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ADAM2 localization and expression in the ductus deferens and male accessory glands of rutting camels (*Camelus dromedarius*)

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ABSTRACT

The testicles and epididymal ducts of several mammals, such as camels, have been shown to have ADAM2 (fertilin) protein. But nothing is known about the existence of this protein in the camel's ductus deferens and male accessory glands. The current study employed immunohistochemical (IHC) and quantitative real-time polymerase chain reaction (qRT-PCR) techniques to elucidate the distribution and expression of ADAM2 in the ductus deferens and male accessory glands in dromedary camels throughout the rutting season. Samples of tissue were collected from the ductus deferens. (initial, middle and ampullary parts), prostate (corpus and disseminated part) and bulbourethral gland from eight mature male camels. IHC result revealed that ADAM2 protein localized in all parts of the ductus deferens with a strong immunoreaction in the ampullary parts. A variety of immunoreactions were recognized in the different parts of the male accessory glands. qRT-PCR results showed that ADAM2 mRNA was expressed variably in all parts of the ductus deferens and male accessory glands; the ampullary part of the vas deferens displayed the highest levels of expression ($P<0.05$). The current study concluded that ADAM2 is found in the ductus deferens and male accessory glands with greater expression in the ampulla and prostate glands where seminal fluids are secreted. Thus, it is believed that these organs aid in the sperm's creation of this protein before ejaculation in the female camel's genital organs.

Introduction

Camel is one of the most valuable animals in Saudi Arabia. It is well adapted to hot, arid areas and can live and breed there. As in other desert countries of Asia, camels are the main source of food such as meat and milk in the Saudi kingdom. Camels are regarded as socially wealthy in the tribes of Arabia (Getahun and Belay, 2002). The major problem of the male reproductive tract of dromedary camels was discussed by several authors (Ali *et al*., 2018; Ali *et al*., 2021). To develop therapies for male infertility, it is essential to understand the proteins involved in male fertility (Beeram *et al*., 2019). Thus, molecular techniques have been designed to precisely assess the expression levels of specific proteins of the sperm that are utilized as indicators of fertility in males (Ashrafzadeh *et al*., 2013). The journey of the sperm to the oocyte goes via several physiological and biochemical changes (Yoshida *et al*., 2008; Chan *et al*., 2009; Sato *et al*., 2010). For instance, numerous proteins released by the epididymis and male accessory glands are attached to the sperm along various portions of the male reproduction system (Aitken *et al*., 2007; Fàbrega *et al*., 2011).

A disintegrin and metalloproteases (ADAMs) are a kind of transmembrane proteins which have three conserved multidomain structures. ADAMs were found in various tissues like the testis and brain (Gupta *et al*., 1996; Sagane *et al*., 1998).

Nevertheless, it remains unclear how most ADAM gene products work, the ADAM gene products are linked to cell migration, cytokine and growth factor shedding, and regulation of membrane fusion (Seals and Courtneidge, 2003). Also, they act in a wide range of biological procedures, such as fertilization (Primakoff and Myles, 2000). ADAM2 (fertilin β) belongs to the prior family and is possibly involved in the eclectic movement of certain proteins of the sperm from the endoplasmic reticulum of testicles germ cells onto the cellular surface (Nishimura *et al*., 2004).

Previous research in guinea pigs indicated that the testes generated ADAM2, which was then metabolized in the epididymal parts (Blobel *et al*., 1990). Whereas many mammalian species showed ADAM2 in their testes and epididymal sperm (Perry *et al*., 1995; Hardy and Holland, 1996; Hunnicutt *et al*., 1997; McLaughlin *et al*., 1997; Waters and White, 1997; Han *et al*., 2009; Fàbrega *et al*., 2011; Cho, 2012; Choi *et al*., 2016). In dromedary camels, ADAM2 was expressed in the sperm, epididymis and testis (Al-Shabebi *et al*., 2021). As of the literature of the moment, no data about the expression of ADAM2 in the ductus deferens, prostate and bulbourethral glands of the camel. For that, this study was done to reveal ADAM2's presence and expression in the ductus deferens and male accessory glands of the dromedary camel throughout the meeting (rutting) season to assist in completing the dispersion of this protein throughout the camel's male genital tract.

Materials and methods

Samples collection

The King Faisal University ethics committee authorized an animal protocol, which was followed for all animal sample operations. In this investigation, fresh ductus deferens and male accessory gland samples were taken from eight clinically healthy adult camels (4≥ years old). Tissue samples were collected immediately from slaughtered animals in Al-Omran abattoir, Al-Ahsa, Saudi Kingdom throughout the rutting season (from October to next April). After the extraction of the reproductive organs, tissue samples were collected from the initial, median and ampullary ductus deferens, compact, and disseminated prostate and bulbourethral glands. Samples were preserved for the immunohistochemistry method (IHC) in 10% buffered formalin. For quantitative polymerase chain reaction (qRT-PCR) analysis, additional samples were obtained from the same organs and stored at 80°C after being frozen in liquid nitrogen for ten minutes.

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Immunohistochemistry Technique

Samples were dehydrated in a series of graded ethanol after being fixed in formalin, cleaned via xylene, and then embedded inside the paraffin wax. Using a microtome, sections thickness of 5 mμ were cut and put on Superfrost slides. Following dewaxing and rehydrating, slices were stained by the avidin–biotin–peroxidase complex technique (Adeghate *et al*., 2001) using rabbit polyclonal antibody against ADAM2 (1:100) (Cat. No. HPA026581, Sigma-Aldrich, USA). To further enhance the epitope access immunostaining signal, buffer for antigen-retrieval (pH 6.0, sodium citrate 50mM) was made and heated to 100°C for 20 min. Ten minutes were utilized for treating the tissue sample slides with 3% hydrogen peroxide (90μL), then, for 10 min with normal goat serum (NGS) (Cat. No. ab7481 by Abcam, Inc.) at a 1:20 dilution, and after that using dilution 1:100 primary antibody for one hour at room temperature (RT). After adding the appropriate biotinylated secondary antibody (1:100), the slides were incubated at RT for 30min. For 20 min, each slide was treated with a streptavidin–horseradish peroxidase HRP (Cat. No. ab64269, Abcam, Inc.). After adding an appropriate quantity of 3,3′-diaminobenzidine tetrahydrochloride chromogen substrate for 5min, the color was produced. Phosphate-buffered saline (PBS) was used for washing processes in between each reagent. Hematoxylin was used to counterstained slides, then, the slide was dehydrated, cleared, and after that put on a coverslip using dibutylphthalate polystyrene xylene (DPX), and examined with a Leica DM6000 B light microscope (Germany). Photomicrographs were taken using a digital camera (Leica DFC420, Germany). The strength of immunostaining was categorized as high (+++), moderate (++), or faint (+).

qRT-PCR technique

Utilizing a Bead Ruptor Homogenizer (OMNI International, NW Kennesaw, GA, USA), a 50 mg tissue sample was obtained. The total RNA had been extracted by TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) by the manufacturer's guidelines. After extracting the total RNA by chloroform and isopropanol, ultrapure diethylpyrocarbonate (DEPC)-treated RNase-free water (Invitrogen, USA) was used to suspend it. Next, the total RNA was examined with BioTek Synergy HTX reader (BioTek, USA) for purity and concentration. After that, with the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA), RNA was reverse-transcribed to cDNA in a 20μL total volume combination containing 4μL iScript® Reaction Mix, 1μL iScript® Reverse Transcriptase, and nuclease-free distilled water. To inactivate the reverse transcriptase, The combination was then left to incubate at 25°C for 5min, 46°C for 20min, and 95°C for sixty seconds in the last step.

The qPCR reaction was performed using the CFX96® Touch Real-time PCR (BioRad, USA) and SsoAdvanced SYBR Green Supermix dye (BioRad, USA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and dromedary camel ADAM2 genes were tested using the gene-specific primers given in Table 1. The 20μL reaction mixture was made up of 10μL of the master mix, 2 μL of each forward and reverse primer pm/μL, 2 μL of the sample's cDNA, and 4 μL of nuclease-free water. The thermal cycling program was as follows: 30 seconds at 95°C; 40 cycles at 95°C for 15seconds; thereafter,30seconds at 60°C, and 10 seconds at 72°C. Fluorescence emission was established using two replicates of each cDNA template. Using the housekeeping GAPDH gene as a reference and the CFX ManagerTM software V3.1 (BioRad, Hercules, CA, USA), the respective levels of expression of the ADAM2 gene were automatically determined.

Table 1. Primer sequences employed in qRT-PCR in the present research.

Gene	Sequence (5'-3')	Accession No.	Amplicon Length (bp)
ADAM2	F: TGA GTG GGG CAA TCC AAT GT R: TTC GCA CTT CGT GTA CCC TG	XM_010999136.1	140
GAPDH	F: CCT GGA GAA ACC TGC CAA ATA R: TCG TTG TCG TAC CAG GAA ATG	XM_010990867.2	207

Statistical analysis

To examine the data, SPSS® 16 software was utilized. The mean± SE of the variance expressions for the various tissues were compared using analysis of variance (one-way) with post hoc analysis.

Results

Immunohistochemistry

In the dromedary camel, all parts of the ductus deferens and male accessory glands showed distinct immunostaining for ADAM2 with varying pattern intensities (Table 2). There was no staining in the control sections. In the vas deferens, ADAM2 protein was detected moderately in the tall columnar and basal cells of the epithelial layer in the initial and middle parts (Figs. 1, A & B), while the lining epithelial cells of the ampullary part showed a strong reaction (Fig. 1C). The secretory epithelial cells in both parts of the prostate gland had moderate immunoreaction to the ADAM2 antibodies (Figs. 2D &E). In contrast, the bulbourethral secretory epithelium showed a weak reaction (Fig. 3A). The submucosa of all previous parts was free of any positive stains. Also, the control sections showed no staining (Figs. 1a, b & c; 2d, e; 3f).

qRT-PCR

The expression of ADAM 2 mRNA in the vas deferens, prostate, and bulbourethral glands of dromedary camels is shown in Table. 2 and Fig. 4. In comparison to the beginning and middle portions of the vas deferens, the ampullary part had considerably higher levels of ADAM2 mRNA expression ($p < 0.05$), while the middle part had the lowest expression in the ductus deferens. In the male accessory gland, a moderate level of ADAM2 expression was seen in the disseminated and compact prostate glands, but the bulbourethral gland had the lowest level of ADAM2 mRNA expression.

Table 2. Distribution of the ADAM2 immunostaining in the ductus deferens, prostate and bulbourethral glands of the dromedary camel.

Organ	DI	DM	DA	PC	PD	BU
ADAM2	++	++	+++	++	++	+

The strength of the positive immunostaining was rated as +++strong, ++ moderate and + weak. (DI) vas deferens initial part, (DM) vas deferens median part, (DA) ampulla, (PC) prostate compact, (PD) prostate disseminated and (BU) bulbourethral gland.

Table 3. Heat map summarizing relative ADAM2 mRNA expression levels in the ductus deferens, prostate and bulbourethral glands of the dromedary camel.

DI	DM	DA	PC	PD	BU
0.58±0.16	0.37±0.09	1.39±0.10	0.95±0.21	0.96±0.25	0.35±0.06

ADAM2 mRNA expression as means ± standard errors. The red indicates the highest expression. While the green indicates the lowest expression level. (DI) vas deferens initial part, (DM) vas deferens median part, (DA) ampulla, (PC) prostate compact, (PD) prostate disseminated and (BU) bulbourethral gland.

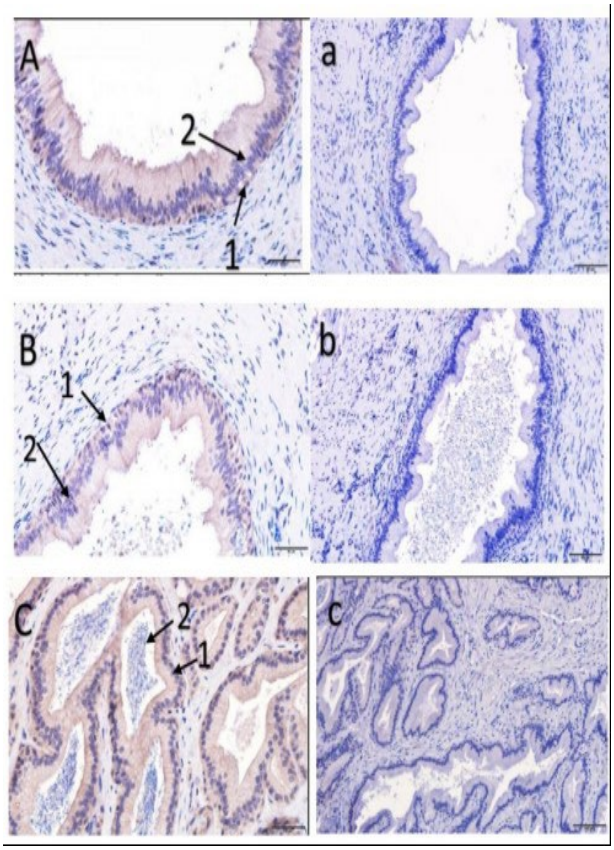

Fig. 1. Photomicrograph of ADAM2 immuno-reactive staining in the vas deferens of the dromedary camel. (A) initial and (B) middle parts showing moderate immunostaining reaction of ADAM2 in the pseudostratified epithelium cells (1, tall columnar cells; 2, basal cells). 20X. (C) Ampullary part displaying strong immunostaining of ADAM2 in the epithelial cells (1). 2, luminal containing. 20X. (a), (b) and (c) negative control 20X.

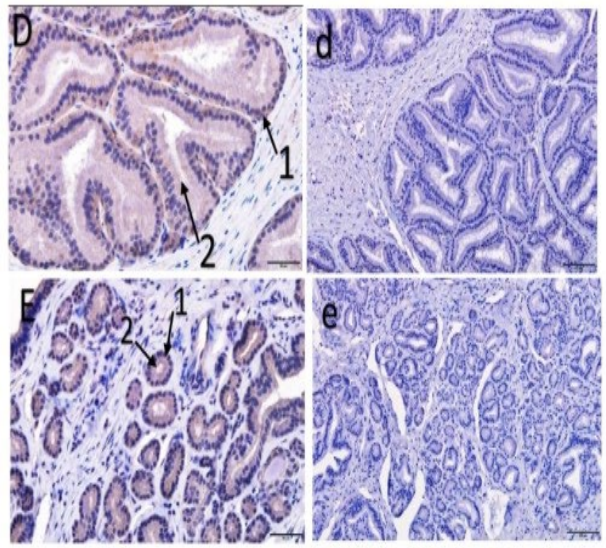

Fig. 2. Photomicrograph of ADAM2 immuno-reactive staining in the prostate gland of the Sahara camel. (D) compact and (E) disseminated prostate showing moderate immunostaining of ADAM2 in the secretory epithelial cells (1). 2, lumen. 20X. (d) and (e) negative control 20X.

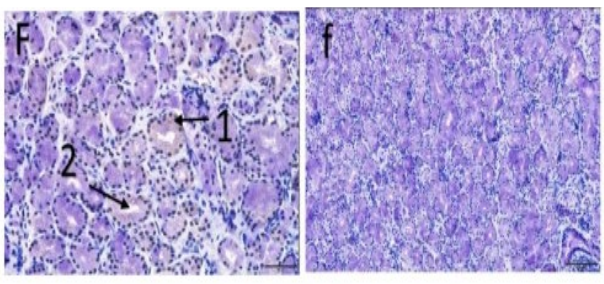

Fig. 3. Photomicrograph of ADAM2 immunostaining in the bulbourethral gland of the Sahara camel. (F) the gland revealing weak immune-reactive staining of ADAM2 in the secretory epithelial cells (1). 2, lumen. 20X. (f) negative control 20X.

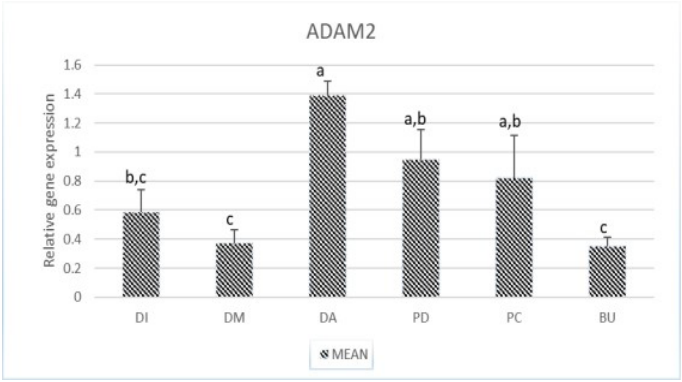

Fig. 4. Expression of ADAM2 mRNA in the Sahara camel's initial, DI; middle, DM; and Ampullary part, DA of the ductus deferens, compact, PC; and disseminated part, PD of the prostate gland and bulbourethral gland (BU). In a comparison of the ADAM2 mRNA expression level in the vas deferens, DA was considerably expressed more highly than in the DI and DM. ADAM2's mRNA expression level in the prostate gland (PC and PD) was moderately seen, while BU had weak expression.

Discussion

Among the male reproductive system of the one-humped camel, the expression of proteins in the ductus deferens has received attention in the last few decades (Saleh, 2006; Alkafafy *et al*., 2011; Althanian, 2023; Al Khodair *et al*., 2023). Although many studies have reported the presence of some proteins within male accessory glands, such as cysteine-rich secretory protein-3 (CRISP-3) in horses (Fedorka *et al*., 2017) and cysteine sulfinate decarboxylase (CSD) in mice (Fan *et al*., 2009), the literature about proteins in the male accessory glands of the camel was scarce (Bogle *et al*., 2018; Althanian, 2023).

A recent study investigated the ADAM2 expression in the testes and epididymis of the dromedary camel (Al-Shabebi *et al*., 2021). As far as the authors know, there is a lack of published research indicating the distribution of ADAM2 proteins and/ or expression of mRNA of ADAM2 in the ductus deferens and/ or male accessory glands of the camel.

The present study showed that a significant amount of ADAM2 was found in the ampulla of the camels' ductus deferens, whereas the initial and middle parts of the vas deferens and both parts of the prostate gland showed a moderate immuno-reaction. Also, ADAM2 antibodies have a limited affinity for the bulbourethral gland. ADAM2 mRNA expression in the preceding organs supported the immunohistochemistry image and revealed that the ampullary part had considerably higher levels than the beginning and middle parts of the vas deferens. In contrast, the prostate glands showed moderate expression, and the bulbourethral gland had the lowest level.

The sperm and semen fluid express different types of ADAM proteins, including ADAM2 (Choi *et al*., 2016; Almhanna, 2019; Al-Shabebi *et al*., 2021). In addition, the quality and quantity of camel semen correlate with changes in the morphology of the testis and accessory sex glands (Al-Bulushi *et al*., 2019).

The ductus deferens' function is an essential organ for sperm transport, viability, storage and protection against reactive oxygen species and proteases. Following this, the male accessory sex gland secretes seminal plasma, 60-90% of the total amount of sperm (Chughtai *et al*., 2005; Dukes, 2005) that contains a wide variety of heterogeneous molecular structures, including proteins (Wang *et al*., 2020). Moreover, the amount and quality of semen in camels are related to changes in the morphology of the testis and accessory sex glands (Al-Bulushi *et al*., 2019). Therefore, the function of these organs in releasing proteins and our earlier research on ADAM2 in the testicles, epididymis, and sperms of the camel support the expression and distribution of ADAM2 in the ductus deferens and male accessory glands of this animal.

Conclusion

The current study's findings revealed that ADAM2 protein is distributed in the ductus deferens and male accessory glands of the camels, which suggests that these organs may have significant effects in the synthesis of this protein in the sperm before ejaculation in the female genital organs of the camel.

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Conflict of interest

The author declare that they have no conflict of interest.

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