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Characterization and immunolocalization of HBP, FA-1 and TIMP-2 like proteins in cattle bull semen: HBP modification in in vitro capacitated spermatozoa

Ranjna S Cheema*, V. Zodinsanga and Amrit K Bansal

Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India.

**Corresponding author: Ranjna.cheema@gmail.com*

Abstract

Characterization and localization of HBP, FA-1 and TIMP-2 like proteins in spermatozoa and seminal plasma of cattle bulls was carried out by immunoblotting and immunofluoresence. Anti-HBP, anti-TIMP-2 and anti-FA-1 reacted with 55, 48, 45, 42, 35, 30, 24, 18, 16 kDa; 65, 45, 24, 16 kDa and 55, 48, 16 kDa sperm proteins on immunoblots. Immunofluoresence indicated that HBP/ TIMP-2 are localized mainly on acrosomal cap, whereas, FA-1 predominantly on post acrosomal cap. Among the bulls, positive for 60, 45, 16 kDa FA-1 like proteins in sperm extracts and 11/ 16 kDa in SP; 7, 8, 6 and 7 bulls also showed higher rate of in vitro AR. Number of bulls positive for 65, 24 kDa-TIMP-2 and with higher rate of AR was more as compared to other anti-TIMP-2 reactive sperm/ SP proteins. Therefore, 60, 45 and 16 kDA-FA-1, 65 and 24 kDa-TIMP-2 like proteins may serve as indicators of higher rate of in vitro AR vis a vis fertility of cattle bulls. Immunoblotting of capacitated and un-capacitated spermatozoa with anti-HBP suggested that removal of 55, 48, 45, 40, 37 and 30 kDa HBP from in vitro acrosome reacted cattle bull spermatozoa allowed heparin to mediate in vitro AR and an increase in intensity of 110, 90 kDa and exposure of 65, 60, 26 and 16 kDa HBP after AR may be important for binding of sperm to ovum, penetration and fertilization.

Keywords: Cattle bull, Spermatozoa, Seminal Plasma, HBP, TIMP-2, FA-1, In vitro acrosome reaction.

Infertility is an important aspect of human / animal reproduction and still presents with much etiological ambiguity. As fifty percent of infertility is related to the male partner, molecular investigations on sperm and seminal plasma can lead to new knowledge on male infertility (Ashrafzadeh *et al.,* 2013). Heparin, a sulfated glycosaminoglycan, induces capacitation and acrosome reaction in mammalian sperm and participation of heparin-binding proteins (HBPs) in mammalian sperm capacitation (Thérien *et al.,* 1995) is well documented. HBPs are produced by the male accessory glands, secreted into seminal fluid (Nass *et al.,* 1990), and upon ejaculation bind to sperm (Miller *et al.,* 1990). Addition of HBP to epididymal sperm induced heparin-stimulated acrosome reaction (Miller *et al.,* 1990). Spermatozoa from high-fertility bulls have a greater frequency of acrosome reaction in response to heparin-like material (Ax *et al.,* 1985; Ax and Lenz, 1987; Lenz *et al.,* 1988) and have a greater binding affinity for heparin (Marks and Ax, 1985) than sperm from less-fertile bulls. The FA-1 antigen is a glycoprotein of 23 kDa (monomer) that has a ligand activity for ZP3 of oocyte zona pellucida (Naz et al, 1991) and causes a reduction in fertility of actively immunized female rabbits. A monoclonal antibody to the human sperm plasma membrane protein, fertilization antigen-1 (FA-1), was tested for its reactivity with bovine spermatozoa and its effects on bovine fertilization in vitro (Coonord et al, 1994). The 24 kDa HBP, purified and characterized from bovine seminal fluid shared 90% identity (McCauley *et al.,* 2001) to a tissue inhibitor of metalloproteinases-type 2 (TIMP-2). The well documented presence and abundance of TIMP-2 in bovine semen and its ability to bind heparin (Calvete *et al.,* 1996; Liberda *et al.,* 2001; and McCauley *et al.,* 2001) suggests that it has an important relationship to reproduction in bulls. The relationship of TIMP-2 to bull fertility has been established by a retrospective method (Dawson *et al.,* 2002). Bulls who possessed TIMP-2 in detergent extracts of sperm were 13% more fertile than TIMP-2 negative bulls. A study was carried out in cross-bred (HF X Red dane X Sahiwal) and pure-bred bulls: to 1) characterize HBP, FA-1 and TIMP-2 like proteins in spermatozoa with commercial antibodies, 2) variation in FA-1 and TIMP-2 like proteins in spermatozoa and seminal plasma of bulls and 3) modification in HBPs of capacitated spermatozoa.

Material and Methods

Procurement of samples: About 500 µl ejaculated semen of 12 cross-bred (CB) , 9 HF, one each jersy (J) and sahiwal (S) bulls was procured from GADVASU dairy farm, Semen station Bhattian (Khanna) and Ropar, Punjab, India were procured at the time of collection.

Processing of semen: Seminal plasma and spermatozoa were separated by

centrifuging semen at 3000 rpm for 5 min. The proteins of washed spermatozoa (twice with PBS, pH 7.4) of 15 bulls (5 CB, 8 HF, 1 each J and S) were extracted with SDS (Cheema et al 2011). About 500 x 106 spermatozoa were washed twice with PBS (pH 7.4) and suspended in 1.0 ml of 2% SDS in 62.5 mM Tris-HCl (pH 6.8) containing protease inhibitors (Cocktail, SERVA). Sperm suspensions were sonicated at 20 Watts for 3 x 20 secs, centrifuged at 10000 rpm for 15 min. Sperm extracts (SE) and seminal plasma were stored in aliquots at -20o C till further use. Total protein was measured in seminal plasma and sperm extracts (SE) by the method of Lowry et al (1951).

SDS-PAGE and Immunoblotting: (Lamelli *et al.,* 1971 and Towbin *et al.,* 1979): Proteins of pooled sperm extracts of crossbred bulls and variably in vitro capacitated/ acrosome reacted spermatozoa were reacted on immunoblots with anti-HBP (anti AZU-1, Sinobilogical) to detect HBP like proteins in cattle bull spermatozoa and to observe modification in HBPs of variably capacitated/ acrosome reacted spermatozoa. Seminal plasma and SE of 23 and 15 bulls (pooled as well as separately) were also reacted with anti-TIMP-2 (TIMP-140, Santa Cruz) and anti-FA-1 (NHP-2 like protein-1 antibody, Pierce) to observe any variation in such fertility associated proteins among the bulls.

Proteins separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose membrane using wet electrophoresis transfer apparatus (Clever Scientific Co, UK, VS10WD) at 100 V for 2.5 hours. Transfer quality was checked by 0.2% ponceau dye and proteins were blocked in 2 % BSA as blocking solution for overnight at 4°C. The membrane was washed with PBS + 0.05% Tween-20, and was incubated in 1: 1000 diluted primary antibodies for 2.5 hours. The membrane was again washed thrice with PBS + 0.05% Tween-20, and incubated with 1:10000 anti-rabbit IgG as secondary antibody for 45 minutes. It was washed thrice with PBS + 0.05% Tween-20 and incubated with substrate (0.05% Diaminobenzidine + 0.015% 4-Chloro Napthol + 0.06% Hydrogen Peroxide) for 10 minutes. Gel images were captured on gel doc (Syngene International Ltd, UK, SYDR4) using Gene Snap image acquisition software and analyzed by using GeneTools gel analysis software.

Immunolocalization of HBP, FA-1 and TIMP-2: Immunolocalization of antigenic proteins was done using FITC labeling (Verdier *et al.,* 2002). Smears of pooled washed spermatozoa were prepared on glass slides, air-dried, and fixed in ethanol for 30 minutes. Slides were then covered with PBS containing 1% BSA for 45 minutes to block nonspecific antibody binding. They were then incubated at room temperature (25±3 °C) in a humidified chamber for 2 hours with Anti-HBP

(1:500), anti-TIMP-2 / anti-FA-1(1:1000). Slides were then washed and incubated for 1 hour with goat anti-rabbit IgG-FITC-conjugated antibody (Sigma) diluted to 1:100. After 3 washings, slides were mounted with PBS-glycerol (1:1 v/v) and observed on a fluorescent microscope (Olympus) and images were captured on digital camera. Negative controls with either primary or secondary antibody were also run.

***In vitro* acrosome reaction:** *In vitro* capacitation and acrosome reaction was performed as per the method of Parrish *et al* (1989). Tyrode albumin lactate pyruvate (TALP) stock solution (mg/100 ml): a) NaCl: 584.4 mg, b) KCl: 23 mg, c) $NaHCO_3$: 210 mg, d) Na_2HPO_4: 8.4 mg, e) $CaCl_2$: 29.5 mg, f) $MgCl_26H_2O$: 6.68 mg, g) Na Pyruvate: 11 mg, h) Na Lactate: 80 µl, i) HEPES: 130 mg. 2). TALP energy medium (mg/10 ml stock TALP): a) Bovine Serum Albumin (BSA): 60 mg, b) Dextrose: 9 mg, c) Heparin: 100 µl of 0.1 % stock solution.

Ten straws of frozen semen were thawed, pooled and mixed with equal volume of energy TALP and centrifuged at 1000 rpm for 5 min to get rid of the extender, re-suspended the pellet in 1.0 ml of TALP and washed twice by centrifugation at 1000 rpm for 5 min. Finally, pellet was suspended in 500 µl of TALP to have a 200 X 10^6 spermatozoa/ml, incubated at 37^o C in an incubator for 6 hrs. Motility was checked every hour and sperm smears were prepared at 0, 4 and 6 hrs of incubation. Giemsa stained slides were observed under oil immersion (1000 X) and about 200 spermatozoa with swollen heads, vesiculated and shedded acrosomes were counted.

Results and Discussion

Characterization of HBP, TIMP-2 and FA-1 in cattle bull spermatozoa: Anti-HBP, anti-TIMP-2 and anti-FA-1 reacted with 55, 48, 45, 42, 35, 30, 24, 18, 16 kDa; 65, 45, 24, 16 kDa and 55, 48, 16 kDa sperm proteins on immunoblots (Fig 1). Proteins of 15-17, 24 and 31 kDa isolated from bovine seminal plasma had been identified as HBP (Miller *et al.,* 1990). Therefore, 16, 18, 24 and 30 kDa proteins detected by anti-HBP in cattle bull sperm may be similar to those detected by Miller et al (1990). A 31 kDa protein was characterized as fertility associated antigen (Bellin *et al.,* 1998), which shared 73-92% identity with DNase-1 like protein (McCauley 1999). Purified FA-1 antigenic molecule is composed of a monomer of 23 kDa and / or a dimer of 47-50 kDa (Naz and Zhu, 1997). NHP2-like protein 1 antibody, which cross-reacted to 3 polypeptides of cattle bull spermatozoa is a 15.5 K antibody and has **synonyms like** FA-1, SPAG12, SSFA1 and sperm specific antigen 1. Therefore, it indicated that 48 and 16 kDa proteins identified by this antibody in cattle bull sperm are sperm specific fertility antigens. Further, 24 kDa

proteins purified and characterized from bovine seminal fluid shares 90% identity to TIMP-2 (McCauley *et al.,* 2001). Strong reaction of anti-TIMP-2 with a protein of 24 kDa in comparison to 65, 45 and 16 kDa proteins confirmed it as TIMP-2 like protein of cross-bred bull sperm. Two seminal plasma proteins (26 kDa, pI 6.2; 55 kDa, pI 4.5) predominated in higher fertility bulls (Killian *et al.,* 1993). Protein characterization revealed that the 55 kDa protein was glycosylated and its identity was as osteoponin (Cancel *et al.,* 1997). Cross-reaction of 55 kDa cattle sperm protein to anti-HBP as well as FA-1 reveals it also as a fertility associated protein of cattle bull sperm.

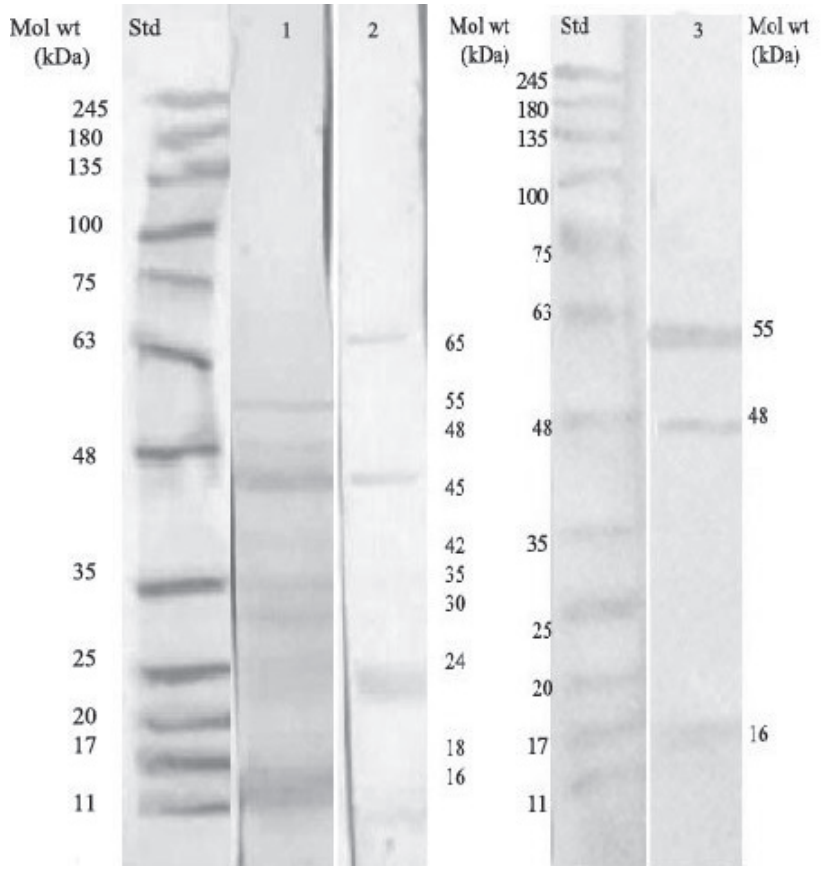

Figure 1. Immunoblotting of cattle bull sperm extracts with commercial antibodies. (1) anti-HBP, (2) anti-TIMP-2 and (3) anti-FA-1. Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane and reacted with anti-HBP, FA-1 and TIMP-2.

Immunolocalization of HBP, FA-1 and TIMP-2 in distinct regions of cross-bred cattle bull spermatozoa: Immunostaining of crossbred cattle bull spermatozoa with anti-AZU-1 (HBP) gave very intense signal on the acrosomal cap and a strong signal on post- acrosomal cap (Fig 2). It indicated that HBP are localized in large amount on only acrosomal cap and also to some extent on the post acrosomal cap. McCauley et al (1996) performed immunofluorescence of M1 to determine localization of HBP on bovine spermatozoa and stated that specific membrane domains containing HBP in acrosomal and post acrosomal regions exist in bovine ejaculated spermatozoa. They indicated that HBP bind to sperm in distinct patterns, which differed among bulls of varying fertility. Dawson (2005) observed that HBP were primarily localized to the posterior head region of ram and bull sperm and acrosome of the stallion sperm.

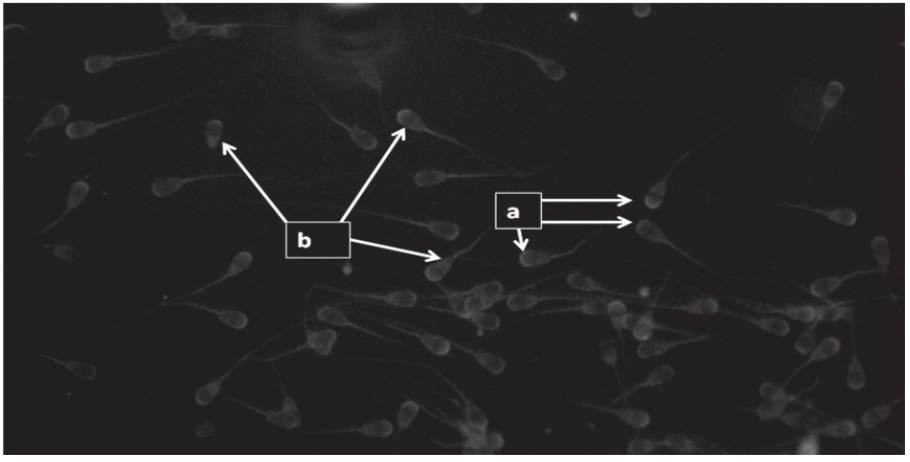

Figure 2. Immunofluoresence of anti-HBP with cattle bull spermatozoa. (a) Intense signal on tip of acrosome, (b) Strong signal on post acrosomal cap

In indirect immunofluoresence, anti-FA-1 could detect FA-1 like proteins predominantly on post acrosomal cap in majority of the spermatozoa and also on acrosomal cap in some spermatozoa in a smear on a slide. Indirect immunofluorescence also revealed that the FA-1 antigen is present in the post-acrosomal region of bovine spermatozoa (Coonord *et al.,* 1994), post-acrosome, mid piece and tail region of methanol-fixed human spermatozoa (Naz *et al.,* 1984; Naz *et al.,* 1986), postacrosomal, mid-piece and tail regions of rhesus monkey sperm (Naz and wolf, 1994).

Signals with anti-TIMP-2 were obtained on acrosomal cap, acrosome and post

acrosomal cap, which revealed that TIMP-2 like proteins were more concentrated on acrosomal cap than acrosome and post acrosomal cap (Fig 3). TIMP-2 was primarily localized to the posterior head of bull and ram sperm, but exclusively found on the acrosomal cap of stallion sperm (Dawson, 2005).

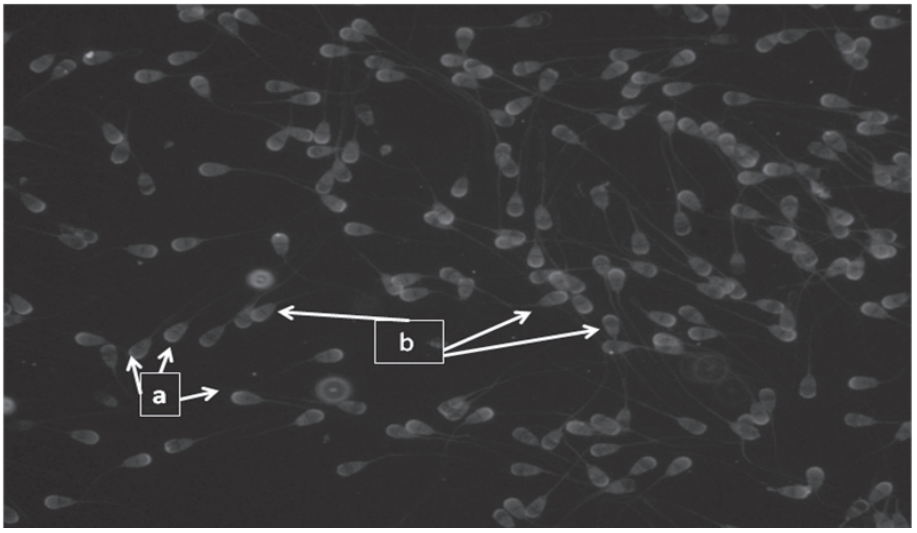

Figure 3. Immunofluoresence of anti-FA-1 with cattle bull spermatozoa. (a) signal on post acrosomal cap (b) Strong signal on acrosomal cap.

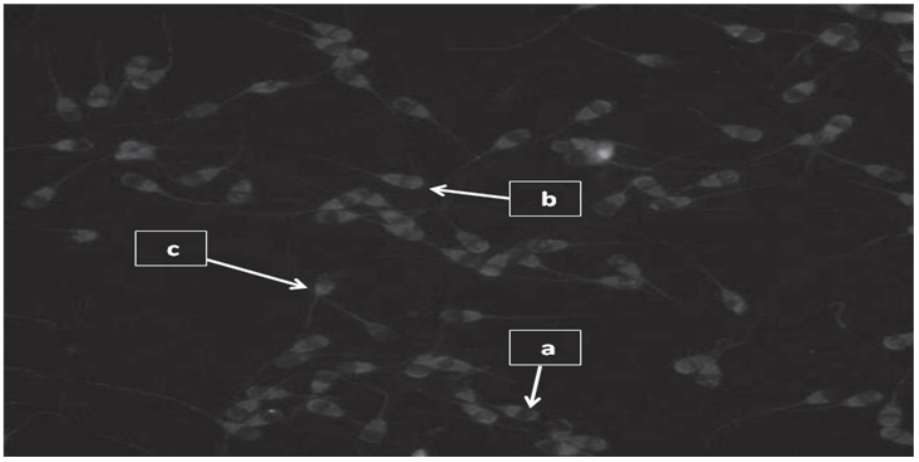

Figure 4. Immunofluoresence of anti-TIMP-2 with cattle bull spermatozoa. Signal on (a) acrosomal cap, (b) acrosome and (c) post acrosomal cap.

Characterization of FA-1 like proteins in seminal plasma and spermatozoa of different bulls and their association with in vitro acrosome reaction: Anti-FA-1 reacted with 11 and 16 kDa SP proteins of only 20 and 11 bulls respectively (Fig 5). In addition to cross reaction of anti-FA-1 to 16 kDa sperm protein of 9 bulls, it also cross reacted with 60 and 45 kDa sperm proteins of 10 bulls (Fig 6). Western blot analysis revealed that the FA-1 mAb to human sperm plasma membrane protein reacted with proteins of similar molecular mass (53 +/- 2 kDa) in human and bovine sodium deoxycholate (DOC)-solubilized sperm extracts (Coonord *et al.,* 1994). These antibodies also specifically recognized a single protein band of 51 +/- 2 kDa, corresponding to the dimeric form of FA-1 antigen, on a western blot of lithium diiodosalicylate (LIS)-solubilized monkey sperm.

Among the bulls, positive for 60, 45, 16 kDa FA-1 like proteins in sperm extracts and 11/ 16 kDa in SP; 7, 8, 6 and 7 bulls also showed higher rate (35.1-54.9%) of in vitro acrosome reaction. It reveals that 60, 45 and 16 kDA FA-1 like proteins may play an important role in capacitation/ acrosome reaction of cattle bull spermatozoa. Anti-FA-1 antibodies, when present in the insemination mixture,

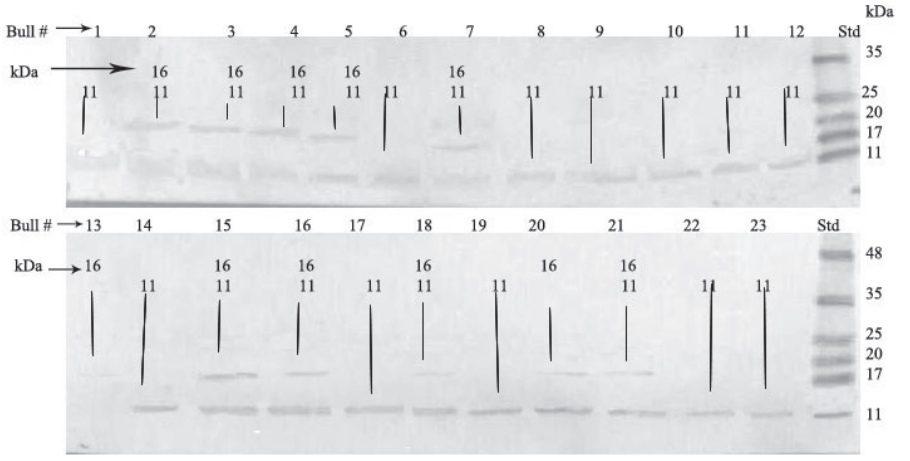

Figure 5. Immunoblting of cattle bull seminal plasma with anti FA-1 antibody. Seminal plasma proteins separated by SDS-PAGE were reacted with anti-FA-1 antibody on immunoblots to detect variation in FA-1 like proteins in seminal plasma of different bulls. Std (Marker) and 1-23 (Bull numbers).

inhibited the in vitro fertilization (IVF) of monkey oocytes (Naz and Wolf 1994) and further Coonord *et al.,* 1994) performed in vitro fertilization (IVF) trials in bovine, using oocytes obtained from slaughterhouse ovaries and concluded that

addition of 20, 40, or 80 µg/ml of FA-1 mAb to the IVF medium resulted in a linear decrease in the fertilization rate from 86.3% in the controls to 54.6%, 21.6%, and 1.8% in the respective experimental groups ($p < 0.01$). It indicates that FA-1 plays an important role in fertilization of mammalian spermatozoa including bovine. Since capacitation and acrosome reaction are pre-requisite for fertilization, therefore, a higher fertility rate can be expected in the bulls positive for 60, 45 and 16 kDa FA-1 like sperm proteins and with higher rate of in vitro acrosome reaction. Coonrod et al (1994) and Kadam et al (1995) were also of the opinion that FA-1 played an important role in fertility of mammalian spermatozoa.

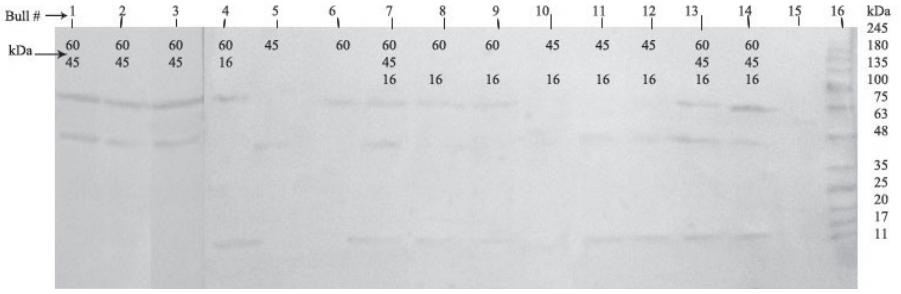

Figure 6. Immunoblting of cattle bull sperm extracts with anti-FA-1 antibody. SDS extracts of 15 bulls were reacted with anti-FA-1 on immunoblots to detect variation in FA-1 like proteins in spermatozoa of different bulls. Track # 16 (Marker) and 1-15 (Bull # 21, 7, 8, 3, 19, 20, 24, 9, 10, 12, 15, 16, 22, 17, 23).

Characterization of TIMP-2 like proteins in seminal plasma and spermatozoa of different bulls and their association with in vitro acrosome reaction: Expression of 65, 55, 48, 35, 24 and 16 kDa proteins was obtained with anti-TIMP-2 in SP of 22, 14, 1, 16, 23 and 2 bulls, respectively (Fig 7). The presence of TIMP-2 in seminal plasma has been documented for bulls (Liberda *et al.,* 2001; McCauley *et al.,* 2001; Calvete *et al.,* 1996), humans (Shimokawa *et al.,* 2003; Baumgart *et al.,* 2002a and 2002b) and in testicular and epididymal fluids from rams and stallions (Metayer *et al.,* 2002). In addition to expression of 65, 55, 48, 35 and 24 kDa proteins in 11, 1, 6, 2 and 12 bulls, expression of 45, 40 kDa proteins was also detectable in sperm extracts of 2 and 6 bulls respectively (Fig 8).

Among the bulls positive for 65, 55, 48, 35, 24 and 16 kDa SP-TIMP-2 like proteins; 11, 7, 1, 7, 11 and 1 bulls also had higher percentage (35.1-54.9%) of in vitro acrosome reacted spermatozoa. Whereas, rate of acrosome reaction was higher (35.1-54.9%) in 8, 2, 1, 5, 8 and 5 bulls positive for 65, 48, 45, 40, 24 and 16 kDa SE-TIMP-2 like proteins. Number of bulls positive for 65, 24 kDa

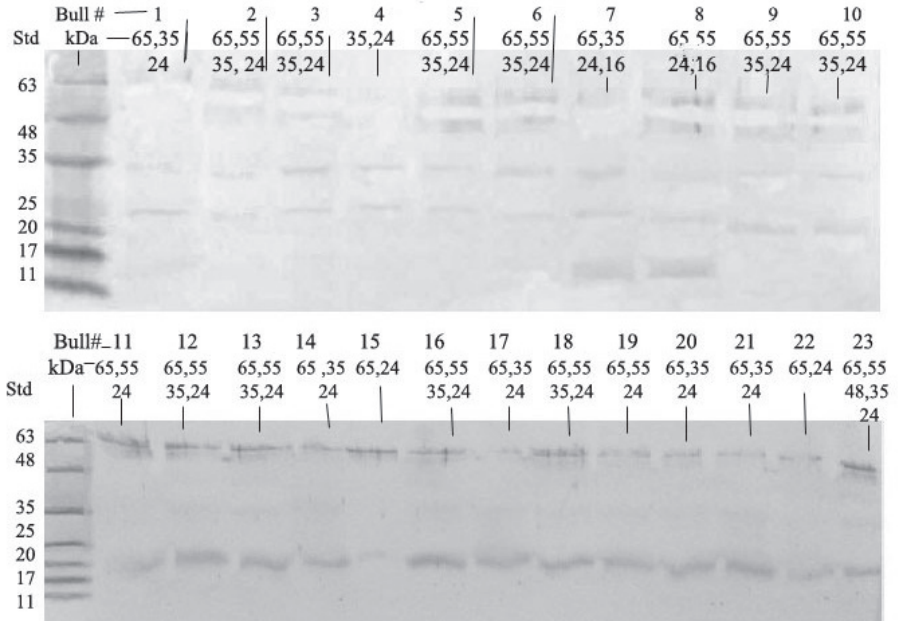

Figure 7. Immunoblotting of seminal plasma with anti TIMP-2 antibody. Seminal plasma proteins separated by SDS-PAGE were reacted with anti-FA-1 on immunoblots to detect variation in FA-1 like proteins in seminal plasma of different bulls. Std (Marker) and 1-23 (Bull numbers).

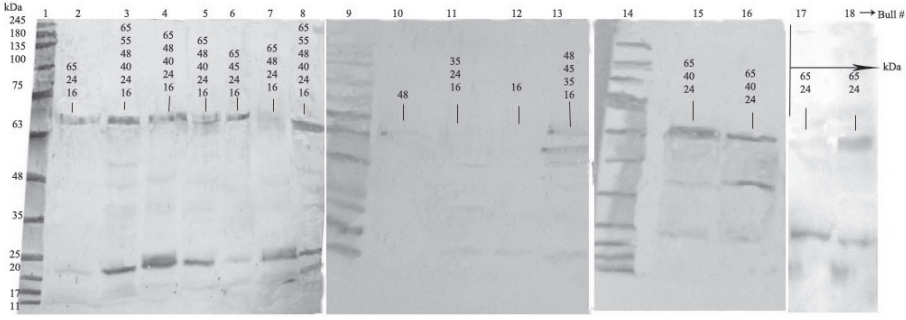

Figure 8. Immunoblotting of cattle bull sperm extracts with anti TIMP-2 antibody. SDS extracts of 15 bulls were reacted with anti-FA-1 on immunoblots to detect variation in TIMP-2 like proteins in spermatozoa of different bulls. Track # 1, 9, 14 (Marker) and 2-8 (Bull # 15, 16, 21, 23, 19, 20, 24); 10-13 (Bull # 3, 7, 8, 9) and 15-18 (Bull # 10, 12, 17, 22).

proteins and with higher rate of acrosome reaction was more as compared to other anti-TIMP-2 reactive sperm/ SP proteins. Therefore, 65 and 24 kDa TIMP-2 like proteins may serve as indicators of higher rate of in vitro acrosome reaction vis a vis fertility of cattle bulls. Newton et al (2009) also identified a 25 kDa sperm protein (TIMP-2) that could serve as a molecular marker of sperm impaired function due to elevated testicular temperature with important implications for fertility prediction.

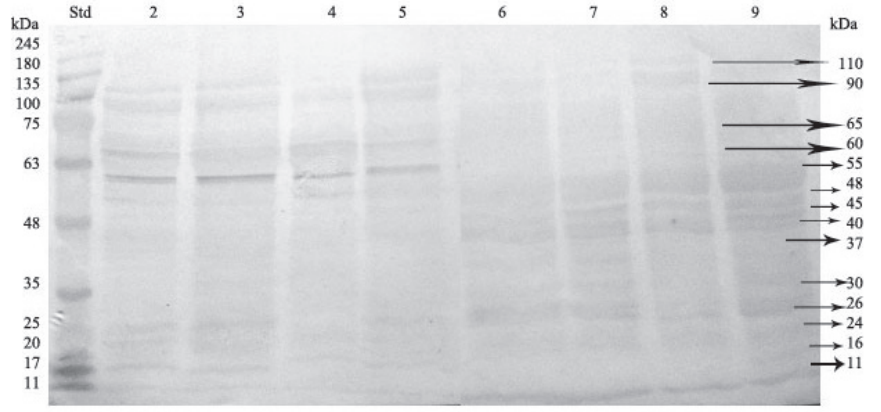

Fig.9. Immunoblotting of SDS sperm extracts of capacitated (2-5) and uncapacitated (6-9) spermatozoa with anti-HBP. SDS sperm extracts of capacitated and un capacitated spermatozoa were reacted with anti-HBP on immunoblots to identify changes in HBP like proteins in cattle bull spermatozoa after capacitation/acrosome reaction. Bulls were grouped on the basis of percent acrosome reaction, i.e. G-1 (51-55%), G-2 (41-50%), G-3 (31-40%) and G-4 (21-30%). Std (Marker). Track # 1/5 (G-1), 2/6 (G-2) and 3/7 (G-3) and 4/8 (G-4).

Alterations in HBP after in vitro capacitation/Acrosome reaction: Immunoblotting of anti-HBP with SE of capacitated spermatozoa revealed the expression of 110, 90, 65, 60, 55, 26, 24 and 16 kDa proteins, whereas, that with uncapacitated spermatozoa resulted in expression of 110, 90, 55, 48, 45, 40, 37, 30 and 24 kDa proteins (Fig 9). Expression of 110 and 90 kDa proteins in capapcitated spermatozoa was strong as compared to uncapacitated, which indicated that these proteins were more exposed in acrosome reacted spermatozoa (Fig 9). Reaction of 65, 60, 26 and 16 kDa proteins of only capacitated/ acrosome reacted spermatozoa with anti-HBP revealed that these HBP are exposed on cattle bull sperm membrane only during capacitation/acrosome reaction. Cross-reaction of 55, 48, 45, 40, 37 and 30 kDa proteins of only uncapacitated spermatozoa with

anti-HBP suggested the deletion of these proteins during capacitation/ acrosome reaction. Immunoblotting of capacitated and uncapacitated spermatozoa with anti-HBP did not show any difference with respect to rate of acrosome reaction (Fig). Heparin-binding proteins (HBPs) are essential constituents of seminal fluid, which bind to sperm lipids containing the phosphorylcholine group and mediate the fertilization process. It has been previously shown that the heparin in seminal fluid stimulates sperm capacitation in bulls and seminal fluid HBPs are supposed to attach themselves to the sperm surface, especially lipids containing the phosphoryl-choline group, thus allowing heparin-like GAGs in the female reproductive tract to activate sperm capacitation (Miller *et al.,* 1990). In bovine non-capacitated sperm, lectin-like heparin binding proteins such as bovine seminal plasma protein family members (BSP A1, BSP A2, BSP A3 and BSP-30-kDa), bind to fucose, an oviduct epithelial trisaccharide, to produce a sperm reservoir in the oviduct prior to ovulation. These sperm binding proteins play a crucial role in fertility by maintaining sperm motility and viability during storage (58, 61-63). HBPs have been viewed as actually favoring capacitation, acrosome reaction, and altering the immune system response toward the sperm, especially fertility associated antigen (FAA, 31 kDa) and the Type-2 tissue inhibitor of metalloproteinase (TIMP-2, 24 kDa, Alvarez-Gallardo *et al.,* 2014). Keeping in view the importance of HBP in bovine semen fertility, it can be predicted that removal of 55, 48, 45, 40, 37 and 30 kDa proteins from in vitro acrosome reacted spermatozoa allowed heparin to mediate in vitro acrosome reaction. Further increase in intensity of 110, 90 kDa and exposure of 65, 60, 26 and 16 kDa HBP after acrosome reaction may be important for binding of sperm to ovum, penetration and fertilization.

Conclusion

It can be concluded that FA-1 (60, 45, 16 kDa) and TIMP-2 (65, 24 kDa) like proteins of cross-bred bull spermatozoa and seminal plasma may act as indicators of fertility and heparin binding proteins gets altered in in vitro capacitated and acrosome reacted spermatozoa.

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