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Cellular distribution of aquaporins in testes of normal and cryptorchid dogs: A preliminary study on dynamic roles

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ABSTRACT

Fluid regulation within the male gonad is an important process for promoting sperm differentiation and maturation. Aquaporins (AQPs) are a family of thirteen integral membrane proteins involved in these processes. The expression of several genes of AQPs occurs in the male reproductive tract of humans and other animal species, although there are few studies on domestic animals. In this study, the localization of AQP7, AQP8, and AQP9 as well as the abundances of protein and mRNA transcripts were examined in normal and cryptorchid dog testes. There was immunohistochemical localization of AQP7, AQP8, and AQP9 in both the tubular and interstitial compartments of the normal and retained testes and crytorchid dogs, albeit there was an obvious difference in cellular localization with the testes from the cryptorchid dogs. These results were supported by western blotting and real-time RT-PCR analyses, there was a lesser AQP7 and greater AQP9 abundance of protein and mRNA transcripts in the cryptorchid testis. These findings indicate combined testicular functions of AQPs in cell volume regulation. In addition, with the cryptorchid condition characterized there was a different cellular distribution of AQPs supporting the thought that early detection is important for controlling possible side effects of cryptorchidism, such as pre-neoplastic and carcinogenic outcomes.

1. Introduction

The regulation of fluid movement across the compartments of the male reproductive system has a primary role in many of the processes involved in spermatogenesis and in its local hormonal regulation (Setchell, 1986; Rato et al., 2010). In the testis, where spermatogenesis occurs, there are morphological and cytological modifications that are accompanied by complex mechanisms responsible for rapid water and solute transport to regulate optimal cell volume (Huang et al., 2006). In the course of germ cell development in the seminiferous epithelium, fluid secretion is essential for an optimal environment for spermatogenesis (Setchell, 1986; Mruk and Cheng, 2004; Rato et al., 2010). The regulation of fluid homeostasis and thus the transport of diverse molecules is a multifaceted process in which several mechanisms are involved. In particular, water efflux from Sertoli cells makes an important contribution to the composition of the fluid secreted in different segments of the seminiferous tubules. This fluid, containing water and electrolytes, is secreted by Sertoli cell (Rato et al., 2010) and surrounds spermatids with the fluid being a source of nutrients for thedevelopment and differentiation into spermatozoa (spermiogenesis; (Sprando and Russell, 1987; Holdcraft and Braun, 2004). In

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addition, the specific fluid composition in different segments of the tubules is adequately balanced from a content perspective for promotion of the maturation, storage, transport, and liberation of sperm and to increase sperm concentration (Huang et al., 2006).

Because of the significant physiological roles of fluid homeostasis in the testicular compartment, and the importance of adequate cell volume regulation during cell development, the potential role of specific water channel proteins, such as aquaporins (AQPs), during these processes has received particular consideration. Data from previous studies indicate that there is a wide and varied distribution of AQPs in the several compartments of the male reproductive system, indicating there is localization in different cell types (Hermo and Smith, 2011; Zhang et al., 2012). In particular, there is the presence of multiple AQP isoforms (AQP0, AQP1, AQP3, AQP7, AQP8, and AQP9) in the testes of humans and laboratory animals (Ma et al., 2000; Badran and Hermo, 2002; Hermo et al., 2004; Yeung and Cooper, 2010; Yeung et al., 2010; Boj et al., 2015).

Results from previous studies do not adequately define the function of AQPs but indicate there are multiple regulatory actions promoted by AQPs. In particular, the main functions attributed are related to the control of spermatogenesis, interstitial fluid formation and its reabsorption. There, however, is regulation of these processes by different mechanisms, particularly from a hormonal perspective. The functions of AQPs are associated with organic transporters and, in particular, to glucose transporters (GLUTs) that in testicles function in glucose metabolism, an important process for sperm maturation (Alves et al., 2013).

Recently, different studies have focused on the distribution of AQPs in domestic animal testes (dog, cat, chicken, buffalo, horse, and sheep) (Domeniconi et al., 2008; Skowronski et al., 2009; Klein et al., 2013; Arrighi et al., 2016; Schimming et al., 2015). The results obtained have been equivocal and in general indicate these channel proteins participate in the mechanisms of transcellular fluid secretion. Further research focused on this fluid regulation function is necessary because this fluid component is an important aspect in spermatogenesis (Yeung et al., 2009). Furthermore, results of other studies suggest that AQPs have a role in conditions where there are concomitant alterations in other aspects of spermatogenesis and thus of fertility (Arrighi et al., 2013; Arrighi and Aralla, 2014; Arrighi et al., 2016). Such variations within the testicular environment might also involve several biochemical processes regulating fluid formation.

In particular, Setchell (1970) reported that the testicular fluid secretion is a complex process that is completed prior to spermatogenesis. The aim of the present study, therefore, was to evaluate the presence of different AQPs (AQP7, AQP8, and AQP9) in testes from either normal and cryptorchid dogs. The second aim was to identify the cell types containing AQPs to clarify the physiological roles of these compounds in male gonads.

2. Materials and methods

2.1. Animals

This study was performed using five adult normal male dogs and five male dogs affected by unilateral cryptorchidism from the surgical unit of the Department of Veterinary Medicine and Animal Productions of the University of Naples

“Federico II”. All of the dogs were medium sized and aged between 2 and 8 years. Testes were collected immediately after bilateral orchiectomy by surgical techniques. All of the procedures for testis collection at the surgical unit were monitored and guaranteed by competent veterinary authorities and approved by the Ethical Animal Care and Use Committee of University of Naples “Federico II”, Department of Veterinary Medicine and Animal Production, Naples, Italy (No. 0,050,377). The owners of the animals gave verbal consent to perform surgical procedures, collection of the samples and animals were not involved in any clinical trials or treatments. Tissue samples were divided in three groups: normal testis (testis from normal dogs), contralateral testis (scrotal testis from dog affected by unilateral cryptorchidism), and the testis retained in the body cavity of the crypotorhid dog (testis affected by unilateral cryptorchidism). For western blot and RT-PCR analyses, fresh segments of testes were immediately frozen on dry ice and stored at −80 °C. For immunohistochemical studies, fresh segments of testes were immediately fixed by immersion in Bouin’s fixative (24–48 h).

2.2. Immunohistochemistry

After the fixation procedure, the samples were processed for paraffin embedding in a vacuum and cut at a thickness of 3–6 μm. Immunohistochemical staining was performed by EnVision system-horseradish antiperoxidase (HRP) (cod. K4002, Dako, Santa Barbara, CA). After dewaxing in xylene and rehydration, sections were washed in phosphate buffered saline (PBS) and then placed in antigen retrieval solution (Citrate buffer, pH 7.4) brought to boil using a microwave. Sections were washed with PBS and treated with 3% H_2O_2 (20 min), washed with PBS (pH 7.4), and incubated in a humid chamber overnight at 4 °C with the relative primary antibodies. The primary anti-sera included rabbit polyclonal directed against AQP7, AQP8, and AQP9 (diluted 1:1000) (AQP7 cod. orb13253; AQP8 cod. orb101163; AQP9 cod. orb10127, Byorbit LLC, San Francisco, USA). The sections were subsequently washed in PBS and incubated with EnVision for 30 min at RT. The sections were washed, and the immunoreactive sites were visualized by incubation for 5 min in a fresh solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Vector). The sections were counterstained with haematoxylin to identify the testicular cytotypes. The specificity of the primary immunoreactions was tested by replacing each antibody with a buffer or preabsorbing the antibody with an excess (100 μg/mL antiserum as the final dilution) of the relative antigen described elsewhere (Valiante et al., 2015). No immunoreaction was detected in control testis. Five slides (one slide selected from every ten slides according to the sequential thickness) for each testis from each animal were independently evaluated by two observers using a Leica DMRA2 microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Preparation of testicular tissue homogenate and western blot analysis

Testes homogenate and western blot analyses were conducted as previously described (Squillacioti et al., 2015). Briefly, testis lysates were prepared by homogenization of collected testes in a RIPA buffer containing protease inhibitors followed by centrifugation at 13,500 rpm at 4 °C. The obtained supernatant was collected and the protein concentration was determined prior of western blot analysis. Samples containing equal protein content (50 μg) were subjected to electrophoresis under reducing conditions in 4%–12% NuPAGE Bis-Tris gradient mini gels (Life Technologies; Grand Island, NY) at 150 V using MES-SDS running buffer (Life Technologies, Grand Island, NY). Separated proteins were electrophoretically transferred onto nitrocellulose membrane by iBlot Dry Blotting System (Life Technologies, Grand Island, NY) at 20 V for 7 min. The membranes were first blocked in TBS buffer (137 mM NaCl and 20 mM Tris) with 0,1% Tween-20 and 5% skimmed milk (1 h at room temperature) and then incubated overnight at 4 °C with specific primary antibodies against AQP7, AQP8, and AQP9 (all diluted 1 : 500 in TBS with 0.1% Tween 20 and 3% skimmed milk). After washing three times for 5 min each time, the membranes were incubated for 1 h at room temperature with 1 : 5000 dilutions of HRP-conjugated secondary antibodies in 5% non-fat dry milk/TBST.

Immune complexes were detected with chemiluminescence reagents (Western-Bright ECL Plus Enhanced chemiluminescence reagent (Sirius, Advasta, CA, USA), and bands were visualized in a C-DiGit® Chemiluminescent Western Blot Scanner (LI-COR Biosciences, Germany). Blots were stripped and re-probed with anti β-actin rabbit antibody (cat n. A4700, Sigma) 1:2000), as loading control. The antibody was diluted 1 : 2000 in blocking solution (Pelagalli et al., 2016).

Densitometric analyses of the western blots were analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to those of β-actin, used as a loading control.

2.4. RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA was obtained after homogenization of tissue samples in ice-cold TRI-Reagent (Sigma) using an Ultra-Turrax homogenizer. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in RNAase-free DEPC water. Quantification of total RNA was performed by an Eppendorf Biophotometer (Eppendorf AG, Basel, Switzerland). For cDNA synthesis, 1 μg of total RNA was retro-transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) using random hexamers as primers. For conventional and real-time PCR reactions, all specific primers used were designed from the published mRNA Genbank gene sequences as reported in Table 1.

The PCR cycle conditions were as follows: 94 °C (30 s), 60 °C (30 s), and 72 °C (1 min) for 35 cycles. The PCR products of dog AQP7, AQP8, and AQP9 were purified using GFX PCR DNA and Gel Purification Kit (28-9034-70, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and sequenced. To study the mRNA transcript profiles of these genes, quantitative RT-PCR was used. The real-time PCR reactions contained 1 μL of cDNA (20 ng/well) and 24 μL of SYBR Green Master Mix (Applied Biosystems) containing specific primers. The PCR conditions were as follows: 50 °C for 2 min and 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. The GAPDH gene was also amplified in separate tubes under the same conditions to serve as an active endogenous reference to normalize the quantification of the mRNA target.

Real-time detection was performed on an ABIPRISM 7300 Sequence Detection System (Applied Biosystem), and data from the SYBR Green I PCR amplicons were assessed with the ABI 7300 System SDS Software. The relative quantification method $2^{-\mathrm{deltadeltaCt}}$ ($2^{-\Delta\Delta Ct}$) was used for the normalization of mRNA transcript abundance as described previously (Squillacioti et al., 2011).

2.5. Data analysis

The data are expressed as mean ± standard deviation (SD). For real-time RT-PCR, significant differences in the relative abundances of AQP7, AQP8, and AQP9 mRNA transcripts between the calibrator sample (normal testis) compared with the contralateral and testis retained in the body cavity of cryptorchid dogs were determined by one-way ANOVA followed by Tukey's HDS test for independent samples. The level of statistical significance was set at $P < 0.05$ for all experiments. For densitometric analysis,

Table 1
Primers for real-time RT-PCR.

Gene	Sequence 5'–3'	Genbank accession number
GAPDH		XM_544701.4
Forward	TGTCCCCACCCCCAATG	
Reverse	TCGTATATTTGGCAGCTTTCTC	
AQP7		XM_531973.5
Forward	GCTGCTGCCACCATCTACCT	
Reverse	TTGTCCGTGATGGCGAAGA	
AQP8		XM_014114532.1
Forward	AAACATCAGTGGAGGACATTTCAA	
Reverse	GCTCCTGGACTGTCACAAAGG	
AQP9		XM_544701.4
Forward	CCTTCCCTGCGAATCACAA	
Reverse	AGGTGCATCGCTTGATGTAGAG	

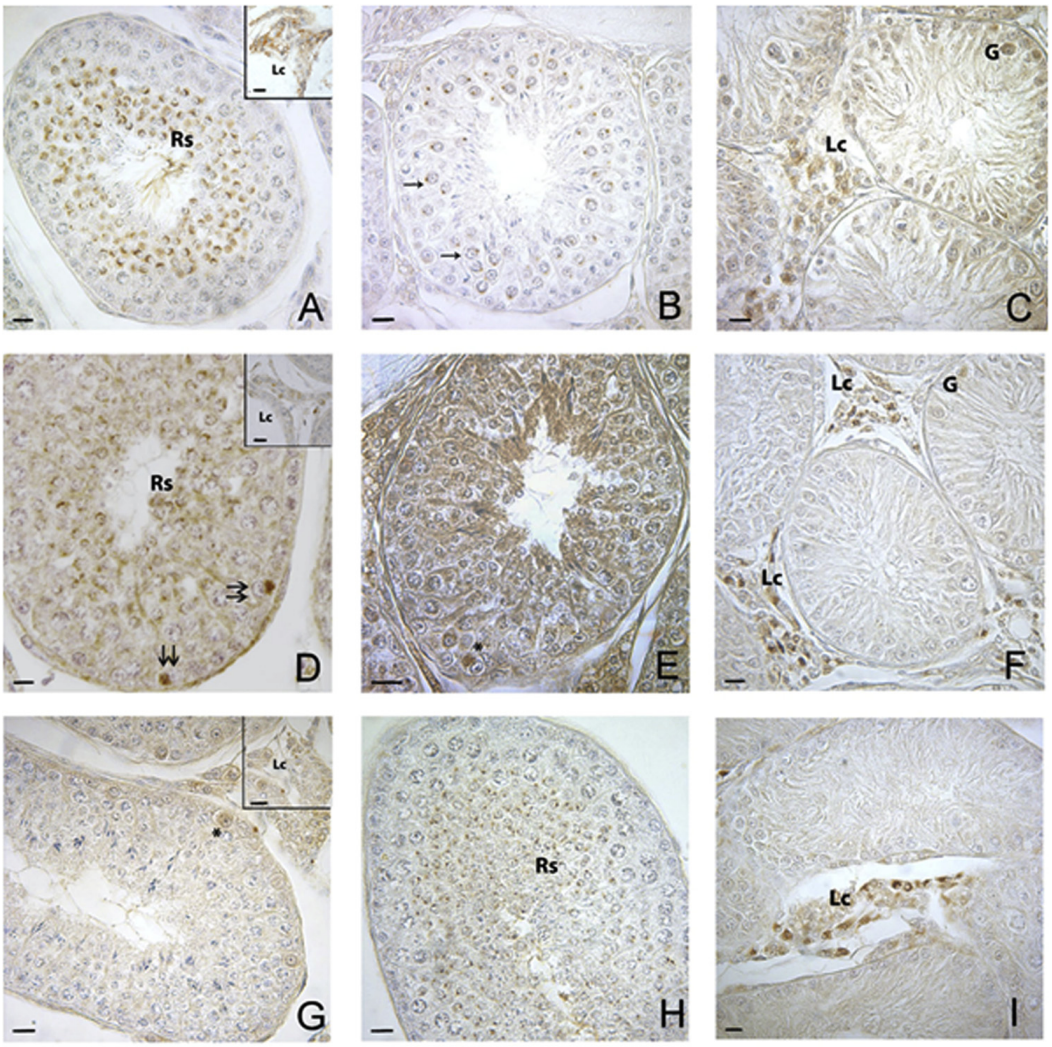

Fig. 1. Distribution of the immunoreactivity (IR) to AQP7 (A–C), AQP8 (D–F) and AQP9 (G–I) in normal (A, B, D, E, G, H) and cryptorchid dog testes (C, F, I); In the normal testis IRs were distributed in the germ cells and interstitial Leydig cells (Lc, inserts); In the retained testis of dogs resulting the cryptorchid condition, there was IR in gonocytes (G) and interstitial Lc; Round spermatids (Rs); preleptotene or resting spermatocytes (double arrows); diakinesis spermatocytes (arrows); spermatogonia (asterisks) Scale bar: 25 μm.

significant differences for these proteins between normal and cryptorchid dogs were determined by using the tests that were previously described in this manuscript.

3. Results

3.1. Immunohistochemistry

In normal testis, the immunoreactivities (IRs) for AQP7, AQP8, and AQP9 were observed in the tubular compartment and in the interstitial Leydig cells (Fig. 1A, B, D, E, G, H). Specifically, AQP7-IR was present in the cytoplasm of round spermatids (Rs) (Fig. 1A) and diakinesis spermatocytes (Fig. 1B, arrows). The immunoreactive material appeared as a perinuclear halo in Rs and a single granule close to the nuclear membrane in the diakinesis spermatocytes. The AQP8 and AQP9-IRs were also present in spermatogonia (Fig. 1E, G, asterisk), preleptotene or resting spermatocytes (Fig. 1D, double arrows), and Rs (Fig. 1H). Spermatogonia and preleptotene spermatocytes were located along the basal membrane of the tubule, and the positive material clustered in one extremity of the cytoplasm of these cells. A few positive Sertoli cells were detected (data not shown). The distribution of AQP-IRs in the contralateral testis was similar to that in the normal testis (data not shown).

In the retained testis of cryptorchid dogs, AQPs-IRs were distributed in the interstitial Leydig cells principally. Furthermore, there was a few gonocytes (G) with weakly positive staining (Fig. 1C, F, I). The immunoreactive material in the interstitial Leydig cells were included in the inserts of (Fig. 1A, D, and G) and data are summarized in Table 2.

Table 2
Distribution of AQP7-, AQP8- and AQP9-IRs in the testis of the normal and cryptorchid dog.

	Normal		Cryptorchid	
	Leydig cells	Germ cells	Leydig cells	Germ cells
Aquaporin 7	+	+ + +	+ +	+
Aquaporin 8	+ +	+ +	+ + +	–
Aquaporin 9	+ + +	+	+ + +	–

– Undetectable, + low density, + + medium density, + + + high density.

3.2. Western blotting analysis

Results from the western blot analysis that all AQPs that were assessed in testes of the present study were present in the testis of both normal and cryptorchid dogs as indicated by the presence of a main band at ˜25 to 35 kDa corresponding to a regular form of the protein (Fig. 2A).Furthermore, other faint bands were observed at molecular weights corresponding to ˜ 48 to 63 kDa. These bands could be indicative of glycosylated forms of the protein, as reported previously to occur in testis tissue (Calamita et al., 2001a; Domeniconi et al., 2007).

Results of the densitometric analysis indicated there were slight differences, albeit not significant, particularly for the abundances of AQP7 and AQP9, with lesser (AQP7) and greater (AQP9) abundances, respectively, in the cryptorchid and normal testis (Fig. 2B).

3.3. Real-time RT-PCR

Results from real-time RT-PCR experiments confirmed that there were AQP7, AQP8, and AQP9 mRNA transcripts in normal and contralateral testes, as well as retained testis of cryptorchid dogs (Fig. 3). The relative abundance of AQP7 mRNA transcript was less in the retained testis of cryptorchid dogs than in the normal and contralateral testes (Fig. 3). The relative abundances of AQP8 and AQP9 mRNA transcripts were greater the retained testis of cryotrochid dogs as compared with normal testis, but this difference was

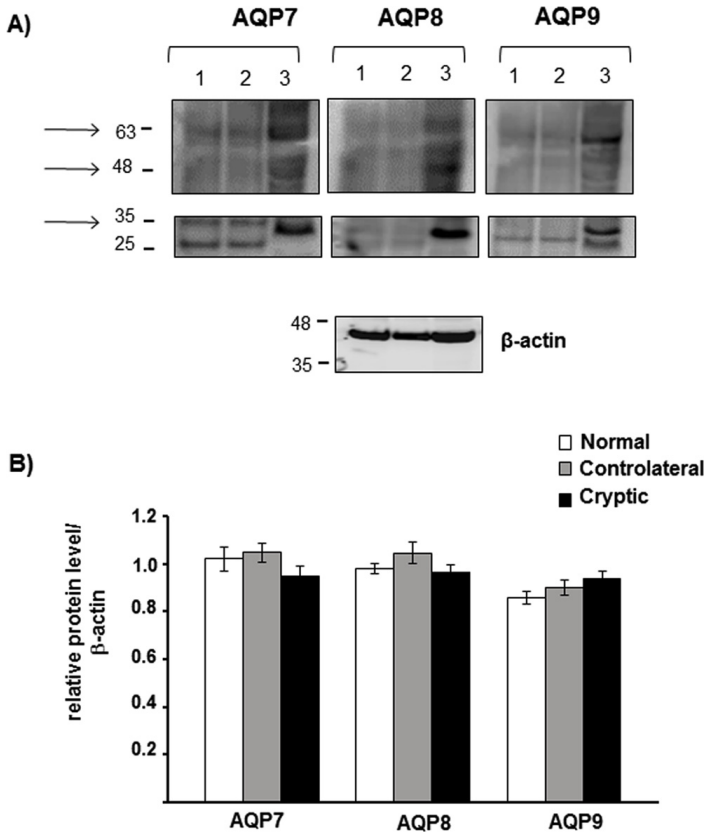

Fig. 2. Aquaporin protein abundances in normal (lane 1), contralateral (lane 2) and the retained testis of cryptorchid dogs that resulted in the condition (lane 3); A. Immunoblots for AQP7, AQP8, AQP9 and actin are shown; For each aquaporin, the protein band was detected at a molecular weight specific for the antibody used (AQP7, 37 kDa; AQP8, 29 kDa; AQP9, 32 kDa); B. Densitometric analysis of AQP7, AQP8 and AQP9 protein abundance; β-actin was used as the loading control; Each plotted value corresponds to the mean ± SD obtained from three independent experiments.

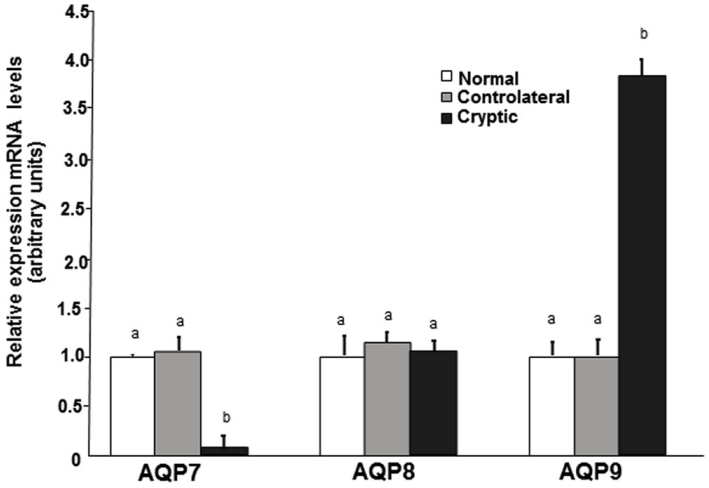

Fig. 3. Relative abundances of AQP7, AQP8 and AQP9 mRNA transcripts in normal, contralateral and the retained testis of dogs resulting in the cryptorchid condition using real-time RT-PCR; Statistical differences in relative abundances of AQP7, AQP8 and AQP9 mRNA transcripts between the calibrator sample (normal testis), and the contralateral and retained testis that resulted in the cryptorchid condition were determined by one-way ANOVA followed by Tukey's HDS test; Different letters depict differences between the examined groups ($P < 0.05$).

not statistically significant.

4. Discussion

In this study, immunohistochemical and molecular approaches were used to characterize the cellular distribution and expression of AQP7, AQP8, and AQP9 in testes from normal and cryptorchid dogs.

Recently, numerous experimental studies focusing on the possible presence of AQPs in the male reproductive system of different animal species, evidenced not only their possible involvement in the spermatozoa production but also a possible regulation of their activity. Such mechanisms not clearly investigated, should be related to the physiology of the cells of the male reproductive system that, in turn, are influenced either by hormones (androgens and oestrogens) or by local factors produced by the seminiferous epithelium itself (Hermo and Smith, 2011).

Regarding our experimental results, all tested AQPs were expressed and localized in the testes of normal and cryptorchid dogs, albeit in the latter condition, several specific changes were observed in cellular distribution. These data are particularly interesting because they are the first demonstration of AQP distribution in the testes of cryptorchid dogs.

In detail, immunohistochemical results showed a widely presence of all tested AQPs in different cytotypes of canine normal testis in either the tubular or interstitial compartments. Differently, in the cryptic testis, AQPs distribution revealed their particular localization exclusively in the interstitial compartment of Leydig cells. AQPs expressions were also confirmed by western blot analysis showing, both in normal and in cryptorchid dogs, a main band (28–35 kDa) corresponding to the regular form of each assayed AQP. The AQP7 and AQP9 protein levels analysed by densitometry showed slightly insignificant differences in the retained gonad with respect to a normal one with lower and higher levels, respectively. Multiple immunoreactive bands were also present, even under reducing conditions, confirming previous data on other AQPs (Van Hoek et al., 1995). In particular, in the testes from cryptorchid dogs, our results, supported by real-time RT-PCR data, demonstrated different expression patterns of AQP7 and AQP9 mRNAs with low and high expression, respectively. This data probably reflect the particular condition of cryptorchidism characterized by abolished germ cell production but also relevant modifications which include also testis microenvironment.

To further elucidate the localization and function of AQPs at the testicular level, other aspects should be considered, such as: i) the specific AQPs permeability and their possible relationship with sperm volume regulation, ii) the particular metamorphosis occurring along the tubule where spermatids maturation that is accompanied by a volume reduction due to osmotically driven fluid efflux, and iii) the singular condition of cryptorchidism characterized by altered spermatogenetic morphology and function.

Our immunohistochemical data on canine normal testis confirm previous AQP characterization studies on several animal species showing a certain AQP distribution in specific cytotypes of both tubular and interstitial compartments or, in some cases, their co-expression in the same cell type as observed in other tissues (kidney, epididymis, etc.) (Da Silva et al., 2006). The slight difference in the AQP distribution with respect to germ line localization could be ascribed probably to the particular role played by the tested channel protein at this level.

In particular, our immunohistochemical results show AQP7 and AQP8 localization at the level of germ cells (spermatids, and primary spermatocytes in the pre-leptotene), with moderate immunoreactivity at this level for AQP8. Differently, slight AQP7 and AQP8 IRs were observed in the Leydig cells. Taken together, such data in agreement with those of previous results from human, rat, and avian species (Kageyama et al., 2001; Saito et al., 2004; Skowronski et al., 2009; Yeung et al., 2010) demonstrate a particular localization of AQP7 and AQP8 in round spermatids. Their possible collaborative role in spermatogenesis become particularly interesting by considering the fact that Sertoli cells play a pivotal role in fluid secretion to facilitate the spermatozoa transportation

from the testis to the epididymis (Setchell et al., 1969; Hinton and Setchell, 1993). Moreover, the presence and the role of multiple forms of Na, K-ATPase either in Sertoli and in germ cells could suggest its interaction with AQPs in the spermatogenesis process and in the fluid secretion such as observed in other body districts (Illarionova et al., 2010).

Regarding AQP9 expression in the normal dog testis, our results demonstrate a certain IR either in the germ line (from spermatogonia to round spermatids), or in the interstitial Leydig cells, confirming previous data obtained in testes from rats, cats, and equine species (Nihei et al., 2001; Arrighi et al., 2013; Klein et al., 2013). However, previous immunohistochemical studies on dog testis demonstrated no AQP9 expression in the seminiferous tubules or rete testis, while AQP9 IR was detected along the entire apical brush border of efferent ducts (Domeniconi et al., 2007). In addition, Arena et al., 2009 reported in humans, a diffuse cell membrane staining of the primary spermatocytes and maturing haploid germ cells (h-GCs), suggesting, for this channel protein, a role in water and lactate transport from Sertoli cells to GCs.

Cryptorchidism is a particular pathophysiological condition

n characterized by a high steroidogenic activity accompanied by a particular morphology of Leydig cells. In particular, these cells appear preferentially clustered around blood vessels or formed by aggregations associated with networks of connective tissue with respect to the contralateral normal rat testis (Risbridger et al., 1987. In addition, a reduced fluid production as well as oxidative stress was demonstrated in cryptorchid testis, confirming either an impaired capacity of Sertoli cells to produce fluid (Setchell, 1970; Hagenas et al., 1976) but also several alterations in endocrine signaling, and germ cell apoptosis.

Our results for cryptorchid condition, showed significant AQP7 and AQP8 IRs modifications, demonstrating their particular localization especially at the level of Leydig cells and some early germ cells. This condition could suggest a different role of these channel proteins related to the profound changes of testis morphology in a similar way to that observed in disorders of the male reproductive system, such as abnormal sperm motility, abnormal epididymis and infertility (seen in cystic fibrosis), and varicocele (Huang et al., 2006).

Similar immunohistochemical data on boar-retained testes describe a clear alteration in Leydig cell activity related to fluid transport, and thus membrane permeability (Pinart et al., 2001). Our immunohistochemical data supported also by western blotting and real-time RT-PCR evidenced AQP7 and AQP8 mRNA levels respectively lower and quite similar to that observed in normal testis.

Furthermore, for AQP9, a different cellular distribution pattern was observed in the cryptorchid condition with respect to normal condition, showing AQP9 IR exclusively in the Leydig cells as previously observed (Elkjaer et al., 2000; Badran and Hermo, 2002). This could suggest its specific role in the transport of water and non-charged solutes in those cells where it has widely demonstrated also a high grade of cell hyperplasian. This assumption, confirmed also the higher level of AQP9 mRNA observed in this condition with respect to that observed for the normal condition, could suggest as evidenced by Rato et al. (2010) a particular AQP9 involvement in the extensive activity of Leydig cells.

Moreover, considering a possible collaborative role of AQPs with Na,K-ATPase, some studies revealed similarly to higher level of AQP9 mRNA, an increase of Na,K-ATPase α1-isoform in the testis of mice made infertile through a deficiency in the production of the early growth response transcription factor, Egr4 (Blanco et al., 2000). This data could further reinforce the idea of a complex mechanism of regulation in this condition and thus, contributing with a particular role in the physiology of the male gonad.

In addition, this different behaviour of AQP9 mRNA with respect to that observed for AQP7 mRNA could be justified by a different hormonal control mechanism. In fact, testicular and epididymal AQP9 displays exclusive hormonal regulation, being controlled by both oestrogen and dihydrotestosterone (DHT) in the efferent ductules, while only DHT influences the activity of the initial segment epididymis (Picciarelli-Lima et al., 2006). On this regard, Wellejus et al., 2008 demonstrated that a condition called “testicular dysgenesis syndrome”, similar to clinical diseases (testicular cancer, cryptorchidism, and hypospadias), determined by a neonatal exposure of the efferent ductules (ED) to diethylstilbestrol, can induce an upregulation of AQP9 and a downregulation of the correspondent mRNA. Similar data were also accompanied by evidence demonstrating that antioestrogen treatment reduced AQP9 expression (Oliveira et al., 2005; Picciarelli-Lima et al., 2006). To reinforce the hypothesis of the existence of intricate regulation mechanisms influencing general fluid homeostasis in cryptorchidism, there are the results of (Hahn et al., 2017) demonstrating an important role of glucose transporter 3 (GLUT3) at level of the Leydig cells. In fact, the evidence of strong GLUT3 IR only at the level of Leydig cells, let to conclude that GLUT3 acts increasing abdominal uptake of glucose into interstitial areas, and thus leading to an increased risk of developing cancer.

The different and more complex regulation of AQP9 at the testis level, as reported in the literature, suggests several factors are involved separately or in combination for its role in the cryptorchid condition (Fig. 4).

In particular, in accordance with Oliveira et al. (2005), who observed an upregulation of oestrogen-evoked AQP9 in the efferent ductule epithelium, we can hypothesize a similar mechanism in the cryptic gonad. As previously reported, an oestrogen increase was revealed in the unilateral cryptorchidism in stallion (Hejmej and Bilinska, 2008), mouse (Bilinska et al., 2003) and dog (Kawakami et al., 1999), suggesting that P450 aromatase enzyme, responsible of the conversion of androgens into oestrogens, was also determining for the decreased testosterone levels. In addition, taking into account that high oestrogen levels observed in cryptorchidism can also cause mast cell migration and proliferation (Mechlin and Kogan, 2012), another possible role of AQP9 could be the facilitation of these mechanisms (Bryce, 2018). Consistent with this observation, recently different papers focused on the possible channel proteins role in the physiology of inflammatory processes (Holm et al., 2015; Meli et al., 2018).

Additionally, our data suggest that AQP9, as well as AQP7 and AQP8, participate in a complex series of events important either for the regulation of fluids and for germinal cell formation and maturation and thus leading to further investigations to clarify the specific transport function of individual AQPs.

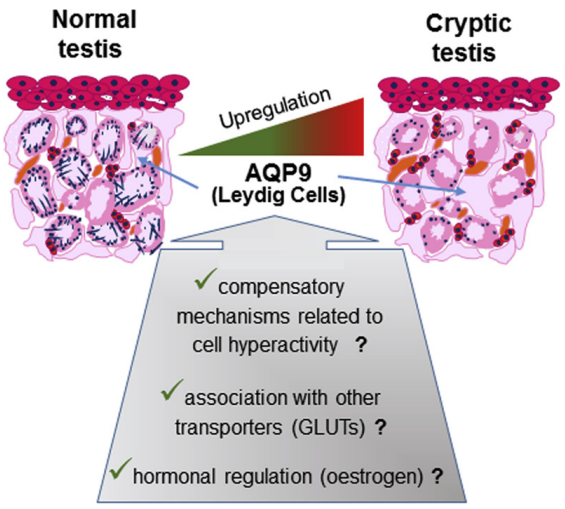

Fig. 4. Schematic diagram of the hypothesized mechanisms regulating testicular abundances of AQP9 in greater amounts when the cryptorchidism condition exists.

5. Conclusions

The findings of the present study confirm the specific functions of AQPs for the normal maturation of the germ cells in the dog testis. The complete elucidation of the possible mechanism of AQP function in spermatozoa development when there are testicular dysfunctions could indicate there are therapeutic strategies to counteract male subfertility/infertility.

Conflict of interest

The authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled: “Cellular distribution of aquaporins in testes of normal and cryptorchid dogs: A preliminary study on their dynamic roles”.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.03.001.

References

Alves, M.G., Martins, A.D., Rato, L., Moreira, P.I., Socorro, S., Oliveira, P.F., 2013. Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochim. Biophys. Acta 1832, 626–635.
Arena, S., Arena, F., Maisano, D., Di Benedetto, V., Romeo, C., Nicòtina, P.A., 2009. Aquaporin-9 immunohistochemistry in varicocele testes as a consequence of hypoxia in the sperm production site. Andrologia 43, 34–37.
Arrighi, S., Aralla, M., 2014. Immunolocalization of aquaporin water channels in the domestic cat male genital tract. Reprod. Domest. Anim. 49, 17–26.
Arrighi, S., Aralla, M., Fracassetti, P., Mobasheri, A., Cremonesi, F., 2013. Aquaporin water channels in the canine gubernaculum testis. Acta Histochem. 115, 541–548.
Arrighi, S., Bosi, G., Accogli, G., Desantis, S., 2016. Seasonal and ageing-depending changes of aquaporins 1 and 9 expression in the genital tract of buffalo bulls (*Bubalus bubalis*). Reprod. Domest. Anim. 51, 515–523.
Badran, H.H., Hermo, L.S., 2002. Expression and regulation of aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development. J. Androl. 23, 358–373.
Bilinska, B., Kotula-Balak, M., Gancarczyk, M., Sadowska, J., Tabarowski, Z., Wojtusiak, A., 2003. Androgen aromatization in cryptorchid mouse testis. Acta Histochem. 105, 57–65.
Blanco, G., Sánchez, G., Melton, R.J., Tourtellotte, W.G., Mercer, R.W., 2000. The_4 isoform of the Na,K-ATPase is expressed in the germ cells of the testes. J. Histochem. Cytochem. 48, 1023–1032.
Boj, M., Chauvigne, F., Cerda, J., 2015. Aquaporin biology of spermatogenesis and sperm physiology in mammals and teleosts. Biol. Bull. 229, 93–108.
Bryce, P., 2018. Aquaporin 9 regulates mast cell activation. Abstract Presented at the 14th Annual Lewis Landsberg Research Day.
Calamita, G., Mazzone, A., Cho, Y.S., Valenti, G., Svelto, M., 2001a. Expression and localization of the aquaporin-8 water channel in rat testis. Biol. Reprod. 64, 1660–1666.
Da Silva, N., Piétrement, C., Brown, D., Breton, S., 2006. Segmental and cellular expression of aquaporins in the male excurrent duct. Biochim. Biophys. Acta 1758, 1025–1033.
Domeniconi, R.F., Orsi, A.M., Justulin Jr., L.A., Beu, C.C., Felisbino, S.L., 2007. Aquaporin 9 (AQP9) localization in the adult dog testis excurrent ducts by immunohistochemistry. Anat. Rec. 290, 1519–1525.
Domeniconi, R.F., Orsi, A.M., Justulin Jr., L.A., Leme Beu, C.C., Felisbino, S.L., 2008. Immunolocalization of aquaporins 1, 2 and 7 in rete testis, efferent ducts,

epididymis and vas deferens of adult dog. Cell Tissue Res. 332, 329–335.
Elkjaer, M., Vajda, Z., Nejsum, L.N., Kwon, T., Jensen, U.B., Amiry-Moghaddam, M., Frøkiaer, J., Nielsen, S., 2000. Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. Biochem. Biophys. Res. Commun. 276, 1118–1128.
Hagenas, L., Ploen, L., Ritzen, E.M., 1976. Blood-testis barrier: maintained function of inter-Sertoli cell junction in experimental cryptorchidism in the rat, as judged by a simple lanthanum-immersion technique. Andrologia 9, 3–7.
Hahn, K.R., Jung, H.Y., Yoo, D.Y., Kim, J.W., Kim, Y.H., Jo, Y.K., Kim, G.A., Chung, J.Y., Choi, J.H., Hwang, I.K., Jang, G., Yoon, Y.S., 2017. Immunohistochemical localization of glucose transporter 1 and 3 in the scrotal and abdominal testes of a dog. Lab. Anim. Res. 33, 114–118.
Hejmej, A., Bilinska, B., 2008. The effects of cryptorchidism on the regulation of steroidogenesis and gap junctional communication in equine testes. Endocrinol. Pol. 59, 112–118.
Hermo, L., Smith, C.E., 2011. Thirsty business: cell, region, and membrane specificity of aquaporins in the testis, efferent ducts, and epididymis and factors regulating their expression. J. Androl. 32, 565–575.
Hermo, L., Krzeczunowicz, D., Ruz, R., 2004. Cell specificity of aquaporins 0, 3, and 10 expressed in the testis, efferent ducts, and epididymis of adult rats. J. Androl. 25, 494–505.
Hinton, B.T., Setchell, B.P., 1993. Fluid secretion and movement. In: Russell, L.D., Griswold, M.D. (Eds.), The Sertoli Cell. Cache River Press, Clearwater, FL, USA, pp. 249–268.
Holdcraft, R.W., Braun, R.E., 2004. Hormonal regulation of spermatogenesis. Int. J. Androl. 27, 335–342.
Holm, A., Karlsson, T., Vikström, E., 2015. Pseudomonas aeruginosa lasI/rhlI quorum sensing genes promote phagocytosis and aquaporin 9 redistribution to the leading and trailing regions in macrophages. Front. Microbiol. 6, 915.
Huang, H.F., He, R.H., Sun, C.C., Zhang, Y., Meng, Q.X., Ma, Y.Y., 2006. Function of aquaporins in female and male reproductive system. Hum. Reprod. Update 12, 785–795.
Illarionova, N.B., Gunnarson, E., Li, Y., Brismar, H., Bondar, A., Zelenin, S., Aperia, A., 2010. Functional and molecular interactions between aquaporins and Na,K-ATPase. Neuroscience 168, 915–925.
Kageyama, Y., Ishibashi, K., Hayashi, T., Xia, G., Sasaki, S., Kihara, K., 2001. Expression of aquaporins 7 and 8 in the developing rat testis. Andrologia 33, 165.
Kawakami, E., Hori, T., Tsutsui, T., 1999. Function of contralateral testis after artificial unilateral cryptorchidism in dogs. J. Vet. Med. Sci. 61, 1107–1111.
Klein, C., Troedsson, M.H.T., Rutllant, J., 2013. Region-specific expression of aquaporin subtypes in equine testis, epididymis, and ductus deferens. Anat. Rec. 296, 1115–1126.
Ma, T., Song, Y., Yang, B., Gillespie, A., Carlson, E.J., Epstein, C.J., Verkman, A.S., 2000. Nephrogenic diabetes insipidus in mice lacking aquaporin-3 water channels. Proc. Natl. Acad. Sci. U. S. A. 97, 4386–4391.
Mechlin, C., Kogan, B., 2012. Mast cells, estrogens, and cryptorchidism: a histological based review. Transl. Androl. Urol. 1, 97–102.
Meli, R., Pirozzi, C., Pelagalli, A., 2018. New perspectives on the potential role of aquaporins (aqps) in the physiology of inflammation. Front. Physiol. 16 (9), 101.
Mruk, D., Cheng, C., 2004. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr. Rev. 25, 747–806.
Nihei, K., Koyama, Y., Tani, T., Yaoita, E., Ohshiro, K., Adhikary, L.P., Kurosak, I., Shirai, Y., Hatakeyama, K., Yamamoto, T., 2001. Immunolocalization of aquaporin-9 in rat hepatocytes and Leydig cells. Arch. Histol. Cytol. 64, 81–88.
Oliveira, C.A., Carnes, K., Franca, L.R., Hermo, L., Hess, R.A., 2005. Aquaporin-1 and -9 are differentially regulated by oestrogen in the efferent ductule epithelium and initial segment of the epididymis. Biol. Cell 97, 385–395.
Pelagalli, A., Squillacioti, C., De Luca, A., Pero, M.E., Vassalotti, G., Lombardi, P., Avallone, L., Mirabella, N., 2016. Expression and localization of Aquaporin 4 and Aquaporin 5 along the large intestine of colostrum-suckling buffalo calves. Anat. Histol. Embryol. 45, 418–427.
Picciarelli-Lima, P., Oliveira, A.G., Reis, A.M., Kalapothakis, E., Mahecha, G.A., Hess, R.A., Oliveira, C.A., 2006. Effects of 3-beta-diol, an androgen metabolite with intrinsic estrogen-like effects, in modulating the aquaporin-9 expression in the rat efferent ductules. Reprod. Biol. Endocrinol. 4 (6).
Pinart, E., Bonet, S., Briz, M.D., Pastor, L.M., Sancho, S., Garcia, N., Badia, E., 2001. Morphologic and histochemical study of blood capillaries in boar testes: effects of abdominal cryptorchidism. Teratol. 63, 42–45.
Rato, L., Socorro, S., Cavaco, J.E., Oliveira, P.F., 2010. Tubular fluid secretion in the seminiferous epithelium, ion transporters and aquaporins in Sertoli cells. J. Membr. Biol. 236, 215–224.
Risbridger, G.P., Drummond, A.E., Kerr, J.B., de Kretser, D.M., 1987. Effect of cryptorchidism on steroidogenic and mitogenic activities in rat testicular interstitial fluid. J. Reprod. Fert. 81, 617–624.
Saito, K., Kageyama, Y., Okada, Y., Kawakami, S., Kihara, K., Ishibashi, K., Sasaki, S., 2004. Localization of aquaporin-7 in human testis and ejaculated sperm: possible involvement in maintenance of sperm quality. J. Urol. 172, 2073–2076.
Schimming, B.C., Pinheiro, P.F.F., de Matteis, R., Machado, C.M., Domeniconi, R.F., 2015. Immunolocalization of aquaporins 1 and 9 in the ram efferent ducts and epididymis. Reprod. Domest. Anim. 50, 617–624.
Setchell, B.P., 1970. The secretion of fluid by the testes of rats, rams and goats with some observation on the effect of age, cryptorchidism and hypophysectomy. J. Reprod. Fertil. 23, 79–85.
Setchell, B.P., 1986. The movement of fluids and substances in the testis. Aust. J. Biol. Sci. 39, 193–207.
Setchell, B.P., Scott, T.W., Voglmayr, J.K., Waites, G.M., 1969. Characteristics of testicular spermatozoa and the fluid which transports them into the epididymis. Biol. Reprod. 1, 40–66.
Skowronski, M.T., Leska, A., Robak, A., Nielsen, S., 2009. Immunolocalization of aquaporin-1,-5, and -7 in the avian testis and vas deferens. J. Histochem. Cytochem. 57, 915–922.
Sprando, R.L., Russell, L.D., 1987. Comparative study of cytoplasmic elimination in spermatids of selected mammalian species. Am. J. Anat. 178, 72–80.
Squillacioti, C., De Luca, A., Liguori, G., Paino, S., Mirabella, N., 2011. Expression of urocortin and corticotropin-releasing hormone receptors in the bovine adrenal gland. Gen. Comp. Endocrinol. 172, 416–422.
Squillacioti, C., De Luca, A., Pero, M.E., Vassalotti, G., Lombardi, P., Avallone, L., Mirabella, N., Pelagalli, A., 2015. Effect of colostrum and milk on small intestine expression of AQP4 and AQP5 in newborn buffalo calves. Res. Vet. Sci. 103, 149–155.
Valiante, S., Liguori, G., Tafuri, S., Pavone, L.M., Campese, R., Monaco, R., Iachetta, G., Assisi, L., Mirabella, N., Forte, M., Costagliola, A., Vittoria, A., 2015. Expression and potential role of the peptide orexin-A in prostate cancer. Biochem. Biophys. Res. Commun. 464, 1290–1296.
Van Hoek, A.N., Wiener, M.C., Verbavatz, J.M., Brown, D., Lipniunas, P.H., Townsend, R.R., Verkman, A.S., 1995. Purification and structure function analysis of native, PNGase F-treated, and endo-beta-galactosidase- treated CHIP28 water channels. Biochemistry 34, 2212–2219.
Wellejus, A., Jensen, H.E., Loft, S., Jonassen, T.E., 2008. Expression of aquaporin 9 in rat liver 658 and efferent ducts of the male reproductive system after neonatal diethylstilbestrol 659 exposure. J. Histochem. Cytochem. 56, 425–432.
Yeung, C.H., Cooper, T.G., 2010. Aquaporin AQP11 in the testis: molecular identity and association with the processing of residual cytoplasm of elongated spermatids. Reproduction 139, 209–216.
Yeung, C.H., Callies, C., Rojek, A., Nielsen, S., Cooper, T.G., 2009. Aquaporin isoforms involved in physiological volume regulation of murine spermatozoa. Biol. Reprod. 80, 350–357.
Yeung, C.H., Callies, C., Tuttelmann, F., Kliesch, S., Cooper, T.G., 2010. Aquaporins in the human testis and spermatozoa – identification, involvement in sperm volume regulation and clinical relevance. Int. J. Androl. 33, 629–641.
Zhang, D., Tan, Y.J., Qu, F., Sheng, J.Z., Huang, H.F., 2012. Functions of water channels in male and female reproductive systems. Mol. Asp. Med. 33, 676–690.