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Characterization of fertility-associated antigens in seminal fluid and their relationship with vital sperm function tests vis-à-vis fertility of breeding buffalo bulls

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ABSTRACT

The present study was undertaken to separate the FAA of seminal plasma and frozen-thawed sperm extracts from 30 buffalo bulls by immunoblotting and determine their relationship with post-thaw sperm function tests vis-à-vis bull fertility. Eight immunoreactive bands in seminal plasma (60, 55, 45, 33, 31, 18, 16 and 14 kDa) and four in frozen-thawed sperm extracts (65, 55, 48 and 18 kDa) were detected in Western blots. The frozen-thawed semen was evaluated for first service conception rate (FSCR), per cent acrosome reaction, HOST, viability, DNA integrity and total motility and linked to FAA. In seminal plasma, the bulls positive for 60, 31 and 14 kDa FAA had significantly higher FSCR (37.0±3.2 vs 0.0±0.0%, 46.7±3.2 vs 22.5±3.3% and 48.6±3.8 vs 26.0±3.0%), respectively, as compared to their negative counterparts. The FSCR was also higher in detectable FAA-33 than in undetectable FAA-33. Almost all seminal parameters were found to be significantly higher in bulls positive for FAA of 60, 33, 31 and 14 kDa than in their negative contemporary mates. In frozen-thawed sperm extracts, the bulls positive for FAA-65, 48 and 18 had significantly higher FSCR, per cent acrosome reaction and total motility in comparison to their negative herd mates. In conclusion, we have identified buffalo bull seminal FAA that bind to spermatozoa; influence semen quality and subsequent fertility of buffalo bulls.

Key words: Buffalo bull, FAA, FSCR, Semen

Fertility-associated antigens (FAA) and their homologs are considered as prominent proteins of seminal fluid which are secreted from accessory sex glands and form the bulk of heparin binding protein group (Kumar *et al.* 2012). Binding of FAA to sperm membrane increased the number of heparin binding sites on the sperm surface and conveyed the capacitating effects of heparin *in vitro* or other heparin-like glycosaminoglycans *in vivo*, thereby influencing sperm fertilizing ability (Divyaswetha *et al.* 2008). Five proteins with molecular weight of 18, 31, 33, 48 and 55 kDa have been identified as members of FAA family and are referred to as fertility-associated antigen-5-complex, with 31 and 55 kDa proteins predominant in complexes with the greatest affinity for fertility (McCauley *et al.* 1999). The FAA have predominately been linked to bull fertility potential. When the semen of FAA positive and negative bulls was inseminated artificially, the FAA positive bulls were 16% more fertile than FAA-negative ones (Dalton *et al.* 2012). Further, immuno-localization studies have revealed FAA labelling over acrosome and posterior head region of bovine spermatozoa and established their relationship to cellular changes during capacitation and the acrosome reaction induced *in vitro* (McCauley *et al.* 1999). Characterizing functionally important FAA is a first step toward better understanding the modulating effects of seminal fluid on fertility of buffalo bulls. In addition, the fertilization potential of the spermatozoa is affected by important semen characteristics viz. acrosome reaction, plasma membrane integrity, viability, DNA integrity and motility (Agarwal and Said 2003, Gamboa and Ramalho-Santos 2005) which involve different signal transduction pathways. Thus, correlative data and direct evidence in practical applications have demonstrated that an increasing number of apparently diverse seminal molecules play an important role in modifying male fertility. Keeping in view of the above facts and also taken into consideration the deficit knowledge of FAA in buffalo bulls, the present study was designed to characterize FAA in seminal plasma and frozen-thawed spermatozoa and determine their role in sperm function tests in relation to fertility of breeding buffalo bulls.

MATERIALS AND METHODS

Semen procurement and preparation of sperm extracts: Both fresh (1–2 ml) and frozen semen (50 straws per bull) from thirty healthy breeding Murrah buffalo bulls were procured from two government semen processing and freezing laboratories in the month of September having

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ambient temperature 30.6°C and relative humidity 92% for the study. The fresh and frozen-thawed semen (20 straws per bull) was centrifuged at 3,000 rpm for 10 min to separate out seminal plasma and dilutor, respectively. The seminal plasma from fresh semen was transferred to cryovials for storage at –20°C until analysis. The dilutor from frozen-thawed semen was discarded. The sperm pellet from frozen-thawed semen 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 ml of 62.5 mM Tris-HCl (pH 6.8, 2% SDS, 1 mM PMSF, 25 mM benzidine), ultrasonicated (3 bursts of 20 sec each) and centrifuged at 15,000 rpm for 30 min. The aliquots of sodium dodecyl sulphate-sperm extracts (SDS-SE) were stored at –20°C till further use.

Molecular weight determination by immunoblotting: The enzyme linked immuno transfer blot was done as per the method of Towbin *et al*. (1979) after electrophoresis of proteins (100 µg) by SDS-PAGE using 10% separating gel and 4% stacking gel. The proteins in seminal plasma and frozen-thawed sperm extracts were reacted with anti-FAA (NHP-2 like protein-1 antibody, Pierce) and the blot 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).

Evaluation of semen parameters: The frozen-thawed semen was evaluated for percent acrosome reaction, HOST, viability, DNA integrity and total motility.

Acrosome reaction: The status of acrosome reaction was calculated according to the protocol described by Yanagimachi (1994). The frozen-thawed semen (10 straws) 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 1,000 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 (100×10^6 sperms/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.

Hypoosmotic swelling test (*HOST*)*:* Functional integrity of the sperm was evaluated through 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.

Sperm viability: The live sperm count was determined by Eosin-Nigrosin staining technique. Briefly, a semen sample (one straw) was washed twice in phosphate buffer solution. One drop of semen was mixed with one drop of stain and a thin smear was prepared using a pre-warmed, clean and grease free glass slide from the semen stain mixture and examined under oil immersion lens of light microscope to determine sperm viability. About 200 spermatozoa were counted under different fields and classified into two categories viz. live sperms with clear bright head and dead sperms with stained and partially stained head and per cent live sperm was calculated.

DNA integrity: The method of evaluation of sperm DNA integrity using Acridine Orange (AO) was determined as described by Lui and Baker (1992). The AO staining stock solution was prepared by adding 6 mg of AO in 1 ml of DDW and stored in the dark (4°C). Washed frozen-thawed semen (200 µl) was added to 400 µl of solution A (0.1% Triton-X-100, 0.08 N HCl, 0.15 M NaCl and 3 µl of AO stock solution) and mixed gently for 30 sec. Then 1.2 ml of ice cold solution B (1 mM sodium EDTA, 0.15 M NaCl, 0.3 M $Na_2HPO_4.7H_2O$ and 0.1 M citric acid at pH 6.0) was mixed gently and allowed to equilibrate for 15 min. Finally, 10 µl of AO mixed semen was gently placed on a glass slide and covered with coverslip. About 200 spermatozoa were evaluated under an epifluorescent microscope (40×). The heads of the sperm cells with normal DNA integrity (double stranded) emitted green fluorescence, whereas those with denatured or single stranded DNA had orange, yellow and/or red fluorescence. The slides were evaluated within one hour after staining.

Sperm motility: A previously validated computer assisted semen analysis (CASA; version Hamilton-Thorne IVOS 12.2) was used to denote the total motility. Briefly, 10 µl of frozen-thawed semen from each straw was mounted on a disposable CASA slide (Leja-8; IMV Technologies, France) to analyze the total motility. Five randomly selected fields were scanned per straw and five straws per bull semen were evaluated to denote the total motility, obtaining 25 scans for each bull.

The mean of 25 scans for the total motility and the mean of three replicates for percent acrosome reaction, HOST, viability and DNA integrity per bull semen was used for the statistical analysis.

Fertility trial: Buffaloes (300) were enrolled for fixed time insemination program ($PGF2_\alpha$-GnRH-$PGF2_\alpha$-GnRH on day –2, 0, 7 and 9, respectively followed by inseminations at 16 and 40 h after last GnRH injection) with frozen semen. All buffaloes were healthy, recently calved (60–80 days earlier), free from physical and genital problems and maintained under identical feeding and management systems. The pregnancy diagnosis was done on day 45 post-insemination and confirmed on day 60 using ultrasonography. The first service conception rate (FSCR) was calculated according to the following formula:

$$\text{FSCR (\%)} = \frac{\text{Number of buffaloes conceived after first insemination}}{\text{Total number of first services}} \times 100$$

Based on FSCR, the percentage of tested frozen-thawed semen samples with > 50% FSCR and those with < 50% FSCR were considered as high fertility and low fertility semen samples, respectively for further comparisons.

Immunolocalization of FAA like antigens on buffalo bull spermatozoa: Localization of FAA on sperm cells was determined with anti-FAA (NHP-2 like protein-1 antibody) as the primary antibody and the conjugated goat anti-rabbit-FITC as secondary antibody (Merck).

Statistical analysis: The statistical analysis was performed with Statistical Package for Social Sciences (SPSS, version 16.0) program. The proportionality data (acrosome reaction and FSCR) were transformed using the arcsine transformation [asin (sqrt (percent/100))] with adjustment to allow for zero values. The mean ± SE were calculated using arcsine transformed data in the software. Duncan's multiple range test and one way analysis of variance (ANOVA) was used for comparing the level of significance among the group of bulls of different gradients (bulls positive and negative for HBP). The mean ± SE were calculated using arcsine transformed data in the software. The minimum significant interaction was considered at 5% level.

RESULTS AND DISCUSSION

Characterization of FAA in seminal plasma and frozen-thawed sperm extracts by immunoblotting: Blot images of protein bands in seminal plasma and frozen-thawed sperm extracts of all 30 bulls have been shown in Figs. 1 and 2. The anti-FAA identified eight proteins (60, 55, 45, 33, 31, 18, 16 and 14 kDa) in seminal plasma and four proteins (65, 55, 48 and 18 kDa) in frozen-thawed spermatozoa of buffalo bulls (Tables 1 and 2). The electrophoretic profiles showed polymorphism among individual semen samples ranging from 3–7 proteins in each tested seminal plasma and 1–4 proteins in each post-thaw semen sample. However, all proteins were not recognized by anti-FA-1 in seminal plasma and frozen-thawed sperm extracts of each bull. These findings are in agreement with the observations of McCauley *et al.* (1999) who named the proteins with molecular weight of 18, 31, 33, 48 and 55 kDa as fertility-associated antigen-5-complex (FAA-5-complex) in sperm extracts and recognized them as a diagnostic indicator of semen fertility. Similar studies (Roncoletta *et al.* 2006) in seminal plasma of Nelore bulls also demonstrated six FAA having molecular weight from 14–65 kDa (14, 18, 33, 45, 60 and 65). Further, Singh (2013) also observed four major FAA in the range of 14–31 kDa (14, 18, 26 and 31 kDa) in buffalo bulls. In seminal plasma, proteins with molecular weight of 60, 55, 45, 33, 31, 18, 16 and 14 kDa were detected in 30, 28, 26, 13, 18, 19, 9 and 15 bulls, respectively; and in SDS-SE of frozen-thawed spermatozoa, 65, 55, 48 and 18 kDa proteins were identified in 14, 28, 13 and 10 bulls, respectively (Figs. 1 and 2). Therefore, qualitative differences (presence or absence of FAA bands) in seminal plasma and frozen-thawed sperm extracts of all bulls were observed. Dalton *et al.* (2012) in cattle bull spermatozoa also showed differences in FAA with molecular mass of 55, 33, 31 and 18 kDa. Moreover, the inherent character of proteins may also contribute toward the difference in number of bands.

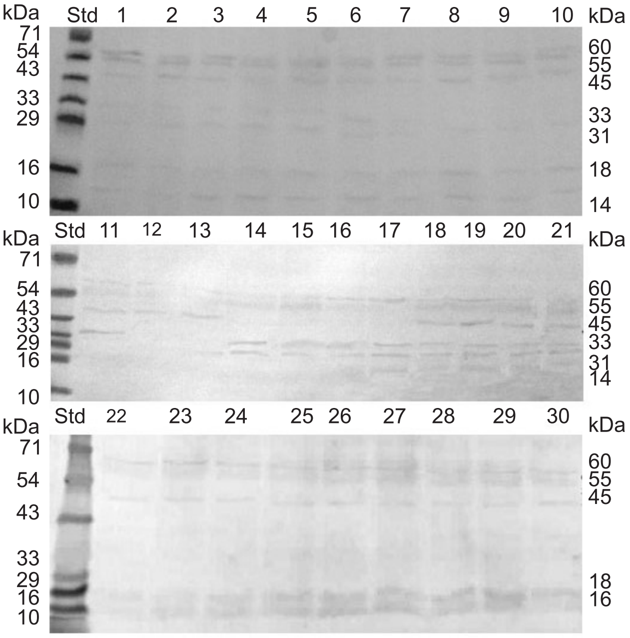

Fig. 1. Immunoblotting pattern of FAA in seminal plasma of buffalo bulls. Lane Std, standard protein marker; lanes 1–30, bull numbers.

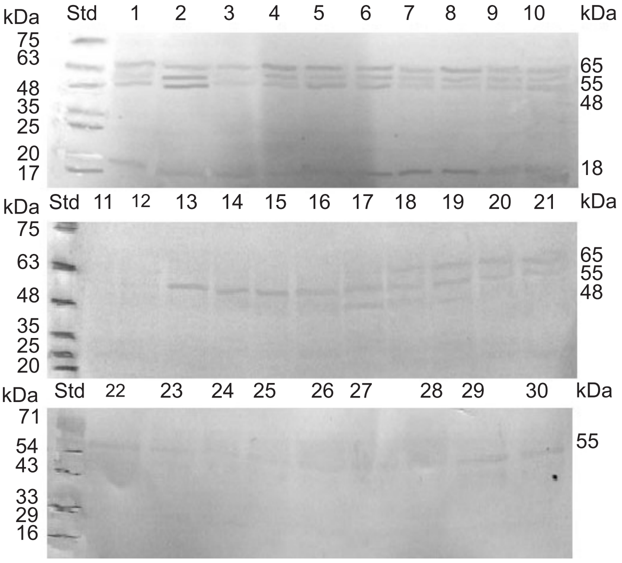

Fig. 2. Immunoblotting pattern of FAA in frozen-thawed sperm extracts of buffalo bulls. Lane Std, standard protein marker; lanes 1–30, bull numbers.

Relationship of different FAA with semen attributes: Measurements of acrosome reaction (51.7±1.8; 32.7–70.6%), HOST (65.1±2.2, 60.2–80.3%), viability (69.3±1.7, 55.8–81.3%), DNA integrity (78.7±1.9, 61.7–95.4%) and total motility (55.5±1.6, 40.4–72.7%) for frozen-thawed semen exhibited wide variation among 30 tested bulls. The association of different FAA (65, 60, 48, 31, 18 and 14 kDa) with semen attributes was in accordance with the observations of Peixoto *et al*. (2012) that semen characteristics viz. HOST, acrosome reaction, DNA integrity and motility are most valuable indicators of semen quality of bulls. In seminal plasma, almost all seminal parameters (per cent acrosome reaction, HOST, viability, DNA integrity and total motility) were found to be significantly ($P<0.01$, $P<0.05$) higher in bulls positive for FAA of 60, 33, 31 and 14 kDa than in their negative contemporary mates. On the other hand, the FAA with molecular weight 16 kDa exhibited significantly ($P<0.05$)

Table 1. Relationship of FAA with spermatozoal characteristics and FSCR in seminal plasma of buffalo bulls (Mean±SE)

Mol. Wt. (kDa)		60	55	45	33	31	18	16	14
Bulls positive for FAA	AR (%)	51.7 ± 1.8$^{\wedge}$	51.4 ± 1.9	51.6 ± 2.1	53.6 ± 2.0	55.5 ± 2.1^{a}	51.7 ± 2.8	44.3 ± 3.3^{a}	56.6 ± 2.3^{a}
	HOST (%)	65.1 ± 2.2$^{!}$	66.1 ± 1.3^{c}	66.4 ± 1.4	69.4 ± 1.9^{c}	68.7 ± 1.5^{c}	65.0 ± 1.6^{c}	61.4 ± 2.3^{c}	67.6 ± 1.9
	Viability (%)	69.3 ± 1.7^{+}	70.4 ± 1.8^{e}	68.6 ± 1.8^{e}	72.5 ± 2.6	72.4 ± 2.1^{e}	68.6 ± 2.1	67.3 ± 3.1	71.2 ± 2.4
	DNAI (%)	78.7 ± 1.9$^{@}$	79.2 ± 2.0	78.2 ± 2.1	82.2 ± 3.1^{g}	81.9 ± 2.3^{g}	77.2 ± 2.4	71.8 ± 3.0^{g}	79.2 ± 2.8
	TM (%)	55.5 ± 1.6$^{\$}$	55.4 ± 1.6	55.7 ± 1.9	57.6 ± 2.1	58.4 ± 1.7^{i}	55.3 ± 2.3	48.6 ± 2.8^{i}	59.2 ± 2.0^{i}
	FSCR (%)	37.0 ± 3.2*	37.5 ± 3.4	37.3 ± 3.7	41.5 ± 3.2	46.7 ± 3.2^{k}	37.9 ± 5.0	16.7 ± 1.8*	48.6 ± 3.8^{k}
	Bulls (%) with ≥ 50.0% FSCR	33.3 (10)$^{\#}$	35.7 (10)$^{\#}$	38.5 (10)$^{\#}$	38.5 (5)$^{\#}$	55.6 (10)$^{\#}$	52.6 (10)$^{\#}$	0.0 (0)$^{\#}$	66.7 (10)$^{\#}$
Bulls negative for FAA	AR (%)	0.0 ± 0.0$^{\wedge\wedge}$	56.3 ± 4.3	51.9 ± 2.7	50.2 ± 2.8	42.8 ± 4.3^{b}	51.5 ± 1.6	54.9 ± 1.8^{b}	46.8 ± 2.2^{b}
	HOST (%)	0.0 ± 0.0$^{!!}$	77.1 ± 1.8^{d}	69.6 ± 3.3	64.9 ± 1.7^{d}	64.1 ± 2.3^{d}	69.1 ± 2.0^{d}	69.2 ± 1.3^{d}	64.8 ± 2.0
	Viability (%)	0.0 ± 0.0^{++}	65.6 ± 2.6^{f}	79.3 ± 1.2^{f}	68.2 ± 2.1	66.6 ± 2.5^{f}	70.6 ± 2.8	71.2 ± 2.0	69.0 ± 2.4
	DNAI (%)	0.0 ± 0.0$^{@@}$	71.1 ± 8.3	81.7 ± 5.5	76.0 ± 2.4^{h}	73.8 ± 3.0^{h}	81.2 ± 3.2	81.6 ± 2.2^{h}	76.3 ± 2.8
	TM (%)	0.0 ± 0.0$^{\$\$}$	56.9 ± 13.3	53.7 ± 1.5	53.8 ± 2.3	51.1 ± 2.7^{j}	55.8 ± 2.1	58.4 ± 1.6^{j}	50.4 ± 1.8^{j}
	FSCR (%)	0.0 ± 0.0**	30.0 ± 10.0	35.0 ± 2.9	33.5 ± 5.0	22.5 ± 3.3^{l}	35.5 ± 1.6	45.7 ± 2.8**	26.0 ± 3.0^{l}
	Bulls (%) with ≥ 50.0% FSCR	0.0 (0)$^{\#}$	0.0 (0)$^{\#}$	0.0 (0)$^{\#}$	29.4 (5)$^{\#}$	0.0 (0)$^{\#}$	0.0 (0)$^{\#}$	47.6 (10)$^{\#}$	0.0 (0)$^{\#}$

Values with different alphabetic superscripts differ significantly ($P<0.05$) in the same column for their respective parameter. Values with different symbolic superscripts differ significantly ($P< 0.01$) in the same column for their respective parameter. Figures in parentheses with symbol $^{\#}$ indicate the number of tested bulls with ≥ 50.0% FSCR. AR, acrosome reaction; HOST, hypoosmotic swelling test; DNAI, DNA integrity test; TM, total motility; FSCR, first service conception rate

Table 2. Relationship of FAA with spermatozoal characteristics and FSCR in frozen-thawed sperm extracts of buffalo bulls (Mean±SE)

Mol. Wt. (kDa)		65	55	48	18
Bulls positive for FAA	AR (%)	56.7 ± 2.5^{a}	51.9 ± 1.9	57.1 ± 2.7^{a}	58.7 ± 3.1^{a}
	HOST (%)	68.5 ± 1.7	66.7 ± 1.4	69.2 ± 1.6^{c}	68.3 ± 1.9
	Viability (%)	70.4 ± 2.4	70.4 ± 1.7	71.0 ± 2.5	69.7 ± 2.9
	DNAI (%)	82.0 ± 2.6^{e}	78.6 ± 2.0	80.6 ± 2.8	82.1 ± 3.1
	TM (%)	59.7 ± 2.0^{g}	55.7 ± 1.7	59.3 ± 2.1^{g}	61.3 ± 2.4^{g}
	FSCR (%)	50.0 ± 3.6^{i}	36.8 ± 3.4	51.5 ± 3.5^{i}	57.0 ± 2.6*
	Bulls (%) with ≥ 50.0% FSCR	71.4 (10)$^{\#}$	35.7 (10)$^{\#}$	76.9 (10)$^{\#}$	100.0 (10)$^{\#}$
Bulls negative for FAA	AR (%)	48.3 ± 2.5^{b}	57.4 ± 12.3	48.5 ± 2.4^{b}	48.3 ± 1.8^{b}
	HOST (%)	65.5 ± 2.0	68.8 ± 2.6	65.1 ± 1.9^{d}	66.1 ± 1.7
	Viability (%)	69.8 ± 2.3	64.9 ± 7.4	69.4 ± 2.3	70.2 ± 2.1
	DNAI (%)	75.8 ± 2.7^{f}	80.3 ± 14.1	77.2 ± 2.7	77.0 ± 2.4
	TM (%)	51.1 ± 2.1^{h}	52.5 ± 3.4	52.5 ± 2.2^{h}	52.6 ± 1.8^{h}
	FSCR (%)	25.6 ± 2.9^{j}	40.0 ± 0.0	25.9 ± 2.7^{j}	27.0 ± 2.4**
	Bulls (%) with ≥ 50.0% FSCR	0.0 (0)$^{\#}$	0.0 (0)$^{\#}$	0.0 (0)$^{\#}$	0.0 (0)$^{\#}$

Values with different alphabetic superscripts differ significantly ($P<0.05$) in the same column for their respective parameter. Values with different symbolic superscripts differ significantly ($P<0.01$) in the same column for their respective parameter. Figures in parentheses with symbol $^{\#}$ indicate the number of tested bulls with ≥ 50.0% FSCR. AR, acrosome reaction; HOST, hypoosmotic swelling test; DNAI, DNA integrity test; TM, total motility; FSCR, first service conception rate poor seminal attributes in FAA positive bulls than in negative ones (Table 1). In the remaining FAA-55, FAA-45 and FAA-18, significant (P<0.05) difference was noticed in one or the other semen characteristics between detectable and undetectable FAA. Marques *et al*. (2000) established a high correlation ($r^2 = 0.68$) of FAA-18, FAA-31 and FAA-48 with per cent HOST, acrosome reaction and motility and presented them as a candidate protein marker for fertility. Further, Rueda *et al*. (2013) also reported the critical role of 31 and 33 kDa proteins in osmotic fragility, DNA fragmentation and acrosome membrane fusion events. Alternatively, poor relationship of a 16 kDa protein with seminal characteristics in bulls positive for FAA as compared to negative ones was in consonance with the results demonstrated by Amours *et al*. (2010) that FAA-16 has a low association with semen characteristics (motility, acrosome integrity and acrosome reaction) of cross-bred bull.

At post-thaw stage, three out of four bands with molecular weight of 65, 48 and 18 kDa had significantly (P<0.05) higher percentage of most seminal parameters (acrosome reaction, percent HOST, DNA integrity and total motility) in bulls positive for FAA than in their negative herdmates (Table 2). Conversely, FAA-55 was the only protein that seemed to exhibit a non-significant (P>0.05) reverse trend for most semen characteristics. Similar studies (Cancel *et al.* 1997, Cormier and Bailey 2003) in Holstein bulls also depicted that freezing and/or thawing decreased the binding of osteopontin (55 kDa) to spermatozoa. Overall, higher percentages of seminal characteristics might possibly be responsible for improved semen quality of bulls positive for FAA.

Relationship of FAA differences with bull fertility: A field fertility trial with frozen-thawed semen was conducted to determine the fertility of 30 bulls and its association with FAA. The results revealed an overall first service conception rate (FSCR) of 37.0±3.2% (10–70%). The presence or absence of FAA in seminal plasma and frozen-thawed spermatozoa was compared with FSCR. In seminal plasma, the overall FSCR (P<0.01; P<0.05) was significantly higher in the bulls positive for 60, 31 and 14 kDa proteins as compared to their negative herd mates (Table 1). A difference of about 37.0, 24.2 and 22.6% in FSCR could be appreciated in bulls positive for FAA of 60, 31 and 14 kDa, respectively than in their counterparts. Likewise, the percentage of bulls with good fertility (> 50.0% FSCR) was higher (33.3, 55.6 and 66.7%) among the bulls positive for FAA-60, FAA-31 and FAA-14 kDa proteins as compared to their negative contemporary mates (0.0, 0.0 and 0.0%). Although non-significant (P>0.05), the FSCR was also higher in bulls positive for FAA of 55, 45, 33 and 18 kDa than in their counterparts and had a difference of 7.5, 2.3, 8.0 and 2.4%, respectively. On the other hand, a highly significant (P<0.01) reverse association with fertility was observed in the bulls with detectable FAA-16. The percentage of bulls with good fertility was also lower (0.0%) in those positive for FAA-16 as compared to their negative herdmates (47.6%). A higher FSCR for 68, 60, 55, 48, 45, 33, 31, 18 and 14 kDa antigens in bulls positive for FAA was in agreement with the findings of previous workers (Divyaswetha *et al.* 2008) who purified and characterized 60, 55, 48, 31 and 18 kDa proteins from bovine seminal fluid and determined their role in fertility of mammalian spermatozoa. Further, Rajeev and Reddy (2004) also characterized fertility associated proteins in human seminal plasma. Conversely, a negative association of FAA-16 with FSCR was in corroboration with the observations of Hung and Suarez (2012) that 16 and 11 kDa proteins in seminal fluid exhibit an inverse relationship with bull fertility.

In SDS-SE of frozen-thawed spermatozoa, the overall FSCR was significantly (P<0.05; P<0.01) higher in the bulls positive for 65, 48 and 18 kDa proteins than in their counterparts (Table 2) with a difference of approximately 24.4, 25.6 and 30.0% over the negative ones. The percentage of bulls exhibiting < 50.0% FSCR and positive for 65, 48 and 18 kDa proteins was 71.4%, 76.9% and 100.0%, respectively, while none of the bull negative for FAA exhibited good fertility. Alternatively, the FSCR was merely 3.2% lower in bulls with detectable FAA-55 than in those with undetectable FAA-55. Nevertheless, the percentage of good fertility was higher (35.7%) in bulls positive for FAA-55 kDa as compared to their negative contemporary mates (0.0%). These observations corroborated with the findings of Moura *et al.* (2006) that FAA-55 kDa protein is associated with higher fertility of bulls.

Alterations in FAA during cryopreservation: The presence of FAA with molecular weight of 18 kDa were identified in seminal plasma of 19 bulls and frozen-thawed spermatozoa of 10 bulls leading to alteration in spermatozoa of 9 bulls (Tables 1 and 2). Proteins (60, 45, 33, 31, 16 and 14 kDa) showed their expression only in seminal plasma, whereas the FAA-65 and FAA-48 were recognized in SDS-SE of frozen-thawed spermatozoa only. Therefore, some alterations on sperm surface occurred during freeze-thaw procedures in these bulls. This variation in FAA of seminal plasma and spermatozoa might possibly be due to difference in expression of these proteins during the process of cryopreservation of semen of different bulls. Different sperm cells exhibit differences in freezing resistance upon cryopreservation resulting in concomitant coating and decoating of proteins on their surface (Druart *et al.* 2009, Leahy and Gadella 2011). Similar differences in protein patterns between ejaculated (17 bands) and cryopreserved (14 bands) spermatozoa using comparative western-blot analysis have been observed in boars (Zigo *et al*. 2013).

Immuno-localization of FAA to distinct regions of buffalo bull spermatozoa: Localization of anti-FAA on frozen-thawed semen has been depicted in Fig. 3. Indirect immunofluorescence of spermatozoa revealed binding of NHP-2 like protein-1 antibody to specific regions of sperm and indicated heterogeneity in the distribution of FAA on the surface of buffalo bull spermatozoa. The acrosomal and post-acrosomal segment of the head displayed punctate fluorescence in majority of the spermatozoa, showing the

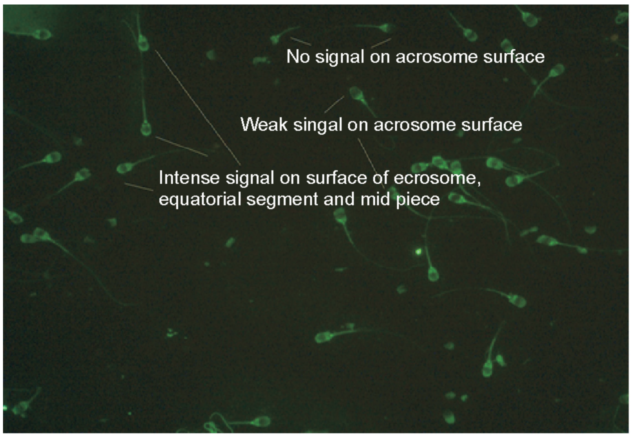

Fig. 3. Immunofluorescence of buffalo bull spermatozoa with anti-FAA (40×).

presence of distinct binding domains for FAA in sperm membranes and representing qualitative assessment of antibody binding. However, in few spermatozoa, poor and/or no fluorescence was observed on the acrosomal cap. This could be due to damage to acrosome and/or loss of FAA during the process of freeze-thawing of spermatozoa. Nevertheless, a decline in the intensity of punctuation on acrosomal cap was observed with decreasing FSCR of bulls. Furthermore, the fluorescence in mid-piece and principal piece regions of the tail was less punctate in appearance compared with acrosomal staining. Indirect immunofluorescence revealed that the FAA is present in the post-acrosomal, mid piece and tail region of bovine, equine and human spermatozoa (Dawson *et al.* 2003). Further, Cormier and Bailey (2003) also reported similar observations in bulls that percentage of acrosome showing fluorescence with FAA was higher in frozen-thawed semen.

In conclusion, immunoblots of buffalo bull seminal fluid demonstrated that FAA binds spermatozoa *in vitro*. Higher fertility bulls can be segregated from lower fertility bulls based on presence of FAA variants (65, 60, 48, 31, 18 and 14 kDa) in seminal fluid. The FAA may play a role in capacitation and thereby modify male fertility potential. Studies are underway to examine and validate the functional relationship between presence of FAA on sperm and increased fertility potential of buffalo bulls.

REFERENCES

Agarwal A and Said T M. 2003. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Human Reproduction Update* **9**: 331–45.

Amours O D, Frenette G, Fortier M, Leclerc P and Sullivan R. 2010. Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes. *Reproduction* **139**: 545–56.

Cancel A M, Chapman D A and Killian G J. 1997. Osteopontin is the 55 kDa fertility associated protein in Holstein bull seminal plasma. *Biology of Reproduction* **57**: 1293–1301.

Cormier N and Bailey J L. 2003. A differential mechanism is involved during heparin and cryopreservation induced capacitation of bovine spermatozoa. *Biology of Reproduction* **69**: 177–85.

Dalton J C, Deragon L, Vasconcelos J L M, Lopes C N, Peres R G F and Ahmadzadeh A. 2012. Fertility-associated antigen on Nelore bull sperm and reproductive outcomes following first-service fixed-time AI of Nelore cows and heifers. *Theriogenology* **77**: 389–94.

Dawson G R, McCauley T C, Oyarzo J N, Grossman J S, Marks S H F and Ax R L. 2003. Immunolocalization of Fertility-associated antigen (FAA) on bovine, equine and human sperm. *Animal Breeding Abstracts* **80**: 240–41.

Divyaswetha P, Bindu N, Abdullah K, Jean M F, Shane C B and Erdogan M. 2008. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *Systems Biology* **2**: 1–14.

Druart X, Congnie J, Baril G, Clement F, Dacheux J L and Gatti J L. 2009. *In vivo* imaging of *in situ* motility of fresh and liquid stored spermatozoa in the ewe genital tract. *Reproduction* **138**: 45–53.

Gamboa S and Ramalho-Santos J. 2005. SNARE proteins and caveolin-1 in stallion spermatozoa: possible implications for fertility. *Theriogenology* **64**: 275–91.

Hung P and Suarez S S. 2012. Alterations to the bull sperm surface proteins that bind sperm to oviductal epithelium. *Biology of Reproduction* **87**(4): 1–11.

Kumar V, Hassan M I, Tomar A K, Kashav T, Nautiyal J, Singh S, Singh T P and Yadav S. 2009. Proteomic analysis of heparin-binding proteins from human seminal plasma: a step towards identification of molecular markers of male fertility. *Journal of Biosciences* **34**: 899–908.

Leahy T and Gadella B M. 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction* **142**: 758–78.

Lui D Y and Baker H W. 1992. Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding and fertilization rates *in vitro*. *Fertility and Sterility* **58**: 1178–84.

Marques V A, Goulart L R and Silva A E D. 2000. Variations of protein profiles and calcium and phospholipase A_2 concentrations in thawed bovine semen and their relation to acrosome reaction. *Genetics and Molecular Biology* **23**: 825–29.

McCauley T C, Zhang H M, Bellin M E and Ax R L. 1999. Purification and characterization of fertility-associated antigen (FAA) in bovine seminal fluid. *Molecular Reproduction and Development* **54**: 145–53.

Moura A A, Koc H, Chapman D A and Killian G J. 2006. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. *Journal of Andrology* **27**: 201–11.

Peixoto G C X, Silva M A, Castelo T S, Silva A M, Bezerra J A B, Souza A L P, Oliveira M F and Silva A R. 2012. Individual variation related to testicular biometry and semen characteristics in collared peccaries (*Tayassu Tajacu L*). *Animal Reproduction Science* **134**: 191–96.

Rajeev S K and Reddy K V R. 2004. Sperm membrane protein profiles of fertile and infertile men: Identification and characterization of fertility associated sperm antigens. *Human Reproduction* **19**: 234–42.

Roncoletta M, Morani E S, Esper C R, Barnabe V H and Franceschini P H. 2006. Fertility-associated proteins in Nelore bull sperm membranes. *Animal Reproduction Science* **91**: 77–87.

Rueda F L, Quimica L, Herrera R F, Arbelaez L F, Garces T, Velasquez H, Pena M A and Cardozo J A. 2013. Increase in post thaw viability by adding seminal plasma protein to Zebu sperm. *Revis. Colomb. Cienc. Pecu.* **26**: 98–107.

Singh M, Ghosh S K, Prasad J K, Kumar A, Ramteke S S and Bhure S K. 2013. Heparin binding proteins of buffalo bulls seminal plasma and their relationship with semen freezability. *Indian Journal of Animal Sciences* **83**: 700–04.

Sprott L R, Forrest D W, Zhang H M, Dyarzo J N, Bellin M E and Ax R L. 2000. Artificial inseminations outcome in beef females using bovine sperm with a detectable fertility-associated antigen. *Journal of Animal Science* **78**: 795–98.

Towbin H, Staehelin T and Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of National Academy of Sciences*, USA **76**: 4350–54.

Yanagimachi R. 1994. Mammalian Fertilization. Knobil E and Neill JD (Eds), Physiology of Reproduction, 2nd Edn, Raven Press, New York. pp 189–317.

Zigo M, Jonakova V, Sulc M and Manaskova-Postlerova P. 2013. Characterization of sperm surface protein patterns of ejaculated and capacitated boar sperm, with the detection of ZP binding. *International Journal of Biological Macromolecules* **61**: 322–28.