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Differential abundances of AQP3 and AQP5 in reproductive tissues from dogs with and without cryptorchidism

Nicola Mirabella [a,1], Alessandra Pelagalli [b,c,1], Giovanna Liguori [a,*], Mohammad Alam Rashedul [d], Caterina Squillacioti [a]

[a] Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Via Delpino 1, 80137, Naples, Italy
[b] Department of Advanced Biomedical Sciences, University of Naples "Federico II", Via Pansini 5, 80131, Naples, Italy
[c] Institute of Biostructures and Bioimages, National Research Council, Via De Amicis 95, 80131, Naples, Italy
[d] Institute for Anatomy and Cell Biology, Department of Medical Cell Biology Justus Liebig University Giessen, Aulweg 123, 35385, Giessen, Germany

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ABSTRACT

Aquaporins (AQPs) are integral transmembrane proteins facilitating transport of water and small solutes, such as glycerol and urea, between cells. In male reproductive tracts, AQPs maintain a milieu conducive for sperm formation, maturation, and storage. The aim of this study was to clarify effects of testicular and epididymal function on male fertility by investigating localisation and abundances of AQP3 and AQP5 in testes and epididymal segments from dogs with and without unilateral cryptorchidism. Immunohistochemistry results indicated AQP3 and AQP5 have different distribution patterns in reproductive tissues of dogs with and without unilateral cryptorchidism. The AQP3, an aquaglyceroprotein, is present in different germ and Sertoli cells in testis of dogs without cryptorchidism. The AQP5 protein was not detected in germ cells but was present in Sertoli and Leydig cells and in endothelia of blood vessels. In cryptorchid dogs, AQP3 was detected in early-developing germ and Sertoli cells, and AQP5 had a distribution pattern similar to testes of dogs without cryptorchidism. In the epididymis, AQP3 and AQP5 were localised in epithelial cells of dogs with and without cryptorchidism in a cell-specific manner. The AQP3 and AQP5 protein was in larger abundance in the gonads from dogs with and without cryptorchidism. In contrast, AQP3 and AQP5 abundance increased in each segment of the cryptorchid epididymis, likely as a compensatory mechanism associated with the pathologic condition. These results indicate involvement of AQP3 and AQP5 in spermatogenesis and sperm maturation. Results from the present study indicate dogs are a useful for comparative reproductive biology studies.

1. Introduction

The flow of water and other small molecules across the epithelium of the male reproductive tract is an important prerequisite to regulate the luminal milieu for spermatogenesis and sperm maturation and for increasing the sperm concentration. This transport is associated with aquaporins (AQPs) in the male reproductive tissues (Carrageta et al., 2020). Aquaporins are small hydrophobic integral transmembrane proteins of a larger family of major intrinsic proteins (MIPs) that are components of pores in the plasma

* Corresponding author.
E-mail address: giovanna.liguori@unina.it (G. Liguori).
[1] The authors contributed equally to the manuscript.

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membrane, facilitating the transport of water, small solutes, and gases between cells (Agre, 2006). On the basis of the permeability of AQPs, these proteins are divided into three groups: 1) water-selective, permeable aquaporins, which are classified as orthodox aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8), that facilitate the transport of water; 2) aquaglyceroproteins, which are permeable to water, glycerol, urea, and other solutes (AQP3, AQP7, AQP9, and AQP10); and 3) subcellular or super-aquaporins, which have a small amount of homology (15 %–32 %) with other AQPs and lack the N-terminal cytoplasmic region (AQP11 and AQP12) (Ishibashi, 2009; Sales et al., 2013; Yeste et al., 2017). Structurally, AQPs are assembled as tetramers that stabilise the position of individual monomers (Agre, 2006), in which each monomer is a functional unit (Preston et al., 1992). Several types of AQPs have been studied in the male reproductive system and each one of of the AQPs has an important function in reproduction, cellular physiology, and gamete cryopreservation (Zhang et al., 2012; Yeste et al., 2017; Carrageta et al., 2020).

Results from previous studies indicated AQPs are present in different tissues of the male mammalian reproductive system. In particular, the AQPs are present in the testes, efferent ducts, epididymis, vas deferens, and accessory glands of humans, laboratory animals, and domestic animals (Mobasheri et al., 2005; Domeniconi et al., 2008; Chen et al., 2011; Arrighi and Aralla, 2015; Pelagalli et al., 2019). In the male gonads, AQPs are present in Sertoli, Leydig, and germ cells, which are important for sperm development, sperm concentration, and epididymal maturation, as well as sperm storage and transport. The distribution of AQPs in different cell types of the mammalian reproductive system (such as germ cells and Sertoli cells in the testis, and principal cells in the different segments of the epididymis) indicates that the functions of the AQPs are cell-specific, and that the regulation of AQP abundances is important in homeostasis as a result of balancing fluid concentration. This balance is directly associated with the production of viable spermatozoa (Boj et al., 2015; Ribeiro et al., 2021).

The purpose of the present study was to evaluate the distribution and functions of AQP3 and AQP5 in the testes and epididymal segments by investigating the presence of these proteins in dogs with and without cryptorchidism. Cryptorchidism is considered the most common birth defect of the male genitalia. It is characterised by the absence of one (unilateral) or both (bilateral) testes in the scrotum (Fawzy et al., 2015) with a reported prevalence of 2.4%–5% in human infants (Amann and Veeramachaneni, 2007; Fawzy et al., 2015) and 1%–11% in dogs, of which 75 % of the cases are of the unilateral type (Amann and Veeramachaneni, 2007). In the cryptorchid condition, the testicular tissues are at a greater temperature, which negatively affects the development and differentiation of somatic and germ cells. Fine histological alterations are observed in epididymal cells; these alterations are characterised by a narrowing of the epidydimal duct, flattening of the epithelial cells, disorganisation of the tubular arrangement, and presence of abundant interstitial tissue throughout the epididymis, with the absence of spermatozoa in the lumen of the duct (Arrotéia et al., 2005). Cryptorchidism can be considered to be a complex disease. This testicular tissues are susceptible from a pathologic perspective with there being the risk of lesser fertility and testicular cancer as a result of the cryptorchid condition. Dogs, therefore, are an important species for conducting comparative and experimental reproductive biology studies.

2. Material and methods

2.1. Animals and tissues

Five adult male dogs without cryptorchidism and five adult male dogs with unilateral cryptorchidism were obtained from the surgical unit of the Department of Veterinary Medicine and Animal Productions of the University of Naples “Federico II”. Dogs without cryptorchidism were healthy (average age = 4.8 ± 1.91 years) and had not previously mated with females and all these dogs were castrated upon request from the owners. In cryptorchid dogs (average age = 4.2 ± 1.64 years), orchiectomy was performed as surgical therapy. The dogs used in this study were of medium size and of different breeds. The testis and epididymis were collected immediately after bilateral orchiectomy using surgical techniques. All the procedures used for tissue collection at the surgical unit were monitored and assured by competent veterinary authorities and were approved by the Ethical Animal Care and Use Committee of the University of Naples “Federico II”, Department of Veterinary Medicine and Animal Production, Naples, Italy (No. 0,050,377). The owners of the animals provided verbal consent for the surgical procedures and collection of the samples. The animals were not involved in any clinical studies nor were they receiving any treatments as a consequence of conducting the surgery. The tissue specimens were divided into two groups: normal testes and epididymis (testes and epididymis from normal dogs), and testes from cryptorchid dogs and epididymis (testes and epididymis collected from dogs with unilateral cryptorchidism). The epididymis was divided into three segments: caput, corpus, and cauda. For Western blot and RT-PCR analyses, all tissues were immediately frozen on dry ice and stored at −80 °C. For immunohistochemical studies, all tissues were immediately fixed with immersion in Bouin’s fixative occurring for 12–24 h.

2.2. Immunohistochemistry: ENVISION system

After fixation for 12–24 h in Bouin’s solution, the samples were paraffin-embedded and cut in slices at a thickness of 3–6 μm. The sections were deparaffinised using 100 % xylene and hydrated using a graded series of ethanol concentrations (100 %, 95 %, 85 %, 75 %, and 70 %). After deparaffinisation, the sections were washed in phosphate buffered saline (PBS), placed in an antigen retrieval solution (citrate buffer, pH 6), and brought to a boil in a microwave. The sections were washed with PBS, treated with 3% H_2O_2 (20 min), washed with PBS once again, and incubated in a humid chamber overnight at 4 °C with the relevant primary antibodies. The primary antibodies used were rabbit polyclonal anti-AQP3 (1:400; rb216020; Biorbyt, San Francisco, CA, USA) and rabbit polyclonal anti-AQP5 (1:400; STJ111968; St John’s laboratory Ltd, London, UK). After washing the sections with PBS, these were incubated with the EnVision system-horseradish antiperoxidase (HRP) (cod. K4002, Dako, Santa Barbara, CA) for 30 min at room temperature. The

slides were subsequently washed with PBS and treated with diaminobenzidine 3, 3′ tetrahydrochloride (DAB) (Vector Laboratories, CA, USA) until there was the desired staining intensity. The tissue sections were then counterstained with haematoxylin for 10 s. The specificity of the primary immunoreactions was tested by replacing each antibody with a buffer or pre-absorbing the antibody with an excess (100 μg/mL antiserum as the final dilution) of the relevant antigen, as described in a previous study (Liguori et al., 2014). No immunoreaction was detected in the control testis tissues. Rat kidney sections were used as a positive control for AQP3, and rat lung sections were used as a positive control for AQP5. Five slides (one slide was selected per ten slides, according to the sequential thickness) for each sample from each animal were independently evaluated by three observers using a Leica DMRA2 microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Western blot and densitometric analyses

Frozen tissue samples were mechanically crushed by using an Ultra-Turrax homogeniser in RIPA buffer containing a protease inhibitor cocktail according to a method described previously (De Luca et al., 2015). The homogenates were centrifuged at 12,000 *g* for 30 min at 4 °C. After centrifugation, the supernatants were collected, and the protein concentration was quantified using a Bradford protein assay (Bio-Rad Laboratories, USA). For western blot analysis, 30 μg of each sample were re-suspended in Laemmli buffer containing beta-mercaptoethanol. The samples were subsequently loaded on a 4%–20% Mini-Protein, TGX stain-free precast gel (Bio-Rad Laboratories, USA). After electrophoretic separation of proteins, the gel was analysed using the ChemiDoc molecular imager (Bio-Rad Laboratories, USA) to confirm whether the proteins had been separated in the gel. The proteins were subsequently transferred to a nitrocellulose membrane using a mini Trans-blot apparatus (Bio-Rad Laboratories, USA), and the transfer was confirmed using the ChemiDoc molecular imager. The nitrocellulose membrane was then blocked using 5% non-fat milk diluted in TBS-T buffer (1.5 M NaCl, 200 mM TRIS−HCL and 0.1 % Tween 20, pH 7.2) at room temperature for 1 h. After washing with TBS-T (10 min each), the membrane was incubated with the primary antibodies diluted by 1:500 overnight at 4 °C (rabbit polyclonal antibodies against AQP3 (rb216020; Biorbyt) and AQP5 (STJ111968; St John's laboratory)). The membrane was washed using TBS-T and incubated with diluted (1:1,000) goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (ImmunoReagents, Raleigh, USA) for 1 h at room temperature. After washing with TBS-T, enhanced chemiluminescence (ECL) (Bio-Rad Laboratories, USA) was used to visualise the proteins, and the images were obtained using the ChemiDoc molecular imager. The standard molecular weight ladder used was Precision Plus Protein™ All Blue Prestained Protein Standards (10–250 kDa, cat. N. 1610373, Bio-Rad Laboratories, USA). Densitometric analysis was performed using the Image lab 6.0 software with normalisation to the total protein concentration for each lane. The results are expressed as the relative intensity to that of the normal tract.

2.4. RNA isolation, cDNA synthesis, and qRT-PCR

Total RNA was extracted from the tissue samples in ice-cold Trizol using an Ultra-Turrax homogeniser. Total RNA (1 μg) was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions, using random hexamers as primers. Specific primers that amplify 200 base pair regions of the AQP3 and AQP5 genes were designed using the published GenBank gene sequences of *Canis lupus familiaris* AQP3 and AQP5 mRNA utilising Primer Express and Primer3. The GenBank accession number and sequences of the primers are listed in Table 1.

The qRT-PCR solution contents were 1 μL of cDNA (50 ng/well) and 24 μL of SYBR green master mix (Applied Biosystems) containing specific primers. The PCR conditions used were as follows: 50 °C for 2 min, 94 °C for 10 min, followed by 40 cycles at 94 °C for 15 s and 60 °C for 1 min. The *GAPDH* gene was amplified in separate wells using the same conditions to serve as an active endogenous reference for normalisation. Real-time detection was performed using an ABIPRISM 7300 Sequence Detection System (Applied Biosystem), and data from the SYBR Green I PCR amplicons was assessed using the ABI 7300 System SDS Software. The relative quantification $2_{-\Delta\Delta Ct}$ method was used for determination of the relative mRNA transcript abundance, as described previously (Squillacioti et al., 2016).

2.5. Statistical analysis

For densitometric and qRT-PCR analyses, data are expressed as the mean ± standard deviation (SD). The differences were determined in protein or mRNA transcript abundances between different segments of the epididymis in dogs with and without cryptorchidism using a one-way ANOVA, followed by use of the Tukey's honestly significant difference (HDS) test for the independent

Table 1
Forward and reverse primer sequences of the target and reference genes.

Primer		Sequence	Accession number
AQP3	Forward	5′-CCC TCT GGA CAC TTG GAC AT-3′	XM_849503.5
	Reverse	5′-ATG GAA GTG CCG ATA ACC AG-3′	
AQP5	Forward	5′-ACT TCT ACC TGC TAT TCC CCA ACT C-3′	XM_543677.4
	Reverse	5′-CAT TTT TTT CCT CCT GTG GTT CA-3′	
GAPDH	Forward	5′-TGT CCC CAC CCC CAA TG-3′	XM_003434387.5
	Reverse	5′-TCG TAT ATT TGG CAG CTT TCT-3′	

samples. The differences between values for variables in tissues of dogs with and without cryptorchidism were determined using an unpaired student's *t*-test. There were considered to be differences when there was a $P < 0.05$ when data were analysed for all experiments. Statistical analyses were performed using the SPSS software version 25.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Immunohistochemical analysis of AQP3 and AQP5 in the reproductive tissues of dogs with and without cryptorchidism

Results from immunohistochemical analysis indicated that in testis of dogs without cryptorchidism, AQP3 was present at all stages of the seminiferous epithelium cycle, from Stages I–VIII, in dogs. There was detection of AQP3-immunoreactivity (IR) in different

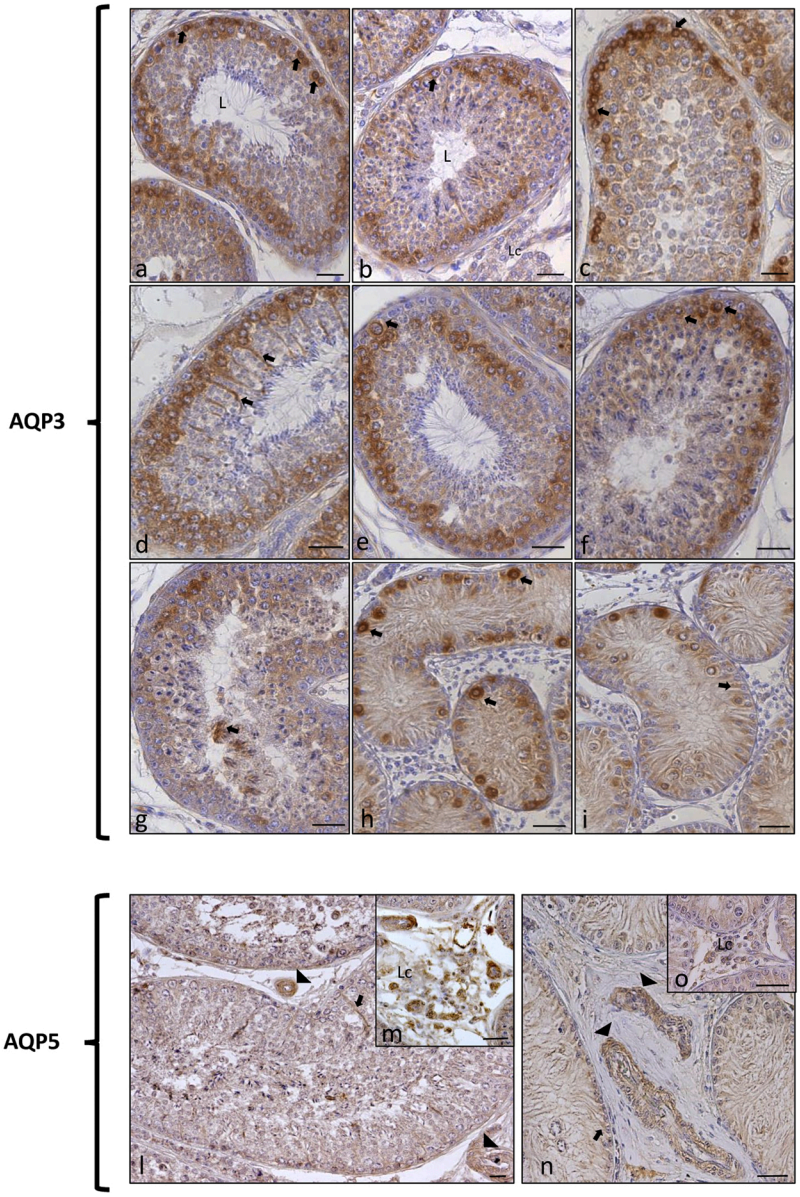

Fig. 1. Immunohistochemical staining of AQP3 (a–i) and AQP5 (l–o) in dog testis without cryptorchidism (a–f; l, m) and with cryptorchidism (h, i; n, o); AQP3 positivity (arrow) was distributed in different cytotypes of the seminiferous tubules; a. Type A spermatogonium; b. Type-b spermatogonium; c. group of pachytene spermatocytes; d. Sertoli cells; e. Pre-leptotene/Leptotene spermatocytes; f: Zygotene spermatocytes; g. elongated or mature spermatids; AQP3 was located in early germ cells (h-I; arrow) and Sertoli cells (I; arrow) of the testis from dogs with cryptorchidism. AQP5 immunostaining was detected in the blood vessels (arrowhead), Sertoli cells (l; arrow), and Leydig cells (Lc) of the testis of dogs without cryptorchidism (l, m) and dogs with cryptorchidism (n, o); L: lumen; Scale bar represents 50 μm.

testicular cytotypes. In particular, Type A and type B spermatogonia, which are localised near the basal lamina of the seminiferous vesicle, were AQP3-positive (Fig. 1a and b). Immunopositivity for AQP3 was also observed in Sertoli cells, which are essential for sperm formation during spermiogenesis (Fig. 1c). The AQP3 protein was also detected in pachytene spermatocytes, located between round spermatids and the basal lamina (Fig. 1d). In addition to these cytotypes, pre-leptotene/leptotene spermatocytes (originating from Type B spermatogonia) also were AQP3 immunopositive (Fig. 1e). The positive staining appeared as small granular fragments in the peri-nuclear cytoplasm. Zygotene spermatocytes that were undergoing transition into pachytene spermatocytes also contained AQP3 in dogs without cryptorchidism (Fig. 1f). There was also staining in the cytoplasm of elongated or mature spermatids localised in the luminal portion of the seminiferous tubules (Fig. 1g).

In testes of dogs with cryptorchidism, AQP3 was detected in undifferentiated germ cells that were in the early developmental stages (Fig. 1h) located within the centre of seminiferous cords, which are in a mitotic arrest state. In addition to these cells, there was AQP3 in some Sertoli cells (Fig. 1i).

The AQP3 protein was localised in the apical portion of the principal cells in all segments of the dogs without cryptorchidism (Fig. 2a–c) and the epididymis of dogs with cryptorchidism (Fig. 2d–f). The AQP3 protein was also detected in basal and narrow cells of the caput epididymis in both dogs with and without cryptorchidism (Fig. 2a, d). Furthermore, there was a small amount of staining of AQP3 in narrow cells in all segments.

There was the largest amount of staining of AQP5 in endothelial cells of the blood vessels and in some Leydig cells (Fig. 1l, m) of the testes in dogs with and without cryptorchidism (Fig. 1n, o), but not in germ cells. In testes of dogs with cryptorchidism, there was only a small amount of AQP5 staining in Sertoli cells, while there was a large amount of AQP5 staining in endothelial cells of the blood vessels (Fig. 1n, o), as well as in interstitial Leydig cells (Fig. 1o). There was staining of AQP5 in all epididymal segments (Fig. 2g–i) of dogs with cryptorchidism (Fig. 2h–m). The staining for AQP5 was distributed in the apical portion of the principal and basal cells. The AQP5 protein was also localised in the narrow cells of the corpus epididymis of dogs without cryptorchidism (Fig. 2h, arrow).

3.2. *Western blot and densitometric analysis of AQP3 and AQP5 in the reproductive tissues from dogs with and without cryptorchidism*

Results from Western blot analysis indicated AQP3 and AQP5 proteins are present in the testes and in all segments of the epididymis in both dogs without and with cryptorchidism. The AQP3 protein was detected as one band (40–42 kDa) in all samples and AQP5 was detected as two bands (37 and 47 kDa) (Fig. 3A). Results from densitometric analysis indicated there were lesser abundances of AQP3

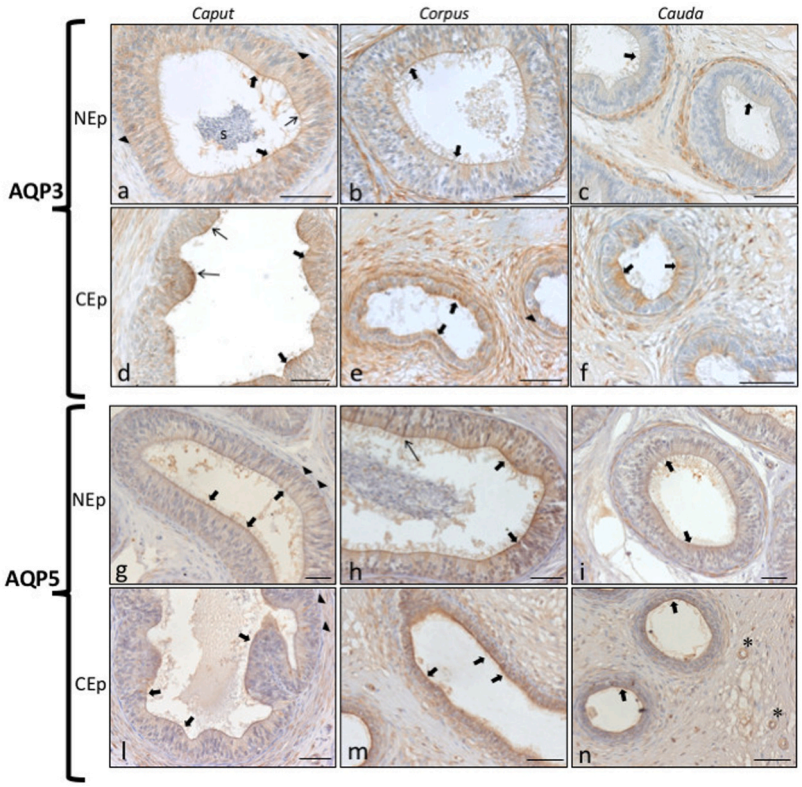

Fig. 2. Immunohistochemical staining of AQP3 (a–f) and AQP5 (g–n) in epididymal tissues of dogs without cryptorchidism (a–c; g–i) and with cryptorchidism (d, f; l–n); The staining was distributed throughout the apical portion of principal cells (arrow), basal cells (arrowhead), some narrow cells (line arrow), and blood vessels (asterisk); S: spermatozoa; NEp: Epididymis of dogs without cryptorchidism; CEp: Epididymis of dogs with cryptorchidism; Scale bar represents 50 μm.

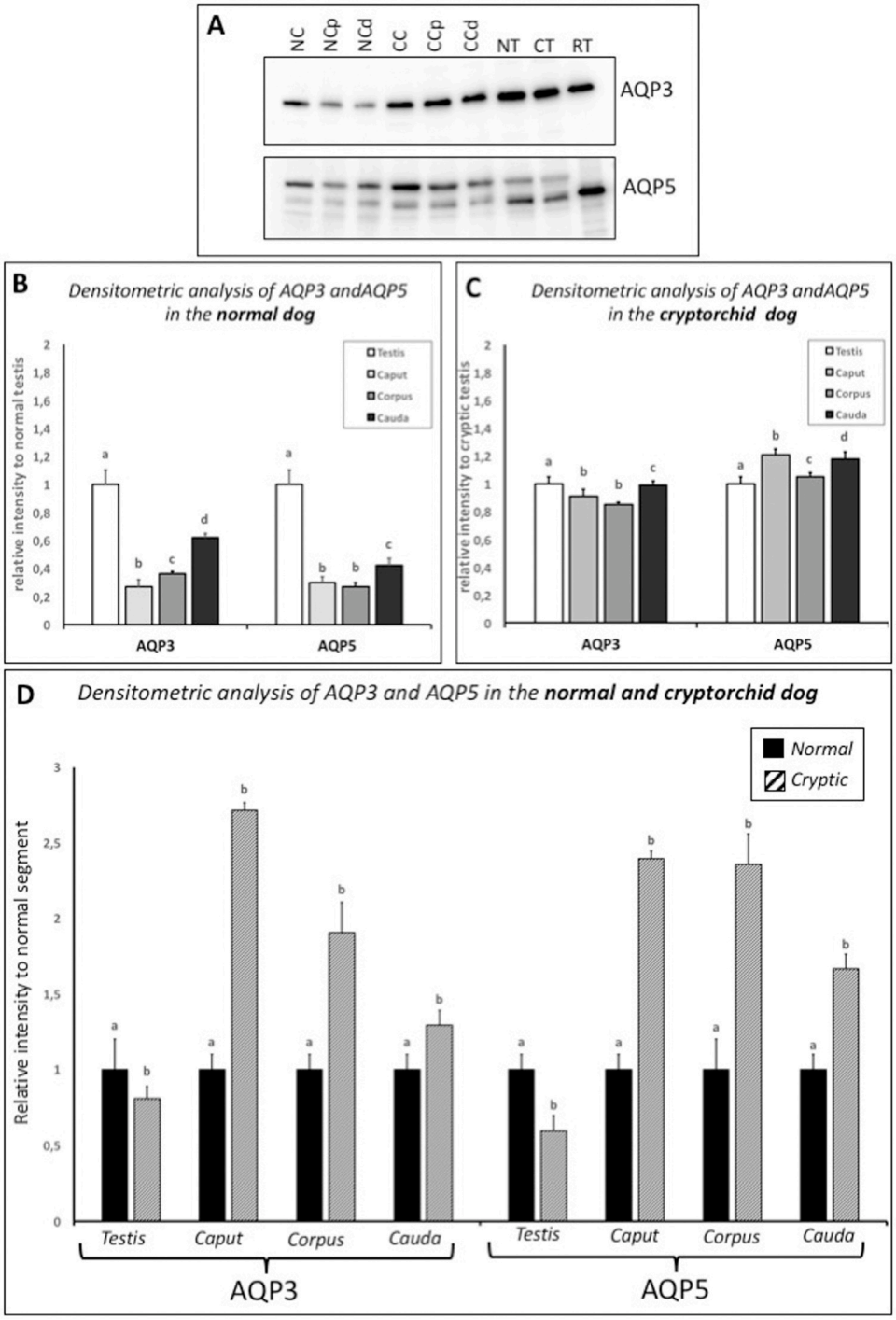

Fig. 3. Western blot (A) and densitometric analysis of the AQP3 and AQP5 abundances in dogs without crypotorchidism (B) and dogs with cryptorchidism (C, D); Densitometric analysis was performed using stain-free technology with normalisation to the total protein density in each lane; Results are expressed as relative intensity; The intensity of the protein band in different samples is relative to that of the testis of dogs without cryptorchidism (B) and to that of the testis of dogs with cryptorchidism (C); The intensity of the band in each segment of dogs with cryptorchidism is relative to that of each segment in dogs without cryptorchidism (D); Each plotted value corresponds to the mean ± standard deviation (SD) obtained from three independent experiments; Different letters depict the differences between the examined groups ($P < 0.05$); NC: caput dogs without cryptorchidism; NCp: corpus dogs without cryptorchidism; NCd; cauda dogs without cryptorchidism; CC: caput of dogs with cryptorchidism; CCp: corpus dogs with cryptorchidism; CCd; cauda dogs with cryptorchidism; NT: testis of dogs without cryptorchidism; CT: testis of dogs with cryptorchidism; RT: rat testis used as a positive control.

and AQP5 protein in different segments of the epididymis in dogs without cryptorchidism, compared with the testicular tissues of dogs without cryptorchidism (Fig. 3B). In addition, there was an increase in the abundance of AQP3 and AQP5 protein from the caput to the cauda sections of the epididymis (Fig. 3B).

In cryptorchid dogs, the abundance of AQP3 protein was similar to that in dogs without cryptorchidism and, there was consistently a larger abundance of AQP5 protein in the epididymis than testis of cryptorchid dogs (Fig. 3C). The results from densitometric analysis of tracts from the dogs without cryptorchidism compared to dogs with cryptorchidism indicated there was a lesser abundance of AQP3 and AQP5 protein in the testis of dogs with cryptorchidism. In contrast, there was a larger abundance of AQP3 and AQP5 in all epididymis segments of dogs with compared to those without cryptorchidism (Fig. 3D).

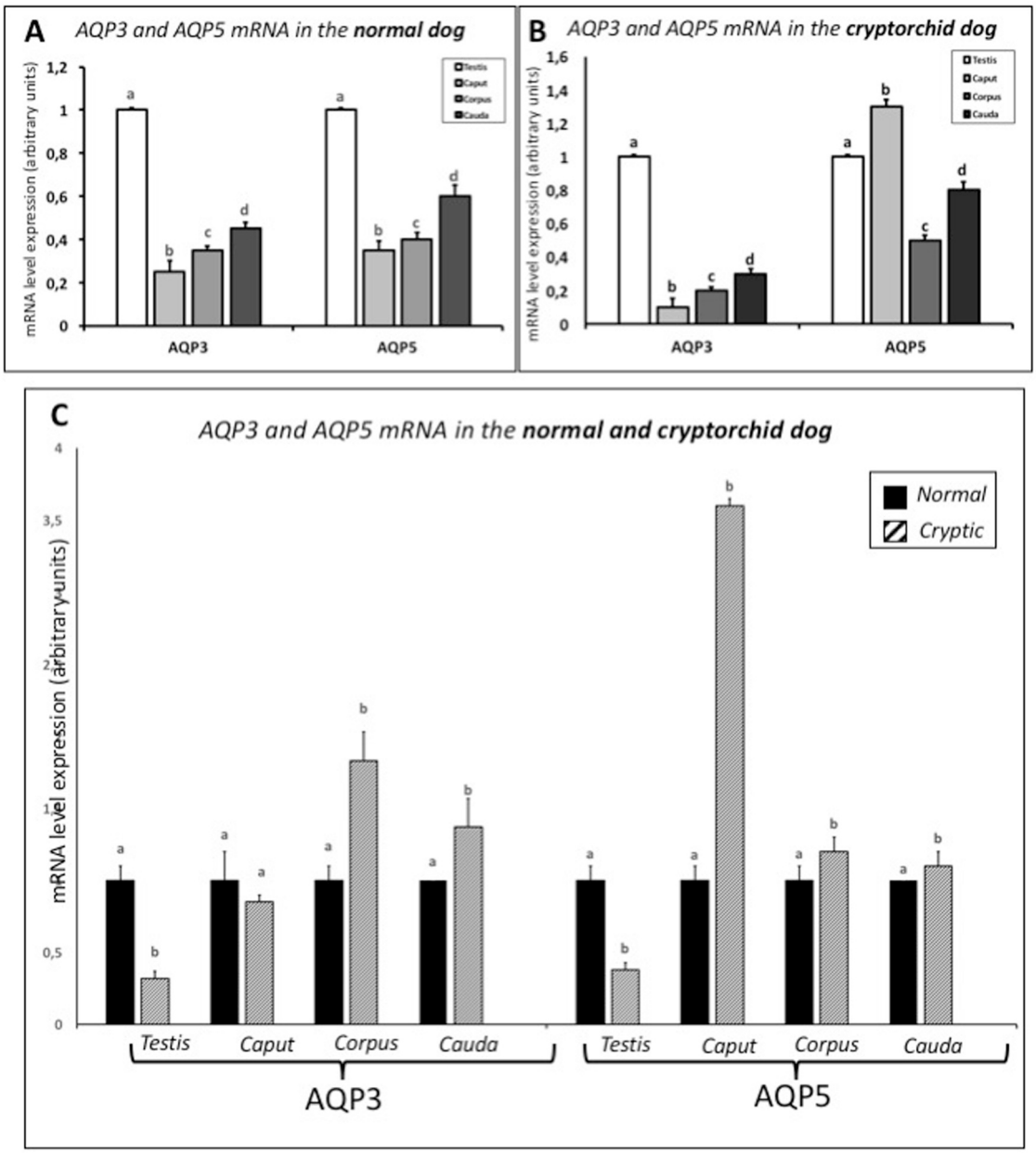

Fig. 4. AQP3 and AQP5 mRNA abundances in dogs with cryptorchidism (A, C) and dogs without cryptorchidism (B, C); A. Normalised to that in the testis of dogs without cryptorchidism; B. Abundance normalised to that in the testis of dogs with cryptorchidism; C. Abundances normalised to that in the segment of testis of dogs without cryptorchidism; Each plotted value corresponds to the mean ± standard deviation (SD) obtained from conducting three independent experiments; Different letters depict differences between the examined groups ($P < 0.05$).

3.3. Abundances of AQP3 and AQP5 transcripts in the reproductive tissues from dogs with and without unilateral cryptorchidism

Results from qRT-PCR analysis indicated the relative abundances of AQP3 and AQP5 mRNA transcripts were less ($P < 0.05$) in the different segments of the epididymis than in the testes of dogs with and without cryptorchidism (Fig. 4a; b). In addition, there was an increase ($P < 0.05$) in abundance of both transcripts from the caput to the cauda sections of the epididymis. In cryptorchid dogs, relative abundance of AQP5 mRNA transcript was larger ($P < 0.05$) in the caput of the epididymis than in the testicular samples (Fig. 4b). Results from the qRT-PCR analysis also indicated there was a lesser AQP3 and AQP5 abundance in the gonads of cryptorchid compared to the dogs without cryptorchidism, and a larger abundance of both AQP3 and AQP5 mRNA transcripts in the different cryptorchid epididymal segments, compared to that in dogs without cryptorchidism (Fig. 4c).

4. Discussion

In the present study, there was investigation of the distribution and abundances of AQP3 and AQP5 protein in the testis and epididymis of dogs with and without cryptorchidism. Results from immunohistochemical analysis indicated AQP3 is present in testis and in all segments of the epididymis of dogs without cryptorchidism. During spermatogenesis, and especially during the maturation process from round to elongated spermatids, one of the most distinct morphological changes is a marked reduction in the germ cell volume. There is a large abundance of AQP3 protein and mRNA transcript in the testes and this protein is important in the regulation of sperm volume and in the differentiation of spermatids into spermatozoa during spermiogenesis (Chen et al., 2011; Yeste et al., 2017). There were different cytotypes in testes of dogs without cryptorchidism. These findings indicate AQP3 is involved in the exchange of water and glycerol across the plasma membrane in the male reproductive tract and this protein likely has important functions during spermatogenesis (Chen et al., 2011; Crisóstomo et al., 2017). In the gonads retained in the body cavity of cryptorchid dogs, there was AQP3 in undifferentiated germ cells during the early developmental stages, as well as in Sertoli cells. In this pathological condition, AQP3 had a distribution pattern similar to that of dogs without cryptorchidism and AQP3 was also in lesser abundance, indicating AQP3 is probably essential for sperm formation. The distribution pattern of AQP5-IR was similar in the testis of dogs with and without cryptorchidism in the present study, however, this distribution was very different from that of AQP3. The AQP5 protein is water-selective and was present in the blood vessel endothelium and Sertoli cells, as well as in interstitial Leydig cells, but not in germ cells of testes of dogs without cryptorchidism. In the gonads retained in the body cavity of cryptorchid dogs, the abundance of AQP5 was similar to that in the testes of dogs without cryptorchidism. In addition, the abundances of AQP5 protein and mRNA was less in the testis of dogs with compared with dogs without cryptorchidism, indicating AQP5 is also involved in spermatogenesis, similar to AQP3. The AQP5 protein has also been reported to be present in avian testis (Skowronski et al., 2009), horse testis and epididymis (Klein et al., 2013), and the rat epididymis; however, the function of this protein in the human male reproductive system is still unknown (Hermo et al., 2008). The function(s) of AQP5 in Leydig cells, including in modulation of endocrine function, is unclear. The function of AQP5 in the testes is thought to be related to water reabsorption/secretion during sperm maturation. The AQP3 and AQP5 may have an important function in fluid maintenance in the male gonad microenvironment in which there is sperm formation and, therefore, the capacity for fertility. Differences in the AQP3 and AQP5 abundances are a result of the cryptorchidism-associated alteration of the expression of the genes encoding for these AQPs (i.e., hormonal regulation, susceptibility to tumorigenesis, and inflammatory processes associated with cryptorchidism). In addition to androgens, oestrogens can regulate the development and function of the male reproductive tract (Hess et al., 1997; Cooke et al., 2017). The androgen-oestrogen imbalance can lead to suppression of spermatogenesis as a result of cryptorchidism (Mizuno et al., 2011). Hormonal alterations in the male reproductive system might be associated with altered AQP abundances and functions.

Dogs with as compared with those without cryptorchidism are at a greater risk of developing testicular cancer later in life. Testicular cancer is the second most common cancer in older male dogs, and the risk of developing testicular cancer in dogs is 13 % greater in dogs with compared with those without cryptorchidism. The most common testicular cancer are Sertoli cell tumours, interstitial cell tumours, and seminomas (Hayes et al., 1985; Reif and Brodey, 1969). Cryptorchidism is also an established risk factor for infertility and testicular germ cell tumours in men (Ferguson and Agoulnik, 2013). The AQPs are associated with carcinogenesis, and specifically, in tumour angiogenesis and tumour cells (Ribatti et al., 2014; Marlar et al., 2017; de Ieso and Yool, 2018). The AQP3 and AQP5 proteins are in marked abundances in cancer tissues. The AQP5 protein facilitates H_2O_2 permeation, affecting the adaption of cancer cells to oxidative stress and inducing cancer cell migration, thus indicating that it is a promising drug target for cancer therapies (Prata et al., 2019; Rodrigues et al., 2019). Results from some studies, however, have indicated there is a correlation between AQP3 and AQP5 abundances and tumour stage and grade, with AQP3 abundances being less or there being an absence of this protein in high-grade tumours and in human urothelial and prostatic carcinoma (Rubenwolf et al., 2014; Bründl et al., 2018). In the present study, there was a lesser abundance of AQP in testis of cryptorchid dogs, indicating these proteins could regulate the cancer biological processes in the testis.

The AQP3 and AQP5 proteins were detected in all epididymal segments from dogs with and without cryptorchidism, indicating these proteins might have important physiologic functions in this organ. The abundances of both proteins were less in the different segments of the normal epididymis, compared to those in the testes. There, however, was an increasing abundance of these AQPs from the caput to the cauda sections of the epididymis. In cryptorchid dogs, the patterns of abundances of these proteins were similar as those in dogs without cryptorchidism. These results are indicative that AQP3 and AQP5 are essential for sperm production, as well as for sperm maturation, transport, and storage in the epididymis. The differential abundances of the AQPs detected in the cauda epididymis may be related to the reabsorption of a large amount of fluid leaving the testes in the distal epidydimal segments to concentrate sperm and enhance the maturation capacity of these gametes (Robaire et al., 2006). In the epididymis, water and solute

transport as a result of the actions of AQPs are essential to establish a milieu that is conducive for sperm maturation and storage (Yeste et al., 2017).

There have been inconsistent results regarding AQP3 abundance in the rat epididymis. Hermo et al. (2004) reported the localisation of AQP3 in epididymal basal cells, whereas Da Silva et al. (2006) did not detect AQP3 in the rat epididymis. The results from the present study are somewhat consistent with the findings that there was AQP3 present in rat epididymal basal cells (Hermo et al., 2004). In addition, in the present study, there was detection of AQP3 in the apical portion of the principal cells, as well as in narrow cells. Because AQP3 is an aquaglyceroporin, its presence in basal cells and principal cells may contribute to the active transport of glycerol from the epithelium to the lumen, where these molecules are essential for sperm maturation (Robaire et al., 2006; Hermo and Smith, 2011; Arrighi and Aralla, 2015; Yeste et al., 2017). Similarly, AQP5 immunolabeling was observed in the cytoplasmic apical portion of the principal, basal, and narrow cells of the epidydimal segments of dogs without cryptorchidism in a region-specific manner. There are also similar reports that there is AQP5 in the apical portion of the principal cells in the rat epididymis (Da Silva et al., 2006) but not in that of cats (Arrighi and Aralla, 2015). Although the presence of AQP5 in narrow cells has not been validated and is not related to its function, these cells might also be involved in fluid secretion and the reabsorption process, in addition to the H^+-secretion capacity. Additionally, there was AQP3 and AQP5 in the basal cells of the epididymal segments of cryptorchid dogs. It is possible that the testicular luminal factor did not have effects on the presence of these AQPs in basal cells; hence, cryptorchidism may not affect the abundances of these proteins in these cells (Arrighi and Aralla, 2015).

In cryptorchid dogs, AQP3 and AQP5 were in larger abundances in each epididymal segment; in particular, AQP5 abundance was markedly greater in the caput. There is differential gene expression in different segments of the epididymis consistent with the specific functions of these proteins. The caput epididymis is the most active segment for protein synthesis and secretion in several species and is the region where there is initiation of spermatozoa maturation (Robaire et al., 2006). There were similar results when there were studies conducted on the horse genital tract (Klein et al., 2013) and in the testes of dogs with and without cryptorchidism (Pelagalli et al., 2019), in which there were differential abundances of AQP isoforms in the different segments, indicating region-specific distributions. Dube et al. (2010) reported there was suppressed expression of different genes, including *AQP5*, in the caput epididymis in humans with obstructive azoospermia.

Based on the results from the present study, it is hypothesised that the cryptorchid condition is characterised by a dysregulation or alteration of the AQP3 and AQP5 gene expression patterns. This alteration depends on whether regulatory molecules are present in the absence of spermatozoa in the lumen of the epididymis of dogs with cryptorchidism and/or when there is an increased temperature environment, which is characteristic of this condition. The interaction of the sperm cells with epididymal epithelial cells is another control factor, in addition to lumicrine factors (Garrett et al., 1990). The functional changes in functions when there was an increased abundance of AQP3 and AQP5 protein in the epididymis of dogs with cryptorchidism could be related to the reabsorption of a large amount of fluid leaving the testes in the epidydimal segments to concentrate the seminal liquid without spermatozoa, as a compensatory mechanism. In the gonadal tissues of dogs with cryptorchidism, there are greater temperatures as a result of core body temperatures being greater than those in testicular tissues located in the scrotum and it could lead to heat-induced changes in the spermatogenic process, which affect sperm production and the expression of relevant genes (Durairajanayagam et al., 2015; Bertolla et al., 2006; Li et al., 2006; Liu, 2010; Rizzoto et al., 2020;). Heat-stress also affects sperm maturation in the rat epididymis as a result of altered expression in the epididymal epithelia and fluids (Wang et al., 2016).

5. Conclusion

The present study is the first on the localisation and abundances of AQP3 and AQP5 in the testis and epididymis of dogs with and without cryptorchidism. The abundances of these proteins were different in the testes of dogs with and without cryptorchidism and in the various epididymal segments of these dogs. In the cryptorchid dogs, these aquaporins are in lesser abundance in the testes but are of a larger abundance in the epididymal segment. These alterations in the abundances of AQP3 and AQP5 can contribute to the pathogenesis of testicular cancers. In future studies, the causes of the difference in the abundances of aquaporins should be further studied and clarified by investigating the pathways involved in heat stress and/or oxidative stress associated with this condition and the pathogenesis of testicular cancer.

Author contributions

NM, AP, GL, and CS contributed to the design and implementation of the research, interpretation of results and to the critical review of the manuscript draft; MRA performed immunohistochemistry, western blotting, and real-time RT-PCR. All authors discussed the results and contributed to writing the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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