

# SPATIAL EXPRESSION OF OSTEOPONTIN IN TESTIS, EPIDIDYMIS AND SPERMATOZOA IN DROMEDARY CAMEL

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## ABSTRACT

The aim of this study is to clarify the function of osteopontin (OPN) through its expression and localisation in male testis, epididymis and sperm cell of dromedary camel during rutting season. Testis and epididymis parts (caput, corpus and cauda) specimens were obtained from 8 mature male camels and semen was collected from 4 another fertile camels for sperms. OPN mRNA expression and its location was analysed using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) techniques, respectively. OPN mRNA expression was significantly ( $P < 0.05$ ) higher in the cauda of epididymis than other epididymal parts and testis. Immunolocalisation revealed high immunoreaction intensity in the elongated spermatids in seminiferous tubules and Leydig cells in interstitial tissue. However, a faint immunostaining was seen in spermatogonia and Sertoli cells. Along the epididymis tract, intense immunoreactivity of OPN was localised in the cytoplasm of the epithelial cells with high concentration in the apical region nearby lumen and the acrosomal part of the cauda epididymal sperms. Immunofluorescence and immunochromogenic staining of OPN was detected on the acrosomal cap and neck of epididymal sperms. These findings suggested that OPN might play an important role in the sperm protection, migration and fertilisation in dromedary camels.

**Key words:** Dromedary camel, epididymis, expression, osteopontin, spermatozoa, testis

Osteopontin (OPN), also named as Secreted Phosphoprotein1 (SPP1), is an indispensable highly phosphorylated glycoprotein in mammalian reproduction. Firstly, it was isolated from the mineralised matrix of bovine bone (Denhardt and Guo, 1993; Franzen and Heinegård, 1985). Thereafter, it was found in different tissues such as brain (Shin *et al*, 2005), kidney (Xie *et al*, 2001) and body secretions like male seminal fluids (Cancel *et al*, 1999). The function of OPN has been determined according to the expressed cells in the tissues and it thought to play a role in cell adhesion (Wai and Kuo, 2004). Moreover, it was observed in different male reproductive tissues such as testis, epididymis as well as sperm (Lin *et al*, 2006; Rodríguez *et al*, 2000; Siiteri *et al*, 1995; Wilson *et al*, 2005; Souza *et al*, 2009; Kang *et al*, 2014). Meanwhile, it has been discovered to contribute in several reproductive processes such as sperm-egg interaction, fertilisation and early embryonic development (Erikson *et al*, 2007; Souza *et al*, 2009; Monaco *et al*, 2009). It has been suggested that the OPN has a potential role in fertilisation (Erikson *et al*, 2007); spermatogenesis and spermatozoa function (Zhang *et al*, 2016) and was considered as one of

the decapacitation factors to prevent premature sperm motility activation (Goncalves *et al*, 2007). In addition, OPN has been stated as a sperm surface molecule; where it was found in testes, epididymis and on the surface of epididymal spermatozoa. Therefore, OPN could play a role in maturation during spermatogenesis (Siiteri *et al*, 1995).

In different mammals, spatial expression of OPN has been thoroughly investigated. The OPN mRNA has been detected in the germ cells of spermatids, epididymis and spermatozoa of the bull (Rodríguez *et al*, 2000); Sertoli cells of the mouse (Wilson *et al*, 2005); both germ and Sertoli cells in the rat testis (Siiteri *et al*, 1995) and in the testicular interstitium, acrosomes of testicular spermatids and epididymis of the sheep (Zhang *et al*, 2016). Meanwhile, immunolocalisation of OPN was detected in different locations; in the epididymal sperm, testis and cauda epididymal fluid of bull (Erikson *et al*, 2007); testes in both rat and mouse (Wilson *et al*, 2005; Siiteri *et al*, 1995); spermatogonia, different stages of spermatocytes, acrosomes of spermatids and few sertoli cells of boar; testis, epididymis and cauda epididymal spermatozoa in the sheep (Zhang *et al*,

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2016) and it has been detected in the seminal plasma of the dromedary camel and its high concentrations was positively correlated with fertility of camels (Waheed *et al*, 2015).

According to previous studies, the expression pattern of OPN has distinct interspecies differences, which indicated that OPN might contribute in several biological processes in various species. Although, there are several studies on detection of OPN in the reproductive system of domestic animal species, but the role of OPN in camel reproductive system has not been reported yet. Therefore, the present study was under taken to clarify the expression and localisation of OPN in dromedary testis, epididymis and epididymal sperm during rutting season to understand its activity in spermatogenesis.

## Materials and Methods

### Animals and sample collection

Twelve male reproductive tracts of clinically healthy dromedary camels (4-12 years) were obtained from local abattoirs in Saudi Arabia during rutting season (November to April). The testes and caput, corpus and cauda of epididymis were collected from 8 animals. First specimen's part was immediately snap freeze in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  for qrt-PCR analyses and the 2<sup>nd</sup> part was fixed in 10% buffered formalin for immunohistochemistry. In addition, epididymal sperms were collected from the cauda epididymis of the rest 4 males. Sperm samples washed with phosphate-buffered saline (PBS) and suspended sperms were smeared on Superfrost slides, air-dried and then stored at  $-20^{\circ}\text{C}$  until use for immunohistochemistry and immunofluorescence.

### Gene expression analysis

Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions, then RNA pellets were resuspended in UltraPure™ DEPC-Treated Water (Invitrogen, USA). BioTek Synergy MX reader (BioTek, USA) was used to measure RNA concentration and purity at optical density value 260:280 nm absorbance, which was between 1.8 and 2.0. Reverse transcription reactions were performed using iScriptcDNA Synthesis Kit (BioRad, Hercules, CA, USA). The total of reaction (20  $\mu\text{L}$ ) was a mixture of 2  $\mu\text{g}$  RNA templates, 4  $\mu\text{L}$  iScript Reaction Mix, 1  $\mu\text{L}$  iScript Reverse Transcriptase and nuclease-free distilled water. Reaction thermocycle was at  $25^{\circ}\text{C}$  for 5 min,  $46^{\circ}\text{C}$  for 20 min, then at  $95^{\circ}\text{C}$  for 60 seconds to inactivate the reverse transcriptase according to manufacturer's protocol. Gene-specific

primers were designed for dromedary camel OPN and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), reference gene, using NCBI primer-blast website (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and Gen-Bank accession numbers and amplicon length of target genes were presented in table 1. Quantitative real-time PCR (qRT-PCR) carried out using CFX96 Touch Real-time PCR (BioRad, USA). SsoAdvanced SYBR Green Supermix (BioRad, USA) was used to detect the fluorescence emission in a reaction volume of 20  $\mu\text{L}$ . All cDNA templates were run in duplicate and relative quantification was done by the  $2^{-\Delta\Delta\text{CT}}$  method. Relative quantifications were calculated to the reference gene (GAPDH) directly via CFX Manager™ software V3.1 (BioRad, Hercules, CA, USA).

**Table 1.** Designed primer sequences, Genbank accession numbers and product sizes used for qRT-PCR.

Gene name	Sequences	Accession number	Amplicon Length (bp)
OPN	F: AAGACA CAC AAG ATG GCC GA	XM_010983105.1	187
	R: TGG CTG TTC CAG TCA GAA GC		
GAPDH	F: CCT GGA GAA ACC TGC CAA ATA	XM_010990867.2	207
	R: TCG TTG TCG TAC CAG GAA ATG		

### Tissues immunostaining

Sections of paraffin block samples (5 $\mu\text{m}$  thick) were cut and placed on Superfrost slides. After deparaffinised and rehydration, antigen-retrieval buffer was applied at  $100^{\circ}\text{C}$  for 15 min. After cooling, the slides were immersed with 3% hydrogen peroxide following by blocking buffer for 10 min after washing 0.1% (v/v) Tween 20 in Tris-buffered saline (TBST: 3 $\times$ 5min). Sections were then incubated with Rabbit anti-Osteopontin (Abcam, Inc., ab8448, 1:100) over night at  $4^{\circ}\text{C}$ . After washing (TBST: 3 $\times$ 5min), the goat anti-rabbit biotinylated secondary antibody (Abcam, Inc., ab64256) was added to the sections for 1 hour at room temperature then were washed again with TBST. Next, the slides were incubated with streptavidin-HRP conjugate (Abcam, Inc., ab64269) for 20 min then washed 3 $\times$ 5 min TBST. Colour was developed by adding a suitable amount of 3,3'-diaminobenzidine tetrahydrochloridechromogen substrate for 5 min. Slides were counterstained with haematoxylin, dehydrated and cleared, mounted by cover slip and visualised using a Leica DM6000 B

light microscope. Primary antibody was omitted in the negative control sections.

### **Sperm immunostaining**

Sperm-smear slides were surrounded by Hydrophobic Barrier Pap Pen, then incubated in PBS for 5 min followed by blocking buffer for 15 min at RT. Slides were then incubated with rabbit anti-Osteopontin (Abcam, Inc., ab8448, 1:50) for 1 hour at RT followed by washing (3×5 min PBS). All slides were incubated with goat anti-rabbit fluorescent secondary antibody (FITC) (Abcam, Inc., ab6717, 1:100) for 2 hours in a dark place at RT. After washing (3×5 min PBS), a cover slip was mounted on the slides using aqueous antifade mounting medium. Then slides were visualised immediately using Leica DM6000 B fluorescent microscope. In addition, chromogenic immunostaining of sperm slides were performed similar to that conducted on tissue slides.

### **Statistical analysis**

Data were analysed using SPSS software version 16. Comparisons were made among different tissues with varying expressions as means ± standard errors via a one-way analysis of variance with post hoc analysis. Data were shown as means ± standard errors.

## **Results and Discussion**

### **Relative gene expression of OPN in the testis and epididymis**

As shown in fig 1, relative gene expression of OPN revealed differences mRNA expression between testis and epididymis parts. Meanwhile, the highest expression of OPN was detected in the cauda of epididymis. The data indicated that OPN mRNA expression in the cauda of epididymis was significantly ( $P < 0.05$ ) higher compared to the other tissues. However, no significant differences were seen between testis, caput and corpus of epididymis.

### **Immunolocalisation of OPN in the testis and epididymis**

In seminiferous tubules, a highly intense immune-reactivity of OPN was observed in the elongated spermatids. However, spermatogonia and Sertoli cells revealed a faint immune-intensity. In addition, Leydig cells in the interstitial tissue showed strong OPN immune-intensity (Fig 2A). The caput, corpus and cauda of epididymis, revealed intense immune-reactivity of OPN in the cytoplasm of epithelial cells with a highly concentrated intensity in the apical region nearby lumen of epididymis (Figs

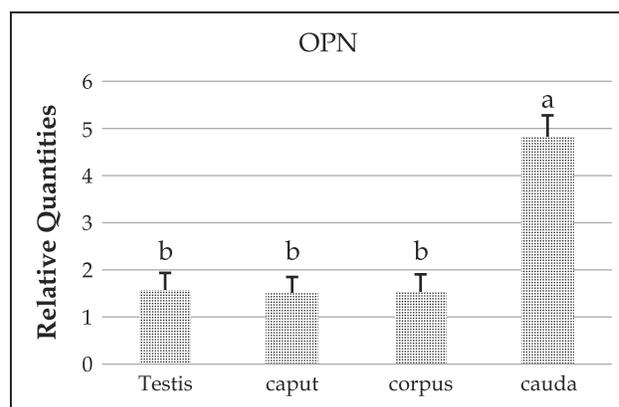
2B, C and D). Meanwhile, a strong immune-reactivity was observed in the sperm's acrosome of cauda epididymidis (Fig 2D). No positive immune reaction was observed in all control sections (Figs 2, A1, B1, C1 and D1).

### **Immunolocalisation of OPN of epididymal sperm**

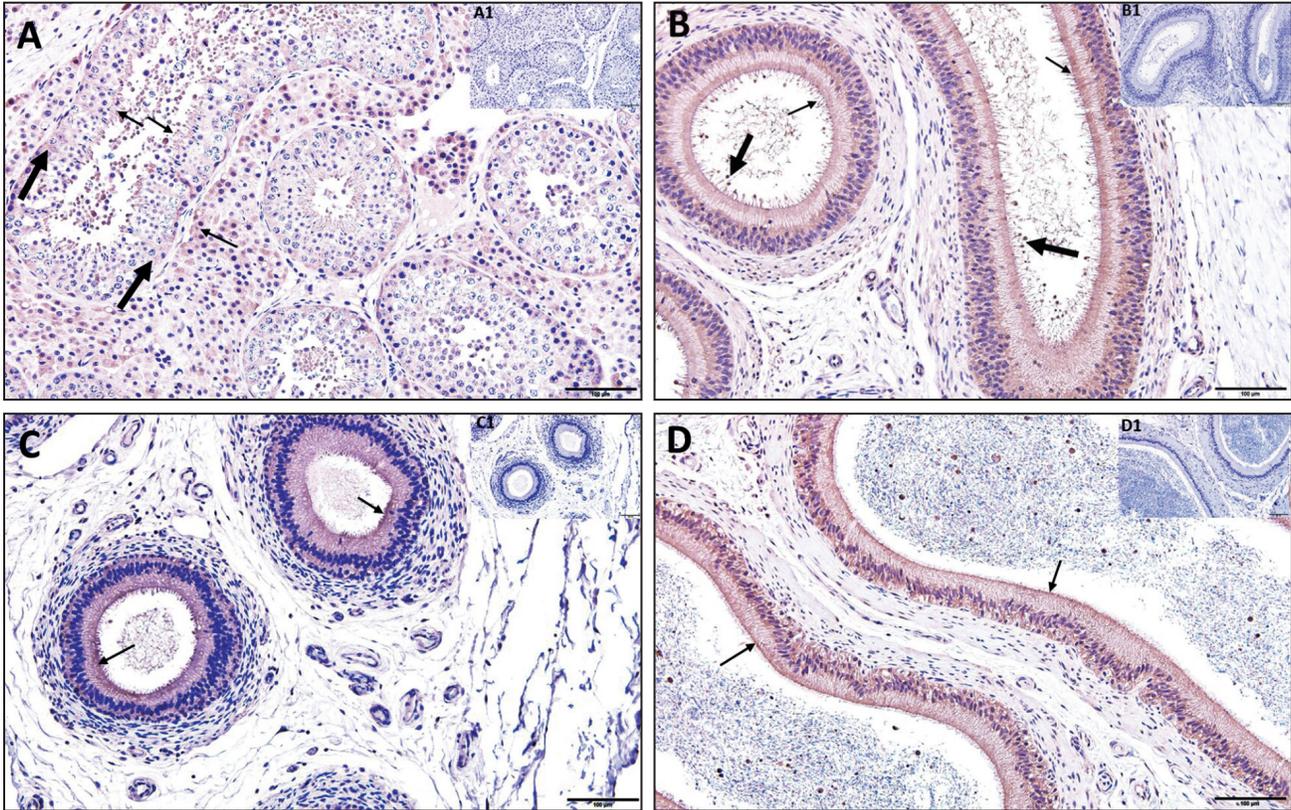
Positive OPN immuno-reactivity was found in the epididymal sperm. Specific immune-localisation of OPN was detected in both acrosomal cap and neck of epididymal sperm in fluorescent (Fig 3A) and chromogenic immunostaining (Fig 3B). However, in other parts of the sperm, no positive immuno-reaction was observed.

OPN is an indispensable highly phosphorylated glycoprotein in mammalian reproduction that was observed in different male reproductive tissues such as testis, epididymis and sperm cells (Lin *et al*, 2006; Rodríguez *et al*, 2000; Siiteri *et al*, 1995; Wilson *et al*, 2005; Souza *et al*, 2009; Kang *et al*, 2014). Our study examined for the first time the spatial expression of OPN in the male dromedary camel reproductive tract.

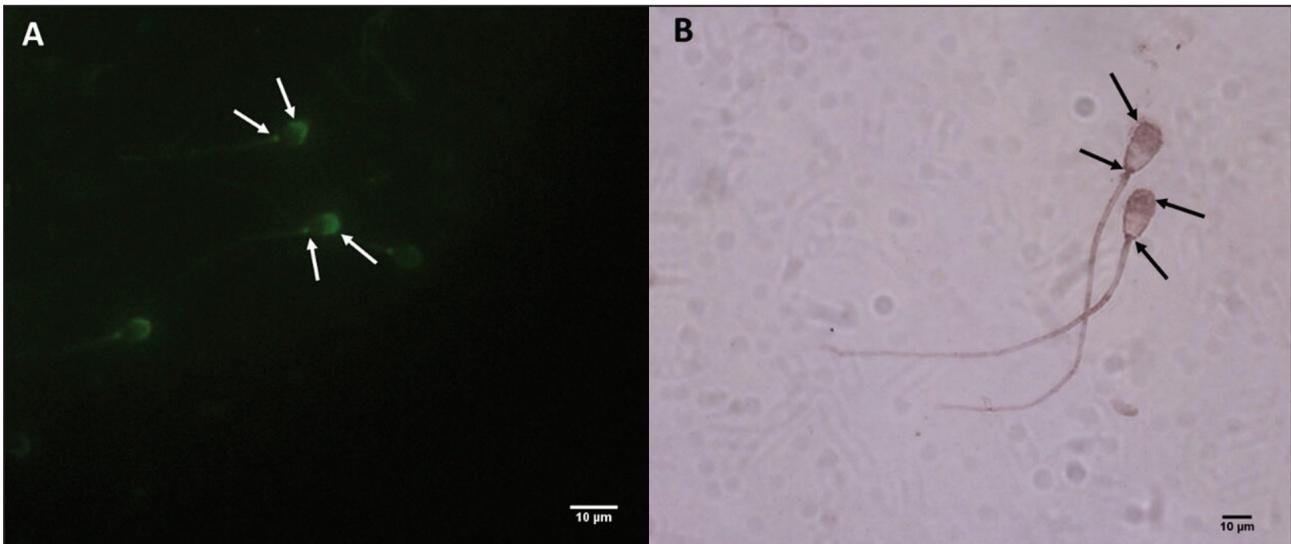
In this study, mRNA transcription of OPN was expressed in the testis and all parts of epididymis. Moreover, the highest expression was in the tail (cauda) of the epididymis. OPN expression in different locations along the reproductive tract implies to the need of this vital protein for the supportive and protective function during sperm journey. However, the concentration of this protein at the last trip (storage) is also suggested to have a strong protective role with integrity adhesion



**Fig 1.** Comparison of OPN expression level in the testis, caput, corpus and cauda epididymis of dromedary camels by qRT-PCR. The gene expressions were normalised to GAPDH and showed as means ± standard errors. The significance was set to  $P < 0.05$  and different letters (a, b) indicate significance among examined tissues. The cauda epididymis showed a high expression in comparison with other reproductive tissues.



**Fig 2.** Immuno-staining of the OPN in the testis and epididymis of male dromedary camels. (A) Testis, showing highly intense immuno-reactivity in both elongated spermatids and interstitial Leydig cells (thin arrows), while less faint immuno-reactivity appeared in spermatogonia and Sertoli cells (thick arrows). (B) Caput epididymis, (C) Corpus epididymis and (D) Cauda epididymis showing highly concentrated intensity in the apical region nearby lumen of epididymis (thin arrows); meanwhile, strong immune-reactivity was observed in the sperm's acrosome of cauda epididymis (thick arrows). (A1-D1) negative control Section. Scale bar, 100  $\mu$ m.



**Fig 3.** Fluorescent (A) and chromogenic (B) immunostaining of OPN in epididymal camel sperm. Both acrosomal cap and neck (arrows) of epididymal sperm showing positive immuno-reactivity of OPN. Scale bar, 10  $\mu$ m.

function that required for sperm maturation, storage and migration. In the same context, similar results were found in other species such as rat and bull by (Rodríguez *et al*, 2000; Siiteri *et al*, 1995).

In previous studies, the OPN immunolocalisation has been detected in the testis and epididymis of different mammals such as boar, bovine and rat (Lin *et al*, 2006; Rodríguez *et al*, 2000;

Siiteri *et al*, 1995). In the present study, OPN was localised on several regions of the camel testis. The highest signals were localised in the elongating spermatids of seminiferous tubules and testicular interstitium (Leydig cells). However, the low signals were observed in some spermatogonia and Sertoli cell. Presence of OPN in various localised stages of testicular tissue particularly the seminiferous tubules might indicate that it may play a key role in spermatogenesis. Previously, it has been documented that the OPN protein binds to a variety of cell surface integrins (transmembrane receptors) which promote cell-cell and cell-ECM adhesion resulting in cytoskeletal organisation and cellular transduction (Burghardt *et al*, 2002; Sodek *et al*, 2000; Johnson *et al*, 2014). Meanwhile, Sertoli cells to germ cells and germ cells to germ cells adhesion and communication are required to provoke and regulate the spermatogenesis (Jégou and Pineau, 1995). Therefore, OPN seems to share in Sertoli-germ cells, germ-germ cells or both adhesion and communication in the seminiferous tubules.

In similar previous studies, OPN was detected in spermatogonia, different stages of spermatocytes, acrosomes of spermatids, some Sertoli cells and Leydig cells in the boar testis (Kim and Shin, 2007). However, in the sheep testis, it was detected in the acrosomes of spermatids near the lumen of the seminiferous tubule (Zhang *et al*, 2016). In addition, OPN has been presented in both sertoli cells and germ cells in the rat (Siiteri *et al*, 1995; Luedtke *et al*, 2002) and mouse (Wilson *et al*, 2005). Interestingly, these results are closely resembling to that found in our present study. However, in contrast, Cancel *et al* (1999) reported that the OPN was undetected in the testis of bovine.

In the present study, the epididymis of the camel showed clear immunostaining of OPN in the epithelial layer. However, highly concentrated intensity was detected in the apical region nearby lumen of epididymis. Meanwhile, a strong immunoreactivity was observed in the sperm's acrosome of cauda epididymidis. Beside cell-cell and cell-ECM adhesion and communication functions, OPN has been reported to promote cell migration, cell death reduction and intracellular calcium alteration (Butler, 1989; Johnson *et al*, 2014). It is therefore, believed that OPN localisation in the epididymis parts particularly in cauda epididymidis and sperm acrosome promote spermatozoa migration and protect it from death during the long journey and may contribute in ova penetration during fertilisation process. In

comparison, similar data have been reported in the rat by Luedtke *et al* (2002). However, in the sheep, it was concentrated on the apical region of the principal cells in the epididymis and on the caudal epididymal spermatozoa (Zhang *et al*, 2016). However, in contrast, OPN was undetected in the epididymis of bovine (Cancel *et al*, 1999).

In the sperm cell, we found OPN in the acrosomal cap and neck of epididymal sperm of camel. Harmonically, Waheed *et al* (2015) reported that the OPN was detected in the seminal plasma of dromedary camel suggesting that OPN has an important role in male's fertility. In another species, OPN was located in the post-acrosomal region and on the midpiece of epididymal sperm of bull (Erikson *et al*, 2007). Furthermore, OPN was observed in the surface of epididymal sperm and epididymal fluid in the rat (Siiteri *et al*, 1995). Therefore, detection of OPN in acrosomal and neck of sperm may provide biofuels to migrate and penetrate during reproductive process.

In conclusion, spatial expression patterns of OPN in testis, epididymis and sperm cell of dromedary camel could play a role in spermatozoal vital functions and spermatogenesis. In addition, the localisation of OPN on the acrosomal cap and neck of camel sperm might carry out a function in the migration, fertilisation and early embryonic development.

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