UNIVERSIDADE ESTADUAL DE CAMPINAS

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"EXPRESSÃO E LOCALIZAÇÃO DE AQUAPORINAS NA VIA ESPERMÁTICA DE CÃO ADULTO, *Canis familiaris*"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Anatomia.

Orientador: Prof. Dr. Antonio Marcos Orsi

Co-Orientador: Prof. Dr. Sérgio Luis Felisbino

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

D712e

Domeniconi, Raquel Fantin
Expressão e localização de aquaporinas na via espermática de cão adulto, *Canis familiaris* / Raquel Fantin Domeniconi. – Campinas, SP: [s.n.], 2007.

Orientador: Antonio Marcos Orsi.
Co-orientador: Sérgio Luis Felisbino.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Condutos eferentes. 2. Epidídimo. 3. Vasos deferentes. 4. Aquaporinas. 5. Transporte biológico. I. Orsi, Antonio Marcos. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(rcdt/ib)

Título em inglês: Aquaporins expression and localization in the adult dog testis excurrent ducts *(Canis familiaris)*.
Palavras-chave em inglês: Efferent ducts; Epididymis; Vas deferens; Aquaporins; Biological transport.
Área de concentração: Anatomia.
Titulação: Doutora em Biologia Celular e Estrutural.
Banca examinadora: Antonio Marcos Orsi, Maria Júlia Marques, Paulo Pinto Joazeiro, Maíra Aparecida Stefanini, Patrícia Fernanda Felipe Pinheiro.
Data da defesa: 01/10/2007.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 01 de outubro de 2007.

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Dedico,

Ao meu Pai,

Pela ternura e companheirismo; incentivo e apoio em todos os momentos; por compreender minhas dificuldades e alegrar-se com as minhas conquistas.

À minha Mãe,

Pelo amor paciente e incondicional que sempre me dedicou; pelas doces lembranças e exemplos de amor e de vida.

AGRADECIMENTOS

Especialmente aos orientadores:

Ao Prof. Dr. **Antonio Marcos Orsi**, pela generosidade com que conduziu minha orientação, pela amizade e por ter me mostrado o caminho para o amadurecimento profissional.

Ao Prof. Dr. **Sérgio Luis Felisbino**, pela amizade, confiança e pelos momentos dedicados à orientação e conclusão desse trabalho.

Àqueles que me guiaram e transmitiram seus conhecimentos e experiências indispensáveis para o meu crescimento profissional e pessoal, o meu profundo respeito e sincero agradecimento.

Aos Professores da Banca Examinadora Profa. Dra. **Maria Júlia Marques**, Prof. Dr. **Paulo Pinto Joazeiro**, Profa. Dra. **Maíra Aparecida Stefanini** e Profa. Dra. **Patrícia Fernanda Felipe Pinheiro**, pelas valiosas sugestões e correções.

Aos **Professores e funcionários do Departamento de Anatomia** do Instituto de Biociências UNESP, Campus de Botucatu, pelo agradável convívio, amizade e por me disponibilizarem os laboratórios.

Aos Professores e funcionários do Programa de Pós-graduação em Biologia Celular, IB, UNICAMP**,** em especial a Profa. Dra. **Shirlei M. Recco Pimentel**, Profa. Dra. **Laurecir Gomes** e **Líliam Alves Senne Panagio** pela valiosa contribuição para a realização desse trabalho.

Aos Amigos, **Luis Antonio Justulin Jr, Daniela Cristina Ferreira**, **Sérgio Pereira, Célia Cristina Leme Beu, Robson Francisco Carvalho, Wellerson Rodrigo Scarano**, pela colaboração em diversas etapas desse trabalho e, sobretudo, pela amizade.

À **Patrícia Mazzolin,** amiga de todos os momentos, pelo respeito e apoio.

Ao companheiro **Marcos Augustinho**, pelo carinho, incentivo e por ter caminhado comigo.

À Fundação de Apoio a Pesquisa no Estado de São Paulo, **FAPESP**, pelo apoio financeiro.

Ao Prof. Dr. **Stélio Pacca Loureiro Luna**, por permitir a coleta do material utilizado nesse trabalho.

Ao senhor **James Welsh**, pela gentileza de ter corrigido a versão em inglês dos artigos que compõem essa tese.

Aos demais **Amigos** e **Colegas** do Curso de Pós-Graduação, e todos aqueles que de forma direta ou indireta, colaboraram para a realização desse trabalho.

SUMÁRIO

RESUMO

Estudos recentes têm identificado família de proteínas denominadas aquaporinas (AQP), relacionadas à alta permeabilidade de água em várias membranas biológicas. As AQP1, AQP2, AQP7, AQP8 e AQP9 são as principais AQPs identificadas no sistema genital masculino, sendo a sua localização espécie-específica e região-específica. Em vista da importância do fluido luminal na via espermática para a integridade morfofuncional dos espermatozóides, bem como dos componentes que os constituem, tais como a água e proteínas, é importante estudar a distribuição das AQPs ao longo da via espermática. Assim, este trabalho teve como objetivos principais estudar no cão as AQP1, AQP2, AQP7, AQP8 e AQP9, visando identificá-las e localizá-las, através de imuno-histoquímica e "Western blotting" na via espermática. No cão, a AQP1 foi notada na rede testicular, ductos eferentes e em vasos, sugerindo sua importância na rápida absorção de fluido testicular. Pela primeira vez a AQP2 foi detectada na rede testicular, ductos eferentes e epidídimo, e a AQP7 no epitélio epididimário e ducto deferente em mamíferos. Porém, o papel funcional dessas AQPs no sistema genital masculino do cão permanece desconhecido. A AQP8 não foi detectada ao longo dos ductos extratesticulares do cão. A AQP9 foi abundantemente expressada ao longo da via espermática do cão, que representa um importante caminho apical para o fluxo transmembrana de água e solutos. Portanto, os resultados confirmam o padrão de expressão espécie-específica e região-específica das AQPs, sugerindo variações de atividades de absorção de fluidos e solutos ao longo da via espermática. O conhecimento destas variações torna-se relevante para estudos clínicos de infertilidade, bem como para tecnologias de reprodução assistida.

ABSTRACT

Recent studies have identified proteins called aquaporins (AQP) related to the fast water permeability in some biological membranes. AQPs are small, intrinsic membrane proteins that are present in many cell types involved in fluid transport. AQP1, AQP2, AQP7, AQP8 and AQP9 had been the main AQPs identified in the male reproductive tract, being their localization species-specific and region-specific. In view of the importance of the luminal fluid to sperm maturation and integrity of the spermatozoa, it is important to study the distribution of the AQPs throughout the spermatic way. Thus, the aim of this study was to examine the expression of AQP1, AQP2, AQP7, AQP8 e AQP9 in epithelial cells in the adult dog efferent ducts, epididymis and vas deferens, using immunohistochemistry and Western blotting methods to characterize the aquaporins in male reproductive tract. In dog, AQP1 was noted in rete testis, efferent ducts and in vessels in intertubular space, suggesting that AQP1 is important for rapid absorption of testicular fluid. For the first time the AQP2 was detected in rete testis, efferent ducts and epididymis and the AQP7 was expressed in the epithelium epididymidis and in vas deferens in mammals. But its functional role in the male dog reproductive tract, remain unknown. No specific staining for AQP8 was detected in epithelial cells of excurrent ducts in dog testis. AQP9 was abundantly expressed in dog male reproductive tract, in which it is an important apical pathway for transmembrane flow of water and neutral solutes. Thus the results confirm that the AQPs are species-specific and region-specific, suggesting activity variations related with the fluid and solute absorption throughout male excurrent ducts. Investigations of AQP biology could be relevant to clinical studies of the male reproductive tract, as well as to technologies for assisted procreation.

INTRODUÇÃO GERAL

Via espermática:

A composição do fluido seminal dos ductos extratesticulares é progressivamente modificada durante a sua passagem ao longo dos ductos eferentes, epididimário e deferente (Robaire e Viger, 1995). A secreção e absorção do fluido são processos vitais na fisiologia do sistema genital masculino, e alterações na homeostase deste fluido estão relacionadas à infertilidade (Russell et al., 1989). A reabsorção de fluido pelo epitélio epididimário implica em aumento da concentração espermática intraluminal, em direção à cauda do epidídimo (Turner, 1991; Robaire e Viger, 1995).

Turner (1984) demonstrou que o ducto epididimário tem grande função reabsortiva com relação às proteínas e íons salinos, principalmente sódio e potássio. Essa função constitui importante processo para o ducto epididimário controlar o seu ambiente luminal. A reabsorção de fluido constitui mecanismo especialmente ativo no segmento inicial epididimário, sendo que aproximadamente 90% do fluido, que parte da rede testicular, é reabsorvido quando alcança a cabeça do epidídimo de rato, e 62% do fluido remanescente da cabeça epididimária é reabsorvido ao alcançar a cauda do epidídimo.

A reabsorção de fluido pelo epitélio epididimário implica em aumento da concentração espermática intraluminal (Turner, 1991; Robaire e Viger, 1995). Durante o trânsito epididimário, os espermatozóides são banhados em vários ambientes bioquímicos sucessivos, específicos a cada região, nos quais ocorrem interações seqüenciais entre a membrana dos espermatozóides e o fluido luminal. Isto conduz à aquisição de capacidade potencial dos espermatozóides para fertilizar os ovócitos (Fouchécourt et al., 2000).

A análise de polipeptídios, em amostras teciduais provenientes do testículo, rede testicular, ductos eferentes e epidídimo de suíno, demonstrou que a maior parte das proteínas encontradas no lúmen epididimário não estava presente no testículo, na rede testicular ou nos ductos eferentes. A análise eletroforética desses segmentos da via espermática mostrou a presença de 187 bandas representando 125 polipeptídios, todos identificados como produtos de secreção do epidídimo. Além disso, observou-se a presença

de 21 proteínas que não eram específicas a nenhuma das partes da via espermática, pois foram detectadas desde o testículo até a porção distal do epidídimo (Syntin et al., 1996).

De acordo com a literatura científica especializada, a importância das proteínas na via espermática não se restringe somente à sua ação direta sobre os espermatozóides, mas também pode estar associada à manutenção e sobrevivência das células constituintes do revestimento epitelial, bem como à formação do micro-ambiente luminal adequado, no qual os espermatozóides ficam imersos (Fouchécourt et al., 2000; Syntin et al., 1996). Assim, nos últimos tempos uma série de substâncias, dentre elas as proteínas, têm sido identificadas e sua atividade é associada à formação do fluido luminal epididimário e também ao fluido presente em outras partes da via espermática.

Aquaporinas:

A água pode ser permeada lentamente por difusão simples através da bicamada lipídica. No entanto, observações de vários "sistemas biológicos experimentais", utilizando membranas de alta permeabilidade à água, sugeriram que a difusão não era a única via para a passagem de água através da membrana plasmática (Calamita, 2005). Várias hipóteses foram sugeridas até a descoberta de uma proteína de transporte específico para a água, a aquaporina-1 (Preston e Agre, 1991; Preston et al., 1992).

AQPs constituem uma família de proteínas intrínsecas de membrana, presentes em muitos tipos celulares envolvidos em transporte de fluidos (Verkman e Mitra, 2000; Agre, 2004). Em células de mamíferos foram identificadas treze isoformas de AQPs, AQP0-12, sendo cada isoforma codificada por um gene distinto (Da Silva et al, 2006). As AQPs são compostas por uma cadeia simples de aproximadamente 270 aminoácidos, que atravessam a membrana seis vezes. Suas extremidades amino e carboxi-terminal localizam-se no citoplasma, e há três alças extracelulares (A, C e E) e duas alças intracelulares (B e D. A representação esquemática da estrutura geral proposta para as AQPs é apresentada na figura A, segundo Agre et al. (1995).

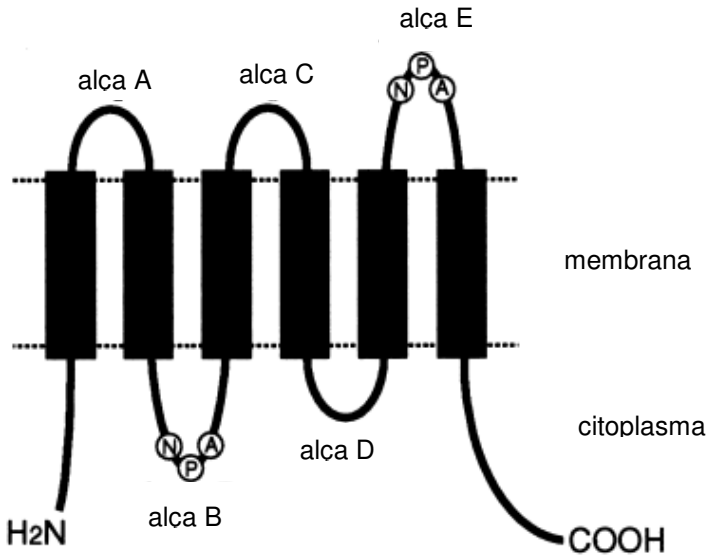

Figura A – Representação esquemática de uma cadeia simples de AQP.

Em regiões epiteliais onde há canais de água mediados por AQP a velocidade de permeação de água é de 10 a 100 vezes maior do que a observada na difusão simples (Agre, 2004). O transporte de água através das membranas, pelos canais de AQPs, não é um processo ativo, pois depende da presença de gradiente osmótico através da membrana. Em vista da relevância das AQPs para o transporte de fluidos em outros tecidos, é provável que essas proteínas desempenhem importante papel nos processos de reabsorção e secreção de fluidos presentes ao longo do sistema genital masculino (Cho et al., 2003).

Poucos estudos foram feitos sobre a presença de AQPs no sistema genital masculino. Atualmente, foram detectadas as aquaporinas AQP1, AQP2, AQP7, AQP8 e AQP9 nesse sistema, principalmente no homem e em roedores laboratoriais (Brown et al., 1993; Nelson et al., 1998; Elkjaer et al., 2000; Stevens et al., 2000; Badran e Hermo, 2002).

AQP1 também chamada CHIP-28 (“channel integral protein, 28 kDa”) foi a primeira das AQPs a ser identificada, tendo sido purificada a partir de células vermelhas do sangue (Preston e Agre, 1991). A AQP1 foi localizada na borda em escova e membrana basolateral de túbulos contorcidos proximais e alça de Henle descendente de rins de ratos e de humanos, além de estar presente nos túbulos retos descendentes. Essas localizações sugerem que a AQP1 funciona como via para a transferência de grandes quantidades de

água do lúmen para o interstício tubular e deste para o compartimento vascular renal. Outras estruturas nas quais a AQP1 foi detectada foram os plexos coróides encefálicos, colangiócitos e endotélio capilar de vários órgãos (vide Matsuzaki et al., 2002 como referência geral).

No sistema genital masculino de ratos a AQP1 foi abundante nos ductos eferentes, estando localizada na borda de microvilos e na membrana basolateral das células não-ciliadas. Reação positiva para AQP1 também foi mostrada na ampola do ducto deferente, na glândula seminal e na próstata de ratos (Brown et al., 1993). Entretanto, segundo Badran e Hermo (2002) a AQP1, nos ductos eferentes, foi localizada em células não-ciliadas e também nas células ciliadas. Porém não estava presente nos testículos ou no epitélio epididimário, embora sua expressão tenha sido mostrada nas células endoteliais dos canais vasculares dos ductos eferentes e do epidídimo. Talvez, a expressão de AQP1 no espaço intertubular esteja relacionada à remoção de água e manutenção do equilíbrio osmótico nesse tecido.

AQP2 também pertence à família de proteínas de canais de água e é de fundamental importância na regulação da excreção renal. No sistema genital masculino de camundongo, sua presença foi demonstrada nas porções apical ou subapical das células principais do ducto deferente e regiões centrais dos túbulos seminíferos, mas não foi detectada em espermatozóides epididimários maduros ou no próprio epidídimo (Nelson et al., 1998). A AQP2 no ducto deferente deve estar envolvida com a modificação do conteúdo luminal, promovendo a concentração de espermatozóides por extração de fluido do lúmen (Matsuzaki et al., 2002). A ação da AQP2 no ducto deferente ocorre de maneira hormônio-sensitiva (Nelson et al., 1998), embora Stevens et al. (2000) tenham afirmado que ela, ao contrário da AQP2 renal, não é regulada pela vasopressina.

A **AQP7** foi abundantemente expressa no testículo e parece estar envolvida no transporte de uréia, glicerol e água (Ishibashi et al., 1997), tendo sido localizada em espermátides e espermatozóides testiculares (Suzuki-Toyota et al., 1999). O glicerol tem sido usado, quase universalmente, como crioprotetor efetivo para os espermatozóides, a permeabilidade dos espermatozóides ao glicerol deve ser um importante determinante do

índice de congelamento desses gametas. Contudo, ainda não foi esclarecido se a atividade da AQP7 é crítica para a criopreservação dos espermatozóides (Ishibashi et al., 1997).

A **AQP8** foi identificada no pâncreas e testículo, bem como nos colos intestinais e em glândulas salivares de rato. A expressão de AQP8 foi demonstrada em todos os estágios da espermatogênese. As propriedades de transporte da AQP8 parecem ser espécie-dependentes, uma vez que ela mostrou permeabilidade à uréia em camundongo, mas não em rato, enquanto que em humanos foi relatada como canal seletivo à água, mas não à uréia e/ou ao glicerol. A AQP8 também foi detectada nas células basais do epidídimo de rato, no entanto, o papel exato nestas células ainda não foi definido, especulando-se que ela esteja relacionada à maturação, diferenciação ou regulação do volume celular (Elkjaer et al., 2001). Nos testículos de rato foi expressa exclusivamente pelas células de Sertoli (Badran e Hermo, 2002). Provavelmente, AQP7 e AQP8 sejam importantes no processo de redução do volume celular, pelo qual, passam as espermátides ao se diferenciarem em espermatozóides durante a espermiogênese (Calamita et al., 2001).

AQP9 tem alta permeabilidade à água e a outros solutos tais como: carbamidas, polióis, purinas e pirimidinas; por isso foi incluída no subgrupo das aquagliceroporinas, ou seja, AQPs permeáveis à água, uréia e glicerol. Inicialmente, a AQP9 foi localizada em leucócitos humanos, posteriormente o RNAm da AQP9 foi encontrado no fígado, testículos, cérebro e pulmões de rato. A localização celular nestes tecidos foi feita através de análise imuno-histoquímica e hibridização *in situ* (Elkjaer et al., 2000).

A AQP9 foi detectada nos hepatócitos, superfície interna dos túbulos seminíferos (Elkjaer et al., 2000), nas células de Leydig, nos microvilos das células não-ciliadas dos ductos eferentes e nos microvilos das células principais, em todas as regiões epididimárias. Nas células epididimárias, a AQP9 mostrou reações de maior intensidade no segmento inicial e na cauda do epidídimo, além de estar presente nas células claras (Badran e Hermo, 2002). Também foi demonstrada a presença desta AQP nos estereocílios do epitélio epididimário, nos quais provavelmente estaria envolvida no transporte de água (e solutos) nestas células. As AQP1 e AQP9, presentes nos ductos eferentes e epidídimo, respectivamente, possivelmente devem estar, possivelmente, envolvidas no processo de

reabsorção de fluidos para promover a concentração de esperma (Brown et al., 1993; Elkjaer et al., 2000).

Os trabalhos realizados no sistema genital masculino mostram que AQPs são encontradas na via espermática intra e extratesticular. A morfologia da via espermática intratesticular (Beu et al., 2003) e de sua seqüência extratesticular, em roedores laboratoriais, tem sido objetivo de estudos em equipe, ligada ao Programa de Biologia Celular e Estrutural da Universidade Estadual de Campinas, sendo que a via extratesticular foi estudada, recentemente, no hamster dourado (Beu et al., 2007) e no gerbilo da Mongólia (Domeniconi et al., 2006; 2007).

Modelo experimental:

As AQPs estão presentes no epitélio tubular da via espermática, sendo a elas sugerida atuação no processo de formação do fluido luminal, cujo mecanismo ainda não está completamente elucidado. Logo, os relatos sobre as AQPs mencionam que a presença delas deve ser região-específica nas partes da via espermática, as quais têm sido demonstradas nos testículos, nos ductos eferentes e nos ductos epididimários e deferentes, conforme já foi referido. Tendo em vista a ênfase dada as AQPs na formação do fluido luminal da via espermática, justifica-se o interesse em identificá-las ao longo desta via no cão, um mamífero de interesse veterinário, e também um notório modelo experimental em pesquisas biomédicas.

Logo, os cães do ponto de vista biomédico constituem uma espécie-chave porque estão sujeitos a muitas das doenças humanas adquiridas ou induzidas experimentalmente. Ademais, as proteínas epididimárias do cão apresentam grande similaridade àquelas presentes no epidídimo humano, além de terem larga distribuição tecidual e relativa abundância (Kirchhoff, 2002). Além disso, estudos prévios (Anderson et al., 2001), mostraram que o cão é um excelente modelo para dar suporte a estudos reprodutivos comparativos, realizados em várias espécies carnívoras, principalmente no que diz respeito ao desenvolvimento de protocolos de criopreservação de espermatozóides e de tecidos do sistema genital masculino de espécies silvestres raras (Anderson et al., 2001).

OBJETIVOS

✓ Caracterizar a expressão das AQP1, AQP2, AQP7, AQP8 e AQP9, por "Western blotting", nas regiões epididimárias do segmento inicial, cabeça, corpo e cauda, e na região proximal do ducto deferente do cão adulto, sem raça definida (s.r.d.), espécie em que as AQPs ainda não foram estudadas.

✓ Localizar a distribuição tecidual das AQP1, AQP2, AQP7, AQP8 e AQP9 através de reações imuno-histoquímicas no testículo (rede testicular e ductos eferentes), no ducto epididimário (segmento inicial, cabeça, corpo e cauda) e na parte proximal ducto deferente, do cão adulto s.r.d., espécie em que as AQPs ainda não foram estudadas.

1° Artigo (Aceito para Publicação): The Anatomical Record

"Aquaporin 9 (AQP9) localization in the adult dog testis excurrent ducts by immunohistochemistry"

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Running title: AQP9 in dog male reproductive tract

Grant Sponsor FAPESP; Grant numbers: 04/05578-1 and 04/05579-8.

Abstract

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 epididymidis. 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.

Key words: efferent ducts, epididymis, vas deferens, aquaporin9, immunohistochemistry, water channel, epithelial transport

Introduction

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 (AQP).

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, the AQP9 represents an important apical pathway for transmembrane movements of water and others solutes − such as carbamides, 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 m RNA has been found in liver, testes and brain. The cellular localization in these tissues was shown by immunostaining and 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 staining 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).

Besides to be 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 which 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 et al. (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 PBS for 24 h. Fixed samples were washed in PBS for 24 h, dehydrated in graded ethanol series, clarified in xylene, and embedded in Paraplast™ (Sigma, St. Louis, MO, USA). Paraplast sections (5µm thick) were stained with Hematoxylin-Eosin (H&E) for general morphological view or pretreated with a citric acid monohydrate antigen retrieval method, and afterwards immunostained with rabbit polyclonal antibody against AQP9 (Chemicon Temecula, CA, USA) at 1:100 dilution. The secondary biotinylated antibody goat anti-rabbit IgG (Santa Cruz Biotechnology, CA, USA) 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 and the proximal part of the vas deferens were homogenized in 50mM Tris buffer (pH 7.5) plus 0.25% Triton-X 100 by Polytron for 30 s at 4º C, centrifuged, and the protein fraction was extracted on

supernatant and quantified as per Bradford (1976). A protein sample (70μg) was loaded into 8% SDS-PAGE under reducing conditions. After electrophoresis, the proteins were transblotted onto a nitrocellulose membrane (Sigma). The blot was blocked with 10% non-fat dry milk in TBST (10mM Tris-HCL, pH 7.5; 150mM NaCl; 0.1% Tween-20) for 1 h. The blot was then incubated overnight at 4ºC with 3% BSA containing 1-1000 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 h at room temperature with peroxidase-conjugated goat anti-rabbit-IgG antibody. The blot was again washed for 20min 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 showed) 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 was observed a strong labeling in the long apical stereocilia from principal cells (Fig. 4) and little intracellular staining was detectable. 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 about 30kDa (Fig. 9), confirming the antibody specificity. The antibody also detected other higher bands that represent differentially glycosilated AQP9 forms confirming by Pastor-Soler et al. (2002)

findings. 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 non-ciliated 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, in order 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).

During 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 yet. Perhaps, the own 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 referred 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 to conclude that the dog apparent to be a good model for comparative and experimental reproductive biology studies, targeting the human andrology.

In conclusion, here we described 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.

Acknowledgements

This work was funded by FAPESP (Sao Paulo State Research Foundation; grants 04/05578-1, to AMO and 04/05579-8, to RFD). This paper is part of the PhD Thesis presented by RFD to the State University of Campinas – UNICAMP, Brazil. Special thanks are due to Dr. Stélio Pacca Loureiro Luna Associated Professor of Veterinary School of UNESP at Botucatu. Also thank are indebted to Dr. Patrícia FF Pinheiro and to Dr. Sérgio Pereira from our Department for collaboration and helpful discussions.

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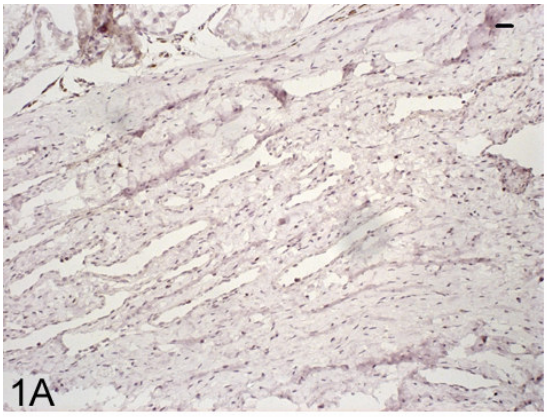
Figure Legends

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

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

Figure 9. Western blot analysis of 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 a 70µg 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 about 30 kDa.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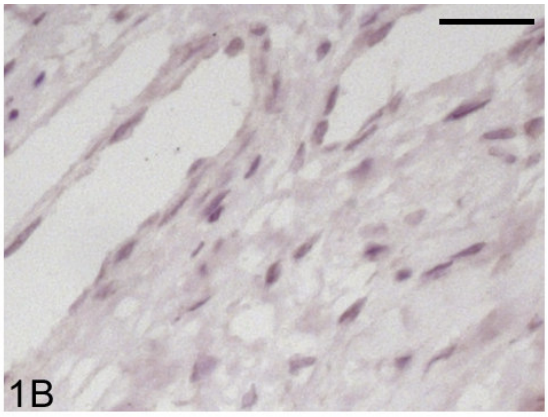
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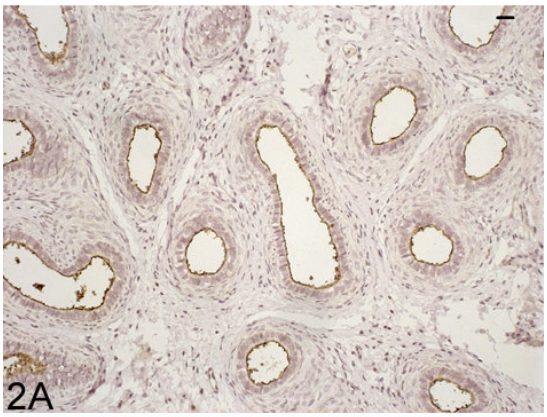
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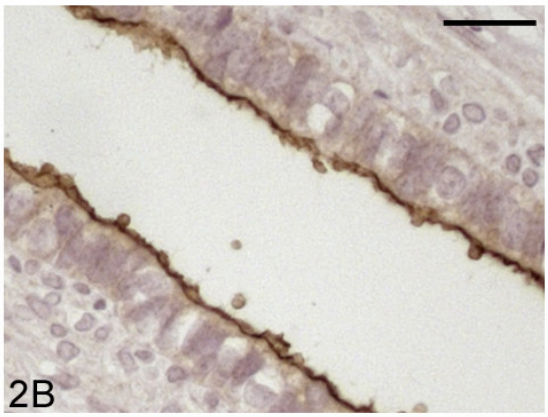
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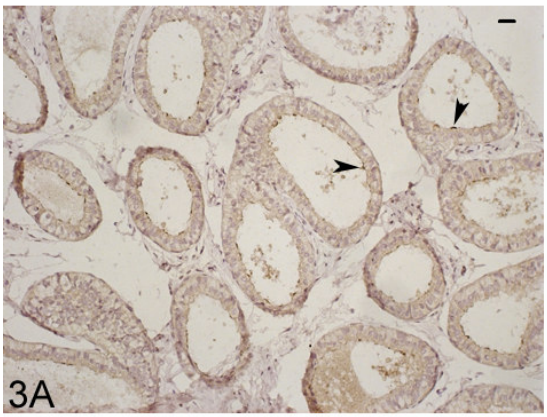
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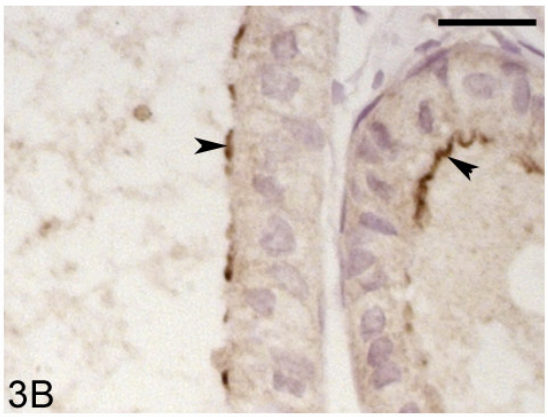
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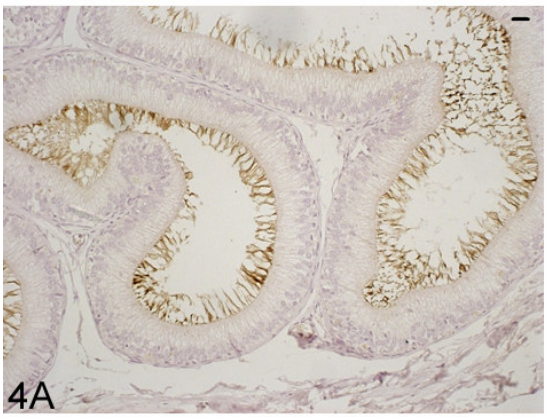
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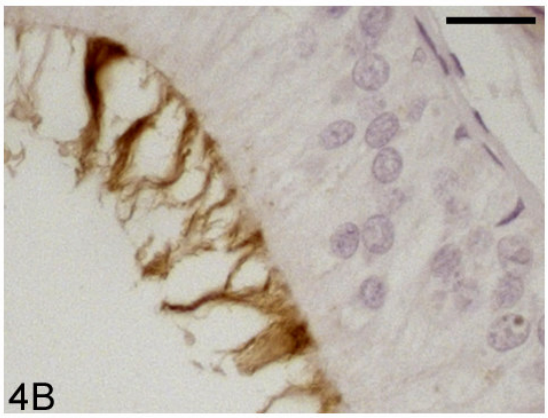
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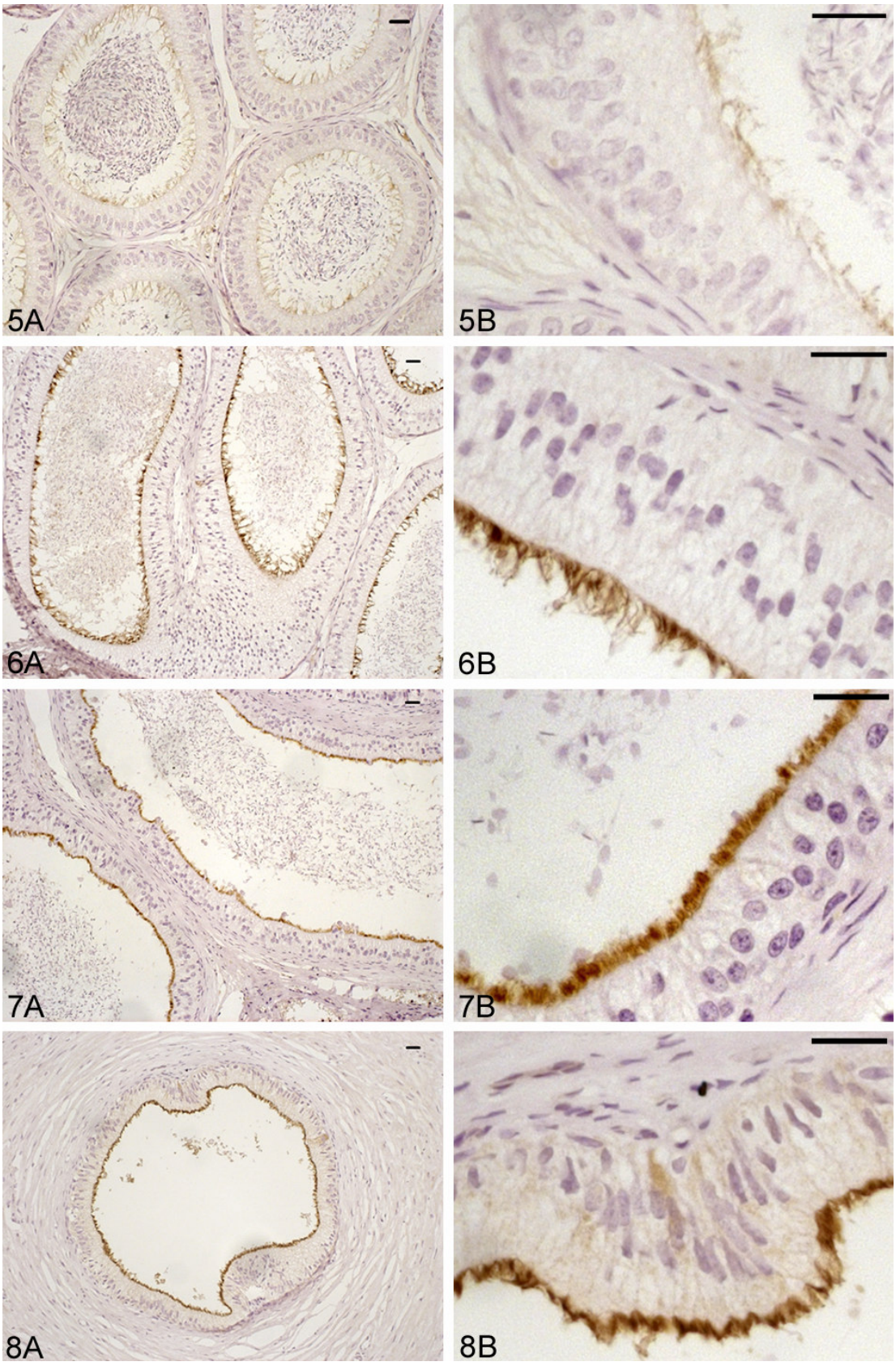
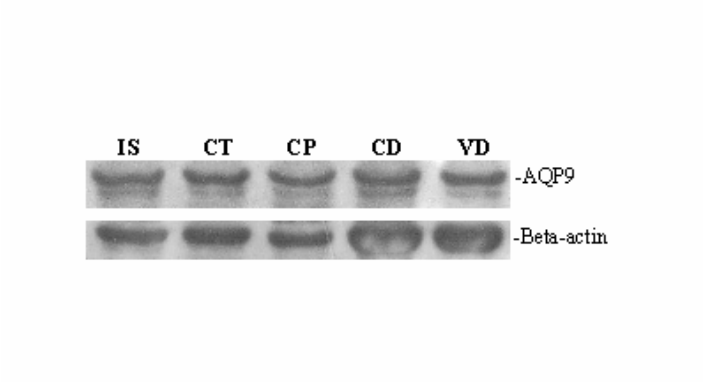

5A
5B
6A
6B
7A
7B
8A
8B

Fig. 9



2° Artigo (Submetido): Cell & Tissue Research

"Immunolocalization of aquaporins 1, 2, 7 and 8 in rete testis, efferent ducts, epididymis and vas deferens of adult dog"

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Running title: Aquaporins 1, 2, 7 and 8 in dog male reproductive tract.

Grant Sponsor FAPESP; Grant numbers: 04/05578-1 and 04/05579-8.

Key words: excurrent ducts, aquaporins, immunohistochemistry, water channel, epithelial transport

Abstract

The transepithelial water movement into the male reproductive tract is an essential process for normal male fertility. Protein water channels, referred to as AQPs are involved in increasing the osmotic permeability of membranes. This study aimed to examine the expression of AQP1, 2, 7 and 8 in epithelial cells in 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 epididymidis and vas deferens were obtained from eight adult mongrel dogs. Immunohistochemistry and Western blotting procedures were used to show localization and distribution of AQPs. AQP1 was noted in rete testis, efferent ducts and in vessels in intertubular space, suggesting that AQP1 is an important channel for rapid absorption of a great amount of testicular fluid that occurs characteristically in efferent ducts. In dog, AQP2 expression was found in rete testis, efferent ducts and epididymis, while AQP7 was expressed in epithelium of proximal regions epididymidis and in vas deferens. This was the first time that AQP2 and AQP7 were observed in these regions of mammals' excurrent ducts, but its functional role in the male dog reproductive tract, remain unknown. No specific staining for AQP8 was detected in epithelial cells of excurrent ducts in dog testis. Investigations of AQP biology could be relevant to clinical studies of the male reproductive tract, as well as to technologies for assisted procreation.

Introduction

In the testis, water is secreted into the lumen of seminiferous tubules by Sertoli cells in order to create a propitious environment for spermatogenesis, and to transport the spermatozoa forward to the efferent ducts (Hinton and Setchell, 1993). Throughout the efferent ducts a great part of testicular luminal fluid is reabsorbed (Clulow et al., 1998). This process appears to concentrate sperm as an initial step in promoting fertility and the motility of spermatozoa during their passage along the epididymal lumen (Hess et al., 2002). Water is important as a vehicle for sperm passage throughout the epididymis (Setchell et al., 1994).

The vas deferens conducts sperm from the epididymis to the urethra. The epithelial cells lining the lumen of vas deferens are responsible for maintaining the environment in which sperm continue their maturation and are stored. The composition of the luminal fluid in which the spermatozoa terminate their maturation and are stored involves a complex regulation of transepithelial water and solute transport (Da Silva et al., 2006a).

Thus transepithelial water movement into the male reproductive tract is a process essential to normal male fertility. Protein water channels, referred to as aquaporins (AQPs), are involved in increasing the osmotic permeability of membranes. AQPs are small, intrinsic membrane proteins that are expressed in a wide variety of cells and tissues involved in fluid transport (Verkman and Mitra, 2000). 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).

AQP1 was first identified from human erythrocyte membranes as an integral membrane protein of 28kDa (Denker et al., 1988) and its DNA was isolated from a human bone-marrow cDNA library (Preston and Agre, 1991). AQP1 in rat male reproductive tract was localized in apical plasma membrane of non-ciliated cells in the efferent ducts as well as in the basolateral membrane of epithelial cells in ampulla of the vas deferens, seminal vesicle and prostate gland (Brown et al., 1993). In rat epididymis, AQP1 was expressed

only in the myoid cells and endothelial cells of vascular channels located in the intertubular spaces (Badran and Hermo, 2002). AQP1 may play a role in the process of water secretion in the seminal and prostatic fluid (Cho et al., 2003), and would serve to remove water from intertubular space and thus maintain water equilibrium in epididymis (Badran and Hermo, 2002).

AQP2 is a member of a family of water channel proteins and is important in regulation of renal water excretion (Nielsen et al., 1995). Only in the male reproductive tract of the mouse was AQP2 expressed in principal cells of the vas deferens and seminiferous tubules, but it was absent in the epididymis. The functional significance of AQP2 in mouse testis remains unclear, as does the reason that studies of rat testis did not reveal AQP2. In vas deferens, the AQP2 may be involved in modifying the luminal fluid content (Nelson et al., 1998). Thus AQP2 would promote the sperm concentration through the luminal fluid extraction into the vas deferens (Matsuzaki et al., 2002).

AQP7 has been found to be permeable to small neutral solutes, such as glycerol and urea in addition to water and was abundantly expressed in the testis (Ishibashi et al., 1997). In the male reproductive tract of adult rat, AQP7 was observed in spermatids and testicular spermatozoa (Suzuki-Toyota et al., 1999). Perhaps AQP7 and AQP8 are responsible for most of the cell volume reduction by which spermatids differentiate in spermatozoa during spermatogenesis (Calamita et al., 2001a).

AQP8 was identified in the pancreas, intestine, salivary glands and testis of the rat (Elkjaer et al, 2001). In the male reproductive tract, AQP8 has been suggested as being expressed by germ cells located next to the luminal compartment of the seminiferous tubules (Calamita et al., 2001b; Elkjaer et al., 2001), and also in Sertoli cells of rat testis (Badran and Hermo, 2002). In the rat epididymis, AQP8 was detected only in basal cells, but its role in these cells remains unclear (Elkjaer et al., 2001).

Therefore, the present study aimed to localize the tissue distribution of AQP1, AQP2, AQP7 and AQP8 in rete testis, efferent ducts, epididymis and proximal vas deferens of adult mongrel dogs using immunocytochemistry and Western blotting analyses. The dog

has been considered an excellent model for comparative studies of reproductive biology (Kirchhoff, 2002).

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 et al. (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 PBS for 24 h. Fixed samples were washed in PBS for 24 h, dehydrated in graded ethanol series, clarified in xylene, and embedded in Paraplast™ (Sigma, St. Louis, MO, USA). Paraplast sections (5µm thick) were stained with Hematoxylin-Eosin (H&E) for general morphological view or pretreated with a citric acid monohydrate antigen retrieval method, and afterwards immunostained with rabbit polyclonal antibody against AQP1, 2, 7 e 8 (Chemicon Temecula, CA) at 1:100 dilution. The secondary biotinylated antibody goat anti-rabbit IgG (Santa Cruz Biotechnology, CA, USA) 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 and the proximal part of the vas deferens were homogenized in 50mM Tris buffer (pH 7.5) plus 0.25% Triton-X 100 by Polytron for 30 s at 4º C, centrifuged, and the protein fraction was extracted on supernatant and quantified as per Bradford (1976). A protein sample (70µg) was loaded into 8% SDS-PAGE under reducing conditions. After electrophoresis, the proteins were transblotted onto a nitrocellulose membrane (Sigma). The blot was blocked with 10% non-fat dry milk in TBST (10mM Tris-HCL, pH 7.5; 150mM NaCl; 0.1% Tween-20) for 1 h. The blot was then incubated overnight at 4ºC with 3% BSA containing 1-1000 dilution of the AQP1, 2, 7 e 8 (Chemicon, USA) or Actin (C-11: sc-1615, Santa Cruz Biotechnology) primary antibodies. The blot membrane was then washed for 20 min three times in TBST and incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit-IgG antibody. The blot was again washed for 20min three times in TBST. Proteins were detected using the Chemiluminescent Peroxidase Substrate (Sigma).

Results

In the adult dog, the rete testis showed an intense and continuous reactivity to AQP1 (Fig. 1). In the epithelium of testicular efferent ducts a strong reaction was observed only in some cells irregularly distributed along the epithelium (Fig. 2). In other epithelial cells of the testicular efferent ducts, reactions were considered weak to moderate and were located in epithelial apex (Fig. 2). An intense reaction also was noted on the apex of the epithelial cells corresponding to the microvilli of the nonciliated cells in the efferent ducts, and a diffuse reaction was presented in lateral plasma membranes of adjacent epithelial cells (Figs. 3 and 4). No reaction was observed over the entire epithelium of any epididymal region or proximal vas deferens of adult dog. Although throughout the entire epididymis (Figs. 5 and 6), efferent ducts and vas deferens, an intense reaction was noted only in endothelial cells of vessels located in interstitial spaces.

AQP2 immunolocalization showed positive reaction in rete testis epithelium (Fig. 7) and a more diffuse reaction in apical and basal regions of the testicular efferent ducts (Fig. 8). In the epididymal efferent ducts and epididymal tubules of the proximal regions, no reaction to AQP2 was detected. In contrast, the epithelial epididymidis cells of the corpus and cauda regions showed the same pattern of reactivity in some tubules, in which was observed a weak reaction in the epithelial apex and in storage vesicles located in the basal region (Figs. 9 and 10). No reaction to AQP2 was detected in proximal vas deferens (data not shown).

AQP7 was not expressed in any cell type of the epithelium of rete testis or efferent ducts in the dog. In the epididymis, AQP7 was presented only in the apical and basal plasma membranes of the principal cells in the initial segment (Fig. 11) and caput (Fig. 12) epididymidis. The initial segment showed a more intense reaction, in relation to the epididymal caput, and the reaction also extended to lateral plasma membrane of the principal cells in this region in the dog epididymis (Fig. 11). With the immunohistochemical methods used, the epididymal tubules of dog corpus and cauda showed absence of reaction to AQP7 (Fig. 13). The dog vas deferens presented positive reaction to AQP7 in apical and basal regions of epithelial cells (Fig. 14). Hitherto, no AQP8 positive reaction was observed in epithelial cells of excurrent ducts of adult dog.

The Western blotting procedure for AQP1, AQP2, AQP7 (Fig.15) and AQP8 (data not shown) made in extracts from different portions of the dog epididymis and vas deferens detected one main band of about 30kDa. Apparently, the immunoreactivity to AQP7 was more intense in samples of initial segment and vas deferens. AQP2 was weakly detected and AQP7 showed intense reactivity in all samples. No bands were noted in immunoblots with anti-AQP8 antibody. The actin protein, used as an internal control of the reaction (Fig.15), showed an equal amount of protein loaded in each lane.

Discussion

AQPs mediate the rapid and efficient water movement across the plasma membrane of different tissues and organs (Matsuzaki et al., 2002). In the present study was noted the presence of some AQPs in the epithelial cell lining of the epididymis and vas deferens, as in the rete testis and efferent ducts of the adult dog. Thus the results on the immunolocalization of AQPs in the male dog reproductive tract confirm that the AQPs are species-specific, because variation was observed in the localization and expression of the isoforms studied when compared with other mammals. Moreover, different isoforms of AQPs were located in the same cell type or in the same membrane domain, in agreement with previous descriptions for the kidney, efferent ducts, vas deferens and epididymis. This redundancy suggests that aquaporins, in addition to facilitating the rapid movement of water across the epithelium, could also be involved in other functions (Da Silva et al., 2006a).

AQP1 was noted immunohistochemically in the epithelium of rete testis in the adult dog while AQP1 was also described in the marmoset monkey (Fisher et al., 1998). However, in contrast to the rat AQP1 was expressed solely in efferent ducts (Fisher et al., 1998). In the dog testicular efferent ducts, AQP1 reaction was diffused in the apex of epithelial cells and was intense in some cells, which had been called oval cells and were described previously in hamsters (Flickinger et al., 1978; Vicentini et al., 1990). The oval cells were described only in testicular efferent ducts in the golden hamster (Flickinger et al., 1978). This cells presented oval and cylindrical shape and the cytoplasm are filled with electron-dense content (Vicentini et al., 1990). The authors described the morphology of this cellular type; however, the oval cell role remains unclear.

In the dog epididymal efferent ducts, AQP1 was noted in lateral plasma membrane and in the apex of nonciliated cells. This pattern for AQP1 was also observed in studies realized with the rat and monkey (Fisher et al., 1998). In addition, AQP1 was not observed in epithelium of dog epididymis; however, throughout the entire epididymis and efferent ducts an intense reaction was noted in endothelial cells of vessels in the interstitial site, data apparently related to the rat (Badran and Hermo, 2002). In the tubular space, AQP1 would

serve to remove water from this site and thus maintain water equilibrium in these tissues (Badran and Hermo, 2002).

The results showed that AQP1 is involved in reabsorption of fluid from efferent ducts in the dog. The presence of AQP1 in rete testis, efferent ducts and in intertubular vessels, and their absence in epididymal epithelium of the dog, suggest that AQP1 is an important channel for rapid absorption of a great amount of testicular fluid that occurs characteristically in efferent ducts (Setchell et al., 1994). The removal of water from efferent ducts plays an important role in concentrating sperm in the initial segment of the epididymis, and thus in providing a better interaction between the sperm surface and the secretory products of its epithelial cells (Badran and Hermo, 2002). These results on AQP1 localization were confirmed by other studies addressing AQPs in the rat (Oliveira et al., 2005; Da Silva et al., 2006a). AQP1 detected by Western blotting methods in distinct regions of the epididymis and vas deferens in dogs could be related to the positive reaction viewed in the vessel cells located in interstitial space around the tubules, and probably not related to the epithelial cells.

AQP2 expression in rete testis, efferent ducts and epididymis has not previously been reported in other mammals. However, in the large white turkey the ductuli efferentes, connecting ductules and ductus epididymidis, were positive for AQP2 (Zaniboni et al., 2004). Here, we showed by the first time that AQP2 is present in the intracellular basal vesicles of the epithelial cells from dog corpus and cauda of the epididymis; however, these results obtained by immunohistochemistry were not frequent. These vesicles may play a role in AQP2 storage, which could be recycled rapidly between the plasma membrane and intracellular vesicles, as occurred in the kidney collection duct (Agre et al., 1995). Thus AQP2 expression as well as its functional role in the male reproductive tract in dogs and in mice (Nelson et al., 1998) remains unknown.

Da Silva et al. (2006b) analyzed the AQP2 expression during the first weeks of postnatal development in rat epithelial cell epididymidis. The results obtained for the epididymis showed variations of this AQP at different ages. AQP2 mRNA was described as abundant in adult epididymidis epithelial cells but the protein is not detectable, either by

immunofluorescence or by Western blotting. The AQP2 protein is transiently expressed in cauda epididymidis during postnatal development and is not expressed in adult epididymis, despite the presence of mRNA (Da Silva et al., 2006b). On the other hand, by the RT-PCR methods it was possible to observe that the coding sequence of AQP2 is present in epididymal epithelial cells and also to show that the AQP2 mRNA expression level is significantly higher in the cauda epididymidis compared to the other epididymidis regions (Da Silva et al., 2006b). This temporal variation in AQP2 might indicate an important role for this AQP during postnatal development (Da Silva et al., 2006a).

AQP2 was undetectable in the dog proximal vas deferens. In rat vas deferens only the apex of the epithelium of the ampulla contained detectable AQP2. In this region, the AQP2 was considered a structural protein of the membrane that is not regulated by vasopressin, in contrast with the kidney AQP2. This suggests that the initial segment of this vas may be less water permeable. The AQP2 function in this region of the male reproductive tract is probably related to the maintenance of an appropriate luminal environment in which spermatozoa could continue their maturation (Stevens et al., 2000). The epididymis and vas deferens are, like the kidney collecting duct, derived from the Wolffian duct during development, and the presence of AQP2 in these tissues may reflect this common embryological origin (Stevens et al., 2000).

In this study, AQP7 was not detected by immunohistochemistry in dog rete testis or efferent ducts. However, it had been identified in elongated spermatids, testicular spermatozoa and in residual bodies of mammalian testis (Suzuki-Toyota et al., 1999; Calamita et al., 2001b). Perhaps, AQP7 and AQP8 are the channel proteins responsible for major volume reduction of testicular cells, by which spermatids differentiate in spermatozoa during spermiogenesis (Calamita et al., 2001a).

In dog epididymis, AQP7 was expressed in epithelium of proximal regions and vas deferens, and in distal regions of the epididymis (corpus and cauda) there was no positive reaction to AQP7 observed. However, in extracts from different portions of the epididymis and vas deferens of the dog, positive reaction to AQP7 was detected by Western blotting methods. Perhaps this reaction could be related to dog spermatozoa as AQP7 was present in

epididymal spermatozoa of rat (Suzuki-Toyota et al., 1999). Therefore, our results showing the presence of AQP7 in epithelial cells of dog epididymis are considered original data related to the investigations realized in the male reproductive tract of mammals.

No specific staining for AQP8 was detected in epithelial cells of excurrent ducts of dog testis, in agreement with a previous thesis showing that the genes coded for AQP8 are not specifically expressed in epididymal epithelial cells of the adult rat (Da Silva et al., 2006b). Other authors also reported the absence of AQP8 in rat epididymis (Calamita et al., 2001b; Badram and Hermo, 2002). However, AQP8 expression was shown in basal cells of rat epididymis (Elkjaer et al., 2001), but this data was not observed in dog epididymis.

Investigations of the AQP biology could be relevant to clinical studies of the male reproductive tract, as well as to assisted procreation technologies (Cho et al., 2003). This supports the necessity of more studies about the functional role of the AQPs in epithelium and in and biological membranes, specifically in distinct structural compartments of animals and human.

Acknowledgements

This work was funded by FAPESP (Sao Paulo State Research Foundation; grants 04/05578-1, to AMO and 04/05579-8, to RFD). This paper is part of the PhD Thesis presented by RFD to the State University of Campinas – UNICAMP, Brazil. Special thanks are due to Dr. Stélio Pacca Loureiro Luna, Associate Professor of Veterinary School of UNESP at Botucatu. Also, we are indebted to Dr. Patrícia FF Pinheiro and to Dr. Sérgio Pereira from our Department for collaboration and helpful discussions.

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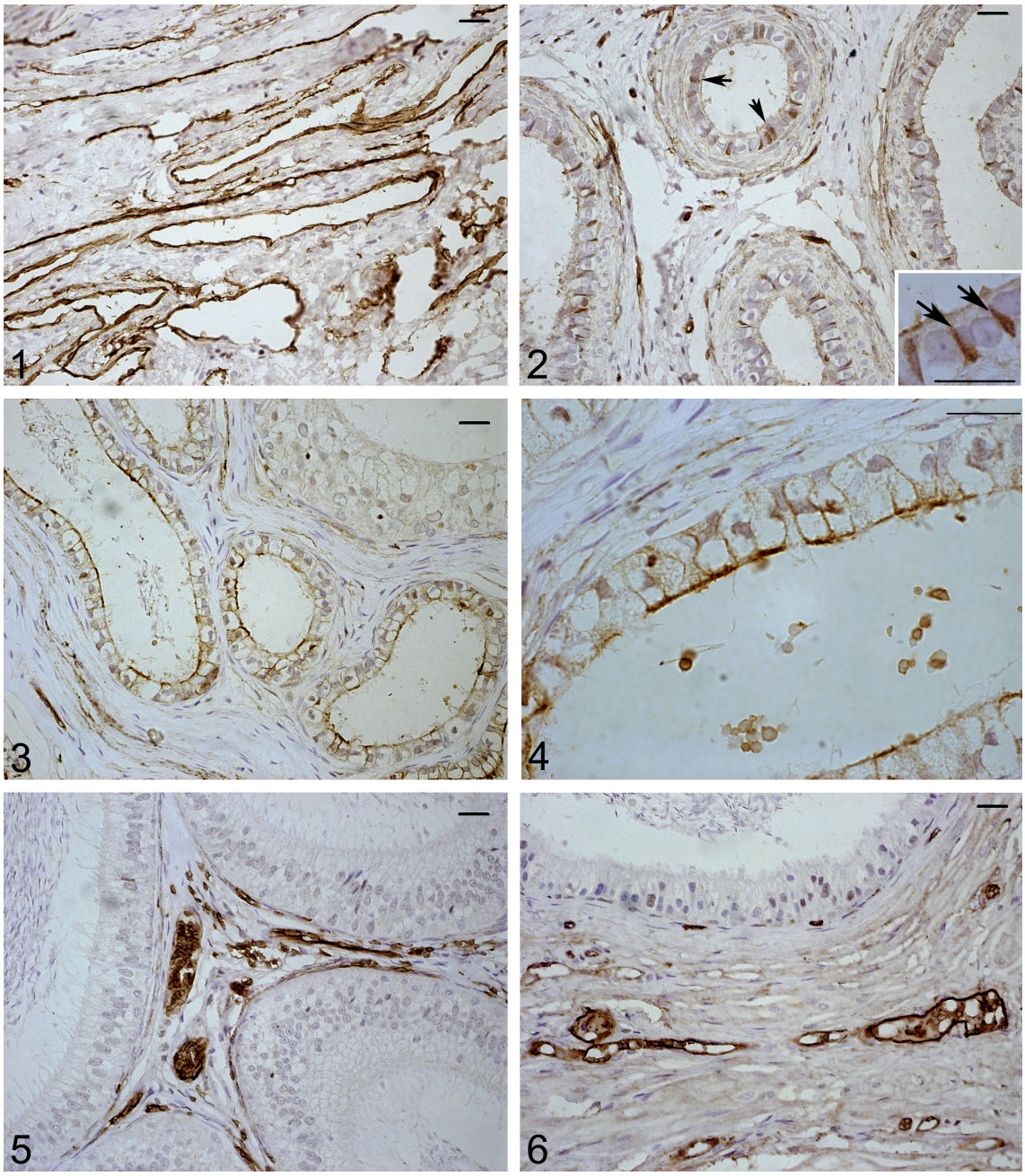
Legends

Figures 1-6. AQP1 immunolocalization in dog excurrent ducts. The reaction was observed in rete testis (1); in the oval cells of testicular efferent ducts (arrow, 2 and detail); in the apical brush border at epididymal efferent ducts (3 and 4); and in vessels throughout the epididymis, in 5 the corpus and in 6 the cauda epididymidis. Bar = 20µm.

Figures 7-10. AQP2 immunolocalization in dog excurrent ducts. The reaction was observed in rete testis (7) and in apical and basal regions of testicular efferent ducts (8); positive reactivity in apical membrane of epithelial cells and basal vesicles, in the corpus (9) and in the cauda epididymidis (10). Bar = 20µm.

Figures 11-14: AQP7 immunolocalization was observed in apical and basal region at initial segment (11) and caput epididymidis (12); AQP7 immunoreaction was absent in corpus (13) of dog epididymis. The reaction was not continuous in apical brush border of vas deferens epithelium, but an intense reaction was observed in the basal vas deferens region (14). Bar = 20µm.

Figures 15. Western blot analysis of AQP1, AQP2 and AQP7 in the initial segment (IS), caput (Ct), corpus (CP), cauda (CD) of epididymis and vas deferens (VD) protein extracts from dog. Each line represents 70µg of protein from different tissues. The actin protein (43kDa) was used as an internal control of the reaction. Each antibody recognized a band of about 30 kDa.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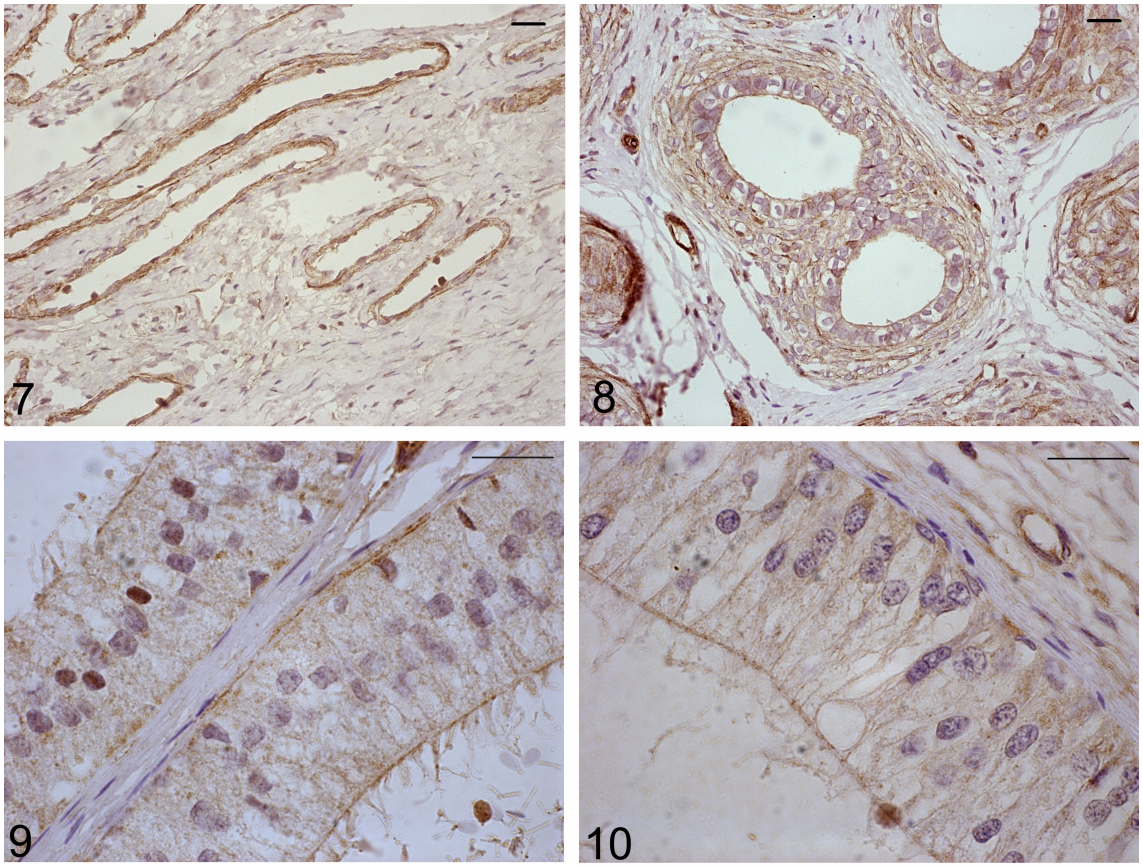
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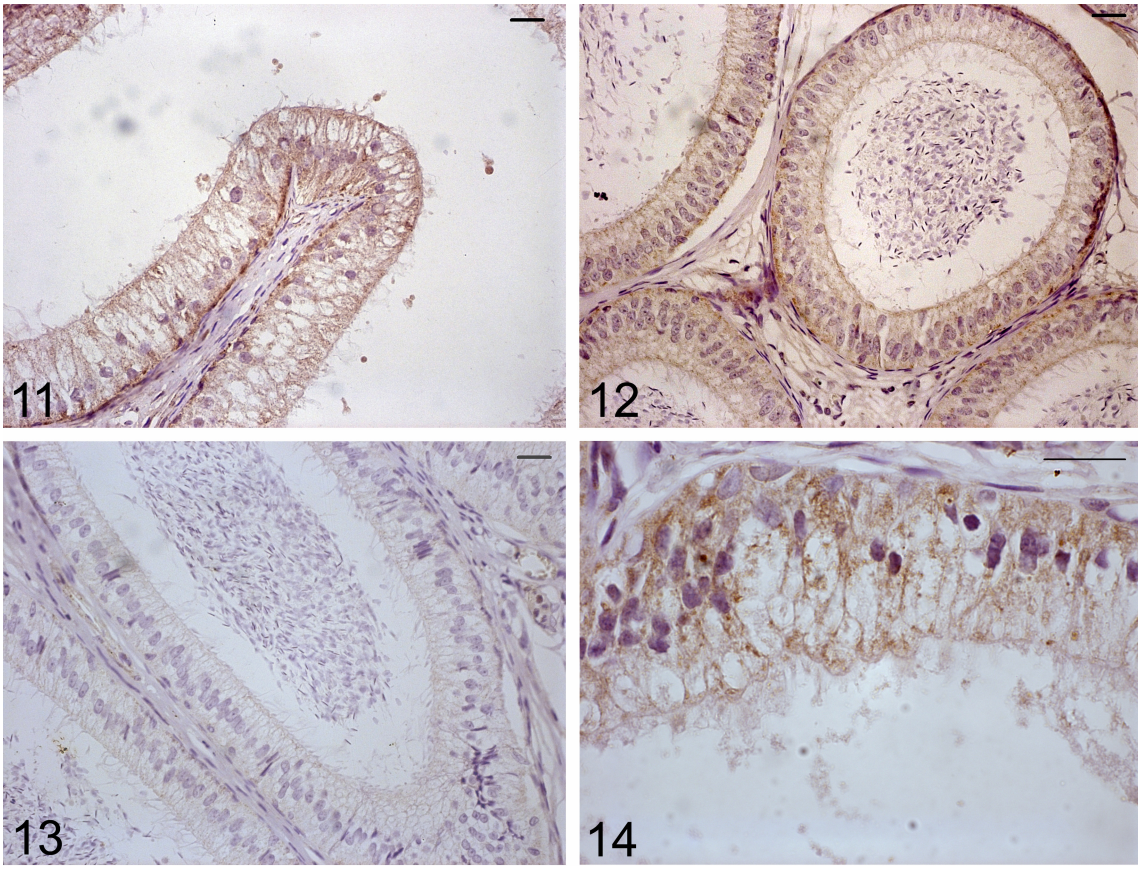
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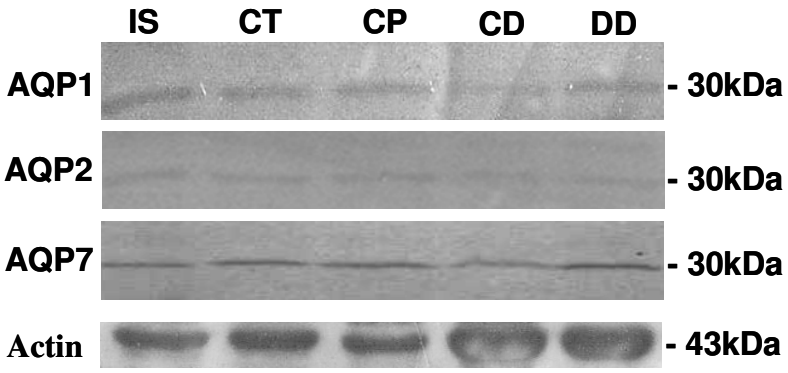
Fig.15



CONCLUSÕES GERAIS

- ✓ Há um padrão de expressão região-específica das AQPs no cão e espécie-específica comparado a outras espécies;
- ✓ A AQP7 foi identificada na porção proximal do ducto epididimário e ducto deferente;
- ✓ A AQP8 está ausente nos ductos extratesticulares do cão adulto;
- ✓ A AQP9 distribui-se amplamente nas diferentes partes da via espermática, exceto na rede testicular;
- ✓ O conhecimento das variações no processo de absorção de fluidos e de solutos, ao longo da via espermática, é relevante para estudos clínicos de infertilidade e para o desenvolvimento de tecnologias de reprodução assistida;
- ✓ A distribuição das AQPs na via espermática do cão comparada ao homem reafirma as discussões a respeito da semelhança dessa via entre ambos.

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Campinas, 01 de outubro de 2007.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de mestrado/doutorado intitulada Expressão e localização de aquaporinas na via espermática de cão adulto, *Canis familiaris*:

( ) não se enquadra no Artigo 1°, § 3° da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo n°_____), intitulado _____

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo n°036/04-CEEA).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo n°_____).

Raquel F. Domeniconi

Aluno(a): Raquel Fantin Domeniconi

Orientador(a): Prof. Dr. Antonio Marcos Orsi

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Ana Maria Azanuda Guaraldo

Nome: Profa. Dra. ANA MARIA A. GUARALDO
Função: Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP

unesp

Universidade Estadual Paulista
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CERTIFICADO

Certificamos que o Protocolo nº 036/04-CEEA, sobre *"Perfil eletroforético de proteínas epididimárias, expressão e localização de aquaproinas na via espermática do cão adulto (Canis familiaris)"*, sob a responsabilidade de **ANTONIO MARCOS ORSI**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de 10 de maio de 2004.

Botucatu, 10 de maio de 2004.

Prof.Dr. Sílvio Luís de Oliveira
Presidente - CEEA

Nádia Jovêncio Cotrim
Secretária – CEEA