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Analysis and Profiling of the Bovine Sperm Proteome in University of Kufa, Al-Najaf, Iraq

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Abstract

This study was intended to identify the different types of amino acids and whole proteins of the healthy bovine sperm. Two methods applied for characterizing the bovine sperm. Firstly, the whole sperm lysate was extracted and digested and then run in mass spectrometry. Secondly, the total sperm lysate was run in SDS page gel, and then the bands were stained with coomassie stain. The bands were cut and digested to extract the whole amino acids. And finally, it was also run in mass spectrometry. The results revealed that several proteins identified in healthy bovine sperm. These were involved in different binding proteins, glycoproteins, transmembrane, and soluble proteins. These proteins might play a role in sperm activity. It might also be related to the behaviour of sperm during the formation process in testis and migration of sperm via the male reproductive tract and also inside the female reproductive tract after mating and fertilization. Interestingly, gel bands had more abundance of protein compared to the whole lysate, which disappeared fewer proteins. Through combing both methods, it was observed that bovine sperm had several proteins which should be investigated in future studies to understand more about the metabolism and physiology of sperm. In conclusion, this study approved that bovine sperm has essential different and uncharacterized proteins which might answer many theoretical aspects of biological and biochemical sperm migrations after the mating process.

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Introduction

Sperm are produced by seminiferous tubules of the testis of the male reproductive system during embryological development. They grow in childhood and develop into mature spermatozoa in adult age. The male reproductive system consists of left and right testis,

epididymis, and vas deferens which receives products of accessory glands and joins into urethral part of urethra in the penis (external male genitalia) (Moore *et al.,* 2011; Netter, 2017; Saladin, 2007).

Testes are surrounded by skin, then serous layer that is tunica vaginalis, beneath it tunica albuginea which is a thick muscular connective tissue. Tunica albuginea is extended into parenchyma of testis and divided into incomplete lobules and constitutes many septa and mediastinum of testis (Mescher, 2013). Internally, testis is composed largely of coiled tubules called convoluted seminiferous tubules that contain germinal epithelium of sperm and are connected to epididymis via efferent ductuli. Different stages of sperm are growing and developed at tubules that are extended from basement membrane of convoluted tubules into lumen, spermatogonia type A and B, primary and secondary spermatocytes, spermatids, as well as free sperm at the lumen (Carlson, 2012). Sertoli cells are irregular in shape and found between spermatogenic cells; they are considered as supporting cells responsible for the nutrition of sperm (Sharma, 2007).

The seminiferous tubules are supported by interstitial connective tissue that consists of loose connective, blood vessels, and lymph. In addition, it has some special cells called interstitial cells (leydig) that produce testosterone hormone which is responsible for the development of sperm and secondary growth of male (Anglade and Oates, 2004; Mori and Christensen, 1980).

Sperms undergo different stages of development before maturing, and these stages continue after sperm is liberated into the lumen of seminiferous tubules; therefore, cilia of lumen of convoluted tubules would push sperm into epididymis via efferent ductuli, and sperm would pass head, body and tail of epididymis (Bedford, 1975; Cooper, 2012; R Jones, 1998).

Furthermore, sperm will undertake remodelling and biochemical changes in the epididymis, resulting in motility and capability to be a motile (Elzanaty *et al.,* 2002; La Vignera *et al.,* 2012). Moreover, head of sperm obtains specific β-defensin protein that supports sperm in the motility, maturation, and antimicrobial activity and it is essential for fertility (Yudin et al., 2008; Zhao *et al*., 2011; Zhou *et al.,* 2004). The sperm are surrounded by plasma membrane which constitutes glycocalyx and carbohydrates such as sialic acid which consist the major sugar of the sperm (Alkhodair *et al.,* 2018; S. Suarez, 2002). These carbohydrates would be mediated and reacted with the female reproductive tract during capacitation process of sperm and also during reservoir of sperm in uterine tube before fertilizations (Roy Jones, 1989; S. S. Suarez, 1998). Moreover, there are different putative recognition proteins expressed on sperm which might play a role in sperm motility and fertilizations (Chapman and Barratt, 1996). Beta-defensin family are expressed and coated sperm which protect antibacterial activity and are involved in sperm capacitation to prepare sperm for fertility (Tollner *et al.,* 2008; Yudin *et al.,* 2005; Zhou *et al.,* 2004). Most proteins identified on sperm included Siglecs family which could mediate sperm activity before fertilizations and may regulate the internal environment for sperm (Alkhodair *et al*., 2018). Many recent studies are interested in characterization of proteins of sperm to learn more about transmembrane proteins activity of sperm in relation with female reproductive tract. Accordingly, numerous proteomic studies have been applied on whole lysate of sperm in different ways to detect its whole proteins and also understand its functions (Martínez-Heredia *et al.,* 2006; Oliva *et al.,* 2009).

Sperm has different types of protein which enclose it and other coated sperm which are acquired from products of accessory glands (Mann and Lutwak-Mann, 1981; Perez-Patiño *et al*., 2019). Many researchers are interested in sperm proteins and they have distinguished different types of protein, which might influence sperm activity such as motility or enable

sperm to avoid the immune response and prevent it via immunosuppression (Kumar *et al.,* 2019). In this study, we focused on whole proteins of bovine sperm used for mass spectrometry. The main aim of this study was to conduct a comprehensive characterisation of proteins of bovine sperm and compare the two methods. Additionally, it attempted to analyse the proteomic behaviour of whole lysates of sperm along with electrophoresis of bovine sperm lysates to augment previous studies.

Material and Methods

Gel formulation

Briefly, Bio-Rad protocol was used for making gel (Hames, 1998). The gel solution was composed of 10%acrylamide/bisacrylamide. The solution cocktail was mixed in a 15 ml Falcon tube, then the TEMED was added and poured between the 0.75mm gap between the thick and thin glass plates, and then left for 25 minutes to solidify. The stacking gel (4% acrylamide/bisacrylamide) was made by mixing the reagents. A comb to create appropriate well volumes was inserted between the plates before pouring the stacking gel on top of the Liquid gel.

Tissue cell lysis buffer

Tissue cell lysis buffer was made according to manufacturer instructions. Before using the buffer, one tablet of protease inhibitor (Roche cat no. 11836170001) was added per 10 ml of lysis buffer, and this solution can store at 4oC for up to two weeks.

Preparation of samples

In this study, two different methods were used:

Method 1

Fresh bovine sperm was collected and diluted approximately 20x106 in Eppendorf and centrifuged for 5 minutes at 4oC and 18400 g rpm. The supernatant was removed and frozen (-20^{o}C). Then, 180 µl of HEPES was added to the pellet and mixed gently by pipetting up and down and centrifuged for 5 minutes at 4^{o}C and 18400g rpm. After discarding the supernatant, the pellet dissolved by the addition of 600 µl of fresh 8M urea and mixed thoroughly. Afterward, 50 µl of the suspension was transferred into a 0.5 ml Eppendorf, and 1µl of DTT (10 mM final concentration) was added, vortex, and incubated for 10 minutes at room temperature. In addition, 2.5 µl of 10mM iodoacetamide was added, followed by 150 µl of 200mM ammonium bicarbonate buffer to adjust the pH to neutral. The pH was checked using a pH strip by taking 5 µl from lysate and put it onto the pH paper until the colour developed corresponded to that for neutral by comparison with the standard guide. Then, 245 µl of reaction buffer plus 5 µl (µg) trypsin was added, and the digestion was incubated overnight at 37^{o}C. Next day, the digested lysate was sent for proteomic analysis by Mass Spec machine (FT-ICR/Orbitrap) in the Conway Institute. University College Dublin.

Method 2

Electrophoresis of bovine sperm lysates was made as follow:
The gels were prepared and set up in the chamber according to the manufacturer's instructions. Then, 40 µl aliquots of sample transferred into large wells, with one well reserved for 5 µl of magic marker protein standards (Catalogue number: LC5602). The samples were run in were electrophoresis for 2 hours at 100 volts. Preparation of lysates of bovine followed the protocol of (Shevchenko *et al.,* 2006). The following were required for the protocol:
Ammonium bicarbonate buffer: 100 mM (to make 100ml, it should dissolve 0.79 g of ammonium bicarbonate in 80 ml dH2O and adjust pH to 7.8, then bring total volume into 100 ml). After gel electrophoresis of the samples and coomassie staining, the excess dye in the SDS polyacrylamide gel was removed by destaining until the protein bands were very clear; this took 3 hours at room temperature with shaking. Fresh buffer containing 100 ml of 100 mM ammonium bicarbonate/acetonitrile (1:1 vol/vol) was made. Then, the selected bands were cut into cubes (ca. 1 x 1 mm). The cubes were placed into an Eppendorf to which was added 250 uM l from the 100 ml of 100 mM ammonium bicarbonate/acetonitrile. The whole buffer was incubated for 30 minutes and mixed gently by vortex 3-4 times during the incubation. After 30 minutes, 1250 µl acetonitrile was added with gentle mixing, and the samples were left at room temperature. The cubes that cut from the gel should have shrunk and been white. Then, 250 µl of the ammonium bicarbonate/acetonitrile was added, and the samples stored at -20 ºC. Next day, the samples were placed on ice bucket, and sufficient trypsin buffer was added to the gel pieces to cover them with an approximate 1:50 ratio µg trypsin: µg proteins and they were left on ice for 2 hours.
The trypsin buffer was made by:

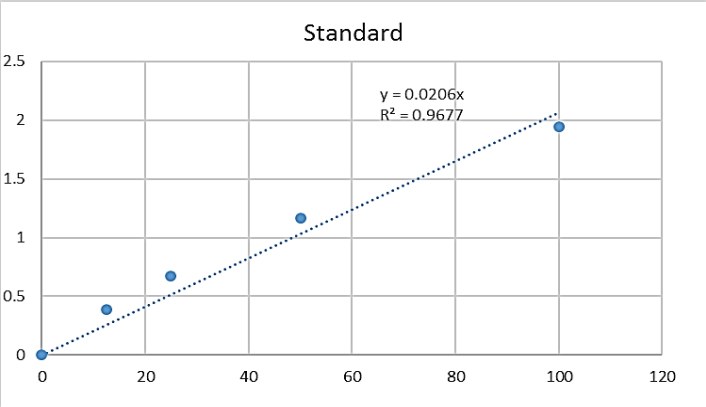
A: 10 % 10 mM ammonium bicarbonate. 10 ml
B: Acetonitrile (HPLC gradient grade). 10 ml
C: 1, µg. Trypsin: 50 µg proteins.

After 30 minutes, the pieces of the gel had absorbed the trypsin buffer, and additional trypsin buffer was added to cover gel matrix. Then it was left for 90 minutes, and 15 µl of 10 mM ammonium bicarbonate was added. The samples were incubated at 37ºC overnight. The following day, the samples were centrifuged at 14000 rpm, 4ºC for 2 minutes and the supernatant removed (keep in 0.5 ml Eppendorf labeled (S1)). Then extraction buffer was added to pieces of gel (Buffer: gel ratio 1:2) for 15 minutes with shaking at 37ºC. The extraction buffer consisted of 5% formic acid in acetonitrile. The supernatant collected and mixed with SI after centrifugation of samples. The samples were dried in a vacuum centrifuge at 45ºC. Afterward, they were resuspended in buffer (97.5% H2O, acetonitrile 2%, and formic acid 0.5%). Eventually, the samples were ready for injection into the mass spectral machine by Mass Spec machine (FT-ICR/Orbitrap) in the Conway Institute. University College Dublin.

Micro BCA assay quantitation of protein in bovine sperm

The Micro BCA Protein Assay Kit (Pierce product code #23235) was used to quantitate the protein in bovine sperm. The Micro BCA Protein Assay Kit (Pierce product code #23235 USA) was used to quantitate the protein content of bovine sperm lysates. The standard curve is shown in Figure 1, lysates containing 23-41 µg/ µl were used for gel electrophoresis after diluting with 5 x sample buffer and used for extraction of the equation.

Figure (1) BSA standard curve: x axis mg/BSA, y axis A562nm Equation y=0.0206 x



Coomassie staining solution

Coomassie Blue Staining (Brilliant Blue) for SDS-PAGE gels was made as below per liter. 1 g of Coomassie Brilliant Blue dye was dissolved in 100 ml glacial acetic acid. Then, 400ml of methanol was added. Finally, 500 ml dH2O was added and mixed well and used twice. Destaining for coomassie stain solution was made by 100ml glacial acetic mix with 200ml methanol, 700ml dH2O per 1 liter of solution.

Staining and destaining of coomassie solution from the gel

The gel was run at 100V, and when the dye front reached the end of the gel, the power was switched off. Then, it was removed from between the glass plates the gel was placed in a suitable tray and rinsed in dH2O. Next, it was incubated in coomassie staining solution with gentle rocking for one hour. After that, the coomassie solution was poured off, and the gel rinsed in dH2O again. Finally, coomassie destaining solution was added for one hour with gentle agitation. After one hour, the gel was rinsed in dH2O, and the fresh destaining solution was added; this was repeated as necessary until the protein bands could be seen against a clear background.

Analysis of data

The data of proteins were collected from Mass Spec machine (FT-ICR/Orbitrap) in the Conway Institute and analyzed by Peak7 studio software which displayed the sequence of amino acids and similarity of each protein (Zhang et al., 2012). The data was uploaded on Peak7 studio software and identified sequences of amino acids and particular proteins and uncharacterized proteins.

Results

In this study, samples were prepared in two methods. The first was using cubes cut from 10% SDS polyacrylamide gels containing lysate of sperm samples, where proteins were trypsin digested in situ with Promega™ Trypsin Gold, Mass Spectrometry Grade product (Manufacturer: Promega™ V5280) then extracted from the gel for analysis. The peptides

from the digest of two methods were resuspended in buffer and run in the Mass Spec machine (FT-ICR/Orbitrap) (Shevchenko *et al*., 2006), the second method used whole lysates of sperm which were directly digested by trypsin (Manufacturer: Promega™ V5280), then run in the Mass Spec, with a minimum of three replicates being used for each sample. Our results revealed that sperm has many different types of proteins including membranes, glycoproteins such as (glycoprotein 2 (Zymogen granule membrane), Leucine-rich alpha-2-glycoprotein 1 and mucin) non-glycosylated proteins and different enzymes in bovine sperm as well as uncharacterized proteins.

The results of both methods detected different types and various proteins of bovine sperm. However, the electrophoresis method gave better isolated and identifications of proteins. Sliced gel or band had more efficiency in recognizing proteins compared to the whole lysate. All biological replications of whole lysate and electrophoresis methods were combined, and data were analyzed. Proteomic of bovine sperm revealed that there are about 26649 peptide sequences, 2950 protein groups, and 3332 proteins (Table (1). Figures (2, 3, and 4).

Major important proteins were selected according to functions in different locations of the body and location in sperm and its behavior in other organs such as the immune system. These proteins include constituents of bovine sperm as follows: uncharacterized proteins approximately: 1705, binding proteins: 44, synthase proteins (enzyme): 25, calcium-binding proteins: 3, transmembrane proteins: 9, mucin: 11, galectin: 2, beta-defensin protein: 3, sperm antigens: 16, glycoproteins: 13, myosin: 9, actin: 10 (additional supplementary 1).

Figure (2). False discovery rate (FDR) curve. X axis is the number of peptide-spectrum matches (PSM) being kept. Y axis is the corresponding FDR.

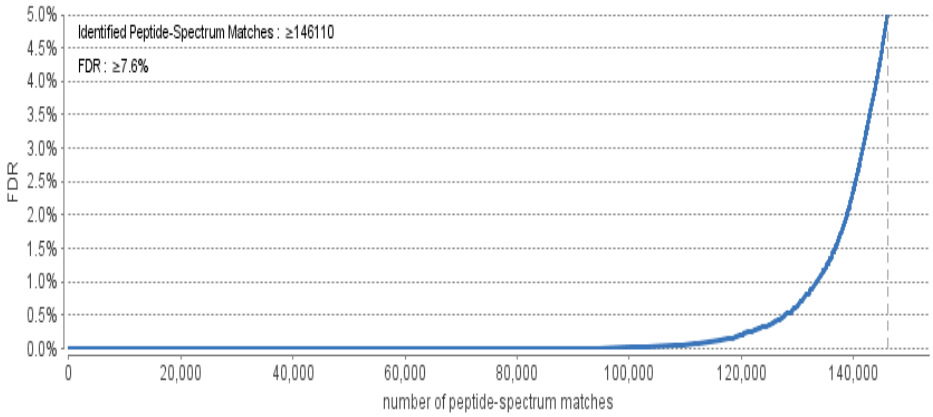


Figure (3). PSM score distribution. (a) Distribution of PEAKS peptide score; (b) Scatterplot of PEAKS peptide score versus precursor mass error.

(a) **(b)**

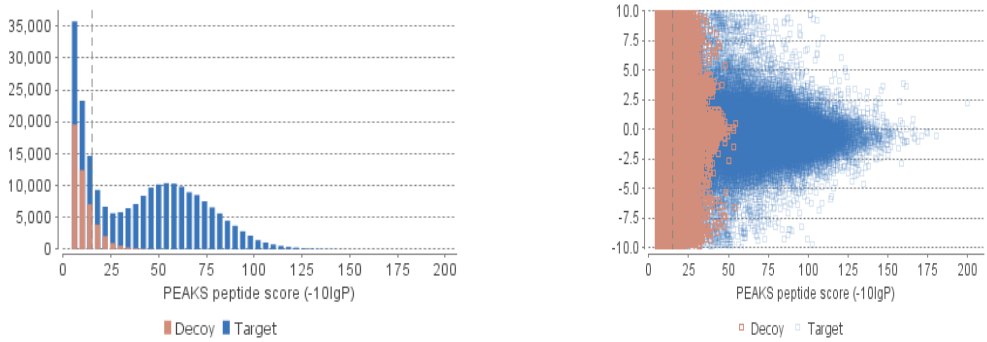


Figure (4). De novo result validation. Distribution of residue local confidence: (a) Residues in de novo sequences validated by confident database peptide assignment; (b) Residues in "de novo only" sequences.

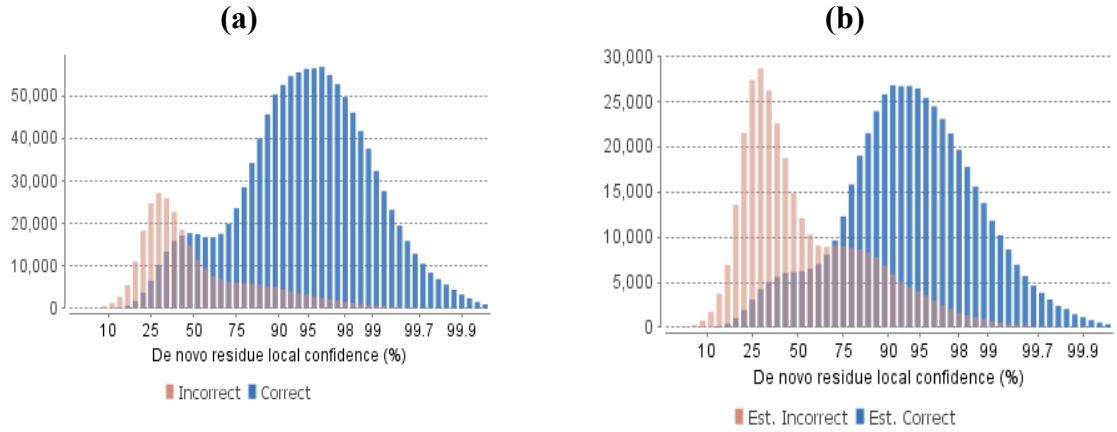


Table 1. Statistics of filtered result

No.	Parameters	Result
1	Peptide-Spectrum Matches	150553
2	Peptide Sequences	26649
3	Protein Groups	2950
4	Proteins	3332
5	Proteins (#Unique Peptides)	1077 (>2); 519 (=2); 1279 (=1);
6	FDR (Peptide-Spectrum Matches)	7.4%
7	FDR (Peptide Sequences)	20.8%
8	FDR (Protein)	83.4%
9	De Novo Only Spectra	65037

Discussion

Mass spectral analysis is a strategy used in proteomic studies to identify the relative abundance of amino acids and proteins by measuring deconvoluted overlapping peptide HPLC peaks to provide relative quantitation based on peak areas (Fenselau, 2007). It has been used to detect relative expression of proteins that characterize different diseases or to identify specific proteins in biological samples (Puchades *et al*., 2003, Zargar *et al.,* 2017, Nielson *et al.,* 2017). The results of this study suggested it is possible to use mass

spectrometry to find out proteins on bovine sperm. Several proteomic and transcriptomic studies of human and bovine sperm have already been conducted, and have identified different types of proteins which also displayed different types of important proteins such as free proteins, transmembrane proteins, glycoproteins and enzymes, motility as well proteins related to immune system in bovine sperm (Amaral *et al*., 2013, Gu *et al.,* 2011, Baker *et al*., 2013). These confirm the findings of the current study.

Interestingly, our proteomic results provided evidence for identifying thousands of proteins of bovine sperm with proteins detected in other proteomics studies and could enhance diagnosis of the disease and fertility by identifying normal proteins of sperm (Lalancette *et al.*, 2008, Peddinti *et al*., 2008). Many researchers have applied for the proteomic technique to assess the fertility between high and low fertilized bulls and identified the large difference between the contents of proteins seminal plasma and bovine sperm in high versus low fertility bulls. In particular, the biological functions for proteins which altered are binding, catalytic, oxidative damage of carbonylated proteins, and receptor activities (Kasimanickam *et al.,* 2019, Mostek *et al.,* 2018). Remarkably, the abundance of sperm proteins bearing X is different from those bearing Y, depending on sex determination of bull sperm; this study confirmed that proteomic studies could distinguish the database of sperm proteins and evaluate the physiological and biochemical functions of sperm (Scott *et al.,* 2018). Therefore, mass spectrometry can be used for identifying specific sperm protein such as PEBP4 of bovine sperm which associates in regulating sperm maturation, functions, and fertility and might be a marker for predicting semen quality and fertility (Somashekar *et al.*, 2017) or assess of quality and activity of cryopreserving sperm after using different treatment of straws (Maciel *et al.,* 2018).

The analyzing of the data obtained from mass spectrometry profiles approved that the sperm surrounded by different types of transmembrane of proteins. It might involve in glycosylation process of sperm during the passage via cervix and uterus and reservoir in the uterine tube before meeting the ovum and fertilization. These are glycosylated and considered as glycoproteins such as mucins and glycoprotein 2 (van den Berg *et al.,* 2001; Cornish *et al.,* 1998; Crocker *et al.,* 2007). Consequently, it might be necessary to use a different type of proteomic methods to profile all proteins in bovine sperm, since mass spectrometry of glycoproteins is limited by technical issues due to the considerably large differences and various chemical properties of individual glycoproteins (Fröhlich and Arnold, 2009, Ulbrich *et al.,* 2013). There are many complicated reasons which can preclude accuracy in the analysis of glycoproteins in biological samples using mass spectroscopy, especially in the case of proteins with low abundance. It is difficult to analyze glycoproteins due to the complexity and heterogeneity of glycan structures, which increases the complexity of the structural analysis of glycoproteins (Liu *et al.,* 2014). The results of this study revealed that in-gel digestion of proteins extracted by gel electrophoresis displayed less number of proteins compared to the whole lysate of bovine sperm (Shevchenko *et al.,* 2006). These results indicate that proteins which are isolated by electrophoresis method and digested by trypsin might extract the majority of proteins in the sperm sample, as the gel will run proteins only without any other carbohydrates such as sugar. In addition, the whole lysate contained proteins and carbohydrates, which could lead to incomplete digestion of whole proteins.

This study concluded that there is a biological diversity of 3332 proteins in bovine sperm which can be expressed as soluble proteins, enzymes, the cytoskeleton of sperm, transmembrane, putative uncharacterized proteins as well as binding proteins. These proteins might play a significant role in sperm motility, reservoir, the internal regulation environment of spermatozoa, immune suppression of female reproductive tract, and finally fertilisations.

In addition, some putative proteins, uncharacterized proteins, and recognized proteins in our data can be used by other researchers in future studies to understand more about the sperm journey in the female reproductive tract, fertilization, and effect of the female reproductive tract on sperm.

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