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Detection of the ADAM proteins in the ejaculated bovine sperm

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***Abstract*---**The current study aimed to detect specific types of ADAM proteins in the bovine sperm. Histology study of tissue of male reproduction was involved with testis, head, body, and the tail of the epididymis. Results showed that the testis composed of the parenchyma of testis contained the seminiferous tubules which are surrounded by two layers externally tunica vaginalis and internally tunica albuginea. PAS was positive in the seminiferous tubules of testis and lumen, epithelium of the head, and body of epididymis, Whereas Alcian blue was very low intensity stains with sections and contents of the head, body and tail of the epididymis. Fresh ejaculated bovine sperms were collected and separated from seminal plasma, and proteins of sperm were extracted and run on an electrophoresis gel. Then, the bands of proteins have been stained with coomassie stain, after that, the pieces of proteins were cut and separated from gels and amino acids were extracted after the digestion of proteins, and uploaded to a mass spectrometry machine. Our data was profiled and analysed and detected characterised ADAM proteins included: ADAM1, ADAM2, ADAM3A, ADAM10 and ADAM32, and uncharacterized ADAM proteins included: ADAM1B, ADAM7, ADAM9, ADAM12, ADAM15, ADAM17. These ADAM on bovine sperms could be transmembrane proteins or constitute the cytoskeleton of cells of sperms. These results concluded that there are many ADAM proteins were expressed in the bovine sperm, so these proteins have different ligands and domains interaction sites, and might be interacted with other glycoproteins, carbohydrates and other proteins in the male

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reproductive tract or external environment in the female reproductive tract, for this reason, ADAM proteins could be mediated many functional duties during growth, maturation of sperms or more roles in the female-male reproductive tract during the fertilization process.

***Keywords*---**ADAM proteins, bovine sperm, mass spectrometry, and transmembrane.

Introduction

Many proteomic studies have been applied to human and bovine sperm for identifying of types of proteins which constitute the internal and transmembrane proteins of sperm(1-3), these studies were detected that there are different types of sperm and might be had a different biological and biochemical function in maturation, growth and fertility of sperms (4-6), because the proteomic studies have found that abundance of some proteins could be the difference in comparison between high and low infertility sperm proteins formation of humans and bulls (6, 7).

Disintegrin and metalloproteinase(ADAM) proteins are groups of transmembrane proteins which belong to the zinc protease superfamily (8, 9). These members of the family are characterized by being rich in cysteine and presence of metalloprotease, C-terminal receptor-binding sites and a cytoplasmic domain it would be active for different cellular transduction proteins (10, 11). Several ADAM proteins are identified even now involved with ADAM1, ADAM2, ADAM3, ADAM7, ADAM8, ADAM9, ADAM10, ADAM11, ADAM12, ADAM15, ADAM17, ADAM18(27), ADAM19, ADAM20, ADAM21(31), ADAM22, ADAM23, ADAM28, ADAM29, ADAM30, and ADAM33(12-21).

These varieties of the ADAM are allowed them for different biological and physiological functions such as protein–protein interactions, cell to cell and proteins with sugar (22, 23). These ADAM are expressed in different parts of tissue and some ADAM have proteases domains and are classified as sheddases, this active domain would cut off or shed extracellular portions of some particular transmembrane proteins which would be allowed to achieve more functional activities of the body (8, 24, 25).

Powerley, ADAM have high diversity and are expressed in the different tissue regions as extracellular transmembrane proteins or cleaved or sheddase, potentially conferring them to interact and have many physiological and biological reactions among cells of tissue (8, 26). This study was designed to detect specific ADAM in bovine sperm, so this result could be helped researchers to find out and investigate more about the roles of these proteins in the male reproductive tract in relation to fertility and more applications studies.

Materials and Methods

Gel formulation

In short, 10%acrylamide/bisacrylamide gel recipe was used by following Bio-Rad protocol (27). Also, a 0.75mm gap between the thick and thin glass plates was used. The stacking gel was 4% acrylamide/bisacrylamide, and a plastic comb was inserted in the stacking gel on top to make well volumes, and leave it for 30 minutes to be polymerized until use.

Bovine sperm lysate

Fresh healthy ejaculated bovine sperm was collected, and then bovine sperm were washed and diluted approximately $20x10^6$ in Eppendorf and centrifuged for 5 minutes at 4°C and 18500 g rpm. The supernatant was discardted, then, added 200 µl of HEPES buffer was to the pellet and mixed vigorously by vortex, after that centrifuged for 5 minutes at 4°C and 18500g rpm. The supernatant was discarded and the pellet was smashed using mortar and pestle using liquid nitrogen. Collected the lysate and spun at 4oC and 18500 g rpm for 5 minutes. The supernatant was taken off and pellets were neglected. The supernatant of bovine sperm was aliquoted and stored at (-20 °C) for the next step.

Electrophoresis and digestion of bovine sperm lysates

For this experiment, the protocol for running the bovine sperm lysate on SDS-page gel was made following (3, 28, 29), briefly, bovine sperm lysate was run on SDS-page gel. Then, the pieces were stained with coomassie blue Staining (brilliant blue) to stain bands of proteins. After that, destaining dilution was used to remove coomassie blue staining from gels and detected the bands of proteins. So, bands of bovine sperm were cut as cubes under extreme conditions to prevent any contamination, transferred to Eppendorf, and added 250 uM l from the 100 ml of 100 mM ammonium bicarbonate/acetonitrile and incubated for 30 minutes. Then, 1250 µl acetonitrile was added with mixed gently at room temperature. So, after shrinking of pieces and becoming white in colour, 250 µl of the ammonium bicarbonate/acetonitrile (1:1 vol/vol) was added, and stored at -20° C. Next day, and trypsin buffer (1:50 ratio µg trypsin: µg proteins) (Manufacturer: Promega™ V5280) was added to the gel pieces to cover them and incubated at 37° C for overnight. The next day, samples were centrifuged at 18500g rpm, 4°C for 2 minutes and discarded the supernatant. Then extraction buffer (5% formic acid in acetonitrile) was added to pieces of gel and incubated for 15 minutes with shaking at 37° C. Afterward, samples were spun and collected in the supernatant, and dried in a vacuum centrifuge at 45oC. Finally, samples were resuspended in buffer (97.5% H2O, acetonitrile 2%, and formic acid 0.5%). To finish samples were loaded on a Mass Spec machine (FT-ICR/Orbitrap) in the Conway Institute. University College Dublin. The parameters and curves were profiled of samples after a run of samples and uploaded on Peak7 studio software.

Table 1. This table showed result filtration parameters after run the samples of bovine sperm

Peptide -10lgP	≥15
Protein -10lgP	≥15
Proteins unique peptides	≥0
De novo ALC Score	≥50%

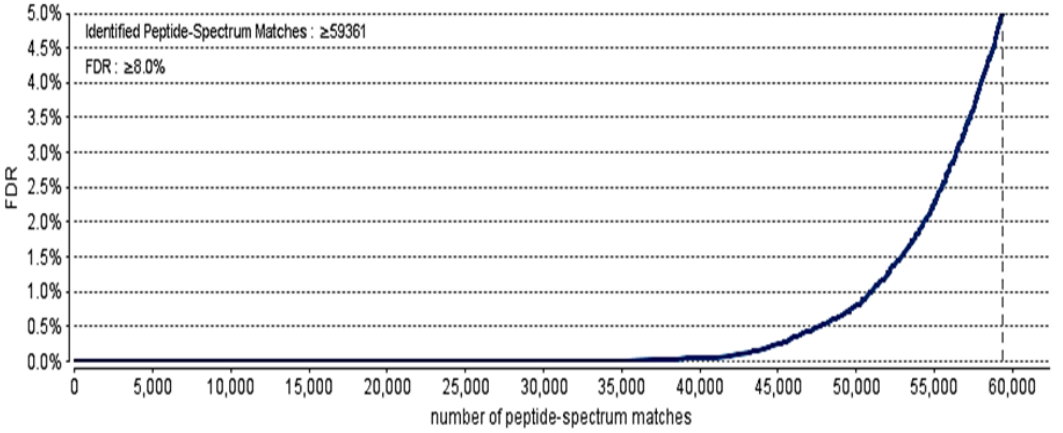

Figure 1. This figure illustrated the false discovery rate (FDR) curve. X axis is the number of peptide-spectrum matches (PSM) being kept. Y axis is the corresponding FDR

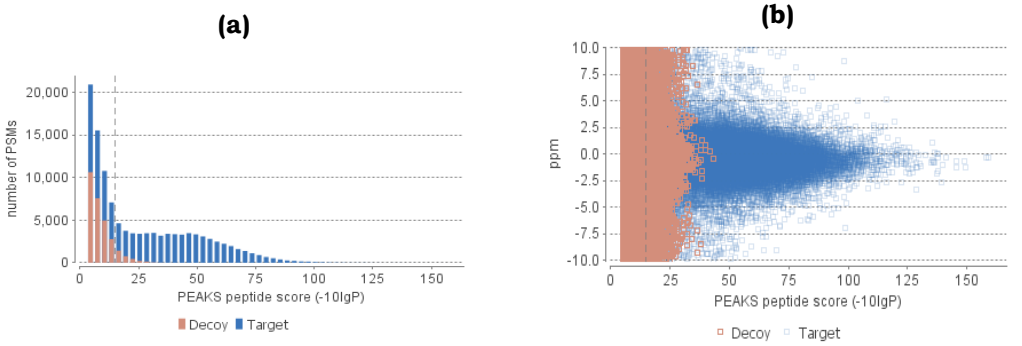

Figure 2. This figure illustrated the PSM score distribution. (a) Distribution of PEAKS peptide score; (b) Scatterplot of PEAKS peptide score versus precursor mass error.

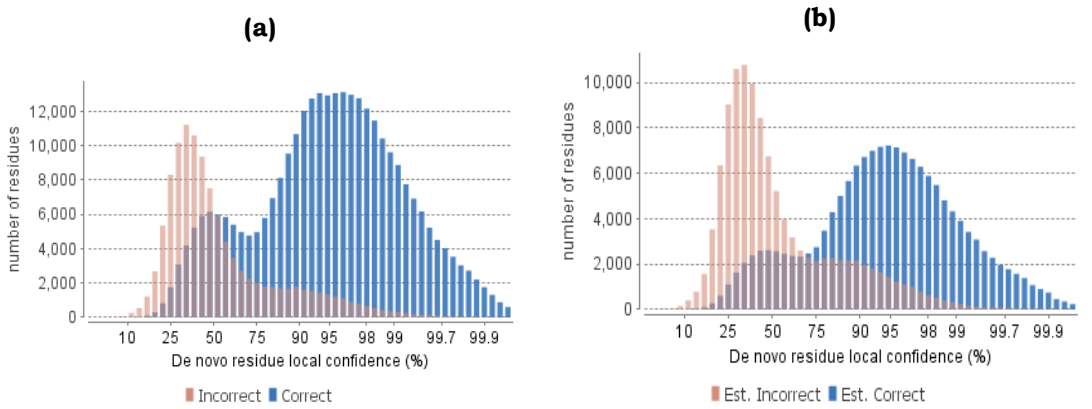

Figure 3. This figure illustrated the De novo result validation. Distribution of residue local confidence: (a) Residues in de novo sequences validated by confident database peptide assignment; (b) Residues in "de novo only" sequences.

Histology of testis and epididymis

Six right and left fresh samples of the adult bull samples were directly collected from the slaughterhouse including the testis, head of the epididymis, body of epididymis, and tail of the epididymis. Samples were fixed in 10% neutral formalin for 48 hours. Then, the tissue process of histology was applied to making sections of tissue. Tissue sections were stained with three stains: H and E, Periodic acid–Schiff (PAS) and Alcian blue stains for identifying the main structures of the testis, head of the epididymis, body of epididymis, tail of the epididymis and to detect glycosylation intensity according to these regions. Sections were examined and images were chosen and snapped using by light microscope model 6300 (Japan) under magnificent 4x10x, 20x, 40x and. 100x.

Data Analysis

The sequences of amino acids of lysate of bull sperm were collected from the Mass Spec machine (FT-ICR/Orbitrap) and analysed by Peak7 studio software. All data were compared to the sequence of amino acids in the proteins data bank to recognise the ADAM proteins in the sperm of the bulls.

Results

ADAM are cellular transmembrane projection and secreted metalloendopeptidases and have domains which could be interacted with other glycoproteins, for this reason, testis and epididymis parts stained with routine stain and specific periodic acid Schiff(PAS) stain to label sugar of glycoproteins, so result displayed that testis was surrounded by tunica albuginea composed of dense collagenous fibrous and extended with a vascular layer which was contained smooth muscle fibres within and covered externally by simple squamous epithelium layer and mesentery formed the tunica vaginalis.

The parenchyma of the testis was divided into lobules by different septali which extended from the capsule of the testis. Seminiferous tubules were embedded inside the testis and lined by stratified epithelium composed of a germinal layer including spermatogonia, spermatocytes, spermatids and spermatozoa and in addition to Sertoli cells, free sperm in the lumen. Also, these tubules were enclosed by interstitial tissue of the testis and spaces which consisted of connective tissue, blood supply and adipose tissue. The epididymis was a highly coiled duct opened and extended with efferent ductules in the testis and consisted of the head, body and tail and drained into vas deferens. Histologically, it was lined by pseudostratified columnar and simple columnar epithelium projected stereocilia inside the lumen, besides, this epithelium was based on a muscular layer consisting of packed smooth muscle fibers and covered by tunica serosa composed of connective tissue.

PAS and Alcian blue were clearly stained the sections tissue of the testis and head, body, and tail of epididymis to display the main structures, glycoconjugates and types of the carbohydrates. The result explained PAS positive result for basal lamina, structures and contents of seminiferous tubules of testis and lumen, epithelium of the head, body of epididymis, while, the tail of epididymis was negative for PAS stain. Whereas, Alcian blue was very low affinity to structures and contents of the head, body, and tail of the epididymis, however, testis was not affinity to this stain (figure 4).

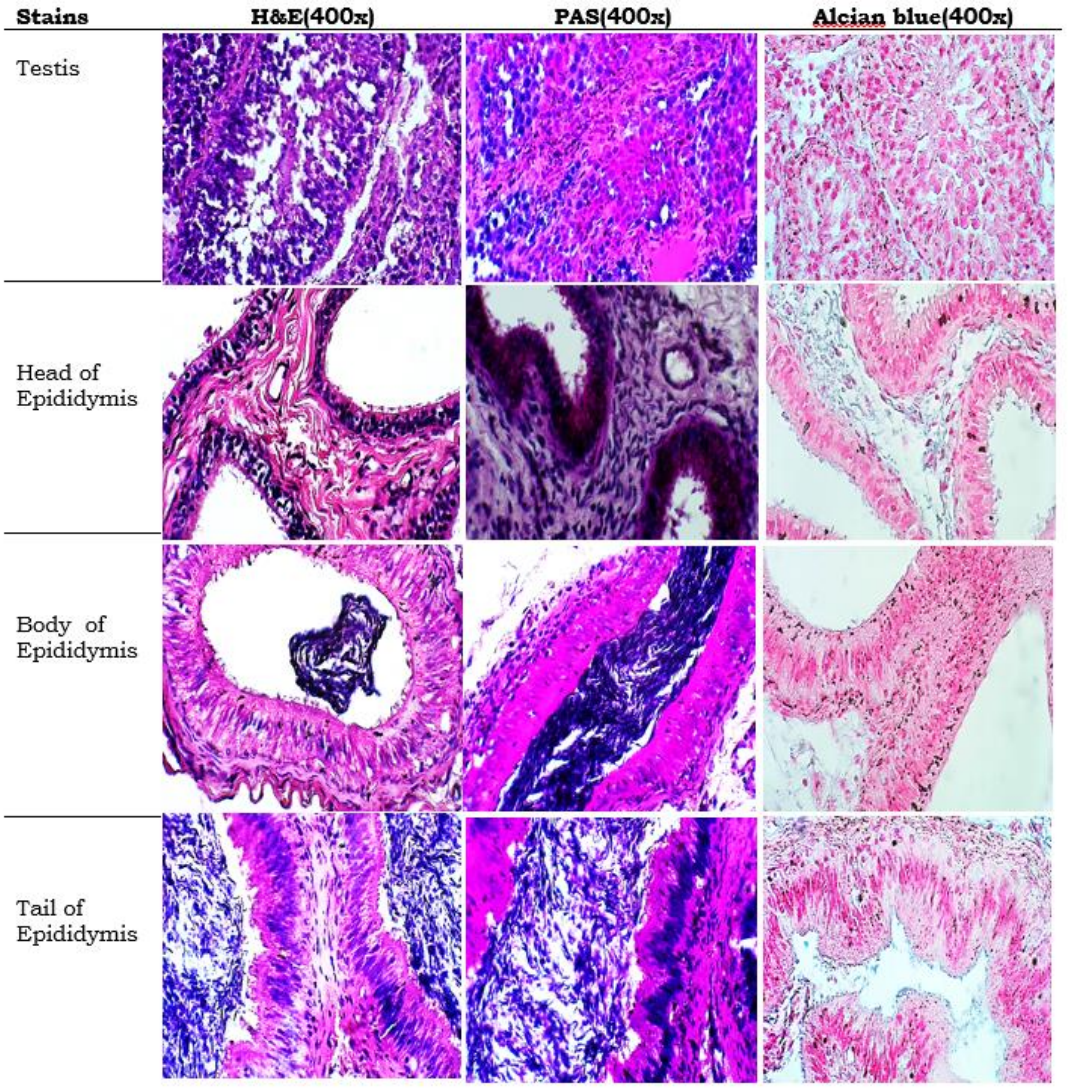

Figure (6): This figure is revealed the testis, head of epididymis, body of epididymis, and tail of epididymis were stained with H&E, PAS, and Alcian blue.

Our proteomic study has been directed on the bovine healthy sperm to distinguish ADAM proteins in the bull sperms using spectrometry essay. So, sperm lysate was run on the 10% SDS polyacrylamide gels and stained with Coomassie staining solution, then destaining of coomassie solution and bands were cut as cubes for lysate of sperm samples from the gel. These bands were proteins of sperm trapped in the network of the gel according to molecular weight, figure (5).

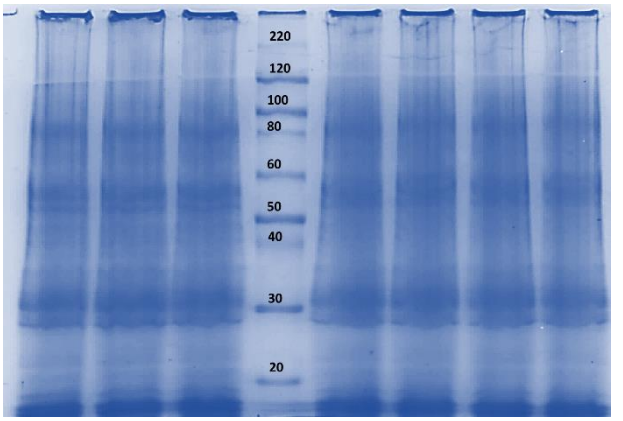

Figure (5): This figure illustrated electrophoresis of bovine sperm lysate on SDS-page gel and stained with Coomassie stain.

Then, these slices of protein bands were digested with trypsin (Promega™ Trypsin Gold, Mass Spectrometry Grade product (Manufacturer: Promega™ V5280), and extracted peptides of sperm lysate were resuspended in reaction buffer and injected into the Mass Spec machine (FT-ICR/Orbitrap) figure (6).

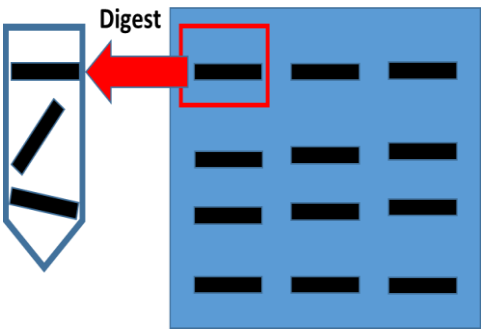

Figure (6): This diagram showed electrophoresis of bovine sperm lysate, cut of bands and then digest the bands.

The profile data of sequences of amino acid of bovine sperm lysate were uploaded on Peak7 studio software to distinguish the characterised ADAM proteins and uncharacterized ADAM proteins. So, results showed there were five types of characterised ADAM proteins including ADAM1, ADAM2, ADAM3A, ADAM10 and ADAM32, and six uncharacterized ADAM proteins included: ADAM1B, ADAM7, ADAM9, ADAM12, ADAM15, ADAM17. These proteins constituted the whole proteins of sperm, involved with transmembrane and internal proteins of sperm. This result is shown in table (2).

Table (2): This table is displayed the Uncharacterized and Characterized of the ADAM proteins

No.	Uncharacterized ADAM proteins	Characterized ADAM proteins
1	ADAM1B	ADAM 1
2	ADAM7	ADAM2
3	ADAM9	ADAM3A
4	ADAM12	ADAM10
5	ADAM15	ADAM32
6	ADAM17	

Discussion

Histologically, testis and epididymis parts in bulls were stained with routine stain and specific periodic acid Schiff(PAS) stain to label sugar of glycoproteins, so the parenchyma of the testis was divided into lobules contained seminiferous tubules which were lined by stratified epithelium composed of a germinal layer including spermatogonia, spermatocytes, spermatids and spermatozoa and in addition to Sertoli cells, free sperm in the lumen. Also, these tubules were enclosed by interstitial tissue of the testis and spaces which consisted of connective tissue, blood supply and adipose tissue, same these structures of the testis of the bulls are described by other researchers (30, 31). The epididymis was lined by pseudostratified columnar and simple columnar epithelium projected stereocilia inside the lumen, besides, this epithelium was based on a muscular layer consisting of packed smooth muscle fibers and covered by tunica serosa composed of connective tissue. This result has confirmed in previous research(32, 33).

PAS were displayed that there was a clear affinity to contents of seminiferous tubules of testis, and lumen, but and Alcian blue stains was negative affinity to testis structures, so same these results were recorded in testis of bovine and mouse (34). Furthermore, the epithelium of the head and body of epididymis were positive to PAS stain, also Alcian blue was positive but it was very low and stained the contents of the head of the epididymis, body of epididymis, and tail of the epididymis which agreed with (35) however, the tail of epididymis in study has no affinity to the contents. Whereas, , however, testis was no stain.

Several papers have identified ADAM1, 1B, 2, 3, 4, 5,6, 7, 12, 15, 17, 18, 20, 21,28, 29, and 32, in the male reproductive tract and sperm (36-45), our data of bovine sperm did not detect all ADAM proteins in bovine sperm, but ADAM1, 1B, 2, 3A, 7, 9,10, 12, 15, 17, and 32 were found in our analysis data in this study. ADAM family have many different interacted ligands including metalloproteinase, transmembrane, growth factor, cytoplasmic signalling and propeptide domains which can be reacted with different types of carbohydrates and glycoproteins(46, 47), therefore, this study was detected different types common sugar and glycoproteins which could be in relevant with ADAM domains activity or reaction between cell to cell interaction or cleaved proteins or other putative functions during sperm development, after mating of bovine and fertilization.

These groups of ADAM1, 1B, 2 were whole ADAM proteins which were distinguished in bovine sperm , so ADAM1, 1B, 2, 7 are considered as integral sperm membranes and formed a fertilin complex which might be engaged in sperm-oocyte membrane interaction (38, 48, 49), and this confirmed that these ADAM proteins have the main role in growth, maturation, sperm motility, and fertilization process.

Also, ADAM3A was detected in bovine sperm which was not recorded by other research, but ADAM3 was expressed in sperm and considered an essential plasma membrane protein for migration of sperm in female reproductive for fertilization(50, 51). So ADAM3 has a large similarity with ADAM3A and should be more studies need to find the relationship between two ADAM3 and 3A. Previous studies were reported that ADAM9 is widely expressed in snake venom metalloproteases and shares the metalloproteinase domain with matrix metalloproteinases (MMPs) (52, 53), so our study has detected ADAM9 and may play important roles in cellular functions and cell-cell fusion between sperm and cell membrane of oocyte reactions.

In addition, ADAM10, and 17 are considered a transmembrane protein and can be shedding in the ectodomain region and proteolytically detach as cleaved molecules and release according to location in tissue to mediate cellular reactions and regulate immune response or growth of cells, and some cells surface proteins functions(54, 55), these studies have confirmed our result that the expression of ADAM 10 and 17 in bovine sperm could be an influence on transmembrane of sperm and mediated sperm reaction in male and female tracts. Moreover, ADAM 12 was expressed in testis, and this suggested that ADAM12 may be related to the spermatogenesis of sperm, therefore our analysis has detected ADAM12 between sperm proteins(56). In addition to ADAM 15 was detected in in the sperm of mice and testis which is agreed with our findings. So, this result explained that ADAM 12 and 15 could be played a main role in sperm maturation and sperm egg-fusion Also, the analysis result of this study has recognised ADAM32 which was identified in a previous study (57, 58) that confirmed ADAM 32 is a large similarity of ADAM2 and ADAM3, and it is expressed in testis and sperm and has an independent function, this study is enhanced that ADAM32 could be had specific functions in growth, maturation of sperm and fertility.

This study concluded PAS and Alcian blue were affinities to testis and epididymis tissue of bulls which confirmed that there are different types of carbohydrates and glycoproteins. Also, the proteomic study can be enhanced in other biological studies in relation to normal and abnormal infertile proteins of sperm and can investigate the presence or absence of different types of ADAM proteins which can be detected in the level of fertilization of bulls. So this study has confirmed that there are several ADAM proteins are existed in bovine sperm and these should be thought interesting for researchers to do more research and studies about the main role of these specific ADAM proteins as membrane-anchored glycoproteins in the male reproductive tract and bovine sperm.

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Conflict of Interest
This research has no conflict of interest.

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