Identification of Heparin Binding Proteins in Frozen-thawed Capacitated/Acrosome-reacted Spermatozoa and Their Relationship with Fertility of Buffalo Bull Semen

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Publication Date: 26 February 2016

Article Link: http://scientific.cloud-journals.com/index.php/IJAVST/article/view/Sci-394

Abstract The present was conducted to separate heparin binding proteins (HBP) of capacitated sperm extracts and determine their relationship with acrosome reaction and semen fertility of bulls (n=30). Fourteen immunoreactive bands in capacitated spermatozoa of bulls (135, 100, 75, 70, 65, 55, 48, 45, 33, 31, 26, 24, 20 and 18 kDa) were detected in western blots following incubation with anti-HBP antibody. The frozen-thawed semen was evaluated for first service conception rate (FSCR) and percent acrosome reaction and linked to HBP. In capacitated spermatozoa, bulls positive for 70, 31, 20 and 18 kDa had significantly higher (P < 0.05) FSCR (42.3 ± 5.1 vs 32.9 ± 3.9%, 45.0 ± 6.5 vs 34.1 ± 3.5%, 60.0 ± 10.0 vs 35.2 ± 3.3% and 42.9 ± 5.1 vs 31.9 ± 3.7%), respectively, as compared to their negative counterparts. Although non-significant, FSCR was reasonably higher (P > 0.05) in bulls positive for 135 and 100 kDa HBP than in their negative contemporary mates and exhibited an increase of 7.4% for the two proteins. A significantly higher (P < 0.05) rate of acrosome reaction was recorded in bulls with HBP of 135, 100, 70 and 18 kDa in comparison to their contemporary mates with a difference of 7.4, 7.4, 8.7 and 9.4%, respectively. Alternatively, FSCR and acrosome reaction of bulls with 75, 55 and 48 kDa were lower (P < 0.05) than their corresponding counterparts. In conclusion, HBP of 135, 100, 70 and 18 kDa did seem to activate in vitro sperm acrosome reaction and subsequent fertility of bulls.

Keywords Acrosome Reaction; Buffalo Bull; Capacitation; HBP; FSCR; Semen

1. Introduction

The heparin binding proteins (HBP) are produced by male accessory sex glands and upon ejaculation bind to the sperm [1]. The HBP bind to sperm membrane choline phospholipids and capacitation factors viz. heparin and glycosaminoglycans at ejaculation resulting in acrosome reaction, sperm-oocyte fusion and fertilization [2]. HBP have predominately been linked to bull fertility potential. Five proteins with molecular weight of 18, 31, 33, 48 and 55 kDa have been identified as members of HBP family and are referred to as fertility-associated antigens [3]. Another HBP (24 kDa) was found to have an amino acid sequence related to tissue inhibitor of metallo-proteinases-2 and played an
important role in buffalo bull fertility [4]. Further, two proteins (BSP-A1 and BSP-A2) exhibited their binding capacity to heparin and could be used as biochemical markers to predict fertility potential of bulls [5]. The HBP protect sperm from stress of freezing and thawing and maintain intracellular protein homeostasis [6]. Characterizing functionally important HBP is a first step toward better understanding the modulating effects of seminal fluid on fertility of buffalo bulls. In addition, fertilization potential of spermatozoa in vitro is determined by capacitation / acrosome reaction status which involve different signal transduction pathways [7]. Keeping in view of above facts and also taken into consideration the deficit knowledge of HBP in buffalo bulls, the present study was designed to characterize HBP in capacitated frozen-thawed spermatozoa and determine their relationship with fertility of buffalo bull semen.

2. Materials and Methods

2.1. Procurement of Semen

Frozen semen (30 straws per bull) from thirty breeding Murrah buffalo bulls were procured from two government semen processing and freezing laboratories in the month of September having ambient temperature 30.6°C and relative humidity 92%. The straws (0.25 ml each) were frozen from same ejaculate on same date and earmarked for the present study.

2.2. In Vitro Capacitation Acrosome Reaction

Semen from ten straws per bull was taken in 15 ml graduated tube and washed twice with the basic TALP medium (2 ml; 92.9 mM NaCl, 4 mM KCl, 25.9 mM NaHCO3, Na2HPO4, 10 mM CaCl2.2H2O, 0.5 mM MgCl2.6H2O, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM HEPES) by centrifuging at 1000 rpm for 5 minutes [8]. The sperm suspension was then re-suspended in the energy rich medium (0.5 ml; 10 ml basic TALP medium, 0.25% glucose, 0.6% bovine serum albumin, 10 mg of streptomycin and 100 µl of 0.1% stock solution of heparin), transferred in eppendorf tubes and placed in the incubator (5% CO2) at 37°C for 6 h. Capacitation status was assessed at every 2 h interval for 6 h. At the end of every 2 h, a 10 μl sperm suspension was removed from the aliquot, smear was prepared, stained using Giemsa stain and assessed for acrosome reaction. The evaluation of the acrosome reaction was carried out by counting 200 spermatozoa from each smear under bright field microscope (100x). The spermatozoa showing complete sequence of capacitation events viz. swelling, vesciculation and shedding at 6 h were considered as acrosome-reacted.

2.3. Semen Fertility

The number of females inseminated per bull semen was ten. Therefore, ten mini straws from each bull were used for the field fertility trial. All the buffaloes (n = 300) enrolled for fixed time insemination program (October to April) were healthy, multiparous (2nd to 5th parity), recently calved (60-80 days earlier), free from physical problems, vaginal discharge and maintained under standard feeding and management systems. Prior to the start of breeding program, clinical assessment of genitalia was done ultrasonographically using a B-mode linear array trans-rectal transducer with 5/7.5 MHz interchangeable frequency (EXAGO, ECM, France) to visualize a cyclic CL twice at 10 days apart and rule out the possibility of reproductive tract infections, if any. The buffaloes were synchronized using double ovsynch protocol (PGF2α-GnRH-PGF2α-GnRH on day -2, 0, 7 and 9, respectively) followed by fixed time inseminations at 16 and 40 h after last GnRH injection, respectively. 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:
2.4. Extraction of Sperm Proteins

The frozen-thawed semen (20 straws from each bull) were centrifuged at 3000 rpm for 10 minutes to separate out and discard dilutor. The remnant sperm pellet obtained from frozen-thawed semen was washed twice with PBS (pH 7.4), suspended in 1.0 ml of 2% SDS in 62.5 mM Tris-HCl (pH 8.0) containing protease inhibitors (Cocktail, SERVA). The sperm suspensions were sonicated at 4°C (20 watts, three times for 20 seconds each) and then centrifuged at 10000 rpm for 15 minutes. The pellet was discarded and sperm extracts (SE) were collected and stored in 0.5 ml fractions of cryovials at -20°C till further use.

2.5. SDS-PAGE and Immunoblotting

Exactly 100 µg of protein was fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel and 4% stacking gel. After electrophoresis, enzyme linked immuno transfer blot was done as per the method of Towbin et al. [9]. The frozen-thawed sperm extracts were reacted with anti-HBP (anti AZU-1, Sino Biological) to correlate such sperm specific proteins of buffalo bull spermatozoa with semen function tests and bull fertility. 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).

2.6. 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 tests 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.

3. Results and Discussion

3.1. Identification of HBP in Frozen-Thawed Capacitated Sperm Extracts by Immunoblotting

Blot images of protein bands in capacitated frozen-thawed sperm extracts of all 30 bulls have been shown in Figure 1. Anti-HBP (anti-AZU-1) recognized fourteen proteins of 135, 100, 75, 70, 65, 55, 48, 45, 33, 31, 26, 24, 20 and 18 kDa in capacitated spermatozoa of 10, 10, 13, 13, 17, 4, 4, 23, 16, 8, 5, 26, 3 and 14 bulls, respectively (Table 1). To the best of our knowledge, this is the first study to characterize HBP in frozen-thawed capacitated spermatozoa of buffalo bulls. The electrophoretic profiles showed polymorphism among individual semen samples ranging from 2-10 proteins in post-thaw capacitated semen. However, no individual tested bull had all bands in the capacitated sperm extracts. Therefore, qualitative differences (presence or absence of bands) were observed in HBP bands of the 30 bull capacitated frozen-thawed sperm extracts. Moreover, the inherent character of the proteins may also contribute toward the difference in number of bands. The findings are in accordance with the observations of Arangasamy [10] and Singh et al. [11] who reported eight (13, 14, 16, 20, 36, 41, 56 and 71 kDa) and nine HBP (14, 15, 20, 24, 33, 40, 55, 70 and 100 kDa), respectively in buffalo seminal plasma. Studies using one dimensional polyacrylamide gel
Electrophoresis detected 35 bands of HBP in seminal plasma of rams and was associated with the sperm capacitation process [12]. Further, Moura et al. [13] detected 52 spots in accessory sex gland fluid of Holstein bulls by 2-dimensional electrophoresis indicating qualitative differences in HBP bands of all the bulls.

**Figure 1:** Immunoblotting Pattern of HBP in Frozen-Thawed Capacitated/Acrosome-Reacted Sperm Extracts of Buffalo Bulls. Lane Std: Standard Protein Marker; Lanes 1-30: Bull Numbers

### 3.2. Relationship of HBP differences with Acrosome Reaction and Fertility in Frozen-thawed Capacitated Sperm Extracts

The FSCR of bulls with HBP of 70, 31, 20 and 18 kDa was significantly (P < 0.05) higher than their counterparts. Although non-significant (P > 0.05), the FSCR was also higher in bulls positive for 135 and 100 kDa HBP as compared to their negative contemporary mates. An increase of 7.4, 7.4, 9.4, 10.9, 24.8 and 11.0% in the FSCR was recorded in the bulls with 135, 100, 70, 31, 20 and 18 kDa HBP, respectively as compared to their counterparts. The percentage of bulls with >50.0% FSCR was also higher (50.0, 50.0, 46.2, 62.5, 66.7 and 50.0%) amongst those positive for 135, 100, 70, 31, 20 and 18 kDa HBP than their respective counter mates (25.0, 25.0, 23.5, 22.7, 29.6 and 18.8%). No significant (P > 0.05) difference in the rate of acrosome reaction was observed between the bulls positive and negative for a 20 kDa HBP whereas, a significantly (P < 0.05) higher rate of acrosome reaction was recorded in bulls with HBP of 135, 100, 70 and 18 kDa in comparison to their contemporary mates with a difference of 7.4, 7.4, 8.7 and 9.4%, respectively. The FSCR of bulls with 75 kDa (P < 0.05), 55 kDa (P < 0.05) and 48 kDa (P > 0.05) was lower than their corresponding counterparts and had a decrease of 11.0, 16.7 and 11.0%, respectively. In addition, the proportion of bulls with good fertility was lower in the bull’s positive as compared to negative ones (15.4, 0.0 and
0.0% vs 61.5, 38.7 and 38.7%, respectively). Likewise, the rate of acrosome reaction of bulls with HBP of 75, 55 and 48 kDa was significantly lower (P < 0.05) than their negative contemporary mates and a respective reduction of 7.3, 14.7 and 11.7% was observed. Similar studies [14] in buffalo bulls showed that heparin is the most potent enhancer of capacitation in buffalo spermatozoa. Further, induction of sperm capacitation in female reproductive tract is aided by HBP secreted by male accessory sex glands [15]. The HBP in seminal fluid attach themselves to the sperm surface, especially lipids containing the phosphoryl-choline group, thus allowing heparin-like glycosaminoglycans in female reproductive tract to activate sperm capacitation [4]. Therefore, seminal fluid HBP play a vital role in spermatozoon survival and the overall fertilization process, and any alteration in these proteins can directly be related to infertility.

**Table 1: Relationship of HBP with FSCR and Acrosome Reaction in Frozen-Thawed Capacitated Semen of Buffalo Bulls (Mean ± SE)**

| Mol. Wt. (kDa) | Overall FSCR (%) | Bulls positive for HBP | Bulls negative for HBP | Overall FSCR (%) | Percentage of bulls with ≥ 50.0% FSCR | Percent acrosome-reacted spermatozoa | Percent acrosome-reacted spermatozoa |
|----------------|-------------------|------------------------|------------------------|-------------------|----------------------------------------|-------------------------------------|-------------------------------------|
| 135            | 40.9 ± 6.9        | 50.0 (5)               | 56.7 ± 2.5             | 33.5 ± 3.4        | 56.7 (5)                               | 25.0 (5)                             | 49.3 ± 2.3                          |
| 100            | 40.9 ± 6.9        | 50.0 (5)               | 56.7 ± 2.5             | 33.5 ± 3.4        | 56.7 (5)                               | 25.0 (5)                             | 49.3 ± 2.3                          |
| 75             | 30.8 ± 4.0        | 15.4 (2)               | 47.6 ± 2.9             | 41.8 ± 4.5        | 47.1 (8)                               | 23.5 (4)                             | 48.0 ± 2.5                          |
| 42.3 ± 5.1     | 46.2 (6)          | 56.7 ± 2.0             | 32.9 ± 3.9             | 32.9 ± 3.9        | 38.5 (5)                               | 51.8 ± 2.9                          |
| 55             | 22.5 ± 6.3        | 0.0 (0)                | 39.3 ± 2.5             | 39.2 ± 3.9        | 38.5 (10)                              | 53.9 ± 1.7                          |
| 48             | 27.5 ± 7.5        | 0.0 (0)                | 41.6 ± 3.9             | 38.5 ± 3.4        | 38.5 (10)                              | 53.3 ± 1.9                          |
| 45             | 37.8 ± 3.7        | 34.8 (8)               | 52.7 ± 2.1             | 34.3 ± 6.5        | 28.6 (2)                               | 48.7 ± 3.8                          |
| 33             | 36.4 ± 4.4        | 25.0 (4)               | 49.9 ± 2.9             | 37.5 ± 4.7        | 42.9 (6)                               | 53.3 ± 2.3                          |
| 31             | 45.0 ± 6.5        | 62.5 (5)               | 56.1 ± 3.3             | 34.1 ± 3.3        | 27.7 (5)                               | 50.9 ± 2.2                          |
| 26             | 38.0 ± 7.3        | 40.0 (2)               | 56.7 ± 4.3             | 36.8 ± 3.6        | 32.0 (8)                               | 50.7 ± 2.0                          |
| 24             | 36.2 ± 3.5        | 30.8 (8)               | 51.0 ± 1.9             | 42.5 ± 7.5        | 50.0 (2)                               | 56.8 ± 5.5                          |
| 20             | 60.0 ± 10.0       | 66.7 (2)               | 49.8 ± 5.1             | 35.2 ± 3.3        | 29.6 (8)                               | 51.9 ± 2.0                          |
| 18             | 42.9 ± 5.1        | 50.0 (7)               | 56.7 ± 2.3             | 31.9 ± 3.7        | 18.8 (3)                               | 47.3 ± 2.3                          |

*Superscripts a,b differ significantly (P < 0.05) in same row for overall FSCR.
*Superscripts c,d differ significantly (P < 0.05) in same row for acrosome-reacted spermatozoa.
*Figures in parentheses with * indicate number of tested bulls with ≥ 50.0% FSCR.

4. Conclusion

In conclusion, immunoblotting demonstrated that HBP are present on surface of capacitated spermatozoa and may play a significant role in regulating reproductive potential of buffalo bull semen. The HBP of 135, 100, 70 and 18 kDa did seem to activate the *in vitro* sperm acrosome reaction vis-à-vis higher conception rate in bulls’ positive for these proteins as compared to their negative counterparts. More studies are clearly required to validate the functional relationship between presence of HBP on capacitated sperm and fertility of buffalo bulls.

References

[1] Manjunath, P., Nauc, V., Bergeron, A., and Enard, M. Major Proteins of Bovine Seminal Plasma Bind to the Low Density Lipoprotein Fraction of Hens Egg Yolk. Biology of Reproduction. 2002. 67; 1250-1258.

[2] Divyaswetha, P., Bindu, N., Abdullah, K., Jean, M.F., Shane, C.B., and Erdogan, M. Comprehensive Proteomic Analysis of Bovine Spermatozoa of Varying Fertility Rates and Identification of Biomarkers Associated with Fertility. Systems Biology. 2008. 2; 1-14.
[3] McCauley, T.C., Zhang, H.M., Bellin, M.E., and Ax, R.L. *Purification and Characterization of Fertility Associated Antigen (FAA) in Bovine Seminal Fluid.* Molecular Reproduction and Development. 1999. 54; 145-153.

[4] McCauley, T.C., Zhang, H.M., Bellin, M.E., and Ax, R.L. *Identification of a Heparin Binding Protein in Bovine Seminal Fluid as Tissue Inhibitor of Metalloproteinases-2.* Molecular Reproduction and Development. 2001. 56; 336-341.

[5] Gwathmey, T.M., Ignotz, G.G., and Suarez, S.S. *PDC-109 (BSP A1/A2) Promotes Bull Sperm Binding to Oviductal Epithelium In Vitro and May be Involved in Forming the Oviductal Sperm Reservoir.* Biology of Reproduction. 2003. 69; 809-815.

[6] Shi, Y., Mosser, D.D., and Morimoto, R.I. *Molecular Chaperones as HSF I-Specific Transcriptional Repressors.* Genes and Development. 1998. 12; 654-666.

[7] Newton, L.D., Krishnakumar, S., Menon, A.G., Kastelic, J.P., Vander-Hoorn, F.A., and Thundathil, J.C. *Na+K+-ATPase Regulates Sperm Capacitation Through a Mechanism Involving Kinases and Redistribution of its Testis-specific Isoform.* Molecular Reproduction and Development. 2010. 77; 136-148.

[8] Yanagimachi, R., 1994: *Mammalian Fertilization.* In: Knobil, E., and Neill, J.D. (Eds.), Physiology of Reproduction, 2nd edn. New York: Raven Press. 189-317.

[9] Towbin, H., Staehelin, T., and Gordon, J. *Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications.* In: Proceedings of National Academy of Sciences, USA. 1979. 76; 4350-4354.

[10] Arangasamy, A. *Isolation of Buffalo Seminal Plasma Proteins and Their Effect on In Vitro Capacitation, Acrosome reaction and Fertilizing Potential of Spermatozoa.* Ph.D. Thesis submitted to IVRI (UP). 2003. India.

[11] Singh, M., Ghosh, S.K., Prasad, J.K., Kumar, A., Ramteke, S.S., and Bhure, S.K. *Heparin Binding Proteins of Buffalo Bulls Seminal Plasma and Their Relationship with Semen Freezability.* Indian Journal of Animal Sciences. 2013. 83 (7) 700-704.

[12] Martins, J.A.M., Souza, C.E.A., Silva, F.D.A., Cadavid, V.G., Nogueira, F.C., Domont, G.B., de Oliveira, J.T.A., and Moura, A.A. *Major Heparin-binding Proteins of the Seminal Plasma from Morada Nova Rams.* Small Ruminant Research. 2013. 113; 115-127.

[13] Moura, A.A., Koc, H., Chapman, D.A., and Killian, G.J. *Identification of Proteins in the Accessory Sex Gland Fluid Associated with Fertility Indexes of Dairy Bulls: A Proteomic Approach.* Journal of Andrology. 2006. 27; 201-211.

[14] Harshan, H.M., Singh, L.P., Arangasamy, A., Ansari, M.R. and Kumar, S. *Effect of Buffalo Seminal Plasma Heparin Binding Protein (HBP) on Freezability and In Vitro Fertility of Buffalo Cauda Spermatozoa.* Animal Reproduction Science. 2006. 93; 124-133.

[15] Kumar, V., Hassan, M.I., Tomar, A.K., Kashav, T., Nautiyal, J., Singh, S., Singh, T.P., and Yadav, S. *Proteomic Analysis of Heparin-binding Proteins from Human Seminal Plasma: A Step towards Identification of Molecular Markers of Male Fertility.* Journal of Bioscience. 2009. 34; 899-908.