SDS-PAGE OF SPERM MEMBRANE-, EPIDIDYMAL TISSUE- AND FLUID PROTEINS OF INDIAN MONGREL: IDENTIFICATION AND CHARACTERIZATIONOF SPERM SPECIFIC PROTEINS

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ABSTRACT
Sperm membrane-, epididymal tissue- and –fluid proteins were characterized by SDS-PAGE in mongrel dog. Sperm membrane extracts, epididymal tissue extracts and partially purified proteins of epididymal fluid were characterized by SDS-PAGE under reducing conditions. The proteins with mol wt of 173, 135, 94, 72, 65, 46, 36, 23, 17 kDa; 55, 46, 36, 28, 23 kDa; 173, 116, 86, 72, 60, 55, 46, 42, 36, 32, 28, 23, 20, 17 kDa and 173, 116, 86, 72, 62, 55, 46, 42, 36, 32, 28, 23, 20, 17 kDa were detected by SDS-PAGE in Triton X-100, DOC, SDS and LDIS extracts of dog spermatozoa respectively. There was strong correlation between quantity of 17 kDa; 23/ 32 kDa; 36 kDa; 46 kDa proteins and % motility; % HOS positive; % live/ % motile and % live spermatozoa respectively. About 7-9 proteins in the range of 17-207 kDa were separated by SDS-PAGE from the tissue extracts as well as luminal fluid of dog. The presence of 46, 42, 38, 28, 23, 17 kDa proteins in tissue as well as luminal fluid indicated their secretory/ structural nature. The detection of 55 and 36 kDa only in the luminal fluid indicated their secretory/functional nature. The detection of 173, 135, 94, 80 and 20 kDa proteins only in the sperm membrane extracts indicated these as acquired or modified proteins during epididymal maturation or ejaculation. The presence of 55, 46, 42, 36, 28, 23 and 17 kDa proteins in the sperm extracts, luminal fluid and tissue extracts depicted these as surface proteins, associated in the maturation of spermatozoa during epididymal transit.

Key words: Dog; Sperm; Epididymal- tissue; -fluid; Proteins; SDS-PAGE

INTRODUCTION

Mammalian spermatozoa undergo morphological and physiological modifications within the epididymis, which has a very active secretory and reabsorption function (1-5). The epididymis creates sequential changes in the composition of luminal fluid e.g. ions, solutes, proteins and lipids (6-10). The epithelial cells of the epididymis form a barrier to create a unique microenvironment in the lumen, where interactions between epithelial cells and spermatozoa take place via the fluid (11, 3, 4, 12, and 13).

Identification of sperm antigens has become the object of increasing attention as a means to understand the molecules involved in fertilization process and to discover potential targets for anti-sperm contraceptive vaccines. For this purpose, the identification of sperm proteins would be necessary for each species.

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An important aspect of epididymal sperm maturation and storage seems to be the interaction of luminal proteins with the sperm surface (14-16). Three proteins are thought to be synthesized post-testicularly by the epithelial cells lining the epididymal duct and secreted apically into the lumen, where they come in contact with or may be adsorbed to the surface of spermatozoa. During the last decade, various methods have been used to characterize these proteins. As not much work has been done on this aspect in dog, therefore sperm membrane-, epididymal tissue- and –fluid proteins were characterized using SDS-PAGE in mongrel dog to find out the sperm specific proteins, which can be worked out for their immune-contraceptive potential later on.

MATERIAL AND METHODS

Collection and evaluation of semen: Four healthy adult mongrel dogs weighing about 16-20 Kg and of 2-3 years were selected for semen collection. Starting 4 weeks prior to semen collection, the dogs were housed in concrete floored kennels with access to outside runs and fed commercial dog feed (nutripet).

The water was available at libitum. Deworming and vaccination against rabies and six viral diseases was done. The semen (entire second sperm rich fraction and part of 3rd fraction) was collected by manual manipulation in clean graduated tube attached to a glass funnel. Five ejaculates were collected from each dog with a minimum interval of 4 days. Semen analysis was done immediately after semen collection.

A drop of semen, diluted in PBS, pH 7.4 was placed on clean pre warmed glass slide, covered with a cover slip. Video recording of motile semen was done at 400 X using Olympus microscope (CH-21) and digital Camera. About 100 motile and non motile sperms in different recorded fields were counted and percentage of motile spermatozoa was calculated. Sperm concentration was evaluated with the help of haemocytometer.

A drop of semen was mixed with a drop of eosin: nigrosin, kept at 37°C for 2 min, smears were prepared on clean glass slides. Air dried slides were examined at 1000 X for live (unstained) and dead (pink) spermatozoa. About 100 live and dead spermatozoa were counted in different fields and percentage of live spermatozoa was calculated.

50µl of semen was mixed with 250µl of 60mM HOS solution and incubated at 37°C for one hour. A control was run in PBS, pH 7.4. A drop of semen covered with a cover slip was examined under microscope at 400 X. About 100 coiled and uncoiled spermatozoa were observed in control as well as HOS solution in different fields and percentage of HOS positive spermatozoa were calculated by using the formula-

No. of coiled spermatozoa in HOS sol - No. of coiled spermatozoa in control X100

Total Spermatozoa

Extraction of sperm membrane proteins (sme): $500x10^6$ Spermatozoa washed twice with PBS (pH 7.4) containing protease inhibitors were suspended in either of the following for extraction of sperm membrane proteins:

1). 1.0 ml of 2% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.4 containing protease inhibitors (1mM PMSF, 25 mM benzidine and10mM aprotinin) and sonicated at 4°C (20 W, thrice for 20 seconds each).

2). 1.0 ml of 15 mM DOC/ 2% SDS in 62.5 mM Tris-HCl, pH 8.0 containing protease inhibitors and sonicated at 4°C (20 W, thrice for 20 seconds each).

3).1.0 ml of 0.3 M LDIS in 50 mM Tris-HCl, pH 8.0 containing protease inhibitors, agitated at room temperature for 30 min and then at 4°C for two hrs. Extracts were dialysed against 0.15M Lithium chloride and 50 mM Tris-HCl, pH 8.0 containing 0.15M NaCl and 1 mM EDTA.

All the sperm suspensions were centrifuged at 16,000 g for 30 minutes and SME were stored in 0.1 ml fractions at -20°C till further use.

Processing of tissue: Epididymii were removed from the testis and divided into three parts i.e. caput, corpus and cauda. All the blood vessels were removed and tissue was thoroughly washed with PBS, pH 7.4. Cuts were given to the respective tissue part and fluid containing spermatozoa was collected with 1.0 ml of PBS. Epididymal fluid and spermatozoa were separated by centrifuging at 10,000g for 30 min. Proteins were further precipitated from the fluid by saturated $(NH_4)_2SO_4$ and precipitated proteins were suspended in known volume of PBS. Thoroughly washed tissue was dried between folds of filter paper and weighed. One gram of tissue was sonicated (20 watts, 2 min) at 4°C in 0.02M Tris-HCl buffer, pH, 7.5, 0.5 mM dithiothretol, 0.2 mM EDTA, 2% SDS and protease inhibitors (as per SME protocol). The sonicated samples were centrifuged at 16,000 g for 30 min and tissue extracts were stored at -20°C till further use.

Protein estimation: Total protein was estimated from SME, SDS tissue extracts and $(NH_4)_2SO_4$ precipitated proteins of luminal fluid by the method of Lowry et al[17].

SDS-page (18): 100 µg protein of SDS- tissue-extracts, $(NH_4)_2SO_4$ precipitated proteins of luminal fluid and SME was fractionated by SDS-PAGE using 10% separating gel and 4% stacking gel. Gels were run at a constant current of 30 mA and maximum 200 V for 2 hrs and were stained with commassie brilliant blue. Gel images were captured on Syngene gel doc using Gene Snap image acquisition software and were analyzed by using Gene Tools gel analysis software (Syngene). At least three gels were run for each sample.

Statistical analysis: Standard error and correlation coefficient between quantities of proteins, semen characteristics was calculated using CPCS-1 software, developed by Department of statistcs, Punjab Agricultural University, Ludhiana, Punjab, India.

RESULTS

Sperm membrane proteins: The proteins with mol wt of 173, 135, 94, 72, 55, 46, 36, 23, 17 kDa; 55, 46, 36, 28, 23 kDa; 173, 116, 86, 72, 60, 55, 46, 42, 36, 32, 28, 23, 20, 17 kDa and 173, 116, 86, 72, 62, 55, 46, 42, 36, 32, 28, 23, 20, 17 kDa were detected by SDS-PAGE in Triton X-100, DOC, SDS and LDIS extracts of dog spermatozoa respectively (Table 1, Fig 1). The results indicated better extraction of dog sperm membrane proteins with SDS and LDIS.

Table1. Protein profile (Mol. Wt., kDa) of sperm membrane extracts of Indian Mongrel.

S. No.	Tr X-100	DOC	SME SDS	SME LDIS
1.	173±1.2	---	173±1.0	173±1.5
2.	135±2.4	---	----	----
3	----	----	116±3.6	116±1.0
4.	94±1.5	---	86 ±1.5	86 ± 0.6
6.	72 ±1.5	---	72 ±1.5	72 ± 1.0
8.	----	---	62 ± 1.2	60 ± 0.9
9.	55 ± 1.0	55±1.1	55 ± 1.0	55 ± 1.0
10	46±0.3	46±2.0	46 ± 1.0	46 ± 1.1
11.	----	----	42 ±0.5	42 ± 0.6
12.	36±1.0	36±1.0	36±1.0	36±1.1
13.	----	----	32 ± 1.3	32 ± 0.8
14.	----	28±0.6	28±0.6	28±1.0
15.	23±2.0	23±1.0	23±0.6	23±1.0
16.	----		20 ± 0.7	20 ± 0.6
17.	17±0.5	----	17±0.6	17±1.0

SDS-page of sds- sme of different dogs: As indicated by SDS-PAGE the proteins with mol wt of 116, 86, 72, 60, 55, 46, 40, 36 and 28 kDa were common in SDS extracts of different dogs (Table 2, Fig 1). A 173 kDa protein was detected only in the SME of dog # 2, which also has less HOS positive spermatozoa. The proteins with mol. wt. of 32; 23; 17 kDa were lacking in dog #1, 2; 1; 2 respectively. There was strong correlation between quantity of 17 kDa; 23/ 32kDa; 36 kDa; 46 kDa proteins and % motility; % HOS positive; % live/ % motile and % live spermatozoa respectively (Table 3). There was not much variation in the sperm membrane proteins of mongrel dog but the quantity of different proteins vary among the dogs.

Table3. Correlation between quantity of sperm membrane proteins and semen characteristic of Indian Mongrel.

Semen Characteristic	Correlation Coefficient: Quantity of Protein (Mol Wt, kDa) 46	36	32	23	17
Motility	+0.20	**+0.57**	+0.11	+0.02	**+0.60**
Live	**+0.55**	**+0.88**	--	+0.18	+0.37
	--	+0.27	**+0.75**	**+0.58**	+0.02

Epididymal tissue and fluid proteins: About 7-9 proteins in the range of 17-207 kDa were separated by SDS-PAGE from the tissue extracts as well as luminal fluid of dog. The presence of 46, 42, 38, 28, 23, 17 kDa proteins in tissue as well as luminal fluid indicated their secretory/ structural nature (Table 4, Fig 2). The detection of proteins with mol wt of 65; 156 and 207

Table2. Protein profile (Mol. Wt., kDa) of SDS-SME of different Indian Mongrel.

S. No.	Dog No. 1	2	3	4
1	---	173±1.0 (11.7±0.7)	---	---
2	116±3.5 (3.9±0.4)	116±4.0 (4.4±0.4)	116±1.5 (10.8±1.8)	116±2.8 (4.9±0.5)
3	86±1.0 (10±2.4)	86±0.6 (19.4±0.3)	86±0.5 (4.6±0.2)	86±3.0 (5.6±0.2)
4	72±1.0 (3..0±2.2)	72±0.7 (2.5±1.2)	72±0.3 (2.8±0.4)	72±0.5 (2.6±0.4)
5	60±1.2 (1.5±0.9)	60±0.8 (3.5±1.2)	60±0.5 (2.9±0.6)	60±0.6 (4.4±0.2)
6	55±1.0 (2.8±1.4)	55±0.6 (2.3±0.3)	55±2.0 (19.0±0.7)	55±1.0 (4.2±0.2)
7	46±1.0 (5.3±0.60)	46±0.6 15.5±0.6)	46±0.6 (2.6±0.1)	46±1.0 (15±0.7)
8	40±1.0 (4.4±1.1	40±1.0 (2.6±0.2)	40±0.6 (19.1±0.4)	40±1.0 (8.9±0.4)
9	36±1.0 (5.3±0.3)	36±1.0 (5.8±0.6)	36±0.6 (3.2±0.4)	36±0.6 (5.3±0.3)
10	-----	----	32±0.6 (5.6±0.5)	32±0.6 (3.7±0.2)
11	28±0.6 (4.4±0.3)	28±0.6 (8.9±0.4)	28±0.3 (9±0.2)	28±0.3 (17.1±0.5)
12	----	23±0.6 (10.9±0.4)	23±0 (7.3±0.2)	23±0.6 (18.4±1.1)
13	20±0.3(21.2±3.3)	20±0.4 (5.1±0.6)	20±0.6 (4.4±0.6)	20±1.1 (8.4±1.0)
14	17±1.0 (35.9±0.5)	---	17±1.0 (12.4±0.3)	17±0.6 (21.7±1.6)

Figures in parentheses represent percent protein

Table4. Protein profile (Mol wt, kDa) of epididymal tissue and fluid proteins of Indian Mongrel.

S. No.	Tissue				Fluid			
	Testis	Caput	Corpus	Cauda	Testis	Caput	Corpus	Cauda
1	----	207± 9.2	207± 7.5	-----	----	----	-----	----
2	----	----	156 ±3	-----	----	-----	-----	-----
3	115±1.1	---	----	-----	----	116 ±4.9	116± 4.5	116±4.3
4	-----	---	-----	-----	72± 1.4	-----	-----	----
5	65±2.1	65± 0.6	65±4.3	65±0.7	----	-----	-----	----
6	-----	---	----	----	55 ±0.7	55 ±0.7	55 ±1.7	55± 1
7	46± 2.3	46± 0	46± 0.2	46 ±0.3	46 ±2.8	46 ±0.7	46± 2	46 ±1.5
8	42± 0.2	42± 1.1	42±0	42± 0.6	42± 2.0	42± 0.1	42± 1.3	42± 2.1
9	38 ±1.5	38 ±0.6	38 ±0.6	38 ±0.8	38 ±1.4	38 ±0.7	38±0.6	38±0.1
10	-----	-----	-----	-----	36±0.1	36±0.0	36 ±1	36 ±0.6
11	28 ±2.5	28 ±0.8	28 ±1.4	28 ±0.1	28 ±2.1	28 ±0.7	28 ±1.5	28±0.3
12	23±0.6	23± 0.5	23± 1.7	23 ±0.5	23 ±0.7	23 ±0.7	23 ±0.6	23 ±1.1
13	17±0.3	17±1.0	17±2.6	17±1.1	17±0.6	17±0.3	17±1.7	17±1.5

kDa mainly in testis, caput, corpus, cauda; corpus; and caput, corpus tissue respectively indicated these as structural proteins of respective regions. The detection of 55 and 36 kDa only in the luminal fluid indicated their secretory/functional nature.

DISCUSSION

About 17 bands in the mol wt range of 17 kDa to 173 kDa were identified by SDS-PAGE in dog-SME, extracted with different detergents. Haden *et al*.[19] resolved at least 14 different swine sperm membrane proteins by 2-D electrophoresis, 13 of which possessed acidic PIS range 4.2-4.8. Verdier *et al*.[20] also recognized 25 protein bands between 10 and 110 kDa by SDS-PAGE in the sperm membrane extrats of fox spermatozoa. The detergents of different polarities selectively extract proteins of different nature and embedded to different extents in the lipid bilayer of membrane. Since DOC & SDS are ionic detergents of different polarity and Tr X-100 & LDIS are non ionic & a mild chaotropic agent respectively (Sundhey *et al* 1992), therefore, SDS with highest polarity and LDIS, a mild chaotropic agent extracted higher number of proteins as compared to DOC and Tr X-100.

A series of events including changes in the composition of membrane lipids and proteins, ion exchange between the extra and intracellular environment of spermatozoa (6, 21, 22, 13) occurs during the epididymal transit. The epididymal epithelium secretes proteins that potentially affect not only sperm maturation (23) but also other aspects of sperm physiology, while these are stored in the cauda (16). These proteins may determine important attributes of the fertilizing capacity of spermatozoa. Electrophoretic analysis of epididymal fluid proteins has been performed in various mammals, mainly rodents. A few major secretory proteins have been identified biochemically, some of which appear to represent sperm surface components. In this study, the proteins in the range of 46, 42, 38, 28, 23 and 17 kDa were identified as structural as well as functional/secretary proteins. Whereas the proteins with molecular weight of 55, 36 kDa were identified as functional/secretory proteins. The presence of 55, 46,

42, 36, 28, 23 and 17 kDa protein in the sperm extracts, luminal fluid and tissue extracts depicted these as surface proteins, associated in the maturation

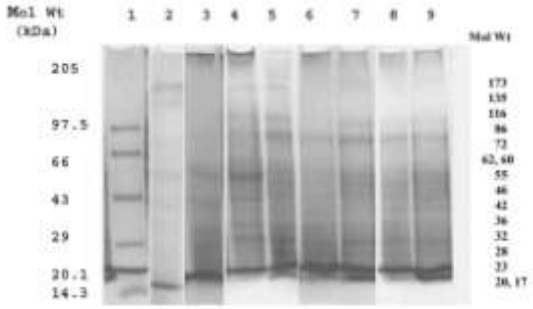

Fig 1. SDS-PAGE of Pooled sperm membrane extracts of dog. 1-std 2- Tr X-100, 3-DOC, 4-LDIS, 5-SDS and HME of different dogs, extracted with SDS:6-dog # 1, 7-dog # 2, 8-dog # 3, 9-dog # 4.

of spermatozoa during epididymal transit. About 32 (24), 17 (25) and 16 (26) protein bands were also identified by SDS-PAGE in the epididymal fluid of goat, ram and rat respectively.

The strong correlation of quantity of 23/ 32 kDa proteins with HOS +ve spermatozoa (a membrane integrity test and one of the fertility markers) indicated their contribution in sperm maturation viz a viz fertility of dog sperm. Olson and Hinton[27] were also of the opinion that major luminal fluid proteins may be associated with cauda spermatozoa. Similarly a rat epididymal glycoprotein of 37 kDa (DE) is synthesized by the epithelium of the proximal segments of the epididymis and associates with the sperm surface during epididymal transit (28). The

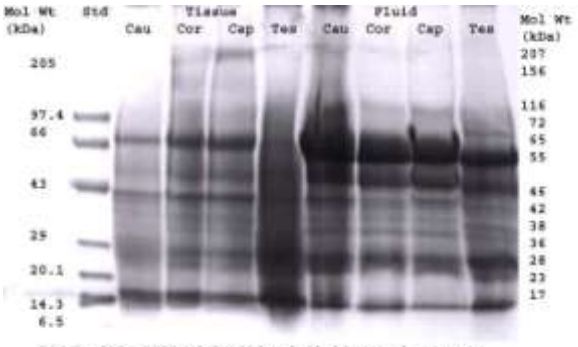

Fig 2. SDS- PAGE of Epididymal-fluid and tissue-proteins of Mongrel dog.

protein with a similar mol wt was also detected in sperm extracts and epididymal fluid of dog spermatozoa, which indicates its synthesis in the proximal region. Guo et al.[29] indicated that ERP-29, a rat precursor is related to secretory protein synthesis and absorbed by spermatozoa, may play a role in sperm maturation during the epididymal transit, particularly in the sperm/ organelle membrane. Moura *et al*.[30] also presented empirical evidence that certain cauda epididymal proteins are significant molecular indicators of bull fertility. The detection of 173, 135, 94, 86 and 20 kDa proteins only in the sperm membrane extracts indicated these as modified proteins during epididymal maturation or ejaculation. The presence of 72 kDa protein only in the testicular fluid and sperm membrane extract (LDIS) indicated it as a protein of testicular region.

The proteins with such molecular weights like FA-1 (31); SP-10 (32); PH-30 (33); SP-17 (34); FAA (35); testis specific antigen ($LDHC_4$,(36) PH-20 (37), sperm associated antigen-9 (SPAG-9, 38) have been reported as sperm specific antigenic proteins. These proteins have a role in different aspects of fertilization and their immunocontraceptive potential has been tested in rabbit, mouse, human, bovine, guinea pig etc. Active immunization of male/ female mice with FA-1, PH-20, SP-17, $LDHC_4$ has been shown to reduce fertility *in vivo.* Therefore, it can be concluded that the surface proteins of 55, 46, 42, 36, 28, 23 and 17 kDa are associated with the sperm maturation and may be associated with the fertility of Indian Mongrel. Therefore, their antigenicity and immunocontraceptive potential is further being worked out in our lab.

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