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Characterization of buffalo bull frozen-thawed sperm proteins through SDS-PAGE and their correlation with HOST and *in vitro* acrosome reaction

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ABSTRACT

Intrinsic factors such as proteins modulate the fertilizing ability of male gametes. The present study was undertaken to separate and compare the protein of sperm SDS-extracts from 30 bulls by SDS-PAGE and correlate quantity of various proteins with hypo-osmotic swelling test (HOST) and acrosome reaction. There were 26 major polypeptides, ranging from 12–23 proteins in each semen sample. These bands were separated in the range of 18–345 kDa in frozen-thawed semen by SDS-PAGE on 10% acrylamide gel with bands 135, 110, 48, 41, 37, 31, 28, 26, 24, 20 and 18 kDa present in all the tested frozen semen. There were positive correlations between 5 protein bands 135, 70, 55, 26 and 18 kDa and HOS positive (r^2 = 0.28, r^2 = 0.19, r^2 = 0.37, r^2 = 0.24and r^2 = 0.31, respectively) and acrosome reacted spermatozoa (r^2 = 0.29, r^2 = 0.38, r^2 = 0.42, r^2 = 0.32 and r^2 = 0.14, respectively). Both qualitative (presence or absence of bands) and quantitative differences (concentration of protein in each amplified band) were observed in fertility associated protein bands. The concentration of fertility associated proteins was relatively higher in sperm extracts of buffalo bulls having ≥ 60% HOS positive and ≥ 50% acrosome reacted spermatozoa than in their counterparts.The concentration of proteins 55 kDa (30.7 ± 6.0 µg/band) and 26 kDa (22.1 ± 2.5 µg/band) was highest of all the protein bands identified through SDS-PAGE using 10% separating gel and 4% stacking gel and were linked to the osteopontin and lipocalin-type prostaglandin D synthetase subunits, respectively, of the bovine semen. HOST and *in vitro* acrosome reaction were indicators of fertility, and therefore, proteins of 55, 31, 26, 24, 20 and 18 kDa may be candidates for protein fertility markers in buffalo bull spermatozoa. In conclusion, 26 proteins were identified in frozen sperm extracts and significantly correlated with HOST and acrosome reaction.

Key words: Acrosome reaction, Buffalo, Frozen semen, HOST, SDS-PAGE

Bull fertility is very complex and exhibits considerable variation in actual semen fertilization capacity (Rodriguez-Martinez 2003). Till date, there is no single objective test to evaluate the bull fertility. Identification of molecular markers of fertility represents a remarkable way for achieving genetic gain in dairy farming. Many sperm components such as lipids, proteins, ions and nucleic acids vary in quantity and/or quality with respect to the male fertility status (Brinsko *et al.* 2007, Lalancette *et al.* 2008). The identification of additional semen quality attributes associated with fertility may provide more accurate methods to predict bull fertility (DeJarnette *et al.* 2004). The bovine sperm proteome is associated with hundreds of different proteins. Functions of some seminal proteins found in spermatozoa, viz. osteopontin (55 kDa), fertility associated antigens (FAA), ovum-binding proteins (26 kDa), heparin binding protein (31kDa) and phospholipase A_2 (bovine 18 and 16 kDa), were found associated with bull fertility (Manjunath *et al.* 2002). Absence and/or presence of specific proteins could alter sperm functions, affecting the semen fertility. Functional integrity of sperm plasma membrane integrity is important in the fertilization process and hypo-osmotic swelling test (HOST) was successfully used for its evaluation. Furthermore, acrosome reaction also determines the fertilization potential of the spermatozoa (Gamboa and Ramalho-Santos 2005). Both HOST and acrosome reaction are triggered by many effectors and involve different signal transduction pathways (Check *et al.* 2001). However, seminal plasma proteins in buffaloes had not been well characterized. Earlier Kumar (2005) identified only 6, Arangasamy (2003) 8 and Singh *et al.* (2013) 9 protein bands. Keeping in view the above facts and also taken into consideration the deficit knowledge of seminal plasma proteins in buffaloes, the present study was undertaken to separate the buffalo bull frozen sperm proteins with SDS-PAGE and to determine correlation between individual proteins with HOST and acrosome reaction.

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MATERIALS AND METHODS

Procurement of frozen semen and preparation of sperm extracts: Frozen semen straws from 30 breeding Murrah buffalo bulls were procured from 2 government semen processing and freezing laboratories. The frozen-thawed semen was centrifuged at 3,000 rpm for 5 min to separate out fluid and spermatozoa. The sperm pellet was washed thrice with PBS, pH 7.4 to get rid of the dilutor. Sperm extracts (SE) were prepared by suspending 1×10^9 spermatozoa in 2.0 ml of 62.5 mM Tris-HCl (pH 6.8, 2% SDS, 1mM PMSF, 25 mM benzidine), ultrasonicated (3 bursts of 20 sec each) and centrifuged at 15,000 rpm for 30 min. The aliquots of SE were stored at –20°C till further use.

Molecular weight determination by SDS-PAGE: Exactly 150 µg of protein was fractionated by SDS-PAGE using 10% separating gel and 4% stacking gel (Laemmli 1970). The gels were run at a constant current of 30 mA and maximum 200 V for 2 h and were stained with commassie brilliant blue. Gel images were captured on Syngene gel doc using Gene Snap image acquisition software and were analyzed for molecular weight and quantity by using Gene Tools gel analysis software (Syngene).

Semen parameters evaluated: Frozen-thawed semen was evaluated for hypo-osmotic swelling test (HOST) and acrosome reaction. Functional integrity of the sperm was evaluated by HOST using hypo-osmotic solution (100 mosm/L). Frozen-thawed semen (20 µl) was mixed with 100 µl of HOS solution and incubated at 37°C for 30 min. A drop of semen on a slide covered with cover slip was observed at 400× under light microscope. A total of 200 sperms each were counted under different fields and percentage of spermatozoa positive to HOS test (having coiled tails) was calculated. Ten straws of each bull were used to determine *in vitro* acrosome reaction. The frozen-thawed semen was mixed with double the volume of TALP (100 mM NaCl, 31 mM KCl, 25 mM $NaHCO_3$, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$ $4H_2O$, 10 mM HEPES, 1 mM Na pyruvate, 0.6 % BSA, 5 mM glucose and 10 µg/ml heparin) and centrifuged at 1000 rpm for 5 min to allow removal of seminal plasma and extender. Sperm pellet was washed twice with TALP and finally suspended in 2 ml TALP (100×10^6sperms/ml) and incubated at 37°C for 6 h. The smears were prepared every 2 h until 6 h and stained with Giemsa. About 200 sperms were evaluated under the light microscope at 400 × for various stages of acrosome reaction, viz. swelling of acrosome, vesiculation and acrosome shedding.

Based on HOST and acrosome reaction, the percentage of tested frozen-thawed semen samples with ≥ 60% HOS positive and ≥ 50% acrosome reacted spermatozoa and those with < 60% HOS positive < 50% acrosome reacted spermatozoa were considered as high fertility and low fertility semen samples, respectively, for further comparisons.

Statistical analysis: The significance of differences among the variables was tested through one-way analysis of variance using student's *t*-test. The significant interactions were determined by Duncan's multiple range test. The Karl Pearson's correlation between semen characteristics and each protein band, as well as total sperm membrane protein concentrations were determined through Statistical Package for Social Sciences (SPSS 16.0, Chicago, IL; 2003) packet program. Correlations with $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Electrophoretic characterization of proteins in sperm extracts by SDS-PAGE: Gel images of protein bands in frozen semen extracts of all 30 bulls are shown in Fig. 1. The electrophoretic profiles showed polymorphism among individual semen samples. Ranging from 12–23 proteins in each post-thaw semen sample a total of 26 major polypeptides were identified. However, no individual tested bull frozen semen had all 26 bands. These bands arranged themselves from the starting line solely in the direction of the negative pole. The bands were separated in the range of 18–345 kDa in all the frozen thawed sperm extracts by SDS-PAGE on 10% acrylamide gel. Chacur *et al.* (2010) found 28 major polypeptides ranging from 15–24 bands in individual Brown Swiss bull. The proteins of 135, 110, 48, 41, 37, 31, 28, 26, 24, 20 and 18 kDa were detected in the sperm extracts of all the bulls, however, the proteins with molecular weight of 345, 75, 60, 55 kDa; 80, 70 kDa; 65 kDa; 345, 45; 230; 50; 33; 180; 100 and 245 kDa were separated in the sperm extracts from bulls 28; 27; 20; 16; 14; 8; 5; 4; 2 and 1, respectively (Fig. 1). Therefore, qualitative differences (presence or absence of bands) were observed in 18 protein bands of the 30 bull sperm extracts. Quantitative differences were also observed in 25 protein bands among the tested frozen-thawed semen. The inherent character of the proteins may also contribute towards the difference in number of bands. The results were in agreement with findings of Marques *et al.* (2000) who observed both quantitative and qualitative differences in proteins with low molecular mass (31, 29, 25, 21, 20, 18 and 16 kDa) in bovine spermatozoa.

Per cent HOST and acrosome reaction: The proportion of HOS and acrosome reactive sperm indicated its membrane intactness and capacitation ability, respectively. The per cent HOST varied from 53.8 to 80.3% and the per cent acrosome reaction was in the range of 32.7 to 70.6% in all the frozen-thawed semen samples. About, 83.3 and 66.7% of the tested semen exhibited ≥ 60% and ≥ 50% HOS positive and acrosome reacted spermatozoa, respectively. A higher proportion of HOS and acrosome reactive sperm in the current study indicated that the sperm plasma membranes were functionally intact to undergo capacitation, thereby reflecting high fertility. Chacur *et al.* (2010) reported that higher fertility rates can be predicted in the bulls possessing high percentage of HOS-positive and acrosome reacted spermatozoa (≥ 50%). Although low, a significant ($P < 0.05$) positive correlation ($r^2 = 0.27$) existed between HOS-positive and acrosome-reacted

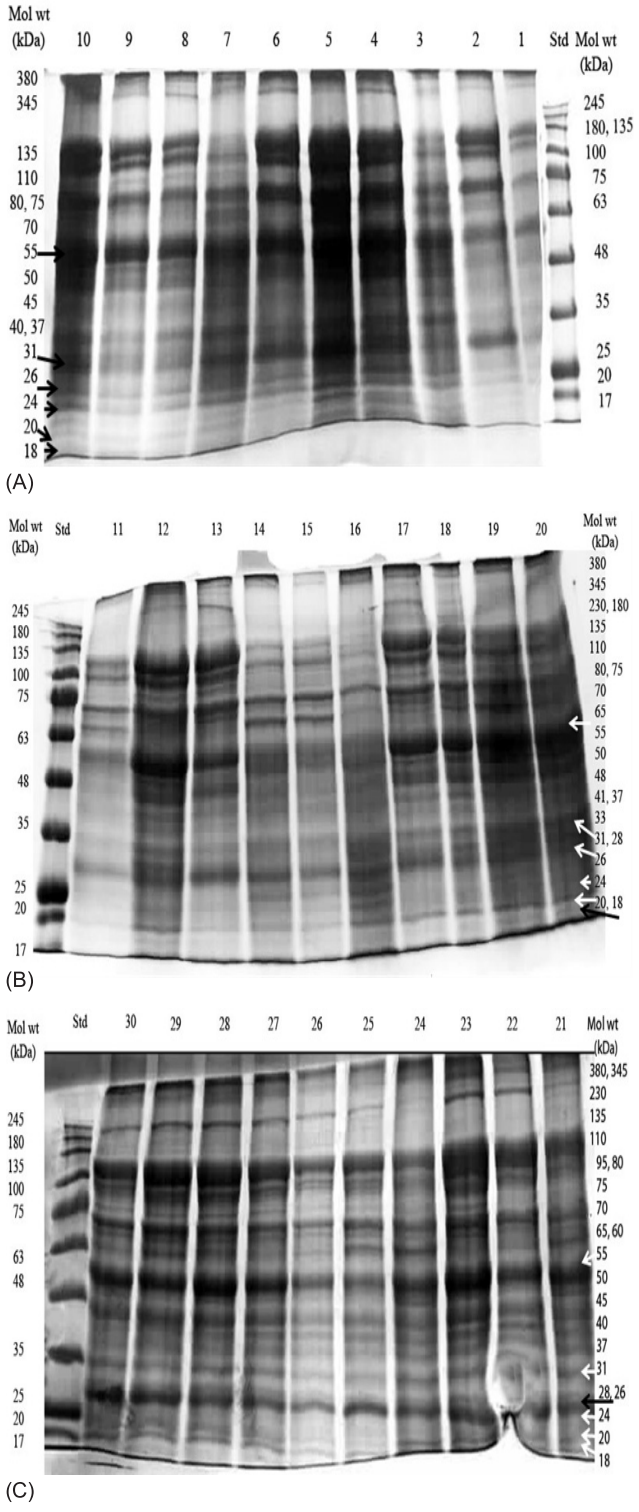

Fig. 1. Separation of SDS-extracted proteins of frozen-thawed buffalo bull spermatozoa by SDS-PAGE on 10% acrylamide gel, stained with commassie brilliant blue. Std, pre stained marker, 1–30 bull numbers (plates A, B and C).

spermatozoa indicating a prognostic value in screening out sub-fertile males with apparently normal spermiogram. A similar correlation (r^2 = 0.29) between the percentage of HOST-positive and acrosome-reacted spermatozoa had been reported both in buffalo and cow bull semen (Lodhi *et al.* 2008). Currently, the data were in agreement with the observations of Peixoto *et al.* (2012) who reported that HOST and acrosome reaction may be valuable indicators of semen quality in bulls. Therefore, some merit still lies in per cent HOST along with acrosome reaction.

Table 1. Correlation between concentration of protein bands (18–135 kDa) and percent HOS-positive and acrosome-reacted spermatozoa

Molecular weight (kDa)	Protein quantity (µg/band, mean ± SEM)	HOS × protein quantity (r^2)	AR × protein quantity(r^2)
135	20.3±0.3^{bc}	0.28	0.29
110	11.0±2.1^{a}	0.17	–0.27
80	7.0±1.0^{a}	–0.21	–0.43
75	15.8±2.7^{bc}	0.08	–0.36
70	11.2±3.0^{a}	0.19	0.38
65	10.7±2.1^{a}	0.02	–0.49
60	15.5±2.9^{bc}	–0.07	0.22
55	30.7±6.0^{d}	0.37	0.42
48	12.8±7.3^{b}	–0.01	–0.32
45	9.3±2.0^{a}	0.07	–0.08
41	7.8±1.0^{a}	–0.18	–0.46
37	9.9±1.8^{a}	0.25	–0.18
31	18.9±3.8^{bc}	0.27	–0.23
28	1.7±1.1^{a}	–0.24	–0.47
26	22.1±2.5^{bd}	0.24	0.32
24	3.8±0.7^{a}	–0.02	–0.53
20	3.9±0.8^{a}	0.16	–0.47
18	2.8±0.3^{a}	0.31	0.14

Values with different superscripts within the column differ significantly (P < 0.05).

Correlation of sperm protein quantity with HOST and acrosome reaction: The concentration of 18 protein bands (18–135 kDa) was found correlated with per cent HOST and per cent acrosome reaction (Table 1). A significant (P < 0.05) difference was observed in the protein concentration of different bands. Of all the bands, 2 proteins with molecular weight 55 and 26 kDa had significantly (P < 0.05) maximum concentration. Correlations (P < 0.05) between the quantity of 12 proteins and per cent HOS positive spermatozoa in general were low. The correlation was (P < 0.05) moderately positive with 5 proteins of 135, 37, 31, 26 and 18 kDa (Table 1). Marques *et al.* (2000) established a high correlation (r^2= 0.71) between low molecular weight protein (31 and 18 kDa) and per cent HOST and presented them as candidate protein markers for fertility. Likewise, a (P < 0.05) moderate and positive correlation existed between per cent acrosome reaction and quantity of sperm membrane proteins with molecular weight 135, 70, 60 55 and 26 kDa (Table 1). A high and positive correlation of acrosome reaction with protein content (70, 65 and 55 kDa) of capacitated spermatozoa (r^2=0.91) was demonstrated by Dhanju *et al.* (2006) in crossbred cattle bull. They further presented a negative correlation coefficient (r = -0.61) for the spermatozoa membrane protein of 37 kDa and acrosome

Table 2. Concentration (µg/protein band) of fertility associated proteins in sperm extracts

Molecular weight of protein band (kDa)	Protein band in frozen-thawed semen with ≥ 60% HOST/ ≥ 50% acrosome reaction	Protein band in frozen-thawed semen with < 60% HOST / < 50% acrosome reaction	Percentage of tested bulls with ≥ 60% HOS-positive and / ≥ 50% acrosome-reacted spermatozoa and higher protein concentration	Percentage of tested bulls with < 60% HOS-positive and / < 50% acrosome-reacted spermatozoa and lower protein concentration
55	71.4 ± 5.0[a]	20.1 ± 4.8[b]	16.7 (5)	16.7 (5)
31	27.4 ± 2.1[a]	8.0 ± 3.6[b]	16.7 (5)	13.3 (4)
26	37.6 ± 4.9[a]	7.4 ± 3.4[b]	16.7 (5)	13.3 (4)
24	7.9 ± 1.2[a]	2.3 ± 0.8[b]	23.3 (7)	20.0 (6)
20	9.4 ± 1.4[a]	1.7 ± 0.6[b]	26.7 (8)	13.3 (4)
18	8.5 ± 4.6[a]	3.2 ± 0.3[b]	23.3 (7)	20.0 (6)

Values with different superscripts within the row and column differ significantly (P < 0.05). Figures in parentheses represent the number of tested bulls.

reaction. Eventually, a negative correlation between the 37 kDa protein and acrosome reaction was observed in the present study. Certain molecules coating the surface of ejaculated sperm were removed or altered during capacitation called decapacitating factors. Amours *et al.* (2010) reported that 37 kDa protein leaked out as a modified protein during capacitation and/or acrosome reaction of spermatozoa of bulls. Few proteins (135, 70, 55,26 and 18 kDa) presented a significantly (P < 0.05) moderate and positive correlation, both with per cent HOST as well as acrosome reacted spermatozoa in the current set of experiments. Rueda *et al.* (2013) reported the critical role of 18 and 135 kDa proteins in osmotic fragility and acrosome membrane fusion events.

Detection of fertility associated protein differences vis-à-vis HOST and acrosome reaction: Of the 26 identified proteins; 6 proteins (55, 31, 26, 24, 20 and 18 kDa) had been recognized as fertility associated antigens in the present study (Table 2). This was in agreement to earlier studies (Killian *et al.* 1993, McCauley *et al.* 2001) who named proteins with molecular weight of 185–5 kDa as fertility associated antigen5-complex (FAA-5-complex). A significantly (P < 0.05) high concentration of fertility associated proteins were obtained in the frozen semen with high percentage of HOS-positive and acrosome-reacted spermatozoa as compared to their counterparts. The bands 55 and 26 kDa had the highest concentrations of all the fertility associated antigens identified through SDS-PAGE using 10% separating gel and 4% stacking gel and, further, were significantly (P < 0.05) high in tested bulls with ≥ 60% HOS-positive and ≥ 50% acrosome-reacted spermatozoa than those with < 60% HOS-positive and < 50% acrosome-reacted spermatozoa (Table 2). Perhaps these bands were linked to the osteopontin and lipocalin-type prostaglandin D synthetase subunits, respectively, of the bovine semen, associated with fertility; however, that requires confirmation with other methods. These proteins were found involved in osmotic fragility and capacitation, and lower levels could jeopardize the sperm ability to reach and fertilize the oocyte. High concentrations of 55 and 26 kDa proteins were present in high fertility bulls (Cancel *et al.* 1997, Gerena *et al.* 1998). In the present study, both 55 and 26 kDa may have considerable homology to a family of heparin binding proteins from bovine bull sperm (Killian *et al.* 1993). Among the tested buffalo bull frozen-thawed semen, 5–8 bulls (16.7–26.7%) displayed high concentration of fertility associated proteins in sperm extracts. Majority of the fertility associated proteins (5 out of 6) were positively correlated, both with HOST and/or acrosome reaction.The presence of fertility associated antigen was shown to be an indicator of superior fertility of bulls based on artificial insemination field fertility trials (Sprott *et al.* 2006). However, more sophisticated studies that allow higher-resolution separation of seminal proteins and more detailed characterization of those proteins, as well as investigation of their physiological role, will further advance knowledge in this area.

Therefore, since HOST and acrosome reaction were correlated to *in vivo* fertility in buffaloes,the sperm membrane proteins identified in the present study had considerable merit in maintaining the membrane integrity and induction of acrosome reaction and may be valuable markers to predict fertility of buffalo bulls. Studies in buffalo bulls are important, both as a model for assisted reproductive technologies as well as for advances in veterinary research.

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