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Immunolocalization of Androgen Receptor and PCNA in Male Reproductive Tract of Dromedary Camel during the Rutting Season

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Abstract

The male reproductive tissues including the testis, epididymis, and prostate gland undergo dramatic changes in seasonal breeders from the breeding to non-breeding seasons. Classically, sex steroid hormones play important roles in the epididymis, and prostate gland morphology and functions. To clarify the relationship between androgen receptor (AR) expression and seasonal changes in the male dromedary camel, the immunolocalizations of AR and proliferating cell nuclear antigen (PCNA) were investigated in the testis, epididymis, ductus deference and prostate gland of the dromedary male camel in the breeding season (October to April) using immunohistochemistry, morphometrical measurements, and blood analysis. The testis showed a positive immunostaining of AR and PCNA. The reactions were observed in spermatogonia of the seminiferous epithelium. In the interstitial compartment, weak reaction was found in Leydig cells. Moreover, the epididymal epithelial cells displayed positive AR and PCNA reaction that was localized in the nucleus and cytoplasm of principal, basal, and dark cells. The most extensive immunostaining of AR was also present in the body segment. However, the staining signals of AR decreased in the in the head and tail. Furthermore, the ampullary glands epithelium and its duct epithelium were positive for AR and PCNA. Comparatively, the epithelial cells lining the ductus deferens in (DI) and (DM) displayed negative staining than those of other cell types. Finally, the immunostaining for AR and PCNA was detected in tubuloalveolar glands epithelium of both proximal part and distal part. However, in the fibromuscular layer, showed negative staining of both proximal and distal part. Strong immunostaining was detected in in the urothelial cells of prostatic urethra.

KEYWORDS
Androgen receptor, PCNA, Camels, Testis, Prostate

INTRODUCTION

Androgens, which include testosterone and dihydrotestosterone (DHT), are male sex hormones essential for the maturation of the male reproductive system and secondary sexual characteristics. The action of testosterone and DHT occurs through the androgen receptor (AR), a ligand-dependent nuclear transcription factor (Chang *et al*., 1995). DHT is more physiologically active than testosterone and has a 2-fold greater affinity for and a 5-fold lower dissociation rate when it comes to binding to the AR (Grino *et al*., 1990).

The AR is divided into three functional domains: the N-terminal transcriptional regulatory domain, the DNA binding domain, and a C-terminal ligand-binding domain (MacLean *et al*., 1997). The ligand-binding domain of AR interacts with the N-terminus of the AR to stabilize bound androgens and shares structural similarities with the ligand binding domains of the other nuclear receptors (Heinlein and Chang, 2002). The camel genitalia' morphological features and histopathological traits exhibit periodic variations during breeding and non-breeding seasons (Abd El-maksoud *et al*., 2010).

The appearance and function of the epididymis of seasonally breeding animals, such as dromedary camels, differ significantly from those of the testicular tissue and comes in response to the changes in testicular activities and changes in the sex hormones secretion levels. The epididymal epithelium in mammalian species consists of four cell types, two main cell types (principal and basal cells), and two accessory cell types (apical cells and intraepithelial leukocytes), whereas dark cells form a fifth cell type found in the epididymis of camels (Alkafafy *et al*., 2011a). The broad range of functional capacity of the epididymal epithelium may be explained by the diversity of its cellular populations. This enables the epididymis to produce complex sequential changes in the luminal fluid's makeup along its entire length. This facilitates the development of young testicular sperm into mature sperm (Alkafafy *et al*., 2011b).

Furthermore, the prostate gland is known to be one of the major target tissues of sex steroid hormones. Through the AR, androgens are essential for controlling the development of the mammalian prostate gland. For their survival and continued functionality, the prostate's secretory epithelial cells that produce AR must be continuously stimulated (Gallardo *et al*., 2009).

Although AR has an important role in regulating gene expression in multiple tissue types, studies to date on AR in the male reproductive system have largely focused on the prostate gland. To improve the understanding of the role of androgens

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in camels, the exact site of action of this sex steroid needs to be established. This study was designed to identify the cell types expressing AR, PCNA in camel testis, epididymis, ductus deference and prostate gland. This research undertook an immunohistochemical study to locate these receptors during the breeding season in male camels.

MATERIALS AND METHODS

Ethical approval

All experimental procedures used in the current study were approved by the guidelines of Ethics Committee at King Faisal University, Saudi Arabia (Ref. No. KFU-REC-2023-FEB-ETHICS614), and the animal slaughter protocols of Ministry of Municipal, Rural Affairs, and Housing, Saudi Arabia.

Animals

A total of 24 adult male dromedary camels (4-15 years old) were used in this study. Male camels were clinically investigated in Al-Ahsa local slaughterhouses, Saudi Arabia. The soundness of animals was evaluated. Male camels without systemic lesions or deformities were classified as suitable for the experiment, and those showing alterations or pathological lesions were excluded. Given that rutting occurs in winter season (October to April), which is considered a short period for achieving a higher conception rate.

Male genital tract from each camel were transported to the laboratory within 1 h in a thermos flask containing physiological sterile 0.9% NaCl solution at 37°C. After transportation, fat, ligaments, connective tissue, and smooth muscles surrounding the testis, epididymis, and vas deferens were carefully trimmed off and removed. For immunohistochemistry (IHC); tissue samples from different regions of the testis (cranial "TC," caudal "TCa," and rete testis "TR"), epididymis (head "EH," body "EB," and tail "ET"), and vas deferens (initial "DI," middle "DM," and ampulla "DA") were placed in 10% buffered formalin.

Immunohistochemical localization of androgen receptor and PCNA in male reproductive tract of dromedary camels.

The formalin-preserved tissues for 24 h at 4°C were processed and embedded in paraffin. Briefly, samples fixed in formalin were thoroughly washed in phosphate buffered saline (PBS), dehydrated in graded ethanol, and embedded in paraffin wax. Then, 5 µm thick sections were cut and placed on Superfrost slides. After deparaffinization in xylene and rehydration in ethanol, antigen retrieval buffer (sodium citrate, pH 6) was heated in a microwave oven for 20 minutes. Sections were then cooled at room temperature (RT) and washed in 0.025% Triton 100 (v/v) in Tris-buffered saline (TBST: 3×5min). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. After washing, goat serum (10%) was used for 10 min to avoid nonspecific reactions. Sections were then incubated with rabbit anti-Androgen receptors (DS-0013-D, Diagnostic BioSystems, Emergo Europe, Prinsessegracht 20 2514 AP, The Hague, The Netherlands) and PCNA (DS-0360-C, Diagnostic BioSystems, Emergo Europe, Prinsessegracht 20 2514 AP, The Hague, The Netherlands) overnight in a humid chamber. After washing (TBST: 3×5 minutes), the sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Abcam, Inc., ab64261, USA) for 30 hours at RT and then washed again with TBST. The slides were then incubated with streptavidin-HRP conjugate for 10 minutes and then washed with TBST three times for 5 minutes. Color development was achieved by using an appropriate amount of the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 minutes. Counterstaining was done with hematoxylin. Slides were then dehydrated, cleaned, and mounted on coverslips. Normal rabbit IgG was used as a negative control using the same procedure. Visualization was performed using digital light microscope (Leica DM6000B, Tokyo, Japan; Filter, 45 mm GIF; Magnification, ×400), and photomicrographs were taken with a digital camera (Nikon Instech Co., Ltd. Tokyo, Japan).

Evaluation of immunostaining intensity

Visual inspection led to the classification of staining strength as negative (absent), mild, moderate, or strong. If a staining did not vary within a slide from that of the negative control portion, it was categorized as negative.

A morphometric analysis of the testicles and scrotum

Scrotal circumferences and testicular dimensions (length, width, height and circumference) were measured in a sitting position using a caliper and a coulter scrotal tape. The testicular volume was calculated using the following equation (volume = length x width x height x 0.71) (Sakamoto *et al*., 2007).

Blood sampling and testosterone assays

The blood samples were collected from the entire animals that were used in the experiments via jugular venipuncture. The blood samples were collected at abattoir. Samples were allowed to clot at room temperature, were centrifuged within half an hour after collection and then were stored at -20°C in Eppendorf tubes. Circulating concentrations of testosterone was determined by the enzyme-linked immunosorbent assay (ELISA) method using commercial kits (Camel Testosterone (T) Elisa Kit; AFG Bioscience, USA). The assays were performed according to the manufacturer's instructions. Detection Range; 0.625 ng/mL-20 ng/mL; Detection Wavelength: 450 nm.

Statistical analysis

The Descriptive analysis of data was performed by using JMP software version 11.0.0 (SAS Institute, Cary, NC, USA).

RESULTS

Androgens receptor (AR) immunolocalization in testis of male dromedary camels

The testis showed a positive immunostaining of androgen receptor (AR). The reactions were observed in both interstitial compartment and seminiferous epithelium (Fig. 1a, b). In the interstitial compartment, specific labeling of AR was found in Leydig cells with variable staining intensity (Fig. 1b). In tubular compartment, AR immunoreactivity was localized in the nuclei of Sertoli cells and peritubular myoid cells. On the other hand, in germ cells' nuclei or cytoplasm (spermatocytes, spermatids and spermatozoa), immunoexpression of AR was not detected (Fig. 1a-c).

The intensity of AR immunolabeling was stage-dependent (Fig. 1). A high variation was still observed at different stages. In cranial part of testis, the somatic cells, such as Sertoli cells, Leydig cells, and peritubular myoid cells are positivity for AR, but nega-

tive staining in remaining germinal epithelium (Fig. 1a), whereas the distal part of testis revealed week expression in the stromal cells (Fig. 1b) with gradual decline in immunoreactivity was observed towards Reti testis (Fig. 1c).

Androgens receptor (AR) immunolocalization in epididymis of male dromedary camels

The immunochemical staining of AR was characterized in epididymis, which enabled a comparison between AR functions in the male reproductive ducts and previous data from normal prostate tissue and ductus deferens. In camel, the epididymal epithelial cells displayed positive androgen receptor immunoreactivity (Fig. 2a, b). AR was localized in the nucleus and cytoplasm of principal, basal, and dark cells. The most extensive immunostaining of AR was also present in the body segment (Fig. 2a, b). However, the staining signals of AR decreased in the in the head and tail. In the majority of tubules in various epididymal areas, no signal was observed against apical cells (Fig. 2c).

Androgens receptor (AR) immunolocalization in ductus deferens of male dromedary camels

The ductus deferens, a cord-like continuation of the tail of the epididymis, had differentiated epithelium. The ampullary glands epithelium and its duct epithelium were positive for AR (Fig. 3c, e). Comparatively, the epithelial cells lining the ductus deferens in (DI) and (DM) (Fig. 3a, b) displayed negative staining than those of other cell types.

Androgens receptor (AR) immunolocalization in prostate gland of male dromedary camels

The prostate gland was enveloped externally by a thick fibromuscular capsule. Fibromuscular septa arose from the deep aspect of the capsule dividing the prostate into variable numbers of lobules of compound tubuloalveolar glands and duct system. Figure 4 shows the changes in AR immunoreactivity of the camel prostate. Immunostaining for AR was detected in tubuloalveolar glands epithelium of both proximal part and distal part (Fig. 4a, b). However, in the fibromuscular layer, showed negative staining of both proximal part and distal part (Fig. 4a, b). Weak specific staining for AR was also evident in the in the epithelium of the proximal part of the glands. Strong immunostaining was detected in in the urothelial cells of prostatic urethra (Fig. 4c).

PCNA expression in testis

Testis showed a positive immunostaining of PCNA. The reactions were observed in spermatogonia of the seminiferous epithelium (Fig. 1a1, b1). In the interstitial compartment, weak reaction was found in Leydig cells (Fig. 1b1). On the other hand,

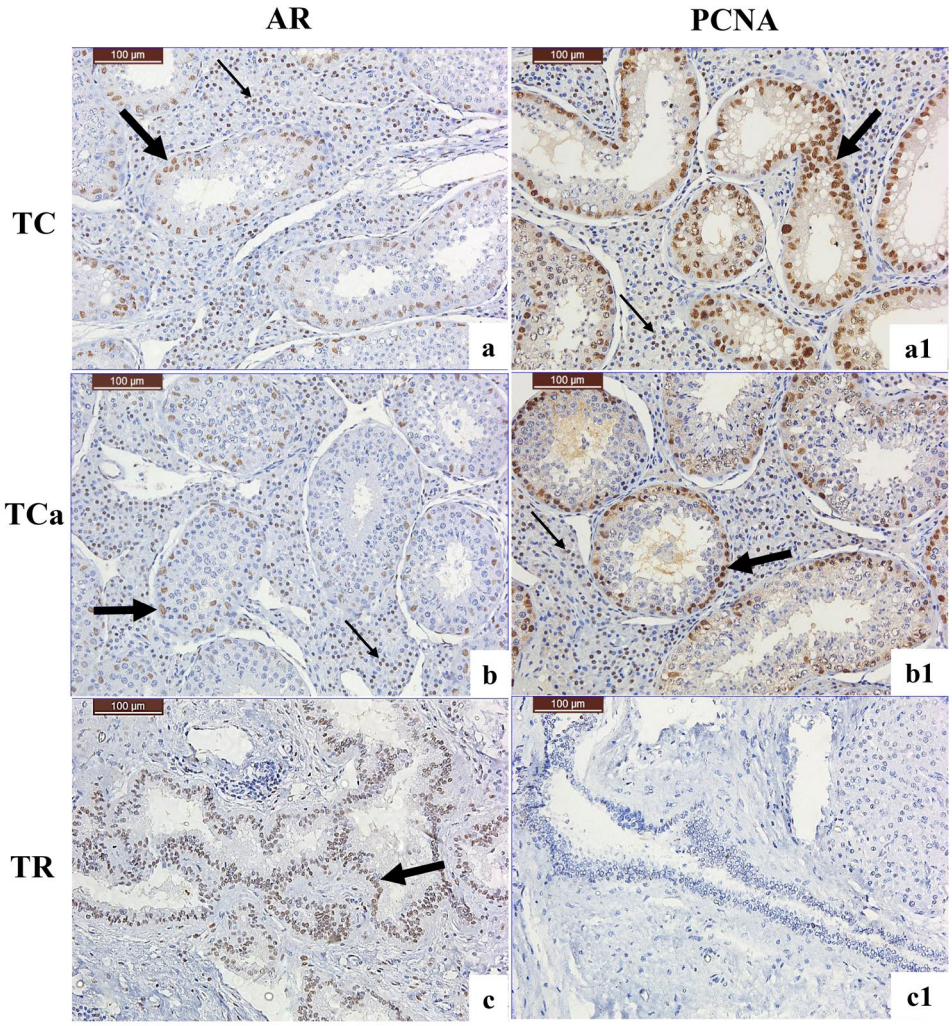

Fig. 1. The expression of AR and PCNA in dromedary camel testis. Arrows indicates positive expression (brown colour). a and a1) Microscopic examination of cranial part displaying faint brown staining in testicular somatic cells, but negative staining in remaining germinal epithelium. b and b1) Microscopic examination distal part of the testis revealed week expression in the stromal cells. c and c1) Microscopic examination reti testis revealed very faint expression in the stromal cells. Arrows indicates positive brown expression.

in germ cells' nuclei or cytoplasm (spermatocytes, spermatids and spermatozoa), immunoexpression of PCNA was not detected (Fig. 1a1-c1).

PCNA immunolocalization in epididymis

In camel, the epididymal epithelial cells displayed positive PCNA reaction (Fig. 2a1, b1). PCNA immunostating was localized in the nucleus and cytoplasm of principal, basal, and dark cells. The most extensive immunostaining of AR was also present in the body segment (Fig. 2b1). However, the staining signals of AR decreased in the in the head and tail. In the majority of tubules in various epididymal areas, no signal or weak was observed against apical cells (Fig. 2c1).

PCNA immunolocalization in ductus deferens

The ampullary glands epithelium and its duct epithelium were positive for PCNA (Fig. 3c, c1). Comparatively, the epithelial cells lining the ductus deferens in (DI) and (DM) (Fig. 3a1, b1) displayed negative staining than those of other cell types.

PCNA immunolocalization in prostate gland

The prostate gland was enveloped externally by a thick fibromuscular capsule. Immunostaining for PCNA was detected in tubuloalveolar glands epithelium of both proximal part and distal part (Fig. 4). However, in the fibromuscular layer, showed negative staining of both proximal part and distal part (Fig. 4). Weak specific staining for PCNA was also evident in the in the epithelium of the proximal part of the glands. Strong immunostaining was detected in in the urothelial cells of prostatic urethra (Fig. 4c1).

Morphometric finding of testis and testosterone assay

During breeding season in male camels, the testicular morphometry and scrotal circumstances are presented in Table 1. The morphometric values for testis were 15.9 ± 0.2 cm length, 5.1 ± 0.1 cm width, and 5.0 ± 0.1 cm height. While the scrotal circumstances morphometry was 26.0 ± 0.3 cm. The mean serum testosterone levels in male camels was 4.6 ± 0.8 ng/ml during breeding season (Table 1).

DISCUSSION

Understanding androgen regulation of spermatogenesis remains enigmatic. Although there is ample documentation to support the hypothesis that androgens are indispensable to complete normal spermatogenesis in all species studied, the vital step(s) regulated by androgens has yet to be identified (Sharpe, 1994). The results of the present investigation confirm those of prior studies in the human testis (Suárez-Quian *et al*., 1999) that

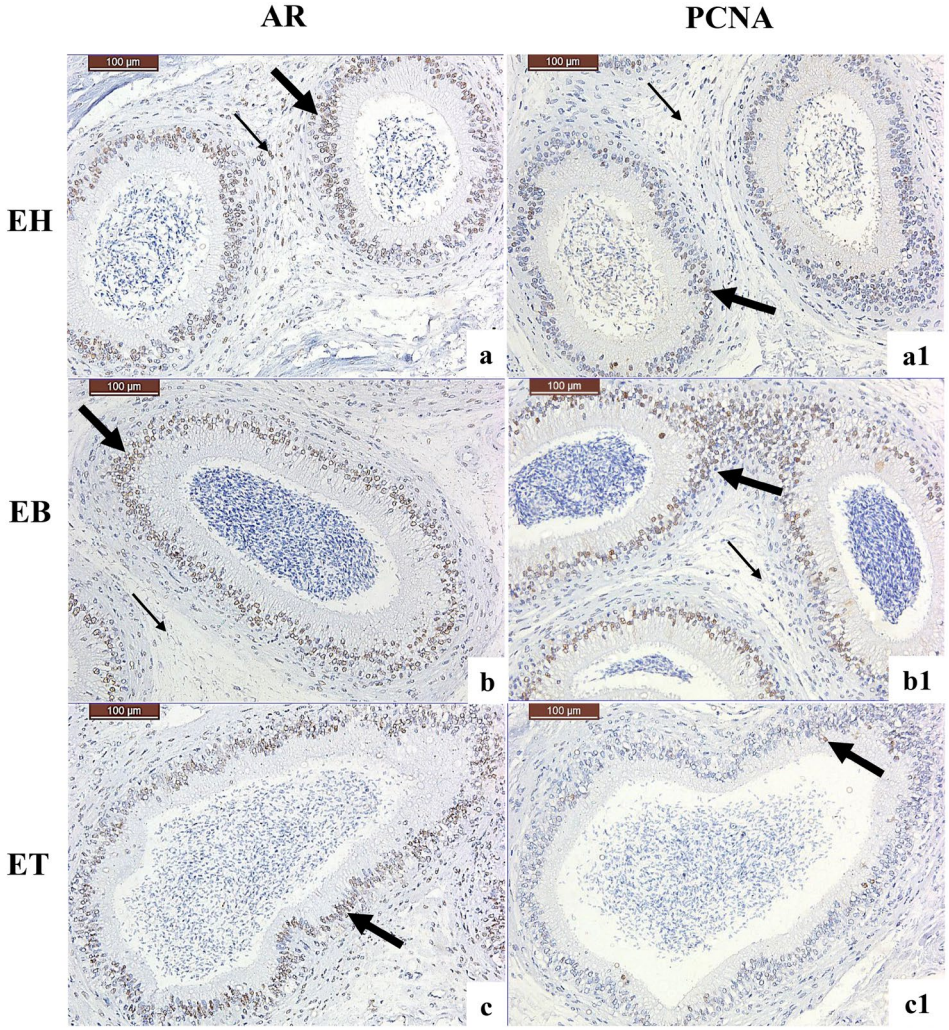

Fig. 2. The expression of AR and PCNA in dromedary camel epididymis. Arrows indicates positive brown expression. A) Microscopic examination the caput segment showed positive immunoreactions to androgen receptor in the epithelial cells. On the other hand, surrounding smooth muscle, connective tissue and blood vessels showed androgen receptor immunonegative signals. B) Microscopic examination of corpus epithelium displaying strong reaction in many epithelial cells. C) Microscopic examination of the cauda epididymis displaying less-intense androgen receptor immunopositive signals epithelium, compared to (caput) epididymis.

only the somatic cells, namely Sertoli, Leydig, peritubular myoid, and smooth muscle cells surrounding the walls of blood vessels, express immunopositive AR. Unlike in the rat and mouse (Zhou, *et al*., 1996) and in two studies in human testis (Kimura *et al*., 1993), immunopositive AR in germ cell populations was not detected. Thus, it is possible to speculate that specific androgen regulation of spermatogenesis occurring via Sertoli and peritubular myoid cells may vary between species.

Table 1. Morphometric assay of the testis, scrotum, and testosterone (means ± standard deviation).

Parameters	Measurement
Testicular length (cm)	15.9±0.2
Testicular width (cm)	5.1±0.1
Testicular height (cm)	5.0±0.1
Testicular volume (cm^3)	284.8±11.5
Testicular circumferences (cm)	13.0±0.2
Sectoral circumferences (cm)	26.0±0.3
Serum testosterone (ng/mL)	4.6±0.8

Strong AR immune reaction was found in epididymis in rutting camel testis. This was in agreement with the previously recorded outcomes from the epididymis of Egyptian camels. During the rutting season, immunohistochemical examination revealed a significant positive positivity in the epithelial cells lining the camel epididymis. During the non-rutting season, however, no immunoreactive signals were detected in the camel epididymis (Ibrahim and Abdel-Maksoud, 2019).

Sperms acquire the ability to fertilize while passing through the proximal portion of the epididymal duct and are subsequently stored in the distal region of the epididymal duct (Abe *et al*., 1983). The present work revealed intense expression of androgen receptors in epididymal epithelium to support sperm maturation and storage efficiency.

In the current study, the epididymal epithelium exhibited a stronger immunoreactivity for AR in the rutting season than that in the non-rutting season. There is considerable evidence suggesting that the seasonal changes in the epididymal epithelium are greatly dependent on the level of androgens. Previous studies show that seasonal changes in the male reproductive system are due to reduced production of androgenic steroids during the nonbreeding season (Lincoln and Short, 1980).

The prostate tissue is androgen dependent. In the present study, nuclear staining for AR was detected in both glandular epithelial and fibromuscular stromal cells. These results are similar to those reported for canine (Gallardo *et al*., 2007) and rat (Pelletier *et al*., 2000) tissues, in which AR have been detected in the nuclei of both glandular epithelial and fibromuscular stromal cells.

The distribution of proliferating cell nuclear antigen (PCNA) in specific somatic and germ cells of the male reproductive system. PCNA staining has proven to be a useful indicator of cells involved in DNA synthesis and repair (Chapman and Wolgemuth, 1994). The localization of PCNA may also help in study of the proliferative activity of testicular renewing stem cells in testis, epididymis, and prostate. PCNA immunostaining is highly specific

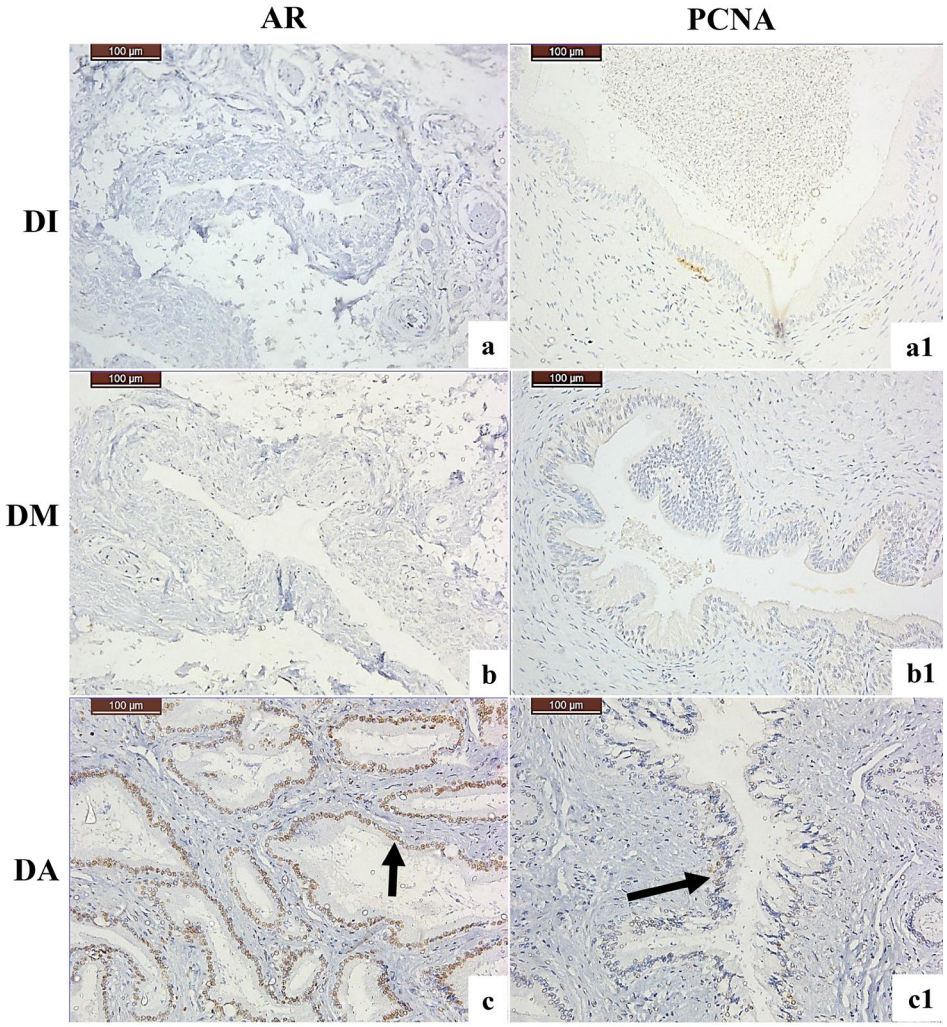

Fig. 3. AR and PCNA immunoreactivity in the ductus deferens of the camel testes. a) Microscopic examination displaying negative reaction in DI. b) Microscopic examination displaying negative reaction in DM. C) Microscopic examination displaying positive reaction in the ampullary glands epithelium and its duct epithelium. Arrows indicates positive brown expression.

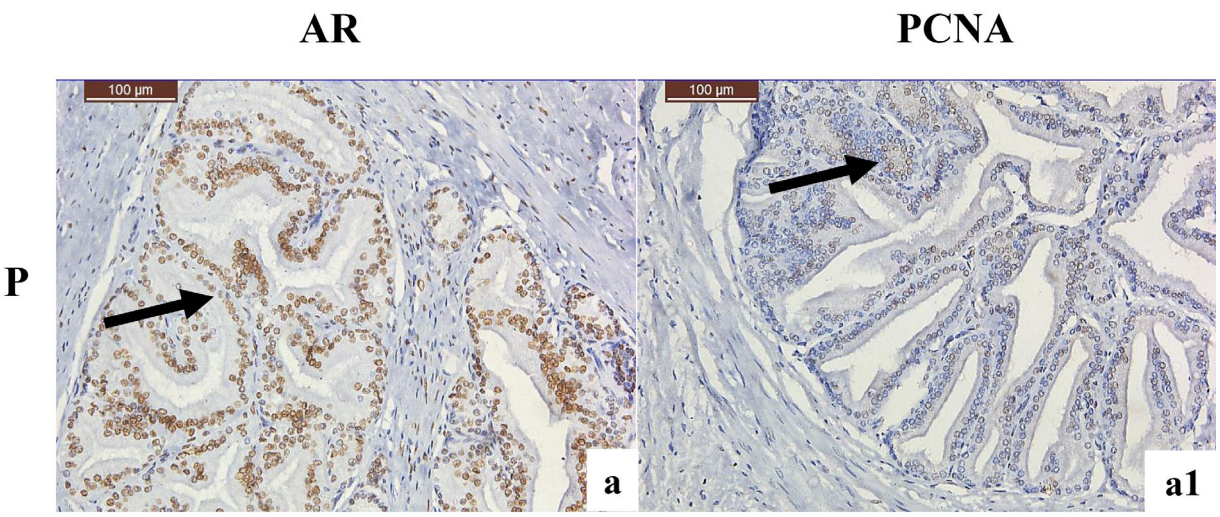

Fig. 4. Immunohistochemical staining for Androgen receptor in camel prostate tissue section. A) Proximal part displaying faint brown staining in the tubuloalveolar glands epithelium, but negative staining in the fibromuscular layer. Arrows indicates positive brown expression. B) Distal part displaying strong positive staining in the tubuloalveolar glands epithelium, but negative staining in the fibromuscular layer. Arrows indicates positive brown expression. C) Prostatic urethra of camel displaying brown staining in the urothelial cells. Arrows indicates positive brown expression.

for cells in the early phase of spermatogenesis, while later stages of germ cells are devoid of label.

Morphometric values for the testis and scrotum were high during breeding season in male camels, which agrees with the results of other authors, who reported that the testicular size and scrotal circumstances were affected by season (Abdel-Rahim *et al*., 1994; Swelum *et al*., 2018; Saini *et al*., 2022). The testis was significantly greater in the breeding than in non-breeding season which led to higher concentration of testosterone in breeding season than in non-breeding season (El-Harairy and Attia 2010)

Males require testosterone in order for spermatogenesis and the reproductive system to function normally. The present data indicate that the means of testosterone levels increased during the breeding season. The increased concentrations of testosterone coincided with radical changes in the behavior of male camels as they became aggressive, extruded the soft palate, smelling the female genital. Similar findings were reported in camel by previous reports (Deen 2008; El-Harairy and Attia 2010; Swelum *et al*., 2018).

CONCLUSION

In the testis, PCNA was detected in the mitotically proliferating spermatogonia, but not in spermatocytes which had just entered meiosis. PCNA staining was again observed in spermatogenic cells in later stages of meiotic prophase, in particular zygotene and pachytene spermatocytes. As these cells are undergoing meiotic recombination, the presence of PCNA in these meiotic prophase cells could reflect a second function of PCNA that of DNA excision repair. Thus, in combination with quantitative assessment (cell counting), PCNA immunostaining could be used as a marker for the number of cells entering spermatogenesis.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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