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Assessment of variations in Indian *Bubalus bubalis* seminal plasma proteins during winter and summer seasons

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Summary

The Indian riverine buffaloes are more susceptible to variations in the environment. These variations affect seminal plasma proteins, which in turn affect fertility. This study was conducted to evaluate variations in the Indian *Bubalus bubalis* seminal plasma protein profile using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) during winter and summer seasons, and its relation with sperm motility and viability. The nine ejaculates from three animals were from winter season while the other nine ejaculates from the same animals were from summer season. The semen samples collected from all bulls in both seasons have similar characteristics of mass activity and total concentration. However, sperm viability was significantly higher in winter season. The 2D-PAGE pattern displayed 42, 29 and 28 protein spots during winter season while 44, 29 and 29 spots during summer season for the first, second and third bull, respectively. Ultra high performance liquid chromatography-mass spectrometry (UPLC-MS) was performed to identify expressed protein spots in winter and summer seasons. During both seasons, four commonly expressed protein spots B6 (pI 8.5, Mr 122.54 kDa), B7 (pI 9.7, Mr 89.98 kDa), B9 (pI 9.3, Mr 19.72 kDa) and B10 (pI 9.7, Mr 16.80 kDa) were identified as glucose phosphate isomerase, epididymal secretory protein E1, peroxiredoxin 5 precursor and tubulin polymerization-promoting protein, respectively. These proteins are involved in either the mechanism of sperm maturation or structural formation. In addition, B37W (pI 6.0, Mr 32.88 kDa), B48W (pI 8.2, Mr 80.14 kDa), B59W (pI 5.6, Mr 90.37 kDa) and B61W (pI 5.8, Mr 15.34 kDa) were differentially expressed protein spots in winter season only. Spots B37W, B48W, B59W, B61W, B6, B7, B9 and B10 were identified as phosphoglycerate kinase LOC538592, androgen regulated protein, hypothetical protein LOC514663, sorbitol dehydrogenase, glucose phosphate isomerase, epididymal secretory protein E1, peroxiredoxin 5 precursor and tubulin polymerization-promoting protein family member. These proteins are known to have modulating properties on sperm motility and viability and also to provide high energy source to sperm. The better fertilizing ability of bull during winter season can be due to the differentially expressed proteins.

Key words: Indian *Bubalus bubalis*, Seminal plasma, Two-dimensional polyacrylamide gel electrophoresis, Silver staining

Introduction

Seminal plasma of animals acts as a carrier for spermatozoa from the male testes to their target, the oocyte (Thomas *et al*., 2003). The protein composition seminal plasma varies among species, and has an important role in the sperm function (Mortarino *et al*., 1998). Some seminal plasma proteins have an influence on sperm motility (Sanchez-Luengo *et al*., 2004), viability and fertilization (Brandon *et al*., 1999). The fertility of animals is affected through changes in semen quality after variations in the environment such as heat stress, high humidity and photoperiod (Trudeau and Sanford, 1986; Kunavongkrit *et al*., 2005). The differences in semen

characteristics and seminal plasma composition among seasons have been examined (Trudeau and Sanford, 1986; Alavi-Shoushtari and Babazadeh-Habashi, 2006; Murase *et al*., 2007). The seasonal influence on the total protein concentration in animal seminal plasma was significantly observed between the breeding and non-breeding seasons, in both the southern (Smith *et al*., 1999) and northern Hemispheres (Perez-Pe *et al*., 2001). The increased protein content of seminal plasma in buffalo bull during winter can be correlated with the survival of the spermatozoa (Singh *et al*., 1969). The low motility, poor survival ability and low fertilizing capacity of the buffalo bull (*Bubalus bubalis*) spermatozoa could be due to lower concentration of total protein, albumin and globulin in buffalo seminal plasma as compared to cattle seminal plasma (Kulkarni *et al*., 1996). Cancel *et al*. (1997) identified osteopontin (55 kDa) protein in seminal plasma of Holstein bull, which can be associated with fertility. Seminal plasma protein profiling studies using two-dimensional polyacrylamide gel electro-phoresis (2D-PAGE) were performed and correlated with fertility in different species like equine (Brandon *et al*., 1999, Novak *et al*., 2010), cattle (Mortarino *et al*., 1998; Gaviraghi *et al*., 2010), sheep (Cardozo *et al*., 2006) and human (Milardi *et al*., 2012).

In the present study, variations in the buffalo bull (*Bubalus bubalis*) seminal plasma protein profile were evaluated during winter and summer seasons. The objective of this study were

1) To resolve proteins found in buffalo bull (*Bubalus bubalis*) seminal plasma utilizing 2D-PAGE

2) To identify proteins using ultrahigh performance liquid chromatography mass spectrometry (UPLC-MS)

3) To identify differential expressed proteins during winter season and its relation with sperm motility and viability.

Materials and Methods

Semen collection and semen quality examination

Semen samples were collected twice a week from three buffalo bulls of Surti breed by artificial vagina method. A total of eighteen ejaculates (three bulls by three ejaculates during two seasons) were studied. The nine ejaculates were collected during winter season while the other nine ejaculates were collected during summer season.

To examine the semen quality, mass activity of semen was recorded under a low power magnification (×10) of a phase contrast microscope. Sperm concentration was determined using a haemocytometer. Percentage of live spermatozoa was estimated by differential staining technique using eosin-nigrosin stain.

Sample preparation

For protein extraction, semen samples were centrifuged at 10000 × g for 15 min at 4°C. Supernatant (seminal plasma) was collected, and protease inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany) was added at the rate of 40 µl in 1 ml seminal plasma. Protein concentration was checked by Mini Bradford method by ND-1000 (Nanodrop Inc., USA) and diluted with Tris-buffer (pH = 7.4) to make the concentration of 4000 ng/µl of seminal plasma protein. The sample was prepared by adding Amberlite 10 mg/ml, 4% CHAPS, 65 mM DTT, 0.8% Pharmalyte, mixture of protease inhibitor cocktail 15 µl and EDTA (All from Sigma-Aldrich, Steinheim, Germany) 15 µl per ml.

The processed seminal plasma was centrifuged at 10000 × g for 15 min at 4°C. Supernatant was collected and protein concentration was rechecked as described earlier. This seminal plasma was kept at -40°C, until rehydration procedure.

Iso-electric focusing in immobilized pH gradient and SDS-PAGE

Sample (60 µl) containing 240 µg protein was loaded in rehydration tray with IPG strip (11 cm, pH = 3 to 10, Sigma Aldrich Germany) in BIO-RAD protean IEF cell for rehydration process. After rehydration, iso-electric focusing procedure was started by activating BIO-RAD protean IEF cell with the following steps:

Step one: Linear gradient - 250 V - 20 min

Step two: Linear gradient - 8000 V - 2 h 30 min

Step three: Rapid gradient - 20000 Vh

After isoelectric focusing, IPG strip was equilibrated by shaking, with equilibration buffer I [2% DL' dithiothreitol (DTT), 20% glycerol, 36% urea, 2% SDS, 1.5 mM Tris-HCl pH = 8.8 (all from Sigma-Aldrich, Germany)] and equilibration buffer II [2.5% iodoacetamide, 20% glycerol, 36% urea, 2% SDS, 1.5 mM Tris-HCl pH = 8.8 (all from Sigma-Aldrich, Germany)] for twenty minutes each.

The IPG strips were then subjected to SDS-PAGE electrophoresis (5% stacking gel and 12% resolving gel) on protean II Xi cell (BIO-RAD, size 16 × 20 cm with cooling unit at 9°C]. A protein molecular marker (Genei, Banglore, India) of medium range (14.4 to 97.4 kDa) was loaded along with loading dye. The electrophoresis was performed at 200 V, 50 mA, 5 W for 9 to 10 h. The second dimension gels were stained with silver nitrate for visualization of proteins. After staining procedure, images of the gels were scanned. The gels were preserved by air drying between two layers of cellophane.

Protein spot analysis by UPLC-MS

Analysis of protein spots of interest was done by UPLC-MS at Mass Spectrometry and Protein Research National Research Council, Plant Biotechnology Institute (NRC-PBI), Canada. Protein spots were manually excised from cellophane-coated silver-stained gels using a scalpel blade and hydrated in one or two drops of de-ionized water. After removing the cellophane pieces by hand, the gel plugs were placed in a 96-well microtitre plate (Sigma, Milwaukee WI, USA). Proteins were de-stained, reduced with DTT, alkylated with iodoacetamide, and digested with porcine trypsin (sequencing grade, Promega, Madison, WI, USA) using a MassPREP protein digest station (Micromass, Manchester, UK). The digest was evaporated to dryness, then dissolved in 12 µl of 0.1% aqueous TFA, of which 6 µL were analysed by UPLC-MS/MS using a nanoACQUITY ultra-high performance liquid chromatography system (Waters, Milford, MA, USA) interfaced to a Q-Tof Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Micromass, Manchester, UK).

The peptide digest sample was loaded onto a C18 trapping column (Symmetry, 180 µm × 20 mm, 5-µm particle size; Waters) and washed for 3 min using 99% solvent A (0.1% formic acid in water) and 1% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 15 µL/min. The flow path was then switched, and the sample eluted onto a C18 analytical column (BEH 130, 100 µm × 100 mm, 1.7-µm particle size; LC Packings) at 0.4 µL/min. Separations were performed using a gradient program in which the solvent composition was changed from 99:1 to 90:10% A:B between 1 and 4 min, held at 90:10% A:B between 4 and 15 min, and changed from 90:10 to 55:45% A:B between 15 and 45 min. The composition was again changed to 20:80% A:B at 46 min and held until 52 min to flush the column, before reverting to 99:1% A:B at 53 min and holding until 60 min to re-equilibrate. Mass calibration of the Q-Tof the instrument was performed using a product ion spectrum of Glu-fibrinopeptide B acquired over the *m/z* range 50 to 1900.

LC-MS/MS analysis was carried out using data dependent acquisition, during which peptide precursor ions were detected by scanning from *m/z* 400 to 1900 in TOF MS mode. Multiple charged (2+, 3+, or 4+) ions rising above the predetermined threshold intensity were automatically selected for TOF MS/MS analysis, and product ion spectra acquired over the *m/z* range 50 to 900. LC-MS/MS data were processed using ProteinLynx v2.15 software (Micromass) and searched against the NCBI(nr) database using MASCOT (Matrix Science Inc., Boston, MA).

Statistical analysis

The analyses were performed with SPSS 16.0 (SPSS Inc., USA). The Mann-Whitney U-test was used to analyse the significant differences in the mass activity, total sperm concentration and live sperm percentages of semen samples between winter and summer seasons.

Results

Microscopic examinations of semen samples

The mean values of mass activity and

total sperm concentration of fresh Indian *Bubalus bubalis* bull semen were within range of normozoospermia in winter and summer seasons. There is no significant difference in mass activity and total sperm concentration in the semen from winter and summer seasons. In addition, the mean live sperm counts were 96.4 ± 0.5 and 93.4 ± 1.4 in the winter season and summer season, respectively. The viable sperm count was significantly high in winter season as compared to that of the summer season (Table 1).

Table 1: Semen characteristics during winter and summer seasons (Mean ± SE)

Parameters	Winter	Summer
Mass activity	2.6±0.4	2.8±0.7
Total concentration	539.2±164.5	700.0±198.2
Live sperms	96.4±0.5*	93.4±1.4

*Significantly higher number of live sperm in the winter season as compared to the summer season ($P<0.05$)

2D-PAGE analysis of seminal plasma

2D-PAGE images showed 42, 29 and 28 protein spots during winter season and 44, 29 and 29 spots during summer season for the first, second and third bull, respectively. Figures 1 and 2 showed the 2D-PAGE pattern (representative sample) of the second Surti buffalo bull (*Bubalus bubalis*) seminal plasma obtained during winter and summer seasons. The protein molecular weight (Mr) and isoelectric point (pI) of these spots were ascertained using molecular weight marker (97.40, 66.20, 45.00, 31.00, 21.50 and 14.40 kDa) and pH range of IPG strip (3 to 10). Identification of spots of interest was carried out by UPLC-MS analysis. The 2D-PAGE patterns displayed 29 protein spots during winter (Fig. 1) and summer seasons (Fig. 2), each.

In the first bull seminal plasma, 29 common protein spots were observed in the winter and summer seasons. The numbers of differentially expressed proteins were 13 and 15 in the winter and summer seasons, respectively. Furthermore, there were 15 common protein spots found during two seasons in the second bull seminal plasma and 14 differentially expressed protein spots were found in each winter and summer season. The common protein spots and differential protein spots in both seasons for the second bull were shown in the Figs. 1 and 2 and listed in the Tables 2 and 3, respectively. In addition, 20 common protein spots were observed in both seasons, while the numbers of differentially expressed protein spots were 8 and 9 in winter and summer seasons, respectively.

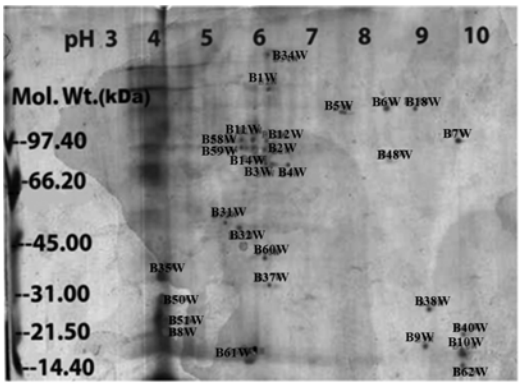

Fig. 1: Different proteins of seminal plasma during winter (W) were separated by 2D gel electrophoresis. At the upper side, pH of IEF gel is denoted and molecular weight for marker are depicted at the left

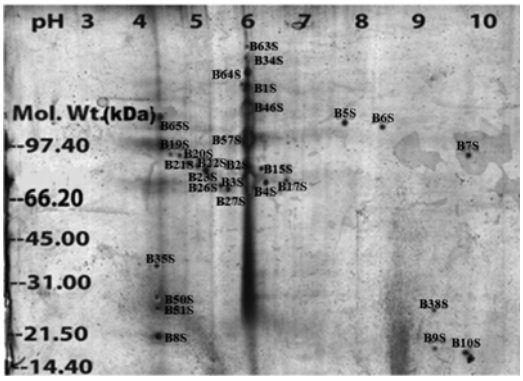

Fig. 2: Different proteins of seminal plasma during summer (S) were separated by 2D gel electrophoresis. At the upper side, pH of IEF gel is denoted and molecular weight for marker are depicted at the left

In Figs. 1 and 2, all the protein spots were expressed in the range of pI 4 to 10, while the range of molecular weight is approximately 14 kDa to 120 kDa. The majority of the differential expressed protein spots were in the range of 14 kDa to 63 kDa in winter season, while those in the range of 66 kDa to 120 kDa were in summer season.

Table 2: Common protein spots observed during two seasons

Serial number	Spot number	PI	kDa
1	B1	6.0	147.07
2	B2	6.0	90.39
3	B3	6.0	83.29
4	B4	6.3	79.20
5	B5	7.8	120.62
6	B6*	8.5	122.54
7	B7*	9.7	89.98
8	B8	4.2	21.85
9	B9*	9.3	19.72
10	B10*	9.7	16.80
11	B34	6.0	186.78
12	B35	4.2	35.89
13	B38	9.3	26.71
14	B50	4.2	29.90
15	B51	4.2	24.85

*Protein spots were sent for UPLC-MS at NRC, Canada

Table 3: Differential protein spots observed during two seasons

Serial number	Spot number	PI	kDa
1	B11W	5.8	91.13
2	B12W	6.0	91.46
3	B14W	5.8	89.37
4	B18W	8.9	122.87
5	B31W	5.4	56.41
6	B32W	5.6	52.11
7	B37W*	6.0	32.88
8	B40W	9.7	21.80
9	B48W*	8.2	80.14
10	B58W	5.6	91.38
11	B59W*	5.6	90.37
12	B60W	6.0	40.69
13	B61W*	5.8	15.34
14	B62W	9.7	13.08
15	B15S	6.3	89.88
16	B17S	6.6	79.90
17	B19S	5.0	93.01
18	B20S	5.1	91.69
19	B21S	5.2	91.04
20	B22S	5.4	90.20
21	B23S	5.5	86.42
22	B26S	5.6	77.25
23	B27S	5.7	73.40
24	B46S	6.0	127.56
25	B57S	6.0	116.47
26	B63S	6.0	203.54
27	B64S	6.0	163.69
28	B65S	4.2	124.05

*Protein spots were sent for UPLC-MS at NRC, Canada

UPLC-MS analysis of selected protein spots

After UPLC-MS analysis, the differentially expressed protein spots B37W, B48W, B59W and B61W of winter season were identified as phosphoglycerate kinase LOC538592, androgen regulated protein arMEP24, hypothetical protein LOC514663 (ribonulease inhibitor-like family) and sorbitol dehydrogenase, respectively. In addition, four commonly expressed protein spots B6, B7, B9 and B10 from winter and summer seasons were glucose phosphate isomerase, epididymal secretory protein E1, peroxiredoxin 5 precursor and tubulin polymerization-promoting protein family member, respectively (Fig. 1 and Table 4).

Discussion

To correlate fertility and seminal plasma proteins, we have carried out 2D-PAGE and UPLC-MS analysis for proteomics study of seminal plasma proteins. One factor affecting fertility is seasonal changes in semen quality (Trudeau and Sanford, 1986) induced by heat stress, high humidity and photoperiod (Kunavongkrit *et al*., 2005). The sperm viability is reduced in summer season (Cardozo *et al*., 2006). The differences in the composition of the semen may be significant in relation to the differences in the viability of their spermatozoa (Singh *et al*., 1969). In this study, sperm viability is significantly lower in summer season than in winter season. This finding suggests that composition in the seminal plasma would play an important role in sperm membrane stability and subsequent sperm viability.

2D-PAGE images showed 42, 29 and 28 protein spots during winter season, and 44, 29 and 29 spots during summer season for the first, second and third bull, respectively. The increase or decrease of the different proteins detected in this study is due to over or under expression or may be due to altered import or export in the spermatozoa from the epididymal, seminal or prostatic fluid (Martínez-Heredia *et al*., 2008). In the Figs. 1 and 2, the second bull showed 29 protein spots in each season. The majority of the differential expressed protein spots were in the range of 14 kDa to 63 kDa in winter season. The reason behind the expression of these proteins in this range of molecular weight is unknown. In contrast, the majority

Table 4: The results of selected protein spots by UPLC-MS analysis

Spot	Season	Accession number	PI	kDa	Identified protein name
B37W	Winter	gi|81674444	6.0	32.88	LOC538592 protein (phosphoglycerate kinase)
B48W	Winter	gi|50024	8.2	80.14	Androgen regulated protein arMEP24
B59W	Winter	gi|114051379	5.6	90.37	Hypothetical protein LOC514663 (ribonulease inhibitor-like family)
B61W	Winter	gi|82617550	5.8	15.34	Sorbitol dehydrogenase
B6	Winter and Summer	gi|94966765	8.5	122.54	Glucose phosphate isomerase
B7	Winter and Summer	gi|27806881	9.7	89.98	Epididymal secretory protein E1
B9	Winter and Summer	gi|27807445	9.3	19.72	Peroxiredoxin 5 precursor
B10	Winter and Summer	gi|78042470	9.7	16.80	Tubulin polymerization-promoting protein family member

of differential expressed protein spots were in the range of 66 kDa to 120 kDa in summer season. The majority of these proteins may be associated with the protection of sperm from heat stress. The winter specific protein spots were selected for UPLC-MS analysis. The semen collected in summer may not be highly fertile. The poor semen quality is associated with the summer infertility. Heat stress causes an increase in abonormal spermatozoa and damaged acrosomes (Murase *et al*., 2007).

Spot B37W was identified as phosphoglycerate kinase LOC538592. This isozyme is highly active in testes, epididymides and semen, which contain spermatozoa (VandeBerg *et al*., 1976). Spot B48W was identified as androgen regulated protein arMEP24. But, there is no relevant reference to this in *Bos taurus* or *Bubalus bubalis* species. This protein belongs to the glutathione peroxidase family (Ghyselinck *et al*., 1991) and protects cells and enzymes from oxidative damage by catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide by glutathione (Miyamoto *et al*., 2003). It may constitute a glutathionine peroxidase-like protective system against peroxide damage in sperm membrane lipids. Mouse spermatozoa and epididymal fluid has glutathionine peroxidase activity, which is the major protective system against oxidative damage (Alvarez and Storey, 1984). This can maintain membrane flexibility and prevent the premature acrosome reaction that would otherwise occur (Ghyselinck *et al*., 1991).

Spot B59W was identified as hypothetical protein LOC514663. This protein is from ribonuclease inhibitor (RI)-like family proteins. Residue sequence motifs of this family participate in protein-protein interactions. Spot B61W was identified as sorbitol dehydrogenase. Seminal plasma contains sorbitol (Kobayashi *et al*., 2002), it is likely that sorbitol dehydrogenase was involved in the polyol pathway, in which sorbitol undergoes oxidation reaction to form fructose for use as an energy source (Kobayashi *et al*., 2002). This enzyme is present in the bovine epididymis, where it is associated with membranous vesicles called epididymo-somes. Based on the distribution of these enzymes, it has been hypothesized that the polyol pathway of semen can modulate sperm motility during the epididymal transit (Frenette *et al*., 2006). These enzymes can play an important role in the motility and viability of the spermatozoa.

Spot B6 was identified as glucose phosphate isomerase. There is evidence that glucose phosphate isomerase acts as a molecular messenger and is essential for glycolysis. Glucose phosphate isomerase (GPI) variants are demonstrated in human semen (Oya *et al*., 1988). Spot B7 was identified as epididymal secretory protein E1. The role of secretory epididymal factors on sperm survival and storage in bovine cauda epididymides is poorly understood. It may get involved in the regulation of the lipid composition of sperm membranes during the maturation in the epididymis.

Spot B9 was identified as peroxiredoxin 5 precursor. This protein is also called thioredoxin reductase. Thioredoxin re-ductase is expressed at low levels in various tissues, which accumulates in testes after puberty (Su *et al*., 2005). The protein is particularly abundant in elongating spermatids at the site of mitochondrial sheath formation but absent in mature sperm (Su *et al*., 2005). Together, thioredoxin reductase and glutathionine peroxidase can serve as a novel disulfide bond formation system. Thioredoxin reductase targets include proteins that form structural components of the sperm, including

glutathione peroxidase (Su *et al*., 2005). Spot B10 was identified as tubulin polymerization-promoting protein. As its name suggests, this protein promotes polymerization of tubulin, a subunit protein of microtubules.

All these protein spots have specific functions and are highly related to semen quality. More specifically, sorbitol dehydrogenase, phosphoglycerate kinase LOC538592, epididymal secretory protein E1, androgen regulated protein arMEP24, peroxiredoxin 5 precursor and glucose phosphate isomerase have a typical role related to strengthening of spermatozoa and motility of spermatozoa. According to function of these four differentially expressed protein spots, seminal plasma of winter season may have higher capacity of providing energy source, modulating property of sperm motility and capacity of sperm protection. Our results indicate that proteomics may be useful to study the role of seminal plasma protein in the sperm function. The physiological importance of these findings calls for more studies to identify, characterize and elucidate seminal plasma protein roles in sperm function, which can conclude the fertilizing ability of semen in winter and summer seasons.

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