Absorptive activities of the efferent ducts evaluated by the immunolocalization of aquaporin water channels and lectin histochemistry in adult cats

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Summary. Ultrastructural and biochemical features of efferent ducts (EDs) are indicative of an intense absorptive activity towards the luminal fluid. This function was investigated by 1) the immunohisto-chemical localization of different aquaporins, integral membrane water channels that facilitate rapid passive movement of water, and 2) the histochemical localization of lectins, known to have specific affinity for glycoconjugate residues. AQP1 was mostly revealed at the apical surface and adluminal cytoplasm of non-ciliated cells and to a minor extent in their lateral plasma membrane, whereas it was absent in ciliated cells. Blood vessels showed AQP1-immunoreactivity, which was present in endothelial cells of venous vessels and capillaries and around the muscular sheath of arteries. AQP9 was expressed in the apical zone of ciliated and non-ciliated cells and in the lateral cell membrane. AQP2 and AQP5 were undetectable. Lectin histochemistry showed that non-ciliated cells contain glycans with terminal Neu5Acα2,3Galβ1,3GalNAc, Neu5Acα2,3Galβ1,4GlcNAc, Galβ1,4GlcNAc, GalNAc (s-PNA, MAL II, RCA$_{120}$, SBA reactivity) and with internal/terminal αMan (Con A affinity) at the luminal surface and the apical region. In addition, non-ciliated cells expressed oligosaccharides terminating with GalNAc and Neu5Acα2,6Gal/GalNAc (SNA reactivity) in the luminal surface and the apical zone, respectively. Ciliated cells revealed glycoconjugates only on cilia, which showed terminal Neu5Acα2,3Galβ1,4GlcNAc (s-RCA$_{120}$ staining) and GalNAc, as well as internal/terminal αMan and GlcNAc (s-WGA, GSA II staining).

Data provide evidence for the involvement of different pathways in the bulk reabsorption of water and low molecular weight solutes by the non-ciliated cell of the cat EDs. AQP-mediated trans-cellular route can be hypothesized, together with fluid phase endocytosis mediated by the glycocalix and a well-developed endocytotic apparatus. Epithelial ciliated cells, whose main function is the movement of luminal content, might also participate in absorptive processes to a lesser extent.

Key words: Aquaporin immunohistochemistry, Lectin histochemistry, Efferent ducts, Domestic cat

Introduction

The cat *ductuli efferentes* (efferent ducts, EDs) are a series of 15-18 tubules (Mobilio and Campus, 1912) through which spermatozoa pass from the *rete testis* to the initial segment of epididymis, propelled downstream by the ciliated epithelium. In mammals the EDs are lined by columnar ciliated and non-ciliated cells (Ilio and Hess, 1994), which build an epithelium mostly implicated in the trans-epithelial movement of luminal fluid and/or ions coming from the testis and rete testis (Hermo et al., 1991; Kujala et al., 2007). This reabsorption activity of the abundant fluid leaving the testis results in a 25-fold increase in sperm concentration (Levine and Marsh, 1971; Clulow et al., 1998). Ultrastructural features of the epithelium lining the EDs in the cat (Wrobel and Gürtler, 2004) as in other mammalian species (Aureli et al., 1984; Hermo and Morales, 1984; Goyal and Williams, 1988; Stoffel et al., 1991; Arrighi et al., 1994; Orsi et al., 1998), are strongly indicative of an absorptive activity taking place towards the intraluminal fluids. Clulow et al. (1998) stated that at

the level of the EDs there are at least three mechanisms involved in the bulk reabsorption of water and low molecular weight solutes from the lumen: i) active solute transport, ii) passive diffusion and iii) fluid phase endocytosis. It appears that reabsorption of luminal fluids leaving the testis is a complex process with multiple components, likely to be controlled by sex steroids (Nie et al., 2002). Synthetic and secretory activities have also been ascertained in EDs (Nixon et al., 2002). Nevertheless, these are possibly "minor" functions of the ED epithelial cells.

The mammalian aquaporins (AQPs) are a family of transmembrane channel proteins that are involved in the passive transport of water in numerous organs (Jung et al., 1994; Agre et al., 2002). AQPs 3, 7, 9, and 10 are also permeable to glycerol and some small solutes and are known as aquaglyceroporins. Their presence and functional significance is important in many of the processes underlying reproduction (Huang et al., 2006). Several aquaporins have been localized in the male genital apparatus up to date: in the testis, efferent ducts and epididymis of rats (Badran and Hermo, 2002; Hermo et al., 2004, 2008; Da Silva et al., 2006a,b), as well as in the genital tract of dogs (Domeniconi et al., 2008). These include also the aquaglyceroporins AQP7 (Domeniconi et al., 2008; Hermo et al., 2008), AQP9 (Pastor-Soler et al., 2001; Badran and Hermo, 2002; Domeniconi et al., 2007; Hermo et al., 2008), AQP3 and AQP10 (Hermo et al., 2004). Some AQP molecules are transmembrane glycosylated proteins (Smith et al., 1994; Hendriks et al., 2003; Buck et al., 2004), or they can exist in glycosylated as well as non-glycosylated form (Lu et al., 2008).

Lectins have a specific binding affinity for the glucidic residues of glycoconjugates due to their individual specific affinity to a particular sugar. Thus, they are useful probes for the intracellular localization of sugar residues of the carbohydrate moiety (Ihida et al., 1991; Danguy et al., 1994) and the characterization of distinct cellular populations, as well as their morpho-functional changes (Spicer and Schulte, 1992; Danguy et al., 1994). Lectin histochemistry has been successfully used to evaluate the composition of the oligosaccharides in the excurrent duct of mammals, such as man (Arenas et al., 1996), mouse (Burkett et al., 1987a,b), horse (Parillo et al., 1997, 1998), dog (Wakui et al., 1996; Schick et al., 2009), alpaca (Parillo et al., 2009a,b), and of non-mammal vertebrates (Labate et al., 1997). A detailed description of the distribution of cellular glycoconjugates is lacking in cat EDs.

The aim of the present study was to investigate the principal function of the EDs, the absorptive one, by means of the immunohistochemical localization of some AQPs and the lectin histochemistry in adult cats. Synthesis and secretion of glycoconjugates have also been demonstrated in EDs of several species (Burkett et al., 1987a; Wakui et al., 1996), and this function was explored as well. Bearing in mind the recent progress towards the application of assisted reproductive technology for the conservation of endangered felids, the domestic cat might be a suitable model for studies regarding the basic male genital tract histophysiology in this family (Farstad, 2000). Artificial insemination, gamete preservation and in vitro fertilization (IVF) are much studied techniques, and also in this respect the implementation of basic knowledge can be important for comparison in valuable pet animals and in threatened wild relatives.

Materials and methods

Tissue preparation

Efferent ducts were obtained from 5 healthy, privately owned domestic cats (aged from 8 months to 2 years) during routine orchiectomy. Fragments of caput epididymis were gently dissected free from testis, and immediately fixed by immersion either in formalin 10% for 24 h at 4°C or in Bouin's fluid for 12 h at room temperature. After fixation, fragments were dehydrated in a graded series of ethanol, clarified in xylene and embedded in paraffin. Tissue blocks were cut serially at 4 μm thickness, and sections were de-waxed and stained with routine Haematoxylin-Eosin technique for general morphological purposes. Other sections were used for the immunohistochemical and histochemical procedures.

AQP-immunohistochemistry

Tris-Buffered Saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) pH 7.6 buffer was used for rinses throughout the whole procedure.

After de-waxing, formalin-fixed sections were washed and immersed in a freshly prepared 3% H_2O_2 solution for 15 min to block the endogenous peroxidase activity, followed by incubation in 1:20 normal goat serum (DakoCytomation, Denmark) in TBS for 30 min to prevent background prior to incubation with primary antiserum. Sections were then incubated overnight in a humidity chamber at room temperature using rabbit polyclonal antibody against rat Aquaporin 1, Aquaporin 2, Aquaporin 5 and Aquaporin 9 (Alpha Diagnostic International, San Antonio, TX; Cat # AQP11-A, AQP21-A, AQP51-A, AQP91-A, respectively) diluted 1:100 (AQP1 and AQP2), 1:30 (AQP5) and 1:300 (AQP9) in specific antibody diluent (DakoCytomation, # S 302283). The sections designed to be incubated with AQP5 antibody were mounted onto poly-L-lysine-coated slides, then pre-treated with two microwave cycles (2x5 min at 450 W in 0.01 M citrate buffer, pH 6.0) in order to unmask the antigen. The sections were then washed and incubated for 30 min with goat anti-rabbit immunoglobulins (DakoCytomation, code # E 0432) diluted 1:200. Either Streptavidin-Biotin Complex (StrepABComplex/HRP, DakoCytomation, code # K 0355) or Envision System Labelled polymer-HRP (DakoCytomation, code # K 4002) were employed as revelation system. Immunoreactive sites were visualized

using a freshly prepared solution of 4 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 10 ml of a 0.5 M Tris buffer at pH 7.6 containing 0,1 ml of 3% H_2O_2 for 13-20 min. Sections were counterstained with Mayers' haematoxylin, dehydrated and mounted using Eukitt.

Controls

The specificity of the immunostainings was tested by negative controls, performed by (1) the use of non-immune rabbit serum (DakoCytomation, code # X 0903) in place of specific antisera; (2) incubating sections overnight at 4°C with antisera preabsorbed with the respective antigens (Alpha Diagnostic International; Cat # AQP11-P, AQP91-P) (10-100 μg/ml); and (3) omission of the first layer. All of them resulted in the absence of immunoreaction.

Lectin histochemistry

For lectin histochemistry Bouin-fixed sections were deparaffinised and treated according to previously published techniques (Desantis et al., 2006). The lectins used are listed in Table 1. The horseradish peroxidase (HRP)-conjugated lectins (PNA, RCA$_{120}$, DBA, SBA, HPA, Con A, WGA, GSA-II, UEA-I, and LTA) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA), whereas biotinylated lectins (MAL II, SNA, and GSA I-B$_4$) were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

De-waxed and re-hydrated tissue sections were immersed in 3% v/v solution of H_2O_2 in methanol 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. After 3 rinsings in TBS, the peroxidase activity of HRP-conjugated lectins

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 room temperature before dehydration and mounting. Tissue sections incubated in biotinylated lectins (MAL II, SNA and GSA I-B4) were rinsed 3 times with 0.05 M phosphate-buffered saline (PBS) and were incubated in streptavidin/peroxidase complex (Vector Lab. Inc., Burlingame, CA, USA) 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).

Enzymatic and chemical treatments

Before staining with MAL II, SNA, PNA, DBA, RCA$_{120}$, and 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 room temperature (Moschera and Pigman, 1975). As controls of the sialidase digestion procedure, sections were incubated in the specific enzyme-free buffer solutions under the same experimental conditions.

The evaluation of either immunohistochemical and lectin staining intensities was based on subjective estimates made in double-blind test by at least two of the authors.

Slides were observed and photographed under an Olympus BX51 photomicroscope equipped with a digital camera and DPsoft software (Olympus, Italy) for

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

Acronym	Source of lectin	Concentration (μg/ml)	Sugar specificity	Inhibitory sugar
MAL II*	*Maackia amurensis*	15	NeuNacα2,3Galβ1,4GlcNAc	NeuNAc
SNA*	*Sambucus nigra*	15	NeuNAcα2,6Gal/GalNAc	NeuNAc
PNA	*Arachis hypogaea*	20	Terminal Galβ1,3GalNAc	Galactose
DBA	*Dolichos biflorus*	20	Terminal GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1	GalNAc
RCA120	*Ricinus communis*	25	Terminal Galβ1,4GlcNAc	Galactose
HPA	*Helix pomatia*	20	Terminal αGalNAc	GalNAc
SBA	*Glycine max*	15	Terminal α/βGalNAc	GalNA
Con A	*Canavalia ensiformis*	20	Terminal/internal αMan>αGlc	Mannose
WGA	*Triticum vulgaris*	20	Terminal/internal βGlcNAc>>NeuNAc	GlcNAc
GSA II	*Griffonia simplicifolia*	20	Terminal D-GlcNAc	GlucNAC
GSA I-B4*	*Griffonia simplicifolia*	25	Terminal αGal	GalNAc
UEAI	*Ulex europaeus*	25	Terminal L-Fucα1,2Galβ1,4GlcNAc,	Fucose
LTA	*Lotus tetragonolobus*	25	Terminal αL-Fuc	Fucose

Fuc, Fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid; *, biotinylated lectins. Non marked lectins were HRP-conjugated.

computer-assisted image acquisition and management.

Results

In the region of epididymis macroscopically indicated as caput, efferent ducts were easily distinguishable from initial segment of epididymal duct for their diameter, epithelium height, and epithelial cell types (Fig. 1a). EDs were lined by simple columnar epithelium constituted by ciliated and non-ciliated cells, surrounding a narrow lumen containing very few spermatozoa. Apical plasma membrane of non-ciliated cells was characterized by a tuft of dense microvilli projecting into the lumen. The cell apex of the ciliated cells showed short cilia (Fig. 1b).

AQP-immunohistochemistry

The AQP1 antiserum intensely immunoreacted with the epithelium of the EDs (Fig. 2a,b), with evident differences in the two epithelial cell types. Strong immunostaining was observed at the apical surface of the non-ciliated cells, corresponding to the microvilli projecting into the lumen and to the underneath adluminal cytoplasm (Fig. 2b), whereas the ciliated cells were unstained. Weak immunoreactivity was present in the lateral plasma membranes of adjacent epithelial cells (Fig. 2b). AQP1-immunoreactivity was constantly evidenced at the level of blood vessel wall. Immunoreactivity was present surrounding the smooth muscle cells of the arteries, and at endothelial level in the veins (Fig. 2c). Capillary endothelium was frequently immunoreactive, and immunostaining of red blood cells was constantly present.

AQP9 was immunolocalized at the apical and lateral cell membranes of both ciliated and non-ciliated cells, as well as in the apical cytoplasm of non-ciliated cells (Fig. 2d).

AQP2- and AQP5-immunoreactivities were undetectable in the cat EDs.

Lectin histochemistry

The results of lectin histochemistry are summarized

Table 2. Lectin staining pattern of the epithelium lining the cat efferent ducts.

Lectin	Ciliated cells	Non-ciliated cells
MAL II	-	++ls/++G
KOH-si-MAL II	-	-
SNA	-	++a
KOH-si-SNA	-	-
PNA	-	-
KOH-si-PNA	-	+++ls/++sn
DBA	-	-
KOH-si-DBA	-	-
RCA$_{120}$		++ls/+sn
KOH-si- RCA$_{120}$	++ci	++ls/+sn
HPA	++ci	++ls
SBA	++ci	++a
Con A	+++ci	+++ls/++sn
KOH-si-WGA	++ci/±G/±sn	-
GSA I-B4	-	-
GSA II	+ci	-
UEA I	-	-
LTA	-	-

a, apical zone; ci, cilia; G, Golgi zone; ls, luminal cell surface; si, sialidase; sn, supra-nuclear cytoplasm; -, negative reaction; ±, faintly visible reaction; +, ++, +++, weak, moderate, strong positive reactions.
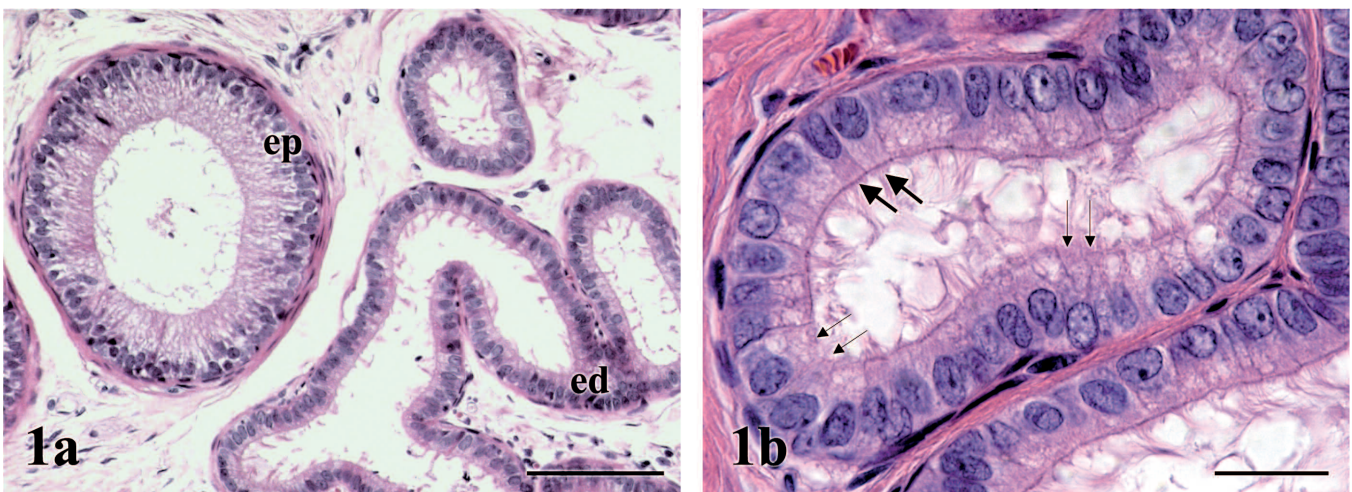
Fig. 1. Cat efferent ducts, Haematoxylin-Eosin staining. **a.** Efferent ducts (ed) are lined with a simple columnar epithelium constituted by ciliated cells and non-ciliated cells. They are easily distinguishable from epididymal duct (ep), which is lined by a taller, pseudostratified epithelium. **b.** Higher magnification shows non-ciliated (thin arrows) and ciliated cells (thick arrows), randomly alternated to constitute the epithelium lining the EDs. Scale bars: a, 50 μm; b, 20 μm.

in Table 2.

MAL II gave a moderate staining at the luminal surface and the Golgi zone of the non-ciliated cells, whereas it did not react with ciliated cells (Fig. 3a). Saponification, followed by neuraminic acid cleavage (KOH-sialidase), abolished non-ciliated cell staining.

SNA showed moderate staining of the apical cytoplasm of non-ciliated cells but no reactivity with ciliated cells (Fig. 3b). The reactivity was abolished after KOH-sialidase treatment.

PNA showed no binding sites along the ED epithelium. After KOH-sialidase pre-treatment this lectin displayed moderate reactivity in the entire supra-nuclear cytoplasm of non-ciliated cells and a stronger one at the luminal surface. No staining was evidenced in ciliated cells (Fig. 3c).

RCA_{120} displayed a weak staining of the supra-nuclear region and a moderate reactivity with the luminal surface of non-ciliated cells. Cilia of ciliated cells were not stained with RCA_{120}, although after KOH-sialidase treatment binding sites were revealed on the cilia (Fig. 3d).

HPA and SBA (Fig. 4a) moderately stained cilia of ciliated cells and apical surface of non-ciliated cells.

Con A strongly reacted with the entire luminal surface of the ED lining epithelium and with the supra-nuclear cytoplasm of non-ciliated cells (Fig. 4b).

KOH-sialidase-WGA (performed to highlight
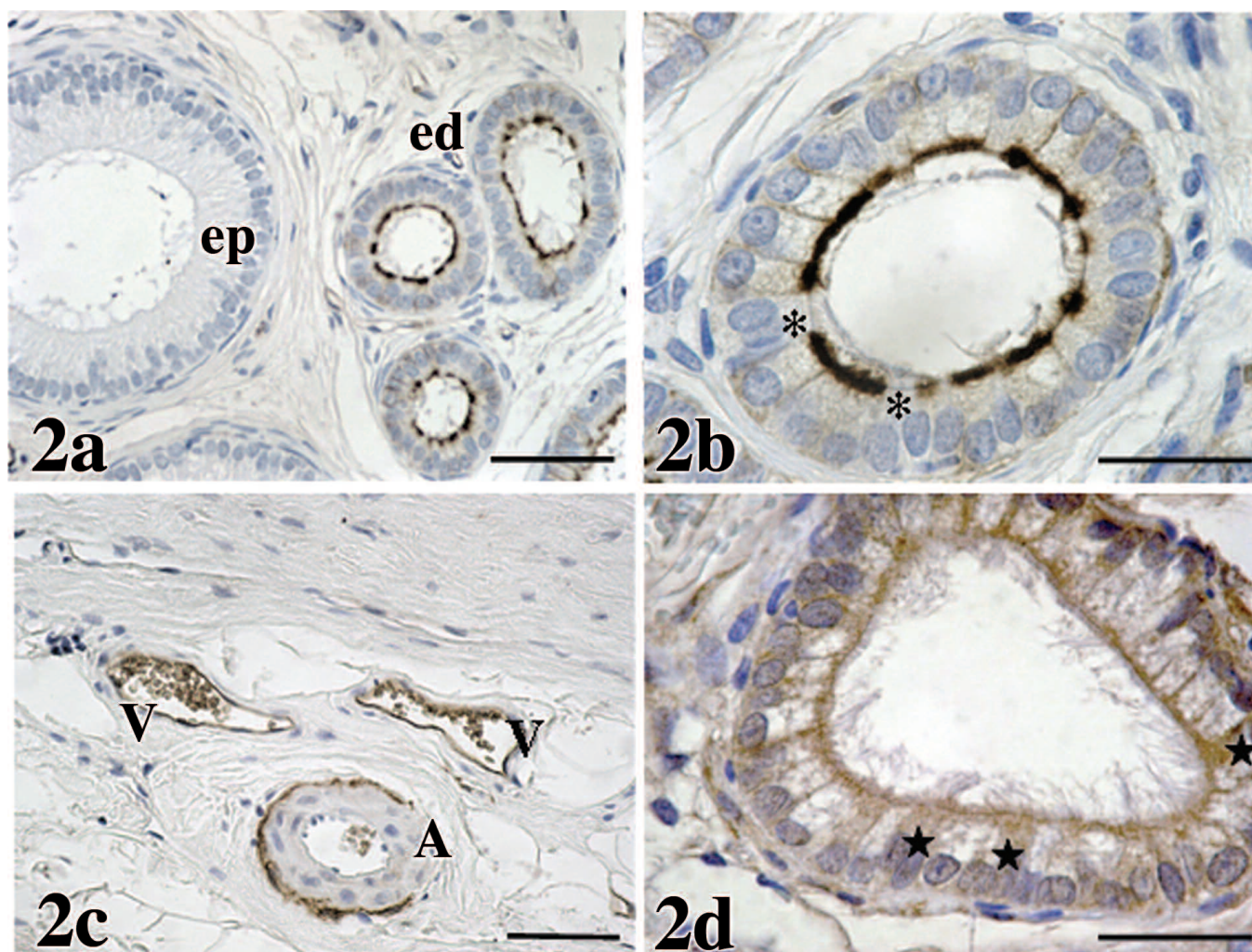
Fig. 2. Immunolocalization of Aquaporins in the cat efferent ducts. **a.** Evident AQP1-immunoreactivity is localized at the apical surface of the epithelium lining the ductuli efferentes (ed). By contrast, the epididymal duct epithelium (ep) is unreactive. **b.** At higher magnification, strong immunostaining is visible at the level of the apical surface and adluminal cytoplasm of the non-ciliated cells. The lateral plasma membranes of adjacent epithelial cells are weakly immunoreactive. The ciliated cells (asterisks) are unstained. **c.** AQP1-immunoreactivity is present around the artery muscular cells (A) and at the level of the veins endothelium (V). Red blood cells are also AQP1-immunoreactive. **d.** AQP9 immunolocalization is evident at the apical and lateral cell membranes of both ciliated and non-ciliated cells, as well as in the apical cytoplasm of non-ciliated cells (stars). Scale bars: a, 40 μm; b, d, 20 μm; c, 50 μm.

ßGlcNAc residue, but not sialic acid) showed unreactive non-ciliated cells and displayed a moderate staining of the cilia and a very faint reaction with the supra-nuclear region and Golgi zone of ciliated cells (Fig. 4c).

GSA II weakly stained the cilia of ciliated cells and displayed no reactivity for non-ciliated cells (Fig. 4d).

DBA (with or without KOH-sialidase pre-treatment), GSA I-B$_4$, UEA I and LTA did not show reactivity with the ED epithelium.

Discussion

The present study investigated by immunohisto-chemistry the expression of aquaporin proteins and by means of lectin histochemistry the glycoconjugate pattern in the epithelium lining efferent ducts, in order to evaluate the main functions of this tract of cat reproductive system.

Several authors focused on the morphological description of the cat epididymal duct, either from a light (Sánchez et al., 1998; Axnér et al., 1999) or electron microscopic point of view (Arrighi et al., 1986; Morales and Cavicchia, 1991), whereas cat EDs have been paid little attention. In a study on the feline urogenital junction, Wrobel and Gürtler (2004) observed that cat EDs are lined by a simple columnar epithelium constituted of ciliated and a unique type of non-ciliated cells. Our investigation confirms the findings of Wrobel and Gürtler (2004). On the basis of the presence of apical secretory cellular components or of endocytotic material, different types of non-ciliated cells were detected in the bull (Goyal and Hrudka, 1981), goat
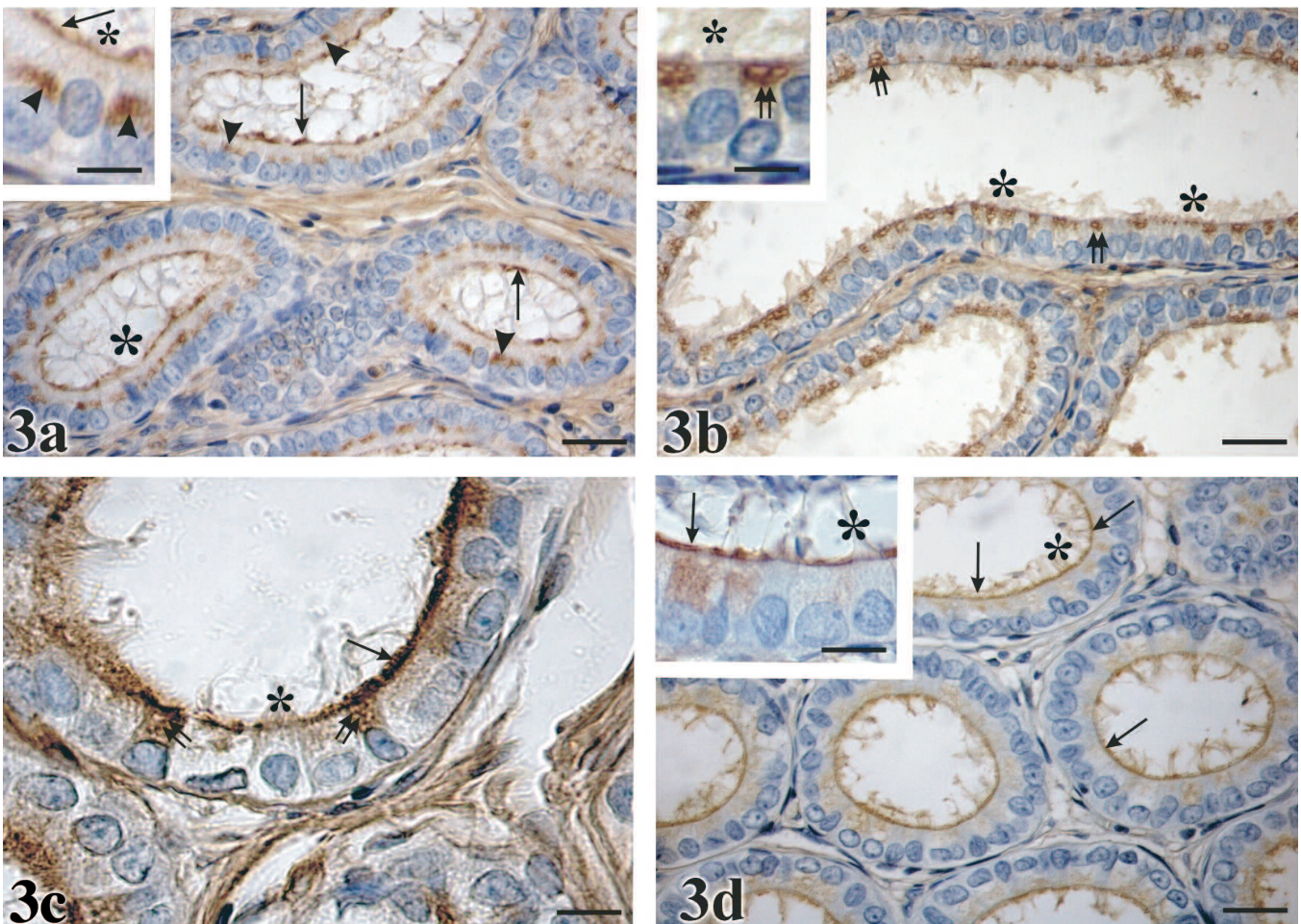
Fig. 3. Lectin histochemistry of cat efferent ducts. MAL II **(a),** SNA **(b)**, KOH-sialidase-PNA **(c)**, and KOH-sialidase-RCA$_{120}$ **(d)** stainings. **a.** MAL II reacts with the microvilli and Golgi zone of non-ciliated cells. A more detailed image is in the inset. **b.** SNA shows binding sites for the apical cytoplasm of non-ciliated cells. A more detailed image is in the inset. **c.** KOH-sialidase-PNA stains the supra-nuclear cytoplasm and the microvilli of non-ciliated cells. **d.** KOH-sialidase-RCA$_{120}$ binds the supra-nuclear region and the luminal surface of non-ciliated cells, as well as the cilia of ciliated cells. Cilia were not stained with RCA$_{120}$ without KOH-sialidase treatment (inset). Arrows, microvilli of non-ciliated cells; arrowheads, Golgi zone; double arrow, supra-nuclear cytoplasm of non-ciliated cells; asterisks, cilia. Scale bars: a-d, 22 μm; insets, 4 μm.

(Goyal et al., 1992), dog (Wakui et al., 1996), alpaca (Parillo et al., 2009a) and human (Ilio and Hess, 1994).

Aquaporin expression is an almost completely unknown item in the male excurrent duct of domestic animal species, studied before only in the dog (Domeniconi et al., 2007, 2008). In cat EDs, all non-ciliated cells showed a strong apical AQP1-immunoreactivity, corresponding to the microvilli. Similar AQP1 expression has been observed in the EDs of marmoset monkey (Fisher et al., 1998), rat (Oliveira et al., 2005), mouse (Lu et al., 2008) and dog (Domeniconi et al., 2008). The likely role of AQP1 at these cellular sites could be related to removal of water from the tubular space. Studies performed by electron microscopy on rat EDs (Badran and Hermo, 2002) showed that gold particles representing AQP1 antigenic

sites were localized not only over the microvilli of the non-ciliated cells, but also over the underlying endosomes, fluid-filled organelles involved in the uptake of substances from the lumen (Hermo and Morales, 1984; Hermo et al., 1988). This could account for the AQP1-immunoreactivity which we observed in the adluminal cytoplasm of ED non-ciliated cells in the cat.

Since AQP1-immunoreactivity was undetected in the epithelium lining the cat rete testis (unpublished results) and caput epididymis (see Fig. 2a), it is possible to infer that in EDs this water channel is of greatest importance in the concentration of testicular fluid, which requires rapid reabsorption (Clulow et al., 1998). In this respect EDs bear a resemblance to the embryologically related renal proximal tubules, which absorb up to 80% of the glomerular ultrafiltrate and where AQP1 is
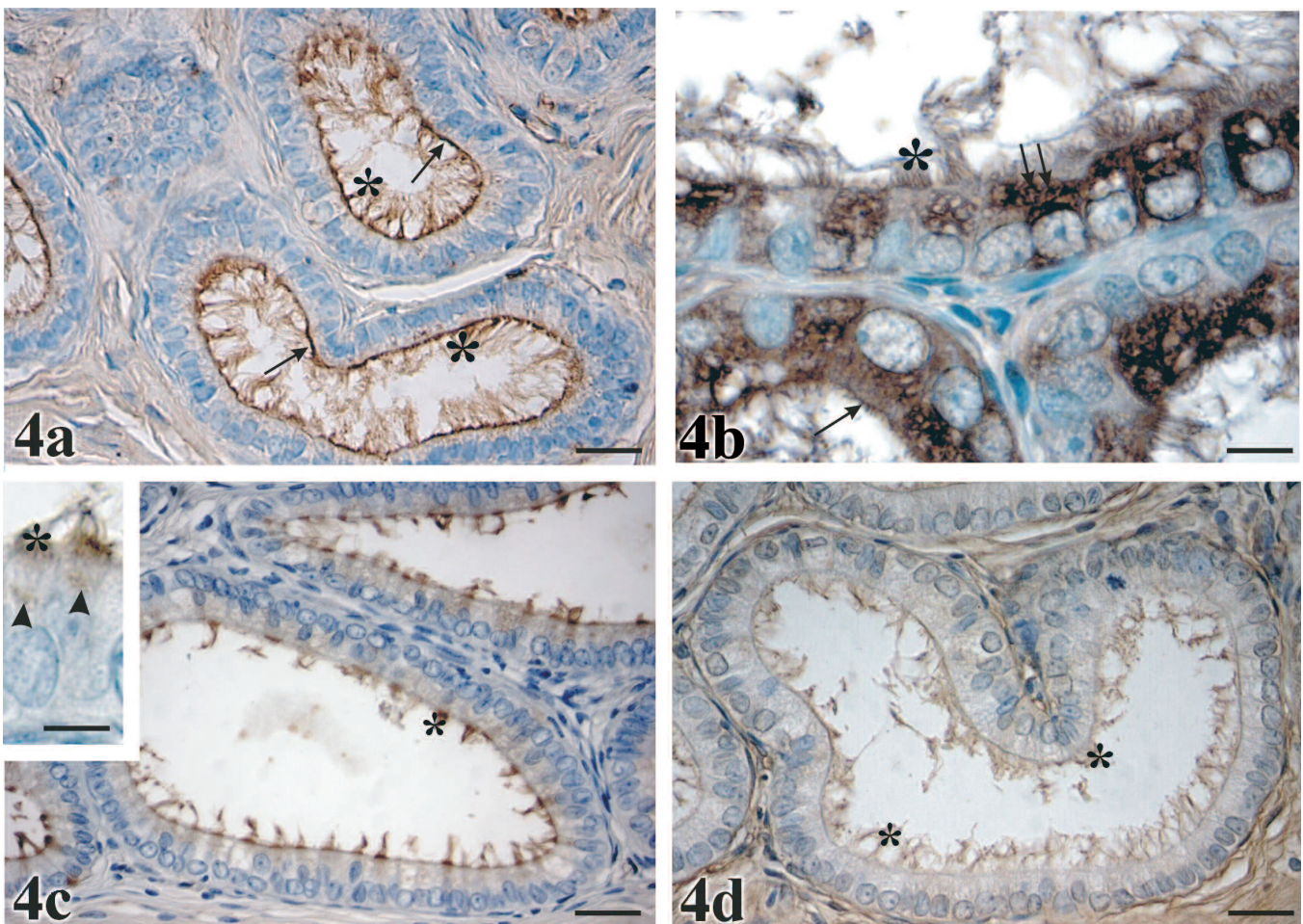
Fig. 4. Lectin histochemistry of cat efferent ducts. SBA **(a)**, Con A **(b)**, KOH-sialidase-WGA **(c)**, and GSA II **(d)** stainings. **a.** SBA stains the cilia of ciliated cells and microvilli of non-ciliated cells. **b.** Con A reacts with the entire luminal surface of the epithelium lining the efferent ducts and with the supra-nuclear cytoplasm of non-ciliated cells. **c.** KOH-sialidase-WGA shows staining of the cilia, the supra-nuclear region, and Golgi zone (inset) of ciliated cells, whereas it does not react with non-ciliated cells. **d.** GSA II weakly stains the cilia of ciliated cells but it does not react with non-ciliated cells. Arrows, microvilli of non-ciliated cells; arrowheads, Golgi zone; double arrow, apical cytoplasm of non-ciliated cells; asterisks, cilia. Scale bars: a, d, 22 μm; b, 10 μm; c, 27 μm; inset in c, 3 μm.

maximally expressed (Schnermann et al., 1998). The weak AQP1-immunoreactivity evidenced at the level of the lateral cell membranes in cat EDs might be indicative of fluid reabsorption taking place by an intercellular route, as well. Also this localization of AQP1 is shared by the epithelial cells lining kidney proximal tubules (Brown et al., 1993; Clulow et al., 1994).

Furthermore, AQP1-immunoreactivity decorated the blood vessels in this part of the cat excurrent ducts. This finding has an obvious implication in water removal from intertubular spaces once water has been transported across the epithelium, in this way maintaining water equilibrium in this tissue (Badran and Hermo, 2002). It is worth mentioning that in other species, such as the rat, AQP1 is found in myoid cells enveloping the tubules, in this way collaborating in the removal of water from the lumen and transport to the interstitium (Oliveira et al., 2005). The AQP1-immunoreactivity which was noticed all around the artery wall in cat EDs could also serve to ensure water equilibrium in this tissue, perhaps acting on the vessel contraction/relaxation mechanism, as was described for the arterial and arteriolar muscular sheath in the canine uterus (Aralla et al., 2009).

AQP1-immunoreactivity observed at the level of red blood cells was an expected result, as aquaporins were first described at this level (Agre et al., 2002).

AQP9-immunoreactivity was identified in the apical membrane of both cell types of cat EDs, along the basolateral cell membranes and, more weakly, in the supra-nuclear cytoplasm of non-ciliated cells. Immunolocalization of APQ9 was also reported in the rat (Pastor-Soler et al., 2001; Badran and Hermo, 2002; Da Silva et al., 2006a,b; Hermo et al., 2008) and dog (Domeniconi et al., 2007). It is interesting to note that AQP9 is an aquaglyceroporin, known to be a channel for neutral solutes, in addition to being a water channel. In this study on cat EDs AQP2 and AQP5 expressions were tested but they were undetectable. Thus, we can affirm that at least two members of the aquaporin family are expressed in cat EDs, AQP1 and AQP9. This apparent overabundance could be explained by the slight difference in AQP functions, especially when an aquaglyceroporin is implicated in addition to an aquaporin, stressing the overall importance of AQPs in a given tissue (Hermo et al., 2004). In the case of EDs, AQP9 allows the passage of glycerol, which has been proposed to serve as a metabolic substrate for sperm to produce CO_2 (Cooper and Brooks, 1981; Da Silva et al., 2006a). Glycerol is derived from glycerylphosphorylcholine, which is presumably synthesized within the epithelium, and both have to be transported to the lumen, where they have a role in relation to sperm maturation (Hermo et al., 2004). In addition to being modulated by sexual steroids (Pastor-Soler et al., 2002; Picciarelli-Lima et al., 2006), quite recently it was demonstrated that AQP9-dependent glycerol permeability of apical membrane of the non-ciliated cells in rat EDs depends on bradykinin, one of the most important peptides regulating water and ionic balance in the body (Belleannée et al., 2009). AQP9 immunoreactivity was also noticed in the apical membrane of ciliated cells. Although the morphology of ciliated cells is mainly indicative of their involvement in propelling the luminal content downstream, it cannot be excluded that this cell type may be involved in absorptive phenomena along with the non-ciliated cells (Arrighi et al., 1994).

The lectin histochemical analysis revealed a different glycoconjugate pattern between ciliated and non-ciliated cells. The non-ciliated cells showed lectin staining mainly along the supra-nuclear region, whereas the basal cytoplasm did not show lectin binding sites. The glycocalyx of microvilli expressed oligosaccharides containing terminal/internal α-D-Man/α-D-Glc (Con A affinity), terminal GalNAc (HPA and SBA reactivity) and sialoligosaccharides terminating with sialic acid linked either via α2,3-6 to penultimate ß-Gal or via α2,6 to subterminal α-GalNAc, as demonstrated with MAL II, SNA, KOH-s-PNA, and KOH-s-RCA$_{120}$. The Con A reactivity suggests the presence of N-linked glycans from high-Man, through intermediate/hybrid, to small bi-antennary complex type, irrespective of bisection (Goldstein and Hayes, 1978; Debray et al., 1981). The high presence of sialoligosaccharides provides the glycocalyx with negative charges, which are involved in the binding and transport of positively charged molecules (Schauer, 2000). This could be the first step in an endocytotic process of intraluminal matter.

The entire supra-nuclear cytoplasm contains oligosaccharides terminating with Galß1,4GlcNAc, sialic acid linked to Galß1,4GlcNAc, sialic acid linked to Galß1,3GalNAc (RCA$_{120}$, KOH-s-RCA$_{120}$, KOH-s-PNA staining). The apical cytoplasm expressed reactivity with SNA and Con A indicating the presence of terminal/internal α-D-Man/α-D-Glc and terminal Neu5Acα2,6Gal/GalNAc residues, respectively. It is interesting to point out that the glycoconjugates contained in the apical cytoplasm of non-ciliated cells belong mainly to N-linked oligosaccharides which, contrary to O-linked ones (mucyn-type glycans), are not typical secretory moieties (Fukuda, 2000). The Con A reaction observed in the supra-nuclear cytoplasm could be associated with the presence of lysosomes, as suggested for Con A reactivity in the apical cytoplasm of Sertoli cells (Arya and Vanha-Perttula, 1985; Arenas et al., 1998; Pastor et al., 2003; Desantis et al., 2006). We agree with this interpretation because the transmission electron microscopy of feline ED non-ciliated cells revealed, below the luminal surface, the presence of an endocytotic apparatus, consisting of small coated pits and vesicles, large vacuoles and irregularly shaped granules, but a lack of secretory granules (Wrobel and Gürtler, 2004). A specialized endocytotic apparatus has been reported in the EDs of rat (Robaire and Hermo, 1988; Hermo et al., 1991), boar (Stoffel and Friess, 1994) and horse (Arrighi et al., 1994). Particularly,

Hermo et al. (1991) demonstrated an endocytotic activity of the testicular sulphated glycoprotein-2 by the non-ciliated cells in the rat ED. In a recent lectin histochemistry study of the alpaca EDs, the endocytotic activity has been proposed for the type II principal (non-ciliated) cells (Parillo et al., 2009a). Involvement of non-ciliated cells of cat EDs in endocytotic phenomena appears more likely than involvement in the synthesis and secretion of glycoconjugates, which was on the contrary hypothesized for alpaca (type I non-ciliated cells) (Parillo et al., 2009a), boar (Wystub et al., 1989), dog (Wakui et al., 1996), equine (Arrighi et al., 1994), goat (Goyal and Williams, 1988), mouse (Burkett et al., 1987a), and lizard (Labate et al., 1997).

A prominent Golgi area has been found in supra-nuclear position of non-ciliated cells in feline EDs by means of transmission electron microscopy (Wrobel and Gürtler, 2004). Our research revealed Golgi zone affinity for Con A, RCA_{120}, MAL II, and PNA after KOH-sialidase treatment, indicating the presence of glycans with terminal/internal α-D-Man/α-D-Glc and terminating with Galß1,4GlcNAc, Neu5Acα2,3 Galß1ß4GlcNac, and sialic acid linked to Galß1,3 GalNAc residues. Lectin binding with the Golgi zones of cells has been interpreted as evidence for synthesis of glycoconjugates. Particularly, Con A reactivity indicates the presence of N-linked glycans. It is well known that N-linked oligosaccharides of glycoproteins complete their synthesis in the Golgi stacks. The presence of N-linked sialyloligosaccharides in the Golgi zone has been reported in ED non-ciliated cells of mouse (Burkett et al., 1987a), canine (Wakui et al., 1996) and alpaca type I principal cells (Parillo et al., 2009a). As several AQPs, including AQP1 and AQP2, contain N-linked glycosylation sites, this type of glycosylation may be important for their trafficking through the Golgi zone (Hendriks et al., 2003; Lu et al., 2008).

Ciliated cells showed binding sites mostly at the level of cilia, which reacted with Con A, KOH-s-WGA, GSA II, HPA, SBA and, after KOH-sialidase treatment, with RCA_{120}. This suggests the presence of N-linked oligosaccharides with terminal/internal αMan, GlcNAc and with terminal GalNAc and sialic acid linked to ß-D-Gal(1-4)-GlcNAc. WGA reactivity in the supra-nuclear cytoplasm could represent the histochemical result of the Golgi zone activity. This cellular compartment has been evidenced at electron microscopy level by Wrobel and Gürtler (2004). Sialic acid linked to Galß1,4GlcNAc revealed with KOH-sialidase-RCA_{120} on cilia indicates the presence of a negatively charged glycocalyx. Because sialic acids implicate negative charges (Schauer, 1985), the cilia can stand separate from one another, maintain motility (Ito et al., 1990), and, consequently, provide the progression of spermatozoa (Hunter et al., 1991).

In conclusion, this study demonstrates that the epithelium lining of cat EDs is implicated in diversified processes of absorption and manipulation of the luminal fluid. These processes might happen: i) by collaboration of different AQP water channels, which permit the rapid removal of the liquids coming from testis secretions, and ii) by the coexistence of lectin-reactive apical cytoplasmic granules and vacuoles, which can be interpreted as components of an elaborate endocytotic apparatus specialized for the uptake of particulate matter from the lumen. Morphological investigations suggested that ED non-ciliated cells can be involved in simultaneous endocytosis of the fluid phase of the luminal content and exocytosis of secretory materials (Goyal and Williams, 1988; Wystub et al., 1989; Arrighi et al., 1994). In the light of the present data it might be hypothesized that synthetic processes, possibly taking place in the cytoplasm of the non-ciliated cells and completed in the stacks of the Golgi apparatus, could serve for the production of the lysosomal vesicles useful for intracellular digestion of absorbed materials, and/or for glycosylation of luminal surface receptors involved in endocytic processes, as well as for glycosilation of aquaporins.

The importance of water movement across the epithelium lining the excurrent duct is exemplified by animal models that are infertile or sub-fertile because of impaired reabsorption of the seminiferous fluid (Da Silva et al., 2006a). The cat is a suitable model for studies regarding reproduction, in addition to habitual laboratory mammals. The knowledge of resorptive phenomena taking place at the level of cat EDs adds information which could be useful in the development of novel compounds aimed at modulating male fertility. Development of specific AQP blockers could sustain investigations of AQPs in reproductive physiology and possible comprehension of the underlying mechanisms of male infertility or, on the contrary, provide methods to adverse undesired fertility (Huang et al., 2006). As a concluding remark, investigations on the domestic cat genital tract may allow possible progress towards the application of assisted reproductive technologies in threatened wild Felidae (Farstad, 2000).

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