Journal of Experimental Biology and Agricultural Sciences, October - 2018; Volume – 6(5) page 800 – 807

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

EXPRESSION OF ADAMTS10 IN MALE REPRODUCTIVE TRACT OF BUFFALOES (*Bubalus bubalis*)

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Received – July 17, 2018; Revision – September 14, 2018; Accepted – September 25, 2018
Available Online – October 5, 2018

DOI: http://dx.doi.org/10.18006/2018.6(5).800.807

KEYWORDS

ADAMTS10

Buffalo

Gene expression

Male reproductive tract

Sperm

ABSTRACT

Large number of proteases have been identified in different parts of the male reproductive tract which play a crucial role during testicular development, epididymal sperm maturation, and sperm-oocyte fusion. The ADAMTS10 is a member of metalloproteinase family, the expression of which is important in sperm-oocyte interaction at the time of fertilization in mouse. In this species, the expression of ADAMTS10 has been demonstrated in testis, and on surface of spermatozoa from caput, corpus and cauda epididymis. Whether ADAMTS10 plays any role in male fertility of any of the domestic ruminant species including buffaloes is not known so far. Thus, a study was conducted to detect the expression of ADAMTS10 in testis, different parts of the epididymis and ejaculated spermatozoa of water buffalo (*Bubalus bubalis*) using real-time PCR, Western blot, and indirect immunofluorescence techniques. The highest expression of ADAMTS10 transcript was observed in corpus epididymis, followed by cauda, caput and testis. The Western blot detection of ADAMTS10 using the heterologous antibody has demonstrated 31-, 44-, 50-, 65 kDa immune-reactive protein bands from testis and 82 kDa protein from both testes and corpus epididymis and a major 36 kDa protein from ejaculated spermatozoa. The indirect immunofluorescence study has demonstrated high fluorescence signal in post-acrosome and the middle piece region of the ejaculated spermatozoa. Thus the differential expression pattern of ADAMTS10 in different parts of male reproductive tract of buffalo indicates their role in sperm maturation process. However, specific role of ADAMTS10 in buffalo male reproduction can only be ascertained after further studies.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Several classes of proteases play the crucial role in mammalian male reproductive events including testicular development, epididymal sperm maturation, sperm-oocyte fusion etc. They are also critical in maintaining the semen fluidity, sperm motility and penetration of the cervical mucus, etc. Among proteases, the Adamalysin-related proteinases (a disintegrin and metalloproteinase, commonly ADAMs) received much attention in its role in sperm–egg interactions at fertilization site because of the disintegrin domain having specific role in cell-cell interaction and shedding of cell surface proteins (Kuno et al., 1997; Yuan et al., 1997). ADAMTS (a disintegrin and metalloproteinase with thrombospondin type 1 motifs) are members of ADAMs and are soluble and secretory in nature (Le Magueresse-Battistoni, 2000). They are zinc dependant metalloproteinases, containing thrombospondin type 1 repeat (Porter et al., 2005). The common functions of these proteases are cell–extracellular matrix (ECM) interaction, tissue remodeling and regeneration. In mice, the role of ADAMTS10 has been reported in fibrillin formation and its function (Mularczyk et al., 2018). However, the Weill-Marchesani syndrome (WMS) in mice and glaucoma in canine has been found to be associated with mutation of ADAMTS10 gene (Cain et al., 2016; Graham et al., 2016). In male reproduction, ADAMTS play an important role in various stages of gonad morphogenesis, gametogenesis and gamete functions involving sperm-egg fusion and implantation of the conceptus (Russel et al., 2015). To date, 19 ADAMTSs have been identified in human tissues (Le Magueresse-Battistoni, 2000). The closely related member, ADAMTS10 has been identified in mouse (Dun et al., 2012). In mouse, the ADAMTS10 is secreted from the testis during the later stages of spermatogenesis and incorporated into the acrosomal domain of developing spermatids. During epididymal maturation, this particular protease is processed and expressed on the surface of the peri-acrosomal region of the spermatids and mature spermatozoa. In mouse, the protease activity of ADAMTS10 has a crucial role in the sperm and zona pellucida interaction. The use of galardin, a broad-spectrum inhibitor of metalloproteinases or anti-ADAMTS10 antibody, demonstrated reduced sperm-zona interaction (Dun et al., 2012). The identity and function of ADAMTS10 have not been elucidated in domestic animals including buffaloes. In the present study, we have demonstrated the relative expression of ADAMTS10 in testis and different segments of epididymis of water buffalo (*Bubalus bubalis*) using real-time PCR, Western blot and immunocytochemistry. The presence of ADAMTS10 in buffalo testis and different segments of the epididymis may help us in assigning its role in male reproduction of this species.

2 Materials and Methods

2.1 Animals

Testes, lungs, kidneys and liver tissues of four healthy, sexually matured adult Murrah buffaloes (*B. bubalis*), about 3–5 years of age, and 450-650 kg of body weight, were collected from the Municipal slaughterhouse, Palakkad, Kerala, following the relevant legislation of the state government. Ejaculated Murrah buffalo bull semen was collected, twice a week from Nandini Sperm Station, Hessarghatta, Bangalore. The tissues and semen were collected in the month of September-February which corresponds to the active period of breeding in buffaloes in India. All the experimental protocols of the study were carried out following the guidelines of Institutional Animal Ethics Committee.

2.2 Collection of buffalo tissues, semen and their processing

The fascia, tunica vaginalis and tunica albuginea were aseptically removed from the testes. Subsequently, the entire epididymis, along with the vas deferens, was separated from the surface of the testes and was blotted on filter paper to remove excess blood and superficial fluid. Initially, the vas deferens was separated from the epididymis and the length of the epididymis was then divided into caput (head), corpus (body) and cauda (tail) segments by following the method of Goyal (1985). The cut pieces of all the tissues were collected in RNA Later (Sigma-Aldrich Co., MO, USA) in a 15 ml tube and brought to the laboratory in a thermos flask containing ice and stored at -86°C. The tissues were processed for extraction of proteins following the method of Kizaki et al. (2008). Briefly, 1g of tissue from each of reproductive and non-reproductive parts of male buffalo were minced with scissors and added with 1.5 ml of protein extraction buffer (50 mM Tris-Cl, pH 7.5 containing 150 mM NaCl, 1 mM $CaCl_2$, 0.05% Brij 35, 10 µg/ml Leupeptin, 1mM PMSF). The tissues were lysed for 3 cycles (10 seconds on and off at 11,000 rpm) on ice in a homogenizer (Polytron, Kinematica AG, Luzernerstrasse 147a, Switzerland). The contents were resuspended well and kept on a rotospin at 10 rpm for 15 minutes at 4°C and centrifuged (Refrigerated centrifuge, Eppendorf, Hamburg, Germany) at 20,817 X g at 4°C for 30 minutes to separate the supernatant. The supernatants were stored at -20°C for future use.

Buffalo semen was collected and processed for extraction of protein as per Gurupriya et al, (2014). The semen was collected with an artificial vagina (IMV Technologies, France) maintained at 40°C at Nandini Sperm Station and assessed the mass activity using light microscopy at 10x magnification. The semen having mass activity of 3.0 and greater (on a scale of 0–4) was used in the study. Within 5 to 10 minutes of collection, buffalo semen

samples (1mL each) were centrifuged in 15-mL polypropylene conical centrifuge tubes at 275 X g for 10 minutes at room temperature to separate seminal plasma and sperm pellets. The sperm pellets were washed thrice in PBS, pH 7.4 (dilution 1:6). The proteins were extracted from sperm pellets using the protein extraction buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 0.05% Brij 35, 0.1% SDS, 1% Triton X-100 and 1% NP-40, 1mM PMSF. The protein concentrations of the extracts from sperm pellet and tissues were determined by Bradford's method (Bradford, 1976), using purified bovine serum albumin as a standard (Fraction V, Sigma-Aldrich Co., MO, USA).

2.3 Western blot detection of ADAMTS10 in testis, epididymis and ejaculated spermatozoa

Buffalo testis, epididymis, lungs, kidney, liver tissues, and spermatozoa proteins were separated using Laemmli's reducing SDS-PAGE on 10% uniform gel (Laemmli, 1970). The separated proteins on the gel were transferred to Immobilon P-polyvinylidene difluoride membrane (pore size, 0.45 µm) adopting a two-step transfer method of Otter et al. (1987). The membranes were processed as per Rana et al. (2017). Since the antibody against buffalo ADAMTS10 was not commercially available, the rabbit anti-human ADAMTS10 polyclonal antibody (1:50 dilution; MyBioSource, Inc, San Diego, USA) was used as primary antibody. The goat anti-rabbit IgG- HRPO conjugate (1: 10,000 dilution; Genei, Merck Biosciences, Bangalore, India) was used as secondary antibody. The bound peroxidase activity was visualized using the Clarity Western ECL Substrate (Biorad Laboratories Inc, Hercules, CA, USA) according to manufacturer's instructions. The chemiluminescence signals on PVDF membranes were captured on X-ray films (Fujifilm Corporation, Tokyo, Japan), and the exposed films were processed and developed in the dark room. Subsequently, the films were photographed by a gel documentation system (LAS-3000; Fujifilm, Tokyo, Japan). The molecular weight of unknown proteins separated on gels or blots was measured by Multi Gauge analysis software version 2.2 (Fujifilm, Tokyo, Japan) using the standard molecular weight markers.

2.4 Immunolocalization of ADAMTS10 in spermatozoa

The buffalo sperm were subjected to immunolocalization procedure according to the method of Divyashree & Roy (2018) for detection of ADAMTS10 protein. The pellets were washed three times in sp-TALPH [100 mM NaCl; 3.1 mM KCl; 0.4 mM EDTA; 0.4 mM $MgCl_2$ $6H_2O$; 0.3 mM NaH_2PO_4 $2H_2O$; 21.6 mm Na lactate; 2 mM $CaCl_2$ $2H_2O_2$; 1 mM Na pyruvate; 40 mM HEPES; 10 mM NaHCO3 and 1mg /mL polyvinyl alcohol (MW: 30–70 kDa)], pH 7.4, by centrifugation at 275 X g for 10 min at room temperature and then resuspended at a concentration of $50X10^6$ cells/mL in sp-TALPH, pH 7.4 An approximate 2-µL aliquot of this suspension was smeared on 18 mm×18 mm glass coverslips (#1), air dried. Briefly, the cells were fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.4 for 20 min at room temperature in a horizontal position on Petri dishes. Cells were permeabilised with 1% (v/v) Triton X-100 in PBS, pH 7.4, for 10 min at room temperature and then washed with PBS. The non-specific protein binding sites were blocked with 5% (w/v) bovine serum albumin (BSA) in PBS at room temperature for 1 h in a humid chamber. The blocking solution was aspirated, and the cells were incubated with rabbit anti-human ADAMTS10 polyclonal antibody (MyBioSource, Inc, San Diego, USA) diluted with 1% BSA in PBS, pH 7.4, for 2 h at room temperature. After being washed in PBS, cells were incubated with corresponding secondary antibodies [goat anti-rabbit IgG–fluorescein isothiocyanate (FITC) conjugate (Genei, Merck Biosciences, Bangalore, India)] diluted 1:50 with 1% BSA in PBS, pH 7.4 for 1 h in the dark. Samples were then washed with 3 mL PBS, pH 7.4, for 3 min and the coverslips were mounted, keeping the smeared surface down, on a clean glass slide with slow fade gold anti-fade reagent with 4', 6'-diamidino-2-phenylindole (DAPI; Life Technologies, NY, USA). Slides were observed under a fluorescence microscope (Eclipse-80i; Nikon Instruments) with excitation (465–495 nm) and emission (515–555 nm) filters. Control slides for immunocytochemistry were prepared by omitting the primary antibody in the procedure to determine the specificity of the secondary antibody used in the study. At least, 4 slides (3 for test and one for control) were prepared per sample per animal and at least 200 spermatozoa were observed for immunolocalization of ADAMTS10.

2.5 Real- time PCR analysis of ADAMTS10 expression in testis and epididymis

Total RNA was extracted from testes, caput, corpus, and cauda epididymis of buffalo using RNeasy Mini Kit (Qiagen, Germantown, USA) according to manufacturer's instructions. The quantification of isolated RNA was performed by a fluorometer (Qubit 2.0 fluorometer, Invitrogen, Carlsbad, CA, USA). Briefly, 1 µl of RNA was mixed with 199 µl of RNA assay buffer containing 1µl of dye (Qubit RNA BR assay kit, Invitrogen, Carlsbad, CA, USA). The fluorescence was measured in fluorometer after incubating the sample– dye–buffer mixture at room temperature for 5 minutes and the RNA concentration was expressed as ng/µl. The purity of isolated RNA was checked using 260/280 ratio in a Nanodrop spectrophotometer (Thermo Scientific Instruments LLC., Verona Road, Madison, USA) and stored at -86°C. The isolated RNAs were converted to cDNA using cDNA reverse transcription (RT) kit (ABI High capacity reverse transcription kit, Thermo Fisher Scientific, Waltham, MA, US) according to manufacturer's instructions. The RT PCR was performed with the following conditions: Step 1: 25°C for 10minutes; Step 2: 37°C for 120 minutes; step 3: 85°C for 5

minutes; step 4: 4℃ forever. The synthesized cDNAs were stored at −20℃ until used for gene expression analysis by real-time PCR.

The expression of ADAMTS10 in testis and different segments of the epididymis were analyzed by real-time PCR. The primers for the selected cDNAs were designed using Primer 3.0 software (https://www.ncbi.nlm.nih.gov/tools/primer-blast). The details of the primer pairs are provided in Table 1. The specificity of the primers was tested using a BLAST analysis against the NCBI nucleotide database. All reactions were performed in a 10 µl reaction volume on the Quant Studio 3 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) using the Sso Fast EvaGreen Supermix (SsoFast EvaGreen Supermix, Bio-Rad Laboratories Inc., Hercules, CA, USA), 5 µM of each forward and reverse primer of ADAMTS10 and 20 ng of cDNA. The β-Actin was used as an endogenous reference gene and testis was used as an internal control. The qPCR cycling was performed as follows: Initial denaturation at 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 5 s; 60 ℃ for 10 s. A melt curve performed at the end of the amplification (initiated at 60℃ with 0.5℃ increment at each step of 10s up to 95℃) was undertaken to confirm that there is only a single product amplified in each reaction. The relative changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.6 Statistical analysis

All experiments were replicated with the tissues collected from at least four different animals and the data are expressed as the mean ± SEM. The significant differences between various groups were determined using analysis of variance (ANOVA) and Tukey's multiple comparison test. Statistical analyses were performed using the SPSS software, version 10.0.1 (SPSS Inc., Chicago, IL) and GraphPad Prism version 4.0 (GraphPad Prism Inc., San Diego, CA). P-values of < 0.05 were considered statistically significant.

3 Results

Under reducing SDS-PAGE, the tissue proteins were resolved into molecular weights ranging from 250-15 kDa whereas the sperm proteins were resolved into 110-15 kDa. Tissue-wise variation in protein profile was also evident (Figure 1A). The Western blot detection using ADAMTS10 heterologous antibody has demonstrated 31-, 44-, 50-, 65 kDa immune-reactive proteins from testis and 82 kDa protein from testes and corpus epididymis and an intense 36 kDa protein from ejaculated buffalo spermatozoa (Figure 1B). The ADAMTS10 expression was also

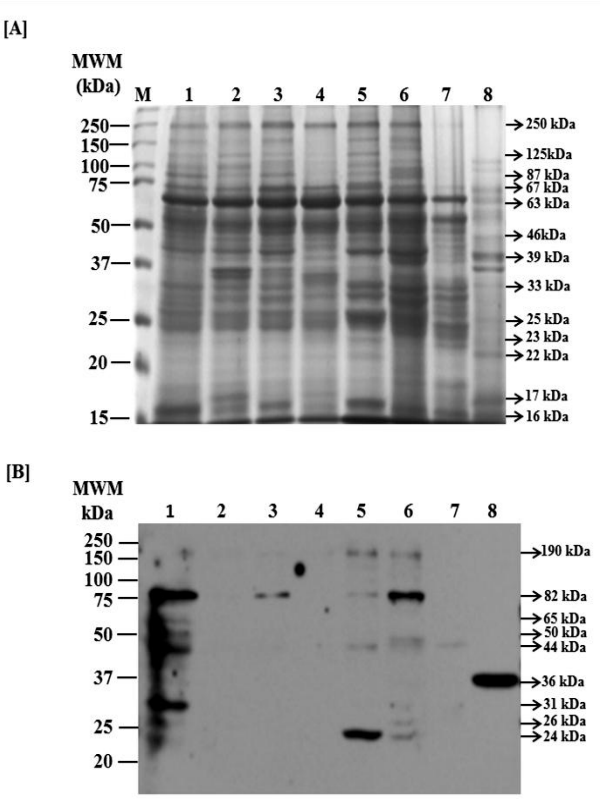

Figure 1A. SDS-PAGE analysis of proteins from different reproductive tissues of male buffalo. Coomassie Brilliant Blue R-250 stained 10% uniform mini gel. 1B. Western blot for detection of ADAMTS10 using rabbit anti-human ADAMTS10 polyclonal antibody using a duplicate gel of Figure 1A. MWM: Standard molecular weight markers expressed in kDa; 1: testes, 2: caput epididymis, 3: corpus epididymis, 4: cauda epididymis, 5: lungs, 6: kidney, 7: liver extracts; 8: buffalo fresh sperm extract. Approximately 35µg of protein was loaded in each well.

Table 1 Primers used for real-time PCR

Gene	Primer Sequence	Amplicon size	Gene bank accession no.	% Identity
ADAMTS10	Forward:5'GCTATGAGATCGCCTTCCCC3' Reverse:5'TTAGCAGGAAGTGGGTGCTG 3'	156 bp	XM_006046800.1	100
β-Actin	Forward 5'CGCAGACAGGATGCAGAAAG3' Reverse: 5' GCTGATCCACATCTGCTGGA3'	148 bp	NM_001290932.1	100

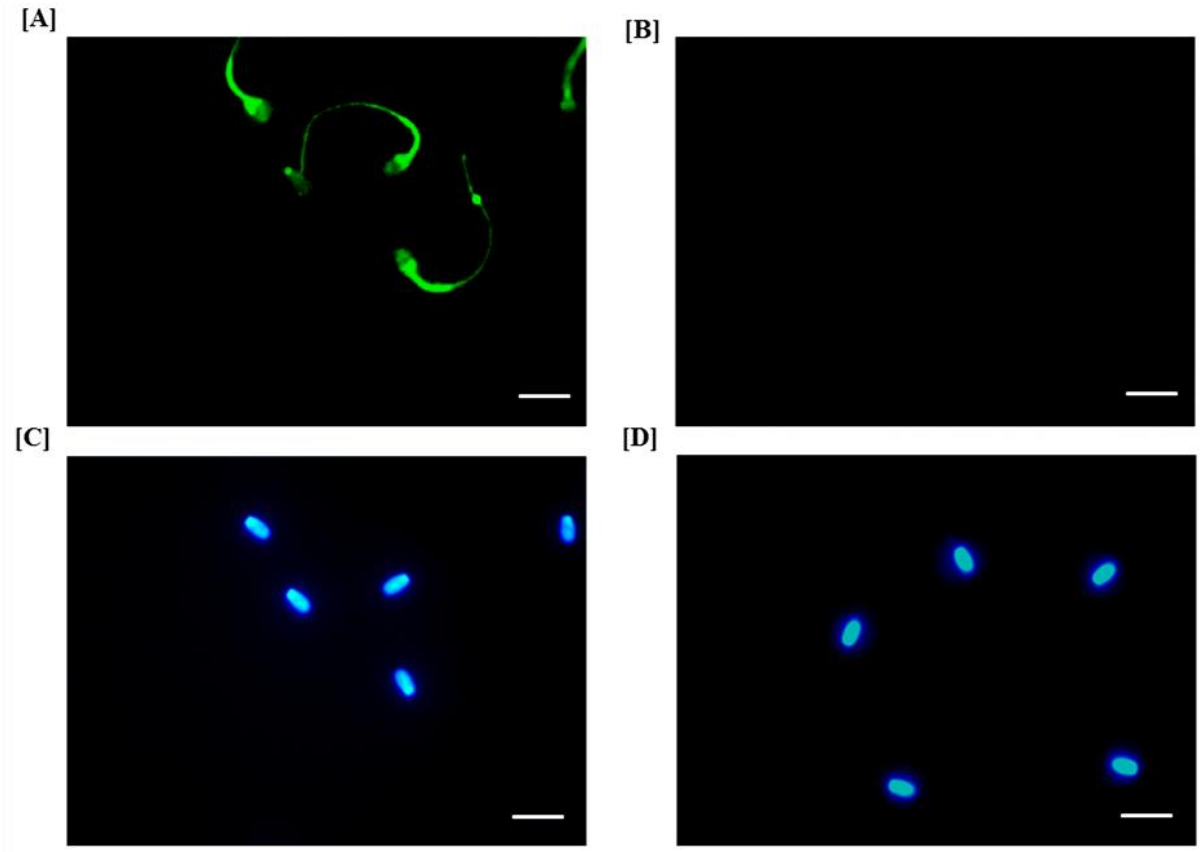

Figure 2 Immunofluorescence detection of ADAMTS10 in ejaculated buffalo spermatozoa. A. ADAMTS10 was localized in fixed, permeabilised ejaculated buffalo spermatozoa using rabbit anti-human ADAMTS10 polyclonal antibody and goat anti-rabbit FITC-conjugated secondary antibodies. B. Ejaculated buffalo spermatozoa labelled with goat anti-rabbit FITC-conjugated secondary antibodies without primary antibody (Negative control). C and D are corresponding images of A and B, counter stained with DAPI (blue). Magnification: 60x, Scale bar = 20µm.

detected in lungs, kidney and liver tissues of buffalo. In the lungs, an intense 24 kDa immune-reactive protein band and 190-, 82- and 44 kDa less intense protein bands were observed. In the kidneys, an intense 82 kDa protein, and 190-, 82-, 44-, 26-, 31-, and 50 kDa less intense proteins were observed. In the liver, only a 44 kDa less intense protein was detected. Immunofluorescence detection of ADAMTS10 in ejaculated buffalo spermatozoa has demonstrated higher fluorescence signal in post-acrosome and the middle piece (mitochondrial) region (Figure 2A). The real time PCR analysis has demonstrated the highest expression of ADAMTS10 transcripts in the corpus epididymis followed by cauda and caput segments of the epididymis and testis (Table 2). However, the level of transcript did not vary between corpus and cauda epididymis (Figure 3).

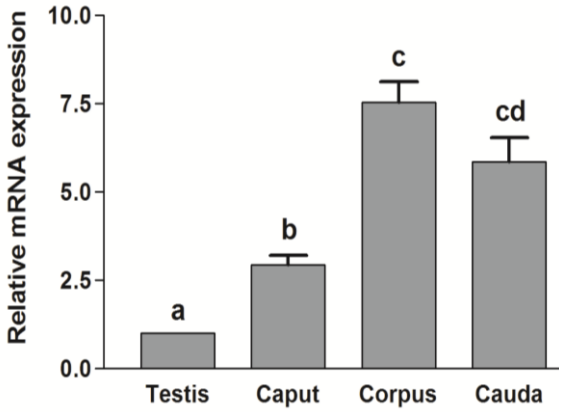

Figure 3 Relative expression of ADAMTS10 transcript in caput, corpus and cauda epididymis as compared to the testis from Table 2. The data are expressed as the mean ± standard error of the mean. Different letters above bars indicate significant differences ($P < 0.05$) between different groups.

4 Discussion

The focus of the present research was to study the expression pattern of ADAMTS10 in the reproductive tract of water

buffaloes so as to ascertain its role, if any, in male reproduction. In mammalian male reproduction, the testis has an important role in spermatogenesis and androgens production and epididymis has a major role in sperm maturation, concentration and storage. Hence studying the expression of any gene in the testis and epididymis will be helpful in ascertaining their role in events associated with male reproduction. In developing mouse embryo, the widespread expression of ADAMSTS10 mRNA has been observed until 12.5 days of gestation, whereas in adult mouse, the protein expression has been reported in the mesenchymal tissues of lung, kidney, gonad, salivary gland, and gastrointestinal tract (Somerville et al., 2004) and in fibrillin formation and its function (Mularczyk et al., 2018). In addition, the Weill-Marchesani syndrome (WMS) in mice and glaucoma in canine has also been associated with mutation of ADAMTS10 gene (Cain et al., 2016; Graham et al., 2016). ADAMTS10 protein has been reported to play a role in sperm function and male fertility events in mouse (Dun et al., 2012). In this species, an ADAMTS10 of 65 kDa was detected in the testes whereas an ADAMTS10 of 50 kDa was detected in the spermatozoa from different segments of the epididymis. A less intense 65 kDa protein was detected in spermatozoa from corpus and cauda epididymis and 120 kDa protein from testis, caput and corpus cells (Dun et al., 2012). In buffalo, the Western blot detection of ADAMTS10 in the extracts of testis, epididymis and ejaculated sperm using heterologous antibody has demonstrated substantially different molecular weight forms (31-, 36-, 44-, 50-, 65-, 82- kDa) as compared to its predicted protein sequence (121 kDa). These forms may be the result of proteolytic processing of the full-length zymogen that occurs during epididymal sperm maturation process as reported earlier (Somerville et al., 2004; Dun et al., 2012). The difference in molecular weight forms of these proteins than that of mice may

Table 2 Real time data analysis of expression of ADAMTS10 in buffalo testis and different segments of epididymis using $2^{\wedge-\Delta\Delta CT}$ method

Tissue	ADAMTS10 CT	β- Actin CT	ΔCT (Avg. ADAMTS10 CT - Avg. β-actin CT)	ΔΔCT (Avg. tissue ΔCT - Avg. testis ΔCT)	$2^{\wedge-\Delta\Delta CT}$ (Normalized ADAMTS10 amount relative to testis)
Testis	30.90	19.60	11.30	0.00	1.00
	30.57	19.60	10.97	0.00	1.00
	30.51	19.78	10.73	0.00	1.00
	31.16	20.22	10.94	0.00	1.00
Average	30.79 ± 0.15	19.80 ± 0.15	10.99 ± 0.12	0.00	1.00 ± 0.00
Caput	30.69	20.76	9.93	-1.37	2.58
	30.29	20.85	9.44	-1.53	2.89
	31.85	22.51	9.34	-1.39	2.62
	31.91	22.87	9.04	-1.90	3.73
Average	31.19 ± 0.41	21.75 ± 0.55	9.44 ± 0.18	-1.55 ±0.12	2.96 ± 0.27
Corpus	31.91	23.24	8.67	-2.63	6.19
	31.16	23.34	7.82	-3.15	8.88
	31.47	23.56	7.91	-2.82	7.06
	31.17	23.24	7.93	-3.01	8.06
Average	31.43 ± 0.18	23.35 ± 0.08	8.08 ± 0.20	-2.90 ±0.11	7.55 ± 0.58
Cauda	32.30	23.14	9.16	-2.14	4.41
	31.45	23.25	8.20	-2.77	6.82
	31.52	23.11	8.41	-2.32	4.99
	31.30	23.22	8.08	-2.86	7.26
Average	31.64 ± 0.22	23.18± 0.03	8.46 ± 0.24	-2.52 ±0.17	5.87 ± 0.69

be attributed to the species difference.

The sperm which are leaving the testis are inactive in nature. During epididymal transport, the spermatozoa undergo a maturation process by proteolytic processing and subsequently acquire the progressive motility and fertilizing ability (Bedford, 1974; Robaire et al., 2006). The expression of any gene in the caput and corpus regions of the epididymis suggests that it is important for early and late maturational events respectively, whereas genes expressed in cauda regions may be important for maintaining sperm function during storage (Cornwall, 2009). The identification and characterization of epididymal proteolytic enzymes may provide valuable information toward our understanding of the epididymal function and sperm maturation process (Cornwall & Hann, 1995). In present study, expression of ADAMTS10 transcripts in corpus epididymis was significantly higher ($P< 0.05$) than that of cauda, caput epididymis and testis. Highest expression in corpus may be related to higher role in later stages of sperm maturation process.

In mouse, ADAMTS10 has been localized in the peri-acrosomal region of both capacitated and un-capacitated spermatozoa from the testis and the different regions of the epididymis; but not in acrosome-reacted spermatozoa. ADAMTS10 expression was also localized in the principal piece of sperm tail, with a punctuate form during epididymal maturation process (Dun et al., 2012). However, in the present study, the localization of ADAMTS10 has demonstrated high fluorescent signal in post-acrosome and the middle piece region of the ejaculated bufflalo spermatozoa may be attributed to species differences.

Conclusion

The present study has demonstrated the expression of ADAMTS10 in buffalo testis and different segments of the epididymis. The ADAMTS10 immune-reactive proteins were observed in testis, corpus and ejaculated spermatozoa. ADAMTS10 localization was observed in post-acrosome and the middle piece region of ejaculated spermatozoa. Thus the differential expression pattern of ADAMTS10 in different parts of male reproductive tract of buffalo indicates their role in the sperm maturation process. However, specific role of ADAMTS10 in buffalo male reproduction can only be ascertained after further studies.

Acknowledgement

The authors are thankful to the Director, National Institute of Animal Nutrition and Physiology (NIANP), Bangalore, India for providing funds and facilities for carrying out the present study. The first author was supported by Junior Research Fellowship from Indian Council of Medical Research, New Delhi. The authors also thank the Director, Indian Veterinary Research Institute (IVRI), Bareilly, India for providing an opportunity to the first author for conducting the present study at NIANP, Bangalore. The authors also thank the Director and staff, Nandini Sperm Station, Hessarghatta, Bangalore for providing the semen samples for the present study.

Conflicts of interest

No conflicts of interests are declared by authors for the contents in this manuscript.

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