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AQUAPORIN 9'S CELLULAR DISTRIBUTION IN THE TESTIS AND EPIDIDYMIS OF CAMELS (*Camelus dromedarius*) DURING AND AFTER RUTTING SEASON

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ABSTRACT

Numerous cell types of the male genital system involved in fluid transport have tiny intrinsic membrane proteins called aquaporins (AQPs). These proteins are necessary to create the ideal luminal environment for sperm formation, maturation, and preservation. Aquaporin 9 (AQP9) allows water to move quickly across the epithelium. The current work used immunohistochemistry to clarify the expression of AQP9 in the testis (cranial and caudal parts and rete testis) and epididymis (caput, corpus, and cauda parts) of dromedary camels in the rutting and non-rutting seasons throughout the year. The results demonstrate that testicular gonocytes, Leydig cells, and rete testis and also the epididymal epithelial cells often exhibit a moderate immunoreaction to AQP9 antibodies during the mid-rutting season. However, these cells also show much higher expression levels at the beginning and end of this season. Throughout the non-rutting season, these organs exhibit intense immunoreactive staining of the AQP9 protein. In conclusion, transmembrane water and neutral solute transport via AQP9 is an essential physiological route in the testis and epididymis of the dromedary camels for spermiogenesis.

Key words: Aquaporin 9, distribution, Dromedary camel, epididymis, testis

Most living things, including people, animals, plants, and even lower species, contain the thirteen subtypes (AQP0–12) of the essential transmembrane protein family known as aquaporins (AQPs) (Azad *et al,* 2021; Shivaraj *et al,* 2017; Verkman, 2012). They regulated numerous physiological processes within cells, such as cell migration and proliferation, body water homeostasis, exocrine fluid secretion, and the transport of nutrients and other functional molecules into cells, along with the removal of metabolic residues (Ribeiro *et al,* 2021; Meli *et al,* 2018; Yu *et al,* 2014; Verkman, 2012). Many AQPs in the male reproductive system may be essential to the ordinary course of reproductive processes (Calamita *et al,* 2001). Furthermore, AQPs have been proposed as potential biomarkers for sperm freezability and fertility in the future (Yeste *et al,* 2017).

AQP9, one of the AQPs found in the male reproductive tract, is thought to play a significant role in the apical pathway for transmembrane fluxes of water and other solutes, including purines, pirimidines, carbimides, and polyols, which are collectively known as aquaglyceroporins (Matsuzaki *et al,* 2002; Tsukaguchi *et al,* 1998). AQP9's expression was inestigated in the testis and epididymis of several species, such as humans, bovines, buffalo bulls, wild ruminant species, dogs, agouti, rats, and adult mice (Oberska *et al,* 2024; Martinez-Madrid *et al,* 2023; Mohamed *et al,* 2022; Schimming *et al,* 2021; Domeniconi *et al,* 2007; Badran and Hermo, 2002; Pastor-Soler *et al,* 2001).

No data exist about this protein's expression in the camel's male reproductive organs. Thus, the current study's goal was to precisely locate AQP9 in various parts of the dromedary camel's testis and epididymis, to investigate the expression of these proteins which vary throughout the rutting season, and to compare this expression to that of non-rutting seasons using immunohistochemistry method (IHC).

Materials and Methods

Sampling

All procedures involving animal samples were conducted under the strict animal protocol approved by the ethical committee of King Faisal University. Thirty-six mature, healthy local bread dromedary camels (aged 4≥ years old or older) from the Al Omran abattoir in Al-Ahsa, Saudi Arabia, provided samples taken every two months for a year. Tissue samples were taken from the testes (cranial and caudal portions of the testis and rete testis) and caput,

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corpus, and cauda of the epididymis. Samples were kept in 10% buffered formalin for the IHC procedure.

IHC procedure

After being fixed in formalin, tissue samples were dehydrated in graded ethanol, cleaned via xylene, and embedded inside the paraffin wax. Sections of 5 µm were cut with a microtome and placed on Superfrost slides. Slides were stained using the avidin-biotin-peroxidase complex method after dewaxing and rehydrating (Adeghate *et al,* 2001). Antigen retrieval was carried out in a microwave oven for fifteen minutes using 0.01M PBS (pH 7.4). Subsequently, the sections were cooled to 25◦ C and given another PBS wash. 3% hydrogen peroxide was used for 30 minutes to suppress endogenous peroxidase. Goat serum (10%) was used for 20 minutes after three rounds of washing in PBS to prevent non-specific responses. After applying the primary antibody, polyclonal rabbit anti-AQP9 (Abcam, dilution 1:200, Cambridge, Cambridgeshire, UK), and the sample was incubated in a wet chamber for the whole night. The sections were treated with biotin-labeled secondary antibody and avidin-horseradish peroxidase (HRP). Dibutyl phthalate polystyrene xylene (DAB) was utilized to identify the positive staining. Section counter-staining was done using haematoxylin stain. The negative control sections follow the identical protocol except for skipping the primary antibody. Slides were examined under light microscopy for immunohistological investigations, and photomicrographs were taken.

Results

In all examined animals, immunohistochemical staining showed the existence of AQP 9 in the testis and epididymis of dromedary camels throughout both the rutting and non-rutting seasons. Tables 1 and 2 represented the localisation and intensity of AQP9 in these organs throughout the rutting and non-rutting seasons, respectively.

Rutting season

At the onset of the rutting season (October), the dromedary camel's testis and epididymis had varying reactions to the AQP 9 antibodies (Figs 1 & 2). In the testis, AQP 9 was localised moderately in the gonocyte and Sertoli cells lining the seminiferous tubules and the interstitial cells (Leydig cells) at the cranial portion and reti testis of the testis, while the protein expressed very strongly in the caudal portion (Fig 1A, 1B, 1C). The stereocilia pseudostratified columnar epithelium of the epididymis showed varying intensities of AQP9 immunoreactivity: weak in caput, very strong in the corpus, and strong in the cauda (Fig 2A, 2B, 2C).

Table 1. Showing AQP9 localisation in various parts of the dromedary camel's testis and epididymis during the rutting season.

Part / Month	T cr	T caud	T ret	EH	EB	ET
October	+ +	+ + + +	+ +	+	+ + + +	+ + +
December	+ +	+ +	+ +	+ +	+ +	+ +
February	+ + +	+ + +	+ +	+ + +	+ + +	+ + +

T cr, cranial part of the testis; T caud, caudal part of the testis; T ret, rete testis; EH, caput of epididymis, EB, corpus of epididymis, and ET, cauda of epididymis; +, weak reaction; + +, moderate reaction; + + +, strong reaction; + + + +, very strong reaction.

Table 2. Showing AQP9 distribution in various parts of the dromedary camel's testis and epididymis during the non-rutting season.

Part / Month	T cr	T caud	T ret	EH	EB	ET
April	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
June	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
August	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

T cr, cranial part of the testis; T caud, caudal part of the testis; T ret, rete testis; EH, caput of epididymis, EB, corpus of epididymis, and ET, cauda of epididymis; +, weak reaction; + +, moderate reaction; + + +, strong reaction; + + + +, very strong reaction.

In the middle of the rutting season (December), the lining epithelial cells of the seminiferous tubules and Leydig cells of the testis and the pseudostratified epithelium of the epididymis displayed moderate staining to the AQP 9 protein (Figs 1D, 1E, 1F, 2D, 2E, 2F). The epididymal sperm in the body and tail also had a positive reaction to this protein (Figs 2E, 2F).

At the end period of the rutting season (February), the lining epithelium of the seminiferous tubules and Leydig cells in the testis, the epithelial cells of all parts of the epididymis, and the epididymal sperm showed expressed AQP 9 strongly, while the rete testis epithelium remained moderately reactive to this protein (Fig 1G, 1H, 1I, 2G, 2H, 2I).

Non-rutting season

In the non-rutting season (April- September), the epithelial cells of the seminiferous tubules and interstitial cells in all parts of the testis and epididymis' epithelium in the dromedary camel displayed strong responses to the AQP 9 antibody with greater affinity in April (Figs 3 & 4). During this phase, epididymal sperm was highly reactive to AQP 9 antibody (Figs 3 & 4).

Fig 1. A photomicrograph of the dromedary camel's testis taken during the rutting season. In October, it showed a moderate immunoreactive of AQP9 in the lining epithelium (arrow) and Leydig cells (red arrow) of the cranial (A) and reti testis (C) parts, while the caudal part (B) showed extreme reaction to the AQP9 antibody. In December, all parts of the testis displayed moderate staining (D, E, F). At the end of the rutting season (February), the lining epithelium of the seminiferous tubules in the testis showed expressed AQP 9 strongly, while the rete testis epithelium remained moderately reactive to this protein (G, H, I). Negative control for the cranial and caudal parts of the testis and rete testis (J, K, L).

Fig 2. A photomicrograph of the dromedary camel's epididymis at the rutting season's beginning, middle, and end. In October, different levels of AQP9 immunoreactivity were seen in the lining epithelium (arrow): faint in the head, highly expressed in the body, and intense in the tail (A, B, C). In December, the AQP 9 protein showed notable staining in the lining epithelium of the epididymis's head, body, and tail (arrow) (D, E, F). The epididymal sperm in the body and tail reacted positively to this protein (arrowhead) (E, F). In February, the epididymal sperm (arrowhead) and the lining epithelium of all parts of the epididymis (arrow) demonstrated significantly expressed AQP 9 (G, H, I). Negative control for the epididymis's head, body, and tail (J, K, L).

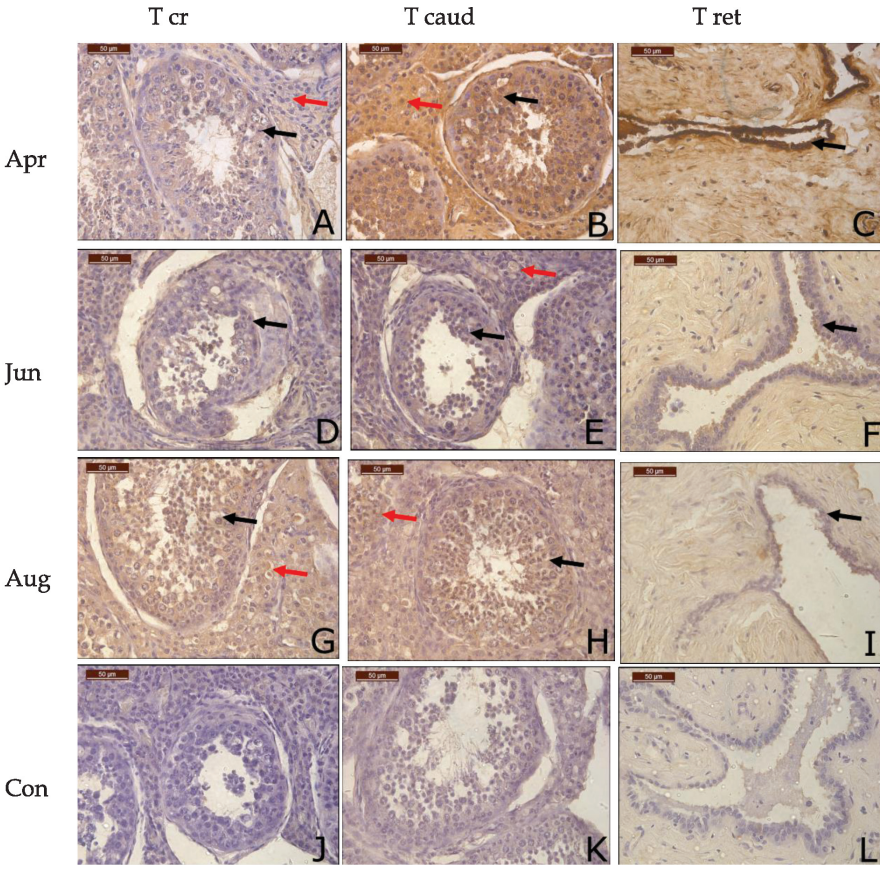

Fig 3. Fig 1: A photomicrograph of the dromedary camel's testis throughout the non-rutting season. In April, AQP9 protein expressed highly significant immunoreactive in the lining epithelium (arrow) and Leydig cells (red arrow) in the caudal (B) and reti testis (C) parts, while in the cranial part (A) is strong. The rest of the period shows a very strong in all parts of the testis (D, E, F, G, H, I). Negative control for the cranial and caudal parts of the testis and rete testis (J, K, L).

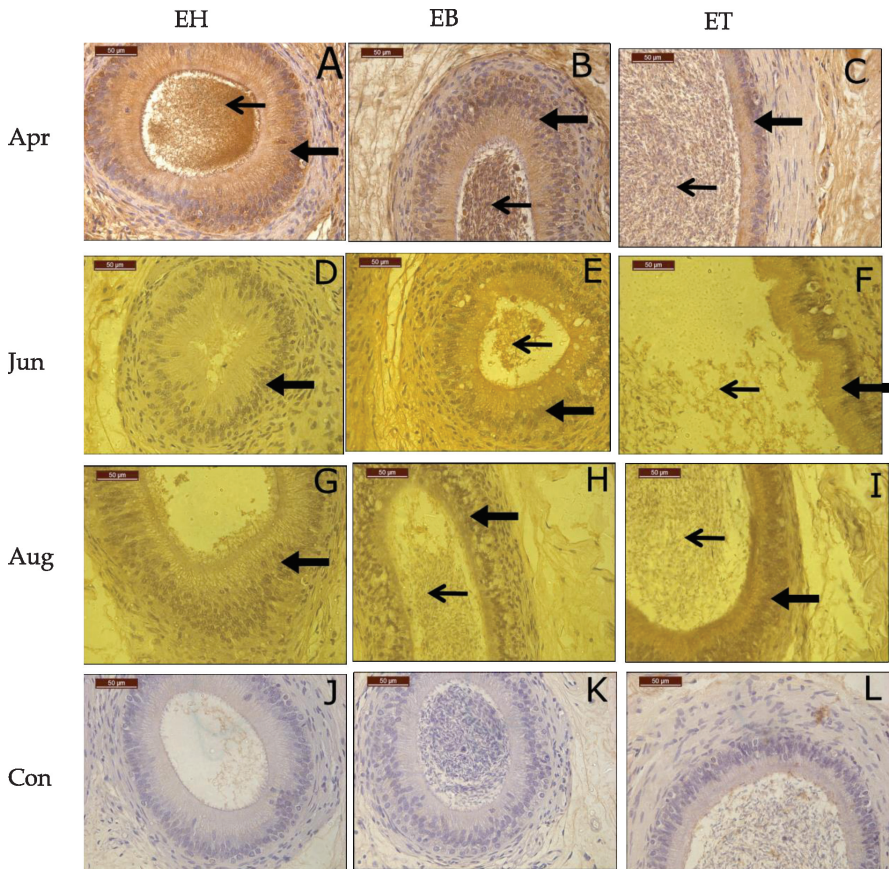

Fig 4. A photomicrograph of the dromedary camel's epididymis throughout the non-rutting season. In April, extreme levels of AQP9 immunoreactivity were seen in the lining epithelium (arrow) and epididymal sperm (arrowhead) in the head, body, and tail (A, B, C). The remaining time duration exhibits a strong in the lining epithelium and luminal sperm of each of the epididymis's parts (D, E, F, G, H, and I). Negative control for the epididymis's head, body, and tail (J, K, L).

Discussion

Our groundbreaking study, the first of its kind, delves into the AQP9 protein expressed in the male genital tract of camels. This research, which unveils the protein's localisation within the target cells, promises to illuminate the intricate roles of AQP9 in crucial fluid secretion and absorption processes, particularly in relation to spermatogenesis, sperm transition, and sperm maturation. The study is also the first to reveal the presence and distribution of aquaporins 9 in the testis and epididymis of dromedary camels, with a segment-specific distribution throughout the year. These findings have significant implications for our understanding of the male reproductive system of this animal and underscore the need for further research in this area.

The current study, conducted with meticulous attention to detail and precision, reveals that testicular and epididymal epithelial cells typically exhibit moderate immunoreaction to AQP9 antibodies in the middle of the rutting season. However, these cells also express significantly higher levels at the beginning and end of this season. Meanwhile, the AQP9 protein shows intense immunoreactive staining in these organs throughout the non-rutting season.

Among the results of this research is the expression of AQP9 in the gonocyte, Sertoli cells and Leydig cells of camel's testis, which, following several publications, that documented the expression of this protein in the testis of mammal species (Oberska *et al,* 2024; Martinez-Madrid *et al,* 2023; Mohamed *et al,* 2022; Schimming *et al,* 2021; Arena *et al,* 2011; Badran and Hermo, 2002; Nicchia *et al,* 2001; Elkjær *et al,* 2000). At the same time, other investigations reported that AQP9 was not found in the testicles of humans, dogs, or mice (Hashem, 2010; Domeniconi *et al,* 2007; Ko *et al,* 1999; Tsukaguchi *et al,* 1999). While the reasons behind these variations remain unclear, they underscore the urgent need for additional research on AQP9 in the dromedary camel and other mammal species to draw definitive conclusions regarding the function of aquaporin in the testis.

According to Setchell (1994), water is crucial in transporting sperm through the epididymis. In addition, transepithelial water flow in the male reproductive tract is critical for proper fertility (Domeniconi *et al,* 2008). As agreed upon by published investigations in mammals (Oliveira *et al,* 2013, 2005; Domeniconi *et al,* 2008, 2007; Badran and Hermo, 2002), the epididymal epithelium of the camel is considerably positive for AQP9 with variety in the distribution in the different parts in both seasons. Thus, possibly explained by the increased AQP9 levels during the slow increase in spermatozoa concentration from caput to cauda, they contribute to the hyperosmolar environment that maintains sperm quiescent. Following similar observations, (Belleannée *et al,* 2009) proposed that AQP9 is the primary water route in the mammalian epididymis.

According to these findings, AQP9 dispersion changed throughout the year in the testis and epididymis of the dromedary camel, which might be associated with variability in androgen and estrogen components across the whole year, as described in camel by Mohamed *et al* (2018). Furthermore, the seasonal variation studied by Althnaian in the camel and Oliveira and colleagues on a big fruit-eating bat confirmed the current research distribution (Althnaian, 2023; Oliveira *et al,* 2013). This seasonal variation could have significant implications for our understanding of the male reproductive system, particularly about the impact of environmental factors on the expression of AQP9.

To sum up, the findings strongly suggest that AQP9 is crucial for the differentiation and maturation of spermatozoa in the testis and epididymis. This implies that AQP9 could potentially serve as a unique biomarker for male fertility and infertility, and a valuable predictor of sperm quality.

Conflict of interest

None declared

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