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Director: Prof. Vittorio Dell'Orto

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**Morphological features of the reproductive organs
in domestic species in relation to water movement
control, hyponutrition and environmental
influences in fetal and prepubertal life, and
metabolism regulatory factors**

Marina Aralla

Tutor:

Prof. ssa Silvana Arrighi

Coordinator:

Prof. Valentino Bontempo

On the cover:

Trasverse section of canine yolk sac expressing Aquaporin 9 immunoreactivity.

Magnification 40X.

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CHAPTER 1

Foreword

1. Foreword

During my PhD I studied mainly the morphological features of some male and female reproductive organs. The studies were carried on in domestic as well as in laboratory species, taking into account the water movement control operated by a peculiar class of proteins, the so-called Aquaporins (AQPs), the effects of hyponutrition and environmental influences in fetal and prepubertal life, and the possible role of metabolism regulator factors, such as leptin, in the reproductive physiology.

The landmark discovery of AQPs starts the era of molecular water channels, which has permeated virtually every branch of biology and medicine. Since multiple isoforms of AQPs are differentially expressed in cells and tissues, their localization to specific cells and membrane domains is critical to understanding the role of each AQP in the transfer of water and small solutes.

A number of recent studies have revealed the importance of mammalian AQPs in both physiology and pathophysiology and have suggested that pharmacological modulation of aquaporin expression and activity may provide new tools for the treatment of variety of human disorders, such as brain edema, glaucoma, abnormal secretion of lacrimal and salivary glands, tumour growth, fertility and obesity in which water and small solute transport may be involved.

On the other hand, the effects of nutrition on reproduction and fertility are well known and widely reported. They occur not only in the ruminant species (Lindsay et al., 1991; Lucy, 2003) but in monogastric species as well (I'Anson et al., 1991; Cox, 1997; Sharpe and Franks, 2001; Hazeleger et al., 2005;). Nutrition affects all aspects of the chain of reproductive events from gametogenesis to puberty in both males and females. The reason for this close association between nutrition and reproduction is to ensure that reproduction is very closely aligned with the food supply. Such an alignment is essential to ensure the success of reproduction by timing lactation and neonatal growth, both of which are intense, energy demanding processes, to the expected availability of a high quality food supply. Several factors should be considered as links between the nutritional status and the reproductive processes. Leptin has been proposed as the most notable modulator in this mechanism (Magni et al., 2000).

1.1 Aquaporin water channels

An appropriate regulation of membrane water-permeability is a fundamental requirement of all living organisms. In contrast to the prevailing view of simple diffusion through a lipid bilayer, studies performed over several decades have predicted the existence of a water-specific channel protein in certain membranes (King and Agre, 1996). Investigation of the Rh protein of the

erythrocyte membrane led to the serendipitous identification of a novel 28 kDa protein (Denker et al., 1988). This protein has been called Aquaporin-1 (AQP1) and has been proved to be the first molecular water channel (Preston et al., 1992).

Eleven mammalian aquaporins have been reported so far (Koyama et al., 1997; Borgnia et al., 1999) (table 1). Each has a unique cellular and subcellular distribution, with little overlap between homologues. The aquaporin family can be divided into two groups on the basis of their permeability characteristics, which generally coincide with specific amino-acid-sequence patterns. Most members of the first group (aquaporins) are only permeated by water, and this group includes AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8. AQP6 and AQP8 are in this group on the basis of sequence analysis, although AQP6 is permeated by anions (Yasui et al., 1999) and AQP8 might be permeated by water and urea (Ishibashi et al., 1997; Ma et al., 1997). Members of the second group (aquaglyceroporins), which includes AQP3, AQP7, AQP9 and AQP10, are permeated by water to varying degrees, but are also permeated by other small solutes, in particular, glycerol. The bacterium *Escherichia coli* has been used as a model for this categorization, as it contains two aquaporin homologues - AqpZ, which is a water-permeable channel, and GlpF, which is a glycerol transporter.

Different cells and organs have diverse requirements for the regulation of water homeostasis. However, across the aquaporin family, differences have been identified in the transcriptional regulation of the genes, as well as in the post-translational modification, stability and polarized distribution of the proteins. The characteristics of permeability differ among some members of the family as well. The complexity in the regulation of expression, membrane targeting and permeability necessitates the existence of more than just a single water-channel gene. It should expect to find new and surprising physiological functions for the aquaporins that require complex patterns of expression and regulation.

Table 1: Permeability characteristics and predominant distribution for the known mammalian aquaporin homologues

Aquaporin	Permeability	Tissue Distribution	Subcellular Distribution
AQP0	Water (low)	Lens	Plasma membrane
AQP1	Water (high)	Red blood cell, kidney, lung, vascular endothelium, brain, eye	Plasma membrane
AQP2	Water (high)	Kidney, vas deferens	Apical plasma membrane, intracellular vesicles
AQP3	Water (high), glycerol (high, urea (moderate))	Kidney, skin, lung, eye, colon	Basolateral plasma membrane
AQP4	Water (high)	Brain, muscle, kidney, lung, stomach, small intestine	Basolateral plasma membrane
AQP5	Water (high)	Salivary gland, lacrimal gland,	Apical plasma membrane

AQP6	Water (low), anions ($\text{NO}_3^- > \text{Cl}^-$)	sweat gland, lung, cornea Kidney	Intracellular vesicles
AQP7	Water (high), glycerol (high), urea (high), arsenite	Adipose tissue, kidney, testis	Plasma membrane
AQP8	Water (high), Urea	Testis, kidney, liver, pancreas, small intestine, colon	Plasma membrane, intracellular vesicles
AQP9	Water (low), glycerol (high), urea (high), arsenite	Liver, leukocytes, brain, testis	Plasma membrane
AQP10	Water (low), glycerol (high), urea (high)	Small intestine	Intracellular vesicles

Table taken from Landon et al., 2004 Review.

1.1.1 The aquaporin structure

Biochemical analyses of AQP1 revealed that the 28-kDa polypeptide that was evident on immunoblots represented the monomeric form of the protein, but that AQP1 (as well as other aquaporins) is present as a tetramer in the cell membrane (Smith and Agre, 1991). Freeze–fracture studies also highlighted a tetrameric arrangement of the protein (Verbavatz et al., 1993).

Monomer structure. The signature sequence motif of the aquaporins is the three-amino-acid sequence NPA (Asn-Pro-Ala). One NPA motif is found in the aminoterminal half of each monomer, and a second NPA motif is found in the carboxy-terminal half (Fig.1). When their amino termini are aligned, the overall percentage sequence identity among the aquaporin-family members is ~25–40%. In addition to the similarity between the different aquaporins, the amino- and carboxy-terminal halves of AQP1 are related by their sequence, although these halves are oriented in opposite directions across the membrane bilayer (Fig. 1). Hydropathy analysis of AQP1 indicated the presence of six transmembrane helices in each monomer (Preston and Agre, 1991).

In addition, mutational analysis of residues around the conserved NPA motifs and structural studies led to predictions of an ‘hourglass’ structure, with the intracellular loop B and the extracellular loop E folding into the membrane to form the pore (Jung et al., 1994) (Fig. 1). In addition, these studies have greatly advanced our understanding of the unique permeability characteristics of the aquaporins.

Tetramer formation. Aquaporins are present in the membrane as tetramers, but, unlike ion channels, the channel for water permeability is not located at the fourfold axis (the centre of the tetramer) because each monomer contains a channel (Jung et al., 1994) (Fig. 1). The helices of each AQP1 monomer that are positioned on the outside face of the tetramer are hydrophobic, whereas those that are placed towards the centre of the tetramer are hydrophilic (Kozono et al., 2002).

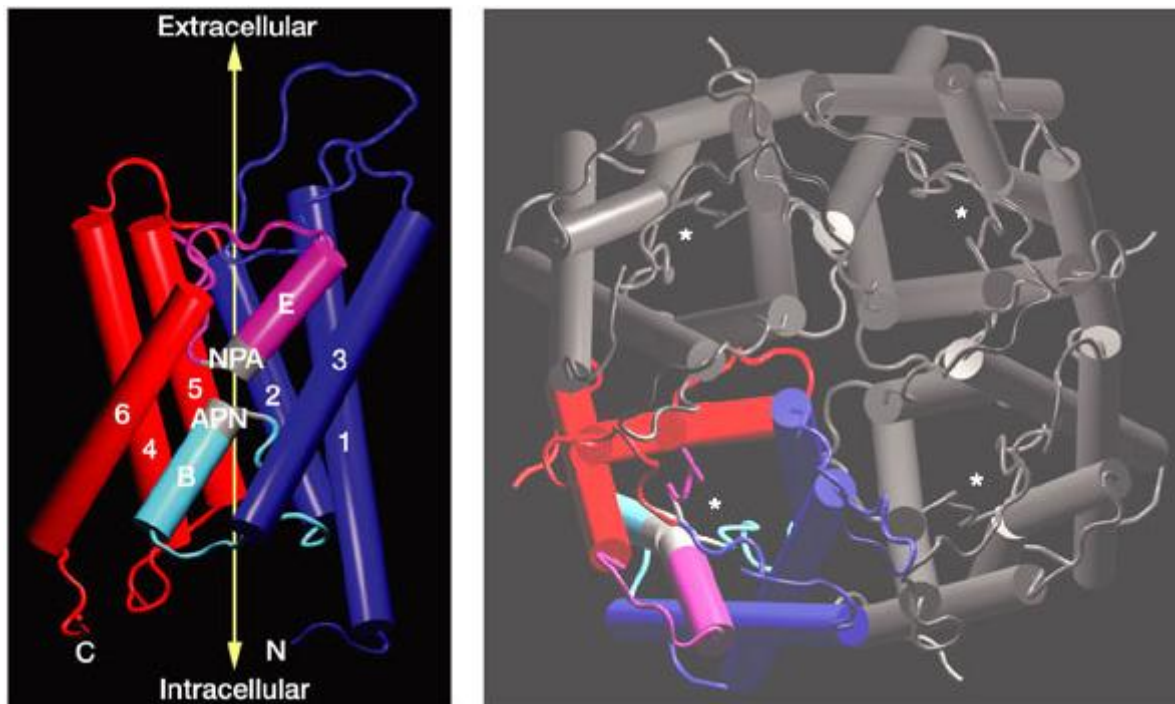


Fig. 1: **Left:** structure of the aquaporin-1 (AQP1) monomer is shown, with membrane-spanning helices numbered 1–6 and displayed as rods. The amino-terminal half of the molecule is shown in purple and light blue, and the carboxy-terminal half is shown in red and pink. Loops B and E, which fold into the membrane to form the pore, are labelled, as are the conserved NPA motifs (shown in light grey). Portions of loops B and E form α -helices, and are therefore shown as rods. The arrow highlights the route taken by water, which can move in both directions through the channel. **Right:** The AQP1 tetramer, as seen from above. Asterisks denote the location of the water pore in each subunit. These structures are based on the Protein Data Bank RCBS. C, carboxyl terminus; N, amino terminus. Fig. taken from Landon et al., 2004 Review.

1.1.2 Channel selectivity and gating

Structural studies revealed that the restriction of AQP1 permeability to water excluding even hydronium (H_3O^+) ions arises from two principal mechanisms (Sui et al., 2001; de Groot and Grubmüller, 2001; Tajkhorshid, E. et al.; 2002; Kozono et al., 2002). First, the channels narrow to a diameter of 2.8 Å approximately 8 Å above the centre of the bilayer, which physically limits the size of molecules that can pass through them. Second, the walls formed from trans-membrane domains TM1, 2, 4 and 5 are hydrophobic, whereas the two highly conserved Asn-76 and Asn-192 in the NPA motifs are juxtaposed, as predicted in the hourglass model, providing the polar residues for hydrogen bonding. In addition, the terminal parts of the pore-forming loops B and E each contain short α -helices which create positive charge in the centre of the membrane. The his-180 residue is uncharged at neutral pH, but becomes protonated at lower pH, providing a second positive charge. The functional separation of water molecules eliminates the possibility of H^+ transfer through a channel. The combination of

size and charge restrictions provides the basis for the unique permeability characteristics of the aquaporins.

Chemical inhibitors of diffusion are not known and diffusion occurs with high Arrhenius activation energy (E_a). In contrast, most mammalian aquaporins are inhibited by mercurials and the E_a is equivalent to diffusion of water in bulk solution ($\sim 5 \text{ kcal mol}^{-1}$) (Agre et al., 2002).

Structural studies and molecular dynamics simulations have brought a remarkably high level of atomic understanding to the process of membrane water transport.

Investigators in diverse fields of biomedical research now recognize that aquaporins provide the mechanism for rapid and selective movement of water through biological membranes.

1.2 Function of Aquaporins in the reproductive systems

More than 10 years ago we had the first reported confirmation of AQP in the female reproductive system achieved by isolating the complementary DNA (cDNA) encoding a water channel from a human uterus cDNA library template. The cloned cDNA had high (99.8%) homology to the 28 kDa human erythrocyte CHIP28 (channel-forming integral membrane protein) water channel gene (Li et al., 1994). The reading frames of both cDNAs were of same length, showing 99% identity, only with valine in place of alanine at position 45 of the human erythrocyte CHIP28 sequence (Li et al., 1994). This variant might be unique to the human uterus (Li et al., 1994). Subsequently, the presence of AQP1 messenger RNA (mRNA) was observed in rat uterus (Li et al., 1997).

To date, at least nine AQP isoforms (AQP1–AQP9) have been confirmed to be expressed in the female reproductive tract or in cells involved in assisted reproductive technology procedures. Their specific expression pattern suggests that they play a role in water movement between the intraluminal, interstitial, and capillary compartments. In addition, there is evidence that the expression of various AQPs can be regulated by steroid sex hormones (Jablonski et al., 2003; Richard et al., 2003; He et al., 2006; Lindsay and Murphy, 2006).

Water and solute movement across the epithelium of the male reproductive tract is responsible for balancing the luminal environment for spermatogenesis; for the maturation, storage, transport and liberation of sperm; and for increasing sperm concentration. At present, multiple AQPs have been recognized in the testis (AQP0, AQP1, AQP7, AQP8 and AQP9), efferent ducts (AQP1, AQP9 and AQP10), epididymis (AQP1, AQP3, AQP9 and AQP10), vas deferens (AQP1, AQP2 and AQP9) and accessory glands (AQP1 and AQP9) of adult mammals. Some of them in specific tissues are regulated by sex steroid hormones (Zaniboni and Bakst, 2004; Zaniboni et al., 2004). The heterogeneous,

segment-specific and developing expression of AQPs along the male reproductive system suggests that fluid transport in these tissues could be locally modulated by physiological regulation of the expression and/or function of AQPs. The pathological expression and regulation of AQPs might cause several disorders of male reproductive system.

Together, these results provide indirect evidence of a role for AQPs in reproductive physiology.

1.2.1 *Aquaporins in female genital tract*

Uterine water imbibition

Dramatic changes in the uterus occur in response to ovarian steroid hormones, including hyperemia (Finlay et al., 1983), increased capillary permeability (Cullinan-Bove and Koos, 1993), recurrent stromal oedema (Okada et al., 2001) and fluctuation of uterine fluid volume (Maier and Kuslis, 1988). To gain insight into the mechanisms responsible for balancing water concentration within the uterus, Jablonski et al. (2003) explored the expression profile and functionality of AQPs in the ovariectomized mouse uterus treated with ovarian steroid hormones. AQP1 is in the myometrium and maybe slightly regulated by ovarian steroid hormones. AQP2 appeared strongly upregulated by estrogen in the epithelial cells and myometrium of the uterus (Jablonski et al., 2003). AQP3 was also detected in the uterine epithelial cells. AQP8 is present in the stroma and myometrium, where it may shuttle water and play an important role in limiting water flow into the stroma and protecting the myometrial layer from oedema (Jablonski et al., 2003). In human the expression of endometrial AQP2 is menstrual cycle-dependent and reaches a high level at the mid-secretory phase, the time of embryo implantation (He et al., 2006) because is correlated to serum E2 and progesterone concentrations (He et al., 2006). AQP5 in rat uterus was shown to be up-regulated by progesterone (Lindsay and Murphy, 2006).

The expression of AQPs in uterus is regulated by ovarian steroid hormones, suggesting that these AQPs may play important roles in hormone-mediated water and other small molecules transport in uterine imbibition.

Moreover, the presence of AQP3, AQP4, AQP5 and AQP8 in the mouse cervix may contribute to the biochemical changes, such as progressive disorganization of the collagen network and increased water content, that occur in the cervical connective tissue during gestation to allow for cervical dilatation during labor (Anderson et al., 2006). Altered expression of AQPs in models of preterm and delayed cervical ripening could also shed light on distinct aspects of cervical water balance during pregnancy and parturition (Anderson et al., 2006).

Aquaporins and amniotic fluid regulation

Amniotic fluid (AF) provides the fluid-filled compartment that is essential for normal fetal growth, movement and development. Disorders of AF volume could be associated with perinatal morbidity and mortality. The sheep has been deeply studied as a model for AF regulation and in this species the transcellular route for water passage has been recognized as a critical regulatory path for amniotic fluid resorption (Hedriana et al., 1995). This way is facilitated by AQPs that increase plasma membrane permeability and provide a route for rapid fluid movement (Verkman et al., 1996; Verkman and Mitra, 2000). To date the expression of only four AQPs (AQP1, AQP3, AQP8 and AQP9) has been demonstrated in mammalian chorioamniotic membranes and placenta. AQP1 is expressed in murine syncytiotrophoblasts and placental and chorionic endothelium (Hasegawa et al., 1994; Johnston et al., 2000). In human, AQP1 was found in amnion and chorion from both membrane locations, but no AQP1 was seen in the trophoblast cells of the chorion (Mann et al., 2002). Moreover, AQP1 was localized in the amniotic epithelium of both the chorionic plate and reflected region of the fetal membranes. The strongest signal was detected in the epithelium of the chorionic plate amnion (Mann et al., 2002). The presence of AQPs in the epithelium suggests how rapid and facilitated could be the water transport between the amniotic cavity and the fetal circulation (Mann et al., 2002). It has been also utilized an AQP1 knockout mouse model to determine what effect the absence of this water channel would have on amniotic fluid volume and composition (Mann et al., 2005). Indeed, with complete knockout of the water channel, the fetal mice showed increases in amniotic fluid volume and decreases in osmolality, further confirming that the AQP water channels are important in the regulation of amniotic fluid volume. AQP3 is expressed in the epithelia of ovine placenta and chorion, but not in amnion (Johnston et al., 2000). AQP3 is also expressed in fibroblasts of the amnion and allantois, but not in the epithelia of these membranes (Johnston et al., 2000). In human, AQP3 protein was not found in the fetal membranes, while AQP3 mRNA was faintly detected in the chorion from both membrane regions but not the amnion (Mann et al., 2002). Another study reported the presence of AQP3 in the apical membranes of syncytiotrophoblast of human term placenta (Damiano et al., 2001). AQP3 is the most highly expressed AQP quantitatively, thus revealing that AQP3 may facilitate water transport across the trophoblast barrier. The similar expression of AQP8 in the ovine trophoblast and membrane epithelial cells was reported (Liu et al., 2004). Wang et al. (2001) have demonstrated that AQP8 is expressed in human epithelial cells of chorion and amnion and of the syncytiotrophoblasts and outer layer trophoblasts of placenta. That is the first study demonstrating the expression of the AQP8 water channel in human chorioamniotic membranes. Using RT-PCR, immunoblotting and

immunohistochemistry, Damiano et al. (2001) found that AQP9 was present in the apical membranes of syncytiotrophoblast of human term placenta. Wang et al. (2005) reported AQP9 mRNA expression in ovine amnion and allantois and AQP9 protein localization in epithelia of amnion and allantois, indicating that AQP9 may be a major water channel for intramembranous amniotic fluid resorption in sheep. Thus, the presence of AQP3, AQP8 and AQP9 represents a molecular mechanism of amniotic water absorption through intramembranous pathways, contributing to the high water permeability of the placenta. Although the functional importance of AQPs in amniotic fluid transport remains to be determined, its presence in human fetal membranes suggests potential therapeutic strategies for the treatment of amniotic fluid volume disorders and inhibitors of AQP water channels could also be useful for managing oligohydramnios and polyhydramnios.

1.2.2 Aquaporins in male genital tract

Water and solute movement across the epithelium of the male reproductive tract is responsible for balancing the luminal environment for spermatogenesis, for the maturation, storage, transport and liberation of sperm and for increasing sperm concentration. At present, multiple AQPs have been recognized in the testis (AQP0, AQP1, AQP7, AQP8 and AQP9), efferent ducts (AQP1, AQP9 and AQP10), epididymis (AQP1, AQP3, AQP9 and AQP10), vas deferens (AQP1, AQP2 and AQP9) and accessory glands (AQP1 and AQP9) of adult mammals. Some of them in specific tissues are regulated by sex steroid hormones. The heterogeneous, segment-specific and developing expression of AQPs along the male reproductive system suggests that fluid transport in these tissues could be locally modulated by physiological regulation of the expression and/or function of AQPs. The pathological expression and regulation of AQPs might cause several disorders of male reproductive system.

Efferent ductules are very important reabsorptive segments of the male reproductive tract, where between 50 and 90% of the luminal fluid secreted by seminiferous tubules is reabsorbed (Levine and Marsh, 1971; Clulow et al., 1994, 1998). The high rate of resorption of luminal fluid requires various water and ion transporters in absorptive epithelial cells of efferent ductules. Besides ion transporters - including basolateral Na⁺/K⁺- ATPase (Ilio and Hess, 1992, 1994), the apical Na⁺/H⁺ exchanger-3 (Hansen et al., 1999; Lee et al., 2001), Cl⁻/HCO₃⁻ exchanger (Lee et al., 2001) and chloride channel cystic fibrosis transmembrane regulator (CFTR) (Lee et al., 2001) - water channels (AQP1, AQP9 and AQP10) may contribute to the transepithelial water movement. AQP1 was expressed intensely [to a degree comparable with that for estrogen receptor (ER α)] in efferent ductules of rats and marmoset monkeys at all ages from late fetal life through puberty to adulthood (Fisher et al., 1998). Once the

epithelial cells had differentiated, AQP1 was mainly localized in the brush-border and, to a lesser extent, the basolateral membranes and apical endosomes of non-ciliated cells and the cilia of ciliated cells (Brown et al., 1993; Fisher et al., 1998; Badran and Hermo, 2002; Oliveira et al., 2005). However, no impaired fluid resorption in efferent ductules was observed in AQP1-null mouse (Zhou et al., 2001), suggesting the existence of other AQPs in this epithelium, proven later. AQP9 was enriched on the microvilli of non-ciliated cells (Pastor-Soler et al., 2001; Badran and Hermo, 2002; Oliveira et al., 2005). Recently, another AQP (AQP10) was found to be expressed over the epithelium in the efferent ductules (Hermo et al., 2004). AQP1 and AQP10 were both intensely detected at the endothelial cells of vascular channels in this region (Badran and Hermo, 2002; Hermo et al., 2004). The expression of AQP1 and AQP9 in the efferent ductules might be regulated by estrogen because the expression of these two isoforms of AQPs was significantly reduced in the efferent ductules of mice deficient in ER α (Ruz et al., 2006). In addition to the efferent ductules, considerable fluid resorption also occurs in the distal segments of the male excurrent duct system, notably in epididymis, where concentration of sperm increases significantly (Wong and Yeung, 1978) and a hypertonic luminal fluid is established (Levine and Marsh, 1971). Several members of AQP family expressed in a region-specific pattern in the epididymal epithelial cells may be of importance in the transepithelial water movement and in postnatal development of this organ as well. AQP9 is the first and main AQP detected in the epididymis. In rat, the stereocilia of the principal cells, not the basal cells, exhibit strong AQP9 protein labelling (Badran and Hermo, 2002; Elkjaer et al., 2000; Pastor-Soler et al., 2001, 2002; Oliveira et al., 2005; Da Silva et al., 2006). Human epididymis (Pastor-Soler et al., 2001) has an expression pattern similar to that found in rats. As a broadly selective neutral solute and water channel, AQP9 probably enables the rapid cellular movement of metabolites required by fast-growing spermatocytes. As for other AQPs, mRNA for AQP2, AQP5, AQP7 and AQP11 are also detected in epididymal epithelium (Da Silva et al., 2006). However, AQP5 co-localized with AQP9 in the apical membrane of a subpopulation of principal cells in the corpus and cauda regions. Surprisingly, AQP2 mRNA is present throughout the development of rats up to adulthood, while the protein was detected in the distal region of the epididymis only in young rats, not in adult ones. Maybe a post-transcriptional mechanism is involved in the regulation of AQP2 expression. As a basolateral water channel, AQP3 is localized exclusively and intensely in basal cells of epididymis (Hermo et al., 2004). The AQP8 expression in epididymis is controversial. Several studies showed that neither AQP8 transcript nor protein was detected (Calamita et al., 2001; Da Silva et al., 2006), but others interestingly demonstrated the localization of AQP8 in the basal cells in the ductus epididymis (Elkjaer et al., 2001). Basolateral expression of AQPs leading to

transepithelial flow remains unknown. Maybe they are involved in the differentiation from basal cells to more differentiated cell types in these epithelia. Moreover, AQP1 is absent from the epithelium of the epididymis, but it was expressed over the endothelial cells of the vascular channels of epididymis (Badran and Hermo, 2002; Oliveira et al., 2005) as well as AQP10 (Hermo et al., 2004). The vas deferens also plays a significant role in transepithelial water resorption of the reproductive tract (Wong and Yeung, 1978; Wong et al., 1978) and is responsible for maintaining the luminal environment during maturation of sperm. AQP1 has been reported to be present at plasma membranes of epithelial cells in the ampulla of the vas deferens, not the cells in proximal parts, in rats (Brown et al., 1993). Unexpectedly, AQP2 was found to be present at the epithelial cells of the vas deferens by a transgenic mouse approach, which was used to examine the mechanism of the principal cell-specific expression of AQP2 within the renal collecting duct. The AQP2 expression in vas deferens was confirmed in normal rats (Nelson et al., 1998). AQP2 (Stevens et al., 2000) began to appear in some epithelial cells of the middle vas deferens and was concentrated on the apical plasma membrane of principal cells in the distal vas deferens (ampulla), where AQP1 (Brown et al., 1993) was highly expressed. No expression was detected in the proximal region, and very little was observed on intracellular vesicles. The variable expression of AQP1 and AQP2 in different regions of the vas deferens may be due to the marked structural differences of principal cells along the vas deferens (Andonian and Hermo, 1999), reflective of diverse functional activities. In contrast, AQP9 is present throughout the entire length of the vas deferens (Pastor-Soler et al., 2001). Therefore, AQP9 is the only AQP detected in the proximal vas deferens.

We have just approached a new research to verify if it could exist a relationship between water imbibitions and the regression of the cranial suspensory ligament during foetal development allowing the descent of the testis. The Leydig insulin-like peptide or relaxin-like factor (INSL3), is supposed to induce the growth of the gubernaculum in male foetuses, thus being directly responsible for the testicular descent in humans and rodents. Arrighi et al. (2009) demonstrated the immunolocalization of INSL3 in the Leydig cells of dog male foetuses and of LGR8 receptor in different tissues of the gubernaculum testis of the same foetuses. AQPs appeared also involved in the water imbibition of gubernaculum testis: dog foetuses gubernacula showed a high expression of AQP1, AQP3, AQP4 and AQP9 respectively in blood vessels, smooth muscles, connective tissue and fat.

1.3 What is the relationship between nutrition and reproduction?

One way of viewing the relationship between nutrition and reproduction is through energy balance. When the animals' net nutrient requirement is more than the net nutrient intake the animals will use their energy stores (glycogen, triglycerides and protein) to meet the deficit. When an animal is in this state, it is in "negative energy balance". Similarly, when the net nutrient requirement is less than the net nutrient intake, the animal will store the excess nutrients (as glycogen and triglycerides) and/or disperse the excess nutrients as metabolic heat. When an animal is in this state it is in "positive energy balance". These metabolic states and the accompanying alterations in appetite and nutrient partitioning within the body are regulated by a series of complex interactions among the blood concentrations of metabolic hormones and various whole-body nutrient fluxes. Many of the metabolic hormones and nutrients that help to maintain whole-body nutrient homeostasis also affect the reproductive system. Consequently, there are well-defined associations between metabolic state and reproduction. The effects of negative energy balance on reproduction are primarily at the hypothalamo-pituitary level of reproductive control (Wade and Jones, 2005) and are characterized by hypoglycaemia, hypoinsulinaemia, suppressed plasma IGF-I and elevated plasma GH, changes that are associated with the inhibition of GnRH pulsatility, anovulation and anoestrus in the female. There is little evidence to suggest that negative energy balance has any direct ovarian effects in the ewe that are independent of its effects on the hypothalamo-pituitary axis (Lozana et al., 2003; Kiyama et al., 2004). However, in the lactating dairy cow there is strong evidence to suggest that negative energy balance has some direct inhibitory effects on folliculogenesis and oocyte quality (Gong et al., 2002; Wates et al., 2003). Positive energy balance leads to increased leptin and insulin concentrations in the blood and increased glucose uptake; these changes appear to affect the ovary directly and are associated with increased folliculogenesis and increased ovulation rate for example in sheep (Scramuzzi et al., 2006). Positive energy balance is also associated with alterations in the hepatic metabolism of steroids (Parr et al., 1987, 1993) that can lead to disturbances in negative feedback between the ovary and the hypothalamo-pituitary system and theoretically, to increased folliculogenesis. There is little evidence to suggest that positive energy balance has a specific stimulatory action on the hypothalamo-pituitary axis (Wade and Jones 2005). It is worth noting that because of the strong negative feedback interrelationship between the hypothalamo-pituitary axis and the ovary, the task of identifying the anatomical sites in the reproductive axis that are influenced by nutrition is proving to be exceedingly difficult. Positive energy balance, if it persists, will inevitably increase body weight. However, the stimulatory effect of nutrition on

folliculogenesis can occur before there is any detectable increase in body weight. The links between the metabolic-nutritional system and the reproductive system almost certainly involves one or more of the metabolic hormones and nutrient fluxes that are an integral part of nutrient utilisation and whole body nutrient homeostasis. Strong contenders among many for the “link” between nutrition and the follicle are the glucose-insulin system, IGF system and leptin system. However, the nutrient requirement for other reproductive events such as foetal growth, lactation and pubertal growth are all very high and significant in the context of whole body energy utilisation. In these later states the effect of nutrition on reproduction is a question of nutrient supply, particularly energy. In these reproductive states, failure to meet the nutrient demand will compromise reproduction. The mechanisms of nutritional effects on folliculogenesis are probably not effects of quantitative nutrient supply per se; it is much more likely that they are specific nutrient signalling effects that link reproduction with favourable environmental conditions for reproduction. For spermatogenesis and folliculogenesis, nutrition acts as a metabolic signalling mechanism.

In summary, dietary nutrients influence expression of metabolic pathways that allow animals to achieve their full genetic potential for reproduction and growth. These pathways are complex and involve appetite regulation along with regulation of the reproductive and growth axis, as well as gonadal function (Muller et al., 1999).

1.3.1 Prenatal needs during pregnancy

The nutritional status of females during pregnancy is essential in the post-natal growth and development of the offspring, often leading to permanent changes (foetal programming, Lucas 1991). This concept, originally related to the propensity of humans to disease, has now been extended and includes the effects of foetal malnutrition on pre- and post-natal development, including the development of the reproductive system before puberty (Engelbrecht et al. 2000; Rhind et al. 2001). Gonadotrophin secretion has been shown to be lower both in underfed lamb foetuses (Deligeorgis et al. 1996). Furthermore, nutritional restrictions can influence the activity of the hypothalamo-pituitary axis and thus reduce Gonadotrophin levels in ram lambs (review Brown 1994). Thus, nutrition during early life could affect the development of the testes and, in some circumstances, have permanent effect on their future capacity to produce spermatozoa.

Sertoli cells appear early in foetal life and divide until pre-pubertal age (rats: Orth 1982; sheep: Hochereau de Reviers et al. 1987). Sertoli cells are a particularly strong candidate for foetal programming of future performance, because the number of Sertoli cells is highly correlated with adult testicular size and the

maximum rate of germ cell production (review: Sharpe et al. 2003). That is, foetal nutrition, particularly during gonadal differentiation, may be a determinant of maximum capacity for sperm output. In undernourished rat pups testes (Bansal-Rajbanshi and Mathur 1985) the cell generation cycle of spermatogonial germ cells and supporting cells (future Sertoli cells) on day 9 of age showed marked prolongation of DNA synthetic phase, and shortening of the pre-DNA synthetic phase indicating a depression in DNA synthesis in undernutrition. However, no information regarding Sertoli cell numbers and adult sperm producing capacity is available.

In a previous study of sheep grazing under rangeland conditions, Bielli et al. tested whether supplementation of the lamb-ewe unit during intrauterine and post-natal life would affect Sertoli cell numbers. In supplemented animals, the male lambs tended to have more Sertoli cells than did controls (Bielli et al. 2001). In a later experiment under better controlled conditions, undernutrition (70% vs 110% of metabolizable energy intake requirements) during pregnancy reduced testicular development in the newborn lamb, the absolute volume of testicular cords and the number of Sertoli cells (Bielli et al. 2002).

Human Amniotic Fluid (AF) results from the diffusion of metabolites and water from foetal plasma through foetal skin, which acts as a permeable barrier. Throughout gestation, there is an increased contribution of foetal urine to amniotic fluid and the concentration of many of its constituents is determined by the synergistic equilibrium between mother and foetus. As AF composition reflects the metabolic status of both mother and foetus during pregnancy, this biofluid is an important target for metabolic studies to monitor mother and foetus's health.

Balancement of water concentration within the uterine environment is important in every reproductive moment of a female mammal life. Disorders of AF volume, either in deficient or excessive amounts, are associated with significant perinatal morbidity and mortality.

AF is created by the flow of fluid from the fetal lung and bladder and reabsorbed in part by fetal swallowing and in part by the transfer across the amnion to the fetal circulation. The amniotic volume absorption across foetal membrane is a significant regulatory path for AF homeostasis, balancing foetal fluid production and resorption and reaching volume homeostasis.

Rapid transcellular water movements are facilitated by Aquaporins that increase plasma membrane permeability. Previous results from our laboratory showed that AQP-mediated water movement between the intraluminal, interstitial, and capillary compartments is crucial in the uterine imbibition mechanism in the bitch, including periodic stromal oedema in preparation for embryo implantation, with evidence that the expression of different AQPs can be

regulated by steroid sex hormones. Also, the importance of the AQP-mediated fluid regulation in uterine environment in the peri-implantation period is known in the woman and laboratory mammals, to assure the successful implantation of blastocyst and its survival. The establishment of pregnancy and its maintenance is dependent on strict synchronization of uterine receptivity with embryonic maturation. Water homeostasis during foetal development is of crucial physiologic importance. Amniotic fluid provides the fluid-filled compartment that is essential for normal foetal growth, movement and development. AF changes during gestation may be dependent on modulations in solute as well as water permeability and changes in AQPs during gestation suggest their role in mediating placental and membrane water flow.

1.3.2 Energy metabolism and Leptin

Leptin is the 167-aminoacids product of the *ob* gene expression and is classically secreted by the adipose tissue. Other organs such as stomach, placenta and ovary are also able to synthesize this important satiety and reproductive hormone. Leptin receptor (ObR) mRNA transcripts has been found in the arcuate (ARC) and ventromedial (VMN) nuclei of the hypothalamus, two areas involved in the regulation of feeding behaviour and reproductive processes (Magni et al., 2000). Leptin circulates in the blood proportionally to the body adiposity becoming the peripheral humoral signal carrying information about energy reserves of the organism. Recent studies demonstrated that leptin is essential in maintaining normal reproductive function as mice deficient in leptin (*ob/ob*) are not only obese but are also infertile (Zhang et al., 1994). After treatment with human recombinant leptin, these *ob/ob* mice recovered fertility (Chehab et al., 1996).

The mechanism by which leptin transfers metabolic information to the reproductive axis is not fully elucidated. Leptin acts in the ARC where the neurons producing neuropeptide Y (NPY), a nervous and peripheral transducers, express leptin receptors. NPY is considered a powerful orexigenic factor and is also involved in reproductive processes (Hauseknecht and Portocarrero, 1998; Li et al., 1999): it has been demonstrated that changes in leptin/NPY ratio may affect the activity of the hypothalamo-pituitary-gonadotropic (HPG) axis hormones, particularly in underfed animals (Caprio et al., 2001).

Leptin protein has been identified in mouse and human oocytes and in preimplantation stage embryos, and appears playing a critical role in early mammalian development (Antczak and Van Blerkom, 1997). Leptin and its receptor have been localized in cultured ovarian cells from the granulosa, cumulus cells of preantral follicles, and mature oocytes from in vitro-fertilized women (Cioffi et al., 1997). Leptin expression has been also recently detected in histological sections of normal and polycystic human (Löffler et al., 2001) and

murine (Ryan et al., 2002) ovaries. Löffler et al. (2001) detected leptin-like immunoreactivity in the developing corpus luteum and also in oocytes, granulosa, and theca, but not in all stages. Ryan et al. (2002) observed strong immunostaining in oocytes and weaker staining in granulosa, stroma, theca, and corpora lutea. Leptin has significant direct effects on ovarian granulosa and thecal cells. *In vitro* leptin stimulates bovine insulin-induced thecal cell proliferation and inhibits insulin-induced progesterone and estradiol production by granulosa cells and insulin-induced progesterone and androstenedione production by thecal cells (Spicer and Francisco, 1998).

Leptin amount in the blood is proportional to body energy stores and/or body mass, so, inadequate nutrition might impair reproductive function leading, for example, to the delayed onset of puberty. Indeed, the onset of puberty in humans and animals is associated with an increase in fat and consequent increase in circulating leptin, suggesting that leptin may be required for normal growth and development of reproductive organs. A similar relationship between nutrition and reproductive efficiency is not understood in the mare where, besides many reports quantifying the correlation of circulating concentration of leptin with body condition scores (Buff et al., 2002; Gentry et al., 2002), only few informations exist about the presence of leptin (Ob) and leptin receptor (Ob-R) in the ovary or in the oocyte.

The direct role of leptin in the ovary of human and mammals remains to be fully elucidated. A better knowledge of the histological localization of this hormone in the ovary may be of particular interest from the functional point of view.

1.4 Animal models

Until the seventies, experiments examining the relationship between nutrition and reproduction used animal models. To date, the main animal models that have been utilised to investigate the impact of maternal diet on long-term programming have been rats and sheeps (McMillen and Robinson, 2005).

Research investigating the early programming of adult metabolic disease has in recent years provided much mechanistic insight into how the early environment impacts on long-term health. It includes studies addressing the roles of intrauterine nutrient availability, which is determined by maternal nutrition, maternal exposure to oxygen, toxic events, and infection; the placental interface; and also the early postnatal environment.

The advantage of using rats for example is their very short gestational length. Appreciable placental growth continues up to term in the rat which is necessary in part to meet the much higher protein demands for fetal growth compared with that seen in human subjects or sheep (Widdowson, 1950). In large mammals, the maximal period of placental growth is early in pregnancy and is

normally necessary to meet the increased fetal nutrient requirements in late gestation when fetal growth is exponential (Symonds and Gardner 2006). Furthermore, rats produce large litters whereas sheep and human subjects normally produce only one (or two) offspring of comparable birth weight per pregnancy.

Otherwise sheep is also a good model particularly for the nutrition-reproduction interactions because of the huge amount of nutritional research for this species and an extensive database on the requirements of sheep for energy, protein and specific dietary components such as amino acids, fatty acids and trace elements. The sheep model has also an intrinsic economic relevance and findings from sheep can be transposed readily to other agricultural mammals. The sheep model is also relevant to human biology, often directly, but also indirectly because it often leads us to question the significance of findings from studies with rodents.

Therefore animal models, including mice, rats, sheep, pigs and rabbits, remain a vital tool for studying the influence of early nutritional events on adult health and disease. Most epigenetic studies have addressed the long-term effects on a small number of epigenetic marks, at the global or individual gene level, of environmental stressors in humans and animal models. They have demonstrated the existence of a self-propagating epigenetic cycle. In parallel, an increasing number of studies based on high-throughput technologies and focusing on humans and mice have revealed additional complexity in epigenetic processes, by highlighting the importance of crosstalk between the different epigenetic marks. In recent months, a number of studies focusing on the developmental origin of health and disease and metabolic programming have identified links between early nutrition, epigenetic processes and long-term illness. Maternal infection, poor or excess nutrition, and stressful events can negatively influence the development of different cell types, tissues and organ systems ultimately predisposing the organism to pathological conditions.

The inappropriate programming of developing organ systems by exposure to excess native or environmental steroids, particularly the contamination of our environment and our food sources with synthetic endocrine disrupting chemicals that can interact with steroid receptors, is a major concern.

1.5 Reproduction, fertility and environmental pollutants

Exposure to pollutants, and in particular to endocrine disrupting compounds (EDCs) and potentially toxic metals (PTMs) can induce adverse physiological changes in animals. Many of the effects pertain to the reproductive system (Paul et al. 2005; Fowler et al. 2008) but other physiological systems can be perturbed by exposure. These include the thyroid gland (Hansen 1998), neuroendocrine

system (Lein et al. 2007) and therefore behaviour (Erhard and Rhind 2004), the immune system (Vine et al. 2000; Daniel et al. 2001) and systems that control adipogenesis and nutrient partitioning (Newbold et al. 2007); disruption of these physiological systems may also impinge on reproductive fitness.

Evidence of relationships between exposure to pollutants and reproductive effects is derived from observational studies of wildlife exposed, unintentionally, to pollutants (Guillette et al. 1994; Institute for Environment and Health 1999) and from empirical studies of laboratory and domestic animals or cell cultures (Gray et al. 2000; Evans et al. 2004).

Developing animals are particularly sensitive to pollutants, even when the embryo comprises only a few cells, e.g. concentrations of a PCB mixture (Arochlor) as low as 0.001 µg/ml have been found to disrupt bovine oocyte and embryo development (Pocar et al. 2001; Gandolfi et al. 2002). Thus, early exposure has the potential to disrupt the development of all tissues, including yet-to-be-differentiated germ cells, and associated gene expression. Furthermore, disruption of the germ cell line in one generation can be expressed in subsequent generations (Edwards and Myers 2007), probably through altered gene methylation, and therefore gene expression, during the period of sex differentiation of the developing gonad (Anway and Skinner 2006).

As already indicated, exposure during development is particularly important in immature animals. 'Food ingestion' for these may be very different from that of adults, e.g. mammalian foetuses are nourished via maternal blood and developing birds by egg yolk and while young mammals consume milk, adults eat animal or plant material. In addition to differences in food source, differences in metabolism with age mean that it is unwise to extrapolate from effects of exposure in adults to those in immature animals, because the relationship between the amounts of pollutants 'ingested' at the different stages and the respective tissue concentrations may differ with differences in metabolism. Furthermore, indirect measures of exposure of immature animals to pollutants, for example, through measurements in mammalian milk or umbilical cord blood may be of less value than tissue concentrations in the foetus/immature animal for the prediction of physiological effects since the former does not take account of effects of metabolism and excretion in the offspring.

While concentrations of pollutants in human (Dekoning and Karmaus 2000; Darnerud et al. 2001) and sheep milk (Rhind et al. 2007), and human umbilical cord blood, amniotic fluid and meconium (Dallaire et al. 2003; Barr et al. 2007) have been measured, there are relatively few reports of foetal tissue

concentrations (Bosse et al. 1996; Dekoning and Karmaus 2000) in any species and so direct assessment of risk to the foetus is not possible.

However, related studies have also shown that very small increases in maternal exposure to pastures fertilized with sewage sludge which contains multiple pollutants, result in changes in structure and function of the foetal testis (Paul et al. 2005) and ovary (Fowler et al. 2008). Collectively, these observations show that the relationships between pollutant exposure and effect are highly complex, that foetal exposure cannot be extrapolated from adult profiles, and that small increases in exposure to multiple pollutants can adversely affect foetal reproductive development.

There is no doubt that exposure to pollutants can adversely affect reproductive function in animals and that food is likely to be the most important route of exposure for many or most species, particularly for those near the top of the food chain. However, assessment of the risk is complicated by the fact that in the 'real world' food contains many different classes of pollutants, each at low and variable concentrations, and exposure to them occurs throughout life, including during developmental stages which are particularly sensitive to their effects. In short, dietary pollutants represent a significant risk factor for reproduction, under certain circumstances, but precise quantification of these risks for individuals is extremely difficult with current knowledge.

1.6 Salivary glands

The plasma membrane is a major barrier to water transport. The apical plasma membrane of the serous cells in the salivary glands is also impermeable to water which is the main component of saliva (King and Agre, 1996). Fluid transport in salivary glands is thought to be driven osmotically in response to transepithelial salt gradients that are generated by ion transport systems localized to the apical and basolateral membranes of the secretory cells. Acinar cells generate a primary fluid containing plasma-like electrolyte concentrations subsequently modified by solute resorption and secretion as it passes along the ductal system, resulting in the final hypotonic solution that enters the mouth. Hence, the principal site of water transport is likely to be the acini with relatively little transepithelial water movement occurring in the ducts.

In situ hybridization showed that AQP5 is expressed in the secretory lobules in the submandibular gland (Raina et al., 1995). By immunohistochemistry, AQP5 is present in the major salivary glands, i.e., the submandibular gland, parotid gland, and sublingual gland (Nielsen et al., 1997; He et al., 1997; Funaki et al., 1998; Matsuzaki et al., 1999). AQP5 was detected in the secretory acinar cells of

submandibular, parotid, and sublingual glands in the rat (Matsuzaki et al., 1999). Expression of AQP5 is restricted to the lobuli; the interlobular tissues including large ducts and blood vessels are negative for AQP5. In the submandibular gland, AQP5 is present in the acinar secretory cells where it is localized at the apical membrane (Matsuzaki et al., 1999). The abundance of AQP5 at the apical membrane of the secretory acinar cells suggests that the secretion of water is carried out transcellularly rather than paracellularly. In the submandibular gland of the rat, much more AQP5 was detected along the apical membrane of the intercalated duct cells in addition to acinar cells (Matsuzaki et al., 1999). This observation suggests that the intercalated ducts may participate in the secretion of water. In the parotid and sublingual glands, AQP5 is restricted to the acinar cells. AQP5 stored in the intracellular compartment moves to the plasma membrane upon stimulation. In the parotid, cholinergic stimulation induced the translocation of AQP5 from the intracellular membrane fraction to the apical membrane fraction (Ishikawa et al., 1998). However, immunohistochemical examination showed that AQP5 is restricted to the plasma membrane, and no intracellular pool of AQP5 has so far been identified. In human parotid, submandibular, sublingual, and labial glands, northern blot analysis showed the abundance of AQP5 transcript (Gresz et al., 2001). Immunoperoxidase labeling of paraffin sections revealed that AQP5 is localized to the apical membrane of both mucous and serous acinar cells. In combination with AQP3 localized along the basolateral membrane, it may provide a pathway for the transcellular osmotic water flow in the formation of the primary saliva (Gresz et al., 2001). To explore the role of AQP5 in saliva secretion, AQP5-null mice were generated (Ma et al., 1999; Krane et al., 2001). The knockout mice have a grossly normal appearance but pilocarpine-stimulated secretion of saliva was reduced by more than 60% (Ma et al., 1999). Marked reduction of osmotic water permeability was seen in the parotid and sublingual acinar cells isolated from AQP5-null mice (Krane et al., 2001). The saliva of AQP5-null mice was hypertonic and more viscous, whereas the secretion of amylase and protein secretion were not affected (Ma et al., 1999; Krane et al., 2001).

All of these observations show that AQP5 plays an important role in the saliva secretion, but data concerning AQP5 expression in domestic animal species are lacking and could be useful to better understand the mechanisms underlying the digestive processes and study the impact on animal performances.

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CHAPTER 2

Objectives

2. Objectives

The aim of this thesis was to carry on studies in domestic as well as in laboratory species, taking into account the water movement control operated by a peculiar class of proteins, the so-called Aquaporins (AQPs), the effects of hyponutrition and environmental influences in fetal and prepubertal life, and the possible role of metabolism regulatory factors, such as leptin, in the reproductive physiology.

AQPs are a class of small, hydrophobic, integral membrane proteins that are able to make the cell membrane 10 to 100 fold more permeable than the membranes lacking such channels. Based on studies with AQP1, it appears that AQPs exist in the plasma membrane as homotetramers. Each AQP monomer contains 2 hemi-pores, which fold together to form a water channel.

2.1 First functional trial: the water transport inhibition in a bovine granulosa cell model

Water transport activity of AQP1 is inhibited by mercurial ion, and the site of mercurial inhibition is the cysteine in the E loop. I have personally verified that using the bovine ovary granulosa cell swelling and shrinkage as a model for mercurial inhibition. The shortest pathway for water to reach the follicle antral cavity is the transcellular pathway directly through the cytoplasm of granulosa cells. In this first experiment we have considered the functional presence of Hg^{2+} -sensitive AQPs in isolated granulosa cells assessing changes in cell volume in presence or absence of HgCl_2 .

2.2 Redistribution of aquaporins in the bitch uterus

Fluid movement through uterine cell membranes is crucial, as it can modulate the tissue imbibition pattern in the different phases of the estrous cycle. To gain insight into the mechanisms underlying steroid-controlled water handling, the presence and distribution of aquaporins (AQPs), integral membrane channel proteins permitting rapid passive water movement, was explored in bitch uterine tissues. Immunohistochemistry and Western immunoblot analysis were used to study the presence of AQP1, AQP2, and AQP5 in the layers of the bitch uterine wall during the different estrous phases. Presence of endothelial nitric oxide-generating enzyme NO synthase (NOS3) was also investigated, as it is known that the vasodilator NOS3 might be involved in the development of uterine edema. We can hypothesize that a functional and distinctive collaboration exists among diverse AQPs in water handling during the different functional uterine phases.

2.3 Role of aquaporins in foetal membranes throughout pregnancy

Balance of water concentration within the uterine environment is important in every reproductive moment of a female mammal life. Previous results from our laboratory showed that AQP-mediated water movement between the intraluminal, interstitial, and capillary compartments is crucial in the uterine imbibition mechanism in the bitch, including periodic stromal oedema in preparation for embryo implantation, with evidence that the expression of different AQPs can be regulated by steroid sex hormones. Also, the importance of the AQP-mediated fluid regulation in uterine environment in the peri-implantation period is known in the woman and laboratory mammals, to assure the successful implantation of blastocyst and its survival. The establishment of pregnancy and its maintenance is dependent on strict synchronization of uterine receptivity with embryonic maturation. Water homeostasis during foetal development is of crucial physiologic importance. Amniotic fluid provides the fluid-filled compartment that is essential for normal foetal growth, movement and development. Amniotic fluid (AF) is created by the flow of fluid from the fetal lung and bladder and reabsorbed in part by fetal swallowing and in part by the transfer across the amnion to the fetal circulation. Placental water flux is an important factor in determining AF volume and fetal hydration, too. In addition fetal membranes might be involved in fluid composition. To understand the mechanisms responsible for maintaining a correct balance of AF volume we evaluated the role of aquaporins (AQPs). Immunohistochemistry were performed to assess the presence of AQP1, 3, 8 and 9 in canine placenta and fetal membranes. Uterine samples were collected on early, middle and late-gestation during ovariohysterectomies (n= 13) performed with owners' acceptance. Changes in AF composition were also analyzed in the different stages of pregnancy. AF changes during gestation may be dependent on modulations in solute as well as water permeability and changes in AQPs during gestation suggest their role in mediating placental and membrane water flow.

2.4 Absorptive activities in the efferent ducts of adult cat studied by Aquaporin immunohistochemistry and lectin histochemistry

AQPs are channel proteins expressed in cell membranes of many epithelia involved in fluid transport and several AQPs have been identified in adult rat excurrent ducts, where considerable fluid resorption occurs. Concerning the morphological changes allowed by the AQP influence in the male reproductive

tract I considered first of all the role of AQP's in the epididymis of a domestic species, the cat, and two animal models, the rat and the lamb.

Ultrastructural features of the epithelium lining the efferent ducts (ED) in the cat, as in other mammalian species, are strongly indicative of an absorptive activity taking place towards the intraluminal fluids. It is well-known that more than 95% of the fluid leaving the testis is reabsorbed by the ED, but the cell structures involved in the resorption processes are still a matter of debate. Previous ultrastructural studies of the epithelium lining the cat epididymal duct indicated that principal cells could be involved in the fluid absorption as well as glycoprotein secretion¹. It is known that modifications of the surface glycoproteins occur during maturation, capacitation and acrosome reaction of spermatozoa, being these glycoconjugates most probably of epithelial origin by a secretory process into the lumen². Therefore, the purpose of the present work was to study the possible absorptive and secretory events taking place in the cat epididymis by 1) the immunohistochemical localization of four different isoforms of Aquaporins (AQP1, 2, 5 and 9), integral membrane water channels that facilitate rapid passive movement of water, and 2) the carbohydrate expression and localization in the epithelial lining, by means of lectin histochemistry, utilizing a panel of 12 lectins in association with sialidase (s) treatment, as previously published³. The study was carried on fragments of adult cat epididymides obtained at orchietomy, fixed in neutral formalin and paraffin embedded.

2.5 Immunolocalization of insl3 in the dog foetal leydig cells and lgr8 receptor in the *gubernaculum testis*

During foetal development, growth of the gubernaculum testis and regression of the cranial suspensory ligament results in transabdominal descent of the testes. INSL3, also known as Leydig insulin-like peptide or relaxin-like factor, is supposed to induce the growth of the gubernaculum in male foetuses, thus being directly responsible for the testicular descent in humans and rodents. This is believed to happen with the LGR8 receptor intervention. This work provides evidence of the immunolocalization of INSL3 in the Leydig cells of dog male foetuses and of LGR8 receptor in different tissues of the gubernaculum testis of the same foetuses. LGR8 was localized at the peripheral cell membrane of muscle and connective cells, as well as of the epithelial cells of the developing excurrent ducts. In parallel, the endocrine compartment of the foetal testis was evaluated for the antimüllerian activity of Sertoli cells and for the androgen-synthetic activity of the Leydig, which were both present in foetuses at term.

2.6 Undernutrition during fetal to prepubertal life affects aquaporin expression in the male genital tract of adult rats

Several AQPs have been identified in adult rat excurrent ducts, where considerable fluid resorption occurs. Their expression in the male excurrent ducts, where water movements mainly take place, is of major importance. It has been demonstrated that testicular and epididymal morphology in adult rats is affected by undernutrition during fetal to prepubertal life (Genovese et al., *Reprod Dom Anim*, 43 suppl 3, 161). To study the influence of pre- and postnatal undernutrition on AQP-expression in the adult male genital tract, 4 pregnant female rats were fed ad libitum (control group) and 4 with 33.5% of gestational feed requirements (underfed group). Feeding restriction of pups born from underfed mothers continued up to weaning (25 days of age), then they were fed ad libitum until 100 days of age, and slaughtered. Epididymides were sampled and processed for aquaporin immunohistochemistry. Results demonstrated a similar expression of AQP1 either in the control and underfed groups of rats, strongly evidenced at the apical and lateral plasma membrane of the efferent ducts non-ciliated cells, in the smooth muscle cells surrounding epididymal duct and at the level of blood vessel endothelium throughout the epididymis. On the contrary AQP9 expression was modified by early life undernourishment, as it was weakly evidenced at the microvilli in the principal cells and completely lacked in the clear cells at the cauda level, in underfed group epididymides. Since it is known that clear cells are involved in luminal fluid acidification, it can be hypothesized that this function might be altered in animals, which were underfed during early life.

2.7 Immunolocalization of leptin (Ob) and leptin receptor (Ob-r) in the ovary and in pre and post in vitro matured horse oocytes: prepuberty and breed effect

Leptin, the hormone product of ob gene expression, is an important endocrine indicator of adipose mass and nutritional status, as well as an important regulator of various aspects of feed intake, growth, metabolism and reproduction. Leptin amount in the blood is proportional to body energy stores and/or body mass, so, inadequate nutrition might impair reproductive function leading, for example, to the delayed onset of puberty. Indeed, the onset of puberty in humans and animals is associated with an increase in fat and consequent increase in circulating leptin, suggesting that leptin may be required for normal growth and development of reproductive organs. A similar relationship between nutrition and reproductive efficiency is not understood in the mare where, besides many reports quantifying the correlation of circulating concentration of leptin with

body condition scores^{1,2}, only few informations exist about the presence of leptin (Ob) and leptin receptor (Ob-R) in the ovary or in the oocyte. Taking this into account, we carried out a study to investigate the immunolocalization of Ob and Ob-R in ovaries and compact cumulus oocytes recovered from fillies and from mares of light or heavy body weight breeds after slaughtering. Both Ob and Ob-R, detected in immature oocytes of all kinds of animals analyzed, were uniformly distributed throughout the ooplasm, but the intensity of reaction was lower either in light weight mares or in fillies oocytes, than in oocytes of heavy weight mares. After IVM, heavy breed mares had a higher proportion of oocytes that reached metaphase II than light mares and fillies ($35.23 \pm 0.006\%$, $19.63 \pm 0.028\%$ and 15.00 ± 0.06 respectively; $P < 0.05$). In matured oocytes both Ob and Ob-R were localized to the oocyte cortex and concentrated at one pole of the oocyte. This distribution within the oocytes was independent from animal group and once again with weaker immunopositivities in light mares and in fillies. Moreover, we localised the presence of Ob in blood vessels within the ovary as well as within the corpora lutea in all kinds of animals analyzed. The results of the present study support the hypothesis that in the horse leptin is differently localized during oocyte IVM showing different immunoreaction intensity related either to the horse breed or to the reproductive puberal development. The presence of leptin in the ovary could suggest a possible involvement in oocyte maturation, angiogenesis, follicle rupture or subsequent corpus luteum formation³. Moreover, leptin may be involved in the determination of the animal pole of the oocyte and in the establishment of the inner cell mass and trophoblast in the embryo.

2.8 The expression of aquaporin 7 in the ovine fetal epididymis is perturbed by in-utero exposure to a cocktail of chemicals contained in sewage sludge fertiliser

Exposure to environmental endocrine disrupting chemicals (EDCs) during pregnancy in animals and humans is associated with reduced sperm counts and increased incidences of testicular cancer and reproductive tract abnormalities. Although male fetuses from pregnant ewes exposed to EDCs contained in sewage sludge fertiliser (a “real life” exposure model) exhibit fewer testicular Sertoli and Leydig cells, the epididymis has not been investigated. The movement of fluids and small solutes in the epididymis is critical for adult sperm maturation and this process is regulated, in part, by the aquaporins. The current study examined the effects of sewage sludge exposure on the expression of aquaporins in the late gestation fetal epididymis.

Pregnant ewes were exposed to sewage sludge fertiliser or control pastures from 0-140 days of gestation. Epididymides were collected post mortem at day 140 of gestation (term = day 145), Bouins-fixed and subjected to immunohistochemistry for Aquaporins 1,4 and 7. Staining intensity was visually assessed (blinded) by 2 observers on an arbitrary 4 point scale. [Animal studies approved by local ethical committee and covered by UK Animals Scientific Procedures (1986)].

Sewage sludge exposure significantly reduced AQP7 immunostaining ($P=0.012$) but had no effect on AQP1 or AQP4 in terms of localisation or immunostaining intensity. These results suggest that exposure to environmental chemicals may affect the capacity of efferent ducts to transport water and small solutes thus influencing fluid resorption, protein synthesis and secretion. Ultimately, this may impact on sperm viability in the adult.

2.9 Preliminary Data on the aqp5 immunodetection in sheep salivary glands related to the pasture vegetative cycle

As well known AQPs are ubiquitarily distributed in the body maintaining all the same the specific function as channels to permeate water and small solutes. I collaborated with the University of Perugia regarding the immunolocalization of AQP5 in the sheep salivary glands in relation to the pasture vegetative cycle. AQP5 is widely expressed in exocrine glands; in particular, in the rat salivary glands AQP5 is highly expressed in the apical plasma membrane of serous acinar cells, in the secretory canaliculi and in the intercalated duct cells, while it is lacking in mucous acinar cells and in striated ducts, indicating its important role in the saliva production (further supported by the observation of markedly depressed rates of salivary secretion in AQP5-deficient mice. Data concerning AQP5 expression in domestic animal species are lacking. In this study the presence of AQP5 was investigated in sheep parotid and mandibular glands, and its expression in the different stages of pasture vegetative cycle was evaluated too. AQP5 was expressed in parotid serous acini and mandibular serous demilunes. In particular, parotid acinar cells showed AQP5 positive sites at the level of apical plasma membrane and intercellular canaliculi; the reactivity was higher when animals were fed on grasslands at the end of vegetative cycle than on grasslands at the maximum of vegetative development. AQP5 was less expressed in the mandibular gland than in the parotid gland and restricted to intercellular canaliculi; variation in reactivity related to diet wasn't observed. AQP5 expression is likely due to its involvement to provide for an appropriate saliva fluidity.

CHAPTER 3

Bovine granulose cell swelling Assay

Report

Bovine granulosa cell swelling assay

Authors: Aralla M¹, Williams L², Luck M²

¹*Department Of Veterinary Sciences And Technologies For Food Safety, Laboratory Of Anatomy, University Of Milan, Italy*

²*School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK*

3.1 Introduction

Gonadotropin stimuli assure the development of primordial follicle into a preantral and antral follicle.

Antrum formation is a very rapid process characterized by a remarkable increase in size that requires a rapid and massive transport of water.

Granulosa cells have been previously characterized as so-called leaky epithelia and water moves from the follicular blood supply to the antrum flowing around them (pericellular transport).

Nevertheless the shortest pathway for water to reach the follicle antral cavity is the transcellular pathway directly through the cytoplasm of granulosa cells. McConnell et al [1] suggest that 70% of fluid movement across the follicular wall occurs by the transcellular route.

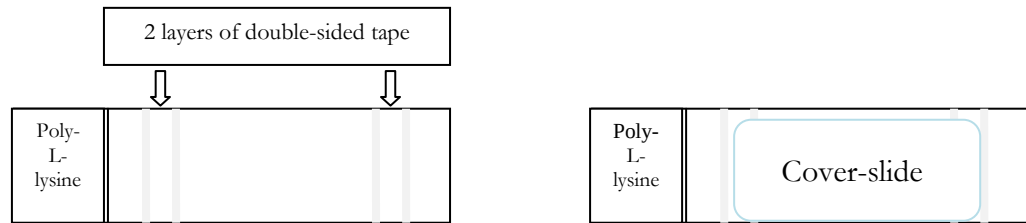
We recognize two mechanisms for transcellular water movement: the simple diffusion, a slow and unregulated process; and through a class of small, hydrophobic integral membrane proteins that facilitate rapid passive movement of water termed aquaporins (AQPs). Identification of ovarian follicular AQPs is incomplete but McConnell et al [1] suggest that fluid movement is mediated by AQPs -7, -8 and -9.

Each of the mammalian AQPs (except AQP4 and -7) contains extracellular mercurial-sensitive sites in which Hg²⁺ binds an extracellular cysteine residue and inhibits water movement through the pore.

In this first experiment we have considered the functional presence of Hg²⁺-sensitive AQPs in isolated granulosa cells assessing changes in cell volume in presence or absence of HgCl₂.

3.2 Materials And Methods

- Make up 16 incubation chambers [2] and name them (A Iso-Hg, B Iso+Hg, C Hypo-Hg, D Hypo+Hg)



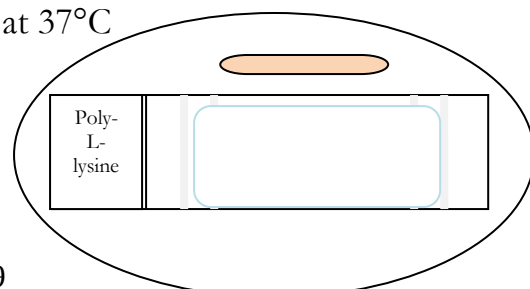
- Prepare 16 dishes, some jars, a loop, some needles and a syringe

Buffers

- ISOTONIC MEDIUM: McCoy's 5a + Hepes (MH)
 - ✓ 100 ml McCoy's 5a
 - ✓ 0,47 g Hepes (20 mM)
 - ✓ 319 mOsm
- HYPOTONIC MEDIUM (5ml)
 - ✓ 70% dH₂O
 - ✓ 30% MH
 - ✓ 95 mOsm
- MERCURIAL AGENT: McCoy's 5a + HgCl₂ (50 µM) 5ml
 - ✓ 7 mg HgCl₂ dil. In 1 ml MH (25 mM) → 10 µl HgCl₂ dil. In 5 ml MH (50 µM)

Granulosa cells collection

- Choose ovaries (growing follicles)
- Choose 4 best ones (→PBS)
- Suck up follicular fluid from the follicle with a syringe
- Pinch the follicle, cut it on the surface and fill it up with MH
- Brush the follicle wall with a loop and suck up the fluid (MH and granulosa cells) with a sterile Pasteur pipette
- Put the fluid into a plastic tube
- Repeat it 4 times and check that none of the aspirated cell preparations coagulate
- Put the whole collected fluid in a single tube and analyze the cellular aspect with a specific count chamber
- Distribute the granulosa cells obtained in each incubation chamber.
- Incubate the cells with MH for 1h at 37°C



- Divide the 16 chambers in 4 groups depending on treatment (Isotonic or Hypotonic medium; with or without incubation with HgCl₂), as shown in the schedule below.

- 4 X ISO→ISO - HgCl₂ A 1-4
- 4 X ISO→ISO + HgCl₂ B 1-4
- 4 X ISO→HYPO – HgCl₂ C 1-4
- 4 X ISO→HYPO + HgCl₂ D 1-4

	A	B	C	D
1	ISO→ISO – HgCl ₂	ISO→ISO + HgCl ₂	ISO→HYPO – HgCl ₂	ISO→HYPO + HgCl ₂
2	ISO→ISO – HgCl ₂	ISO→ISO + HgCl ₂	ISO→HYPO – HgCl ₂	ISO→HYPO + HgCl ₂
3	ISO→ISO – HgCl ₂	ISO→ISO + HgCl ₂	ISO→HYPO – HgCl ₂	ISO→HYPO + HgCl ₂
4	ISO→ISO – HgCl ₂	ISO→ISO + HgCl ₂	ISO→HYPO – HgCl ₂	ISO→HYPO + HgCl ₂

- Medium → HgCl₂ → Medium
1 h 15' 30"
Iso +/- Hypo
- Start off with 1 group (A1, B1, C1, D1...and so on)
- Take a picture of the earlier state (Isotonic medium for all of the slides) and after 15 mins (+HgCl₂, -HgCl₂). Wash the slides with isotonic medium any treatment change. When the hypotonic medium is infused into the chamber (C and D groups) take the second photograph 30 second after infusion.
- Ensure that photographs contain an appropriate scale marker
- Measure the diameter of six cells for each photograph taken
- Analyse the combined data with Two Way ANOVA

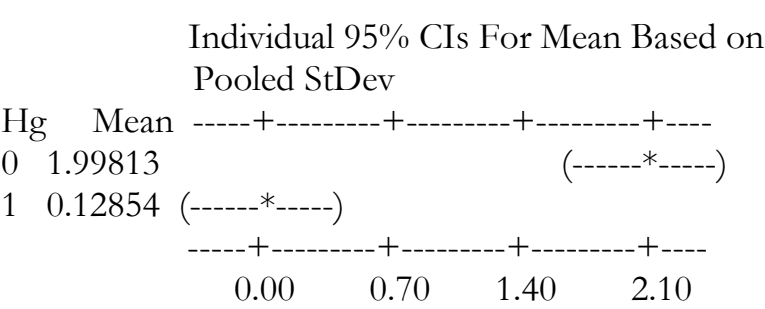
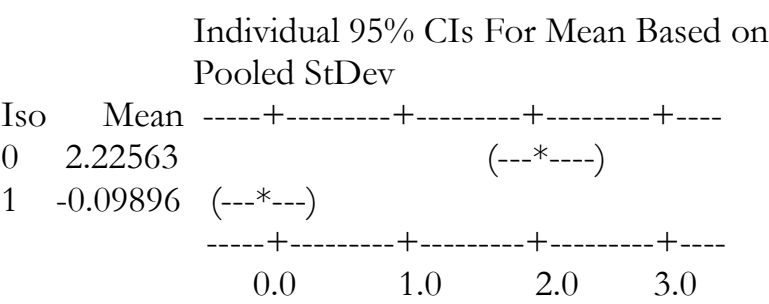
3.3 Results

Two-way ANOVA: Diameter versus Isotonic Medium and in presence of Hg

Source	DF	SS	MS	F	P
Iso	1	21.6148	21.6148	64.07	0.000

Hg	1	13.9814	13.9814	41.45	0.000
Interaction	1	11.4695	11.4695	34.00	0.000
Error	12	4.0481	0.3373		
Total	15	51.1137			

S = 0.5808 R-Sq = 92.08% R-Sq(adj) = 90.10%

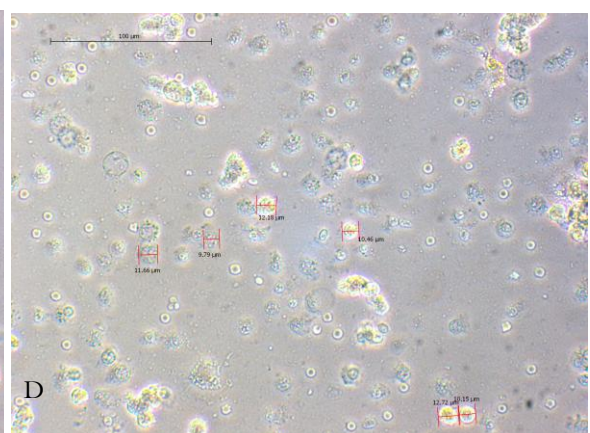
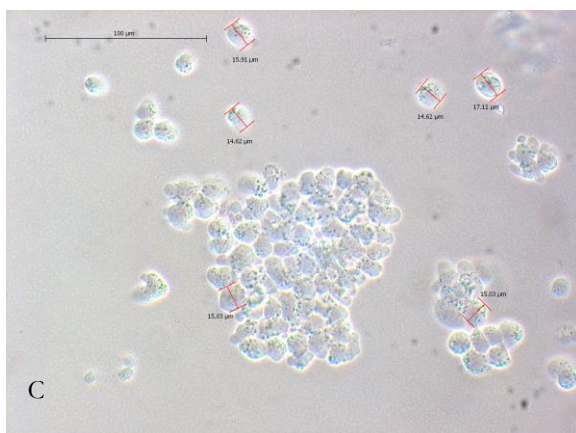
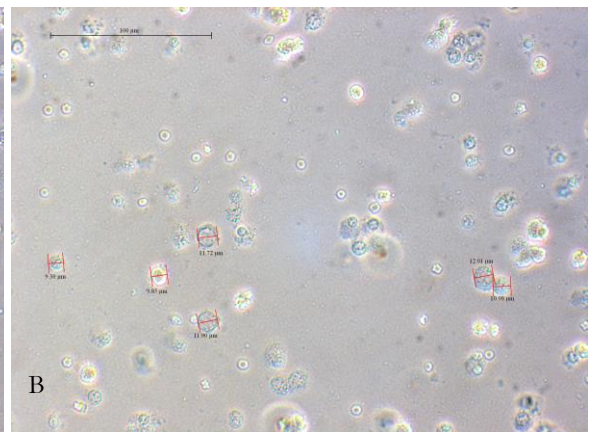
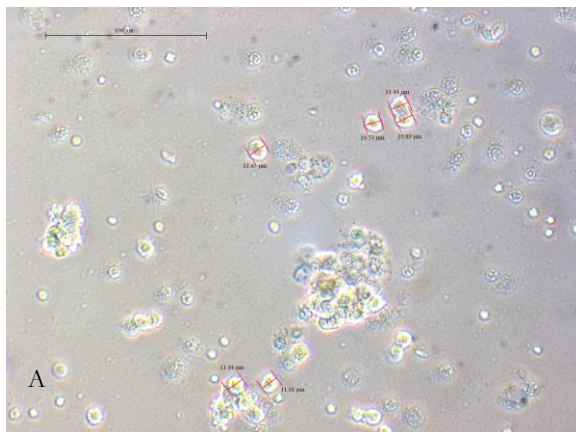
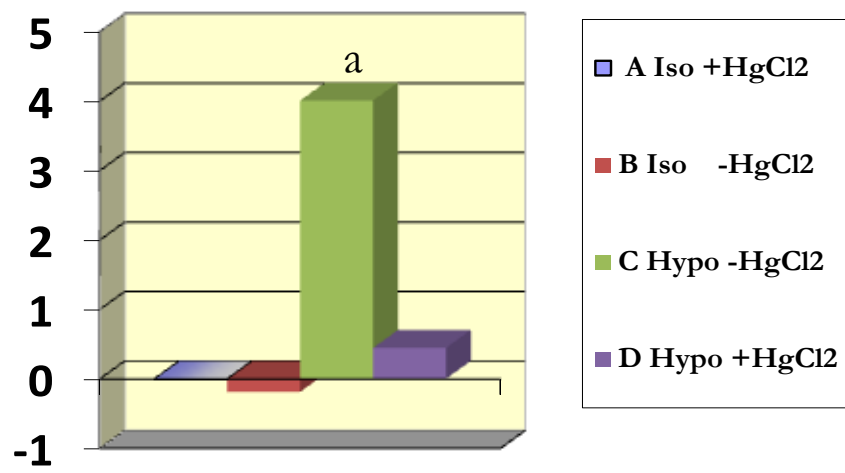


LEAST SIGNIFICANT DIFFERENCE TEST (LSD)

$$LSD = \left[\sqrt{\frac{\text{Error MS}}{n}} \times 2 \right] \times t_{\alpha} = 0.41067 \times t_{\alpha}$$

t 12 =	2.179	P<0.05	0.8949
	3.055	P<0.01	1.2546
	4.318	P<0.001	1.773

A Average	-0.01083
B Average	-0.18708
C Average	4.007083
D Average	0.444167



3.4 Discussion

Granulosa cells showed a significant ($p < 0.001$) swelling when incubated with hypotonic medium in absence of HgCl₂ (group C).

HgCl₂ prevented granulosa cell swelling in hypotonic medium as showed in D group in all likelihood because of mercurial inhibition on Aquaporins action.

Nevertheless HgCl₂ was unable to totally block the swelling in D group suggesting an alternative remaining way of simple diffusion of water through the lipid bilayer. Despite that data demonstrated that the changing in volume in D group is not statistically relevant in comparison to A and B group.

Our data confirmed functional activity of Aquaporins in granulosa cells as previously verified by McConnel et al. in rats [1].

3.5 References

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CHAPTER 4

A collaboration of aquaporins handles water transport in relation to the estrous cycle in the bitch uterus

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A collaboration of aquaporins handles water transport in relation to the estrous cycle in the bitch uterus

M. Aralla^a, V. Borromeo^b, D. Groppetti^c, C. Secchi^b, F. Cremonesi^c, S. Arrighi^{a,*}

^a Department of Veterinary Sciences and Technologies for Food Safety, Laboratory of Anatomy, University of Milan, I-20134 Milan, Italy

^b Department of Veterinary Pathology, Hygiene and Public Health, Biochemistry and Physiology Unit, University of Milan, I-20134 Milan, Italy

^c Department of Veterinary Clinical Science, Reproduction Unit, University of Milan, I-20134 Milan, Italy

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Abstract

Fluid movement through uterine cell membranes is crucial, as it can modulate the tissue imbibition pattern in the different phases of the estrous cycle. To gain insight into the mechanisms underlying steroid-controlled water handling, the presence and distribution of aquaporins (AQPs), integral membrane channel proteins permitting rapid passive water movement, was explored in bitch uterine tissues. Immunohistochemistry and Western immunoblot analysis were used to study the presence of AQP1, AQP2, and AQP5 in the layers of the bitch uterine wall during the different estrous phases. Presence of endothelial nitric oxide-generating enzyme NO synthase (NOS3) was also investigated, as it is known that the vasodilator NOS3 might be involved in the development of uterine edema. The results demonstrated the following: (1) AQP1, AQP2, and AQP5 were present in the uterus of cycling bitches. (2) AQP1 was localized within uterine mesometrial, myometrial, and endometrial blood vessels and in the circular and longitudinal layers of myometrium. AQP1 localization and expression were unaffected by the estrous cycle. (3) The estrogenic milieu was probably at the basis of AQP2 expression in the glandular and luminal epithelium of the endometrium. (4) AQP5 water channels were present in the apical plasma membrane of uterine epithelial cells in coincidence with plasma progesterone increase. (5) NOS3 was localized in the myometrial and epithelial tissues as well as in blood vessels indicating a contribution of this vasoactive peptide to the uterine imbibition processes. Thus, we can hypothesize that a functional and distinctive collaboration exists among diverse AQPs in water handling during the different functional uterine phases.

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Keywords: Aquaporins; Bitch uterus; Estrous cycle; Immunohistochemistry; Western blot

1. Introduction

In the endometrium at the cellular level, hydrostatic and osmotic forces regulate water balance across membrane barriers via specific ion pumps, ion channels, and solute transport or exchange proteins [1]. Water transport across an epithelial barrier can occur by

transcellular or paracellular methods. The paracellular pathway of fluid transport is especially regulated by the tight junction complex [2]. Transcellular, osmotically driven fluid transport across the lipid bilayer of cells can occur by diffusion alone or through specialized transporters, known as aquaporins (AQPs). Aquaporins are a class of small, hydrophobic, integral membrane proteins that facilitate rapid passive movement of water [3]. Structurally, the AQP protein contains six transmembrane helical domains spanned through the lipid bilayer in such a way to form two hemichannels, each with a highly conserved NPA (asparagine, proline,

* Corresponding author. Tel.: +39 0250315741; fax: +39 0250315746.

E-mail address: silvana.arrighi@unimi.it (S. Arrighi).

alanine) sequence. Both N- and C-termini are intracellular. Hemichannels are formed by loops B and E and give origin to an hourglass-shaped channel [4] through which water may pass. The NPA motif ties and then releases the water molecules permitting the crossing through the pores in the opposite direction, driven by an osmotic gradient through the membrane. AQPs exist in plants, bacteria, insects, and several members of the animal kingdom. To date, 13 mammalian AQPs have been identified, and all are highly permeable to water. AQPs 3, 7, 9, and 10 are also permeable to glycerol and some small solutes and are known as aquaglyceroporins. AQPs are located in various cells and tissues, such as kidney, lung, pancreas, brain, gastrointestinal tract, eye, ear, immune system, skin, adipose tissue, muscles, uterus, and testis [5–10]. Their presence and functional significance in female and male reproductive systems was recently reviewed by Huang et al. [11].

Previous studies on the localization of aquaporins in the female reproductive tract, in pregnant mouse and rat uterus and cervix proved that AQP1, AQP2, and AQP5 are involved in uterine fluid homeostasis, and their expression can be regulated by steroid sex hormones [11–15]. In the rat, AQP1 water channels increase in blood vessels of the endometrial stroma to provide a mechanism for the formation of the stromal edema [16]. Recently, AQP1 was found to be present in the rat circular myometrial layer and might be slightly regulated by ovarian steroid hormones [17]. AQP2 expression was demonstrated in human uterine endometrium, correlated with serum 17 β -estradiol (E2) and progesterone concentrations [18]. Moreover, AQP5 in rat uterus was shown in the apical plasma membrane of luminal epithelial cells in response to progesterone and provides a mechanism for the reabsorption of luminal fluid [16]. Thereby, movements of fluids from intracellular to extracellular compartments and back, being a fundamental aspect of the physiologic phases of the reproductive cycle, hesitate in different degrees of tissue imbibition.

The cycling endometrium undergoes recurrent uterine stromal edema in response to hormonal stimuli and several vasoactive mediators. The ovarian steroid hormones play an important role in changes that occur during the estrous cycle in the cervix, too. Changes in cervical connective tissue, such as reorganization of the collagen network, increase of cervical water content, and a shift in glycosaminoglycan composition, take place during gestation, with a relative increase in hyaluronic acid and decrease in chondroitin and dermatan sulfate, thus allowing cervical dilation at labor [19]. It is hypothesized that AQPs intervene in these events, too [15].

Yet further, previous studies have suggested that in the development of uterine edema, a role is also played by nitric oxide (NO), a potent vasodilator [20]. Roberto da Costa et al. [21] have proved that endothelial NOS is present in endometrial glands, endothelial cells, fibroblasts, blood, and lymphatic vessels. Endometrial endothelial Nitric Oxide Synthase (eNOS) expression is the highest in the follicular and mid-luteal phases.

Hence, the aim of this study was to investigate the presence of AQP1, AQP2, AQP5, and endothelial NOS in the bitch uterus, likely to be responsible for balancing water concentration during different phases of the estrous cycle. Serum progesterone levels were monitored in parallel to precisely time the estrous cycle of the bitches the day of ovario-hysterectomy and in search of a possible relationship with the ovarian steroid hormone levels, known to be responsible of the morphologic modifications in the uterus. Even considering the differences existing in the uterine physiology among species, the purpose of this work was also to add general information to the mechanisms underlying the steroid-stimulated water transport in the female reproductive tract.

2. Materials and methods

2.1. Animals

Uterine tissues were obtained from 18 healthy bitches of different breeds (aged from 6 months to 5 years) presented for routine ovario-hysterectomy at the Reproduction Unit of the Department of Veterinary Clinical Science of the University of Milan. The functional phase of the estrous cycle was determined by accurate clinical examination, vaginal cytology, plasma concentration of progesterone, macroscopic inspection of the surgically removed organs, and histologic analysis of the endometrium. Vaginal smears were stained by a modified Romanovsky stain set (Hemaquick[®]; Biomed, Munich, Germany).

Upon assessment of all the parameters, the animals were assigned to the following estrous categories: prepuberal, $n = 1$; proestrus, $n = 3$; estrus, $n = 3$; diestrus, $n = 8$ (4 of which in late diestrus); anestrus, $n = 3$.

2.2. Plasma progesterone measurement

Blood samples for analysis of progesterone were taken into heparinized tubes. The plasma progesterone (P4) concentration was determined using an enzyme linked fluorescent assay (ELFA) quantitative test (MiniVidas; BioMérieux, Craponne, France), which is a combination of an enzyme immunoassay competition method with a final fluorescent detection [22]. The intra-assay coefficient of variation was 4.5% and the interassay coefficient of variation was 7.6%.

2.3. Histology

Immediately after ovario-hysterectomy, tissue specimens were collected from different uterine regions, one sample from the middle part of each uterine horn, another at the body bifurcation, and a final one from the cervix. Both transverse and longitudinal sections were taken. Pieces were fixed in formalin 10% for 24 h at 4 °C. After fixation, fragments were dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections were cut at 4 µm thickness, de-waxed, and stained with routine hematoxylin and eosin (H&E) for histologic examination.

2.4. Immunohistochemistry

Tris-buffered saline (TBS; 0.05 M Tris/HCl, 0.15 M NaCl) buffer was used for rinses throughout the whole procedure.

After de-waxing, formalin-fixed serial sections (4 µm thick) were washed and immersed in a freshly prepared 3% H₂O₂ solution for 15 min to block the endogenous peroxidase activity, followed by incubation in 1:20 normal goat serum (DakoCytomation, Glostrup, Denmark, code no. X 0907) 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 antibodies against rat aquaporin 1, aquaporin 2, aquaporin 5 (Alpha Diagnostic International, San Antonio, TX, USA; cat. no. respectively AQP11-A, AQP21-A, AQP51-A) and human NOS3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; N-20; cat. no. sc-653) diluted 1:100 (AQP1 and AQP2), 1:30 (AQP5), and 1:50 (NOS3) in specific antibody diluent (DakoCytomation; code no. S 302283). The sections designed to be incubated with AQP5 and NOS3 antibodies were mounted onto poly-L-lysine-coated slides then pretreated with two microwave cycles (2 × 5 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 biotinylated goat anti-rabbit immunoglobulins (DakoCytomation; code no. E 0432) diluted 1:200. Either streptavidin-biotin complex (StrepABCComplex/HRP; DakoCytomation; code no. K 0355) or Envision System Labeled polymer-HRP (DakoCytomation; code no. K 4002) were used as revelation system. Immunoreactive sites were visualized using a freshly prepared solution of 4 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, Milan, Italy) in 10 mL of a 0.5 M Tris buffer at pH 7.6 containing 0.1 mL 3% H₂O₂ for 13 to 20 min. Sections were counterstained with Mayers' hematoxylin, dehydrated, and mounted using Eukitt (Bioptica, Milan, Italy).

2.4.1. Staining controls

Sections of rat uterus, similarly processed as above, served as positive controls for immunoreactions to AQP1 and AQP5 antisera. Positive control for AQP2 immunoreaction was performed using sections of dog bladder and kidney. The specificity of the immunostainings was tested by negative controls, performed by (1) use of nonimmune rabbit serum

(DakoCytomation; code no. X 0903) in place of specific antisera; (2) incubating sections with antisera preabsorbed with the respective antigens (Alpha Diagnostic International, cat. no. AQP11-P, AQP21-P, AQP51-P; Santa Cruz Biotechnology, Inc., NOS3 blocking peptide, code no. sc-653 P); and (3) omission of the first layer. All of them resulted in the absence of immunoreaction.

The evaluation of staining intensities was based on subjective estimates of all the authors. Slides were observed and photographed under an Olympus BX51 photomicroscope equipped with a digital camera and DP software (Olympus, Tokio, Japan) for computer-assisted image acquirement and managing.

2.5. Western immunoblot analysis

Full-thickness cross sections of various uterus regions were frozen immediately after ovario-hysterectomy and stored at –80 °C until assayed. For protein extraction, we selected three specimens of uterine horns for each estrous category (proestrus, estrus, diestrus, and anestrus). The endometrium was separated from the myometrium by careful scraping using a scalpel blade, and 50 mg of each sample was homogenized in 500 µL Radio Immuno Precipitation Assay (RIPA) lysis buffer containing 50 mM Tris-HCl pH 8, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5 % deoxycholate, and protease inhibitors (10 µg/mL leupeptin, 10 µg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was incubated on ice for 40 min and was then centrifuged at 30,000 × g for 15 min. The insoluble fraction was discarded. The protein content of each fraction was determined by the method of Lowry et al. [23]. Proteins were diluted in sample buffer (2.5% SDS, 0.05 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol) and heated at 100 °C for 3 min. Samples at 32 µg/lane were separated on a 7% to 15% self-cast polyacrylamide gradient gel (Mighty Small II SE-250; Hoefer, Holliston, MA, USA) according to Laemmli [24]. Molecular weight markers were Precision Plus Protein WesternC standards (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, proteins were transferred by electroblotting to nitrocellulose sheets (pore size, 0.2 µm; Schleicher & Schuell, Dassel, Germany). After incubating for 1 h with blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in TBS), blots were incubated overnight with agitation at 4 °C with the respective primary antibodies in blocking buffer as follows: AQP1 (1:1000), AQP2 (1:200), and AQP5 (1:100) (Alpha Diagnostic International). The membranes were then washed in TBS-T buffer, incubated for 60 min with a 1:3000 dilution of donkey anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK; cat. no. 711035152) in blocking buffer, washed in TBS-T, and the signals detected using the enhanced chemiluminescence technique (Immun-Star HRP substrate kit; Bio-Rad Laboratories) and the ChemiDoc XRS imaging system (Bio-Rad Laboratories). As negative controls, the primary antibody incubation was omitted, and no signal was observed in any of the control blots. As control for loading, the membranes were also overnight incubated with monoclonal anti-β-actin antibody (Clone

AC-15; Sigma; cat. no. A5441) at 1:20,000 dilution at 4 °C, and an anti-mouse secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulins; DakoCytomation, Glostrup, Denmark) was used at a dilution of 1:10,000.

2.5.1. Densitometry and statistical analysis

Protein expression levels were determined by densitometry of the bands using Quantity One software (Bio-Rad Laboratories). Normalized densities expressed as arbitrary units were determined with ratio of band density of each AQP to band density of β -actin, which was used as an internal reference. All data are presented as the mean $\pm$ SEM ($n=3$). The significance of differences between groups was calculated by a one-way analysis of variance followed by Bonferroni test using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

3. Results

Figs. 1–5 summarize the immunohistochemical results according to the uterine functional moments, each of them characterized by the histologic aspect of the endometrium (Figs. 1A, 2A[a], 3A, 4A, 5A), the vaginal smear (Figs. 2B, 3B, 4B, 5B), and the blood progesterone level, as indicated in the figure legends.

Figs. 6–8 show representative immunoblot images assessing the presence of AQP1, AQP2, and AQP5 in the endometrium and myometrium of uterine horns during the estrous cycle and the total mean aquaporin expression levels measured by densitometry.

3.1. AQP1 immunoreactivity

Anti-AQP1 immunoreactivity was found in all phases of the bitch estrous cycle, in every uterine

region considered, and differently distributed in the layers of the uterine wall.

At the level of the uterine horn and body, AQP1 immunoreactivity was present in several anatomic structures, including the blood vessel wall, the glandular epithelium of the endometrium, and the smooth muscle cells of the myometrium.

AQP1-IR blood vessels were present in every layer of the uterine wall, including the endometrial lamina propria, myometrium, meso- and perimetrium. AQP1 immunoreactivity was present in venous and arterial vessels, as well as in capillaries. Endothelial cells showed immunoreactivity in venous vessels (Figs. 1B, 2C, 5C) and capillaries (Figs. 1B and 3C), whereas in arteries and arterioles immunoreaction was present in the smooth muscle cells of the tunica media (Figs. 2D and 5C).

Cells of the endometrial glands showed a faint cytoplasmic AQP1 immunoreactivity.

In the myometrium, immunoreactivity was shown in both the longitudinal and the circular layers, localized at the plasmatic membrane of the smooth muscle cells, in the form of tiny immunoprecipitates (Figs. 1B and 3C).

In the uterine cervix, AQP1 immunoreactivity was present at the levels of blood vessels and musculature (Fig. 4C), and the staining was also evident in the epithelial cells lining the lumen and in the mucosal glandular cells.

The expression of AQP1 was confirmed by immunoblot analysis of uterine wall protein extracts. In Western blotting, the anti-AQP1 antibody recognized a single band, with calculated size of 63 kDa (Fig. 6A), which most likely is the AQP1 dimer. In fact, all AQPs

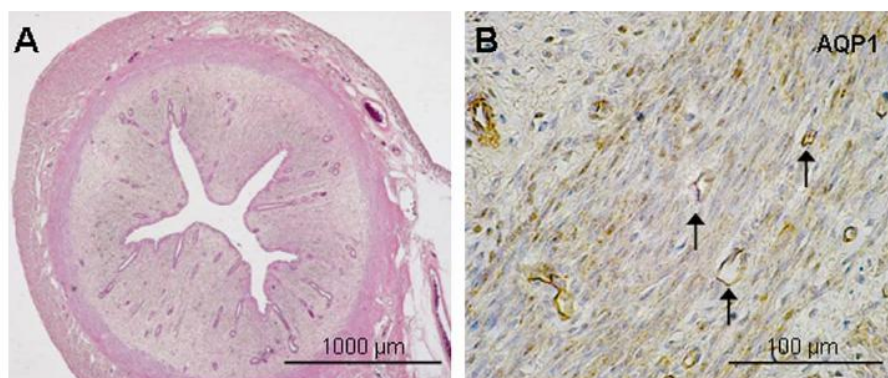


Fig. 1. Uterine horn, sexually immature bitch. Progesterone level <1 ng/mL. (A) Transverse section shows an immature endometrium with few glands, delicate stroma, and poorly developed myometrium. Hematoxylin and eosin stain. Scale bar = 1000 μ m. (B) AQP1 immunoreactivity is present in the circular layer of the myometrium at the level of smooth muscle cells and capillaries (arrows). Scale bar = 100 μ m.

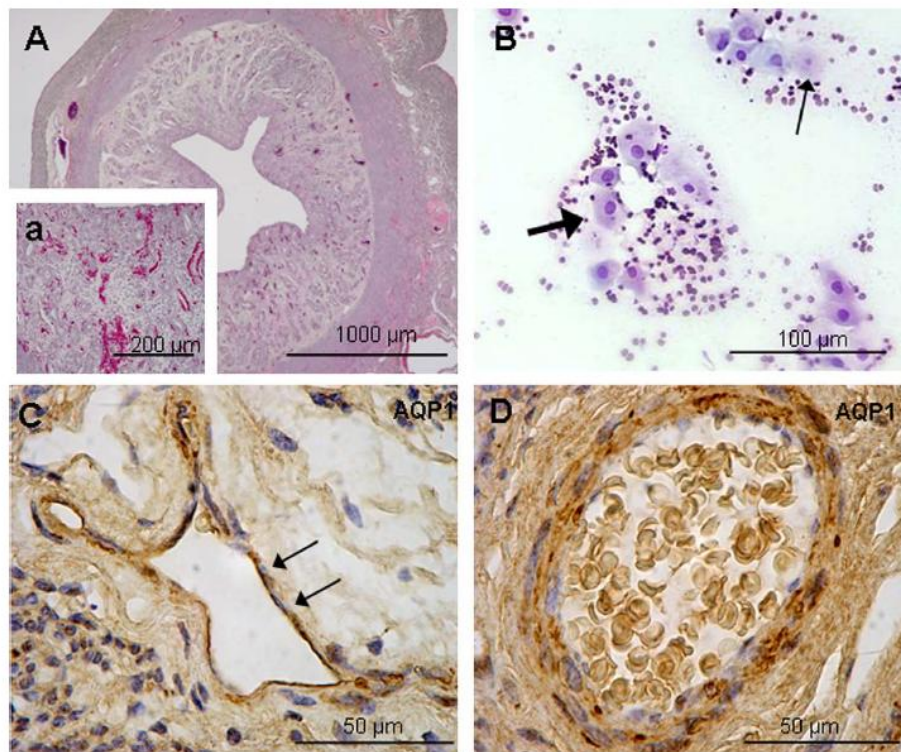


Fig. 2. Uterine horn, proestrus. Progesterone level 2.6 ng/mL. (A) Typical “X-shaped” lumen can be seen in transverse section. Proliferating endometrial glands can be seen throughout the tunica mucosa, correlated with early increase of progesterone levels. Slight stromal edema of endometrium is also present. Hematoxylin and eosin stain. Scale bar = 1000 µm. Inset (a): In the endometrium, hyperemia is associated with extravasation of erythrocytes from capillaries. Scale bar = 200 µm. (B) Vaginal smear: A small number of parabasal cells (thick arrow) and early increase of intermediate cells (thin arrow) can be seen. Typically, red blood cells are present in large numbers and neutrophils are commonly observed. Hemaquick stain. Scale bar = 100 µm. (C) Vascular endothelial cells are strongly positive for AQP1 protein (arrows). Scale bar = 50 µm. (D) AQP1 protein is present in the smooth muscle cells of the arterial tunica media and in red blood cells. Scale bar = 50 µm.

are organized *in vivo* as homotetramers of single-chain subunits of around 29 kDa, and incomplete breakdown to monomers in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) has been reported in several papers on mammalian AQPs (see Ref. [25] and references therein). How AQPs are able to maintain a dimerized state in denaturing gels is unclear. AQP1 signal was present in both the proteins extracted from the endometrium and myometrium, and there were no significant differences in the abundance of AQP1 expression during the various phases of the estrous cycle (Fig. 6B).

3.2. AQP2 immunoreactivity

AQP2 immunoreactivity was only present in the uterus of bitches in proestrous/estrous phases, having

low progesterone levels. Immunoreaction was found in every uterine tract considered, localized at the apical membrane of the glandular epithelial cells (Fig. 3D), as well as in the smooth muscle cells of the myometrium (Fig. 3E). Blood vessel musculature was immunoreactive, too, limited to the estrous phase of the cycle. Uterine samples in diestrous or late diestrous phases, when levels of hematic progesterone are higher, exhibited no AQP2 staining. Moreover, the prepuberal and the anestrus bitches with progesterone levels lower than 1 ng/mL showed no AQP2 immunoreactivity.

In Western blotting analysis of uterine wall protein extracts, the AQP2 appeared as single band of a calculated molecular weight of 58 kDa (Fig. 7A). This size most likely corresponds with the AQP2 dimer, as discussed above for AQP1. AQP2 band was detected in

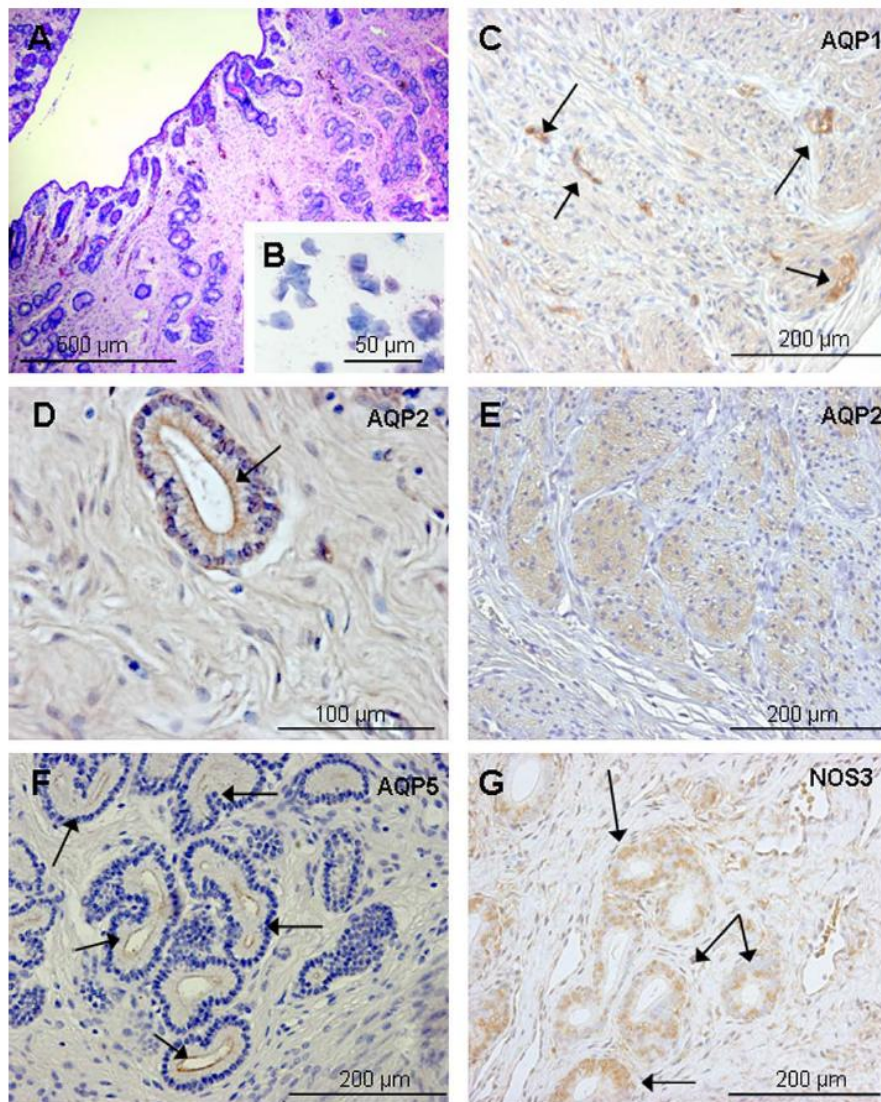


Fig. 3. Uterine horn, estrus. Progesterone level $4.5 < x < 18.18$ ng/mL. (A) After ovulation, stromal edema decreases; uterine glands are numerous and hypertrophic. Hematoxylin and eosin stain. Scale bar = 500 μ m. (B) Vaginal smear shows anucleated cells (flake cells); rare erythrocytes and leukocytes. Hemaquick stain. Scale bar = 50 μ m. (C) AQP1 is present in the longitudinal layer of the tunica muscularis in the form of tiny cytoplasmic immunoprecipitates. There is evidence of immunostaining in the myometrial capillaries (arrows). Scale bar = 200 μ m. (D) Immunohistochemical localization of AQP2 in the glandular epithelium of endometrium: The apical cell membrane is intensely AQP2-immunoreactive (arrow). Scale bar = 100 μ m. (E) AQP2 immunostaining is present in the longitudinal layer of myometrium. Scale bar = 200 μ m. (F) Glandular epithelia show faint immunostaining for AQP5 in the estrous phase (arrows). Scale bar = 200 μ m. (G) Uterine edema coincides with increased NOS3 immunohistochemical localization, which can be seen in glandular epithelia (arrows), in blood vessels, and red blood cells. Scale bar = 200 μ m.

all the estrous cycle phases considered, both in the endometrium and myometrium.

Densitometric analyses (Fig. 7B) showed that in the myometrium, the AQP2 expression levels were inde-

pendent from the estrous cycle phase, whereas in the endometrium the AQP2 quantity was significantly higher in the follicular phase than in the luteal phase (proestrus vs. anestrus, and estrus vs. anestrus, $P < 0.05$).

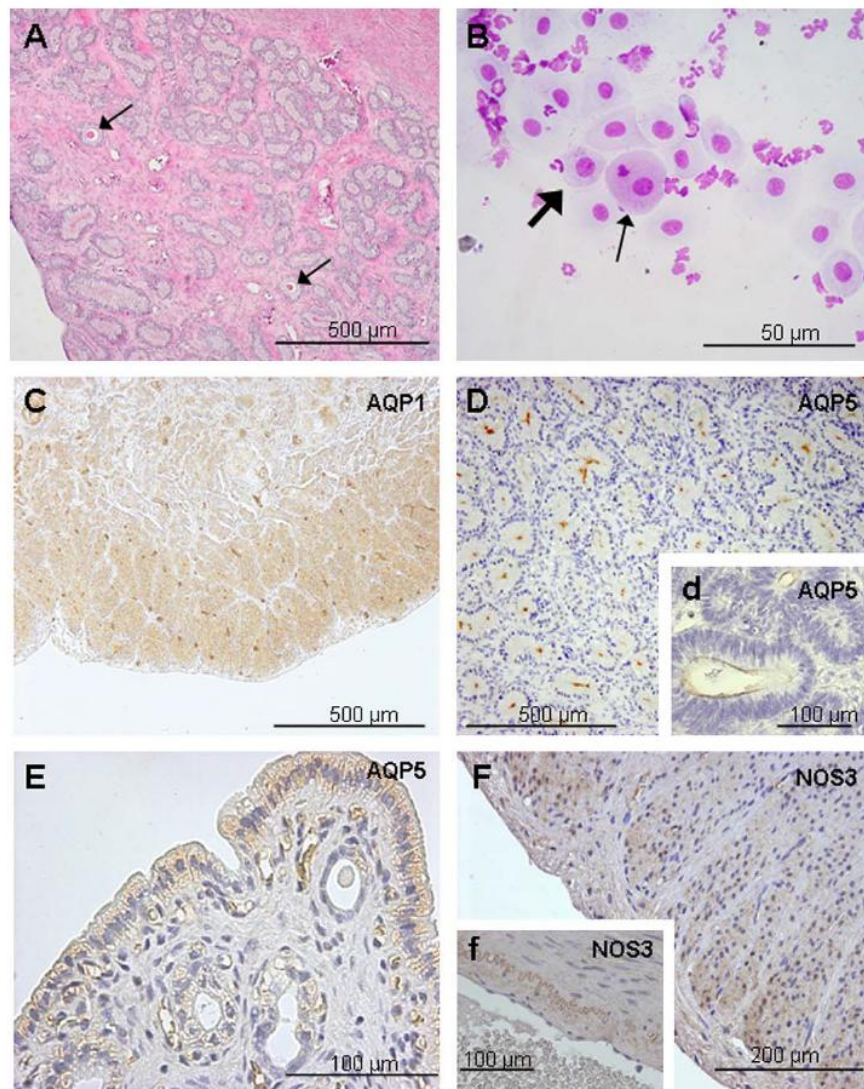


Fig. 4. Uterine horn and cervix, diestrus. Progesterone level 44.73 (early diestrus) $> x > 3.08$ ng/mL (late diestrus). (A) Early diestrus. The endometrium of the uterine horn shows numerous well-differentiated, basally coiled glands and luminal crypts with abundant cytoplasm. Arrows indicate secretory products. Hematoxylin and eosin stain. Scale bar = 500 μ m. (B) The onset of diestrus is marked by a precipitous decline in the number of intermediate cells (thin arrow) and reappearance of intermediate/parabasal cells (thick arrow) in the vaginal smear. Neutrophils are abundant. Hemaquick stain. Scale bar = 50 μ m. (C) Cervix, late diestrus. AQP1 immunoreactivity can be seen in blood vessels and capillaries and in myometrial musculature. Scale bar = 500 μ m. (D) Early diestrus, high level of serum progesterone. Very coiled endometrial glands show intense AQP5 staining in the uterine horn. Scale bar = 500 μ m. Inset (d): Detail of AQP5 immunostaining in the apical membrane of uterine glandular epithelium. Scale bar = 100 μ m. (E) Cervix, middle diestrus. Immunolocalization of AQP5 in the cytoplasm of the epithelial luminal cells. Scale bar = 100 μ m. (F) Late diestrus. Typical pattern of endothelial NOS immunoreactivity in the smooth muscle cells of the longitudinal layer of myometrium. Scale bar = 200 μ m. Inset (f): Immunolocalization of NOS3 in the tunica media of an arterial blood vessel and in the red blood cells. Scale bar = 100 μ m.

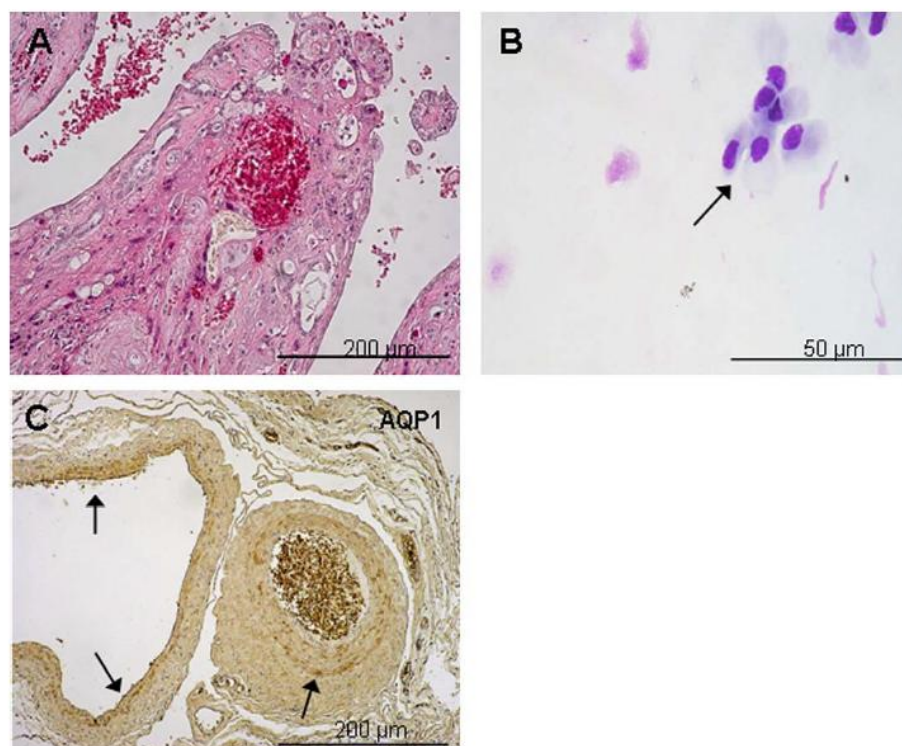


Fig. 5. Uterine horn, anestrus. Progesterone level <1 ng/mL. (A) The surface epithelium consists of cubic cells with big round nuclei; extravasation of blood cells into the stroma is remarkable. Hematoxylin and eosin stain. Scale bar = 200 μ m. (B) Parabasal cells (arrow) predominate in smears taken during anestrus. Superficial cells are absent. There is no evidence of neutrophil granulocytes and bacteria. Hemaquick stain. Scale bar = 100 μ m. (C) Slight AQP1 immunostaining can be seen in blood vessel walls (arrows) and capillaries. Scale bar = 200 μ m.

3.3. AQP5 immunoreactivity

AQP5 immunoreactivity was detected in the apical membrane of both glandular and luminal epithelium of the endometrium (Figs. 3F, 4D[d]). There was no evidence of AQP5 immunostaining in the myometrium or stromal vasculature. The staining of AQP5 was highly restricted to specific hormonal stimulation. The immunolocalization occurred only in diestrus (progesterone ranging from 44.73 ng/mL to 3.08 ng/mL) and was specially pronounced in response to high levels of progesterone.

Also in the cervix, AQP5 was present in the early and middle diestrus. AQP5 staining was marked in the epithelium lining the luminal surface (Fig. 4E). The staining pattern was consistent in the cytoplasm and cell membranes of the epithelial cells.

Protein extracts of the uterine horns separated by SDS-PAGE and probed with anti-AQP5 antibody

showed a single band, with an apparent size of 45 kDa (Fig. 8A). It has been previously suggested that a 42- to 45-kDa band detected by Western blotting of membrane proteins of cells stably transfected with AQP5 represents dimers of AQP5 [26,27]. Highly hydrophobic integral membrane proteins such as AQPs often atypically run in SDS-PAGE [28]. The AQP5 signal was rather low, and it was evident only in the uterine endometrium. Increasing the concentration of the anti-AQP5 antibody over the 1/100 dilution only led to an increased background signal. The replicate Western blot analyses to measure the AQP5 expression levels in various estrous cycle stages were therefore carried out at the 1/100 dilution just on the endometrium specimens (Fig. 8B). AQP5 was detected in all estrous cycle phases, and its expression levels in the endometrium were higher in estrus and diestrus than in proestrus and anestrus ($P < 0.05$) (Fig. 8B).

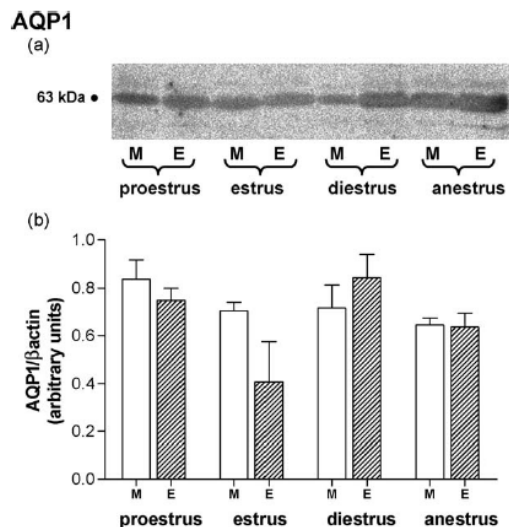


Fig. 6. Expression of AQP1 assessed by Western blotting in the uterine horn wall during estrous phases. (A) Each lane was loaded with 32 mg of protein extracted from the myometrium (M) or the endometrium (E), and blots were probed with anti-AQP1 antibodies. (B) Densitometric analyses of AQP1 protein levels in the myometrium and endometrium normalized against β -actin. Values are the mean $\pm$ SEM (n = 3).

3.4. NOS3 immunoreactivity

Endothelial NOS was immunohistochemically localized in the luminal and glandular epithelia of the bitch endometrium (Fig. 3G) and in the layers of the blood vessel wall (Fig. 4F[f]). The smooth muscle cells of the longitudinal layer of myometrium were positive, too (Fig. 4F). This pattern was principally observed during the luteal phase. In particular, the evidence of endometrial NOS3 was weaker or absent in the early luteal phase than in mid and late diestrus.

In the cervix, NOS3 immunoreactivity was well represented in cell membranes and cytoplasm of cervical luminal epithelium.

4. Discussion

This study demonstrates the presence of AQP1, AQP2, and AQP5 in the uterus of cycling bitches. These three AQPs were chosen on the basis of the few data existing in the literature dealing with rat, mouse, and human uterus (for review, see Ref. [11]). Based on our observations, we suggest that, in the bitch uterus, they display a specific pattern in relationship with the estrous cycle.

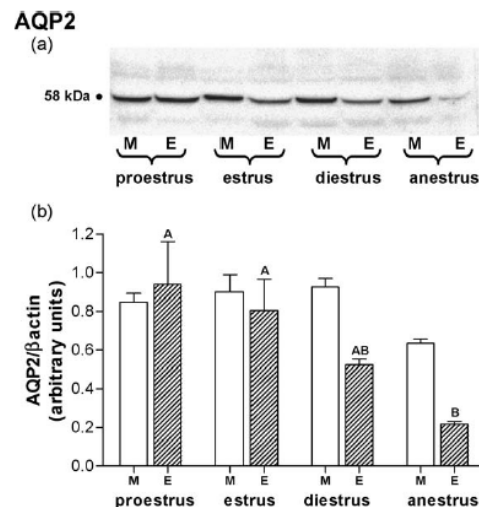


Fig. 7. Expression of AQP2 assessed by Western blotting in the uterine horn wall during estrous phases. (A) Each lane was loaded with 32 mg of protein extracted from the myometrium (M) or the endometrium (E), and blots were probed with anti-AQP2 antibodies. (B) Densitometric analyses of AQP2 protein levels in the myometrium and endometrium normalized against β -actin. Values are the mean $\pm$ SEM (n = 3). ^{A,B} Means with no common superscript letter are significantly different.

It has been known for many years that water moves from the vascular compartment to the stroma and into the lumen of the uterus under the control of ovarian estradiol-17 β [29]. The hormonal control of uterine luminal fluid secretion was confirmed in more recent years [30] using an established regimen of steroid treatment in ovariectomized rats. These authors suggested a mechanism responsible for the control of uterine fluid movement linked to ion transport at the level of Na⁺ and Cl⁻ channels, subject to tissue-specific regulation within the uterus [31].

At the level of the uterine tissue, water imbibition occurs through increased hyperemia and capillary permeability. Nitric oxide is a potent vasodilator molecule that induces uterine edema [20]. In the current study, the uterine endothelial NOS was highly evident in mid- and late-luteal phase, at the level of the endometrial and glandular epithelium, as well as in the blood vessel wall. This is in agreement with studies of women [32,33], sows [34], and mares [21]. The increase in NO synthesis by the bitch endometrium under the influence of P4 might suggest the participation of NO in vasodilation in view of the implantation process [21]. In addition, a role and involvement of NO in the maintenance of the

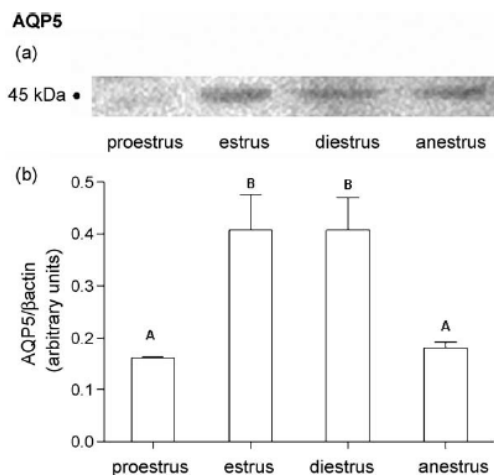


Fig. 8. Expression of AQP5 assessed by Western blotting in the endometrium of uterine horn wall during estrous phases. (A) Each lane was loaded with 32 mg of protein, and blots were probed with anti-AQP5 antibodies. (B) Densitometric analyses of AQP5 protein levels normalized against β -actin. Values are the mean $\pm$ SEM ($n = 3$). ^{A,B}Means with different superscripts are significantly different.

myometrial quiescence during pregnancy was also hypothesized [35].

AQP water channels may contribute to the fluid trafficking in the uterine tissues by transcellular pathways. In the preimplantation period, these molecules could help to shuttle water from the myometrial layer, providing for the rapid transfer to the lumen through the stroma, thus resulting in the stromal imbibition [13].

The staining pattern of AQP2 proteins in the bitch luminal and glandular epithelium and in the myometrium is uniquely observed in estrus, exactly when maximal imbibition takes place. Moreover, Western immunoblot evidences the endometrial presence of AQP2 principally in proestrous phase, when the level of estrogens is higher. In the ovariectomized mouse endometrium, upon estrogen treatment, the AQP2 acts as an exclusive water channel facilitating water movement into the lumen of the uterus [12]. The water movement in the uterus is not restricted to imbibition. Under estrogen dominance, the uterus has an obvious fluid-filled lumen. In the bitch, as in other species, we can suggest that the AQP2 proteins, which are expressed in the apical portion of the cell membrane of the glandular epithelial cells, are appointed to this goal.

In the human endometrium, the expression of AQP2 was higher mainly in the secretory phase, showing a positive correlation with both serum estrogen and progesterone levels [18]. In the physiology of women,

as known, the serum estrogen levels increase gradually in the follicular phase, the first peak appearing at the preovulation time. In humans, progesterone is secreted by the granulosa cells undergoing luteinization, after ovulation. A serum progesterone peak appears at the mid-secretory phase, almost the same time when a second peak of estrogen appears [18]. In the bitch, on the contrary, the serum progesterone level starts to increase before ovulation and, because of preluteinization [36], granulosa cells abruptly discontinue production of estrogens with, most probably, the consequence of blocking AQP2 expression and action completely as diestrus begins.

Moreover, the increase of NO in the endometrium during the luteal phase might explain the involvement of NO in regulating the uterine blood flow and the prostaglandin release, implicated in the production of edema owing to their inflammatory nature [37]. NO stimulates soluble guanylate cyclase, which in turn increases cyclic GMP, likely exerting its relaxant effect on smooth musculature [38–40]. The NO-cGMP system, besides playing an important role in uterine and feto-placental blood flow and in myometrial quiescence, is known to be involved in the mechanism of cervical ripening [39,40].

Although there is a great amount of information on the structural and biochemical changes in the ripening cervix, the mechanisms underlying the cervical water balance are unknown. Prostaglandins increase hyaluronic acid and water content in the cervix, owing to their potent vasoactive action while steroid hormones, estrogen and progesterone, affect vascular permeability and tissue edema. Relaxin, too, carries out cervical softening and increases water content in pregnant rats, mice, and most other species studied [15].

In the nonpregnant uterine cervix of diestral bitches, AQP5 staining was observed at the level of the apical membrane of the epithelial cells. We can suggest that AQP5 might be responsible for early water influx as cervical softening progresses, due to the increase in hyaluronic acid concentration occurring in the meanwhile [19].

In this study, we proved the immunohistochemical evidence of AQP5 proteins localized in the apical plasma membrane of the luminal and the glandular cells of the endometrium in postovulation days. In response to high levels of progesterone and in addition to the osmotic gradient seen during diestrus, the tight junctions are known to become “more tight,” thereby the apical localization of AQP5 water channels provide a possible mechanism for the transcellular pathway of luminal fluid reabsorption [41].

We observed that the influence of steroid hormones on AQP1 immunoreactivity was not meaningful. In fact, we noted that the AQP1 staining was clearly visible in all the phases of the estrous cycle in the smooth muscle cells of myometrium and the blood vessels, more marked in proestrus and estrus than in diestrus and anestrus. In the myometrium, AQP1 proteins were localized in the inner circular and longitudinal layers of smooth muscle. This immunoreactivity seems to be stronger in the follicular phase than in the luteal phase. Western blotting confirmed these data, further evidenced by quantitative analyses the absence of statistically significant differences among phases. In agreement with the hypotheses put forward by Richard et al. [13], it is conceivable that, in response to estrogen, the myometrial AQP1 water channels promote the water flow from myometrium to endometrium, in preparation for the possible blastocyst implantation. An upregulation of estrogen on AQP1 mRNA was shown also by Li et al. [42] in the rat uterus.

In conclusion, this study shows for the first time the presence of AQP1, AQP2, and AQP5 in uterine-specific structures of cycling bitches. In particular, we found that AQP1 protein is localized within uterine mesometrial, myometrial, and endometrial blood vessels and in the circular and longitudinal layers of myometrium. These localizations were present at all stages of the estrous cycle. Moreover, we observed that the estrogenic milieu is probably at the basis of cytoplasmic immunostaining of AQP2 protein in the glandular and luminal epithelium of the endometrium. Finally, we demonstrated that in coincidence with hematic progesterone increase, AQP5 water channels are present in the apical plasma membrane of uterine epithelial cells.

NOS3 immunolocalization in the myometrial and epithelial tissues as well as in blood vessels indicates a contribution of this vasoactive peptide to the uterine imbibition processes, leading to uterine edema. Nevertheless, interaction mechanisms between NOS3, AQPs, and other putative molecules implicated in water flow are unknown.

We can hypothesize that a functional and distinctive collaboration exists among diverse AQPs in the water handling during the different functional uterine phases. The next step of this research will deal with the possible significance of AQP water channels during pregnancy in the same species, as well as in other domestic carnivores.

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CHAPTER 5

Expression of aquaporin water channels in placental and fetal membranes during canine pregnancy: the regulation of production and absorption of amniotic fluid

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Expression of aquaporin water channels in placental and fetal membranes during canine pregnancy: the regulation of production and absorption of amniotic fluid

Marina Aralla¹, Ali Mobasheri², Debora Groppetti³, Fausto Cremonesi³, Silvana Arrighi¹

¹*Department Of Veterinary Sciences And Technologies For Food Safety, Laboratory Of Anatomy, University Of Milan, Italy*

²*Faculty of Medicine and Health Sciences, University of Nottingham Sutton Bonington Campus, Leicestershire, UK*

³*Department Of Veterinary Clinical Science, Reproduction Unit, University Of Milan, Italy*

5.1 Abstract

Amniotic fluid (AF) is created by the flow of fluid from the fetal lung and bladder and reabsorbed in part by fetal swallowing and in part by the transfer across the amnion to the fetal circulation. Placental water flux is an important factor in determining AF volume and fetal hydration, too. In addition the fetal membranes might be involved in fluid composition. To understand the mechanisms responsible for maintaining a correct balance of AF volume we evaluated the role of aquaporins (AQPs). AQPs are a class of integral membrane proteins permitting rapid passive transcellular water movement. Immunohistochemistry was performed to assess the presence of AQP1, 3, 5, 8 and -9 in canine placenta and fetal membranes. Uterine samples were collected on early, middle and late-gestation during ovariohysterectomies performed with owners' acceptance. Changes in AF volume and composition were also evaluated. Our results show distinct expression pattern in both membranes and placenta in relation to pregnancy period. AQP1 was localized in placental vessel endothelia, allantochorion, yolk sac and the strongest signal was detected in the amnion. AQP3 was present at the level of placenta, amnion and yolk sac. AQP8 was evident on the trophoblast, allantois and yolk sac. AQP9, a water channel highly permeable to water and urea, was observed in epithelia of amnion, allantois and yolk sac. AF changes during gestation may be dependent on modulations in solute as well as water permeability and changes in AQPs during gestation suggest their role in mediating placental and membrane water flow.

5.2 Introduction

Aquaporins (AQPs) are a class of small, hydrophobic, integral membrane proteins that are able to make the cell membrane 10 to 100 fold more permeable than the membranes lacking such channels.

They organize in the cell membranes as tetramers, however, each monomer form a hydrophilic pore in its centre and functions independently as a water channel. Selectivity is a central property of the channel (Borgnia et al., 1999; Landon et al., 2004). To date up to 11 different types of AQPs have been identified in mammals, although this number pales in comparison to the greater than 100 related proteins found in plants and bacteria. On the basis of their permeability characteristics, that normally correspond with a particular amino-acids-sequence pattern, aquaporin water channels are divided into two groups: the members of the first group, Aquaporins, are only permeated by water and they are AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8. AQP6 is an intracellular channel gated and permeated by anions as well as water (Yasui et al., 1999); AQP8 might be permeated by water and urea. They are both in this group because of the sequence analysis (Ishibashi et al., 1997; Ma et al., 1997). The members of the second group are the Aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) which are permeable to small uncharged solutes such as lactate, glycerol and urea in addition to water.

AQPs regulate water flux dependent upon cellular location. Aquaporin1 (AQP1) is the first aquaporin to be named and was originally discovered in human erythrocytes. Further studies demonstrated that AQP1 is widely distributed throughout the body, including kidney, brain, heart and lung (Knepper et al., 2003). Aquaporin 3 (AQP3), permeable to water, glycerol and urea, was originally identified in the rat kidney to be expressed in the basolateral membrane of the renal collecting duct (Ishibashi et al., 1994). AQP3 is also expressed in skin, lung, colon, skeletal muscle, salivary glands, peritoneum, and eyes (Hamman et al., 1998). The Aquaporin 8 (AQP8) transcript has been identified in testis, pancreas and liver by homology cloning (Ishibashi et al., 1997; Koyama et al., 1997). Northern blot analysis revealed that Aquaporin 9 (AQP9) transcripts are present in the rat liver, testis and brain (Tsukaguchi et al., 1998) and in human leukocytes, liver, lung and spleen (Ishibashi et al., 1998).

We have already verified the importance of AQPs on water homeostasis in the bitch uterus (Aralla et al., 2009) and recent studies have demonstrated AQP1,3,8,9 expression in human, sheep and mice fetal membranes (Wang et al., 2005; Beall et al., 2006; Shioji et al., 2006; Zhu et al., 2009). These results suggest that these channels may all contribute to the regulation of gestational water flow. Dogs possess endotheliochorial placentation. The endotheliochorial placenta is characterized as having a complete erosion of the endometrial epithelium and

underlying interstitium in such a way to offer a total exposure of maternal capillaries to the chorial epithelial cells.

The chorion is the fetal contribution to the placenta. The functional unit of the fetal placenta is the chorionic villus. The tiny villi protrude away from the chorion toward the uterine endometrium.

The dog zonary placenta includes a prominent region of exchange that forms a broad zone around the chorion equatorially disposed around the conceptus. A second zone consists of a pigmented ring and is made up of small hematomas (marginal hematoma). This zone is also called paraplacenta, an important pathway in iron transport from the dam to the foetus. A third region is the transparent one on the distal ends of the chorion that has poor vascularity and that could be involved in absorption of materials straight from the uterine lumen (Senger 2005).

It is well-known that the placenta regulates the exchange between the foetus and dam. Gases and water pass from high to low concentrations by simple diffusion, sodium and potassium traverse thanks to active transport pumps, as well as calcium. Glucose and other important molecules such as amino acids are carried on by facilitated diffusion with specific carriers. Lipids do not cross the placenta, but placenta is able to hydrolyze triglycerides and maternal phospholipids and to synthesize new lipid materials available for the foetus. Fat soluble vitamins do not cross the placenta easily, while water soluble vitamins such as B and K pass through the placenta quite simply. Nutrients move by pinocytosis and phagocytosis. Areolae form from the chorion over the openings of the uterine glands and are thought to absorb the secretions of these glands.

The regulation of placental water transfer and intramembranous resorption are imperfectly understood.

Amniotic fluid (AF) volume is essential for healthy foetal movement, growth and development. During embryogenesis, AF volume increases faster than embryonic size. Disorders of AF volume are associated with significant perinatal morbidity and mortality. After 35 days, in the dog, increased amniotic fluid volume distends the vesicles and makes them confluent, obscuring their characteristic shape and turgidity (Root Kustritz 2005).

Amniotic fluid is created by the flow of fluid from the fetal lung and bladder and reabsorbed in part by fetal swallowing and in part by the transfer across the amnion to the fetal circulation.

Five pathways of exchange have been identified between the amniotic space and the surrounding tissues. Intramembranous absorption takes place against a hydrostatic gradient and had been alleged to be determined by passive diffusion due to an osmotic gradient. Furthermore, studies illustrate that passive diffusion accounts for only part of the intramembranous fluid absorption and that many solutes diffuse in the opposite direction (from fetus to AF). It is possible that a

good deal of transfer of fluid and solutes occur by massive transport of AF into the fetal circulation perhaps via a trans-cellular transport mechanism. In this way aquaporin water channels should play a pivotal role as their expression demonstrated in fetal membranes (Underwood et al., 2005).

To understand the mechanisms responsible for maintaining amniotic fluid volume in the dog the proposal is to demonstrate the presence of AQP1, 3, 5, 8, 9 in canine placenta and fetal membrane and seek the correlation of AQPs with AF volume and composition changes across gestation in the dog, a species with endotheliochorial placenta.

5.3 Materials and Methods

The canine placenta and fetal membranes were obtained with the owners' approval during natural delivery, ovario-hysterectomies performed at any stage of undesired pregnancies and in case of caesarean sections due to dystocic parturitions, at the Reproduction Unit of the Faculty of Veterinary Medicine of Milan (Italy). The specimens were collected from 12 pregnant bitches of medium sized breed. Gestational age estimate was obtained by the day of mating and by ultrasonography measurement of uterine and fetal diameters, according to Yeager et al. (1992), England et al. (1990) and Kutzler et al. (2003). As such, the specimens were included into three groups: aged up to 35 days of gestation (N=4), from 35 to 45 days of gestation (N=3), from 45 days of gestation to delivery (N= 5).

Immediately after ovario-hysterectomy, AF was gathered by needle aspiration. Soon after, tissue specimens were collected from placenta (fetal and maternal side) and from fetal membranes after careful separation onto amnion, allantois, allantochorion and yolk sac. A part of the pieces were fixed in formalin 10% for 24 h at 4 °C. After fixation, fragments were dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections were cut at 4 µm thickness, de-waxed, and stained with routine hematoxylin and eosin (H&E) for histologic examination. Another part of the pieces were instantly frozen and stored at -80°C to perform protein extraction and western blot analysis as described below.

5.3.1 Immunohistochemistry

Immunohistochemical staining was performed using a DakoCytomation EnVision + Dual Link System-HRP (DAB+) kit (code K4065, DakoCytomation, Denmark), a two-step technique that takes advantage of the superior sensitivity of a horseradish peroxidase-labeled polymer conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin so

that non-specific staining resulting from endogenous avidin-biotin activity in tissues is eliminated.

After de-waxing, formalin-fixed serial sections (4 μ m thick) were washed and immersed in a freshly prepared 3% H₂O₂ solution for 15 min to block the endogenous peroxidase activity, followed by incubation for 30 min at room temperature with 2% protease-free bovine serum albumin (BSA) (Sigma-Aldrich) in PBS-Tween to block non-specific antibody binding. Sections were then incubated overnight in a humidity chamber at 4°C using rabbit polyclonal antibodies against rat AQP1 (Alpha Diagnostic International, San Antonio, TX, USA; cat. no. AQP11), AQP3 (Chemicon International), AQP5 (Alpha Diagnostic International, San Antonio, TX, USA; cat. no. AQP15) AQP8 (developed in the laboratory of Prof. Mobasher) and AQP9 (developed in the laboratory of Prof. Mobasher) diluted 1:200 in PBS containing 2% of BSA. Subsequently, the sections were rinsed 3 X 5 min in PBS-Tween before incubation with horseradish peroxidase-labeled polymer conjugated to affinity purified goat anti-rabbit and goat anti-mouse immunoglobulins for 30 min at room temperature. The sections were washed 3 X 5 min with PBS before applying liquid DAB+ Chromogen (DAKO; 3,3'-diaminobenzidine solution) for up to 10 min. Sections were counterstained with Mayers' haematoxylin, dehydrated, and mounted using Eukitt (Bioptica, Milan, Italy).

5.3.2 Staining controls

Sections of dog kidney, salivary gland, pancreas and liver, similarly processed as above, served as positive controls for immunoreactions respectively of AQP1 and AQP3, AQP5, AQP8 and AQP9 antisera. The specificity of the immunostainings was tested by negative controls, performed by (1) use of non-immune rabbit serum (DakoCytomation; code no. X0903) in place of specific antisera; and (2) omission of the first layer. All of them resulted in the absence of immunoreaction.

The evaluation of staining intensities was based on subjective estimates of at least two authors. Slides were observed and photographed under an Olympus BX51 photomicroscope equipped with a digital camera and DP software (Olympus, Tokio, Japan) for computer-assisted image acquirement and managing.

5.3.3 Amniotic Fluid Biochemical Analysis

AF was collected from vesicles at day 19th, 30th, 40th, 55th, 60th, 62th and 70th. After AF collection, a part of the samples (2-3 mL) were centrifuged (177.4 x g, 22 °C, 10 min.) and supernatants collected and stored at -70 °C for up to 1 months until determination of urea, electrolytes and total protein content using the Cobas Mira Plus Chemistry Analyzer (Roche Diagnostic, Italy). AF Na⁺,

K⁺ and protein content and osmolality were not evaluated at day 19 as the AF volume was insufficient at early pregnancy.

The other part of the AF samples (1 mL) were not centrifuged and used for measuring Osmolality by freezing-point depression (Automatic osmometer Osmomat model 030 Gonotec).

All samples were analyzed in one batch.

Data obtained were evaluated with ANOVA using the PROC GLM of SAS package (9.2 version).

5.4 Results

5.4.1 Immunohistochemistry

AQP1

As shown in Fig. 1 in the dog AQP1 expression is strongly expressed in the vascular endothelium of placenta (A), in the amnion (B), allanto-chorion (C) and yolk sac epithelia (D) at all the stages of pregnancy.

AQP3

AQP3 was associated with trophoblast cells (Fig. 2 A) in the labyrinth and a strong staining was found in amniocytes (Fig. 2 C and D) and in the basolateral membrane of allantois (Fig. 2 E).

AQP5

Immunoreactivity of amniocytes (Fig. 3 A) has been detected with anti-AQP5 antibody throughout pregnancy. AQP5 is also present at the level of basolateral membrane of allantochorion (Fig. 3 B, C, D).

AQP8

A slight intracellular immunostaining for AQP8 was found in amniotic (Fig. 4 C) and allantoic sac (Fig. 4 D), while AQP8 is diffusely expressed in the placenta (Fig. 4 A and B) and in the trophoblast.

AQP9

Immunohistochemical staining with anti-AQP9 antibody has been detected in basolateral membranes of epithelial cells of amnion (early, middle and late pregnancy, Fig. 5 A, B, C respectively), allantois and yolk sac, but there is no evidence of AQP9 immunoreactivity in any structure of the placenta.

In the allantochorion AQP9 is expressed at the level of apical cell membrane and also in cytoplasmatic vesicles.

Table 1
Canine amniotic fluid composition

	From d30 to d40	From d40 to d62
Osm (mOsm)	296±17.45	260.63±7.44
Na	144.38±9.71	122.22±4.14 ^a

(mEq/dl)		
K (mEq/dl)	4.9±0.89	5±0.38
PT (g/dl)	0.28±0.18	0.34±0.08
Urea (mg	46.5±6.9	38.23±2.9

Mean ± SEM amniotic fluid (AF) at middle (day 30 to 40) and late (day 40 to 62) pregnancy.

^a Significant difference from middle pregnancy (p= 0,05)

Data obtained by ANOVA using the PROC GLM of SAS package (9.2 version).

5.4.2 Osmolality, Solutes, urea and Total protein of Amniotic Fluid

Data (mean ± SEM) for each solute in amniotic fluids are presented in Table 1.

Amniotic fluid osmolality decreases when the end of the pregnancy is approaching but the means are not significantly different. Regarding Na and K solutes only the Na amount is significantly higher (p=0,05) in the middle pregnancy despite the last period. Na concentrations in intra- and extracellular fluids tend to vary inversely with the concentration of K.

Amniotic urea concentration is higher in mid than in late pregnancy while total protein concentrations increase at the end of gestation. In both cases no statistically significant differences have been demonstrated.

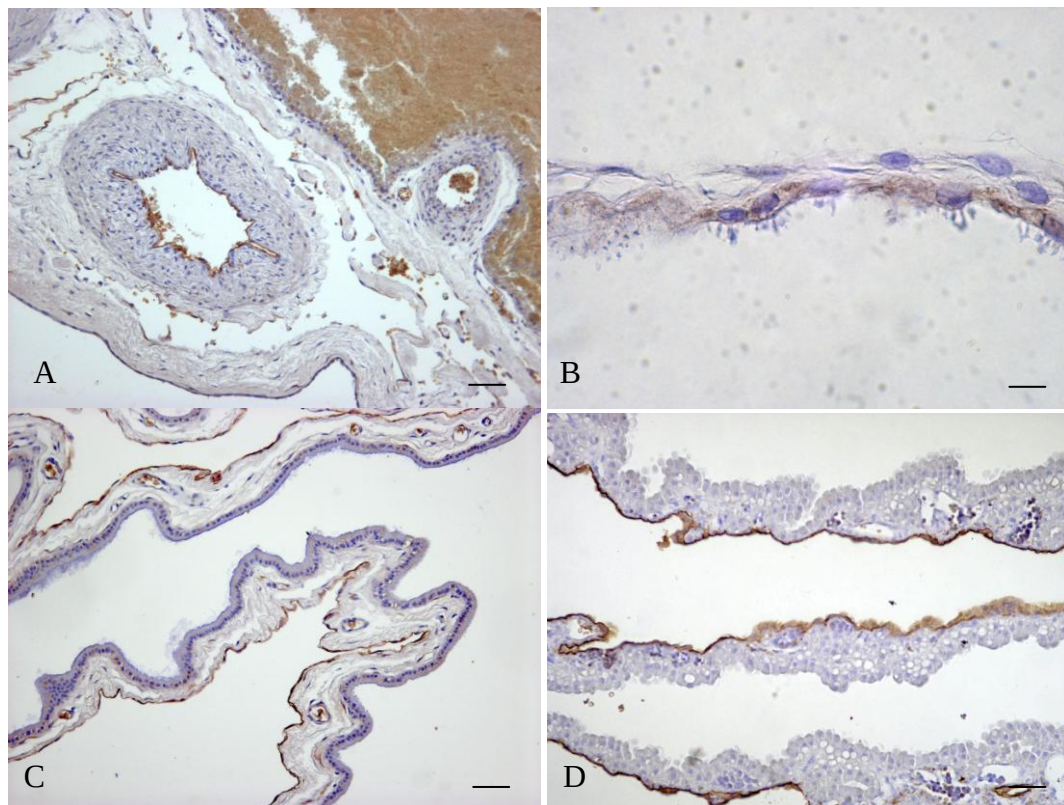


Fig. 1: Aquaporin 1 (A) Transverse section of labyrinth shows AQP1 immunoreactivity of the endothelium of a huge foetal blood vessel. Scale bar = 1000 µm. (B) AQP1 immunoreactivity is present in the amniocytes that at the stage of early pregnancy appear like flattened cells. Scale bar = 100 mm. (C) AQP1 is evident on the allantois side of the allantochoirion. Scale bar = 1000 µm. (D) At about 55 days of gestation the cuboidal cells of the yolk sac appear supported by a thin layer of

connective tissue. AQP1 is strongly expressed on the basal membrane of cuboidal cells. Scale bar = 1000 μ m.

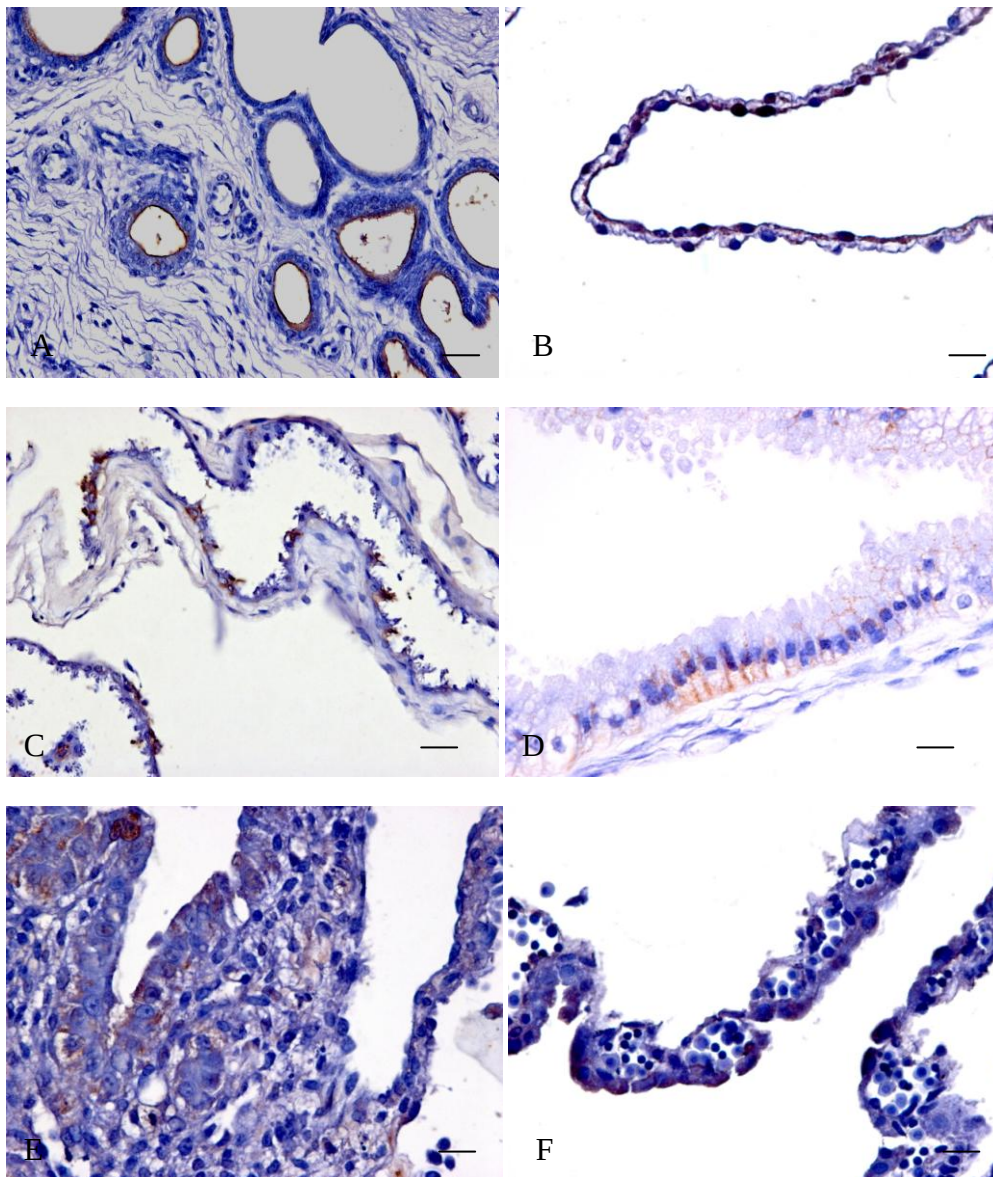


Fig. 2: Aquaporin 3. (A) Middle pregnancy. AQP3 is expressed at the level of apical cell membrane of the placental deep glands. Scale bar = 200 μ m. (B) About 22 days of gestation: early pregnancy. AQP3 immunoreactivity in amniocytes. Scale bar = 200 μ m. (C) Trasverse sections of amnion collected at time of parturition (62 days): amniocytes (positive for AQP3) become cuboidal as pregnancy progresses and the number of microvilli increases allowing fluid exchange. Scale bar = 200 μ m. (D) Middle pregnancy. Specific AQP3 staining of the basolateral membranes of cylindrical cells of allantois. Scale bar = 100 μ m. (E) Middle pregnancy. AQP3 is present in the allantochorion. Scale bar = 100 μ m. (F) Early pregnancy. In the yolk sac are visible blood vessels plentyful of aemocyctoblasts and lymphocytes. The cuboidal cells are positive for AQP3. Scale bar = 100 μ m.

5.5 Discussion

This study demonstrates dynamic changes in placental and fetal membranes aqp expression throughout the dog pregnancy related to the increasing fetal water needs.

Our results suggest that aqp1-3-5-8-9 may all contribute to the regulation of the intramembranous water flow.

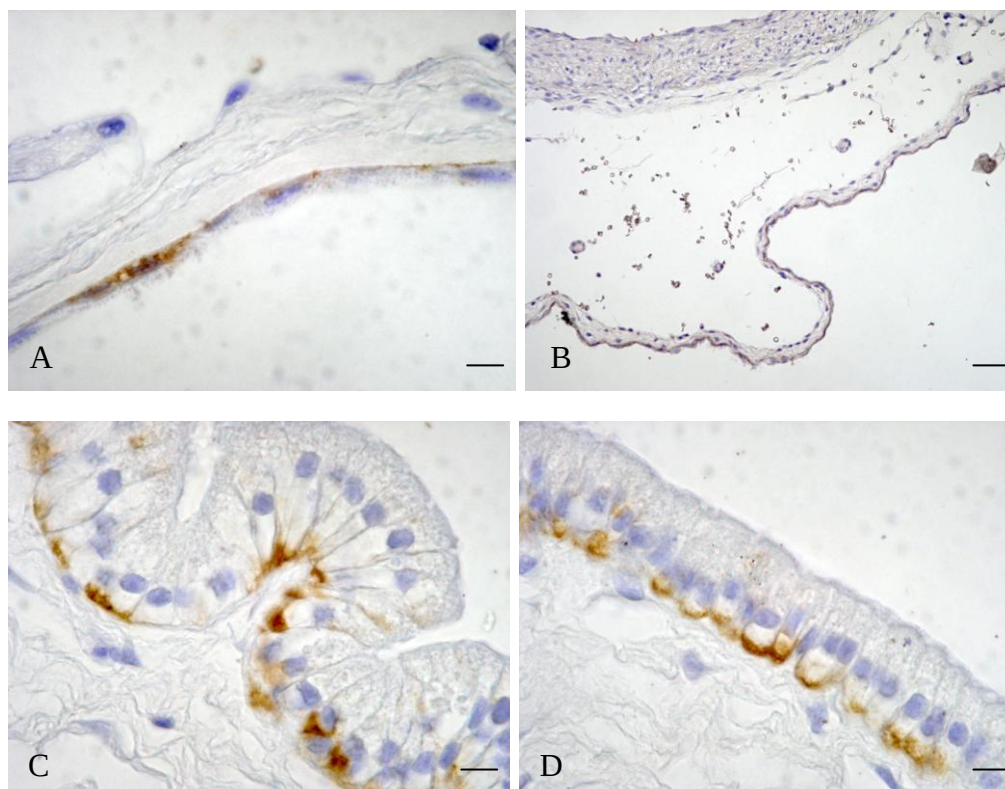


Fig. 3: Aquaporin 5. (A) Early pregnancy. AQP5 immunostaining of amniocytes. Scale bar = 50 μm . (B) AQP5 is expressed on the allantois cells. Scale bar = 1000 μm . (C) and (D) Very clear and specif staining at the level of basal membrane of cyllindric cells in the allantochorion. Scale bar = 50 μm .

Water permeability in the placenta appears to be regulated at the level of syncytiotrophoblat and to vary with gestational age (AQP1, blood vessels, AQP3 and AQP8).

Once in gestational vesicle water is exchanged between the fetus, placenta and AF.

Urea is the secreted end product of nitrogen metabolism and is made in the liver from ammonia by an energy-requiring process. Urea concentrations in allantoic and amniotic fluid and in fetal and maternal plasma were essentially similar; thus, urea formed by the fetus is removed constantly and disposed of through the maternal circulation. We found increasing AF urea concentration at the 45th day of gestation. As this increase occurred at a time when the amniotic fluid volume

was also increasing, this represented a marked increase in the total amount of urea in the AF, likely a result of the increased contribution of fetal urine to the amniotic cavity.

Besides, the permeability of canine amnion to urea decreases with advancing gestation, while AF urea concentration increases. Water and solutes may be absorbed into fetal vasculature after crossing the canine amnion epithelial cells.

In the dog, we found urea in both, amniotic and allantoic sacs.

The observations that AQP3 and 9, both permeable to urea, are expressed in canine amnion, allantois, chorion are consistent with the notion that these AQPs are important for both AF and allantoic fluid water and solute absorption across fetal membranes. Concerning the expression of AQP8 in fetal membranes we first demonstrated in the dog that is diffusely expressed in the epithelial cells of the amnion, allantois, and trophoblast according to Wang et al. that found AQP8 expression in human membranes. They in fact suggested that AQP8 may mediate amniotic fluid absorption by the intramembranous pathway.

Amniotic fluid is 98-99% water. The chemical composition of its substances varies with gestational age. The fluid composition changes differently with stage of gestation suggesting that the primary determinants of amniotic and allantoic fluid composition are the fetal components that directly contribute to the fluid. Differences in the permeability of the two membranes to various solutes also could influence the fluid composition.

Both Na and K, because of their relative abundance, are the most important electrolytes regulating the movement and retention of body water, whereas Cl tends to track with Na transport. The ability to regulate intra- and extracellular Na, K, and Cl levels is an intrinsic requirement for fluid homeostasis.

When fetal urine begins to enter the amniotic sac, amniotic osmolarity decreases slightly compared with fetal blood. After keratinization of the fetal skin, amniotic fluid osmolarity decreases further with advancing gestational age, reaching values of 250-260 mOsm near term. The low amniotic fluid osmolarity, which is produced by the inflow of markedly hypotonic fetal urine, provides a large potential osmotic force for the outward flow of water across the intramembranous and transmembranous pathways. Within certain limits, amniotic fluid mirrors the metabolic status of the fetoplacental unit; for that reason, a study of its components and their respective variations in the different periods of pregnancy provides useful indications for a correct assessment of fetal maturation.

Placental water flow may be equally important in regulating AF volume, as the majority of fetal water is derived from the maternal circulation through the placenta. In human placental permeability has been observed to increase during gestation, with a decrease near term (Jansson et al., 1999), and the structure limiting placental water flow is presumed to be the trophoblast based on

membrane permeability studies. In the present study, AQP3 expression was evident in the placenta at middle pregnancy and less expressed near term, AQP8 expression also increased with advancing gestation, consistent with facilitation of fluid flow to support fetal requirements. AQP1 expression was found in the endothelia of placental blood vessels but AQP9, a urea as well as water channel, was not evident in the placenta, while may contribute to increasing of AF urea concentrations seen in mid gestation. A recent study found urea flux in human placenta to be associated with expression of AQP9, although placental water flux did not demonstrate an AQP9 association (Damiano et al., 2006).

AQP5 has never been demonstrated in foetal membranes. We obtained a specific staining of AQP5 in the amniocytes and in cylindric cells of allantochorion. Interestingly the basolateral immune-localization of AQP5 in the allantoic epithelium is in contrast with the apical expression of AQP9 in the same membrane, suggesting the collaboration of these two aquaporins on transporting large amount of water.

In conclusion, AQPs are expressed in canine placenta and fetal membranes similar to findings described in other species, such as sheep (Johnston et al., 1999) and mice (Beal et al., 2007), suggesting a common mechanism for regulating placental and membrane water flow.

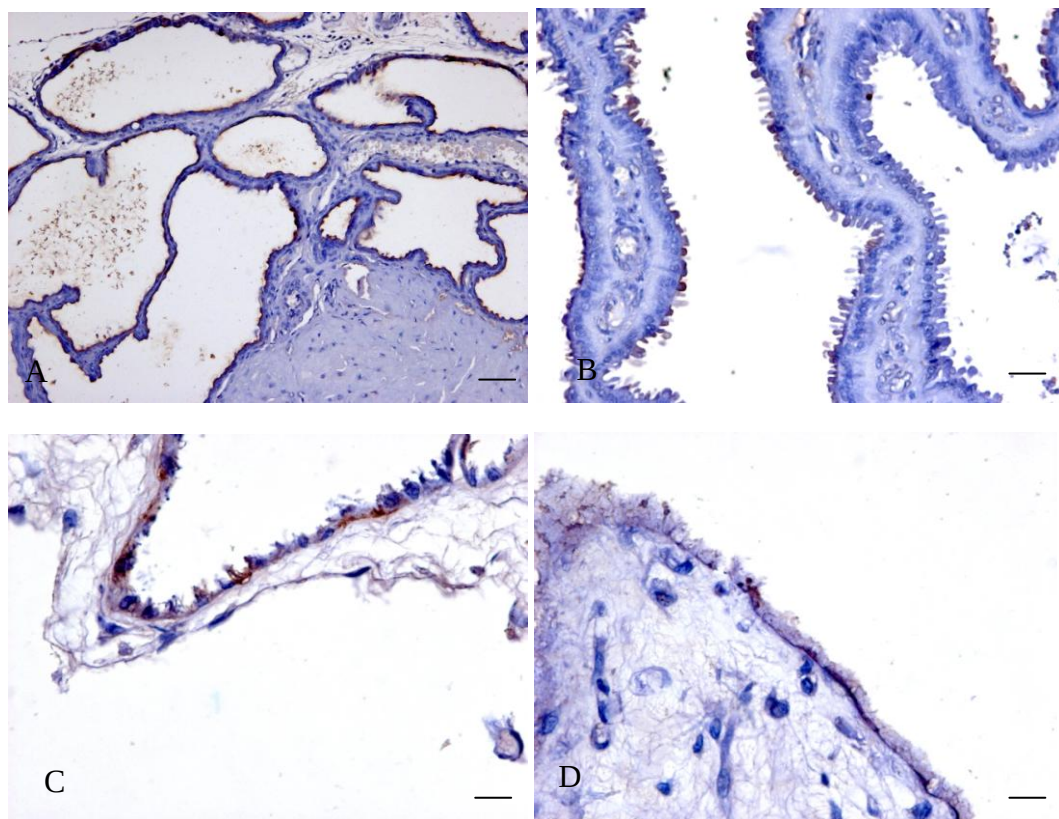


Fig. 4: Aquaporin 8. (A) AQP8 immuno-expression in the deep glandular layer in a placenta at the last stage of pregnancy. Scale bar = 1000 μ m. (B) Late pregnancy: AQP8 is expressed at the level of apical

membrane of epithelial cells belonging to the glandular structures of the placental spongy zone. Scale bar = 200 μm . (C) Late pregnancy. AQP8 staining is evident in the amniocytes. Scale bar = 100 μm . (D) Middle pregnancy. Faint immunostaining of AQP8 in the allantoic membrane. Scale bar = 100 μm .

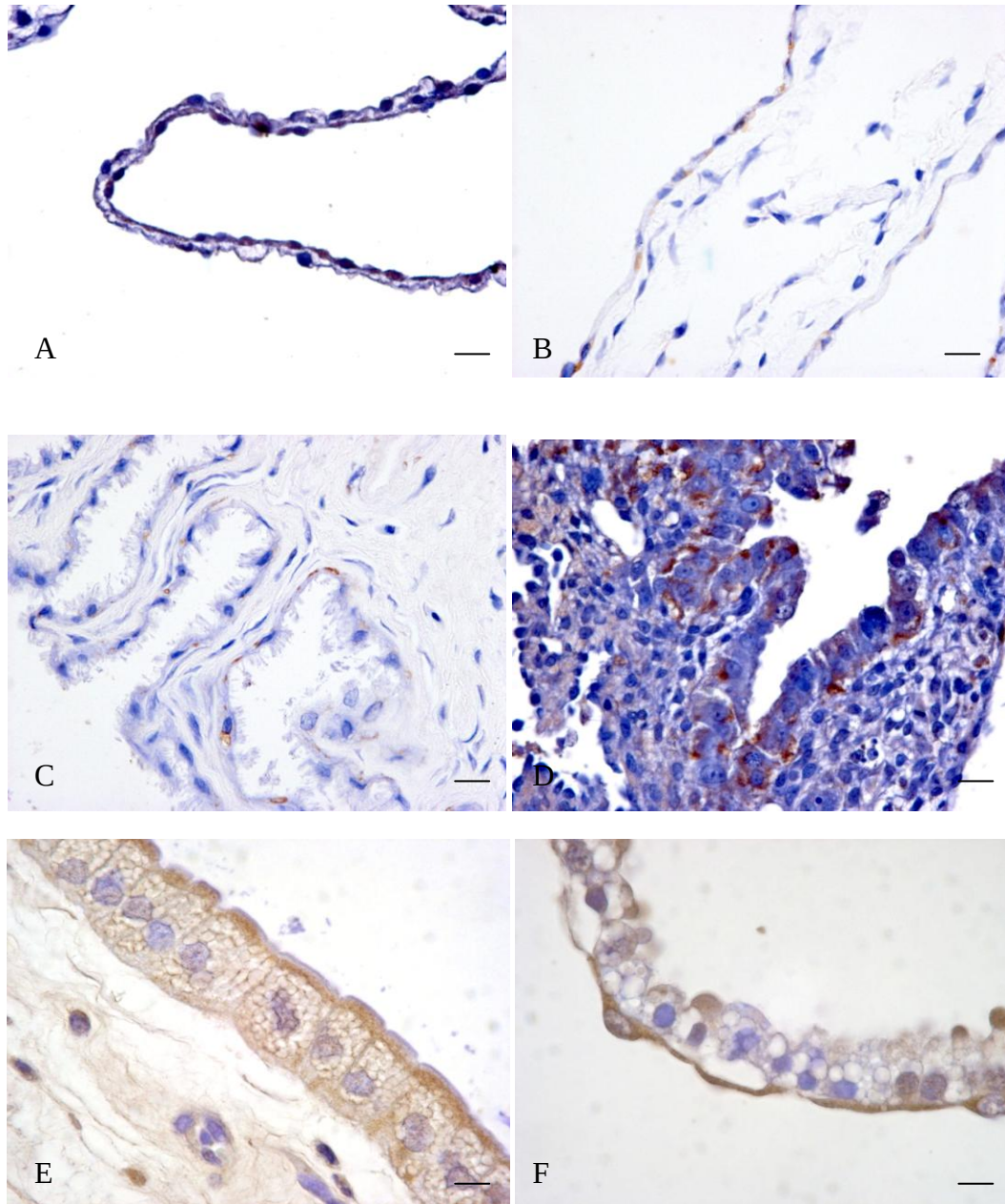


Fig. 5: Aquaporin 9. (A) Early pregnancy: AQP9 is strongly expressed in the cell membrane surrounding amniocytes. Scale bar = 200 μm . (B) and (C) Middle and late pregnancy respectively: specific but faint staining for AQP9 in the amniocytes. Scale bar = 100 μm . (D) Middle pregnancy: basolateral cell membrane of allantochorion strongly expressed AQP9 immunoreactivity. Scale bar = 100 μm . (E) Strong and specific immunostaining for AQP9 at the level of apical cell membrane and in the cytoplasmatic vesicles of the allantochorion. Scale bar = 50 μm . (F) Middle pregnancy: AQP9 immunoreactivity in the epithelial cuboid cells of the yolk sac. Scale bar = 100 μm .

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CHAPTER 6

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

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

S. Arrighi¹, G. Ventriglia², M. Aralla¹, S. Zizza², A. Di Summa² and S. Desantis²

¹Department of Veterinary Science and Technologies for Food Safety, Faculty of Veterinary Medicine, Università degli Studi di Milano

and ²Department of Animal Health and Well-being, Faculty of Veterinary Medicine, Università degli Studi di Bari, Italy

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 immunohistochemical 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 Neu5Ac α 2,3Gal β 1,3GalNAc, Neu5Ac α 2,3Gal β 1,4GlcNAc, Gal β 1,4GlcNAc, GalNAc (s-PNA, MAL II, RCA₁₂₀, SBA reactivity) and with internal/terminal α Man (Con A affinity) at the luminal surface and the apical region. In addition, non-ciliated cells expressed oligosaccharides terminating with GalNAc and Neu5Ac α 2,6Gal/GalNAc (SNA reactivity) in the luminal surface and the apical zone, respectively. Ciliated cells revealed glycoconjugates only on cilia, which showed terminal Neu5Ac α 2,3Gal β 1,4GlcNAc (s-RCA₁₂₀ staining) and GalNAc, as well as internal/terminal α Man 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 glycocalyx 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

Offprint requests to: Prof. Silvana Arrighi, DVM, Università degli Studi di Milano, Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare - Laboratorio di Anatomia, Via Trentacoste 2, 20134 Milano, Italy. e-mail: silvana.arrighi@unimi.it

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% H₂O₂ 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 (StrepABCComplex/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% H₂O₂ 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, RCA₁₂₀, 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-B₄) were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

De-waxed and re-hydrated tissue sections were immersed in 3% v/v solution of H₂O₂ 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% H₂O₂ 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-H₂O₂ 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 haptan sugar (0.2-0.5 M in Tris buffer).

Enzymatic and chemical treatments

Before staining with MAL II, SNA, PNA, DBA, RCA₁₂₀, 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 CaCl₂. 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*	<i>Maackia amurensis</i>	15	NeuNacα2,3Galβ1,4GlcNAc	NeuNAC
SNA*	<i>Sambucus nigra</i>	15	NeuNACα2,6Gal/GalNAc	NeuNAC
PNA	<i>Arachis hypogaea</i>	20	Terminal Galβ1,3GalNAc	Galactose
DBA	<i>Dolichos biflorus</i>	20	Terminal GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1	GalNAc
RCA120	<i>Ricinus communis</i>	25	Terminal Galβ1,4GlcNAc	Galactose
HPA	<i>Helix pomatia</i>	20	Terminal αGalNAc	GalNAc
SBA	<i>Glycine max</i>	15	Terminal α/βGalNAc	GalNA
Con A	<i>Canavalia ensiformis</i>	20	Terminal/Internal αMan>αGlc	Mannose
WGA	<i>Triticum vulgaris</i>	20	Terminal/Internal βGlcNAc>>NeuNAC	GlcNAc
GSA II	<i>Griffonia simplicifolia</i>	20	Terminal D-GlcNAc	GlucNAC
GSA I-B4*	<i>Griffonia simplicifolia</i>	25	Terminal αGal	GalNAc
UEAI	<i>Ulex europaeus</i>	25	Terminal L-Fucα1,2Galβ1,4GlcNAc,	Fucose
LTA	<i>Lotus tetragonolobus</i>	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	-	++s/++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.

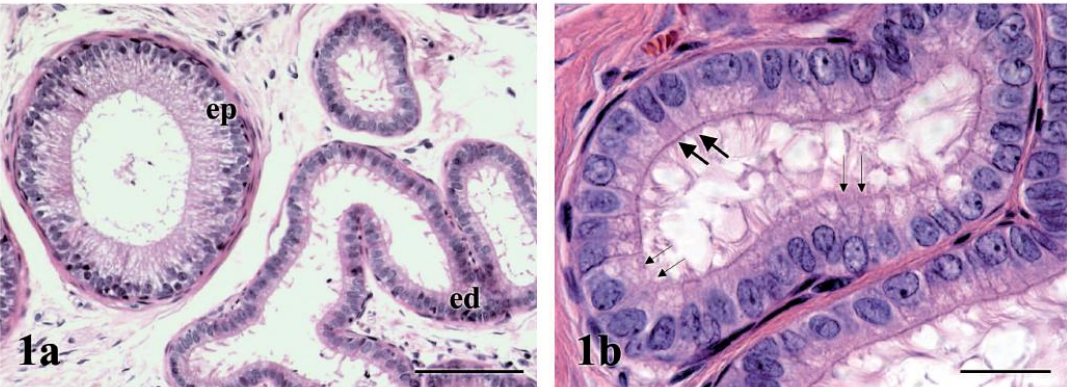


Fig. 1. Cat efferent ducts, Haematoxylin-Eosin staining. a. Efferent ducts (ed) are lined with a simple columnar epithelium constituted by ciliated cells and non-ciliated cells. They are easily distinguishable from epididymal duct (ep), which is lined by a taller, pseudostratified epithelium. b. Higher 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).

RCA₁₂₀ 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 RCA₁₂₀, 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

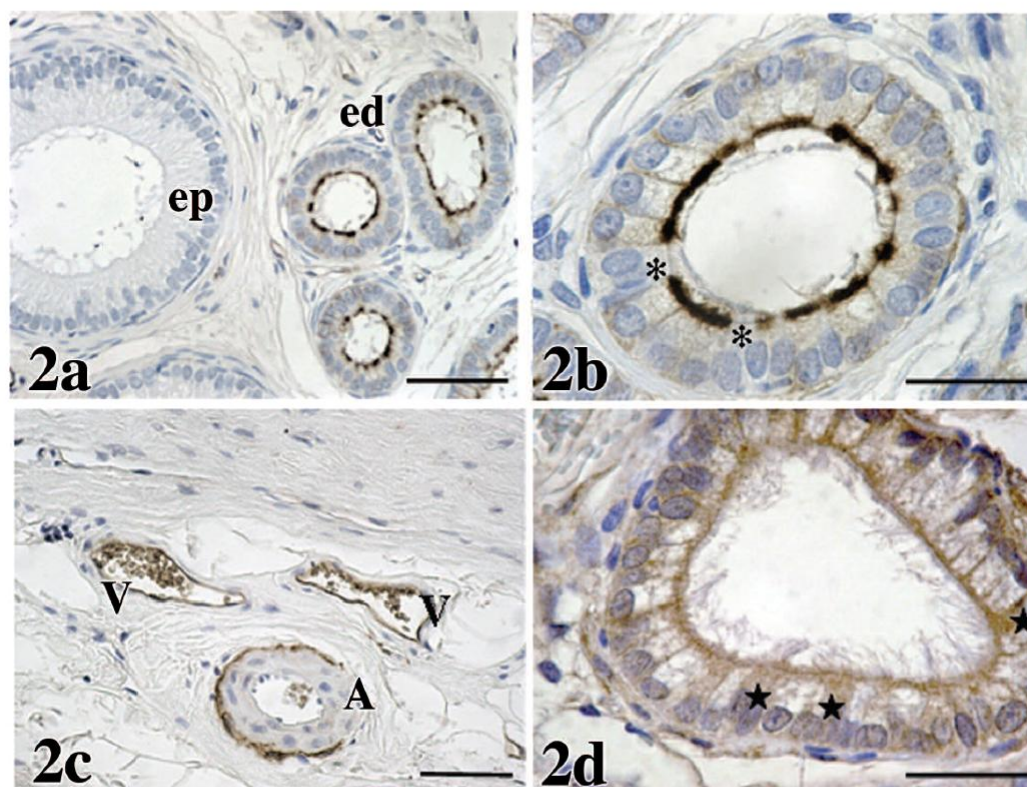


Fig. 2. Immunolocalization of Aquaporins in the cat efferent ducts. a. Evident AQP1-immunoreactivity is localized at the apical surface of the epithelium lining the ductuli efferentes (ed). By contrast, the epididymal duct epithelium (ep) is unreactive. b. At higher magnification, strong immunostaining 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 μ m; b, d, 20 μ m; c, 50 μ m.

β GlcNAc 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-B₄, UEA I and LTA did not show reactivity with the ED epithelium.

Discussion

The present study investigated by immunohistochemistry 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; Axner 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

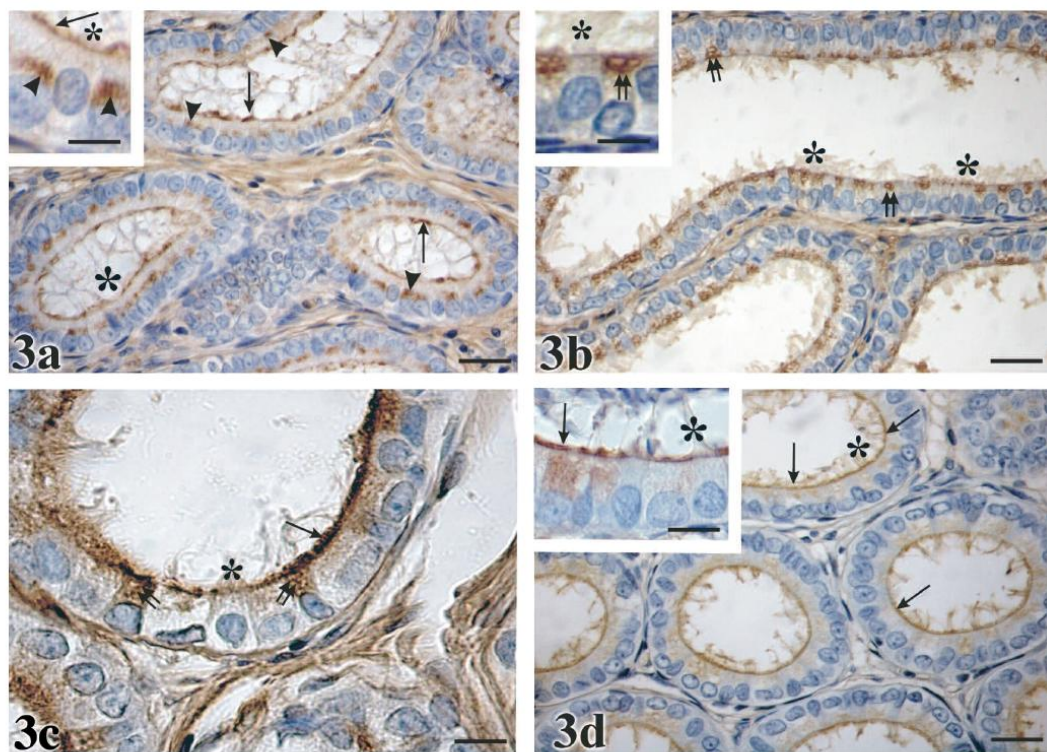


Fig. 3. Lectin histochemistry of cat efferent ducts. MAL II (a), SNA (b), KOH-sialidase-PNA (c), and KOH-sialidase-RCA₁₂₀ (d) stainings. a. MAL II reacts with the microvilli and Golgi zone of non-ciliated cells. A more detailed image is in the inset. b. SNA shows binding sites for the apical cytoplasm 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-RCA₁₂₀ 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 RCA₁₂₀ 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 μ m; insets, 4 μ m.

(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

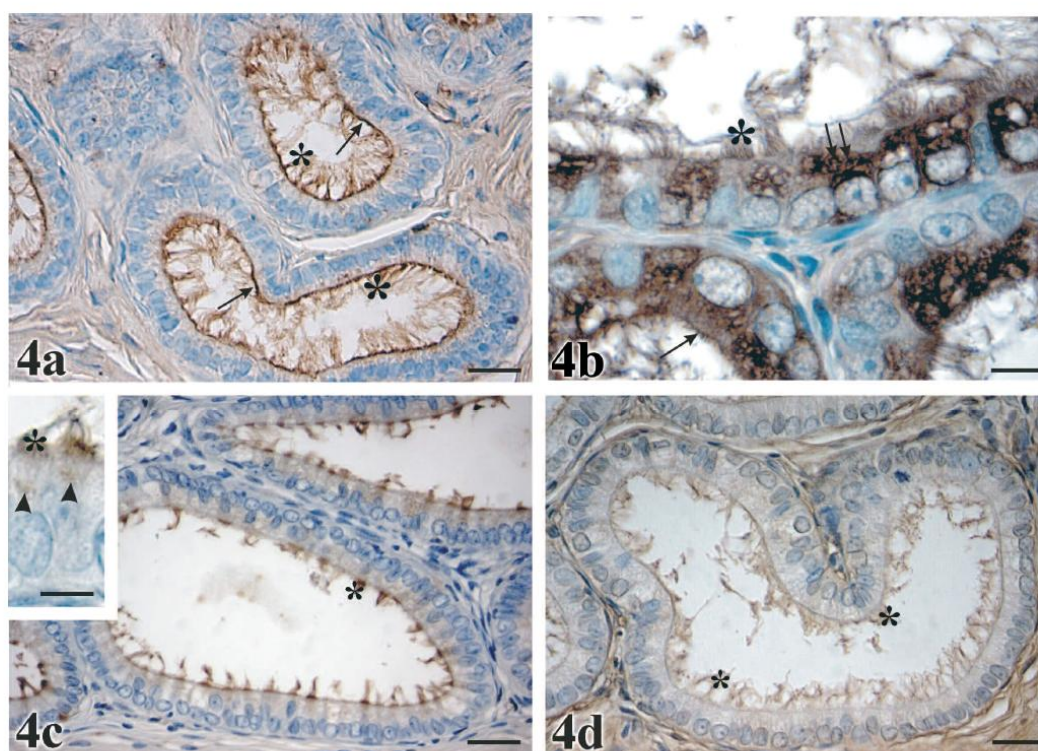


Fig. 4. Lectin histochemistry of cat efferent ducts. SBA (a), Con A (b), KOH-sialidase-WGA (c), and GSA II (d) stainings. a. SBA stains the cilia of ciliated cells and microvilli of non-ciliated cells. b. Con A reacts with the entire luminal surface of the epithelium lining the efferent ducts and with 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 μ m; b, 10 μ m; c, 27 μ m; inset in c, 3 μ m.

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 AQP9 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 CO₂ (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 α 2,3-6 to penultimate β -Gal or via α 2,6 to subterminal α -GalNAc, as demonstrated with MAL II, SNA, KOH-s-PNA, and KOH-s-RCA₁₂₀. 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 GalB1,4GlcNAc, sialic acid linked to GalB1,4GlcNAc, sialic acid linked to GalB1,3GalNAc (RCA₁₂₀, KOH-s-RCA₁₂₀, 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 Neu5Ac α 2,6Gal/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 (mucin-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, RCA₁₂₀, 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 Gal β 1,4GlcNAc, Neu5Ac α 2,3Gal β 1,4GlcNAc, and sialic acid linked to Gal β 1,3GalNAc 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 RCA₁₂₀. This suggests the presence of N-linked oligosaccharides with terminal/internal α Man, GlcNAc and with terminal GalNAc and sialic acid linked to β -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 Gal β 1,4GlcNAc revealed with KOH-sialidase-RCA₁₂₀ 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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CHAPTER 7

An insight into testis and gubernaculum dynamics of INSL3-RXFP2 signalling during testicular descent in the dog

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An insight into testis and gubernaculum dynamics of INSL3–RXFP2 signalling during testicular descent in the dog

S. Arrighi^{A,C}, G. Bosi^A, D. Groppetti^B, M. Aralla^A and F. Cremonesi^B

^ADepartment of Veterinary Sciences and Technologies for Food Safety, Laboratory of Anatomy, Università degli Studi di Milano, Italy.

^BDepartment of Veterinary Clinical Science, Reproduction Unit, Faculty of Veterinary Medicine, Università degli Studi di Milano, Italy.

^CCorresponding author. Email: silvana.arrighi@unimi.it

Abstract. Insulin-like 3 (INSL3) plays a prominent role in male development and is supposed to induce the growth of the *gubernaculum testis* (*g.t.*), thus being directly involved in testicular descent in humans and rodents. This happens through activation of the RXFP2 receptor (GREAT or LGR8). The INSL3–RXFP2 complex is reputed to play an additional paracrine role in the testis, possibly acting as part of an autocrine feedback loop. The present work provides evidence of the immunolocalisation of INSL3 in the Leydig cells of canine fetuses and of the expression of RXFP2 receptor in different tissues of the *g.t.* of the same specimens. RXFP2 was localised at the cell membrane of *g.t.* muscle and connective cells, as well as in the epithelial cells of the developing excurrent ducts. Notably, RXFP2 immunoreactivity of the *g.t.* was limited to fetuses at ~35–45 days of gestation, which is also the fetal period when the endocrine compartment of the dog testis is active endocrinologically, as confirmed by the anti-P450c17 and anti-INSL3 immunoreactivities of the fetal Leydig cells, and by anti-Müllerian hormone immunoreactivity of the Sertoli cells. The same immunoreactivities were also evaluated in the testes of cryptorchid dogs of different ages. RXFP2 immunoreactivity was absent from genital tracts of cryptorchid testes and *g.t.* remnants.

Additional keywords: anti-Müllerian hormone, cryptorchidism, GREAT, RLF, testicular ligaments.

Introduction

During fetal life, sexual dimorphism occurs as concerns the development of the two mesentery ligaments attached to the developing gonad, the cranial suspensory ligament (CSL) and the *gubernaculum testis* (*g.t.*), respectively, which develop in different ways in the male and female fetus. In early embryonic development the position of the gonad is identical in male and female fetuses, attached to the abdominal wall by the CSL and the *g.t.* Thereafter, sexual differentiation progresses in such a way that the developing ovary remains in a high abdominal position: the CSL develops further to sustain the ovary caudal to the kidney and *g.t.* development is impaired. In contrast, during male differentiation, supposedly triggered by expression of the SRY gene on the Y chromosome, the CSL regresses and *g.t.* growth assumes importance for the transabdominal migration of the testis into the scrotum (testicular descent, TD). In fact, the enlargement of the *g.t.*, which keeps the testis anchored securely to the inguinal region, is responsible for the first, transabdominal, phase of TD accompanied by regression of the CSL. The second, inguinoscrotal phase is under the influence of testicular androgens and finalises at different ages depending on the species (Zimmermann *et al.* 1999; Hutson and Hasthorpe 2005; Amann and Veeramachaneni 2007).

As many as twenty years ago, a low molecular weight growth factor was isolated from the testis, and called Descenderin (Fentener van Vlissingen *et al.* 1988). It was documented to be secreted in an androgen-independent way and to be at the origin of the selective growth of gubernacular mesenchymal cells. Most probably, researchers were dealing with the insulin-like hormone that was tentatively called Leydig insulin-like (Ley I-L) few years later (Adham *et al.* 1993), and then conclusively Insulin-like 3 peptide (INSL3). This is a member of the relaxin family of hormones and growth factors, and is therefore also known as relaxin-like factor (RLF), as it was demonstrated in the fetal as well as adult Leydig cells in many animal species (Pusch *et al.* 1996; Ivell and Bathgate 2002). During the first, transabdominal phase of TD, INSL3 is reputed to stimulate the expansion of the *g.t.* through the receptor leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8) (Scott *et al.* 2005; AgoulNIK 2007). The LGR8 receptor, formerly called GPR106 or GREAT (G protein-coupled receptor affecting testis descent) and now named RXFP2 (relaxin/insulin-like family peptide receptor 2) is a G protein-coupled receptor (GPCR) (Kumagai *et al.* 2002). The acronym RXFP2 will be used in the present paper from this point onward. Experimental support was given to the implications of a role of activation of the

ligand–receptor complex in testicular descent (review in Feng *et al.* 2009). It has been demonstrated that inhibition of RLF induces a cryptorchid phenotype (Büllesbach *et al.* 2008) and that targeted ablation of the mouse *Ins13* or insulin-like peptide 3 receptor gene *Rxyp2* caused cryptorchidism (Nef and Parada 1999; Zimmermann *et al.* 1999; Overbeek *et al.* 2001; Gorlov *et al.* 2002). Alterations of the ligand-binding domain of RXFP2 have been reputed to inhibit expression of the receptor on the cell membrane and might be responsible for testicular maldescent in human patients (Bogatcheva *et al.* 2007). Moreover, the INSL3–RXFP2 complex was also implicated as an additional paracrine hormone–receptor system in the testis, able to convey information about Leydig cell status to germ cells, and possibly part of an autocrine feedback loop (Anand-Ivell *et al.* 2006; Ferlin *et al.* 2006).

Other possible factors are hypothesised to be involved in TD including androgens from fetal Leydig cells (Emmen *et al.* 2000; Amann and Veeramachaneni 2007) as well as anti-Müllerian hormone (AMH or MIS) from Sertoli cells (Kubota *et al.* 2002). Additionally, a chemiotactic effect in *g.t.* migration exerted by calcitonin gene-related peptide (CGRP) contained in the genitofemoral nerve was hypothesised (Yamanaka *et al.* 1993; Sugita *et al.* 1997). In turn, androgens are necessary to ‘pre-program’ the proliferative response of the *g.t.* to CGRP (Shenker *et al.* 2006). CGRP is also a neuropeptide with trophic effects, which might be involved in synaptogenesis and morphogenetic processes (Herlenius and Lagercrantz 2001).

The molecular regulation of TD in the canine species is still not well understood even though the gross morphological processes of sexual differentiation, fetal development and testicular descent, for instance, have been described. Although much growth and refinement of function occurs later, differentiation of the indifferent gonad with sexual bipotentiality into a testis producing hormones and growth factors is completed at approximately gestational Day (GD) 34 in dogs (Amann and Veeramachaneni 2007). Thereafter the testes are endocrinologically active (GD 36–46) (Johnston *et al.* 2001), a period during which abdominal migration of the testes proceeds. The testis passes the inguinal canal on the 3rd to 4th day postpartum and reaches the ultimate scrotal localisation by the 35th postnatal day (Baumans *et al.* 1981).

It is well known that errors during TD can occur with significant frequency in the dog. Recently, Yates *et al.* (2003) recorded an incidence of cryptorchidism of between 3.3 and 6.8 per cent among a dog population undergoing castration, and a significantly higher frequency in pedigree dogs. In some purebred dogs such as the chihuahua, boxer and German shepherd, the frequency of the disease was reported as high as 30.4, 20.6 and 14.0 per cent, respectively. Such anomalies, as well as the different forms of *ectopia testis*, probably have a multifactorial genesis. They might be accompanied by other disturbances of the testicular phenotype, which are known collectively as testicular dysgenesis syndrome (TDS), all of them possibly having a common underlying cause from improper development of fetal testes (Amann and Veeramachaneni 2007). Though specific gene defects have not been characterised, the canine *insl3* gene was isolated by Truong *et al.* (2003), who demonstrated that it is well conserved and encodes a peptide with the same structure

and the same receptor-binding domain as the other mammalian INSL3 peptides. Therefore, this research was undertaken, with the aim of: (1) describing the presence and distribution of INSL3 in the canine fetal testis, and investigating the localisation of the RXFP2 receptor in the *gubernaculum testis* of the same animals; (2) evaluating, in parallel, the fetal endocrine compartment with respect to expression of anti-Müllerian hormone by the Sertoli cells and the expression of a key enzyme regulating androgen synthesis by the Leydig cells; (3) analysing the CGRP-utilising innervation, at gubernaculum level; and (4) comparing the same parameters in testes and gubernaculum remnants recovered from cryptorchid dogs of varying ages.

Materials and methods

Animals

The canine specimens employed in this study were obtained, with the owners' approval, both during ovariohysterectomies performed at any stage of undesired pregnancies and, in the case of Caesarean sections, due to dystocic parturitions, at the Reproduction Unit of the Faculty of Veterinary Medicine of Milan (Italy). Fetal testes and the respective *gubernacula* were dissected from 28 male fetuses, obtained from nine pregnancies of medium-sized breed bitches. Since previous clinical and endocrinological remarks were not available, gestational age estimate was obtained by ultrasonography measurement of uterine and fetal diameters, according to Kutzler *et al.* (2003). In such a way, the fetuses were arranged into three groups: aged up to 35 days of gestation ($n=7$), from 35 to 45 days of gestation ($n=6$) and from 45 days of gestation to delivery ($n=15$).

Gonads were also collected from female fetuses ($n=8$). Testes from healthy adult dogs ($n=4$) served as controls for reactions directed to the endocrine compartment. Testes and gubernaculum remnants were also collected from adult cryptorchid dogs ($n=9$), after bilateral orchiectomy.

Histology

Immediately after surgery, accurate dissection of fetuses was performed to collect tissue specimens of testis and *g.t.* In the case of healthy and cryptorchid adult dogs the testes were promptly collected and the epididymides were carefully dissected free. Pieces were fixed in 4% paraformaldehyde in PBS (Sigma, Milano, Italy) for 24 h at 4°C. After fixation, fragments were dehydrated in a graded series of ethanol and embedded in paraffin. Of each pair of gubernacula, one was embedded longitudinally and the other one was cut and embedded in transverse sections. Sections cut at 4-µm thickness were dewaxed and stained with routine hematoxylin and eosin (HandE) for histological examination.

Immunohistochemistry

Serial sections were employed for the immunohistochemical demonstration of reactivities to the following polyclonal rabbit antibodies, diluted in a specific antibody diluent with background-reducing components (DakoCytomation, Glostrup, Denmark): anti-INSL3 (1:500; Phoenix Pharmaceuticals, Burlingame, CA, USA), anti-LGR8 (RXFP2) (1:400; Phoenix Pharmaceuticals), anti-AMH (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-P450c17 (1:5000; kindly supplied by Alan J. Conley, University of California Davis School

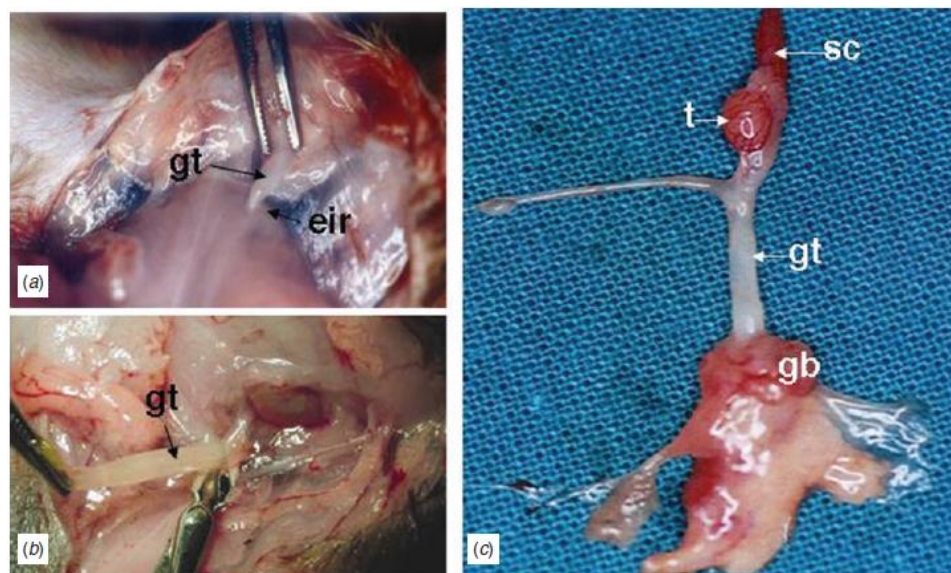


Fig. 1. Dissection of a mature canine fetus showing the macroscopic anatomy of the *gubernaculum testis* (*g.t.*). (a) The short part of the ligament going from the external inguinal ring (*eir*) into the scrotum. (b) The ligament connects the intra-abdominal gonad (retained by a pair of pincers) to the inguinal area. (c) Once dissected, the *g.t.* appears as a jelly-like cord attached to the testicular pole (*t*) opposite to the one where the spermatic cord (*sc*) is forming. At the inguinal end the ligament is connected to the knob-like and gelatinous gubernacular bulb (*gb*).

of Veterinary Medicine, Davis, CA, USA), anti-CGRP (1 : 500; Peninsula Laboratories Inc., San Carlos, CA, USA), employing the techniques in use in our laboratories. The percentage of homology of canine and human INSL3 and RXFP2 was examined by Blast (Basic Local Alignment Search Tool) and determined to be sufficiently high (72 and 90% identity, respectively) to assure good reliability of the data obtained in the canine species by use of commercial antibodies raised against human peptides.

After de-waxing, sections were washed and immersed in a freshly prepared 3% H_2O_2 solution for 15 min to block endogenous peroxidase activity, followed by incubation in 1 : 20 normal goat serum (DakoCytomation) in TBS for 30 min to prevent background before incubation with primary antiserum. Sections were then incubated overnight in a humidity chamber at room temperature with the primary antibody. The sections were then washed and incubated for 30 min with biotinylated goat anti-rabbit immunoglobulins (1 : 200; Vector Laboratories, Inc., Burlingame, CA, USA). Streptavidin-biotin-horseradish peroxidase complex (Vectastain ABC Kit; Vector Laboratories) was employed for detection. Immunoreactive sites were visualised using a freshly prepared solution of 4 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in 10 mL of 0.05 M Tris-buffered saline pH 7.6 (0.05 M TRIS-HCl, 0.15 M NaCl) containing 0.1 mL of 3% H_2O_2 for 13–20 min. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted in DDMount (DDK, Milano, Italy).

Staining controls

Sections of healthy adult dog testis served as positive controls for immunoreactions to INSL3 and P450c17 antisera. Immunoreactivity in fetal Leydig cells served as RXFP2 positive control (Anand-Ivell *et al.* 2006). Positive control for CGRP immunoreaction was performed utilising sections of dog intestine. The specificity of the immunostaining was tested by negative controls, performed by using non-immune rabbit serum (DakoCytomation) in place of primary antisera, which resulted in the absence of immunoreaction. The specificity of AMH and CGRP immunostaining was tested also by incubating sections with the antisera pre-absorbed overnight at their working dilution with the respective antigen at the concentration of $10 \mu\text{g mL}^{-1}$ (AMH protein: Santa Cruz Biotechnology; CGRP protein: Peninsula Laboratories).

Slides were observed and photographed under an Olympus BX51 photomicroscope equipped with a digital camera and DP software (Olympus, Segrate, Milano, Italy) for computer-assisted image acquisition and management.

Results

Macroanatomy

Dissection of the testis–*gubernaculum* complex from a mature canine fetus at the end of pregnancy is illustrated in Fig. 1. Macroscopically, the *g.t.* appeared as a pyramid-shaped structure

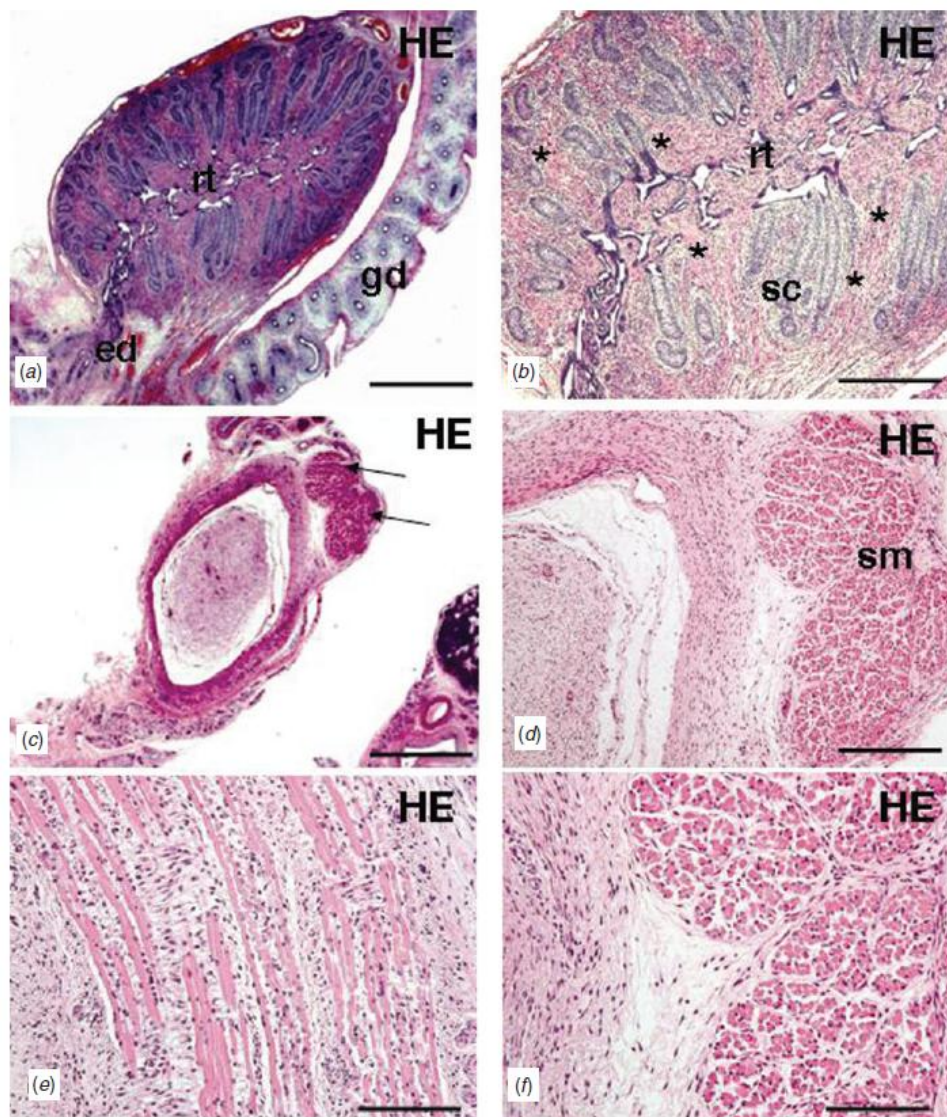


Fig. 2. Microanatomy of the testis and gubernaculum of a mature dog fetus, stained with HandE. (a) The longitudinal section of the testis shows the sperm cords converging towards the *mediastinum* where the *rete testis* (rt) can be seen. At one of the testicular poles the connection between intra- and extra-testicular genital ducts can be noticed, with well-recognisable forming efferent ducts (ed). The developing genital ducts (gd) are present beside the testis, covered by a dedicated peritoneal fold. Scale bar = 2 mm. (b) At higher magnification, the testicular parenchyma shows spermatic cords (sc) surrounded by abundant interstitial tissue (asterisks). The *rete testis* (rt) is well recognisable in the *mediastinum*. Scale bar = 1000 μ m. (c) The transverse section of the gubernaculum testis shows a cord-like structure, with a concentric arrangement of cells and tissues enveloped by a peritoneal fold. The core is made up of connective tissue surrounded by a denser connective sheath. Two strings of skeletal muscle are asymmetrically placed along the main cord (arrows). Scale bar = 2 mm. (d) At higher magnification, the inner core shows a hydrated appearance, with a loose extracellular matrix and poorly differentiated fibroblasts, embedded into an essentially fibrous structure rich in collagen and elastic fibres. sm = skeletal muscle cords. Scale bar = 200 μ m. (e) Longitudinal and (f) transverse aspect of the muscles making up part in the structure of g.t. The muscle cells have an immature aspect and small diameter. Scale bar = 100 μ m.

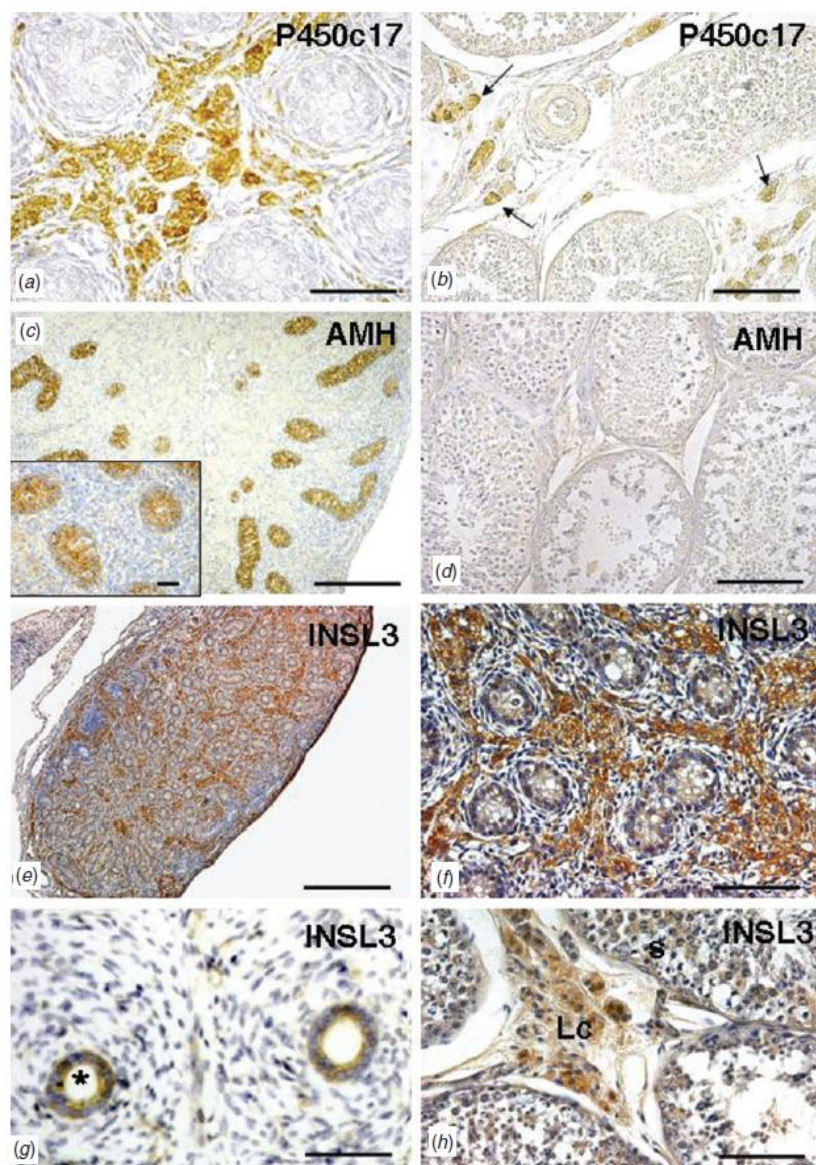


Fig. 3. Immunohistochemical results relative to P450c17, AMH and INSL3 expression in the fetal and adult testes. (a) Fetal and (b) adult Leydig cells (arrows) are stained by anti-P450c17. Scale bars: (a) = 50 μ m, (b) = 100 μ m. (c) Fetal Sertoli cells react with anti-AMH antibodies. Scale bars: (c) = 200 μ m, insert = 50 μ m. (d) AMH immunoreactivity is absent in adult Sertoli cells. Scale bar = 100 μ m. (e, f) Interstitial cells show strong anti-INSL3 immunoreactivity in the testis of a fetus in the intermediate stage of gestation. Scale bars: (e) = 1000 μ m, (f) = 100 μ m. (g) INSL3 immunolocalises in the epithelial cells lining the developing epididymis in a pre-term fetus (asterisk). Scale bar = 50 μ m. (h) In the adult testis, the Leydig cells (Lc) as well as spermatogenic cells (s) show INSL3 immunoreactivity. Scale bar = 200 μ m.

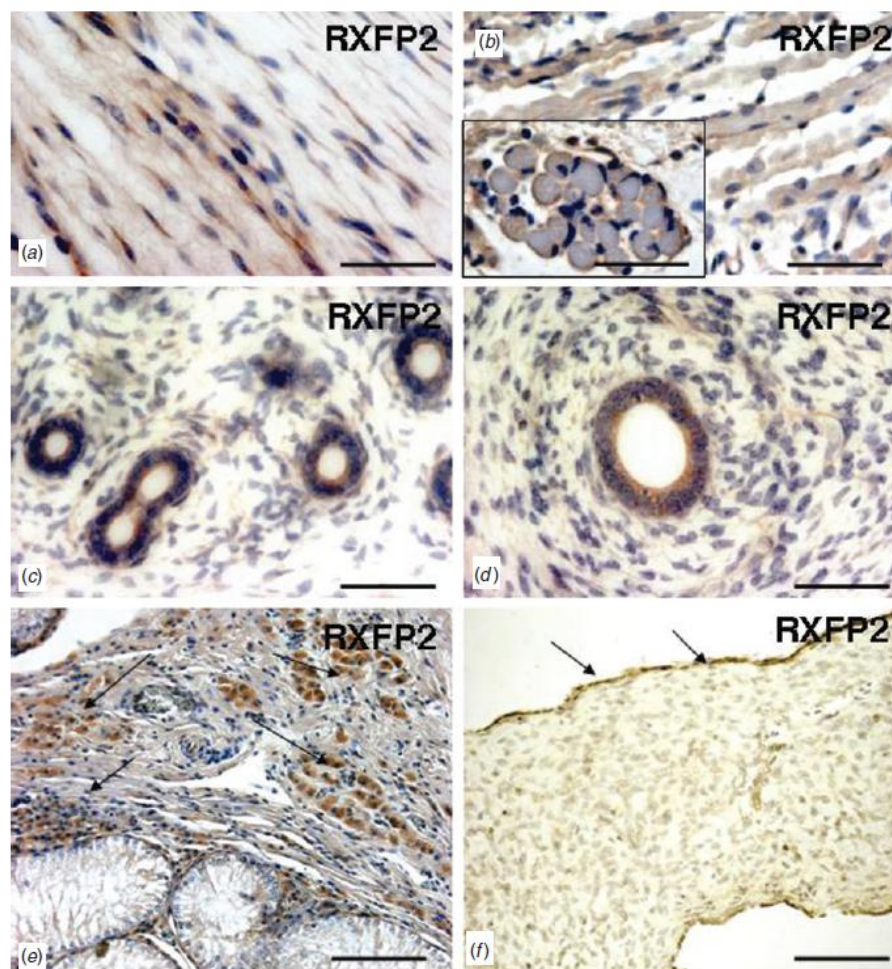


Fig. 4. Immunohistochemical results relative to RXFP2 expression. In the *gubernaculum testis* of a dog fetus at mid-pregnancy (35–45 days of gestation) RXFP2 immunoreactivity can be seen in (a) fibroblast-like cells and in (b) skeletal muscle cells. The insert clearly shows the localisation of the immunoreactivity at the level of the cell membrane. Scale bars = 100 μ m. (c, d) RXFP2 immunoreactivity is present at the level of the epithelial cells covering the developing (c) epididymis and (d) vas deferens in a fetus at term. Scale bars = 100 μ m. (e) RXFP2-immunopositive fetal Leydig cells can be seen (arrows). Scale bar = 200 μ m. (f) In the peritoneum covering the *g.t.* the mesothelium is RXFP2-immunoreactive (arrows). Scale bar = 100 μ m.

suspended in folds of peritoneum, which connected the intra-abdominal gonad to the inguinal area (Fig. 1a, b), attached within the depths of the scrotum. Once dissected, the *g.t.* appeared as a jelly-like, hydrated cord attached to the testicular pole opposite the location of the forming spermatic cord. At the inguinal end, the gubernaculum cord was connected to the knob-like and gelatinous gubernaculum bulb (Fig. 1c).

Microanatomy

Histology of the testis–*gubernaculum* complex in a mature canine fetus at the end of pregnancy is shown in Fig. 2. The ovoid fetal testis was surrounded by a peritoneal sheath, under which several blood vessels distributed to a tunica albuginea (Fig. 2a). Well developed Wolffian derivatives were present to one side, which were recognised as forming genital ducts. The

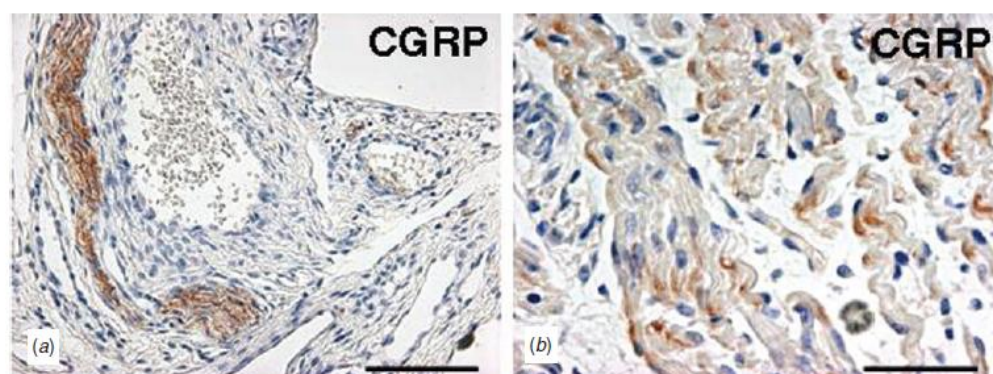


Fig. 5. Immunohistochemical results relative to CGRP in the dog fetus. (a) Large CGRP-immunoreactive nerve fibre bundles can be seen in the gubernaculum testis. Scale bar = 200 μ m. (b) Single subtle CGRP-immunoreactive nerve fibres make contact with the skeletal muscle cells of the g.t. Scale bar = 100 μ m.

gonad was sexually fully differentiated, with sperm cords converging towards a mediastinum where the rete testis was present (Fig. 2a, b). At one of the testicular poles the connection between intra- and extra-testicular genital ducts could be seen, with well-recognisable forming efferent ducts (Fig. 2a).

The g.t. showed a concentric arrangement of cells and tissues (Fig. 2c), comprehensive of a loose connective core of mesenchymal aspect enveloped by a more dense connective sheath. Two cords of skeletal muscle cells were asymmetrically placed beside the cylindrical core (Fig. 2c, d). The inner core had a hydrated appearance, with a loose extracellular matrix and poorly differentiated fibroblasts, embedded into an essentially fibrous structure rich in collagen and elastic fibres (Fig. 2d). The muscles forming part of the structure of the g.t. had an immature aspect with small-diameter cells (Fig. 2e, f). A peritoneal fold enclosed the entire cord-like structure (Fig. 2c).

Immunohistochemistry

Figs 3, 4 and 5 show the results of the P450c17, AMH, INSL3, RXFP2 and CGRP immunoreactions.

In the fetal testes, Leydig and Sertoli cells were stained by anti-P450c17 (Fig. 3a) and anti-AMH antibodies (Fig. 3c), respectively, regardless of the gestational ages considered. Fetal interstitial cells were immunoreactive to anti-INSL3 (Fig. 3e, f), with increasing intensity throughout gestation. P450c17 (Fig. 3b) and INSL3 immunoreactivity (Fig. 3h) were evidenced also in adult interstitial cells, whereas AMH immunoreactivity (Fig. 3d) was absent in adult Sertoli cells. INSL3 was immunolocalised also in the epithelial cells lining the developing epididymis (Fig. 3g) at all gestation ages examined, as well as in cells undergoing spermatogenesis in the adult seminiferous tubules (Fig. 3h).

Anti-RXFP2 antibody immunoreacted with different elements of the fetal g.t., such as the connective cells (Fig. 4a) and the skeletal muscle cells (Fig. 4b). Typically, immunoreactivity was localised at the cell periphery, at the level of the cell membrane. RXFP2 immunoreactivity of the connective and muscle

cells was limited to fetuses at mid-pregnancy (35 to 45 days of gestation). Other immunolocalisations of RXFP2 were noticed, which were present in the fetal structures up to the end of pregnancy. These were at the level of the epithelial cells covering the developing genital tract (Fig. 4c, d), in the endothelium lining numerous blood vessels, in the fetal Leydig cells (Fig. 4e) and in the mesothelium lining the peritoneal covering (Fig. 4f).

CGRP-utilising nerve-fibre bundles were present along the g.t. (Fig. 5a). Single subtle CGRP-immunoreactive nerve fibres accompanied the skeletal muscular component of the g.t. (Fig. 5b).

Results in cryptorchid dogs

Data concerning the macroscopic aspect and immuno-phenotype of cryptorchid testes can be seen in Fig. S1 available as an Accessory Publication to this paper. The retained testicle is usually smaller than the descended, scrotal one. Histologically, the diameter of seminiferous tubules of cryptorchid testes was reduced, and the epithelial lining was composed of Sertoli cells, which did not show AMH immunoreactivity. Spermatogenesis was absent. The interstitial cells were present, with most of them showing p450c17 immunoreactivity, especially those located in extraparenchymal spaces in the tunica albuginea. Leydig cells also demonstrated irregular INSL3 immunoreactivity. RXFP2 immunoreactivity was absent from the genital tracts of cryptorchid testes and g.t. remnants of orchiectomised animals. Otherwise, RXFP2-positive Leydig cells were present in most cases.

In the gonads of female fetuses, which were analysed as well, no immunoreactivity was detected by any of the antibodies tested.

Discussion

Development of the testes in mammalian fetuses is generally considered to be dependent on the presence of a gene on the Y chromosome that signals production of testis-determining factor, although SRY-negative XX sex reversal syndromes in dogs have

been extensively described (Kothapalli *et al.* 2005). Subsequent development of male external and internal genitalia is dependent on testosterone secreted by fetal Leydig cells and AMH (also known as Müllerian inhibiting substance or MIS) secreted by fetal Sertoli cells. The testicular endocrine compartment, comprising these two cell types, in the canine species maintains the hormonal increment between the 36th and the 46th day of fetal development (Johnston *et al.* 2001).

The present study confirms the presence of an active endocrine compartment in male dog fetuses of mid-gestational age, as anti-P450c17 reactivity was immunohistochemically demonstrated in fetal Leydig cells, indicating active androgen synthesis, and Sertoli cells showed AMH immunoreactivity. Moreover, strong INSL3 immunoreactivity was noticed in fetal dog Leydig cells.

In addition, the present study provided immunohistochemical evidence of the presence of the receptor for INSL3 in the dog gubernaculum testis. Diverse cell types in the fetal dog organs showed the presence of RXFP2 receptor. Differentiating skeletal muscle cells as well as fibroblasts in the genitoinguinal ligament showed a typical immunoreactivity on the cell-surface membrane, with a temporal window of expression for RXFP2 protein in mid-gestation that is consistent with a role in trans-abdominal migration. No immunoreactivity for RXFP2 was evident in later stages of fetal development. In general, localisation of the receptor in the gubernaculum is of great interest and significance, as RXFP2 antibodies are not always informative across species. To this end, the present study represents an extensive examination of many fetuses at different pregnancy stages yielding data consistent with a role in testicular trans-abdominal migration between 35 and 45 days of gestation. This period of time might possibly be compared with embryonic Day 14.5 of murine species, when it is known that the expression of *rxfp2* is most prominent in the gubernaculum ligaments (Agoulis *et al.* 2007).

The pattern of expression of Leydig cell insulin-like (Ley I-L) peptide has been extensively studied in the Leydig cells of the adult testis and through testicular development and differentiation (Balvers *et al.* 1998). The peptide was immunolocalised in the normal canine postpubertal testis and epididymis, noting that cryptorchidism did not affect its expression (Klonisch *et al.* 2001). A hormonal function was supposed and, in addition to its potential systemic effects, it was suggested that canine RLF possesses autocrine and paracrine functions within the reproductive tract. INSL3 is equally present in fetal as well as adult Leydig cells, even though it is now well known that they are different cell populations (Haider 2004). Given that the adult Leydig cell population is initially INSL3-negative, the suitability of INSL3 as a differentiation marker has also been assessed (Balvers *et al.* 1998; Anand-Ivell *et al.* 2006). A paracrine role was hypothesised for INSL3 in a sexual dimorphic way, in the mature testis as well as in the ovary (Ferlin *et al.* 2006). Recently it has been demonstrated that RXFP2 is expressed in meiotic and particularly post-meiotic germ cells and in Leydig cells, though not in Sertoli or peritubular cells (Ivell *et al.* 2005). The recent finding of RXFP2 expression in germ cells and the suppression of apoptosis through binding of INSL3 to RXFP2 suggest a paracrine role of INSL3 in germ cell survival (Kawamura *et al.* 2004).

Other immunolocalisations of RXFP2 receptor apart from the expected reactivities at the *g.t.* level were present in fetal structures up to the end of pregnancy. The epithelial cells covering the developing genital tract reacted positively, and Leydig cells also showed RXFP2 immunoreactivity, as previously reported for other species (Kumagai *et al.* 2002; Anand-Ivell *et al.* 2006; Feng *et al.* 2007). RXFP2 immunolocalisation was also seen at the level of the endothelium lining numerous blood vessels and in the mesothelium lining the peritoneal covering. These localisations are possibly part of the INSL3–RXFP2 hormone–receptor system, remaining for a longer time after the developmental period of life (Anand-Ivell *et al.* 2006; Ferlin *et al.* 2006).

As a final comment on INSL3 and RXFP2 immunopositivities seen in dog specimens using commercial antibodies raised against human peptides, it can be assumed that canine INSL3 and RXFP2 display 72 and 90% identity, respectively, with the human ones, according to the results obtained from Blast (Basic Local Alignment Search Tool).

It was suggested that the neuropeptide CGRP plays an important role in testicular descent, either as a chemo-attractant for gubernaculum migration to the scrotum (Sugita *et al.* 1997) or permitting gubernaculum cell proliferation in an androgen-dependent manner (Shenker *et al.* 2006). Recently Chan *et al.* (2009) suggested that CGRP is a survival factor, inhibiting apoptosis in neonatal rat gubernaculum *in vitro*. The present study demonstrated the presence of CGRP-utilising nerves in the canine *g.t.*, also.

Results obtained on canine cryptorchid testes might be of additional significance. The testicular interstitial compartment was found to be capable of androgen synthesis, and the presence of INSL3 was also demonstrated in the Leydig cells. Other structures accompanying the retained gonad, which can be referred to as gubernaculum remnants and genital ducts, didn't show the presence of the receptor. We are aware that these data may be insufficient to define the interrelationship between the INSL3–RXFP2 complex and cryptorchidism in the dog since observations among adult cryptorchids are uninformative. However, it is impossible at the present time to study the *g.t.* of fetuses which are destined to become cryptorchid.

Association between human testis maldescent phenotype and *insl3* and *rxfp2* gene mutations have been identified, in particular at the level of Amino Acid 222 of *rxfp2* (Bogatcheva *et al.* 2007). An INSL3 sequence error involving Amino Acid 22 of the INSL3 dog precursor protein was recently described in a Dalmatian dog suffering from bilateral cryptorchidism and hypospadias (Cassata *et al.* 2008). However, recent research aimed at the identification of polymorphisms within the canine *rxfp2* gene, possibly associated with cryptorchidism, demonstrated complete homology between cryptorchid and healthy dogs, that is, no nucleotide variations exist (see Fig. S2) (Sironi *et al.* 2007). Topical studies carried out in humans suggest several other risk factors for cryptorchidism, including endocrine-disrupting chemicals that have oestrogenic or anti-androgenic effects (Virtanen and Toppari 2008). This hypothesis could be an attractive field of research in the canine species, as well.

In conclusion, the present study demonstrated that in the dog, as in other species, INSL3 likely controls the first intra-abdominal stage of testicular descent by way of the cognate

receptor RXFP2, the expression of which was found in the *gubernaculum testis* in a temporal window at mid-pregnancy only. The canine species may provide a suitable experimental model of relevance to cryptorchidism in other species, based on the relative availability of fetal specimens and the dimensions of the *g.t.*, which is large enough to be easily collected and examined, at specified stages of pregnancy. Moreover, the canine species might be involved in the experimental cryptorchid model, inducing this event surgically, hormonally or genetically (Baumans *et al.* 1982). As Husmann (1998) stated, the most ideal animal models for human cryptorchidism are supposed to be dogs and primates. Unfortunately, the experimental expense and concerns of the institutional animal care committees significantly impact on their use for investigational purposes.

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CHAPTER 8

Undernutrition during foetal to prepubertal life affects aquaporin 9 but not aquaporins 1 and 2 expression in the male genital tract of adult rats

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Undernutrition during foetal to prepubertal life affects aquaporin 9 but not aquaporins 1 and 2 expression in the male genital tract of adult rats

S. Arrighi^{a,*}, M. Aralla^a, P. Genovese^b, N. Picabea^b, A. Bielli^b

^a Department of Veterinary Sciences and Technologies for Food Safety, Laboratory of Anatomy, Università degli Studi di Milano, Italy

^b Department of Morphology and Development, Veterinary Faculty, University of Uruguay, Montevideo, Uruguay

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Abstract

Expression of aquaporin water channels (AQPs) in the male excurrent ducts, is of major importance for local water movements. To study the influence of pre- and postnatal undernutrition on AQP-expression in the adult male genital tract, 4 pregnant female rats were fed *ad libitum* (control group) and 4 with 33.5% of gestational feed requirements (underfed group). Feeding restriction of underfed group pups continued up to weaning (25 days of age), then all pups were fed *ad libitum* until slaughtered at 100 days of age. Epididymides were sampled and processed for aquaporin immunohistochemistry. Expression of AQP1 was similar either in the control and underfed groups of rats, strongly evidenced at the apical and lateral plasma membrane of the efferent ducts non-ciliated cells, in the smooth muscle cells surrounding epididymal duct and in blood vessel endothelium throughout the epididymis. AQP2-immunoreactivity was present in the corpus and cauda regions, strongly expressed in the principal cells of both groups of rats. In contrast, AQP9 expression was modified by early life undernourishment, as it was weakly evidenced at the microvilli in the principal cells and strongly diminished or completely lacked in the clear cells of the cauda, in underfed group epididymides. Since it is known that clear cells are involved in luminal fluid acidification, this function might be altered in adult animals, which were underfed during early life.

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Keywords: AQP (aquaporin); Efferent ducts; Epididymis; Vas deferens; Foetal programming

1. Introduction

Although Robert A. McCance in 1962 [1] was the first to demonstrate, in animals, that early nutrition could also have lifetime “programming” effects, growing interest about the concept of foetal and developmental programming began to rise only twenty years ago after the epidemiological observations of David J. Barker and his co-workers, demonstrating in the human an inverse relation between weight in infancy and death

from ischemic heart disease [2]. Since those observations, Barker developed a theory, now known as the “Barker hypothesis” [3], proposing that adverse events *in utero* induce compensatory responses in the foetus that reflect “developmental plasticity” during this critical period [4] and persist permanently. Each individual’s phenotype, even if based on a specific genetic programme, is influenced by epigenetic and environmental factors. During development there are critical windows of vulnerability to suboptimal conditions when programming may permanently modify adult phenotype and disease susceptibility [5] and the altered phenotype will persist throughout a lifetime.

* Corresponding author. Tel.: +39.0250315741; fax: +39.0250315771.
E-mail address: silvana.arrighi@unimi.it (S. Arrighi).

Alterations in foetal developmental programming can direct adult health consequences by way of epigenetic factors acting in diversified ways [6]. Programming involves structural changes in different organs, such as altered cell number, imbalance in distribution of different cell types within the organ, and altered blood supply or receptor numbers. Compensatory efforts made after birth to reverse the consequences of programming may have their own unwanted consequences, thus carrying a price, as postnatal conditions can be different from those for which the foetus prepared. Effects of programming may pass across generations by mechanisms that do not necessarily involve structural gene changes. Programming often has different effects in males and females [5].

Maternal undernutrition and, under certain circumstances, overnutrition, before or during pregnancy or during early postnatal life can alter reproductive function of the offspring [7]. Developmental maturation of the hypothalamic-pituitary-gonadal axis has been shown to be susceptible to foetal programming, too [8,9]. It is suggested that single nutrients and/or metabolites are unlikely to have direct impacts on the pattern of development of the reproductive system and it is postulated that multiple endocrine and metabolic signals are involved. Early life nutritional influences modify many components of the hypothalamic-pituitary-gonadal system, such as gonadotrophins and GnRH secretion. The normal ontogeny of gonadal development and function can be disrupted by undernutrition or by the influence of endocrine-disrupting compounds either in a direct way or by an altered endocrine status. Hormones such as insulin, triiodothyronine (T_3) and leptin have the potential to directly mediate the effects of nutrition on the gonads or to influence programming of the developing brain of the foetus, and in particular the hypothalamus, with potential indirect consequences for gonadal development and function [9].

Aquaporins (AQPs) are channel proteins facilitating the rapid passive movement of water throughout the cell membranes [10]. Aquaporin water channels are found in a subset of epithelia with a 10- to 100-fold higher capacity for water permeation in comparison with those devoid of these proteins [11]. The presence of AQPs in the male genital tract, where significant movements of fluids and molecules useful to sperm maturation are known to take place [12], is therefore of primary importance [13]. Many AQPs are known to be present in the rat male genital tract (AQP1, AQP2, AQP5, AQP8) [14–18], also belonging to the aquaglyceroporin group (AQP3, AQP9, AQP10) [19,14–16],

known to be permeable even to small solutes such as urea and glycerol. Since some AQP molecules are known to be sexual steroid-dependent [20,14,21–23], it is possible that their expression could be induced by androgens present during prenatal and perinatal development, either in the plasma or *in situ* in the epididymis [21]. Therefore it is of particular interest to study their expression in undernourished rats, in which affected testicular structure and reduced number of Sertoli cells have already been demonstrated [24]. Thus the aim of this research was to study if undernutrition during foetal to prepubertal life up to weaning affects aquaporin expression in the male genital tract of adult rats.

2. Materials and methods

2.1. Animals

Animal breeding and experimental procedures are described in detail in [24]. Briefly, twelve adult (5 month aged) virgin Sprague–Dawley rats (mean body weight 272.5 ± 28.74 g) housed at the 'Instituto de Investigaciones Biológicas Clemente Estable', Montevideo, Uruguay, were bred all with the same male (body weight 257.5 g) in groups of 1 male and 3 females until all became pregnant. Pregnant rats (gestation diagnosed by vaginal smears) were divided into two groups. Control group (CG, $n = 4$), kept in individual cages, were fed a standard rat diet (3 Cal/g, 21% protein, 10% fibre, 7% total minerals) *ad libitum*, during gestation and lactation (until 25 day post-partum). Litters were adjusted to eight pups per lactating dam. Another group of pregnant dams was underfed (UG, $n = 7$) by 33.5% of *ad libitum* intake of CG pregnant rats. Water was always offered *ad libitum* to dams. All the rats were weighed weekly. After parturition, dams were offered feed *ad libitum*, but litters in UG (which could not reach feed) were adjusted to 14 pups. All pups (from both CG and UG) were weighed daily until weaning. At 25 days of age pups were weaned and housed (4 animals per cage), fed *ad libitum* and weighed weekly until 100 days of age.

At 100 days of age, male pups (CG, $n = 10$; UG, $n = 7$) were weighed and euthanized with 25 mg Sodium thiopental *i/p*. Animal management and euthanasia procedures were approved by the Animal Welfare Committee at the Veterinary Faculty, University of Uruguay. Testes were promptly collected and the epididymides were gently dissected free. Both organs were weighed, then fragments of caput (including efferent ducts, EDs), corpus, cauda epididymis, and proximal vas deferens were dissected free from testis, and

immediately fixed by immersion in Bouin's solution for 24 h. Samples were stocked in ethanol 70%, then dehydrated and embedded in paraffin. Blocks were sectioned at 6 μ m thickness and stained with Haematoxylin–Eosin for morphologic purposes or processed for immunohistochemistry.

2.2. AQP-immunohistochemistry

Previously described immunohistochemical methods [25,26] were used for this study. Tris-buffered saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) buffer was used for rinses throughout the whole procedure. After de-waxing and stocking in ethanol 70% overnight at 4 °C, Bouin-fixed sections were washed and immersed in a freshly prepared 3% H₂O₂ solution for 15 min to block the endogenous peroxidase activity, followed by incubation in 1:20 normal goat serum (DakoCytomation, Denmark, code # X 0907) in TBS for 30 min to prevent background prior to incubating with primary antiserum. Sections were then incubated 2 h in a humidity chamber at room temperature using rabbit polyclonal antibody against rat aquaporin 1, aquaporin 2 and aquaporin 9 (Alpha Diagnostic International, San Antonio, TX; Cat # AQP11-A, AQP21-A and AQP91-A respectively) diluted 1:200 (AQP1), 1:30 (AQP2) and 1:250 (AQP9) in a specific antibody diluent (DakoCytomation, code # S 302283). The sections were then washed and incubated for 30 min with biotinylated goat anti-rabbit immunoglobulins (DakoCytomation, code # E 0432) diluted 1:200. Streptavidin-Biotin Complex (Vectastain® ABC Kit, code # PK-4001, Vector Laboratories, Inc., UK) was 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% H₂O₂ for 13–20 min. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted using Eukitt® (Biopica, Milan, Italy).

2.3. Staining controls

Sections of adult rat kidney served as positive controls for immunoreactions to AQP1 (Fig. 1A) and AQP2 antisera. Positive control for AQP9 immunoreaction was performed utilizing sections of rat liver (Fig. 3A). The specificity of the immunostainings was tested by negative controls, performed by (i) the use of non-immune rabbit serum (DakoCytomation, code # X 0903) in place of specific antisera; (ii) incubating sections with antisera pre-absorbed with the respective antigens (Alpha Diagnostic International; Cat # AQP11-P, AQP21-P, AQP91-P); and

(iii) omission of the first layer. All of them resulted in the absence of immunoreaction.

The evaluation of staining intensities was based on subjective, double-blind estimates of two of the authors.

Slides were observed and photographed under an Olympus BX50 photomicroscope equipped with a digital camera and DP software (Olympus, Tokyo, Japan) for computer-assisted image acquirement and managing.

3. Results

3.1. Control rats

Strong AQP1 immunoreactivity (IR) was observed in the epithelium of the EDs, localized at the apical surface of the non-ciliated cells, corresponding to the microvilli projecting into the lumen (Figs. 1B, 1D). Intense IR was present in the lateral plasma membranes of adjacent non-ciliated cells (Fig. 1D). Few ciliated cells were recognized, which remained unstained (Fig. 1D). No reaction was observed over the epithelial cells of any epididymal region or proximal vas deferens of adult rats. Otherwise, immunostaining at the level of the smooth muscle cells surrounding the duct was noticed, with decreasing intensity from the caput region to the vas deferens (Figs. 1B, 1E, 1F). Intense AQP1-IR was evidenced in the endothelial cells of blood vessel walls throughout all the genital tract from EDs to vas deferens (Fig. 1F).

Immunohistochemical expression of AQP2 was evident at the level of epididymal principal cells, at the corpus and cauda level, and in the vas deferens. Intense IR was present both in the plasma membranes and in the cytoplasm (Fig. 2A). Clear cells remained unstained. No reaction was present in the EDs and the caput epididymidis.

AQP9 was immunolocalized at the microvilli in the non-ciliated cells of EDs. At the level of epididymal principal cells AQP9-IR was noticed in the stereocilia, showing increasing intensity from the caput (Fig. 3B) to the corpus (Fig. 3D) and cauda (Fig. 3F), up to the vas deferens (Fig. 3H). In the corpus region AQP9-IR was inconstantly noticed in the clear cells (Fig. 3D), whereas immunoreaction was strongly present in this cell type in the cauda region (Fig. 3F). The entire cytoplasmic area of this cells type was AQP9-IR.

3.2. Underfed rats

AQP1-IR was unaffected in rats undernourished during foetal and postnatal development. It was present with similar localizations and intensities as in the con-

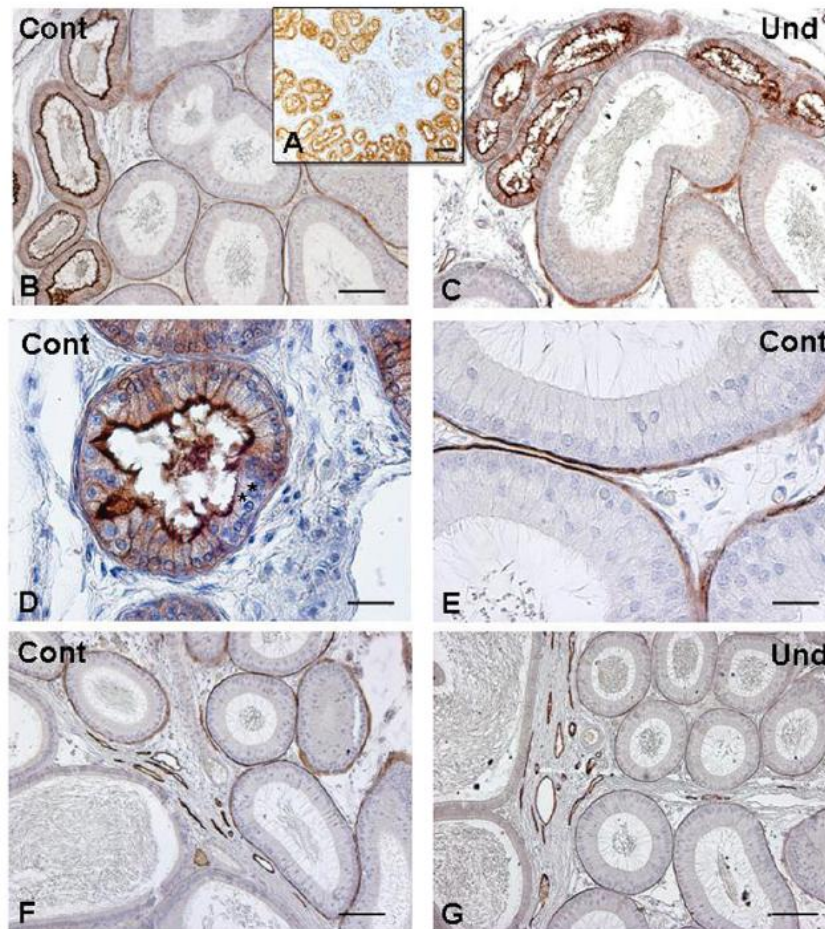


Fig. 1. AQP1-immunoreactivity in adult rat kidney (A), control (B, D, E, F) and undernourished (C, G) rats. (A) In the section of rat kidney used as positive control, AQP1-immunoreaction is expressed in the plasma membranes of the proximal tubule. Scale bar = 50 μ m. (B) In control rats, AQP1-IR is strongly present in efferent ducts (ED) epithelium, especially at the luminal surface. Otherwise, epithelial cells lining the epididymis (ep) are AQP1-negative, whereas AQP1-IR is present in the smooth muscle cells surrounding the epididymis. Scale bar = 100 μ m. (C) AQP1-IR is detectable in similar localizations in undernourished rats. Scale bar = 100 μ m. (D) In the epithelium of EDs the strong AQP1-IR is mainly localized at microvillar level of non-ciliated cells and at the laterobasal surface of adjacent cells. Ciliated cells (asterisks) remain almost unstained both at the luminal surface and at lateral membranes. Scale bar = 20 μ m. (E) Smooth muscle cells surrounding the epididymis are decorated with intense AQP1-IR. Scale bar = 20 μ m. (F–G) AQP1-IR is present with similar intensity in blood vessel endothelium either in control (F) and in underfed (G) rats. Immunoreactivity of epididymal smooth muscle cells can be seen in both groups of rats, with decreasing intensity from caput (on the right in both pictures) to corpus (on the left in both pictures). Scale bars = 100 μ m.

trols in the EDs (Fig. 1C) and the epididymis (Figs. 1C, 1G) as well as in the endothelial cells of blood vessels (Fig. 1G).

The expression of AQP2 in the rat epididymis was not perturbed by *in utero* and postnatal undernourishment. The localization at epididymal corpus and cauda

levels and in the vas deferens remained unvaried (Fig. 2B).

AQP9-immunolocalization was evidently affected by early life undernourishment. At the level of EDs immunoreaction was almost absent in the microvilli of undernourished rats (Fig. 3C). In the caput epididymis

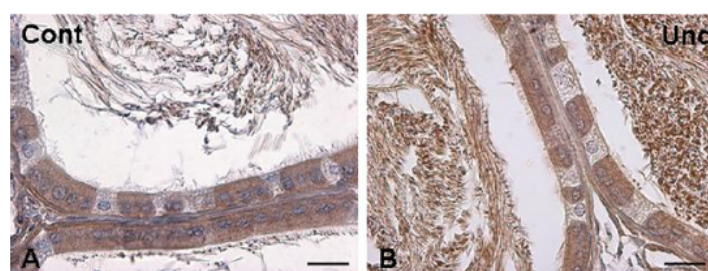


Fig. 2. AQP2-immunoreactivity in control (A) and undernourished (B) rats. AQP2-IR is strongly present in the membranes and cytoplasm of principal cells, either in control (A) and in underfed rats (B). Clear cells are unstained. Scale bars = 20 μ m.

AQP9 expression was only slightly diminished (Fig. 3C). Dramatically different AQP9-IR was noticed in comparison with control rats either in the corpus and cauda regions, where a weak positivity was present in the microvilli in undernourished rats (Figs. 3E, 3G). Clear cells were either very weakly positive or in most cases were negative to AQP9 antiserum in undernourished rats (Figs. 3E, 3G). Also in vas deferens AQP9-IR was hypo-expressed in undernourished rats (Fig. 3I).

4. Discussion

It has been previously demonstrated that testicular and epididymal morphology in adult rats is affected by undernutrition during foetal to prepubertal life. Testicular weights were lower and structure was modified. The long lasting effects observed at testicular level, in particular the reduced number of Sertoli cells per testis, strongly suggested lower daily sperm production in the undernourished male offspring once they had become adult [24]. Epididymides from such animals were lighter even if they showed minor changes in histological structure [27].

In the present study the same experimental sample of rats was utilized to verify the possible influence of pre- and postnatal undernutrition on AQP expression in the adult male genital tract. The choice of these molecules to be studied was done bearing in mind that the presence of AQPs, channel proteins that facilitate rapid passive movement of water across cell membranes, is of major importance in the male excurrent ducts, where water movements mainly take place. Several authors pointed out that an impaired AQP expression can be a consequence of hormonal failure [14,22,23], which in turn is likely to happen in case of undernutrition.

It is known that rat efferent ducts are lined by ciliated and non ciliated cells and rat epididymal epithe-

lium is made up by five cytotypes: principal, basal, halo, apical, and clear cells. The main function of ED non-ciliated cells is the absorptive one, whereas the ciliary movement of the ciliated cells is obviously devoted to the movement of sperm, which are immotile at this level [26]. Although many epididymal functions are still considered an enigma (such was the word used by David W. Hamilton in 1975 about the puzzling basal cells) [28], nowadays a specific function is tentatively appointed to each cell type [29]. Principal cells are the major absorptive and secretory cells; basal cells, far from being stem cells as hypothesized for many years, possibly play a protective and scavenger role [30]; halo cells are involved in immune functions, conceivably being T lymphocytes according to Flickinger et al [31]; apical and clear cells are proton secreting cells, the last ones implicated in luminal acidification [32] as well as in taking up luminal components. The acquisition that epididymis might have a pivotal role in male fertility control has been known for many years [33].

In our experiment, AQP1-IR obtained in the rats belonging to the control group corroborated previously published results acquired on the male genital tract of adult rats [14,22,17,18,23]. AQP1-IR was noticed in the microvilli and laterobasal membranes of the non-ciliated cells in the EDs, whereas the epithelium did not show any immunoreactivity in the different regions of the epididymal duct. AQP1-IR was otherwise present at the level of muscle cells surrounding the duct, with decreasing intensity toward the vas deferens. In addition, AQP1-IR decorated blood vessel endothelia throughout the genital tract. The absorptive function related to these reactivities is possibly exerted mainly at the level of the EDs where the concentration of testicular fluid happens, which requires rapid reabsorption. The AQP1-IR of blood vessel endothelia has an obvious implication in water removal from intertubular spaces once water has been transported across the ep-

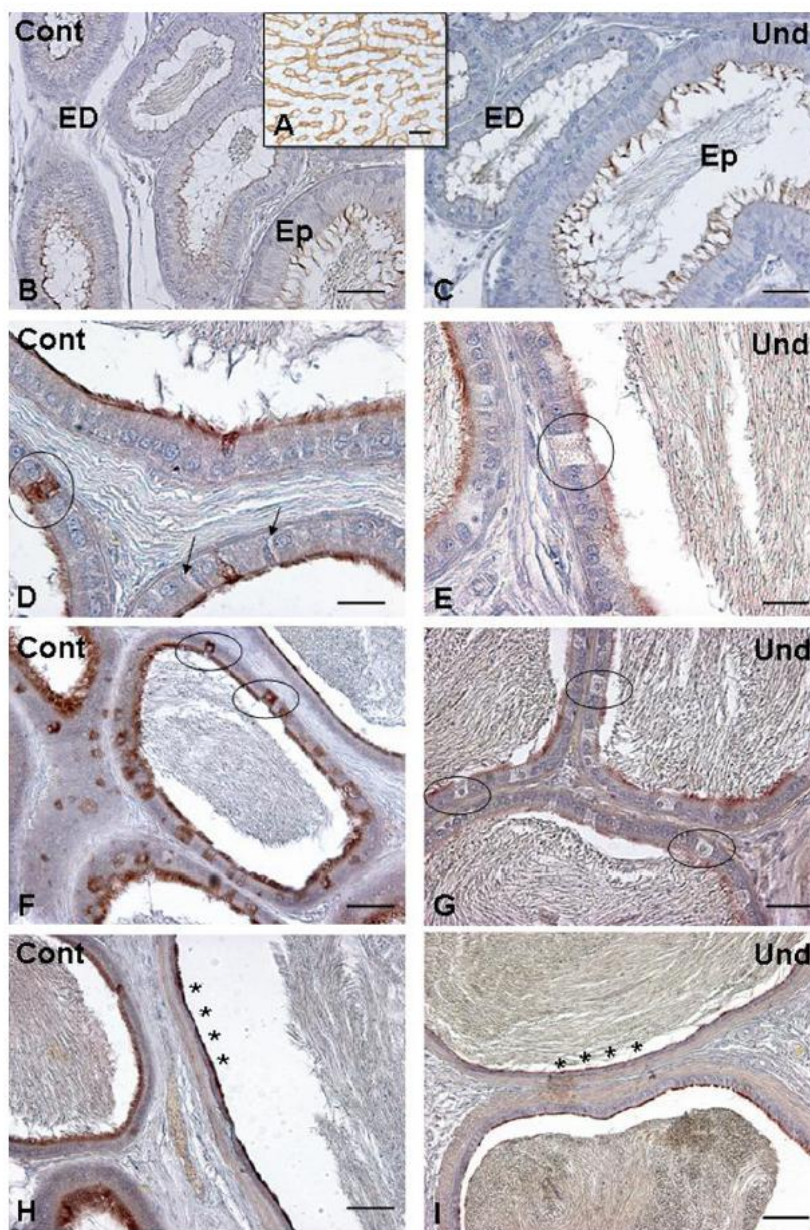


Fig. 3. AQP9-immunoreactivity in adult rat liver (A), control (B, D, F, H) and undernourished (C, E, G, I) rats. (A) In the section of rat liver used as positive control, AQP9-immunoreaction is expressed at the sinusoidal plasma membrane. Scale bar = 20 μ m. (B–C) Rat epididymis, caput region. AQP9-IR is present in the microvilli of the non-ciliated cells of EDs, with a lesser intensity in underfed (C) than in control (B) rats. At the level of epididymal principal cells (Ep) AQP9-IR stereocilia can be seen, with a similar immunoreactivity in control (B) and in undernourished (C) rats. Scale bar, B = 50 μ m. Scale bar, C = 100 μ m. (D–E) Corpus region. In control rats (D) AQP9-IR is strongly localized in the microvilli of principal cells. Some clear cells (encircled) show intense AQP9-IR, whereas others are negative (cell portions indicated by arrows). In underfed rats (E) AQP9-IR appears weaker in the microvilli and clear cells (encircled) are either negative or very weakly positive. Scale bars = 20 μ m. Stronger differences can be seen in the cauda epididymis (F, control rats; G, underfed rats) and vas deferens (H, control rats; I, underfed rats). Scale bars = 50 μ m.

ithelium, and continues throughout all the male tract, in collaboration with the presence of AQP1 in the smooth muscle cells surrounding the duct [22].

AQP2 was thought for a long time to be expressed exclusively in kidney collecting duct principal cells [34]. At the level of the male genital tract, AQP2 immunohistochemical expression was found only in the dog EDs and epididymis [35] and detected by immunofluorescence in the rat vas deferens [18], where it is constitutively expressed in the apical membrane [35]. The rapid recycling between the plasma membrane and intracellular vesicles may account for immunolocalization throughout the cytoplasm [36], which was the pattern we observed in rat samples. Some discrepancies in the published membrane distribution of AQP2 in the male genital tract [18] may reflect differences in rat strains.

Although AQP9-IRs in the rat male genital tract have long been discussed in the literature, deeply different results from diverse laboratories have been reported. A paper by Hermo et al [16] ultimately evidenced clear relationships between methods of fixation/immunodetection as well as antisera employed and AQP revealability in the epididymal clear cells. When using the same antibody employed in our study [14] a clear AQP9-IR can be seen in the microvilli of the ED non-ciliated cells and of the epididymal principal cells, increasing from the caput to the cauda region. Clear cells are weakly to strongly reactive at corpus level while they are constantly AQP9-immunoreactive in the cauda region. AQP9 is an aquaglyceroporin, known to be a channel for neutral solutes, in addition to being a water channel. It allows the passage of glycerol also, which has been proposed to serve as metabolic substrate for sperm to produce CO₂ [17]. Glycerol is derived from glycylphosphorylcholine, 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 [15]. The presence of AQP9 in clear cells further stresses this function. Moreover, Badran and Hermo [14] attribute the cytoplasmic reaction recorded for the clear cells to the abundance of endocytic organelles in these cells and the resulting amplification of the diaminobenzidine reaction product.

In rats which had been undernourished during foetal to prepubertal life only AQP9-immunoreactivity was dramatically affected, whereas AQP1- and AQP2-immunoreactivity remained mostly unvaried. AQP9-immunoreactivity consistently decreased in the ED and epididymal microvilli and almost completely disap-

peared from the clear cells. It is known that AQP1 and AQP2 are constitutively expressed in the male genital tract, while AQP9 is affected by hormonal regulatory factors [22]. It is possible that AQP9 expression could be induced by androgens present during prenatal and perinatal development in the male either in the plasma or *in situ* in the epididymis [21]. It is known that the epididymis (and maturing spermatozoa) are hormonally supplied by at least three different pathways: i) via blood vessels/systemic circulation; ii) directly from the lumen, since testicular androgens bombard the epididymis as lumicrine factors, bound to the androgen binding protein produced by the Sertoli cells; iii) by synthesis by the epididymal epithelium itself, through the action of 5- α -reductase. All these sources might be affected by undernourishment, thus influencing AQP9 expression in the diverse tracts and cell types of the genital tract. In addition, androgen receptors in the epididymal epithelium might be affected, as well. Linked to the importance of these factors altogether, the diminished number of Sertoli cells which was observed in the adult rats of this experiment [24] seems maximally relevant.

Other authors observed a diminished number of Sertoli cells after *in utero* undernutrition in sheep, either in newborn lambs [37] or in adult rams [38], and inferred a possible negative rebound upon daily sperm production, as the number of Sertoli cells is highly correlated with adult testicular size and maximum rate of germ cell production [39]. Sertoli cells appear early in foetal life and divide until pre-pubertal age. Maximum Sertoli cells replication takes place in a temporal window between the 9th and the 16th day of foetal life in rats and they stop replicating within the 4–16th postnatal day [40].

Despite the possible negative outcomes on daily sperm production which could be supposed as a consequence of an alteration in the number of Sertoli cells, Rae et al [41] stated that semen quality at 20 months of age was unaffected by pre-natal undernutrition in sheep. Moreover, homozygous AQP9^{-/-} knockout mice were demonstrated to be fertile with normal sperm motility and morphology [42]. This is why our future purposes include, in the hypothesis to repeat this experiment improving it, monitoring the semen quality of adult rats which had been undernourished during their pre- and postnatal development. Other future items to be studied could be the observation of other AQPs expression, study of receptors for steroid hormones as well as for molecules known to create a link between nutrition, metabolism and reproduction, as for instance

leptin or ghrelin. Investigation of possible critical windows of vulnerability to suboptimal conditions during gestation as well as testing different diets to evidence the effect of diverse nutritive components could be of great utility. Finally, the possibility of monitoring the possible effects on successive generations could add important information useful to understand other topics of the foetal programming theories.

In conclusion, our study demonstrated that AQP9 expression was modified in adult rat epididymis as a likely consequence of a diminished hormonal supply due to foetal to prepubertal undernutrition. AQP9-IR diminished in the microvilli of epididymal duct principal cells and was very diminished or completely lacked in the clear cells of the cauda epididymis. Since it is known that clear cells are involved in luminal fluid acidification, it can be hypothesized that this function might be altered in adult animals, which were underfed during early life.

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A.B. and S.A. conceived, designed, and coordinated the experiment. A.B., P.G., and N.P. performed the experimental procedures concerning managing of the animals and sample processing, providing the rat specimens employed in the immunohistochemical study. S.A. and M.A. planned the immunohistochemical study. M.A. carried out the immunohistochemical procedures. S.A. and M.A. evaluated and photographed the slides, and arranged the figures. S.A. wrote the manuscript. All the Authors participated in critical reading and final approval of the manuscript.

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CHAPTER 9

Immunolocalization and expression of leptin (Ob) and leptin receptor (Ob-r) in the ovary and in pre and post *in vitro* matured horse oocytes: prepuberty, body weight and breed effect

Ready for Submission

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“Immunocytochemical localization of leptin (Ob) and leptin receptor (Ob-R) in preovulatory and in vitro matured horse oocytes related to prepuberty and different weight breeds”

A. Lange Consiglio, S. Arrighi, G.P. Bosi, M. Aralla, F. Cremonesi. - Reproduction in Domestic Animals. – 2008; 43 suppl 3: 107-107

Immunolocalization and expression of leptin (ob) and leptin receptor (ob-r) in the ovary and in pre and post *in vitro* matured horse oocytes: prepuberty, body weight and breed effect

Lange Consiglio A.¹, Dell'Aquila M.E.², Fiandanese N.³, Pocar P.³, Aralla M.⁴, Bosi G.P.⁴, Arrighi S.⁴, Cremonesi F.¹

¹*Università degli Studi di Milano, Reproduction Unit, Large Animal Hospital, Faculty of Veterinary Medicine, Via dell'Università 6, 26900 Lodi, Italy*

²*Università degli Studi di Bari, Production Animal Department, Faculty of Veterinary Medicine, S. Prov. per Casamassima, km 3 – 70010 Valenzano (Bari), Italy*

³*Università degli Studi di Milano, Department of Animal Pathology, Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, Via Celoria 10, 20133 Milano, Italy*

⁴*Università degli Studi di Milano, Department of Veterinary Science and Technologies for Food Safety, Laboratory of anatomy, Via Trentacoste 2, 20134 Milano, Italy*

9.1 Abstract

Several factors, including leptin, could be considered as links between nutritional status and reproductive processes. In the horse, no information about the presence of leptin (Ob) and leptin receptor (Ob-R) in the ovary or in the oocyte is available. The present study investigated the immunolocalization of Ob and Ob-R and the profile expression of Ob and Ob-R mRNA in immature and matured compact cumulus oocytes recovered after slaughtering from fillies and from mares of light or heavy body weight breeds. Moreover, we examined the immunolocalization of Ob and Ob-R in ovaries tissue from these three different kind of animals. Our results showed that both Ob and Ob-R, detected in all immature oocytes, were uniformly distributed throughout the ooplasm, but the intensity of reaction was lower in light weight mares and in fillies oocytes compared to oocytes of heavy weight mares. After IVM, heavy breed mares had higher proportion of oocytes that reached the metaphase II than light mares and fillies and both Ob and Ob-R were localized to the oocyte cortex. This distribution within the oocytes was independent from the animal group and again with weaker immunopositivities in light mares and in fillies. These intensities are confirmed by the profile expression of Ob and Ob-R mRNA. Moreover, we detected the presence of Ob in blood vessels within the ovary as well as within the corpora lutea in all kinds of animals analyzed. The results of the present study support the hypothesis that, in the horse, the different

immunoreaction intensity can be related either to the horse breed or to the reproductive puberal development and that the presence of leptin in the corpora lutea could suggest a possible involvement in oocyte maturation, angiogenesis, follicle rupture or subsequent corpus luteum formation.

9.2 Introduction

Leptin, a lately discovered hormone, is the 167-aminoacids product of the ob gene expression (Zhang et al., 1994) which takes part in the regulation of energy metabolism. It is mainly synthesized by fat cells in the adipose tissue but also in the stomach, liver and placenta (Meier and Gressner 2004).

Leptin may be found in the circulation in the free form or complexed with leptin binding proteins, and this characteristic appears to be species-specific and dependent upon physiological status (Garcia et al., 2002; Houseknecht et al., 1996; Zieba et al., 2005).

Circulating leptin concentrations have been reported to closely correlate with both the body mass index and the total amount of body fat (Banks, 2004; Fruhbeck et al., 1998; Fruhbeck, 2001) and this underlines the important role of leptin as a peripheral humoral signal carrying information about energy reserves of the organism (Maffei et al., 1995). The reproductive system of mammals is very sensitive to the availability of energy from an external environment. Acute changes in the energetic status of animals affects the hypothalamo-pituitary-gonadotropic (HPG) axis activity (Cunningham et al., 1999). It has been documented that fasting or food restriction resulted in the suppression of pulsatile luteinizing hormone (LH) secretion in rodents and farm animals including sheep, cows and horses (Cunningham et al., 1999). Similar changes were also observed in humans (Cameron et al., 1991). Food restriction, acting via influence on the HPG axis hormones, resulted in a delay of the onset of puberty and evoked temporary or permanent infertility (Foster and Olster 1985; Garcia-Lopez et al., 2005). Fasting-induced LH suppression, is believed, to be a consequence of a reduced release of GnRH from the hypothalamus (Kile et al., 1991). Several studies showed that also leptin is involved in the control of reproductive functions. In 1996, Chehab et al. (1996) studied ob/ob mice (mice with a mutated leptin gene and resulting hormone insufficiency). Both male and female ob/ob mice were obese and infertile but food limitation and loss of weight did not lead to reinstatement of fertility. On the contrary, fertility was reinstated with the administration of recombinant leptin (Barash et al., 1996; Wade et al., 1997; Chehab et al., 1996; Mounzih and Chehab, 1997). Administration of leptin to normal mice prior to puberty resulted in the onset of their reproductive function with subsequent maturation of their reproductive system 9 days earlier. This remark led researchers to the conclusion that leptin, in

addition to its role in the normal function of the reproductive system of mature mice, could also fire the processes for the onset of puberty (Chehab et al., 1997; Goumenou et al., 2003). In humans and animals the onset of puberty is associated with an increase in fat and consequent increase in circulating leptin, suggesting that this hormone may be required for normal growth and development of reproductive organs. So, inadequate nutrition might impair reproductive function leading, for example, to the delayed onset of puberty (Short and Bellows, 1971; Day et al., 1986), induction of anestrus (Richards et al., 1989; Rhodes et al., 1996), and prolonged postpartum anestrus (Wiltbank et al., 1964; Selk et al., 1988; Gentry et al., 2002). Moreover, it is well known that increasing feed intake and ad libitum feeding of gilts or sows for 11–14 days before ovulation, a process known as flushing, increases ovulation rate (Cox et al., 1987; Flowers et al., 1989; Zak et al., 1997; Ashworth et al., 1999) also if it is not known whether this increase is a response to a higher intake per se or to an increased intake of a specific nutrient. It is proposed that the flushing response is mediated by an increase in IGF-I release and augmented clearance of steroids by the liver, which would mediate effects on follicular fluid composition and on oocyte maturation. Metabolic hormones such as insulin, IGF-I, and leptin are likely mediators between nutritional status and reproductive performance. Leptin may serve as a signal of nutritional status and may act directly on the brain or may possibly have a direct effect on the ovary and on the oocyte, as leptin receptors have been found in the pig (Ruiz-Cortes et al., 2000; Fergusson et al., 2003) and mouse (Ryan et al., 2003) ovary and in pig (Craig et al., 2004) and mouse (Kawamura et al., 2003) oocytes.

The relationship between nutrition and reproductive hormones is less understood in the mare (Hines et al., 1987). In this species there are some works about the possible role of leptin during transition from seasonal anestrus and throughout the oestrous cycle (Robalo et al., 2001), in the seasonal anovulatory period (Gentry et al., 2002), on maintenance of ovarian activity during seasonal anestrus (Fitzgerald et al., 2002; McManus and Fitzgerald, 2003) and during foaling and lactation (Heidler et al., 2003; Ferreira et al., 2005). These reports quantify the correlation of circulating concentration of leptin with body condition scores. For example, Kearnes (2006) reported that level of plasma leptin is proportional to adiposity in the horse: 1.90 ± 0.34 ng/ml in weaning fillies of 4 months and 3.47 ± 0.50 ng/ml in mature horse of 10 years.

Gentry (2002) showed that mares with high body condition score (BCS) (7.5 to 8.5) continued to ovulate or have follicular activity during the winter, whereas mares with low BCS (3.0 to 3.5) entered into the anovulatory period. In this study leptin concentrations were very low in mares with low BCS but higher in well-fed mares.

In this context is known that, also in the horse, leptin may serve as an important endocrine signal of nutritional status and body fat-mass that is reflected on the reproductive characteristics (Fitzgerald et al., 2000; Buff et al., 2002; Ferreira et al., 2005; Gentry et al., 2002) but there are no informations whether leptin has a direct role in the reproductive physiology of the adult ovary and if Ob and Ob-R are present in the ovary or in the oocyte.

The aim of the present study was examined the immunolocalization of Ob and Ob-R in ovaries tissue recovered after slaughtering from fillies and from mares of light or heavy body weight breeds, and investigated the immunolocalization of Ob and Ob-R and the profile expression of Ob and Ob-R mRNA in immature and matured compact cumulus oocytes of these three different kind of animals, for the purpose of correlating nutritional status with reproductive efficiency.

9.3 Materials and methods

Chemicals were purchased from Sigma Chemical (Milano, Italy) unless otherwise indicated.

9.3.1 Animals

The ovaries of 111 mares of heavy body weight breeds, 17 mares of light body weight breeds and 24 fillies were obtained from a commercial abattoir.

The animals were weighed before slaughtering and body condition score was determined for each animal.

Rump fat thickness was measured using B-mode ultrasonography (Aloka SSD-500) used to calculate percent body fat (% fat) as previously published (Kearns et al., 2001, 2002a,b,c). The site was determined by placing the probe over the rump at approximately 5 cm lateral from the midline at the centre of the pelvic bone (Westervelt et al., 1976). The region was scanned and the position of maximal fat thickness was used as the measured site. Percent fat was estimated from the equations of Kane et al. (1987).

The animals were categorized into fat (average BW of 800 Kg with body condition score ≥ 6 mean; age = 10-12 yr) or thin (average BW of 450 Kg with body condition score ≤ 5 ; mean age 7-10 yr).

9.3.2 Recovery and culture of oocytes

Horse ovaries were transported to the laboratory within 6h in phosphate buffer saline (PBS) supplemented with antibiotics (100 IU/ml penicillin and 100 μ g/ml streptomycin sulphate) at 30°C.

Upon arrival, ovaries were washed in PBS containing antibiotics and any extra tissue and most of the tunica albuginea were removed. Thereafter, ovaries were maintained at 30°C in PBS with penicillin/streptomycin while being processed.

Cumulus oocytes complex (COCs) were harvested by opening and scraping all the surface readily detectable follicles and afterward, the ovary was bisected along its midline at 10 mm intervals, from ovulation fossa to the greater curvature, and any newly exposed follicles were scraped. Only compact cumulus oocytes (CCO) with at least three layers of cumulus cells were used for this study.

Recovered CCO oocytes in all kinds of animals analyzed were washed three times in basic TCM199 medium, HEPES modification with Earle's salts, L-glutamine, supplemented with 10% fetal bovine serum and 25 µg/ml gentamicin (M199) and then were divided randomly into different groups for immunocytochemistry analysis and for analysis of expression of leptin and its receptor: one group of oocytes were denuded of cumulus cells and assigned to be examined immediately; another group of oocytes with a complete, compact, multilayered cumulus investment were selected for in vitro maturation performed in 100 µl droplets (15-20 oocytes for droplet) of the appropriate medium under mineral oil, at 38.5°C under an atmosphere of 5% CO₂ in air with maximum humidity. The maturation medium (IVM) consisted of M199 supplemented with 0.1 IU/ml of each FSH (Folltropin, Bioniche Teo, Inverin, Co.Galway, Ireland) and LH (Lutropin, Bioniche, Teo, Inverin, Co.Galway, Ireland), 1 µl/ml of ITS (insulin, transferrin, sodium selenite), 1 mM sodium pyruvate, 100 ng/ml of IGF1 and 50 ng/ml of EGF.

At the end of maturation also this oocytes were denuded of cumulus and assigned to be screened for the evaluation of immunolocalization of leptin (Ob) and leptin receptor (Ob-R) or for analysis of expression of leptin and its receptor.

9.3.3 Expression profile of leptin and leptin-receptor mRNA

Oocytes mRNA isolation and cDNA synthesis

Polyadenylated [poly(A)+] RNA from pooled compact immature and in vitro matured oocytes was extracted using the Dynabeads mRNA DIRECT kit (Deutsche Dynal, Hamburg, Germany) according to the methods of Pocar et al. (2004). Briefly, compact COCs underwent cumulus and corona cells removal by incubation in TCM 199 containing 80 IU hyaluronidase/mL and aspiration in and out of finely drawn glass pipettes. Pools of 30–40 oocytes were lysed for 10 min at room temperature in 50 µl lysis buffer [100 mmol Tris-HCl (pH 8.0), 500 mmol LiCl, 10 mmol EDTA, 1% (wt/vol) sodium dodecyl sulfate, and 5 mmol dithiothreitol]. After lysis, 10 µl prewashed Dynabeads-oligo(deoxythymidine)25 were pipetted into the tube, and binding of poly(A)+ RNAs to oligo(deoxythymidine) was allowed for 5 min at room temperature. The beads were then separated with a Dynal MPC-E magnetic separator and washed twice with 50 µl washing buffer A [10 mmol Tris-HCl (pH 8.0), 0.15 mmol LiCl, 1 mmol EDTA, and 0.1% (wt/vol) sodium dodecyl sulfate] and three times with

50 μ l washing buffer B [10 mmol Tris-HCl (pH 8.0), 0.15 mmol LiCl, and 1 mmol EDTA]. Poly(A)+RNAs were then eluted from the beads by incubation in 11 μ l diethylpyrocarbonate-treated sterile water at 65 C° for 2 min. Aliquots were immediately processed for RT using the PCR Core Kit (PerkinElmer, Wellesley, MA), employing 2.5 μ mol random hexamers to obtain the widest array of cDNAs. The RT reaction was carried out in a final volume of 20 μ l at 25 C° for 10 min and 42 C° for 1 h, followed by a denaturation step at 99 C° for 5 min and immediate cooling on ice.

Semiquantitative PCR

To normalize signals from different RNA samples, GAPDH transcripts were co-amplified as an internal standard. The amplification reaction was stopped before leaving the exponential phase. Amplifications were performed on 1 μ l first strand cDNA in a 30- μ l final volume containing 0.2 μ M of the primer combinations listed above, 1 U Taq polymerase (Invitrogen S.R.L. Milano, Italy), 0.2 mM deoxy-NTPs, 1.5 mM MgCl₂, and 1x PCR buffer. Amplification cycles comprised a 30-sec step at 94 C° for denaturation, a 30-sec step at 57 C° for annealing, and a 45-sec step at 72 C° for elongation. A water control was included to identify possible contamination. In addition, all samples were amplified with an intron-exon spanning primer pair to detect possible genomic DNA contamination. Nested PCR was performed for both, Leptin and Leptin-R at the same conditions as above, as sample 1 μ l of a 1:1 dilution of primary PCR product was added to a total volume of 30 μ l PCR mix. Thermocycling conditions were the same as for the primary reactions.

Oligonucleotide primers are shown in table 1.

A volume of 20 μ l/reaction was subjected to electrophoresis on a 1.3% agarose gel in Tris-acetate-EDTA buffer, containing 0.2 μ g/ml ethidium bromide. After separation, the fragments were visualized on a 312-nm UV transilluminator. The image of each gel was digitalized using a CCD camera, and the intensity of each band was quantified by densitometric analysis using a computer-assisted image analysis system (Bio-Rad, Quantity One, US). The relative amount of the mRNA of interest was calculated as a percentage of the intensity of the GAPDH band for the corresponding sample. For each mRNA, experiments were replicated at least three times.

Table 1: Sequence of primers for horse leptin, leptin receptors and GAPDH.

Target gene		Sequence	Product size (bp)
Leptin	sense	5'-TCCGAAAAGTCCAGGATGAC-3'	347
	antisense	5'- CTCAGGGCTACCACCTCTGT-3'	
Leptin - nested	sense	5'-GACTTCATTTCCTGGGCTTCA-3'	258
	antisense	5'-GGCTACCACCTCTGTGGAGT-3'	
Leptin R	sense	5'-GTGAATGCCGAGTGCCTGTG-3'	309
	antisense	5'-TGCCCACCAGCAGAGATGTC-3'	
Leptin R - nested	sense	5'-GTGAATGCCGAGTGCCTGTG-3'	211
	antisense	5'-GGTAAAAGTGTTGGGCTGGA-3'	
GAPDH	sense	5'-TCACCATCTTCCAGGAGCG-3'	572
	antisense	5'-CTGCTTCACCACCTTCTTGA-3'	

9.3.4 Immunohistochemistry

Appropriate portions of three ovaries of all kinds of animals were fixed in 10% formalin for 24 hours, dehydrated and included in paraffin.

After de-waxing, formalin-fixed serial sections (4 µm thick) were washed and immersed in a freshly prepared 3% H₂O₂ solution for 15 min to block the endogenous peroxidase activity, followed by incubation in 1:20 normal goat serum (DakoCytomation, Denmark, code # X 0907) in Tris-Buffered Saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) 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 anti-leptin (A-20) and anti-leptin receptor (M-18) affinity purified rabbit polyclonal antibody (sc-842 and sc-1834 respectively; Santa Cruz Biotech. Inc., Santa Cruz, CA) diluted 1:50 in TBS. The detection system used was Envision System Labelled polymer-HRP (Dakocytomation code # K 4002). 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 TBS at pH 7.6 containing 0,1 mL of 3% H₂O₂ for 13-20 min. Sections were counterstained with Mayers' haematoxylin, dehydrated and mounted using Eukitt.

To ensure antigenic specificity, two types of controls were performed: 1) no primary antibody, replaced with PBS-NGS; and 2) preincubation of antibody with peptide raised against the primary antibody to compete for binding sites. Three sections were examined for each ovary.

Sections were observed under an Olympus BX51 photomicroscope connected to a digital camera and equipped with the AxioVision Rel 4.5 (Zeiss, Germany) software for image analysis.

9.3.5 Immunocytochemistry

According to the procedures described by Kim et al. (2006) a group of immatures and matured denuded oocytes of all kinds of animals were fixed for 2 h in 3.7% paraformaldehyde at 4°C. Unless otherwise stated, incubations were carried out at 4°C. Oocytes were washed for 20 min in four changes of PBS containing 1% Triton X-100 (PBS-T). Oocytes were placed in a blocking solution overnight. Blocking solution consisted of 0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered nonfat dry milk, 0.5% BSA, and 0.02% sodium azide in PBS. Primary rabbit antibody for leptin was raised against the N-terminal region of the ob gene product of human and to a lesser extent, mouse and rat (sc-842; Santa Cruz Biotech. Inc., Santa Cruz, CA) and primary antibody for leptin receptor was raised against a recombinant protein corresponding to amino acids 541-840 mapping within an internal domain of OB-R of human origin (sc-1834, Santa Cruz Biotech. Inc., Santa Cruz, CA). After blocking, oocytes were incubated with the primary antibodies diluted to 1:100 in PBS-T overnight. Oocytes incubated without primary antibody were used for the negative control. Oocytes were then washed for 15 min in four changes of PBS-T and placed in secondary antibody [goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugate, 1:100 in PBS-T, Santa Cruz Biotech Inc., Santa Cruz, CA] for 4 h.

9.3.6 Fluorescence and confocal laser scanning microscopy (CLSM)

Oocytes for CLSM analysis were mounted on microscope slides with glycerol and, to avoid excess pressure being exerted on the oocytes, the coverslips were supported with thick droplets of a Vaseline-wax mixture placed in each corner. Oocytes were examined using a laser scanning confocal microscope Olympus Fluoview 300, provided with an argon–krypton ion laser for excitation of FITC using 488nm excitation/barrier filter combination.

9.3.7 Statistical analysis

The statistical significance of the results was evaluated by the Chi-square-test with the Yates correction for continuity and by Fisher's exact test. Fisher's exact test was used when a value of less than 5 was expected in any cell. Proportions of matured were compared between animal groups. Values with $P < 0.05$ were considered to be statistically significantly different.

9.4 Results and discussion

9.4.1 *In Vitro* Maturation

After IVM, heavy breed mares had a higher proportion of oocytes that reached metaphase II than light mares and fillies (35.23% vs 19.63 and 15% respectively). The maturation rates of oocytes collected from examined animal groups are presented in table 2 and Figure 1.

Table 2: maturation rate of CCO oocytes after *in vitro* maturation

ANIMALS	N° oocytes in IVM	% maturation (MII) $\pm$ DS
Heavy body weight breeds	427	35.23 $\pm$ 0.006 ^a
Light body weight breeds	25	19.63 $\pm$ 0.03 ^b
Fillies	84	15.00 $\pm$ 0.06 ^b

Percentages with different superscripts statistically differ at $P < 0.05$ (Chi-square-test).

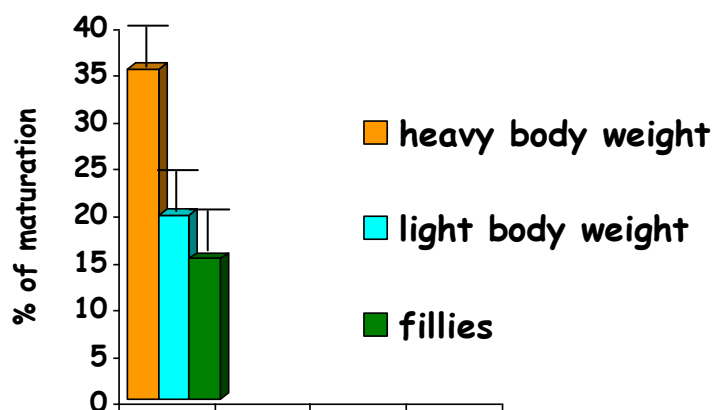


Fig. 1: maturation rate of CCO oocytes after *in vitro* maturation

9.4.2 *Expression profile of leptin and leptin-receptor mRNA*

Ob-R mRNA was detectable in oocytes during *in vitro* maturation in heavy breed mares. Furthermore, during IVM of oocytes from heavy breed mares, Ob-R was expressed in a significantly higher level of MII-stage oocytes (3.2 fold compared to GV). In contrast, expression of Ob-R in light weight mares and fillies was not detectable by primary PCR at any of the stages investigated. However, further analyses demonstrated that Ob-R mRNA was also detectable in light weight mares and fillies oocytes via nested RT-PCR at all stages of oocyte maturation investigated (Fig. 2A, B).

To study whether Ob is locally available in an autocrine/paracrine way, expression of Ob mRNA was analyzed in immature equine oocytes and after *in vitro* maturation. Results indicated that Ob transcript is expressed in the oocytes from

heavy breed mares at both GV and MII stages. Furthermore, Ob transcript was detected by primary PCR in MII light weight mares' oocytes. Finally, analysis by nested RT-PCR demonstrated that Ob mRNA was also detectable in immature oocytes from light weight mares and fillies oocytes, both at GV and MII stages (Figure 2C).

Figure 2. A: expression profile of leptin receptor; B: semi-quantitative analyse of Ob-R in immature and matured oocytes of heavy mares; C: expression profile of leptin.

9.4.3 Immunohistochemistry

The presence of Ob in blood vessels within the ovary (Figure 3A) as well as within the corpora lutea (Fig. 3B,C) was found in light and heavy mares, indicating that there is a high level of immunoreactive leptin within these structures.

Staining for leptin protein was negative in oocytes included in follicles of primordial stage in all kind of animals and for thecal cell layer of preovulatory follicles of light and heavy mares (Fig. 3D,E).

Ob-R was not identified in all samples analyzed.

The intensity of expression and the positivity were unrelated to kind of animal.

The negative controls did not stain for Ob Ob-R (Fig. 3F).

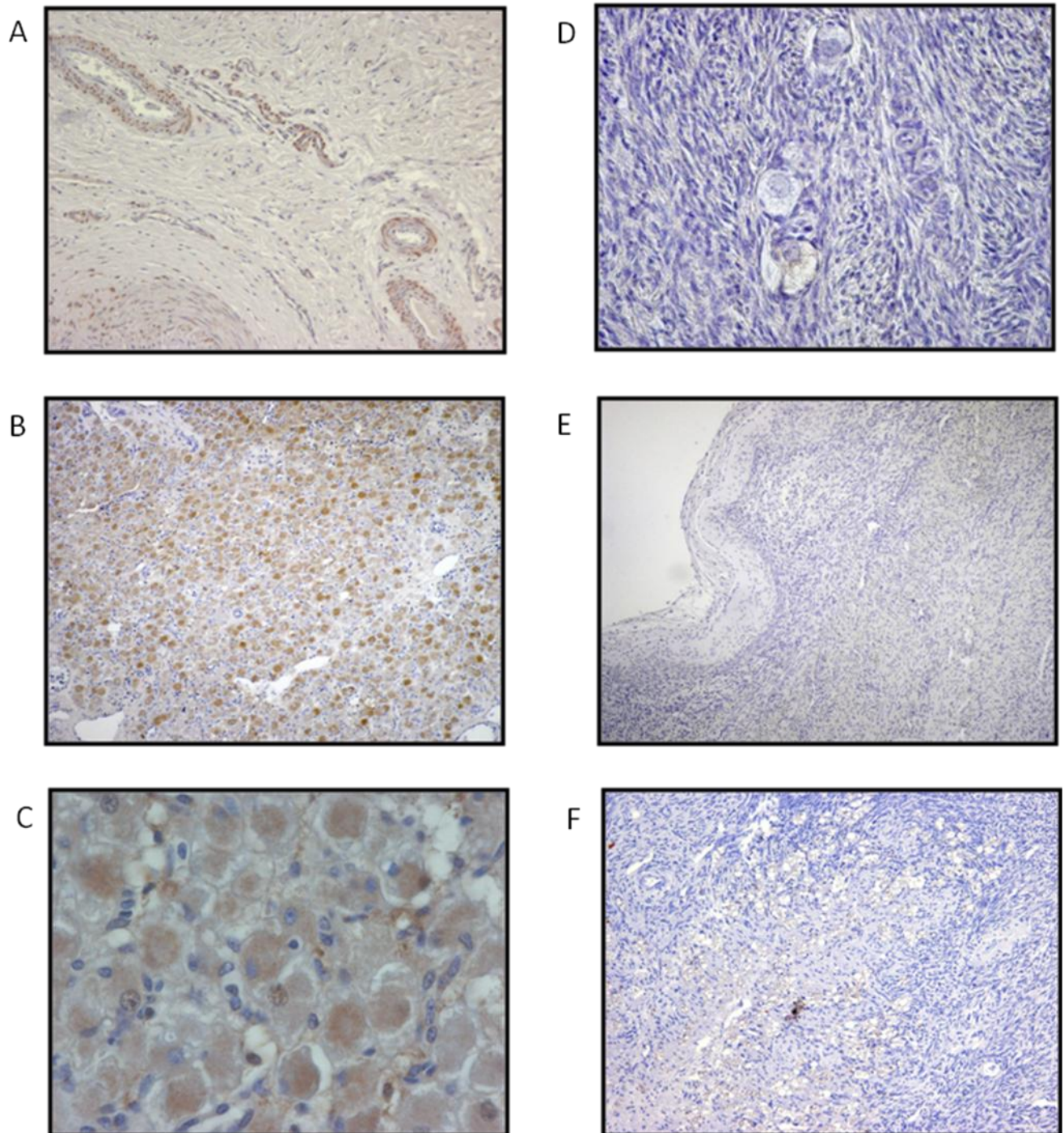


Fig. 3. A: section of a horse ovary with positive staining of blood vessels to leptin protein. Original magnification 200x; B: presence of Ob in corpora lutea. Original magnification 200x; C: presence of Ob in corpora lutea. Original magnification 400x. D: negative oocytes to Ob in primary follicles. Original magnification 400x; E: negative thecal cell layer of preovulatory follicle to Ob. Original magnification 200x; 3F: negative ovary controls. Original magnification 200x.

9.4.4 Immunocytochemistry

Both Ob and Ob-R, detected in immature oocytes of all kinds of animals analyzed, were uniformly distributed throughout the ooplasm, but the intensity of reaction was lower either in light weight mares or in fillies oocytes, than in oocytes of heavy weight mares (Fig. 4 a,b,c).

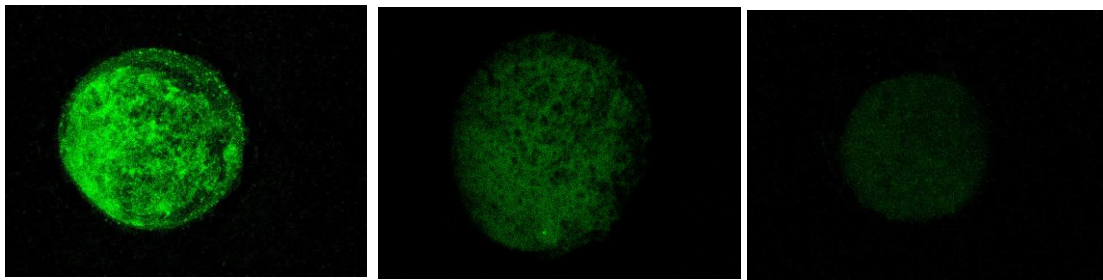


Fig. 4: Confocal laser scanning photomicrographs of immunocytochemical analysis of leptin ligand (Ob) in immature compact cumulus horse oocytes. a) immature oocytes of heavy weight mares b) immature oocytes of light weight mares. c) immature oocytes of fillies. Bars = 50 μ m.

In matured oocytes both Ob and Ob-R were localized to the oocyte cortex and concentrated at one pole of the oocyte. This distribution within the oocytes was independent from animal group and once again with weaker immunopositivities in light mares and in fillies (Fig. 5 a,b,c).

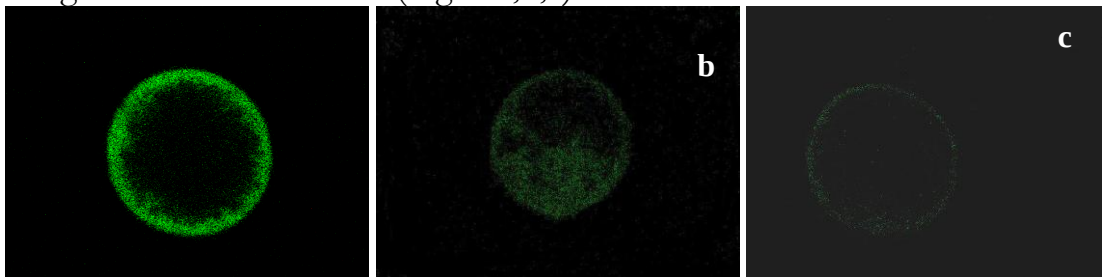


Fig. 5: Confocal laser scanning photomicrographs of immunocytochemical analysis of leptin ligand (Ob) in matured compact cumulus horse oocytes. a) matured oocytes of heavy weight mares b) matured oocytes of light weight mares. c) matured oocytes of fillies. Bars = 50 μ m.

9.5 Discussion

To our knowledge, this is the first report that studies the immunolocalization of leptin (Ob) and leptin receptor (Ob-R) and of their expression in horse ovary and in oocyte for the purpose of correlating nutritional status with reproductive efficiency.

We localized the presence of Ob immunohistochemically and found that there were high levels of leptin in blood vessels within the ovary and within the corpora lutea of recently ovulated ovaries, while leptin receptor was not identified in all samples analyzed although the literature reported that the Ob-R is found on many cells within the ovary, including theca, granulosa and oocytes (Tartaglia et al., 1995; Kim et al., 1999).

There is currently speculation about the role of leptin within the ovary, without any clear evidence to indicate its precise function in this organ. It is known that leptin induces angiogenesis (Bouloumie et al., 1998) and affects ovulation *in vivo* and *in vitro* as well as oocyte maturation, indicating that this hormone has broad-ranging effects within the ovarian environment (Ryan et al., 2003). Barkan et al.

(2005) have shown that leptin, *in vivo*, in unovulating mice due to GnRH deficiency, rapidly induces follicular development and formation of corpora lutea and ovulation concluding that in addition to its role in inducing GnRH, leptin can also mimic LH in its actions on the reproductive system. These findings suggest a possible use of leptin in treatment of female infertility, particularly in subjects who do not respond properly to LH.

It was suggested that leptin acts on the ovary via its specific receptors at ovulation and/or immediately after ovulation and may act on 1) the oocyte, to promote postovulation maturation in readiness for fertilization; 2) the ovarian macrophages or infiltrating monocytes, to promote cytokine production and phagocytosis that occurs during tissue remodelling throughout ovulation and corpus luteum production; and 3) the blood vessels within the ovary, to promote angiogenesis, follicle rupture or subsequent corpus luteum formation (Ryan et al., 2003).

In our study, there was no Ob in horse oocytes included in histological sections of primordial follicles stage but in the scraped immature oocytes, of all kinds of animals analyzed, Ob and Ob-R were immunocytochemically detected with uniform distribution throughout the ooplasm. Ob and Ob-R were also showed in horse oocytes matured *in vitro*, but with cortical distribution.

Ob was highly expressed in the mouse oocytes at all stages of follicular development (Ryan et al., 2002), in all stages of porcine IVF embryos (Craig et al., 2005), in human matured oocytes and in all stages of human embryos (Cioffi et al., 1997; Antczak and Van Blerkom, 1997).

Ob-R is expressed in pig oocytes from all stages of follicular development and oocyte maturation, with the highest level of expression occurring in oocytes from medium follicles and at germinal vesicle breakdown (GVBD), indicating that its expression is dependent on follicular stage and oocyte maturation (Craig et al., 2004). In human oocytes, Ob-R was not detected but leptin receptor-specific epitope was identified by immunofluorescence in the cytoplasm and nuclei of GV-stage oocytes and cleavage stage embryos (Antczak and Van Blerkom, 1997). Moreover, leptin receptor mRNAs were detected in mouse oocytes, 1-cell, 2-cell, morula, blastocysts and hatched blastocyst stage embryos (Kawamura et al., 2002). In mouse preimplantation embryos, two types of leptin receptor (OB-Ra and OB-Rb) mRNAs was expressed (Kawamura et al., 2002), and exogenously secreted leptin was shown to cause ligand receptor-mediated effect mainly through OB-Rb.

Our results showed that in immature and matured horse oocytes, leptin and leptin receptor were expressed and detected by immunocytochemistry but with different distribution. The localization of Ob-R within the cytoplasm of immatured oocytes could simply represent the nascent receptor protein even if cannot be excluded that it could have other biological functions, while the Ob-R

localized at the plasma membrane in matured oocytes could correspond to a small fraction of the total Ob-R and could represent the mature form of the receptor. It has been estimated that, under normal conditions, only 5–25% of the total OB-R isoforms are located at the cell surface, with the majority contained in intracellular pools (Barr et al., 1999). Although *in vitro* studies with transfected Ob-R have shown the existence of a large intracellular receptor pool (Barr et al., 1999; Lundin et al., 2000; Sweeney, 2002), the functional meaning of this distribution and the exact *in vivo* trafficking dynamics have not been fully established.

As pointed out previously, in the horse oocytes, leptin and leptin receptor are present and are differently localized during oocyte IVM but they also showed different immunoreaction intensity related either to the horse breed or to the reproductive puberal development: the intensity of reaction was lower either in immature or matured oocytes of fillies and light weight mares than in oocytes of heavy weight mares. The analyse of expression of Ob and Ob-R mRNA confirmed these data because immature and mature oocytes of heavy mares and mature oocytes of light mares showed expression of Ob and Ob-R immediately by PCR, but the positivity in other oocytes stage and kinds of animals was obtained only after nested PCR. These analysis evidence that after maturation the expression of Ob and Ob-R increased respectively in matured oocyte of heavy and light, mares while remained lower in fillies oocytes.

The increase of Ob-R expression in matured oocytes of light mares may be necessary to a greater transport of leptin into the oocyte during the maturation. The leptin receptor is known to mediate transport of leptin across cellular membranes *in vitro* (Uotani et al., 1999; Hileman et al., 2000; Craig et al., 2004). The increase in Ob expression in matured oocytes of heavy mares demonstrates the need of oocytes to store leptin probably required for oocyte maturation, steroidogenesis alteration or macrophage functionality at ovulation.

Little information is available concerning the effect of nutrition and puberal development on ovulation and rates of oocytes maturation in the horse. After IVM, in our study, heavy breed mares had a higher proportion of oocytes that reached metaphase II than light mares and fillies and had also more intensity of immunoreaction of leptin and leptin receptor .

We did not measure the serum leptin concentration in the animals under our experiments but, from literature, is well documented that adipocytes secrete leptin (Hamilton et al., 1995; Lonnqvist et al., 1995), that body mass index and serum leptin levels are highly positively correlated (Butzow et al., 1999; Maffei et al., 1995; Swain et al., 2004) and that *in vivo* leptin levels are influenced by the energy balance with an elevation in plasma leptin following an increase in the plane of nutrition. Increased dietary intake for a relatively short time prior to mating increased the ovulation rate and oocyte quality, and consequently

produced good quality embryos in sheep (Blache et al., 2000, 2000a). These data suggest that leptin may be an important factor in regulating early pregnancy but the requirement of this hormone appears to be within a limited range, with too much or too little being detrimental to the oocyte quality and embryo development. Indeed, Herrid et al. (2006) in cattle showed that the effect of the feeding level on oocyte quality is dependent on the body condition of the animal, with the high level of feeding being beneficial to oocytes from animals of low body condition but detrimental to oocytes from animals of moderately high body condition. Also *in vitro*, physiological leptin doses enhance oocyte maturation effect (Paula-Lopes et al., 2007; Lange-Consiglio et al., 2009) and differentially regulate gene expression in oocytes and cumulus cells while higher doses have no effect (Paula-Lopes et al., 2007). It is probably that only at physiological concentrations leptin exerts a beneficial effect during maturation of oocytes.

As with the other mammalian species examined, plasma leptin concentrations in horse are related to body fatness and level of feeding (Huff et al, 2008). Buff et al. (2002) showed that serum concentrations of leptin tended to be greater in the fat animals (6.99 ± 1.77 ng/ml of leptin and body condition score >6) than in the thin animals (2.33 ± 1.88 ng/ml of leptin and body condition score <5) and that there was a linear relationship between body condition score and serum concentration of leptin in all animals. Also Kearns et al. (2006) positively correlated plasma leptin concentration to percent body fat.

Body condition and level of feeding influence a number of factors that regulate ovarian follicular development, granulosa cell proliferation and steroidogenesis.

The highest rate of maturation in the present study were for the heavy breed mares that we had classified with BCS >6 . It is tempting to speculate that moderate plasma concentrations of this metabolic hormone are associated with improved oocyte quality and maturation rate whereas low concentrations, as in light mares, are associated with impaired *in vitro* maturation rate (Adamiak et al., 2005). Our data suggest that moderate concentrations of leptin in these animals may have had a more direct, positive impact on the follicle enclosed oocyte, thus improving the rate of maturation. The precise mechanisms of such an effect are not understood but Kim et al. (2006) showed that the coincubation of leptin with pig oocyte-granulosa cell complexes derived from preantral follicles improved the oocyte developmental competence in a dose-dependent manner and this observation confirms the stimulating effect of leptin on oocyte maturation and it is broadly in keeping with that reported here. The improvement of maturation rate of oocytes may be related to some potential action mechanisms exerted by leptin on oocyte cytoplasmic maturation. These mechanisms may include direct or indirect cumulus cell-mediated effects such as restructuring oocyte cytoskeleton, reprogramming protein synthesis, or inhibiting

apoptosis (Almog et al., 2001). In bovine, it can be hypothesized that leptin may rescue oocytes that could potentially undergo apoptosis (Boelhauve et al., 2005). The beneficial effect of leptin during oocyte maturation suggests a role for leptin as a survival factor minimizing cellular damage to oocyte and/or cumulus cells.

The developmental competency of the oocyte is acquired during the follicular growth period (Sun et al., 2001; Yoon et al., 2000; Marchal et al., 2002), and follicular development is dependent on the action of hormones, growth factors, and cytokines (Craig et al., 2004). In our contexts, maturation rates and immunoreaction intensity seem to be related with the nutritional status and different serum concentration of leptin in heavy and light body weight mares.

In fillies, the serum concentrations of leptin is low: Buff et al. (2002) show that this concentration tend to increase with age of the horse ($P = 0.08$), with mean concentrations consisting of 2.38 ± 0.32 , 2.64 ± 0.55 , 2.93 ± 0.39 , and 3.67 ± 0.38 ng/ml for horses less than 2 yr, 2 to 4 yr, 5 to 12 yr, and greater than 12 yr in age, respectively. Young horses are in the growth phase of development and thus lower concentrations of leptin would be indicative of an animal with lower body fat and requiring greater nutritional resources. Mature horses generally have greater body fat mass and only need resources to match their level of activity (Buff et al., 2002). Consequently, in fillies the low rate of maturation and the low expression of leptin and leptin receptor can be considered as physiological compared to the sexually mature animals.

At last, in matured oocytes both Ob and Ob-R were localized to the oocyte cortex and concentrated at one pole of the oocyte. The finding that leptin immunofluorescence is localized to the oolemma and immediate subplasmalemmal cytoplasm in mature oocytes suggests that some putative cytoplasmic rotations may significantly affect or alter the distribution of this protein. However, Antczak et al. (1997) suggested the possibility that leptin may be involved in the determination of the animal pole of the oocyte and in the establishment of the inner cell mass and trophoblast in the preimplantation stage embryo.

In conclusion, to our knowledge, for the first time, the present study identified and localized Ob and the Ob-R in the horse ovary and oocytes, and evidenced that the effects of oocyte quality are dependent on the nutritional status of the animal: high levels of feeding improve rates of *in vitro* maturation and impaired oocyte quality is associated with low nutritional status in the horse. The data presented here enable an experimental assessments of future dietary effects on oocyte quality in horse and form the basis for further detailed studies into the underlying mechanisms of such effects (Adamiak et al., 2005).

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CHAPTER 10

The expression of aquaporin 7 in the ovine fetal epididymis is perturbed by in-utero exposure to a cocktail of chemicals contained in sewage sludge fertiliser

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The expression of aquaporin 7 in the ovine fetal epididymis is perturbed by in-utero exposure to a cocktail of chemicals contained in sewage sludge fertiliser

Marina Aralla¹, Ali Mobasheri², Stewart M. Rhind³, Paul A. Fowler⁴, David Marples⁵, Kevin D. Sinclair², Silvana Arrighi¹, Richard G. Lea²

¹*University of Milan, Italy*

²*Schools of Veterinary Medicine and Science and Biosciences, University of Nottingham, UK;*

³*Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen, UK*

⁴*Institute of Medical Sciences, University of Aberdeen, UK*

⁵*Institute of Membrane and Systems Biology, University of Leeds*

10.1 Abstract

Introduction: Exposure to environmental endocrine disrupting chemicals (EDCs) during pregnancy in animals and humans is associated with reduced sperm counts and increased incidences of testicular cancer and reproductive tract abnormalities. Although male fetuses from pregnant ewes exposed to EDCs contained in sewage sludge fertiliser (a “real life” exposure model) exhibit fewer testicular Sertoli and Leydig cells, the epididymis has not been investigated. The movement of fluids and small solutes in the epididymis is critical for adult sperm maturation and this process is regulated, in part, by the aquaporins. The current study examined the effects of sewage sludge exposure on the expression of aquaporins in the late gestation fetal epididymis.

Methods: Pregnant ewes were exposed to sewage sludge fertiliser or control pastures from 0-140 days of gestation. Epididymides were collected post mortem at day 140 of gestation (term = day 145), Bouins-fixed and subjected to immunohistochemistry for Aquaporins 1,4 and 7. Staining intensity was visually assessed (blinded) by 2 observers on an arbitrary 4 point scale.

Results and Discussion: AQP1 immunoreactivity was detected in the apical and lateral plasma membranes of non-ciliated cells in efferent ducts; smooth muscle cells surrounding epididymal ducts and microvascular endothelia throughout the epididymis. AQP4 was immunolocalised to the efferent ducts and throughout the epididymis and AQP7 was detected primarily in the efferent ducts. Sewage sludge exposure significantly reduced AQP7 immunostaining ($P=0.012$) but had no effect on AQP1 or AQP4 in terms of localisation or

immunostaining intensity. These results suggest that exposure to environmental chemicals may affect the capacity of efferent ducts to transport water and small solutes thus influencing fluid resorption, protein synthesis and secretion. Ultimately, this may impact on sperm viability in the adult.

10.2 Introduction

It is well known that each individual's phenotype, even if based on a specific genetic programme, is influenced by epigenetic and environmental factors, most of them occurring earlier in life, either in utero and/or during infancy. The so-called "Barker hypothesis" (Barker, 1997) proposes that adverse events in utero induce compensatory responses in the foetus that reflect "developmental plasticity" during this critical period (Bateson et al., 2004) and persist permanently. Among these factors, early-life diet and endocrine exposure may predispose or induce late-onset disease during postnatal life. As far as reproduction is concerned, a relationship was demonstrated between fecundity and early-life nutrition (Gardner et al., 2009). Developmental maturation of the hypothalamic-pituitary-gonadal axis has been shown to be susceptible to foetal programming, too (reviews in Rhind et al., 2001; Rhind, 2004).

Exposure to environmental endocrine disrupting chemicals (EDCs) during pregnancy is associated with reduced sperm counts, increased incidence of testicular cancer and reproductive tract abnormalities either in human (for a review see Main et al., 2010) and in laboratory (Welsh et al., 2008; Jacobsen et al., 2010) as well as domestic mammals (Rhind et al., 2010). Among reproductive organs, the testis is the most studied in consideration of its obvious function in gamete production and in endocrine secretory activity. The male genital tract was almost neglected by the literature on this item, however it has a relevant and pivotal role in male fertility control, which has been known for many years (Hamilton, 1973). Far from being unvarying conduits, the different portions of the extratesticular excurrent duct are environments with highly specialized regional differentiation at the basis of modulated secretory and absorptive functions. Next to a starting portion known to be highly absorptive, i.e. ductuli efferentes, the very elongated epididymal duct follows, whose proximo-distal histophysiologic variations have been a matter of discussion for a long time in the different animal species (for reviews see Gatti et al., 2004; Cornwall, 2009). Sperm maturation, a process which may require different microenvironments in relation to specific needs, takes place in the duct lumen, under the hormonal control. It is known that the epididymis (and maturing spermatozoa) are hormonally supplied by at least three different pathways: i) via blood vessels/systemic circulation; ii) directly from the lumen, since testicular androgens bombard the epididymis as lumicrine factors, bound to the androgen

binding protein produced by the Sertoli cells; iii) by synthesis by the epididymal epithelium itself, through the action of 5-alpha-reductase.

The movement of fluids and small solutes in the epididymis is critical for adult sperm maturation and this process is regulated, in part, by the aquaporins. Aquaporins (AQPs) are channel proteins facilitating the rapid passive movement of water throughout the cell membranes (Jung et al., 1994). Aquaporin water channels are found in epithelia with a 10- to 100-fold higher capacity for water permeation in comparison with those devoid of these proteins (Agre et al., 2002). The presence of AQPs in the male genital tract, where significant movements of fluids and molecules useful to sperm maturation are known to take place (Levine and Marsh, 1971), is therefore of primary importance (Huang et al., 2006). Many AQPs are known to be present in the rat male genital tract (AQP1, AQP2, AQP5, AQP8) (Badran and Hermo, 2002; Hermo et al., 2004, 2008; Da Silva et al., 2006a,b), also belonging to the aquaglyceroporin group (AQP3, AQP7, AQP9, AQP10) (Pastor-Soler et al., 2001; Badran and Hermo, 2002; Hermo et al., 2004, 2008), known to be permeable even to small solutes such as urea and glycerol. AQPs have been detected also in the genital tract of other mammals, such as the dog (Domeniconi et al., 2007, 2008) and the cat (Arrighi et al., 2010b). Some AQP molecules are known to be sexual steroid-dependent (Fisher et al., 1998; Badran and Hermo, 2002; Pastor-Soler et al., 2002; Oliveira et al., 2005; Picciarelli-Lima et al., 2006; Aralla et al., 2009). Impaired AQP expression can be a consequence of hormonal failure, as was hypothesized for AQP9 in rats suffering from diminished hormonal supply caused by hyponutrition during the fetal and neonatal life (Arrighi et al., 2010a). The potential adverse reproductive effects of in utero and lactational exposure to EDCs were investigated in rats with emphasis on epididymis (Scarano et al., 2010), including immunohistochemical investigation of AQP9 expression, which was not significantly altered.

Male fetuses from pregnant ewes exposed to EDCs contained in sewage sludge fertiliser (a “real life” exposure model) exhibit fewer testicular Sertoli and Leydig cells (Lea et al., 2010).

It is conceivable that a minor hormonal contribution from these cell types might have worse rebounds over the epididymal histophysiology, which could be a further, indirect cause of diminished fertility in exposed subjects. The current study examined the effects of sewage sludge exposure on the expression of aquaporins in the late gestation fetal epididymis.

10.3 Materials and Methods

10.3.1 *Animals*

Pregnant ewes were exposed to sewage sludge fertiliser pastures from 0 to 140 days of gestation. Never-exposed pregnant ewes served as controls. [Animal studies approved by local ethical committee and covered by UK Animals Scientific Procedures (1986)].

10.3.2 *Tissue collection and processing*

Testes were promptly collected post mortem at day 140 of gestation (term = day 145) from both exposed and control fetuses, after Cesarean deliver. Fetal epididymides were gently dissected free, then fragments of caput (including efferent ducts, EDs), corpus, cauda epididymis and proximal vas deferens were immediately immersed in Bouin's fluid and fixed for 24 h. Samples were stocked in ethanol 70%, then dehydrated and embedded in paraffin. Blocks were sectioned at 6 µm thickness and serial sections were stained with Haematoxylin–Eosin for morphologic purposes or processed for immunohistochemistry.

10.3.3 *AQP-Immunohistochemistry*

Previously described immunohistochemical methods (Aralla et al., 2009; Arrighi et al., 2010a,b) were used for this study.

Tris-buffered saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) buffer was used for rinses throughout the whole procedure.

After de-waxing and stocking in ethanol 70% overnight at 4°C, Bouin-fixed sections were washed and immersed in a freshly prepared 3% H₂O₂ solution for 15 min to block the endogenous peroxidase activity, followed by incubation in 1:20 normal goat serum (DakoCytomation, Denmark, code # X 0907) in TBS for 30 min to prevent background prior to incubating with primary antiserum. Sections were then incubated 24 hours in a humidity chamber at 4°C using the following antisera diluted in a specific antibody diluent (DakoCytomation, code # S 302283): rabbit polyclonal antibody against rat AQP1 (Alpha Diagnostic International, San Antonio, TX; Cat # AQP11-A) diluted 1:500; rabbit polyclonal antibodies against rat AQP4 produced in partnership with Sigma-Genosys in the laboratory of Dr. Ali Mobasheri (Nottingham University), diluted 1:1000; rabbit polyclonal antibodies against rat AQP7 produced in partnership with Sigma-Genosys in the laboratory of Dr. Ali Mobasheri (Nottingham University), diluted 1:500.

The sections were then washed and incubated for 30 min with biotinilated goat anti-rabbit immunoglobulins (DakoCytomation, code # E 0432) diluted 1:200. Streptavidin-Biotin Complex (Vectastain® ABC Kit, code # PK-4001, Vector Laboratories, Inc., UK) was 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% H₂O₂ for 13-20 min. Sections were counterstained with Mayers' haematoxylin, dehydrated and mounted using Eukitt® (Bioptica, Milan, Italy).

10.3.4 Staining controls

Sections of adult sheep kidney served as positive controls for immunoreactions to AQP1. Positive control for AQP4 immunoreaction was performed utilizing sections of lamb lung (Fig. 2E). Sections of lamb spleen were employed as positive control for AQP7 immunoreactions (Fig. 3E). The specificity of the immunostainings was tested by negative controls, performed by (i) the use of non-immune rabbit serum (DakoCytomation, code # X 0903) in place of specific antisera; (ii) omission of the first layer. All of them resulted in the absence of immunoreaction (Figs. 1F, 2D, 3D).

10.3.5 Evaluation of staining intensity

Slides were observed and photographed under an Olympus BX50 photomicroscope equipped with a digital camera (Axiocam MRc5) and AxioVision software for computer-assisted image acquirement and managing. The evaluation of staining intensities was based on subjective, blind estimates of two observers and visually assessed on an arbitrary 4 point scale (0 = negative, no staining; 1= very faint staining; 2= stain; 3= strong staining; 4= very strong staining).

T test was employed to evaluate whether the means of the staining intensity values for the Exposed Group and the Control Group were statistically different from each other. Differences were considered to be significant at $P < 0.05$.

10.4 Results

10.4.1 AQP1-immunoreactivity (Fig. 1)

- **CONTROL GROUP**

Strong AQP1 immunostaining was observed in the epithelium of the efferent ducts (EDs) (Figs. 1A, B, C), localized at the apical surface of the non-ciliated cells, corresponding to the microvilli projecting into the lumen. AQP1 immunoreactivity IR was also present in the lateral plasma membranes of adjacent non-ciliated cells (Fig. 1C). No reaction was observed over the epithelial cells of any epididymal region or proximal vas deferens of foetal sheep (Fig. 1B, D). Otherwise, a decreasing intensity of immunoreactivity from the caput region to the vas deferens was noticed at the level of the smooth muscle cells

surrounding the duct (Fig. 1D). Intense AQP1-immunoreactivity was evidenced in the endothelial cells of blood vessels throughout all the genital tract from EDs to vas deferens (Fig. 1B, D, E).

- EXPOSED GROUP

AQP1-immunostaining was present with the same localizations observed in the Control Group, e.g. in the apical surface epithelium of the non-ciliated cells in the EDs, and at the level of the smooth muscle cells surrounding the epididymal duct, from the caput region to the vas deferens. As in the Control Group, intense AQP1-immunoreactivity was evidenced in the endothelial cells of blood vessels throughout all the genital tract from EDs to vas deferens.

10.4.2 *AQP4-immunoreactivity* (Fig. 2)

- CONTROL GROUP

Intense immunoreactivity was present in the lateral plasma membranes of ED epithelial cells (Fig. 2A, arrows) and, most of all, a strong AQP4 immunostaining encircled the cell nuclei all around.

No staining was observed in the region of the caput and corpus of epididymis (Fig. 2C).

- EXPOSED GROUP

Intense immunoreactivity was present in the lateral plasma membranes of ED epithelial cells (Fig. 2B, arrows), encircling the cell nuclei all around, similarly to the Control Group.

No staining was observed in the region of the caput and corpus of epididymis.

10.4.3 *AQP7-immunoreactivity* (Fig. 3)

- CONTROL GROUP

The ED epithelium showed an intense immunoreactivity, largely localized at the apical membrane and supranuclear cytoplasm (Fig. 3A). In the epididymis, AQP7-immunoreactivity was mainly expressed in the epithelium of the corpus region, localized in the baso-lateral membrane of the principal cells (Fig. 3C).

- EXPOSED GROUP

In the ED epithelium, no staining was present in the apical surface of the principal cells (Fig. 3B).

In the epididymis, faint AQP7-immunoreactivity was expressed in the epithelium of the corpus region, localized in the baso-lateral membrane of the principal cells.

10.4.4. Evaluation of staining intensity

AQP1, 4 and 7-immunoreactivities were evaluated at a level of blood vessels, efferent ducts, head, body and tale of epididymis and vas deferens, and also in myoid cells and connective tissue in the Exposed Group and the Control Group. Fig. 1 shows the statistical difference of the staining intensity values in the two. Differences were considered to be significant at $P < 0.05$.

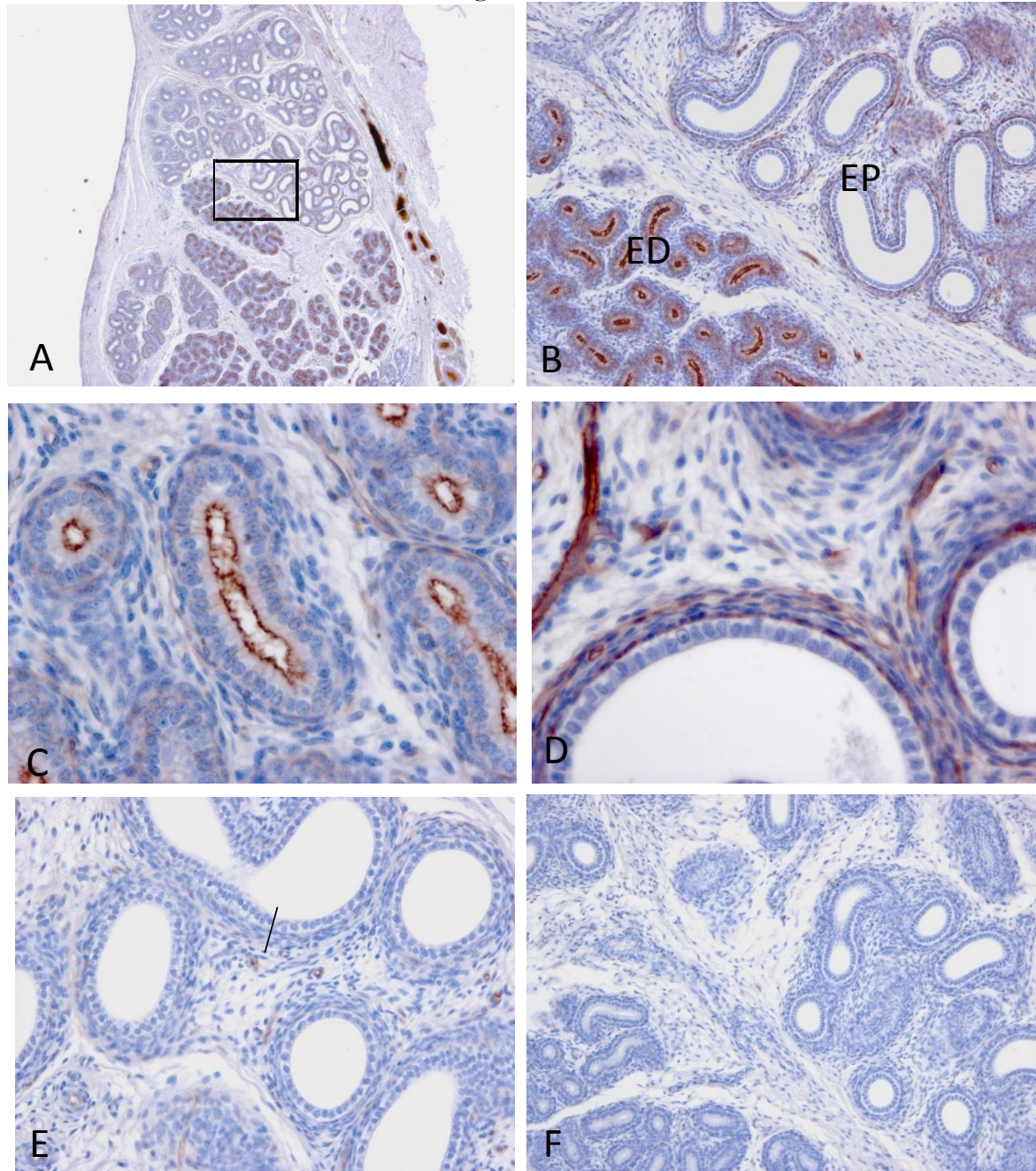


Fig. 1 AQP1 expression in Exposed and Control Groups:

A, B, C: Aqp1 is expressed on the apical surface of Efferent Ducts non-ciliated cells and lateral plasma membranes; D: muscular layer surrounding epididymal duct shows AQP1-immunoreactivity; B, D, E: AQP1 is evident in the endothelial cells of blood vessels from EDs to vas deferens; F:negative control

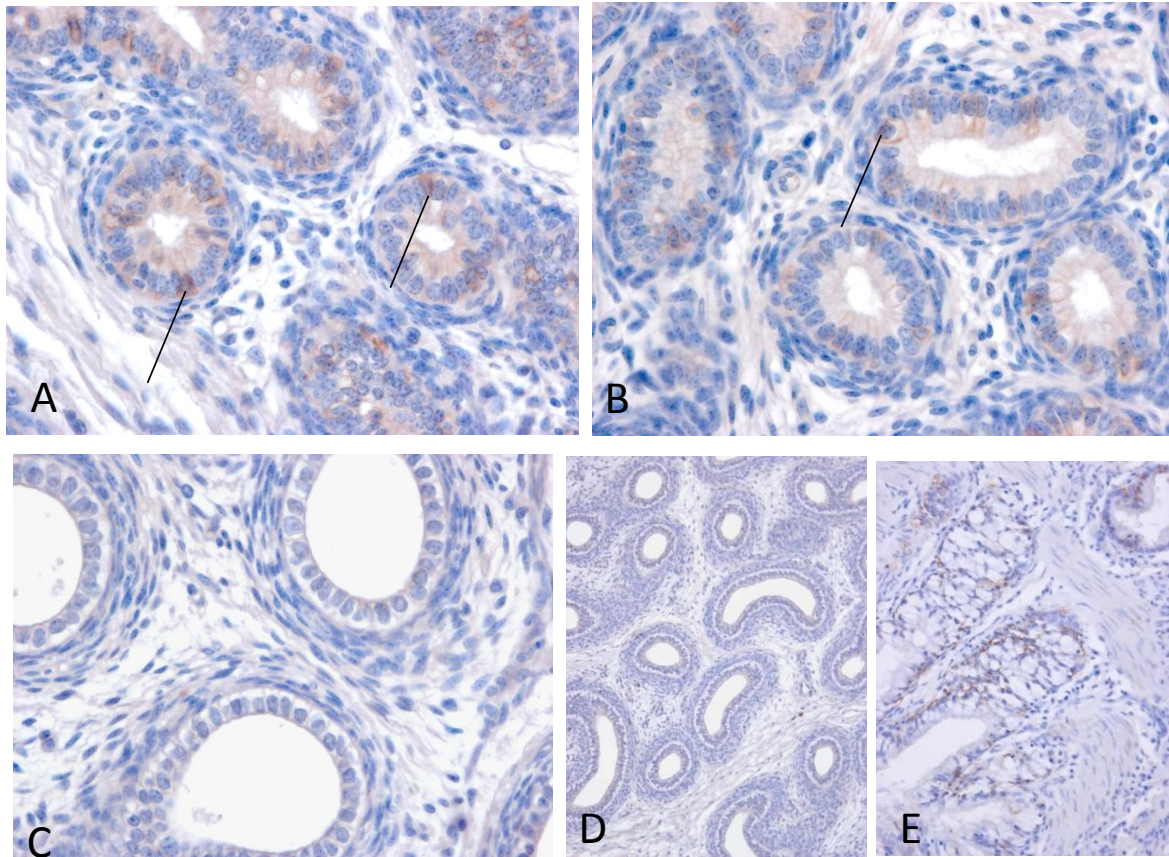


Fig 2: AQP4 expression in Exposed and Control Groups:

A and B: Efferent Ducts (ED): epithelial cells showed an intense immunoreactivity of the lateral plasma membrane either in control (A) and in exposed group (B). Strong AQP4 immunostaining seemed to encircle epithelial nuclei all around (arrows); C: No staining was observed in the region of the caput and corpus of epididymis; D: negative control; E: lamb lung, positive control.

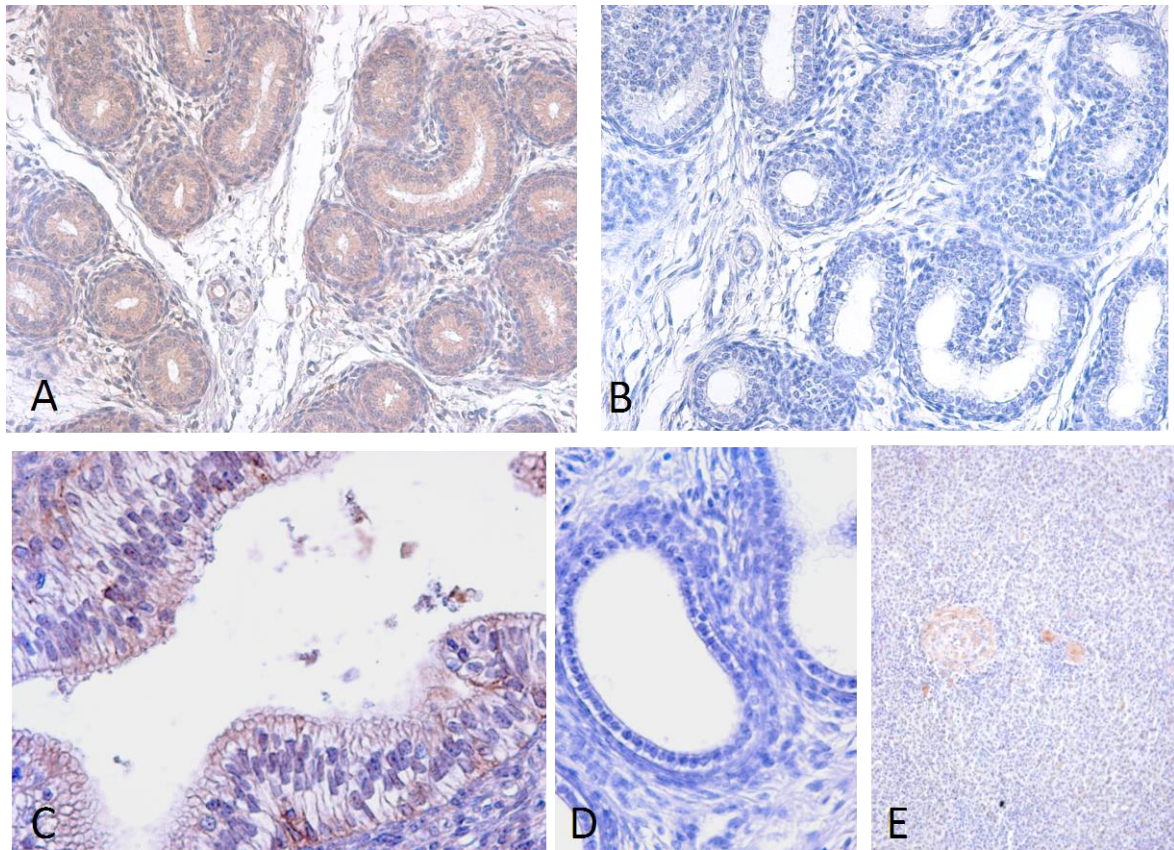


Fig. 3: AQP7 expression in Exposed and Control Groups:

A: Efferent Ducts (ED) of the Control Group show intense immunoreactivity of the apical membrane; B: no staining is present in the Efferent Ducts (ED) of the Exposed Group; C: Body of Epididymis: immunoreactivity of the baso-lateral membrane of the principal cells; D: negative control; E: lamb spleen, positive control.

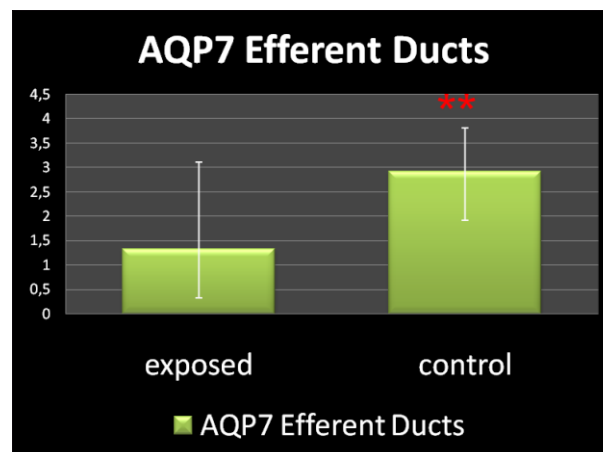


Fig. 4: The staining intensity of AQP7 was evaluated significantly stronger ($p=0,012$) in the ED of the Control Group.

10.5 Discussion

Environmental chemicals similar in structure to estrogen or androgen can bind to steroid receptors and interfere with the endocrine functions. The effects of the exposure to these chemicals in the fetal stage are responsible of reproductive abnormalities in adulthood.

The presence of Aquaporins (AQPs) in the male genital tract, where significant movements of fluids and molecules useful to sperm maturation take place, is of primary importance and it seems regulated by estrogen (Fisher et al., 1998).

In this study we supposed that the exposure to environmental chemicals during gestation could modulate hormonal functions and influence Aquaporins expression in male sheep fetal epididymis.

AQP1 immunoreactivity was detected in the apical and lateral plasma membranes of non-ciliated cells in efferent ducts; smooth muscle cells surrounding epididymal ducts and microvascular endothelia throughout the epididymis. For the first time in sheep we reported that AQP4 was immunolocalised to the efferent ducts and throughout the epididymis and AQP7 was detected primarily in the efferent ducts.

The high incidence of reproductive abnormalities in human males at birth (cryptorchidism and hypospadias) and in adulthood (e.g. low-sperm counts), together with the evidence of temporal changes in incidence of these disorders, especially of falling/low-sperm counts (Swan et al., 2000), is at least consistent with environmental impacts (Skakkebaek et al., 2001).

The first report (Saito et al., 2004) on AQP7 expression in the human testis and ejaculated sperm from fertile men and from infertile patients suggests the possible roles of AQP7 in male infertility. AQP7 may be involved in the maintenance of sperm motility, and a lack of AQP7 expression in sperm may be an underlying mechanism of male infertility. The role of AQP7 in the transfer of water and/or glycerol in sperm differentiation and function is still not clear.

Our data give the first indication of the presence of AQP7 in EDs of sheep; this must be confirmed by additional appropriate controls but can be considered as original with respect to investigations carried out on the male reproductive tract of ruminants. Efferent ductules are very important reabsorptive segments of the male reproductive tract, where between 50 and 90% of the luminal fluid secreted by seminiferous tubules is reabsorbed (Levine and Marsh, 1971). In our study we found that the expression of AQP7 in the Control Group was significantly higher than in the Exposed ones. We suppose that the exposure to EDCs may affect hormonal regulation decreasing functional reabsorptive activity in the EDs. Studies on effects of EDC mixture in rodents have not so far addressed effects on sperm counts/sperm production, although the reported adverse effects on foetal testis development including suppression of androgen

production/ action would be expected to reduce Sertoli cell proliferation in foetal life (Scott et al., 2008). Similar results have also been found in the foetal sheep after pregnant ewes were reared on pasture fertilised with sewage sludge (Paul et al., 2005); specifically, foetal blood testosterone levels were reduced alongside of reductions in Leydig, Sertoli and germ cell numbers. Although these effects were considerable, they did not allow dissection of mechanisms and of cause and effect relationships. Fetal plasma testosterone measurements could be useful. On the basis of rodent studies, it would be expected that reduced intratesticular testosterone concentrations during development would result in reduced Sertoli cell number in the adult (Scott et al., 2007). However, studies in the rat have also shown that even when Sertoli cell number is reduced at birth by 40% to 50%, compensation occurs rapidly after birth so that normal Sertoli cell numbers are restored by puberty and maintained into adulthood (Scott et al., 2008). However, this recovery occurred following cessation of the causal treatment (in this instance, dibutyl phthalate) and so it could be argued that continued EDC exposure through foetal and postnatal life, which is more akin to 'real world' exposures, might interfere with compensatory Sertoli cell proliferation; this possibility remains to be tested.

AQP1 was mainly localized in the apical membrane of non-ciliated cells (Badran and Hermo, 2002) at all ages from late fetal life through puberty to adulthood (Fisher et al., 1998). Fisher et al. (1998) also demonstrated in marmoset the role of estrogen in modulating fluid resorption from the EDs during fetal/neonatal development and in development of EDs and *rete testis*. Our results did not show significant differences in expression of AQP1 staining between Exposed and Control Group. It should be important in the future compare the expression of ER α and ER β with AQP1.

All the results obtained suggest that exposure to environmental chemicals may affect the capacity of efferent ducts to transport water and small solutes thus influencing fluid resorption, protein synthesis and secretion. Ultimately, this may impact on sperm viability in the adult

10.6 References

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CHAPTER 11

Immunodetection of aquaporin 5 in sheep salivary glands related to pasture vegetative cycle

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IMMUNODETECTION OF AQUAPORIN 5 IN SHEEP SALIVARY GLANDS RELATED
TO PASTURE VEGETATIVE CYCLE

Paola Scocco¹, Marina Aralla², Andrea Catorci¹, Carlo Belardinelli³ and Silvana Arrighi²

¹School of Environmental Sciences, Università di Camerino, Italy

²Department of Veterinary Sciences and Technologies for food safety, Università degli Studi
di Milano, Italy

³Department of prevention, Unit of Hygiene of animal origin food (ASUR10), Camerino,
Italy

Short running head: AQP5 in sheep salivary glands

Correspondence:

Prof. Silvana Arrighi

Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare

Laboratorio di Anatomia - Via Trentacoste 2, 20134 Milano, Italy

tel +39.0250315741

fax +39.0250315746

E-mail: silvana.arrighi@unimi.it

Abstract

Mammalian aquaporins (AQPs) are a family of at least 13 integral membrane proteins expressed in various epithelia where they function as channels to permeate water and small solutes. AQP5 is widely expressed in exocrine gland where it is likely involved to provide for an appropriate amount of fluid to be secreted with granule contents.

As regards AQP5 expression in salivary glands, literature is lacking of data concerned with domestic animal species. This study is chiefly aimed to immunohistochemically investigate the presence and localization of AQP5 in sheep mandibular and parotid glands. In addition AQP5 immunoreactivity was comparatively evaluated in animals fed with forages containing different water amounts related to pasture vegetative cycle, in order to shed light to the possible response of the gland to environmental modifications.

Moderate AQP5-immunoreactivity was shown at the level of the lateral surface of mandibular serous demilune cells, not affected by the pasture vegetative cycle and water content. On the contrary, the parotid gland showed AQP5-immunoreactivity at the level of apical and lateral plasma membrane, which was slight to very strong according to the pasture vegetative development and the interannual climatic variations. AQP5 expression is likely due to its involvement to provide for an appropriate saliva fluidity. Indeed, the lowest AQP5 immunoreactivity was noticed when food water content increased.

Key words: AQP5, sheep, salivary glands, pasture vegetative cycle, immunohistochemistry.

Introduction

Mammalian aquaporins (AQPs) are a class of at least 13 integral membrane proteins that facilitate rapid passive movement of water, characterized by having a sequence of about 270 amino acids, forming six transmembrane helical domains spanned through the lipid bilayer, in such a way to form two hemichannels, giving origin to an hourglass-shaped channel through which water may pass [1-3].

AQPs exist in plants, bacteria, insects and several members of the animal kingdom. They are expressed in various epithelia where function as channels allowing the permeability of water and small solutes. Subsets of epithelia where AQP water channels are expressed have a 10- to 100-fold higher capacity for water permeation in comparison with those devoid of these proteins [4]. AQPs 0, 1, 2, 4, 5 and 6 are mainly water selective, while AQPs 3, 7, 9 and 10 also transport glycerol, urea and neutral solutes and are called aquaglyceroporins [4,5]. AQP8 might permeate urea in addition to water [6]. AQP11 and AQP12 belong to a new subfamily, the superaquaporins [7].

AQPs represent constitutive elements of the plasma membrane in most secretory cell types of exocrine glands [8-14]. Recently their presence has been demonstrated in secretory granules and vesicles indicating an involvement in volume regulation of these structures [15].

In recent times, researches have been carried on human and laboratory mammals, aimed at identifying aquaporins and aquaglyceroporins in the digestive system, where it was demonstrated that AQP water channels are involved in the physiologic processes of digestion and absorption of the ingesta [16,17]. Moreover, AQPs have been indicated as co-factors in the etiopathogenesis of some gastroenteric disorders [18-21]. The presence of channels specific for water, urea and other solutes at membrane level of gastrointestinal glandular cells can be modulated, or can influence in turn, hormones, enzymes and pro-enzymes, mediators

and other chemical substances, such as vasopressin, Vasoactive Intestinal Peptide, capsaicin, ethanol, histamine and pepsinogen [16,17, 22-29].

In the salivary glands AQP5 is likely involved to provide for an appropriate amount of fluid to be secreted [15,30]. In particular, in the rat salivary glands AQP5 is highly expressed in the apical plasma membrane of serous acinar cells, in the secretory canaliculi and in the intercalated duct cells, while it is lacking in mucous acinar cells and in striated ducts, indicating its important role in the saliva production [14] further supported by the observation of markedly depressed rates of salivary secretion in AQP5-deficient mice [31]. The significance of AQP5 to human disease may include important disorders of lung, airways, sweat glands, lacrimal glands of the eye and salivary glands [13,31,32]. A defective trafficking of AQP5 has been found in salivary gland biopsies of some patients with Sjögren's syndrome [33] where an abnormal distribution of AQP5 in salivary gland acini is likely to contribute to the deficiency of fluid secretion.

Literature on salivary gland AQP5 expression is lacking of data concerned with domestic animal species, so this study was chiefly aimed at immunohistochemically investigating the presence of AQP5 in sheep mandibular and parotid glands. In addition, its expression was evaluated in animals fed with three different forages depending on the pasture vegetative cycle and containing different water amounts. This investigation is part of a wider, multidisciplinary study focussed on the morphofunctional variations of the digestive apparatus related to pasture vegetative cycle, aimed at shedding light on the response of biological systems, represented in this case by the salivary glands, to environmental modifications, especially the climatic ones, and to their repercussions on farming management planning.

Material and methods

Animal feed, grassland features and forage water amount detection

Animals utilized for this study were fed dry hay and cereals all over the winter season, then nourished with fresh grass during the pasture vegetative cycle. While freely grazing, no additional feed was given to the animals during the permanence on the pasture. Animals were allowed to water twice a day at both sheep-fold and natural pasture. The natural pasture is composed by *Bromus erectus* dominated stands, in a sub-mediterranean contest of Central Apennines (Italy). Forage is usually full-dried in summer because of drought stress and lacking of leaves re-growth. The seasonal length of leaves re-growing lack depends on the amount of annual rainfall, so it usually changes year by year.

Two consecutive pasture vegetative cycles (years 2007 and 2008) were considered for this study to obtain aboveground phytomass samples by mowing performed every fifteen days on fenced permanent plots (5x5 m) during the grazing season (from end of April to end of October). Mowing plots were part of the pasture areas and were representative of the natural plant communities mosaic where animals used for the experiment were allowed to graze [34]. For each plot and in each time point considered for the experiment, 5 pasture samples were harvested. The specimens obtained were kiln-dried at 90°C until reaching the constant weight [35]. Samples of dry hay and rehydrated cereals used for animal nourishment during the winter season were similarly treated.

Tissue collection and processing

Fragments of mandibular and parotid glands were collected at slaughtering from 18 adult female sheep of the Fabrianese breed. Animals had been fed dry hay and cereals all over the winter season, then nourished with fresh grass during the pasture vegetative cycle. Sheep were slaughtered in different moments of the year, thus salivary gland specimens were collected from animals belonging to three experimental groups: 1) at the end of the winter

season (May), exclusively fed with sheep-fold diet (N=3), 2) 15 days after the maximum pasture flowering (begin of August) (N=3), and 3) 15 days after the maximum pasture dryness (half of October) (N=3). The experiment was repeated over two years (2007 and 2008) utilizing the same sampling protocol.

Samples were immediately fixed by immersion either in Carnoy's fluid followed by a post-fixation with calcium acetate 2%-paraformaldehyde 4% [36] and in paraformaldehyde 4% [13], dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Both fixatives were freshly prepared every time.

AQP5-Immunohistochemistry

Sections of all specimens and controls included in the study were simultaneously processed in the same session of immunohistochemistry. Antibodies, buffer and revelation solutions were made fresh for the experiment.

After de-waxing, serial sections (4 μ m thick) obtained from specimens fixed with either fixatives were washed and immersed in a freshly prepared 3% H₂O₂ solution for 15 min to block the endogenous peroxidase activity. This was followed by incubation in 1:20 normal goat serum (DakoCytomation, Denmark, code X0907) in Tris-Buffered Saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) 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 antibodies against rat AQP5 (Alpha Diagnostic International, San Antonio, TX, USA; Cat # AQP51-A) diluted 1:100 in TBS containing 2% of normal goat serum. Subsequently, the sections were rinsed 3 X 5 min in TBS and incubated for 30 min at room temperature with biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Inc., CA, USA, code BA1000) diluted 1:200. Streptavidin-Biotin/Horseradish Peroxidase Complex (Vectastain® ABC Kit, code PK-4001, Vector Laboratories, Inc., CA, USA) was employed for detection. The sections were washed 3 X 5 min with TBS before

applying liquid 3,3'-diaminobenzidine solution (DAB+ Chromogen, DAKO code # K3467) for up to 10 min. Sections were counterstained with Mayers' haematoxylin, dehydrated, and permanently mounted using Eukitt® (Biotica, Milan, Italy).

Staining controls

Sections of human and rat salivary gland (respectively acquired from University tissue collection and experimental laboratory animals facility of Milan Veterinary Faculty) served as positive controls for AQP5-immunoreactions. The specificity of the immunostainings was tested by negative controls, performed by (1) use of non-immune rabbit serum (DakoCytomation; code # X 0903) in place of specific antisera; and (2) omission of the first layer. All of them resulted in the absence of immunoreaction.

The evaluation of staining intensities was based on subjective estimates independently performed by three authors. Slides were observed and photographed under an Olympus BX51 photomicroscope equipped with a digital camera and DP software (Olympus, Tokio, Japan) for computer-assisted image acquirement and managing.

Results

Forage water amount detection

Data obtained from drying of pasture samples are reported in Fig. 1. The trend in water content was similar in the two examined years, with a decrease of water percentage in the forage during the summer period, lasting from the end of July to the beginning of September. During 2007, a rather droughty year, a sudden decrease in the water content occurred until the end of September. During 2008, a rather rainy year, an increase in water content percentage was observed from the half of August. The variation in water content among the samples from the same time point ranged from 1.2 to 2.7 %.

The dry hay and rehydrated cereals used for animal nourishment during the winter had a content of water of 25%.

Immunohistochemistry

The best immunohistochemical results were obtained in Carnoy's-fixed tissues. They are summarized in Table 1 and shown in Fig. 2. In both examined glands AQP5-immunoreactivity was present in the secretory components only, whereas epithelial cells lining any ductal segment never showed immunoreaction. Mandibular gland showed moderate AQP5-immunoreactivity at the level of the lateral plasma membrane of the serous demilunes (Fig. 2A, inset). No reactivity was detected in the mucous adenomeres of the gland (Fig. 2A). The slight AQP5 immunoreactivity detected in the mandibular gland was not affected by the pasture vegetative cycle and relative water content.

The parotid gland showed AQP5-immunoreactivity at the level of the apical and lateral plasma membranes of the acinar cells (Fig. 2B, inset). AQP5-immunoreactivity was very strong in the parotid gland of group 1 sheep, immediately after going out from the sheep-fold (Fig. 2B).

Moreover, AQP5-immunoreactivity was slight to strong in the parotid gland according to the vegetative development and the interannual climatic variations. During the first year of the experiment (2007), a decreased AQP5-immunoreactivity was observed in the parotid gland of group 2 sheep, which were nourished for all the duration of the maximum flowering of the pasture (Fig. 2C) if compared with the reactivity shown immediately after going out from the sheep-fold (group 1 sheep, Fig. 2B). AQP5-immunoreactivity was strong in the parotid glands of group 3 sheep, analyzed after a period of nourishment throughout the maximum dryness of the pasture (Fig. 2C, inset). During the second year of the experiment (2008) results were similar to those obtained in 2007 even if the expression of AQP5 was lower. In fact, AQP5-immunoreactivity was slight in samples collected from group 2 sheep after feeding throughout

the maximum flowering of the pasture (Fig. 2D) and moderate in samples collected from group 3 sheep after feeding for the duration of the maximum dryness of the pasture (Fig. 2D, inset). No appreciable variability of staining intensity was shown among the samples of the animals pertaining to the same group.

Discussion

In a surprising manner, the best immunohistochemical results were obtained in Carnoy's-fixed tissues. Indeed, formaldehyde is one of the most popular fixatives in immunohistochemistry because of its low cost and manageability and, mainly, because of its aptitude on preserving morphological features with few artefacts. Nevertheless formaldehyde fixation results in a variably loss of immunoreactivity by its hiding or damaging the antibody binding sites. Enzyme digestion or heat-induced epitope retrieval may help revealing unavailable sites [37,38] but are invariably harmful to the tissue integrity and might reduce the intensity of the immunostaining. Alcohol and alcohol-based fixatives such as Carnoy's are often superior to formaldehyde in retaining antigenicity [39,40] and this is further demonstrated by the results of our experiment (not shown).

In both examined glands AQP5-immunoreactivity was present in the serous secretory components only; mandibular gland showed AQP5-immunoreactivity restricted to lateral plasma membrane of the serous demilunes, while parotid gland showed AQP5-immunoreactivity at both apical and lateral plasma membrane of the acinar cells.

The movement of fluid occurs in the cell plasma membrane through both the transcellular and paracellular pathway [28,41]. Transcellular water movement can be successfully attained thanks to the presence of water channels in both apical/lateral and basal membranes. In this study AQP5 was present in the apical and lateral membrane of serous cells in the sheep salivary glands. The basolateral membrane is believed to have high permeability in the

salivary gland acinar cells, and AQP5 at luminal surface of the acinar cells is implied to serve as a rate-limiting step in the water transport across the cells [42,43]. Murakami et al. [28] showed that in the rat submandibular gland AQP5 is involved in the control of the junctional fluid transport system (JFT system) as an osmosensor able to influence the paracellular pathway. On the other hand, Krane et al. [31] demonstrated an AQP5 involvement in the water transcellular transport in mouse salivary glands. Our results in the sheep salivary glands show that AQP5 is abundantly expressed in the luminal membranes of serous acinar cells. The positive immunoreactivity, likely localized at the level of intercellular canaliculi that radiate from the luminal space of the serous acini of the parotid and mandibular glands, indicate that the presence of AQP5 might increase the functionality of this small apical surface area. As parotid gland concerns, data also agree with recent findings [13,44] indicating the role of AQP5 in the secretive process at apical membrane level in human parotid acinar cells.

The moderate reactivity of mandibular gland didn't change either among the different moments of the year or between the two examined years. Instead, parotid gland showed an AQP5 reactivity ranging from slight to very strong according to the vegetative development and the interannual climatic variations. In particular, during 2007 animals grazed on a pasture which contained, at the maximum of flowering, a water percentage ranging from 65 to 30, whereas in the period of maximum of dryness the water content varied from 30 to 45%, with a period of about 70 days in which the water content was lesser than 50%. During 2007 the AQP5 expression in sheep parotid gland was higher than in 2008. Actually, 2008 was a particularly rainy year and the pasture percentage of water ranged from 70 to 40 in the period of maximum flowering and from 40 to 65 in the period of maximum dryness, in which the content of water in the pasture was lesser than 50% only for 40 days.

Saliva is the principal means to mix food as well as to dissolve the sapid substances and its secretion can be increased by different kinds and intensity of stimuli; the food ingestion causes a saliva secretion whose amount is inversely linked to the food water content; in addition, Ruminant parotid glands perform a continuous secretion [45,46]. Thus, it is not surprising that only the sheep parotid gland was affected by the hydration of the pasture.

The required additional saliva supply when the food water amount is low, might be assured by an increased AQP5 expression. This hypothesis is further supported by our findings showing that:

- AQP5 staining intensity decreased in the maximum pasture flowering period, when the water amount is higher than in both the winter season and maximum pasture dryness period, when samples showed highest AQP5 reactivity;
- the variability in AQP5 immunoreactivity was higher during 2007 than 2008 year, due to the required additional saliva supply linked to the highest length of pasture dryness period.

Actual findings and acquired knowledge on salivary glands [36,47-50], allow us to hypothesize for the sheep parotid gland a role in the production of the amount of saliva mainly involved in the lubrication and mixing of chewed food, while the mandibular gland produce a saliva more involved in defence functions. Thus, it is not surprising that only parotid gland was affected by the hydration of the pasture.

It is noteworthy the close relation between the salivary gland histophysiology and the pasture biological system evidenced by the rapid functional response of parotid gland to the environmental modifications. This consideration adds to other evidences gathered from the study of morphofunctional modifications of the sheep digestive system linked to environmental variations [51-53], and should be taken into account in the natural farming system management.

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Legends to figures

Fig. 1. Percentages of water content are shown, detected from drying of pasture samples. The graphs were obtained by elaborating the results acquired in the two examined years at the dates indicated at the bottom of the picture.

Fig. 2. Immunohistochemical analysis of AQP5 distribution in sheep salivary glands. **A.** In the sheep mandibular gland AQP5 is present at the level of the serous demilunes while it lacks at the level of mucous acini (*); in particular, AQP5 immunoreactivity has been evidenced in the lateral plasma membrane of serous cells (†), see inset. **B.** Parotid gland: AQP5-immunoreactivity of the acinar cells detected at the end of the winter season (group 1 sheep) was very strong. In particular, AQP5 is apically expressed in the serous acinar cells (†) and in the lateral plasma membrane (▲), see inset. **C.** In the parotid gland collected 15 days after the maximum pasture flowering during the first year of the experiment (group 2 sheep) moderate AQP5-immunoreactivity can be noticed, lower in comparison with group 1. During the maximum pasture dryness AQP5 reactivity increased (see inset). **D.** The AQP5-immunoreactivity was slight in the group 2 sheep samples during the second year of the experiment and moderately increased in group 3 ones (see inset). Scale bars: A,B,C,D, = 20 μ m; insets in A,B = 20 μ m; insets in C,D = 40 μ m.

Table 1. AQP5-immunoreactivity in sheep salivary glands related to pasture vegetative cycle

Sheep salivary gland AQP5-immunoreactivity	Mandibular Gland			Parotid Gland	
	Mucous	Serous	Ducts	Serous	Ducts
	acini	demilunes		acini	
Sheep Group 1 year 2007/2008	-	+/++	-	+++	-
Sheep Group 2 year 2007	-	+/++	-	+/++	-
Sheep Group 3 year 2007	-	+/++	-	++	-
Sheep Group 2 year 2008	-	+/++	-	+	-
Sheep Group 3 year 2008	-	+/++	-	+/++	-

Intensity of immunoreaction is arbitrarily identified as: + slight, +/+ moderate, ++ strong, +++ very strong, - no immunoreactivity detected.

Figure 1

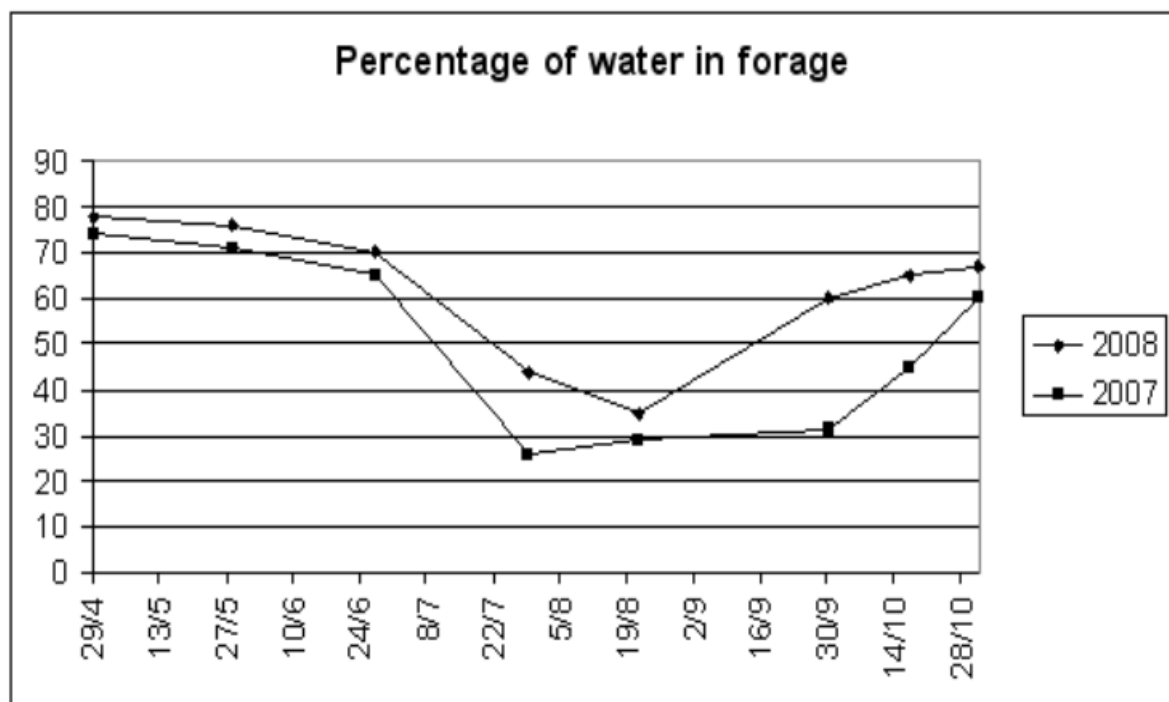
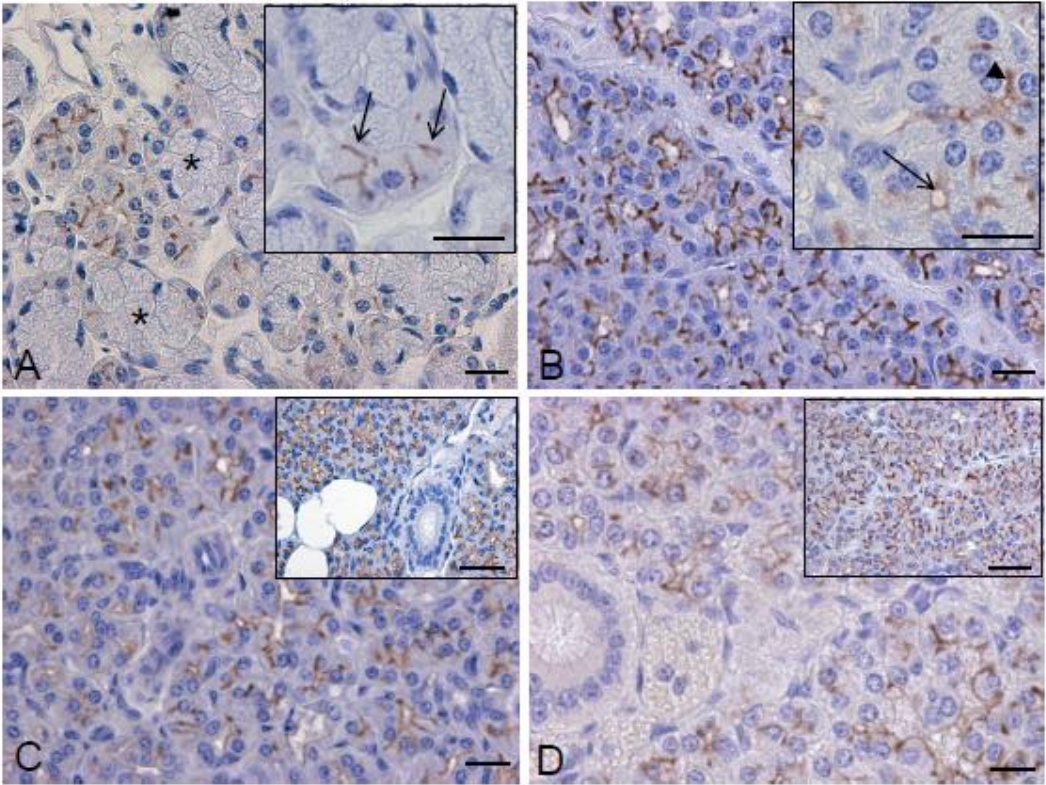


Figure 2



CHAPTER 12

Summary

12. Summary

The discovery of aquaporins has greatly enhanced our understanding of the molecular basis of the transport of water across membranes, and has prompted a reconsideration of the determinants of the water permeability of membranes. It is now evident that membrane water permeability can be regulated independently of solute permeability. Elucidation of the structural determinants of pore specificity should facilitate the design and discovery of channel-modulating agents. Studies of gene expression, as well as studies of protein stability, might reveal mechanisms for manipulating the level of aquaporin expression. The degree to which aquaporins determine membrane water permeability in specific tissues probably varies depending on both the organ and the context. However, as their physiological roles are defined, it will become increasingly appealing to consider aquaporins as specific therapeutic targets for various pathophysiological conditions in which the disruption of water homeostasis is a principal manifestation.

The landmark discovery of AQPs starts the era of molecular water channels, which has permeated virtually every branch of biology and medicine.

A number of recent studies have revealed the importance of mammalian AQPs in both physiology and pathophysiology and have suggested that pharmacological modulation of aquaporin expression and activity may provide new tools for the treatment of variety of human disorders, such as brain edema, glaucoma, tumour growth, fertility and obesity in which water and small solute transport may be involved.

Since multiple isoforms of AQPs are differentially expressed in cells and tissues, their localization to specific cells and membrane domains is critical to understanding the role of each AQP in the transfer of water and small solutes.

Balancement of water concentration within the uterine environment is important in every reproductive moment of a female mammal life. Rapid transcellular water movements are facilitated by AQPs that increase plasma membrane permeability. The results from my research showed that AQP-mediated water movement between the intraluminal, interstitial, and capillary compartments is crucial in the uterine imbibition mechanism in the bitch, including periodic stromal oedema in preparation for embryo implantation, with evidence that the expression of different AQPs can be regulated by steroid sex hormones. Also, the importance of the AQP-mediated fluid regulation in uterine environment in the peri-implantation period is known in the woman and laboratory mammals, to assure the successful implantation of blastocyst and its survival. The establishment of pregnancy and its maintenance is dependent on strict synchronization of uterine receptivity with embryonic maturation. Water homeostasis during foetal

development is of crucial physiologic importance. Amniotic fluid (AF) provides the fluid-filled compartment that is essential for normal foetal growth, movement and development. Placental water flux is an important factor in determining AF volume and fetal hydration, too. AF changes during gestation may be dependent on modulations in solute as well as water permeability and changes in AQP's during gestation suggest their role in mediating placental and membrane water flow.

AQP's are channel proteins expressed in cell membranes of many epithelia involved in fluid transport and several AQP's have been identified in adult rat excurrent ducts, where considerable fluid resorption occurs. Concerning the morphological changes allowed by the AQP influence in the male reproductive tract I considered first of all the role of AQP's in the epididymis of a domestic species, the cat, and two animal models, the rat and the lamb.

Ultrastructural features of the epithelium lining the efferent ducts (ED) in the cat, as in other mammalian species, are strongly indicative of an absorptive activity taking place towards the intraluminal fluids. It is well-known that more than 95% of the fluid leaving the testis is reabsorbed by the ED, but the cell structures involved in the resorption processes are still a matter of debate. The purpose of the present work was to study the absorptive pathways in the ED of adult cats by means of 1) the immunohistochemical localization of different isoforms of the AQP's and 2) the localization and the carbohydrate characterization of the endocytotic apparatus by means of the lectin histochemistry. AQP-mediated trans-cellular route together with fluid phase glyocalix-mediated endocytosis.

Maternal undernutrition and, under certain circumstances, overnutrition, during pregnancy or during early postnatal life can alter reproductive function of the offspring. It is known that epididymal morphology in adult rats is affected by undernutrition during fetal to prepubertal life. For this study I tested the influence of pre- and postnatal undernutrition on AQP-expression in the adult male genital tract.

Exposure to environmental endocrine disrupting chemicals (EDCs) during pregnancy in animals and humans is associated with reduced sperm counts and increased incidences of testicular cancer and reproductive tract abnormalities. Although male fetuses from pregnant ewes exposed to EDCs contained in sewage sludge fertiliser (a "real life" exposure model) exhibit fewer testicular Sertoli and Leydig cells, the epididymis has not been investigated. The movement of fluids and small solutes in the epididymis is critical for adult sperm maturation and this process is regulated, in part, by the AQP's. I examined the effects of sewage sludge exposure on the expression of AQP's in the late gestation fetal epididymis.

The results I obtained suggest that exposure to environmental chemicals may affect the capacity of efferent ducts to transport water and small solutes thus influencing fluid resorption, protein synthesis and secretion. Ultimately, this may impact on sperm viability in the adult.

Another research item was the study of some factors which control the growth of the gubernaculum testis and regression of the cranial suspensory ligament during foetal development, resulting in transabdominal descent of the testes. INSL3, also known as Leydig insulin-like peptide or relaxin-like factor, is supposed to induce the growth of the gubernaculum in male foetuses, thus being directly responsible for the testicular descent in humans and rodents. This is believed to happen with the LGR8 receptor intervention. A study led during my PhD provides evidence of the immunolocalization of INSL3 in the Leydig cells of dog male foetuses and of LGR8 receptor in different tissues of the gubernaculum testis of the same foetuses. AQPs are also involved in the water imbibition of gubernaculum testis: dog foetuses gubernacula showed a high expression of AQP1, AQP3, AQP4 and AQP9 respectively in blood vessels, smooth muscles, connective tissue and fat.

As well known AQPs are ubiquitarily distributed in the body maintaining all the same the specific function as channels to permeate water and small solutes. I collaborated with the University of Perugia regarding the immunolocalization of AQP5 in the sheep salivary glands in relation to the pasture vegetative cycle.

AQP5 is widely expressed in exocrine glands; in particular, in the rat salivary glands AQP5 is highly expressed in the apical plasma membrane of serous acinar cells, in the secretory canaliculi and in the intercalated duct cells, while it is lacking in mucous acinar cells and in striated ducts, indicating its important role in the saliva production (further supported by the observation of markedly depressed rates of salivary secretion in AQP5-deficient mice. I studied the presence of AQP5 in sheep parotid and mandibular glands, and its expression in the different stages of pasture vegetative cycle. AQP5 is expressed in parotid gland and the reactivity was higher when animals were fed on grasslands at the end of vegetative cycle than on grasslands at the maximum of vegetative development.

Ultimately I collaborated to a study created by the Reproduction Unit of the University of Milan regarding the relationship between nutrition and reproductive efficiency in the mare.

Leptin, the hormone product of *ob* gene expression, is an important endocrine indicator of adipose mass and nutritional status, as well as an important regulator of various aspects of feed intake, growth, metabolism and reproduction. Leptin amount in the blood is proportional to body energy stores and/or body mass, so, inadequate nutrition might impair reproductive function leading, for example, to the delayed onset of puberty. Indeed, the onset of puberty in humans and

animals is associated with an increase in fat and consequent increase in circulating leptin, suggesting that leptin may be required for normal growth and development of reproductive organs. In the mare, besides many reports quantifying the correlation of circulating concentration of leptin with body condition scores^{1,2}, only few informations exist about the presence of leptin (Ob) and leptin receptor (Ob-R) in the ovary or in the oocyte. The results support the hypothesis that in the horse leptin is differently localized during oocyte IVM showing different immunoreaction intensity related either to the horse breed or to the reproductive puberal development. The presence of leptin in the ovary could suggest a possible involvement in oocyte maturation, angiogenesis, follicle rupture or subsequent corpus luteum formation. Moreover, leptin may be involved in the determination of the animal pole of the oocyte and in the establishment of the inner cell mass and trophoblast in the embryo.

CHAPTER 13

Acknowledgements

13. Acknowledgements

*“Quis potis est dignum pollenti pectore carmen
condere pro rerum maiestate hisque repertis?
Quisve valet verbis tantum, qui fingere laudes
pro meritis eius possit, qui talia nobis
pectore parta suo quaesitaeque praemia liquit?
Nemo, ut opinor, erit mortali corpore cretus.
Nam si, ut ipsa petit maiestas cognita rerum,
dicendum est, deus ille fuit, deus, inclyte Memmi,
qui princeps vitae rationem invenit eam quae
nunc appellatur sapientia, quique per artem
fluctibus et tantis vitam tantisque tenebris
in tam tranquillo et tam clara luce locavit”*

Lucrezio, De Rerum Natura – Liber V

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Our love is the best *discovery* ever.