations in glycoconjugates observed during anoestrus, oestrus and pregnancy could be linked to fluctuations in the hormonal state and may reflect functional changes in the mucosa.

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