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Identification and characterization of proteins in testis and epididymal sperms of *Rattus norvegicus*

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Abstract
The present study was conducted to identify and characterize proteins in the testis and sperm membrane abstract of male *Rattus norvegcius*. Male albino rats were dissected to collect spermatozoa from the testis and epididymis. Sperm membrane extract (SME) was prepared using deoxycholate detergent (DOC) (Naz *et al* 1986) [1]. Quantitative and qualitative analysis of total protein from testis and epididymal sperm membrane using was carried out by the method described by Lowry *et al* 1951 [2] and SDS-PAGE (Laemmli 1970) [3]. It can be concluded for the present study that the protein content was observed to be highest in caput and lowest in the cauda epididymis due to the maximum and minimum secretory activities respectively in these regions. The SDS-PAGE profile revealed 13 bands corresponding to molecular weight of 17, 20, 24, 35, 44, 45, 47, 55, 60, 80, 95, 115 and 133 kDa in testis; 20 bands corresponding to molecular weights 17, 20, 24, 26, 28, 33,35, 44, 45, 47, 55, 60, 64, 72, 80, 95, 115, 133, 135 and 170 kDa in caput;19 bands corresponding to molecular weights 17, 20, 24, 26, 28, 33,35, 44, 45, 47, 55, 60, 72, 80, 95, 115, 133, 135 and 170 kDa in corpus and 17 bands corresponding to molecular weights 17, 20, 24, 26, 33,35, 44, 45, 47, 55, 60, 72, 80, 95, 115, 133 and 135 kDa in the cauda epididymal sperm membrane extract.

Keywords: Epididymis, *Rattus norvegicus*, sperm membrane extract, testis, SDS-page

1. Introduction
Male fertility relies not only on the production of spermatozoa in the testes but also on their structural and functional maturation during their passage through the epididymis. Sperm released from the testes are immature and lack fertilizing ability. As they transit through the caput, corpus and cauda regions of the epididymis, they progressively acquire motility, fertilizing competence and membrane stability (Cooper 1996; Robaire and Hinton, 2015) [134, 141]. This transformation is driven by the specialized environment of the epididymis, which provides region-specific secretions and reabsorptive activities (Turner 1991; Cornwall 2009) [142, 136]. Proteins play a central role in this maturation process. Epididymal secretions and membrane remodeling alter the sperm protein landscape, with some proteins being removed while others are acquired or modified post-translationally (Belleannée *et al* 2012; Cooper and Yeung 2006) [131, 135]. Many sperm proteins are directly involved in key reproductive events, such as motility regulation, zona pellucida recognition, acrosome reaction and sperm-egg fusion (Naz and Zhu 1997; O'Rand *et al* 2004) [139, 140]. Characterized examples include SP-17, Fertilin and SPAM1, which participate in zona binding, sperm-egg adhesion, and enzymatic activities crucial for fertilization (Kong *et al* 1995; Blobel *et al* 1992; Cherr *et al* 2001) [138, 132, 133]. Rodents, particularly *Rattus norvegicus*, are frequently used as models for reproductive studies due to their physiological resemblance to higher mammals in sperm maturation processes (Jones and Lopez 2004) [137]. Although individual sperm proteins have been identified in different species, limited information is available on the comparative protein profile of sperm membranes across testis and different epididymal regions in rats. Understanding these molecular variations is essential for clarifying the mechanisms that regulate sperm maturation and fertility.
The present study was therefore undertaken to identify and characterize proteins in the testis and epididymal sperm membrane extracts of male *Rattus norvegicus*. By combining biochemical protein estimation with SDS-PAGE analysis, this work aims to provide insights into region-specific protein dynamics and their potential roles in sperm maturation.

2. Materials and Methods

2.1 Procurement and maintenance of animals

Twenty sexually mature albino rats weighing between 130-150 g were procured from Disease Free Animal House at Lala Lajpat Rai, University of Veterinary and Animal Sciences (LUVAS), Hisar, a registered animal breeding centre after getting permission from Institutional Animal Ethics Committee (IAEC). The animals were acclimatized to laboratory conditions for 10 days before beginning of experiments. The rats were kept in polypropylene cages having wheat straw as bedding. The animals were fed a standard pelleted diet and water was provided in glass water bottle feeder with a rubber cork. During the experiment, standard laboratory conditions were maintained including ambient temperature (22-25 °C) and 12- hour light: 12- hour dark cycle. The rats were maintained according to the Indian Committee for the purpose of Control and Supervision of Experiments with animals (CPCSEA) standards.

2.2 Quantitative and qualitative analysis of total protein from epididymal sperm membrane

2.2.1 Dissection of Rats

Sexually mature adult male rats (n=20) were weighed, anaesthetized in CO_2 chamber and then dissected under hygienic conditions to take out testes and epididymii from both the sides. The organs were transferred into Phosphate buffer saline (PBS) kept at 37°C in petri dishes. The epididymis was split into three regions: caput (head), corpus (body) and cauda (tail) and each region was suspended in 1ml PBS. Each region of the epididymis was given a small incision and the epididymal fluid containing spermatozoa was collected. The various morphological parameters were analysed using epididymal fluid. The epididymal fluid containing spermatozoa was collected and centrifuged at 3000 rpm for 10 minutes to get spermatozoa in the pellet and the epididymal fluid (supernatant) was collected in separate vials. From the spermatozoa, sperm membrane extract (SME) was extracted using deoxycholate detergent and buffer and the fluid was processed for the partial purification of proteins with ammonium sulphate $(NH_4)_2SO_4$.

2.2.2 Partial purification of epididymal fluid proteins

A saturated solution of ammonium sulphate $(NH_4)_2SO_4$ was prepared by dissolving 7.67 g of ammonium sulphate in 10 ml of distilled water. 4 ml of ammonium sulphate was added to 1ml of epididymal fluid with continuous stirring with glass rod. The solution was kept overnight at 4°C. Precipitates of proteins were formed which were then separated by performing centrifugation at 10,000 g for 10 min at 4 °C. The precipitated proteins were then dissolved in PBS (pH 7.4) and dialysed against respective buffer to remove traces of ammonium sulphate.

2.2.3 Extraction of testis and epididymal sperm membrane proteins

The sperm membrane proteins of testicular and epididymal fluid were extracted with sodium dodecyl sulphate (SDS) (Jones *et al* 1983) and Deoxycholate (DOC) (Naz *et al* 1986). The fluid from testis and epididymis containing spermatozoa was centrifuged at 3000 rpm for 10 min. The pellet containing the spermatozoa was then given two PBS (pH 7.4) washes. The sperms were then suspended in 1 ml of 15 mM DOC/ 2% SDS in 62.5 mM Tris- HCl (pH 8.0) containing protease inhibitors (10 mM aprotinin, 1mM phenyl methyl sulphonyl fluoride, 25mM benzidine). Following this the sperm suspension was sonicated for 3 × 20 sec at 20 Watts in a cold ice bath. It was then centrifuged at 16,000 g for 40 minutes at 4 °C. The supernatant containing the sperm membrane extract (SME) was collected in aliquots and stored at -20 °C till further use.

2.2.4 Quantitative analysis of proteins (Lowry *et al* 1951)

Protein estimation was carried out by the method of Lowry *et al* 1951 in SME. The protein content was expressed as mg/10^9 spermatozoa.

$$\text{Total soluble protein} = \frac{\text{Conc. of standard}}{\text{O.D of standard}} \times \frac{\text{O.D of sample}}{\text{Vol. of sample}} \times \frac{\text{Total volume}}{\text{Weight of sample}}$$

Where; O.D is optical density

2.2.5 Qualitative analysis of proteins by Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) [3]

For the purpose of separating sperm membrane proteins, the extracted SME was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) [3].

2.3 Statistical analysis

Values were calculated as Mean ± S.E. Significance of difference was determined between different parameters at 5% level of significance by one way-ANOVA followed by Duncan Test using SPSS 16.0 version software.

3. Results

3.1 Quantitative analysis of total protein content in epididymal sperm membrane extract

The protein content in the caput, corpus and cauda sperm membrane extract was recorded to be 6.30±0.16, 5.73±0.16 and 5.47±0.36 mg /10^9 spermatozoa respectively as shown in (Table 1). Significant difference was observed in the protein content in all the three different regions of epididymis. According to the values obtained it was observed that the protein content was highest in caput and consecutively decreased in corpus and was found to be lowest in the cauda epididymis. The results obtained could be due to the maximum secretory activities in the caput (head) region remarking beginning of the process of sperm maturation and minimum secretory activities in cauda (tail) region depicting the presence of mature spermatozoa in this region. The concentration of sperm in the caput, corpus and cauda region of epididymis was 85.4±1.86, 55.13±1.42 and 125.1±2.69 × 10^6/ml respectively (Table 1). Sperm concentration was observed to be highest in the cauda region as it is the storage site for mature spermatozoa.

Table 1: Sperm concentration and total protein concentration of sperm membrane extract in male *Rattus norvegicus*

Regions of Epididymis	Sperm concentration (× 10^6 spermatozoa/ml)	Total protein concentration in sperm membrane extract (mg/10^9 spermatozoa)
Caput	85.4±1.86^a	6.30±0.16^a
Corpus	55.13±1.42^b	5.73±0.13^{ab}
Cauda	125.1±2.69^c	5.47±0.36^b

The values are expressed as mean ± standard error observations from pooled samples of 4 groups of 5 rats each Different superscripts (a, b and c) indicate significant difference ($p \leq 0.05$) along the column according to one-way ANOVA followed by Duncan Test

3.2 Qualitative analysis of total protein extracted from testis, caput, corpus and cauda epididymal sperm membrane extract by Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE indicated the presence of 13 bands corresponding to molecular weight of 17, 20, 24, 35, 44, 45, 47, 55, 60, 80, 95, 115 and 133 kDa in testis; 20 bands corresponding to molecular weights 17, 20, 24, 26, 28, 33, 35, 44, 45, 47, 55, 60, 64, 72, 80, 95, 115, 133, 135 and 170 kDa in caput; 19 bands corresponding to molecular weights 17, 20, 24, 26, 28, 33, 35, 44, 45, 47, 55, 60, 72, 80, 95, 115, 133, 135 and 170 kDa in corpus and 17 bands corresponding to molecular weights 17, 20, 24, 26, 33, 35, 44, 45, 47, 55, 60, 72, 80, 95, 115, 133 and 135 kDa in cauda epididymal SME (Figure 1). An increase in intensity of bands has been observed from caput to cauda, high intensity bands were recorded in cauda region, consequently lower intensity bands in corpus and lowest intensity bands were observed in the caput region. Although the number of protein bands recorded were higher in caput region but their intensity was low. The trend for peak height, raw volume and % raw volume was not same for all the proteins in standard protein ladder, testis, caput, corpus and cauda sperm membrane extract as shown in the tables 3-7 and graphs for variation in protein intensity between the different epididymal regions are shown in figure 2-6.

The difference in band patterns on gel indicates that some proteins are lost and some are acquired during the spermatozoa's journey through the epididymis. The protein bands of molecular weight 26, 28, 33, 64, 72, 135 and 170 kDa were absent in the SME of testis but were present in the SME of different regions of epididymis. These proteins are thought to be acquired by the spermatozoa during epididymal maturation. A 64 kDa protein was absent in corpus and cauda region but were present in the caput region of epididymis depicting that possibly these proteins were absorbed during their transition through the epididymis. The protein bands with molecular weight 28 and 170 kDa were absent in cauda region but were present in caput and corpus region of epididymis indicating that these proteins were probably reabsorbed during their transition (Table 2). The presence or absence of certain protein bands in different regions of the epididymis is because, during the sperm's passage through the epididymis, its surface membrane undergoes sequential protein modifications.

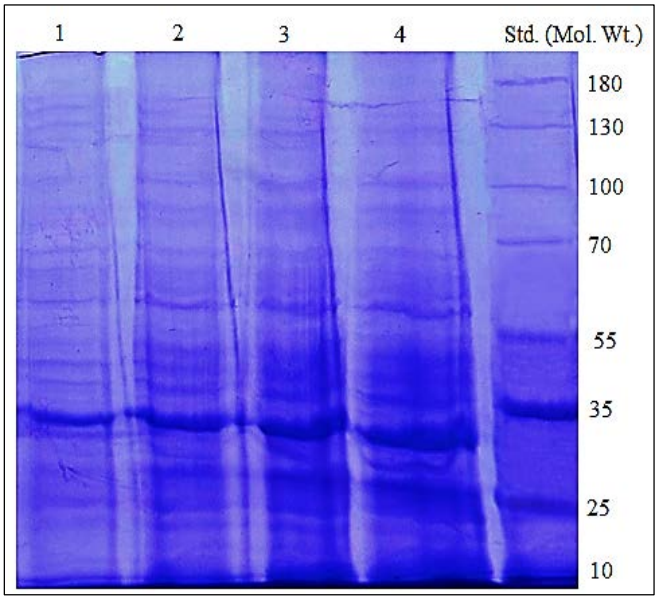

Fig 1: Protein profile of sperm membrane extract from testis and epididymis separated on SDS-PAGE; Lane 1- SME Caput epididymis; Lane 2- SME Corpus epididymis; Lane 3- SME Cauda epididymis; Lane 4- SME Testis; Lane 5- Standard

Table 2: Proteins separated on SDS-PAGE from SME of testis and different regions of epididymis

Molecular weight (kDa)	Testis	Caput	Corpus	Cauda
170	-	+	+	-
135	-	+	+	+
133	+	+	+	+
115	+	+	+	+
95	+	+	+	+
80	+	+	+	+
72	-	+	+	+
64	-	+	-	-
60	+	+	+	+
55	+	+	+	+
47	+	+	+	+
45	+	+	+	+
44	+	+	+	+
35	+	+	+	+
33	-	+	+	+
28	-	+	+	-
26	-	+	+	+
24	+	+	+	+
20	+	+	+	+
17	+	+	+	+

(+) Indicates presence of protein; (-) Indicates absence of protein

Table 3: Analysis of protein bands obtained in standard ladder on SDS-PAGE

S. No.	Mol. Weight (kDa)	Height (on graph)	Raw volume	% Raw volume
1	180	21.938	14188.620	22.600
2	130	16.292	3046.230	4.852
3	100	16.147	6201.625	9.878
4	70	23.475	8267.617	13.169
5	55	16.837	6226.109	9.917
6	35	21.294	7914.859	12.607
7	25	16.408	2877.883	4.584
8	10	8.653	4765.344	7.590

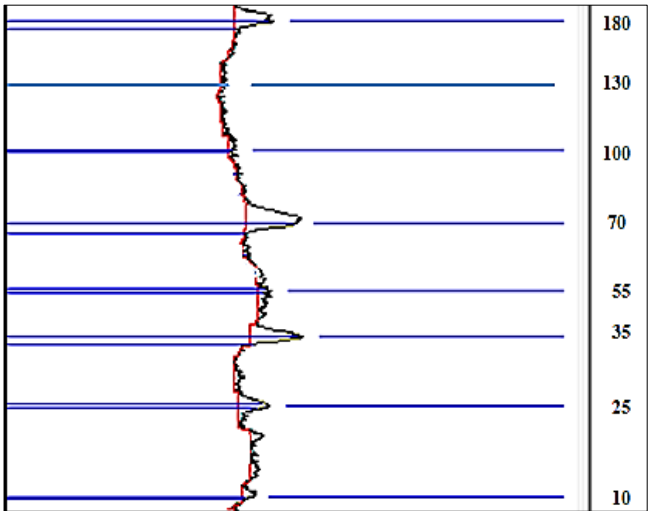

Fig 2: Peak heights of proteins obtained in standard ladder on SDS-PAGE

Table 4: Characterization of proteins of testicular SME separated on SDS-PAGE analysis

S. No.	Mol. Weight (kDa)	Height (on graph)	Raw volume	% Raw volume
1	133	30.764	71583.60	13.618
2	115	34.663	47114.57	8.963
3	95	19.169	18863.18	3.445
4	80	26.615	21250.73	3.881
5	60	27.416	26553.60	4.850
6	55	18.879	16928.46	3.092
7	47	14.743	14267.17	2.606
8	45	15.423	5296.57	0.967
9	44	12.205	4465.63	0.885
10	35	27.290	43358.92	7.919
11	24	12.790	4319.38	0.858
12	20	17.577	36007.14	6.576
13	17	18.740	40465.69	7.375

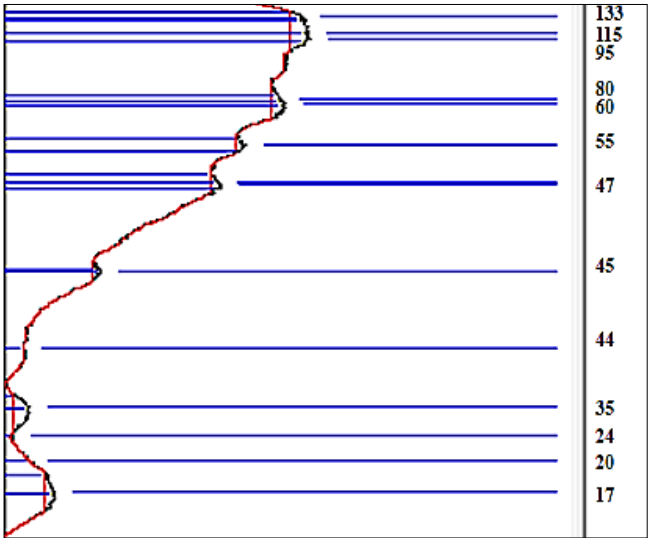

Fig 3: Peak heights of testicular SME proteins separated on SDS-PAGE

Table 5: Characterization of proteins of caput SME separated on SDS-PAGE

S. No.	Mol. Weight (kDa)	Height (on graph)	Raw volume	% Raw volume
1	170	62.369	100190.92	16.593
2	135	77.759	79164.27	13.111
3	133	60.725	65332.74	12.478
4	115	17.500	12808.34	2.070
5	95	23.793	22671.20	3.755
6	80	27.582	7482.00	1.239
7	72	44.272	102132.03	16.914
8	64	15.730	2878.04	0.477
9	60	11.411	19628.60	3.251
10	55	8.892	2425.80	0.402
11	47	16.347	8080.60	1.338
12	45	15.746	8982.93	1.452
13	44	4.045	734.63	0.119
14	35	30.390	34487.30	5.712
15	33	13.761	21056.80	10.175
16	28	28.831	61752.25	10.227
17	26	33.628	52908.73	8.762
18	24	5.440	1092.14	0.181
19	20	26.339	20041.68	3.319
20	17	74.022	87829.63	14.546

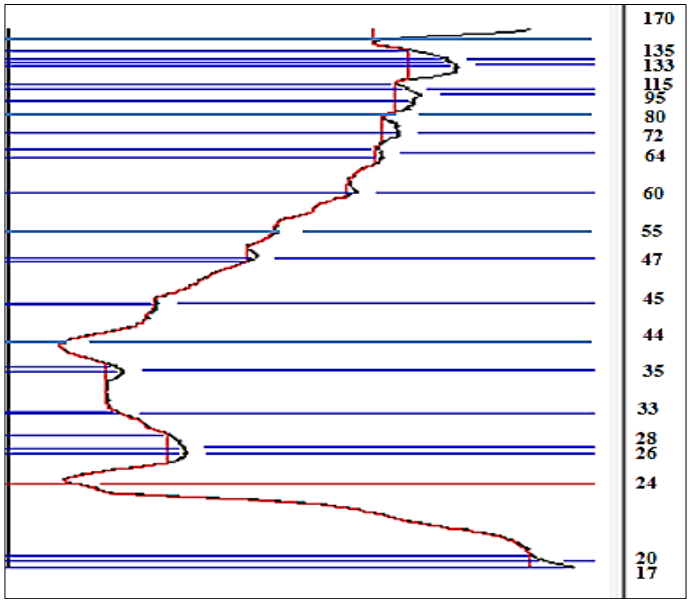

Fig 4: Peak heights of proteins of caput SME separated on SDS-PAGE

Table 6: Characterization of proteins of corpus SME separated on SDS-PAGE

S. No.	Mol. Weight (kDa)	Height (on graph)	Raw volume	% Raw volume
1	170	63.486	162470.25	16.929
2	135	88.884	255034.13	26.574
3	133	32.246	54166.70	5.582
4	115	35.971	24507.16	2.526
5	95	41.832	69404.41	7.152
6	80	47.067	54607.68	5.690
7	72	44.826	5599.17	5.167
8	60	33.863	81664.48	3.365
9	55	16.896	11624.22	10.169
10	47	27.991	39249.49	4.090
11	45	16.039	13029.35	1.316
12	44	13.056	8164.42	0.841
13	33	15.082	11492.21	1.175
14	35	18.737	41843.84	4.617
15	28	14.590	54588.21	4.717
16	26	23.458	6893.94	6.894
17	24	20.461	5294.12	4.510
18	20	3.046	788.20	0.081
19	17	47.067	54607.68	5.628

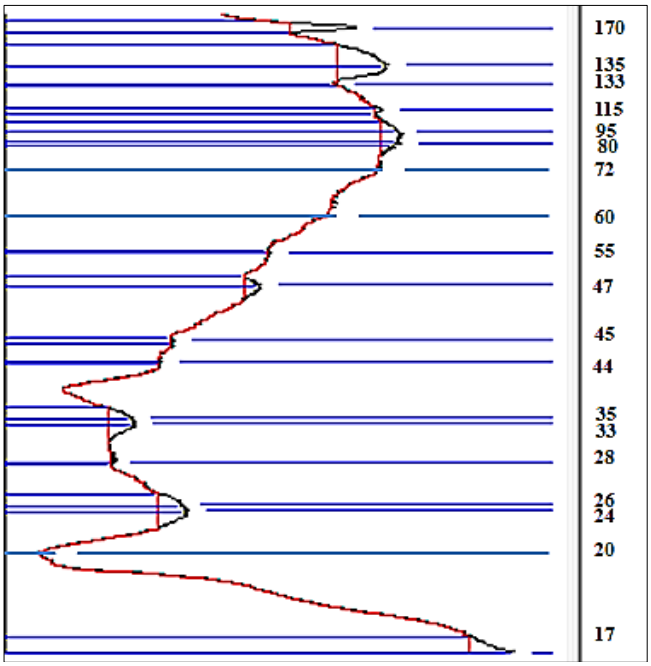

Fig 5: Peak heights of proteins of corpus SME separated on SDS-PAGE

Table 7: Characterization of proteins of cauda SME separated on SDS-PAGE

S. No.	Mol. Weight (kDa)	Height (on graph)	Raw volume	% Raw volume
1	135	15.684	70973.88	7.493
2	133	35.126	38491.12	4.686
3	115	37.931	43924.07	5.347
4	95	29.625	42259.36	5.145
5	80	23.090	3696.26	2.085
6	72	17.081	16180.67	1.970
7	60	24.756	15951.52	1.938
8	55	32.456	30958.25	3.761
9	47	39.202	53835.43	6.554
10	45	9.376	3952.75	0.481
11	44	13.156	1428.82	1.052
12	35	31.463	43888.34	5.332
13	33	27.913	34287.91	4.174
14	26	33.293	31814.91	3.865
15	24	14.090	16802.66	2.041
16	20	47.401	79248.73	9.627
17	17	69.942	60712.45	7.376

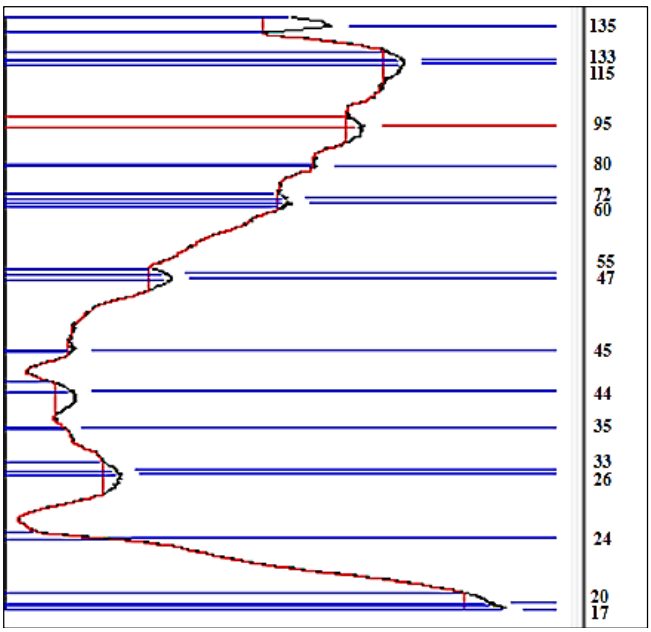

Fig 6: Peak heights of proteins of cauda SME separated on SDS-PAGE

Table 8: Characterization of proteins separated on SDS-PAGE according to related studies

S. No.	Sperm antigen	Molecular weight and their corresponding function	Reference
1	Sperm Protein (Rabbit sperm antigen) RSA-1, RSA-2 and RSA-3/SP17	13±2 kDa 17 kDa (SP17) - participates in sperm- zona pellucida (ZP3) binding	Kong *et al* (1995); Richardson *et al* (1994) [138, 113]
2	NZ-1	14-18 kDa - involved in ZP binding and exhibits tyrosine phosphorylation activity	Naz and Zhu (1997) [139]
3	BS-17	17.5 kDa - participates in acrosome reaction	Wang *et al* (1994) [115]; Wei *et al* (1995) [9]
4	Sperm agglutination antigen SAGA-1/ SP-19	15- 25 kDa - participates in sperm- ZP binding	Homyk and Herr (1992) [10]; Diekma *et al* (1997) [11]
5	Epididymal Protein-20 (EP-20)	20 kDa - participates in sperm- ZP binding	Zong *et al* (1992) [12]
6	Rabbit Sperm Membrane Protein-B (rSMP-B)	20.1 kDa - helps in spermatogenesis and is involved in sperm- ZP binding	Wang *et al* (1986) [13]
7	Testis Specific Antigen-1 (TSA-1)	24 kDa - plays role in sperm functionality, acrosome response and sperm egg binding	Santhanam and Naz (2001) [117]; Trivedi and Naz 2002 [15]
8	Epididymal Protease Inhibitor (Eppin)	26 kDa - microbicidal properties protects sperms, regulates sperm transition to motile spermatozoa and helps in capacitation	O'Rand *et al* (2004) [140]; Sun *et al* (2010) [17]
9	Mouse Sperm antigen	24-28 kDa - functions as intra- acrosomal protein	Naz and Vanek (1998) [18]
10	Sperad	33 kDa - role in cell adhesion and cell signalling	Quill and Garbers (1996) [19]
11	Lactate dehydrogenase-C (LDH-C)	35 kDa - serve in glycolytic pathway catalyzing reversible conversion of pyruvate and lactate, ATP metabolism, sperm motility and hyperactivation	Odet *et al* (2011) [20]; Goldberg (2021) [21]; Farhana and Lappin (2023) [22]
12	Human Equatorial Segment Protein (hESP)	38 and 48-kDa in mouse, 38 kDa in hamster, 34 kDa in humans - associated with acrosome biogenesis and sperm- egg binding	Toshimori *et al* (1992) [22]; Noor and Moore (1999) [24]; Auer *et al* (2000) [25]; Wolkowicz *et al* (2008) [26]
13	Fertilin β	44 kDa - mediates membrane fusion and sperm-egg interaction	Yuan *et al* (1997) [27]; Cho *et al* (1998) [28]; Gundogan *et al* (2022) [29]
14	Sperm Protein-10 (SP-10)	~45 kDa (polypeptide cleaves into ~32, ~30, ~28 and ~26 kDa peptide) ~18-34 kDa in mouse ~ 28 kDa in human - responsible for acrosomal activities, sperm-zona binding	Anderson *et al* (1987) [30]; Herr *et al* (1990) [31]; Foster *et al* (1994) [32]; Suri (2004) [33]; Buffone *et al* (2008) [34]; Venkatanagaraju (2021) [35]
15	Fertilization antigen-1 (FA-1)	~23 kD (monomer) ~47±2 kD (Dimer) - glycoprotein with auto-phosphorylating activity, role in capacitation/acrosome response, binding of sperm to ZP	Naz *et al* (1984) [36]; Naz and Ahmad (1994) [37]; Coonrod *et al* (1994) [38]
16	Cytotestin	55 kDa - mediates sperm-oocyte membrane adhesion process	Cho *et al* (1998) [28]
17	Fertilin α	60 kDa - involved in sperm-egg adhesion	Blobel *et al* (1992) [132]; Cho *et al* (2000) [40]
18	Sperm Adhesion Molecule1 (SPAM1)	64 kDa - during fertilization, the hyaluronidase enzyme SPAM1 plays three primary roles: ZP binding, cumulus penetration and Ca^{2}+ signalling during acrosomal exocytosis	Lathrop *et al* (1990) [41]; Hou *et al* (1996) [42]; Holland *et al* (1997) [43]; Ten Have *et al* (1998) [44]; Cherr *et al* (1999) [45]; Cherr *et al* (2001) [46]; Day *et al* (2002) [47]
19	YLP-12	72 kDa - plays role in acrosome reaction	Naz and Packianathn (2000) [48]
20	Human Sperm Antigen (HAS)	80 kDa - functions in sperm motility	Khobarekar *et al* (2008) [49]
21	Spermatid A kinase anchor protein (S-AKAP)	80 kDa - essential for the assembly of fibrous sheath of sperm, sperm motility	Chen *et al* (1997) [50]; Miki *et al* (2002) [51]; Brown *et al* (2003) [52]
22	Testicular differentiation antigen (TDA-95)/CA-12/BC-7	95 kDa - Cell surface glycoprotein	Koshimizu *et al* (1993) [53]
23	Sperm Flagella Protein (SFP-2)	100-115 kDa (monomer), 220-230 kDa (dimer) - functions in sperm motility and viability	Khan *et al* (2009) [54]
24	Flagellar protein 130 (FP-130)	130 kDa - possess phosphorylating activity and has role in sperm motility	Tash and Brcho (1999) [55]; Nishigaki *et al* (2000) [56]
25	Phospho protein phosphatase (PPase M-1)	170 kDa - possess phosphorylating activity, plays role in acrosome reaction, capacitation, sperm motility and interaction with zona pellucida	Barua *et al* (1999) [57]; Sepideh *et al* (2009) [58]; Majumder *et al* (2012) [59]

4. Discussion

4.1 Quantitative analysis of total protein content in testicular and epididymal sperm membrane extract

The total protein content in the sperm membrane extract was found to decrease when the spermatozoa passes from caput to cauda epididymis. The current experiment's result coincides with the results of previous researches. Several workers have reported changes in the protein concentration of spermatozoa during their transit from caput to cauda region of the epididymis (Lavon *et al* 1971, Brooks and Higgins 1980, Olson and Danzo 1981) [60, 61, 62].

A spermatozoon can only become fertile when it has undergone a series of post-testicular alterations in both the male and female reproductive tracts. In order to acquire motility and the capacity to fertilize, the sperm goes through a number of biochemical and functional changes as it passes through the epididymis (Jones and Lopez 2004 [63], Cooper and Yeung 2006 [135], Robaire *et al* 2006 [135], Robaire and

Hinton 2015) [141]. After the seminiferous tubules produce sperm, the tubule's lumen releases the sperm, which then travels to the rete testis and eventually the efferent ducts via the flow of fluids (Hess *et al* 2001, Cooper *et al* 2003, Shaw and Renfree 2014) [67, 68, 69]. The sperm are then retained until ejaculation after passing through the caput (head), corpus (body) and cauda (tail) regions of the epididymis (Robaire *et al* 2006) [135]. Therefore, the epididymis performs three key roles: storage, transit and most importantly sperm maturation.

Each component of the epididymis serves a different physiological purpose. The sperm in each area have unique structural characteristics. The maturation of sperm is carried out by the caput and corpus, while the cauda epididymis serves as a reservoir for sperm (Cornwall 2009, Belleannée *et al* 2012) [136, 131]. The epididymal epithelium plays a role in absorption, secretion, synthesis and creates an environment that is appropriate for acquiring sperm motility and its fertilizing ability (Turner 1991, Hinton *et al* 1995) [73]. This has also been reported by several other workers that epididymis can change the composition of luminal fluid through its secretion (Kirchhoff 1998, Nixon *et al* 2002, Saez *et al* 2003, Frenette *et al* 2004, Gatti *et al* 2005) [75, 76, 77, 78] and all the major changes in the protein are due to epididymal secretions (Dacheux and Voglmayr 1983, Clulow *et al* 1996, Syntin *et al* 1996, Mital *et al* 2011) [79, 80, 81, 82].

As spermatozoa travel through the epididymis, proteins are known to be acquired, deleted or post-translationally altered. Research has demonstrated that this is a crucial step in determining their capacity to fertilize the oocyte (Cooper 1996, Turner 2005, Cooper and Yeung 2006, Shum *et al* 2011, Dacheux and Dacheux 2014) [134, 84, 135, 86, 87]. The epididymis secretes proteins in a highly regulated and regionalized manner from different regions of the epididymis. The secretory activity of the head epididymal region is 6-8 times greater than that of the tail region in boar. Caput contributes 83%, corpus contributes 16% and cauda contributes mere 1% to the overall epididymal secretions (Dacheux *et al* 2005) [78]. Maximum secretory activities are observed in caput region representing the beginning of maturation process of spermatozoa in this region. Some of the synthesized and secreted proteins from the epididymis become associated with the sperm plasma membrane (Yeung *et al* 1997, Cooper 1998, Holland and Nixon 1998) [89, 90, 91]. The proteins in the plasma membrane are embedded partially or completely in the lipid bilayer and can protrude out from their cytoplasmic surface and have different characteristics depending on their functions (Etemadi 1989) [92]. These membrane proteins can be selectively extracted using various detergents such as SDS and DOC (Ahuja *et al* 1985, Kinger *et al* 1989) [93, 94]. These detergents are of varying polarities; the detergents with different polarities selectively extract proteins of different nature and are embedded to different extents in the lipid bilayer of plasma membrane (Sundhey *et al* 1992) [95].

4.2 Qualitative analysis of total protein from testis, caput, corpus and cauda epididymal sperm membrane by Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to ejaculation, functionally mature sperm cells are stored in the cauda epididymis (Cornwall 2009) [136]. The cauda's epithelial cells release substances that support the luminal environment intended to keep sperm in quiescent stage during storage (Robaire *et al* 2006) [135]. Upon ejaculation, sperm exit this quiescent state and their metabolic activity increases by 3-5 times (Jones 1999) [97]. Sperm maturation is a crucial process that takes place during epididymal transit and is necessary for normal male fertility. During this period, sperm go through a number of maturational changes, but most significantly, they gain motility through interaction with epididymosomes which are released by the epididymal epithelium and plays role in sperm competency (Topfer-Petersen 2000, Sullivan and Saez 2013, Gervasi and Visconti 2017) [98, 99, 100]. Epididymosomes are known to contain various proteins from different functional classes. Furthermore, the various locations of the epididymis have distinct protein compositions of epididymosomes (Girouard *et al* 2011) [101]. Sperm come into direct contact with the materials in the epididymal lumen environment during the maturation phase (Cornwall 2009) [136].

The sperm maturation is believed to be the result of modification of pre-existing sperm components and absorption of extracellular substances from epididymal secretions and incorporation of epididymal fluid peptides (Olson and Orgebin- Crist 1982) [102]. Testicular spermatozoa released into the seminiferous tubules are not yet mature and do not possess fertilizing capacity. They become mature and acquire the potential to fertilize due to its interaction with epididymal secretory proteins. The majority of the testicular sperm surface proteins are gradually lost or altered in immature gametes, while new transient or permanent proteins are added to well-organized sperm membrane protein domains. It has been shown that these alterations are necessary for male gametes to acquire motility and fertility. The epididymal microenvironment surrounding the spermatozoa is maintained by active secretion and reabsorption throughout the tract and by the presence of a continuous blood barrier formed by tight junctions in the epithelial cells of epididymis (Pardyak *et al* 2020, Gibb *et al* 2021) [103, 104]. The interaction may result in removing, masking or unmasking pre-existing surface compounds (Voglmayr *et al* 1982) [105]. Sequential protein modifications occur on the sperm surface during their transit through the epididymis: immature gametes progressively loose or modify most of their testicular surface proteins and gain new transient or permanent proteins in well-organized sperm membrane protein domains. These stages have been demonstrated to be essential for acquisition of motility and fertility by the male gametes. Disruption in the proteins in any of the stage will ultimately affect the process of fertilization (Belleannée *et al* 2011, Dacheux and Dacheux 2014) [106, 107]. Evidences from earlier studies have demonstrated that specific secretory proteins in the epididymis interact with the sperm surface to influence sperm maturation. The epididymis synthesizes and secretes various proteins some of which becomes associated with the sperm plasma membrane and some epididymal glycosidases are absorbed in the plasma membrane which are having role in fertilization (Vernon *et al* 1982, Hammerstedt and Parks 1987, Jones 1998, Gervasi and Visconti 2017) [107, 108, 109, 110].

The different protein bands that appeared on the acrylamide gels correspond to different sperm proteins. Many of these proteins have been characterized by different researchers that support the present study. Some of the characterized proteins are as follows:

A protein band corresponding to molecular weight ranging between ~14-18 kDa is of NZ-1. The testis-specific proteins were isolated from human and mouse sperm antigens. The sperm antigens are involved in ZP binding and exhibits tyrosine phosphorylation activity. Mice immunized with a recombinant form of the NZ-1 antigen showed decreased rates of reproduction (Naz and Zhu 1997) [139]. A protein band corresponding to molecular weight ~17 kDa is of sperm Protein (SP-17). Sperm specific proteins namely RSA-1, RSA-2 and RSA-3/SP17 with low molecular weight ~13±2 kDa have been identified from rabbit sperm (O'Rand and Porter 1982) [110]. Cross-reactions between mouse, baboon and human sperm were observed with monoclonal antibodies against RSA antigens. These antibodies prevented human spermatozoa from penetrating hamster oocytes that were zona-free (O'Rand and Irons 1984) [111]. The most significant antigen among RSA-1, RSA-2 and RSA-3/SP17 is SP-17 with molecular weight corresponding to ~17 kDa, shown on the surface of sperm following an acrosome reaction in human, rabbit and mouse. The protein is localized in the equatorial section and participates in zona pellucida (ZP) binding *in vitro* (Kong *et al* 1995, Richardson *et al* 1994) [113]. Studies conducted *in vitro* demonstrated SP-17's selectivity for ZP3. Antibody titres were increased in both male and female mice immunized with a chimeric peptide including SP-17 and T-cell epitope. The peptide's immunogenic properties were validated by increase in antibody titre in both the sexes.

A protein band corresponding to molecular weight ~17.5 kDa is of BS-17. It is one of the antigens that were found in infertile women's serum samples. A 758 bp cDNA fragment was obtained from the positive clone when polyclonal anti-BS-17 antibodies were used to probe the human testis λgt11 cDNA expression library. The protein possibly participates in acrosome reaction (Wang *et al* 1994, Wei *et al* 1995) [115, 9]. Human sperm were unable to penetrate and fertilize zona-free hamster eggs when polyclonal antibodies targeting the BS-17 antigen were present (Wei *et al* 1994). Given that BS-17 and calpastatin had a 99.7% homology, it was hypothesized that the anti-BS-17 antibodies were responsible for destabilising the calpastatin-calpain complex. Following this destabilisation, calpain may cause the sperm acrosome reaction prematurely, before the sperm reach the ovum, which would diminish the sperm's capacity to fertilize the ovum.

Sperm agglutination antigen or SP-19 corresponding to molecular weight ~ (15- 25 kDa) was first identified and isolated from human spermatozoa. The presence of SAGA-1 on the entire sperm surface was demonstrated by immunolocalization using electron microscopy, which also indicated the reactivity of S-19 monoclonal antibody to human sperm surface (Homyk and Herr 1992) [10]. Sperm agglutination/immobilization and prevention of penetration in zona-free hamster ova were the outcomes of *in vitro* incubation of antibodies produced against SAGA-1 (Diekman *et al* 1997) [11]. A protein band corresponding to molecular weight ~20 kDa is similar to Epididymal Protein-20 (EP-20). It is a glycoprotein that was identified from rabbit cauda epididymal fluid. Polyclonal antibodies specific to EP-20 were used to perform immune localization of the protein, demonstrating its presence in the testis and epididymis. The protein is testis specific as strong staining was only seen in the human testes whereas germ cells, interstitial cells and nine other tissues remained unstained. In addition to immobilising and agglutinating human sperm, polyclonal antibodies produced against EP-20 prevented human sperm from penetrating zona-free hamster eggs *in vitro* (Zong *et al* 1992, Nixon *et al* 2002) [12, 76].

Rabbit Sperm Membrane Protein-B (rSMP-B) - A protein corresponding to molecular weight ~20.1 kDa is of rSMP-B, extracted from the tail of a rabbit sperm (Wang *et al* 1986) [13]. Using polyclonal antibodies against rSMP-B, the immunolocalization of rSMP-B on spermatozoa and somatic cells was examined. The findings implied that during spermatogenesis, germ cells produced this antigen. It was shown that monoclonal antibodies produced against rSMP-B could prevent human sperm from penetrating and fertilizing zona-free hamster eggs *in vitro*. When male rabbits were immunized with rSMP-B protein, spermatogenesis was blocked, leading to azoospermia. Additionally, it was discovered that 83.3% of immunized female rats showed infertility following mating when they were immunized with the rSMP-B 230 peptide (Kamada *et al* 1999) [116].

Testis Specific Antigen-1 (TSA-1) - A protein corresponding to molecular weight ~24 kDa is of TSA-1. Human and mouse sperm express this protein. The expression of TSA-1 in testis was detected using the northern blot technique. Acrosomal, equatorial, midpiece and tail regions of human sperm were identified by antibody (Santhanam and Naz 2001) [117]. *In vitro* tests revealed that sperm egg binding and acrosome response were both blocked by anti-recombinant TSA-1 antibodies (Trivedi and Naz 2002) [15]. These results suggested that a protein unique to the testis and sperm plays a role in sperm funtionality. A protein band corresponding to molecular weight ~26 kDa is of Epididymal Protease Inhibitor (Eppin) which is an epididymal protein (O'Rand *et al* 2004) [140] and was used to immunize male monkeys. 78% of the monkeys that had high anti-Eppin antibody titres following immunization lost their ability to reproduce, while 71% of the monkeys were able to conceive again after the immunization was stopped. Subsequent researches revealed that active immunization with Eppin reduced animal populations to a maximum of 90%. A recombinant version of Eppin was also tested in an attempt to increase its sensitivity. In contrast to native protein, immunization with recombinant protein only produced a 70% decrease in fertility (Sun *et al* 2010) [17].

A protein band corresponding to molecular weight ~24-28 kDa is of mouse sperm antigen. The protein was reported in testis, corpus and cauda and shows 60% homology with human SP-10 at protein level and expresses at the stage of post-meiotic germ cells in mouse and functions as intra-acrosomal protein (Naz and Vanek 1998) [18]. A protein band corresponding to molecular weight ~33.3 kDa is of Sperad. An extensive family of potential cell adhesion molecules known as biliary glycoproteins bears a close resemblance to the sperad. The protein is first expressed by haploid spermatid and is confined to the plasma membrane covering the acrosome along with the role in cell adhesion/signaling. Sperad is lost from the sperm cells when the acrosome reaction is induced, but its molecular weight does not appear to change (Quill and Garbers 1996) [19].

A protein band in the gel corresponding to molecular weight ~35 kDa is of LDH-C data obtained from National Center for Biotechnology Information (NCBI). LDH-C protein family members characteristically are distributed in tissue and cell in specific patterns and serve as the terminal enzyme of glycolysis, catalyzing reversible oxidation-

reduction between pyruvate and lactate (Farhana and Lappin 2023) [22]. They are present as tetramers and only one family member, LDH-C is found abundant in spermatocytes, spermatids and spermatozoa. Modest amounts of LDH-C are also found in oocytes. Lactate dehydrogenase-C gene (Ldhc) gene is localized to human chromosome 11. The gene is expressed exclusively during the first meiotic division of spermatogenesis and is distributed throughout the principal piece of the sperm tail consistent with flagellar location for its enzymatic role in the glycolytic pathway. While other glycolytic enzymes including LDH-A are tightly bound to the fibrous sheath, LDH-C localizes throughout the flagellum presumably for ATP production to power sperm motility (Goldberg 2021) [21]. Previous studies have demonstrated that disruption of germ cell-specific Ldhc led to male infertility due to defects in sperm function, including a rapid decline in sperm ATP levels, a decrease in progressive motility and failure to develop hyperactivated motility (Odet *et al* 2011) [20].

Sperm Protein-38 (SP-38) - A protein band corresponding to molecular weight ~38 kDa was isolated from boar sperm. The protein participates in sperm binding to a 90-kDa family of zona pellucida glycoprotein. According to the amino acid sequence inferred from the cDNA sequence, SP-38 starts off as a 350-residue precursor protein. After post-translational modification the mature protein is formed with 299 residues form the SP-38 precursor. Sperm cell immunostained with an antibody raised against SP-38 fusion protein revealed that it is intra-acrosomal in location and is released after post-acrosome reaction (Mori *et al* 1993) [118].

The molecular weight ~38 and ~48-kDa in mouse, 38 kDa in hamster and 34 kDa in humans are similar to human Equatorial Segment Protein (hESP). It is a sperm-specific protein known as human equatorial segment protein that was initially identified in 2003 (Wolkowicz *et al* 2003) [119]. Human ESP and mouse ESP (mESP) share 81% homology. It is connected to acrosome biogenesis and is restricted to the equatorial segment of the acrosome. In hamster egg penetration assay, the application of antiserum generated against recombinant hESP prevented the binding and fusion of human sperm (Wolkowicz *et al* 2008) [26]. Fertility was dramatically decreased when mice were immunized with polyepitope antigens. Subsequently a study was conducted to identify the significant portion of mESP that is essential for conception. The complete mESP was broken up into three pieces: P1, P2 and P3. Only the presence of anti-P1 and anti-P2 antibodies inhibited sperm-egg interaction; anti-P3 antibodies did not exhibit any inhibition (Wolkowicz *et al* 2008) [26].

A protein band corresponding to molecular weight ~45 kDa is of Sperm Protein-10 (SP-10). It is a sperm-specific acrosomal protein (Suri 2004) [33] that was first identified in humans (Anderson *et al* 1987) [30] and has also been detected in several species including mouse (Buffone *et al* 2008) [34]. In humans the SP-10 protein was found to be a stable protein with a molecular weight of ~28 kDa (Venkatanagaraju 2021) [35]. A full-length SP-10 protein with molecular weight around 45 kDa was found in the testis extracts, along with additional immunoreactive SP-10 peptides weighing 32, 30, 28, and 26 kDa. Furthermore, 25-18 kDa SP-10 peptides were discovered for the first time in caput epididymal sperm extracts. These peptides most likely came from the proteolytic processing of 45 kDa and 32-26 kDa SP-10 peptides in the early section of the caput epididymis. The extracts of cauda epididymis, ejaculated or capacitated sperm does not contain SP-10 protein indicating that the 32-18 kDa SP-10 peptides were not further processed during epididymal transit, ejaculation or capacitation (Foster *et al* 1994) [32]. Hence, the protein is responsible for acrosomal activities. Decrease in the concentration of the protein will alter acrosomal reactions. Several enzymes are present in the acrosome including acid hydrolases and other enzymes specific to spermatogenic cells. During the acrosome reactions, the contents are released by calcium mediated exocytosis that helps sperms to penetrate the zona pellucida surrounding the oocyte.

The antibodies generated against SP-10 recognised sperm from baboons, macaques and pigs but not from rabbits, bulls, rats, guinea-pigs or cats concluding that SP-10 is a testis but not a species-specific protein (Herr *et al* 1990) [31]. SP-10 was cloned and sequenced from a human cDNA expression library and the protein was discovered to have 1117 bp of 256 aa (Wright *et al* 1990) [120]. The mRNA expression of this protein is primarily in the round spermatid stage and is expressed in all the six stages of spermiogenesis. SP-10 proteins were detected only in germ cells and SP-10 RNAs were not detected in any of the tested somatic tissue (Batista *et al* 2022) [121]. *In vitro* incubation of anti-SP-10 antibodies prevented bovine fertilization by lowering sperm-zona binding. An attenuated strain of *Salmonella* sp. that expressed human SP-10 was used to assess the immunogenic potential of SP-10 *in vivo*. In mice, antigen was administered orally; for monkeys, it was administered intramuscularly. Antibodies specific to SP-10 were detected in both the cases (Kurth *et al* 1997) [122]. Since SP-10 is localised within the acrosomal compartment and the outer acrosomal membrane complex, and is thus only accessible to antibodies after the acrosome reaction has been initiated. Hence, antibody levels in the oviduct are particularly relevant in this circumstance.

A protein band corresponding to molecular weight ~47 kDa is of Fertilization antigen-1 (FA-1). It is a glycoprotein with auto-phosphorylating activity, produced in the testis in the later stages of spermatogenesis (Naz *et al* 1984) [36]. The molecular weight of its monomeric form is ~23 kDa and that of dimer is ~47±2 kDa. During the human sperm capacitation/acrosome process, tyrosine phosphorylation takes place (Naz and Ahmad 1994) [37]. Therefore, it was proposed that FA-1 plays a significant role in the capacitation/acrosome response, which may also have an impact on the binding of sperm to ZP. The presence of a complementary sequence of the FA-1 antigen on the mouse oocyte demonstrated the specificity of the antigen towards ZP3. Additionally, to determine the phenomenon behind the decrease in fertilization, monoclonal antibodies produced against the FA-1 antigen were employed (Coonrod *et al* 1994) [38]. Antibodies against FA-1 have been found to impede fertilization by decreasing sperm-zona binding and altering capacitation/acrosome responses. Similar outcomes were seen in the cases of mice, calves, humans and primates. Mice actively immunized with rFA-1 had fertility reductions ranging from 64-70% when compared to control groups. When the titres reached control levels, the animals gave birth to healthy offspring without any change in litter size, indicating that the effect caused by antibodies was reversible and long-lasting (Naz and Zhu 1997) [7].

A protein band corresponding to molecular weight ~60 kDa and ~44 kDa belongs to Metalloprotease/ Disintegrin/

Cysteine-Rich (MDC) protein family. The MDC proteins are an expanding family of integral membrane proteins that are mostly produced in the testes of mammals. A transmembrane domain (also called the ADAM family), a cysteine-rich domain, a disintegrin-like domain, a metalloproteinase-like domain and a prodomain are among the unique conserved properties shared by the proteins (Nishimura *et al* 2004) [123]. Few of these proteins are known to be expressed on mature sperm or male germ cells. Among these, cyritestin (tMDC I) and fertilin α and β (together referred to as fertilin) are noteworthy.

Fertilin is a protein linked with sperm that was earlier known as PH-30. It was first isolated from caudal epididymal sperm of guinea pigs. Fertilin α and β, two related subunits, with molecular weights of ~60 kDa for Fertilin α and ~44 kDa for Fertilin β in guinea pigs, according to results from reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE). Fertilin α's function is linked to sperm-egg adhesion; however it is still unclear if this contributes to membrane fusion (Blobel *et al* 1992) [132]. The existence of a cysteine-rich domain, which seems to be involved in cell adhesion, justifies its role in adhesion. The sperms lacking Fertilin α are still capable of fusing with the egg (Cho *et al* 2000) [40]. One of the earliest "cellular disintegrins" to be discovered was fertilin β. The proteins of Fertilin β are observed in three regions of epididymis. This protein is a sperm- associated that are proteolytically cleaved and activated by various proteases during the epididymal transition. It is a dimeric sperm antigen and antibodies produced against it react with fertilin α. It is well known for mediating membrane fusion and sperm-egg interaction (Yuan *et al* 1997, Cho *et al* 1998, Gundogan *et al* 2022) [27, 28, 29]. Fertilin β seems to use its disintegrin loop sequence to interact with the egg membrane, as evidenced by its inhibition in the presence of antidisintegrin loop antibodies. Fertilin β antibodies prevent fertilization *in vitro* but immunization against it has little effect on fertility *in vivo* (Ramarao *et al* 1996) [124]. The Cyrn gene produces cytotestin, which has an apparent molecular weight of 110 kDa but is processed during its transfer through epididymal region, resulting in an approximate molecular weight of ~55 kDa. It functions in a way similar to fertilin in the sperm-oocyte membrane adhesion process and likewise binds via its disintegrin loop. Mice lacking the ciritestin gene demonstrated impaired sperm-zona binding with no abnormality in sperm-oocyte membrane fusion (Cho *et al* 1998) [28].

A band with molecular weight ~64kDa is of Sperm Adhesion Molecule 1 (SPAM1). It is a glycosyl-phosphatidylinositol (GPI)-linked protein and is expressed in the testis, epididymis, sperm and epididymis luminal fluid (Deng *et al* 2000, Zhang and Martin-DeLeon 2003) [125, 126]. During fertilization, the hyaluronidase enzyme SPAM1 has three primary roles. These consist of ZP binding, cumulus penetration (Cherr *et al* 2001) [138] and Ca^{2}+ signalling during acrosomal exocytosis (Cherr *et al* 1999) [45]. The protein has been cloned from numerous species including fox (Ten Have *et al* 1998) [44]; guinea pig (Lathrop *et al* 1990) [41]; pig (Day *et al* 2002) [47]; rat (Hou *et al* 1996) [42]; rabbit (Holland *et al* 1997); monkey and man (Lin *et al* 1993) showing that the protein is highly conserved among different mammals. Sperm-ZP binding decreased when sperms were incubated with antibodies against SPAM-1. 100% infertility was observed in male and female guinea pigs immunized with SPAM-1 purified from guinea pigs (Primakoff *et al* 1997) [129]. In case of immunized males, autoimmune diseases were linked to infertility, due to loss of normal sperm in the epididymis (Tung *et al* 1997). Infertility in females was caused by antibodies that prevented sperm from binding with the eggs. Since, fertility was resumed in both cases within 6 to 15 months, it was reported that the infertility brought on by vaccination was reversible.

A protein band corresponding to molecular weight ~72 kDa was observed in the caput, corpus and cauda epididymal sperm membrane. The observed protein corresponds to YLP-12. Using phage display technology, a dodecamer sequence (YLPVGGLRRIGG) known as YLP-12, was discovered on human sperm. In human and mouse sperm, the YLP-12 peptide sequence is mainly found on the acrosome and tail region of sperm. An extensive database search yielded no results for homology or similarity with any known sequence, suggesting that the peptide is unique. According to immunoblotting research, antibodies produced after YLP-12 immunization identified a particular 72 kDa protein band in the testis. The acrosome response (AR) was dramatically decreased when spermatozoa were incubated with YLP-12 Fab *in vitro*. Antibodies against YLP-12 were found in the sera of immunoinfertile patients. Utilising a synthetic YLP-12 peptide conjugated with the binding domain of recombinant cholera toxin subunit B (rCTB), *in vivo* investigations were conducted in a mouse model. Two distinct methods of immunizing female mice were used: intramuscular and intranasal. The overall decline in fertility for both methods was 70.3% (intranasal) and 61.4% (intramuscular), respectively (Naz and Packianathan 2000) [48].

A protein band corresponding to molecular weight ~80 kDa is of Human Sperm Antigen (HSA) which was identified from human sperm extract by western blot analysis. The glycoprotein was discovered to be preserved and restricted to the testis and epididymis, with no presence in other somatic tissues (Bandivdekar *et al* 2001) [130]. Native protein was used to carry out active immunization in male and female rats, resulting in infertility in both the sexes. A synthetic version of 80 kDa HSA was also experimented with an attempt to increase immunogenicity. The male rabbits immunized with peptide-1 and peptide-NT showed the greatest suppression of fertility. Six of the seven male marmosets actively immunized with synthetic peptide-1 were infertile and 7 out of 9 marmosets developed an antibody response. The semen samples of treated animals revealed a total loss of progressive motility. It was discovered that the impact was reversible when the animal's fertility returned and the antibody titre decreased 8-10 weeks following the final booster shot. After that, there was no discernible impact on regular physiological functions (Khobarekar *et al* 2008) [49].

A protein band corresponding to molecular weight ~95 kDa is of testicular differentiation antigen (TDA-95)/CA-12/BC-7 which is a cell surface glycoprotein. Using two monoclonal antibodies (mAbs), a mouse cell surface antigen demonstrating stage-specific expression during spermatogenesis was identified. These mAbs were able to identify the population of spermatogenic cells that were situated close to the edge of seminiferous tubules throughout stages I-VI and XII. These cells were later identified as zygotene and early pachytene spermatocytes. In germ cells

at more advanced stages of spermatogenesis, such as late pachytene spermatocytes and round spermatids, the antigen's expression was temporary and absent. Studies using immunoprecipitation and immunoblotting revealed that the antigenic molecule interacted with both the mAbs. Prepubertal mice appeared to experience spermatogenic disruption following intraperitoneal injection of mAb. These findings revealed that the unique cell surface glycoprotein is a crucial component of the early meiotic prophase of spermatogenesis (Koshimizu *et al* 1993) [53].

A protein band corresponding to molecular weight ~130 kDa belongs to a lipoprotein binding Flagellar protein (FP-130). The protein has been identified in sea urchin and bull spermatozoa that has phosphorylating activities and plays role in sperm motility (Tash and Brcho 1999, Nishigaki *et al* 2000) [55, 56]. A protein band corresponding to molecular weight ~ 170 kDa is of Phospho-protein phosphatase (PPase M-1) which was isolated and partially characterized from the plasma membrane of the goat cauda-epididymal sperm. It dephosphorylates the serine and threonine residues of histones. PPase M-I is an ecto-enzyme that dephosphorylates the phosphoproteins on the sperm outer membrane, which may have a significant impact on sperm physiology. It plays role in acrosome reaction, capacitation, sperm motility and sperm- zona binding (Barua *et al* 1999, Sepideh *et al* 2009) [57, 58].

5. Conclusion

It can be concluded for the present study that the protein content was highest in caput due to the maximum secretory activities in this region remarking the beginning of sperm maturation and its concentration was lowest in the cauda epididymis because of minimum secretory activities in this region illustrating the presence of mature spermatozoa. The SDS-PAGE profile of sperm membrane extract of testis and different regions of epididymis revealed the presence of difference proteins of varying molecular weights. 13 bands corresponding to molecular weight of 17, 20, 24, 35, 44, 45, 47, 55, 60, 80, 95, 115 and 133 kDa in testis; 20 bands corresponding to molecular weights 17, 20, 24, 26, 28, 33,35, 44, 45, 47, 55, 60, 64, 72, 80, 95, 115, 133, 135 and 170 kDa were detected in caput;19 bands corresponding to molecular weights 17, 20, 24, 26, 28, 33,35, 44, 45, 47, 55, 60, 72, 80, 95, 115, 133, 135 and 170 kDa were noted in corpus and 17 bands corresponding to molecular weights 17, 20, 24, 26, 33,35, 44, 45, 47, 55, 60, 72, 80, 95, 115, 133 and 135 kDa was recorded in cauda epididymal sperm membrane extract.

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7. Declarations

Ethics approval and consent to participate: permission was taken from Institutional Animal Ethics Committee (IEAC) for the use of animals as experimental model with protocol no. (GADVASU/2023/IAEC/67/18).

Author Contribution: The paper was conceived and designed by Harpreet Kaur and Nisha Vashishat. Both the authors have read and approved the final manuscript.

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