Aquaporin 9 (AQP9) Localization in the Adult Dog Testis Excurrent Ducts by Immunohistochemistry

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ABSTRACT

Aquaporins (AQPs) are small, intrinsic membrane proteins that are present in many cell types involved in fluid transport. AQP9 is a major apical water channel that is expressed throughout the efferent ducts, epididymis, and vas deferens, as well as in other regions of the human and rodent male reproductive tract. The target of this study was to examine the expression of AQP9 in epithelial cells in the adult dog efferent ducts, epididymis, and vas deferens. Samples of dog male reproductive tract comprising fragments of the testis; initial segment, caput, corpus, and cauda of the epididymis; and vas deferens were obtained from eight adult mongrel dogs. Immunohistochemistry and Western blotting procedures were used to show AQP9 localization and distribution. AQP9 expression was not detected either in dog seminiferous tubules or rete testis. However, apical labeling for AQP9 was detected in the different regions of epididymis and vas deferens, with the reaction being less intense in the caput epididymis. Thus, AQP9 is abundantly expressed in dog male reproductive tract, in which it is an important apical pathway for transmembrane flow of water and neutral solutes. Anat Rec, 290:1519–1525, 2007.

Key words: efferent ducts; epididymis; vas deferens; aquaporin 9; immunohistochemistry; water channel; epith elial transport

The composition of the luminal fluid is modified during its passage throughout the efferent ducts, epididymis, and vas deferens (Robaire and Viger, 1995). Significant fluid reabsorption occurs in the efferent duct and epididymis (Wong et al., 1978; Clulow et al., 1994). Fluid secretion and absorption are vital processes in the physiology of male reproduction and alterations in fluid homeostasis are related to infertility (Russell et al., 1989). Fluid reabsorption by epididymal epithelium causes high spermatozoa concentrations forward to the cauda epididymis (Turner, 1991; Robaire and Viger, 1995).

Water can slowly permeate the lipid bilayer by simple diffusion. However, some specialized cell membranes show higher water permeability, suggesting the existence of additional pathways for water moving through the membranes (Agre, 2004; Matsuzaki et al., 2002). Some hypotheses have been suggested until the discovery, by Preston et al. (1992), of a set of proteins involved in water transport in erythrocytes, the aquaporins (AQPs).

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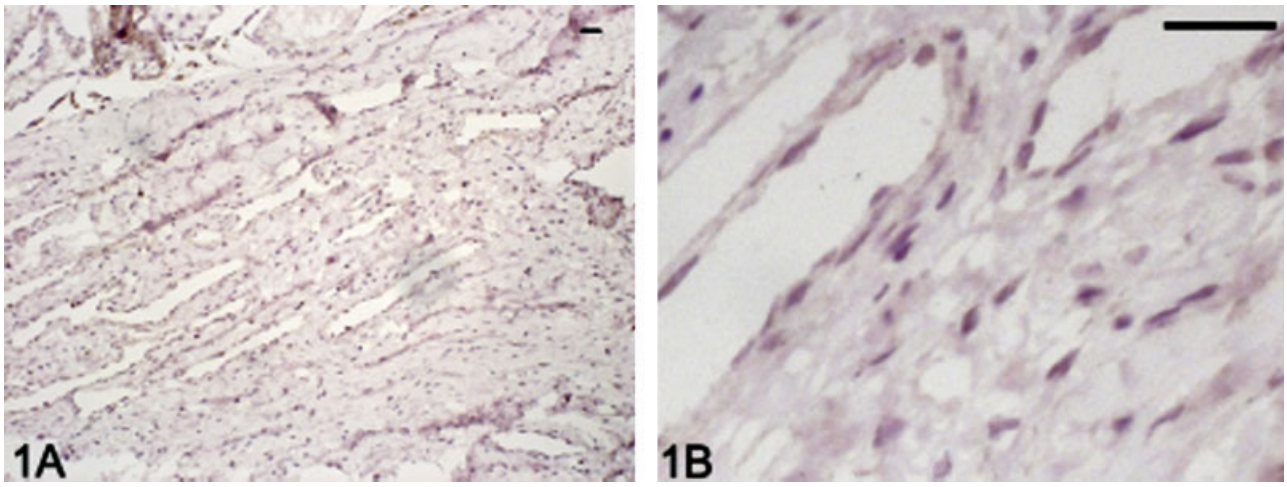

Figure 1.

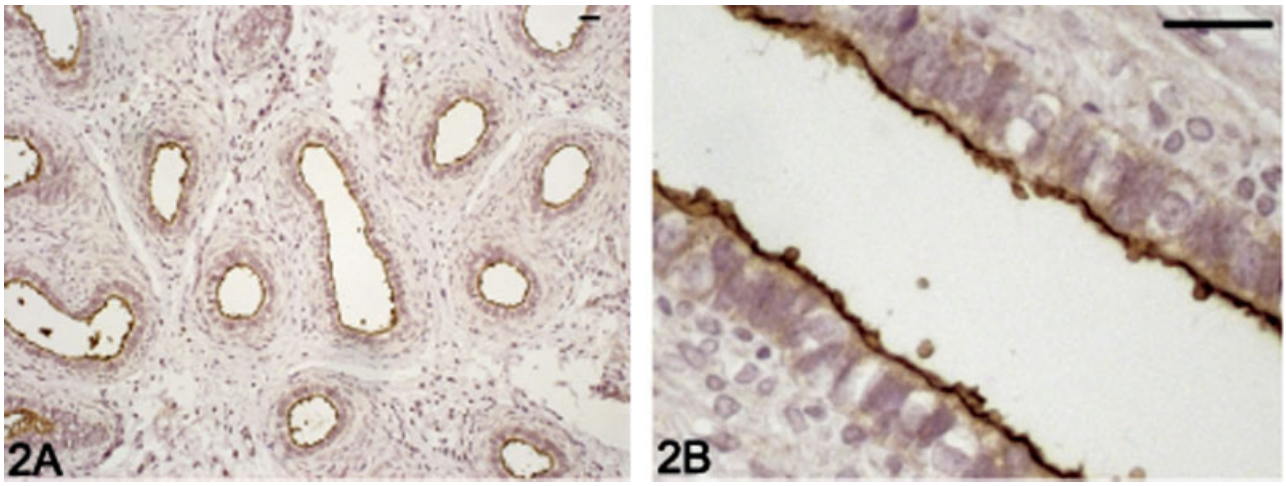

Figure 2.

AQPs are small, intrinsic membrane proteins that are present in many cell types involved in fluid transport (Verkman and Mitra, 2000). Thirteen isoforms of AQPs, AQP0 to AQP12, have been identified in mammalian cells and different genetic codes in each isoform. The AQPs are composed of a single chain of approximately 270 amino acids, which spans the membrane six times and the amino- and carboxyl-terminal ends are both in the cytoplasm (Agre et al., 1995; Da Silva et al., 2006).

The AQPs make the membrane 10- to 100-fold more permeable to water than membranes lacking such channels (Agre, 2004). The movement of water across cell membranes by AQPs is accomplished by bulk flow driven by an osmotic gradient through the membrane. Given the relevance of the AQPs for fluid transport, they have been described in many other tissues, such as leukocytes, liver, kidney, brain, lung, small and large intestines, skin, and in the male reproductive tract (Cho et al., 2003; and for review, see Da Silva et al., 2006).

Until now, many AQPs have been detected in the male reproductive tract, being AQP1, AQP2, AQP7, AQP8, and AQP9 the most investigated (Brown et al., 1993; Nelson et al., 1998; Elkjaer et al., 2000; Stevens et al., 2000; Badran and Hermo, 2002). Among the AQPs detected in male reproductive tract, AQP9 represents an important apical pathway for transmembrane movements of water and others solutes—such as carbimides, polyols, purines and pirimidines—which are sometimes referred as aquaglyceroporins (Tsukaguchi et al., 1998; Matsuzaki et al., 2002).

The rat and the human AQP9 genes were cloned in 1998 (Ishibashi et al., 1998). AQP9 mRNA has been found in liver, testes, and brain. The cellular localization in these tissues was shown by immunostaining and also by in situ hybridization to be in hepatocytes, in seminiferous tubules and Leydig cells (Tsukaguchi et al., 1998). In the efferent ducts, the reaction was noted over the apex of the nonciliated cells corresponding to the stain-

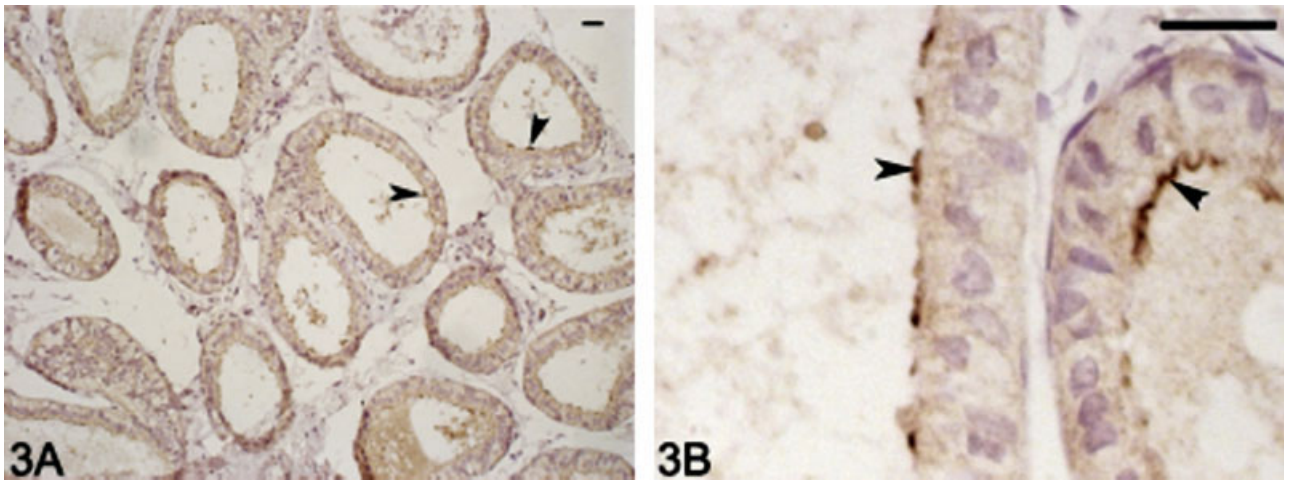

Figure 3.

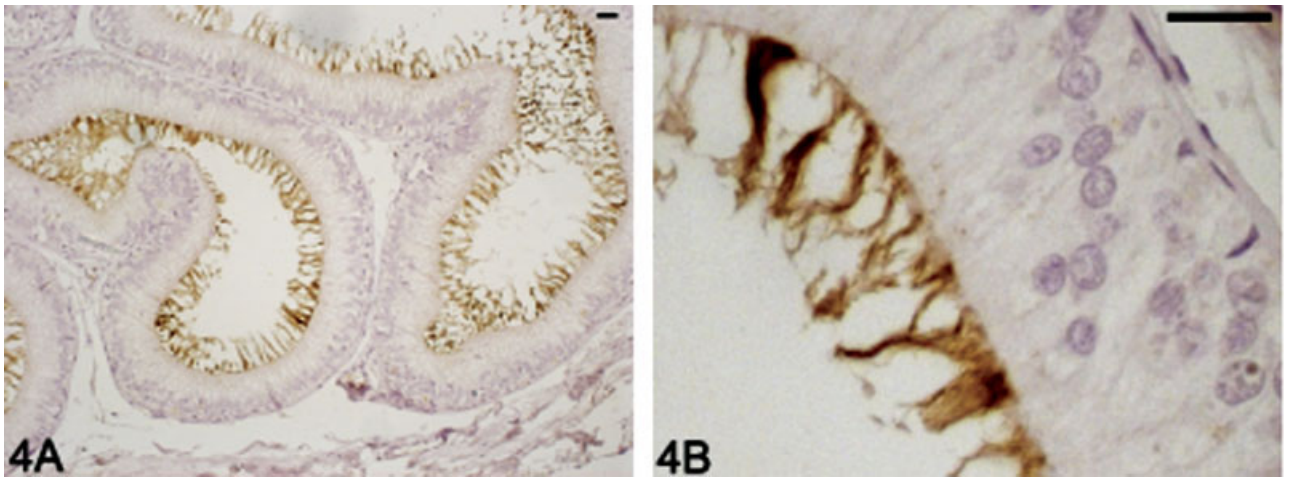

Figure 4.

Figs. 1–4. Aquaporin 9 (AQP9) immunolocalization in dog excurrent ducts. The reaction was observed at the apical pole of the cells. AQP9 immunoreaction was absent in rete testis (Fig. 1A,B), but an intense immunostaining was observed in the apical brush border at testicular efferent ducts (Fig. 2A,B). In the epididymal efferent ducts, only nonciliated cells (arrow) showed a positive immunostaining for AQP9 (Fig. 3A,B). AQP9 was located in the long apical stereocilia at the initial segment epididymidis (Fig. 4A,B). Scale bar = 20 μm.

ing of their microvilli. In the epididymis, reactivity to anti-AQP9 was noted over the microvilli of the principal cells, although the intensity of the AQP9 expression was cell- and region-specific (Badran and Hermo, 2002).

In addition to being expressed in epididymis efferent ducts, AQP9 was present along the entire length of the rat vas deferens (Pastor-Soler et al., 2001). Thus, AQP9 represents a major apical water channel that is expressed throughout the efferent ducts, epididymis, and vas deferens, as well as in other regions of the male reproductive tract.

So, the aim of this study was to examine the expression of AQP9 in epithelial cells in the efferent ducts, epididymis, and vas deferens from adult dogs. It is known that the dog is a biomedical key species, being a model for study of many human diseases, some of them spontaneous and others experimentally induced. Moreover, the canine epididymal proteins are similarly organized to the human epididymis, also having a similar tissue distribution (Kirchhoff, 2002). A previous work also has shown that the dog is an excellent model for comparative reproductive studies about development of sperm cryopreservation protocols (Anderson et al., 2001).

MATERIALS AND METHODS

Animal and Tissues

Samples of dog male reproductive tract were obtained from eight adult mongrel dogs (*Canis familiaris*), during castration surgery realized in the Clinical Hospital of the Veterinary Medical School of UNESP at Botucatu. Fragments of testis; initial segment, caput, corpus and cauda of the epididymis, and vas deferens were collected. A previous histological epididymal zonation of the dog was accomplished according to Schimming and

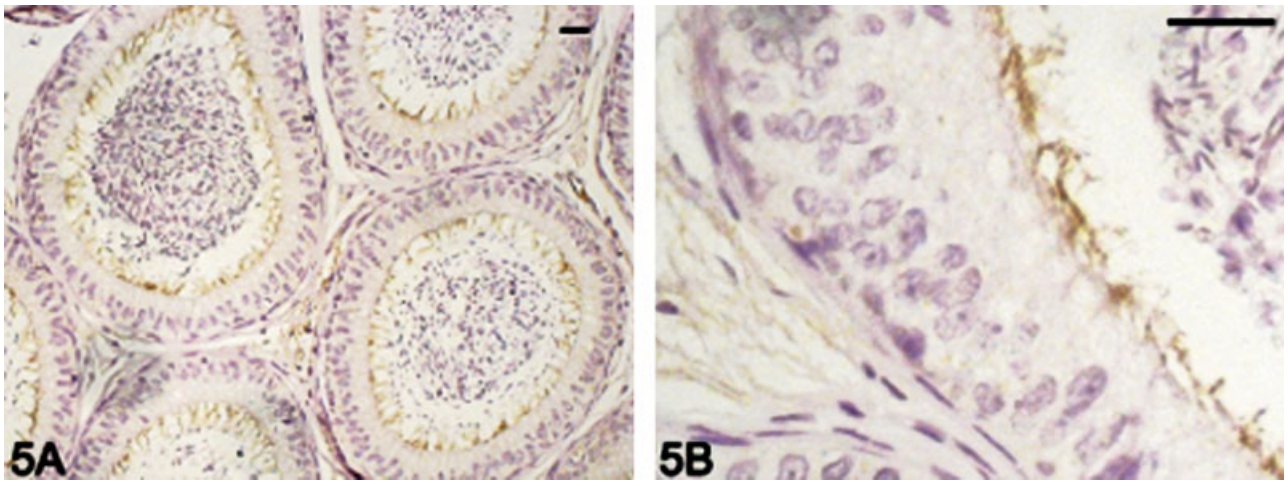

Figure 5.

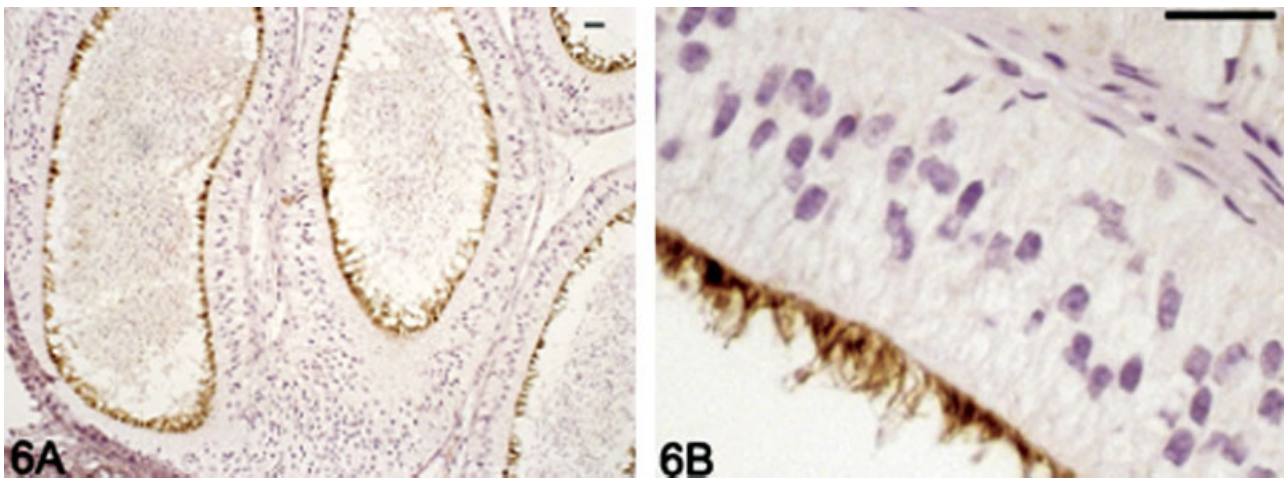

Figure 6.

Vicentini (1997). The biological samples were immediately immersed in fixative, and some alternate samples were randomly assigned and snap frozen.

Immunohistochemistry

The tissues were fixed in 4% paraformaldehyde dissolved in phosphate buffered saline (PBS) for 24 hr. Fixed samples were washed in PBS for 24 hr, dehydrated in graded ethanol series, clarified in xylene, and embedded in Paraplast™ (Sigma, St. Louis, MO). Paraplast sections (5 μm thick) were stained with hematoxylin–eosin (H&E) for general morphological view or pretreated with a citric acid monohydrate antigen retrieval method, and afterward, immunostained with rabbit polyclonal antibody against AQP9 (Chemicon Temecula, CA) at 1:100 dilution. The secondary biotinylated antibody goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:70. The reaction was visualized with diaminobenzidine tetrachloride as a chromagen, and sections were counterstained with hematoxylin. Negative controls were obtained from reactions performed without the primary antibody incubation step. The sections were analyzed in an Olympus BX 41® Microscope connected to a Olympus DP12 camera, and the images digitalized using image analyzer Olympus Image - ProExpress Windows™.

Western Blotting

Frozen samples from the different epididymal regions focused on and the proximal part of the vas deferens were homogenized in 50 mM Tris buffer (pH 7.5) plus 0.25% Triton X-100 by Polytron for 30 sec at 4°C and centrifuged; the protein fraction was extracted on supernatant and quantified as per Bradford (1976). A protein sample (70 μg) was loaded into 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins were transblotted onto a nitrocellulose membrane (Sigma). The blot was blocked with 10% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Tween-

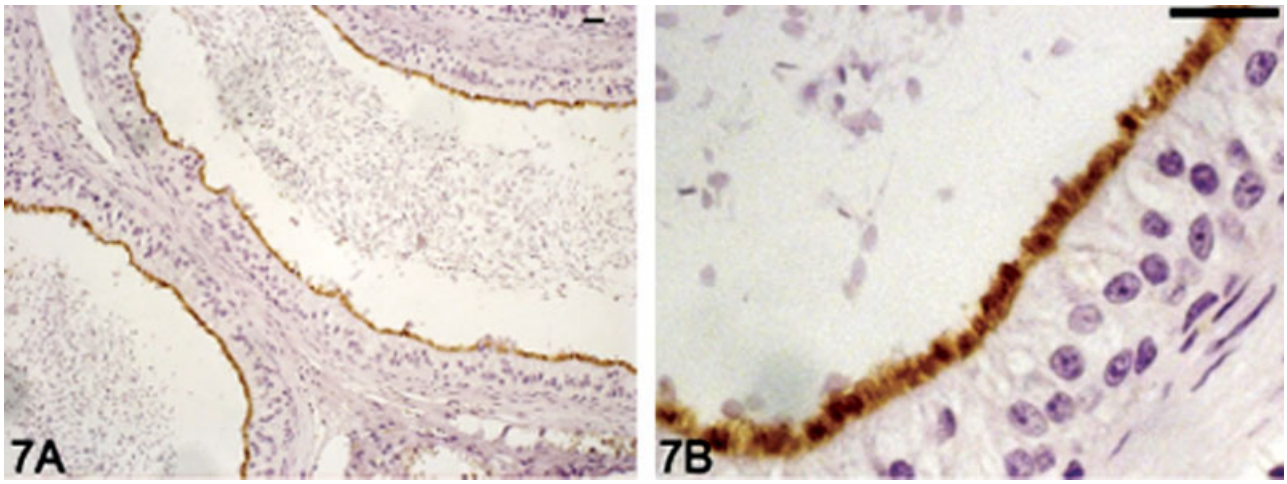

Figure 7.

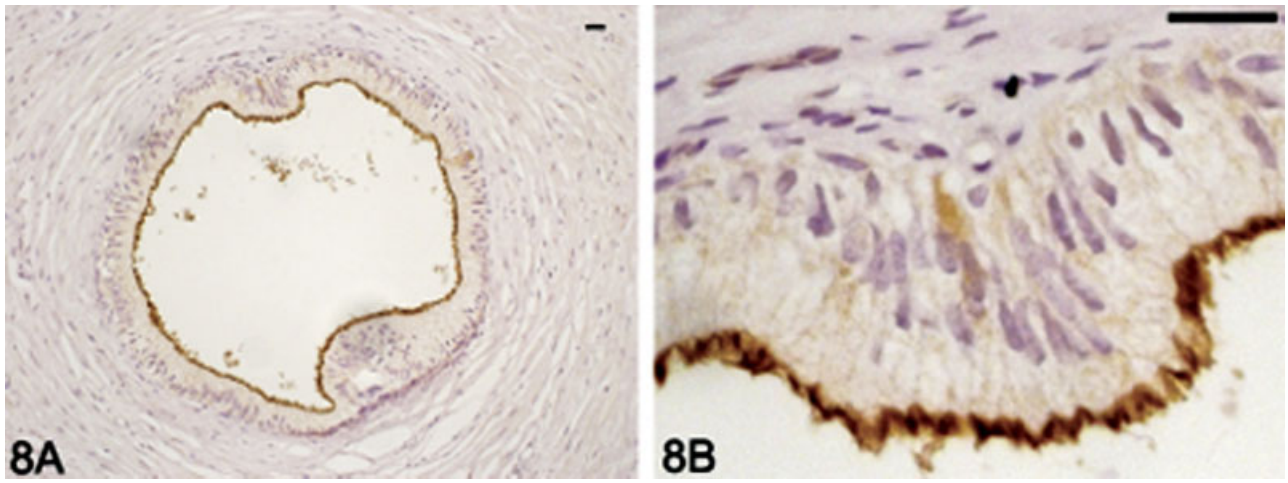

Figure 8.

Figs. 5–8. Aquaporin 9 (AQP9) immunolocalization in dog excurrent ducts. The apical region of caput epididymidis showed a weak to moderate intensity of AQP9 immunostaining (Fig. 5A,B). Immunostaining intensity of AQP9 in the cellular stereocilia increases in the corpus (Fig. 6A,B) and cauda (Fig. 7A,B). AQP9-positive immunoreaction was present on the cellular stereocilia in the proximal region of the vas deferens (Fig. 8A,B). Scale bar = 20 μm.

20) for 1 hr. The blot was then incubated overnight at 4°C with 3% bovine serum albumin containing 1–1,000 dilution of the AQP9 (Chemicon, USA) or β-actin (Santa Cruz Biotechnology) primary antibodies. The blot membrane was then washed for 20 min three times in TBST and incubated for 1 hr at room temperature with peroxidase-conjugated goat anti-rabbit IgG antibody. The blot was again washed for 20 min three times in TBST. Proteins were detected using the Chemiluminescent Peroxidase Substrate (Sigma).

RESULTS

Strong apical labeling for AQP9 was detected by immunohistochemistry in many regions of the excurrent ducts in the adult dog. However, AQP9 expression was not detected in dog seminiferous tubules (not shown) or rete testis (Fig. 1). In testicular efferent ducts, the staining was expressed in the entire apical brush border (Fig. 2) and in epididymal efferent ducts, the staining was restricted to the apical brush border of nonciliated cells (Fig. 3).

In the initial segment of the epididymis, a strong labeling was observed in the long apical stereocilia from principal cells and little intracellular staining was detectable (Fig. 4). The reaction was less intense in the caput epididymis (Fig. 5) than in the corpus (Fig. 6) and cauda epididymidis (Fig. 7). AQP9 staining was abundantly expressed on apical stereocilia of principal cells in the initial portion of the vas deferens (Fig. 8). Basolateral staining or intracellular staining for AQP9 was not detectable in the epithelial cells of vas deferens.

The Western blotting procedure for AQP9 in the extracts from different portions of the epididymis and vas deferens of dog detected one main band approxi-

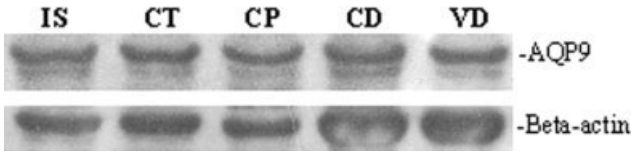

Fig. 9. Western blot analysis of aquaporin 9 (AQP9) in the initial segment (IS), caput (Ct), corpus (CP), cauda (CD) of epididymis and vas deferens (VD) protein extracts from dog. Each line represents 70 μg of protein from different tissues. The beta-actin protein was used as an internal control of the reaction. The antibody recognized a main band of AQP9 of approximately 30 kDa.

mately 30 kDa (Fig. 9), confirming the antibody specificity. The antibody also detected other higher bands that represent differentially glycosylated AQP9 forms confirming the findings of Pastor-Soler et al. (2002). The beta-actin protein, used as an internal control of the reaction, showed an equal amount of protein loaded in each lane.

DISCUSSION

AQPs mediate the efficient movement of water across the cell membranes in different tissues. However, much has been elucidated concerning from molecular structure to cellular distribution in AQPs, hitherto the real knowledge of AQPs is not sufficient to obtain a comprehensive view of their role as channel proteins acting on the overall physiology of cell membranes (Matsuzaki et al., 2002).

Especially the AQP9 is a water channel that allows the passage not only of water but also of neutral solutes (Pastor-Soler et al., 2001). Here, AQP9 was detected in efferent ducts, in different parts of the epididymis and vas deferens epithelium in the adult dog by immunohistochemistry and by Western blotting methods. Our present data show that the AQP9 is abundantly expressed in excurrent ducts of the testis in adult dog, where it could represent an important apical pathway for transepithelial water flow (Pastor-Soler et al., 2001).

AQP9 in testicular efferent ducts was detected in the entire apical brush border. In epididymal efferent ducts, the staining was restricted to the apical brush border of nonciliated cells in adult dog. These findings are in agreement with those reported for rodents, in which AQP9 has been identified in the apical membrane of nonciliated cells of the efferent ducts (Fisher et al., 1998; Pastor-Soler et al., 2001; Badran and Hermo, 2002).

Although AQP1 plays the major role in the fluid resorption of the seminiferous tubules and in the efferent ducts (Clulow et al., 1994), it is possible that the presence of AQP9 in the efferent ducts takes part in resorption. So AQP9 perhaps might compensate, at least partially, for the loss of AQP1 from those tissues in the AQP1 knockout mice aiming to preserve their fertility (Da Silva et al., 2006). Furthermore, AQP9, to facilitate the rapid movement of water across the epithelia, could also be involved in other functions, such as the passage of glycerol, which has been proposed as a source of metabolic substrate for sperm (Cooper and Brooks, 1981; Da Silva et al., 2006).

In the testis, the interstitial cells have been demonstrated to express AQP9 (Elkjaer et al., 2000; Badran and Hermo, 2002). However, in the present study, as shown for human testis by Tsukaguchi et al. (1999), AQP9 was not detected in dog testis. On the other hand, AQP9 is an abundant apical membrane protein in all regions of the dog epididymis. Its reactivity was less intense in the caput epididymidis than in the corpus and cauda epididymidis, showing a pattern similar to that described for rats (Pastor-Soler et al., 2001) and humans (Tsukaguchi et al., 1999). In these tissues, AQP9 appears to be a constitutive epithelial membrane protein that may be responsible for apical membrane permeability of water and solutes (Pastor-Soler et al., 2001).

AQP9 was abundantly expressed in the dog vas deferens at the apical membrane of principal cells along the proximal region of this duct. In the rat, AQP9 staining was also found throughout the entirety of the vas deferens while intracellular or basolateral staining was absent (Pastor-Soler et al., 2001). In addition to AQP9, both AQP1 and AQP2 are also present in the rat vas deferens, suggesting that the composition of the luminal compartment, in which spermatozoa terminate their maturation and are stored (Robaire and Hermo, 1988), involves a complex regulation of transepithelial water and solute transport (Pastor-Soler et al., 2001; Da Silva et al., 2006).

For some years, the vas deferens was considered to be simply a tubular organ whereby sperm exit the epididymis at the time of ejaculation. Although, presently there are a large number of investigations concerning to the structure and functions of the vas deferens epithelium cells, which regulate the vas deferens role on the sperm emission and storage throughout its luminal compartment (Robaire and Hermo, 1988; Hermo et al., 1994).

Moreover, the vas deferens presents regional differences in its morphology and functions, as well as in the tissular distribution and cellular-specific location of the three AQPs (AQP1, AQP2, AQP9) cited, in accordance to Stevens et al. (2000). Nevertheless, the mechanism for transepithelial fluid that occurs in the vas deferens still remains to be elucidated. Perhaps, the vas deferens must play a role to provide in its microenvironment the functional conditions necessary for continuous spermatozoa the maturation, as well viability and protection of sperm during their passage and storage into the duct, as proposed by Hinton et al. (1996).

Our data also showed that AQP9 distribution along the male reproductive tract in dog is very similar to that verified in humans, allowing one to conclude that the dog apparently could be a good model for comparative and experimental reproductive biology studies, targeting the human andrology. In conclusion, here we described by immunohistochemistry and Western blotting that AQP9 is abundantly expressed along the male reproductive tract of the dog, being an important apical pathway for transmembrane water and neutral solutes flow.

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