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Age-related Localization and Expression of Orexin A and its Receptor 1 in Dromedary Camel Testis

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ABSTRACT

Background: The orexin (OX) is a hypothalamic neuropeptide that exists in two forms, orexin A (OXA) and orexin B (OXB), which bind two receptors (OX1 and OX2). While orexin B shows selectivity for OX2, OXA is potent at both receptors. Orexins are also reported in other mammalian organs where they undergo many regulatory functions. However, their detection in the dromedary camel reproductive organs remains to be revealed.

Methods: We investigated the immunoreactivity of orexin OXA and OX1 in the testis of young (6-12 month), pre-pubertal (2-3 years) and adult (6-15 years) dromedary camels. We also studied the relative expression of hypocretin neuropeptide precursor (HCRT) in the testicular tissue of these age groups.

Result: Positive immunoreactions of OXA and OX1 were shown in both the tubules and interstitium of dromedary camel testis in the young animals. While positive OXA and OX1 immune-labeling was detected in the spermatogonia, Sertoli cells and Leydig cells in all ages, adult camels also showed positive OXA and OX1 signals in the primary spermatocytes, spermatozoa and interstitial blood vessels. In testes at pre-pubertal and adult stages, spermatogenic cells, Sertoli cells and Leydig cells showed stronger OXA and OX1 reactions than in young camels. Real time PCR analysis also revealed that HCRT was expressed in the testis of dromedary camels with minimal expression in young camels. In conclusion, the current study provides the first evidence for the presence of OXA and OX1 in the testis of adult and younger dromedary camels. Before puberty, the binding of OXA and OX1 in the camel testis might be important for development and proliferation of spermatogenic cells, Sertoli cells and Leydig cells; in adult animals they may play a role in steroidogenesis and spermatogenesis.

Key words: Animal tissue, Dromedary camel, Histology, Immunohistochemistry, Orexin A.

INTRODUCTION

The dromedary camel (*Camelus dromedarius*) is a productive and economically important animal in desert environment (Benyagoub *et al*., 2022; Demlie *et al*., 2023; Tidjani *et al*., 2025). Recently, its economic value has increased following emerging interest in the racing camels (Skidmore, 2005) and camel beauty festivals (Tharwat and Al-Hawas, 2024) in the Arabian Gulf. However, there is a lack of research addressing their poor fertility and productivity problems.

Orexin (OX)/hypocretin (HCRT) is a neuropeptide family with two members, OXA and OXB, which bind two receptors (OX1 and OX2); OX1 is specific for OXA, whereas OX2 binds both peptides with equal affinity (Wang *et al.,* 2018). While orexins and their receptors were first discovered in rat hypothalamus, they have also been detected in other mammalian organs, performing various behavioral and physiological functions (Zhang *et al*., 2005; Xia *et al*., 2023; Sardar *et al*., 2023; Wojciechowicz *et al*., 2023). The role of orexins in the regulation and control of testicular functions has previously been reported in mammals (Barreiro *et al*., 2004, 2005; Assisi *et al*., 2012; Nurmio *et al*., 2010; Costagliola *et al*., 2024). The immunohistochemical localization and biochemical expression of orexins in the male genital organs has been reported in the different mammalian species (Karteris *et al*, 2004; Barreiro *et al*., 2005; Zhang *et al*., 2005; Tafuri *et al*., 2010; Liguori *et al*., 2012; Liguori *et al*., 2014; Joshi and Singh, 2016, 2017; Liguori *et al*., 2017a,b; Liguori *et al*., 2018 a,b; Squillacioti *et al*., 2022; Yadav *et al*., 2023; Sahoo *et al*., 2024). Moreover, the immunoreactivity of orexins during the different developmental stages has been applied in the testis of rats (Barreiro *et al*., 2005) and mouse (Joshi and Singh, 2016, 2017). Similar studies seem to be lacking from the literature reviewed. Thus, our current study aimed to investigate the localization of OXA and OX1 by immunohistochemistry and to assess the relative HCRT expressions at mRNA level using reverse transcription polymerase chain reaction (RT-PCR). Complementary general histology of the camel testis was

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also shown using H and E stain. The study might reveal the possible involvement of orexins in the testicular functions and could add to our poor knowledge regarding dromedary camel's physiology and reproductive performance.

MATERIALS AND METHODS

Animals

Thirty male dromedary camels (*Camelus dromedarius*) slaughtered at Buraydah Central Slaughterhouse, Buraydah, Saudi Arabia were used in this study. The camels were apparently healthy according to ante-and post-mortem examination performed by the slaughterhouse veterinarians. Animals were slaughtered for human consumption under the laws of the Saudi Veterinary Authority that guarantees strict animal welfare regulations during animal transport, handling and slaughtering. All experiments were conducted at the College of Veterinary Medicine, Qassim University, during the period from May 2024 to April 2025.

Experimental design and sample collection

Testicular samples from three age groups (ten animals each) representing young camels (6-12 months), pre-pubertal camels (2-3 years) and adult camels (6-15 years) were used in this investigation. Camels' age was determined following the method described by Schwartz and Dioli (1992). For general histology and immunohistochemistry, fresh samples (1 cm by 1 cm by 0.5 cm) were randomly taken from left or right testis of each camel group and fixed in 10% buffered formalin. Fresh samples (0.5 mm square) were also taken from four animals of each group and placed in RNA-later solution (Thermo-Fisher Scientific) and stored at -80°C until being processed for total RNA isolation.

Histological and immunohistochemical analyses

Formalin fixed samples were processed and 5 micrometer thick paraffin sections were prepared and then conventionally stained using hematoxylin and eosin (H and E). The sections were examined under light microscope for general histological observations.

Ten paraffin sections from each animal group were de-waxed in two changes of xylol and rehydrated using descending concentrations of ethanol; the sections were then washed in distilled water and phosphate buffer saline (PBS). Retrieval of antigens was carried out in a microwave (750 W) in 0.01 M sodium citrate buffer with pH6.0 for 20 minutes. Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (ab64264; Abcam, UK) were used for OXA and OX1 immunostaining as described by the manufacturer's instructions. Sections were incubated overnight in a humidifying chamber with rabbit anti-orexin A (ab255294) and rabbit anti-orexin A receptor (ab224368) primary antibodies (Abcam, Cambridge, UK). Both antibodies were diluted (1:200) in a universal diluent (ab79995; Abcam, UK). Negative control immune reaction was done by skipping the primary antibody which showed negative staining. The reaction intensity was quantified using ImageJ software.

RNA extraction and cDNA synthesis

For the relative expression of testicular HCRT gene Real-Time PCR was applied in the three animal groups. The total RNA was extracted from the testicular samples kept in the RNA-later using Trizol solution (Therom-Fisher Scientific) following the manufacturer's protocol. The integrity and purity of the extracted RNA was determined using 1% agarose gel electrophoresis and Nanodrop spectrophotometer (Nano-Drop 2000/2000c, Therermo-Fisher Scientific). The isolated RNA was then used to generate cDNA by High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher, Catalog Number 4368814) following the manufacturer's instructions.

Primer design

NCBI webtools were used to obtain HCRT Reference Sequence (XM_031468756.2) to design specific primers for relative expression testing. Primer 3 web-tool was used to design the primer from the reference sequences using the website parameter guideline (Untergasser *et al*., 2012). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer set was used as positive control to check the reliability of other primer sets (Ibrahim *et al.,* 2020). Table 1 represents the primer sets details including primer sequences, product size and annealing temperature.

Real time qPCR

Generated cDNA samples were used Real time PCR after being diluted to 25 Nano-gram/microliter. The reaction was conducted using Applied Biosystems 7500 Real-Time PCR Instrument (Applied Biosystem); the 20 microliter total reaction consisted of 1 unit of QuantiTect SYBR Green PCR Master Mix (Qiagen), 10 picomole forward and reverses primers, 50 nanogram cDNA. Real-time cycler conditions were 50°C for 2 minutes, 94°C for 15 minutes as initial activation step, 40 cycles at 94°C for 15 seconds denaturation, 55°C for 30 seconds annealing and 72°C for 30 second extension. HCRT gene critical threshold (Ct) quantity was normalized against GAPDH (internal control gene's Ct) quantity (Ibrahim *et al*., 2020).

Statistical analysis

The obtained data (mean±SEM) were analyzed using One-Way Analysis of Variance (ANOVA) which was followed by

Table 1: Primer design for HCRT and GAPGH genes including their product sizes and annealing temperatures.

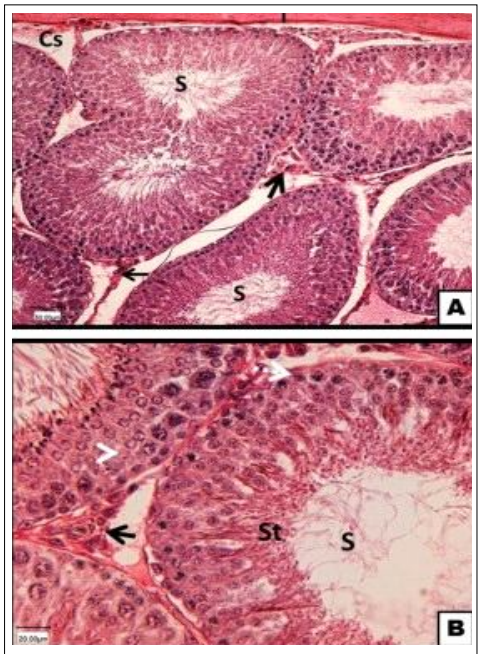
Gene ID	Forward primer 5′-3′	Reverse primer 5′-3′	Product size	Annealing temp.
HCRT	CTCTGCAAAGGTCTTCTGGG	TCTTGCCCAGCGTGAGGAT	194	56
GAPDH	CCATGTTTGTGATGGGCGTG	CATCTTCCCAGAGGGGCCAT	200	59

the Student t-test. Differences at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

General histological observations

The dromedary camel testis was covered by a thick layer of collagenous fibres (tunica albuginea) that sent septa forming small lobules (Fig 1A). The lobules contained small seminiferous tubules surrounded by interstitial connective tissue with Leydig cells; the latter cells were polyhedral and arranged in clumps or cords separated by small blood vessels (Fig 1A). Each seminiferous tubule was surrounded by a basal lamina and contained spermatogenic and supporting cells (Sertoli cells) (Fig 1B). In young camels, Spermatogonia were the only type of spermatogenic cells, whereas in adult ones there were spermatogonia, primary spermatocytes, spermatids and spermatozoa. Spermatogonia had rounded shape and dark nuclei and rested directly on the basal lamina; primary spermatocytes were large with large nuclei; spermatids were rounded or elongated and located near the luminal surface; spermatozoa occupied the tubular lumen. Sertoli cells were tall and irregular with large and oval nuclei; they extended between the spermatogenic cells from the basement membrane to the tubular lumen (Fig 1B).



A: tunica albuginea (T), connective tissue septa (Cs), Leydig cells (arrow) and seminiferous tubules (S). B: Leydig cells (arrows), Sertoli cells (arrowhead), spermatogonia (broken arrow), spermatid (St) and seminiferous tubules (S). Bars: A: 50 µm; B: 20 µm.

Fig 1: H and E-stained testicular sections of camels.

Immunohistochemical observations

The immunohistochemical reaction of OXA and OX1 in the testis of the camel groups is summarized in Fig 2, 3 and 4 and Table 2.

In young camels, OXA (Fig 2A) and OX1 (Fig 2B) immunoreactions were observed in the testicular interstitial tissue and seminiferous tubules. In the interstitium, OXA and OX1 immunoreaction was seen in Leydig cells, while inside the tubules these reactions were observed in Sertoli cells and spermatogonia. The immunoreactivity of OXA was more intense in the spermatogonia than in Sertoli cells and Leydig cells, whereas OX1 reactivity was more intense in the spermatogonia and Leydig cells than in Sertoli cells. The interstitial blood vessels appeared with weak to negative OXA and OX1 reaction at this age.

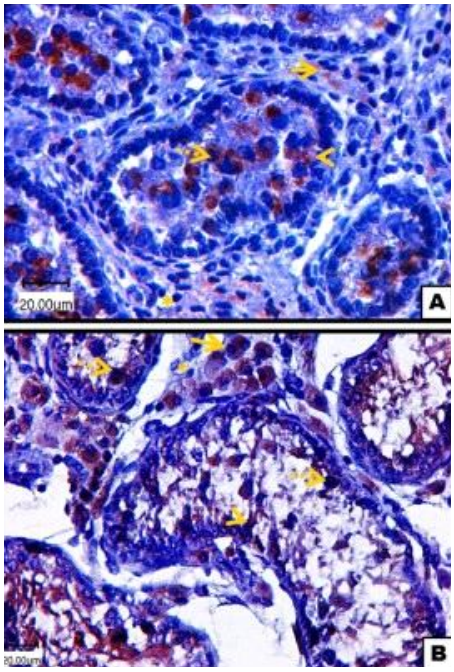
In testes of pre-pubertal camels, OXA (Fig 3A) and OX1 (Fig 3B) immune-labelling was observed in Leydig cells, Sertoli cells and spermatogonia. Strong immunopositive signals were noted in both OXA and OX1, especially in the Leydig cells and spermatogonia. Also at this age, the reaction was less intense in Sertoli cells and spermatogonia.

In the sections of adult camels' group, OXA (Fig 4A) and OX1 (Fig 4B) positive immunoreactions were seen in Leydig cells, Sertoli cells, spermatogonia, primary spermatocytes and spermatozoa. For both proteins, Leydig cells exhibited very strong immunoreaction, while spermatogonia, primary spermatocytes and spermatozoa showed lower intense immunoreactivities. In the Leydig cells the OX-immunoreaction was more prominent than OX1-immunoreaction. Sertoli cells showed moderate to weak OXA and OX1 immuno-reaction at this age.

Table 2: Immunohistochemical reactions of OXA and OX1 in the testis of the three camel age groups.

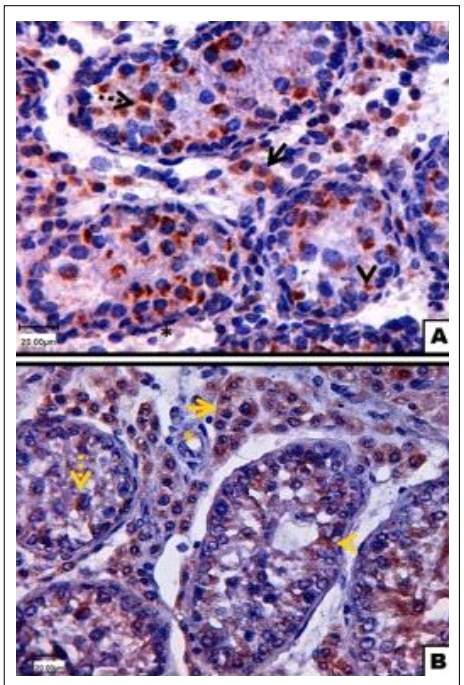
Protein	Animal group	Testicular tissue					
		LCs	SCs	SMG	PSCs	SPZ	IVs
OXA	Young animals	++	++	+++			+-
	Pre-pubertal animals	+++	++	+++			+
	Adult animals	++++	++	+++	+++	+++	+++
OX1	Young animals	+++	++	+++			+-
	Pre-pubertal animals	+++	++	+++			+
	Adult animals	++++	++	+++	+++	+++	+++

(LCs)- Leydig cells; (SCs)- Sertoli cells; (SMG)- Spermatogonia; (PSCs)- Primary spermatocytes), (SPZ)- Spermatozoa; (IVs)- Interstitial blood vessels; (-)- Negative; (+)- weak; (++)- moderate, (+++)- strong; (++++)- Very strong.



Immunoreactions of Leydig cells (arrow), Sertoli cells (arrowhead), spermatogonia (broken arrow) and interstitial blood vessels (asterisk). Bars: 20 µm.

Fig 2: Immunoreactions of OXA (A) and OX1 (B) in testicular sections of young camels (6-12 month).



Immunoreactions of Leydig cells (arrow), Sertoli cells (arrowhead), spermatogonia (broken arrow) and interstitial blood vessels (asterisk). Bars: 20 µm.

Fig 3: Immunoreactions of OXA (A) and OX1 (B) in testicular sections of pre-pubertal camels (2-3 years).

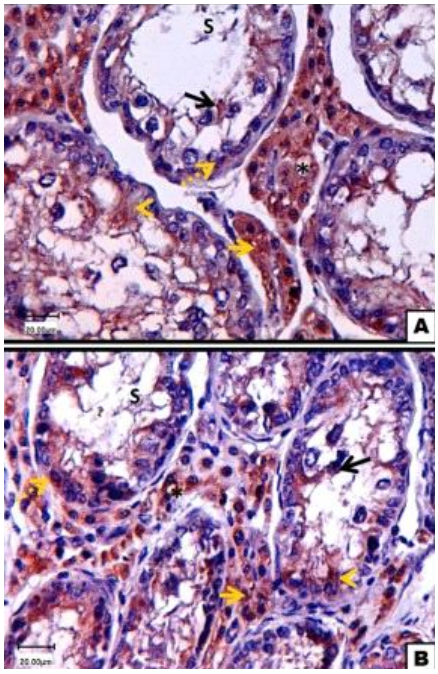
PCR amplification and real time qPCR relative expression

The regular PCR conducted on generated cDNA revealed clear amplification of testicular HCRT with the absence of genomic DNA contamination. The testicular tissue of the young, pre-pubertal and adult dromedary camels showed HCRT gene expression in all samples. The testis of adult group appeared with significant higher expression than the young and pre-pubertal ones; further the expression in the pre-pubertal group was higher than in young group (Fig 5).

Our present study reports, for the first time, the immunohistochemical localization and distribution of the OXA and OX1 in the seminiferous tubules and interstitium of young, pre-pubertal and adult dromedary camels. Positive OXA and/or OX1 immunoreactions have also been reported in the testis of mice (Liguori *et al*., 2012; Joshi and Singh, 2016, 2017), rats (Barreiro *et al*., 2005; Tafuri *et al*., 2010), human (Karteris *et al.,* 2004), sheep (Zhang *et al*., 2005) and alpaca camels (Liguori *et al*., 2012). Liguori *et al*. (2012) thought that the mammalian seminiferous tubules contain a cellular complex that is capable of internalizing and/or secreting OXA. Orexins contribute to the regulation of steroidogenesis and spermatogenesis both systemically through the pituitary gonadotropins and locally through substances, including OXA, which is synthesized in the testis (Nurmio *et al*., 2010). Similarly, it could be suggested that OXA and OX1 might be synthesized in the dromedary testis where they could exert regulatory and steroidogenic effects.

The current investigation showed that OXA and OX1 immuno-positive signals in the young and pre-pubertal camels were detected in the spermatogonia, Sertoli cells and Leydig cells. However, immuno-signals of both peptides were less intense in the Leydig cells of young animals, especially for OXA. Similar findings have also been shown in the testicular tissue of premature mice (Joshi and Singh, 2017). According to the latter authors, the presence of positive immunoreactivities for OXA and OX1 in spermatocytes of premature animals is due to a possible role of orexin A and its receptor in the resumption of mitotic activity in these cells. Positive immunoreactivity signals for OXA and OX1 were observed in the interstitial tissue including Leydig cells and blood vessels; the signals were less intense in young camels compared to pre-pubertal camels. The immunoreactivity of OXA and OX1 in young and pre-pubertal camel testis may indicate possible role of orexins in Leydig cells proliferation and differentiation. It has been previously reported that the proliferation and development of Leydig cells in mice begin at low rates before birth, gradually increase after birth and reach a peak shortly before or at puberty (Vergouwen *et al*., 1991). Further, a sharp increase of steroidogenic capacity in Leydig cells was observed in mice testis at pre-pubertal age (25 days after birth) (O'Shaughnessy and Sheffield, 1991), while increased production of androgens was reported in Leydig cell in mouse testis at the same age (Chase and Payne, 1983).

It has been reported that strong OX1 immunoreactive in Leydig cells of pre-pubertal and pubertal mice suggests the presence of increased binding sites for OXA (Joshi and Singh, 2017). It is known that Sertoli cells proliferation starts during fetal life and shows with maximal proliferative activity just before birth; after birthing their activity decreases gradually and completely stops before puberty (Kluin *et al.,* 1984). As explained by Joshi and Singh (2017), the presence of positive OXA and OX1 immune signals in the testicular tubules in young rats might help in germ cell development and Sertoli cell proliferation.



Immunoreactions of Leydig cells (brown arrow), Sertoli cells (arrowhead), spermatogonia (broken arrow), primary spermatocytes (black arrow) and interstitial blood vessels (asterisk). Bars: 20 µm.

Fig 4: Immunoreactions of OXA (A) and OX1 (B) in testicular sections of adult camels (6-15 years).

The adult animal group in the present study showed positive immunoreactivity for both OXA and OX1 in Sertoli cells, Leydig cells, spermatogonia, primary spermatocytes, spermatids and interstitial blood vessels; the reaction was very strong in Leydig cells and spermatocytes. Positive OXA and OX1 immunosignals have also been detected in adult animals, including mice (Joshi and Singh, 2017), rat (Barreiro *et al*., 2004), dog (Liguori *et al*., 2018), human (Karteris *et al.*, 2004) and alpaca camel (Liguori *et al*., 2012). As stated by Liguori *et al.* (2012) OXA and OX1 immune reactions were shown in numerous Leydig cells, but rarely in Sertoli cells and spermatogenic cells in the testis of adult alpaca camel (Liguori *et al*., 2012). While Joshi and Singh (2017) reported that strong OX1 immuno-positive signals in the testicular interstitium of mature mice could be an indication of much existence of OXA binding sites, Nurmio *et al*. (2010) suggest probable role in the regulation of steroidogenesis. According to Jöhren *et al*. (2001) the expression of orexin receptors in the Leydig cells is consistent with the testosterone role as found in the rat adrenal system at mRNA level. Moreover, Barreiro *et al.* (2004) claimed that the expression of OXs in rat testes is age dependent and the maximal expression occurs in adulthood. Additionally, Costagliola *et al.* (2024) concluded that the linking between orexins and their receptors in Leydig cells could regulate the production of testosterone.

Further, the results of real time PCR analysis also confirm the expression of mRNA coding for HCRT in the testis of dromedary camels with the level of its expressions being remarkably increase with the age increase. The mRNA expression of HCRT has also been shown in the testis of rat (Tafuri *et al*., 2010), epididymis of camelid alpaca

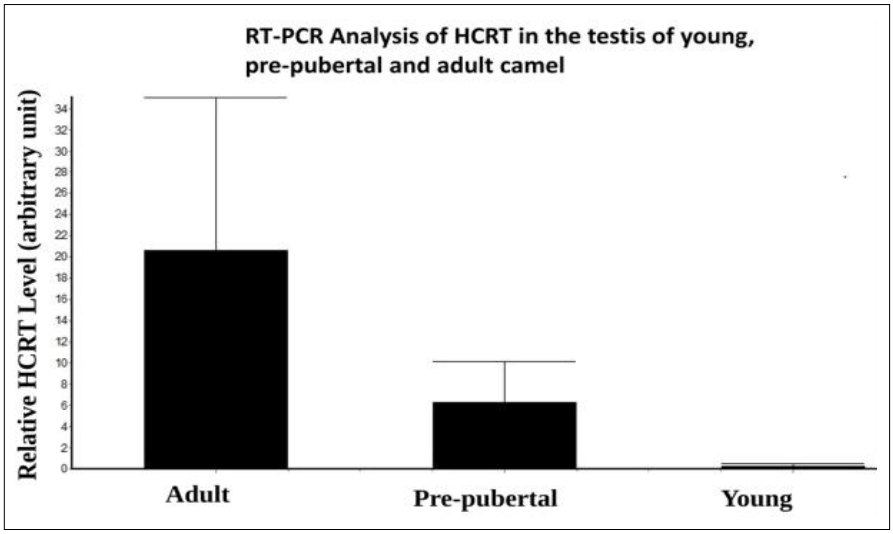


Fig 5: The expression of testicular HCRT at the transcript level is significantly ($p<0.05$) higher in adult than pre-pubertal and young camels and significantly ($p<0.05$) higher in pre-pubertal than young camels.

(Liguor *et al*., 2014) and prostate of human. According to Joshi and Singh (2017), the expression of OXA and OX1 in the mice testis attains gradual increase from the time of birth to reach maximal expression in adult animals. Differential mRNA expression of orexins in the testis could confirm the presence of peripheral orexin system in dromedary camel reproductive organs and consequently, their possible impact on reproductive activity.

CONCLUSION

As far as we know, the present investigation shows, for the first time, the evidence for the presence of orexin A, OX1 and HCRT in the dromedary camel testis; the study confirms the immunohistochemical distribution of OXA and its OX1 in both the interstitial and tubular compartments of the testis of young, pre-pubertal and adult dromedary camel; the presence of OXA and OX1R in the testis of young and pre-pubertal animals suggests a role in germ cells proliferation and development Sertoli cells, germ cells and Leydig cells and they could also be important in spermatogenesis and steroidogenesis during adult life of dromedary camels.

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Conflict of Interest

All authors declared that there is no conflict of interest.

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