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[1]Department of Zoology & Fisheries and [2]Department of Animal Breeding & Genetics, Punjab Agricultural University, Ludhiana, India

CHARANJIT KAUR DHANJU [1], RAVINDER KAUR [1] and RANJNA S. CHEEMA [2]

Protein-leakage during heparin – induced *in-vitro* capacitation of bull sperm

Abstract

Freshly ejaculated washed spermatozoa of six cross-bred bulls were capacitated by incubating in TALP medium supplemented with BSA, heparin and hepes buffer at a sperm concentration of 6 X 10^9 s/ml at 37^o C for 6 h. A control was also run in 0.85% saline. Percentage motility was observed at two h interval. Sperm smears were stained with giemsa and observed under oil emulsion to study different stages of acrosome reaction. After six h of incubation, sperm membrane proteins were extracted from capacitated- and uncapacitated- spermatozoa using deoxycholate in Tris-HCl (0.02M). Spermatozoa, incubation media (IM) and sperm membrane extract (SME) were analyzed for total proteins which were further fractionated by SDS-PAGE. Antisera raised against SME of bull # 1, 2 and 3 were used for identification of antigens in SME of all the bulls using immunodiffusion technique.

After incubation of spermatozoa for 6 h, there was 15-21% and 55-62% decline in motility of capacitated- and uncapacitated- spermatozoa respectively. A total of 62-87% spermatozoa showed acrosome reaction (swelling, vesiculation and shedding of acrosome) in all the bulls. A statistically significant ($P<0.05$) decrease in protein content of spermatozoa was observed during capacitation. The percentage extraction of protein and percentage leakage of proteins into medium showed a significant correlation with percentage acrosome reaction in respective bulls. A higher and positive correlation of rate of acrosome reaction with protein content of capacitated- and uncapacitated- spermatozoa and IM indicates that percentage capacitation in correlation to fertility can be predicted from the protein content of spermatozoa and proteins released into the medium. Protein profile revealed that from 12.5 to 70 kDa proteins get exposed during capacitation. There was leakage of 4-7 proteins, mainly of medium (70-130 kDa) and low mol wt (<12.5-35 kDa) from capacitated spermatozoa, whereas only 2-5 proteins of high (160->240 kDa) and low (<12.5-25 kDa) mol wt proteins get leaked out from uncapacitated spermatozoa. Immunodiffusion reaction of SME of bull # 1, 2 and 3 also suggested the removal/exposure of antigens from sperm surface during capacitation and acrosome reaction.

Key Words: sperm, capacitation, protein, leakage, cattle bulls

Zusammenfassung

Titel der Arbeit: **Proteinfreisetzung während der Heparin-induzierten *in vitro* Kapazitierung von Bullenspermien**

Frisch ejakuliertes Sperma von sechs Kreuzungsbullen wurde gewonnen und einer Kapazitierung von 6 Stunden unterzogen. Die Kapazitierung erfolgte durch Inkubation in TALP Medium supplementiert mit BSA., Heparin und Hepes-Puffer bei 37 °C. Als Kontrolle diente eine Probe in physiologischer Kochsalzlösung. Der Anteil motiler Spermien wurde in Intervallen von 2 Stunden bestimmt. Die Spermienausstriche wurden mit Giemsa angefärbt um die Akrosomenreaktion zu bestimmen. Sechs Stunden nach Inkubation wurde das Protein der Membranen aus kapazitierten und nicht kapazitierten Spermien extrahiert. In Spermien, Inkubationsmedium (IM) und dem Extrakt der Spermienmembran (SME) wurde das Gesamtprotein bestimmt, welches dann weiter mit DSD-PAGE fraktioniert wurde. Antiseren gegen SME der Bullen 1,2 und 3 wurden für die Identifikation der Antigene im SME genutzt (Immunodiffusionstechnik). Nach der Inkubation der Spermien für 6 h konnte ein Rückgang der Spermien Motilität um 15-21 % bei kapazitierten und 55-62 % bei nicht kapazitierten Spermien beobachtet werden. Insgesamt zeigten 62-87 % der Spermien eine Akrosomenreaktion (Schwellung, Vesikelformation, Akrosomenverlust) bei allen Bullen. Während der Kapazitierung wurde eine Verringerung des Proteingehaltes beobachtet ($P<0,05$). Die hohe positive Korrelation der Akrosomenreaktion mit dem Proteingehalt von kapazitierten und nicht kapazitierten Spermien und dem Inkubationsmedium zeigen, dass der prozentuale Anteil der Kapazitation gegenüber der Fruchtbarkeit anhand des Proteingehaltes in den Spermien

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und dem Inkubationsmedium vorhergesagt werden kann. In kapazitierten Spermien wurden hauptsächlich Proteine mit einem mittleren (70-130 kDa) und niedrigem (<123,5-35 kDa) Molekulargewicht freigesetzt. In nicht kapazitierten Spermien waren es Proteine mit einem Molekulargewicht von 160->240 kDa und <12,5-35 kDa.

Schlüsselwörter: Sperma, Kapazitation, Protein, Proteinverlust, Bullen

Introduction

Capacitation is a post-testicular developmental and maturational process of mammalian spermatozoa occurring during their transit through the female reproductive tract. Capacitation is a collective term involving biochemical modifications of membrane characteristics, enzymatic activity and motility changes. It is a multifaceted phenomenon correlated with changes in spermatozoal metabolism, plasma membrane fluidity and thus membrane reorganization, intracellular ion and cAMP concentrations, pH and reactive oxygen species. Cholesterol efflux occurs and an asymmetric phospholipids distribution is established during capacitation. In addition, sperm surface proteins are modified, added or removed and an array of proteins have been shown to undergo tyrosine phosphorylation in different species (CARRERA et al., 1996; LUCONI et al., 1996; GALANTINO et al., 1997). However, so far the substrates of tyrosine phosphorylation particularly in human and cattle has not yet been investigated. Therefore sperm surface proteins involved in capacitation in cattle bull need to be identified to understand this process in this important dairy animal and furthermore, establishment of their antigenicity can be of significant value in developing fertility markers to evaluate their reproductive status in early years of reproductive life and prevent losses to our dairy industry due to their declining fertility.

Material and methods

Freshly ejaculated semen of six cross-bred cattle bulls (HHS, Holstein Friesen x Sahiwal; FC, Fresian Crosses; 1F and 4F first and fourth generation of inter-re-breeding) with more than 76% motility of spermatozoa was procured from dairy farm, Punjab Agricultural University Ludhiana. The samples were transferred to the lab at 37^{o} C, and immediately processed for capacitation and acrosome reaction.

Capacitation and acrosome reaction:

Washed spermatozoa were suspended in TALP medium (NaCl-92.9mM; KCl- 4mM; $NaHCO_3$-25.9 mM; $CaCl_2$.$2H_2O$- 10mM; $MgCl_2$.6 H_2O- 0.5mM; sodium lactate-7.6mM; sodium pyruvate- 1.3mM; HEPES- 20mM; glucose- 0.25%; heparin-200µg/ml and BSA- 0.6%) at concentration of 6 X 10^9 spermatozoa/ml. Similarly a control was also run in 0.85% saline. Spermatozoa, suspended in TALP medium and 0.85% saline were incubated at 37^{o} C for 6 h. Percentage motility was observed at zero h, 2 h, 4 h and 6 h of incubation. Smears were prepared after 6 h of incubation and stained with giemsa and examined under oil emulsion using binocular microscope and percentage acrosome reaction of spermatozoa of six bulls was compared.

Extraction, estimation and analysis of sperm membrane proteins:

Two ml of deoxycholate (DOC) detergent in 0.02M Tris-HCl buffer, pH 8.0 was added to 2.0 X 10^9 spermatozoa. The sperm suspension was incubated for 1 h at 37^{o} C in a metabolic shaker, centrifuged at 6000 rpm for 30 min at room temperature. The pellet was discarded and supernatant was stored at -20^{o} C for protein analysis.

Capacitated-, uncapacitated- spermatozoa, their respective incubation media and DOC extracts were analysed for total proteins (LOWRY et al., 1951). Proteins were fractionated from incubation media and DOC extracts of capacitated- and uncapacitated- spermatozoa by SDS-PAGE (DAVIS, 1964). Proteins were assigned molecular weight on the basis of relative mobilities.

Raising of antisera :

Polyclonal antibodies were raised in rabbit against sperm membrane extract (SME) of bull # 1, 2 and 3 separately. The presence of antigens in SME of all the bulls was checked by immunodiffusion (OUCHTERLONY, 1949).

Results

Morphological changes during capacitation and acrosome reaction:

There was a 15 to 21% and 55 to 62% decline in percentage motility of spermatozoa, incubated in TALP medium and 0.85% saline respectively. The motility of spermatozoa, incubated in TALP medium was mostly forward. The spermatozoa started showing darting and zig-zag movement after 3-4 h of incubation. The percentage of vesiculated and acrosome shedded spermatozoa observed microscopically in stained sperm smears was considered as percentage of acrosome reaction. A total of 62%, 68%, 79%, 87%, 66% and 70% of spermatozoa showed acrosome reaction in bull # 1, 2, 3, 4, 5 and 6 respectively.

Table 1
Protein concentration (mg/10^9 s) in uncapacitated-, capacitated-, spermatozoa, sperm membrane extracts (SME) and incubation media (IM) (mean±S.D.) (Proteinkonzentration in nicht- und kapazitierten Spermien, Extrakt der Spermienmembran und des Inkubationsmediums)

Bull #	Uncapacitated			Capacitated			Leakage of protein into medium due to capacitation and acrosome reaction
	Spermatozoa	SME	IM	Spermatozoa	SME	IM	
1	4.47±0.71	0.82(18.28)	1.60±0.51(26.35)*	2.36±0.38(47.2)^	0.54(23.01)	3.79±0.86(61.63)*	2.19(35.28)**
2	4.95±1.38	0.78(15.82)	1.13±0.44(18.59)*	3.20±0.78(35.4)^	0.64(19.88)	3.23±0.12(50.23)*	2.10(31.64)**
3	8.22±0.53	0.70(8.5)	1.07±0.16(11.52)*	4.32±0.5(47.5)^	0.58(13.38)	5.27±0.64(54.95)*	4.20(43.43)**
4	6.78±0.69	0.62(9.17)	1.08±0.36(14.42)*	4.07±0.88(39.9)^	0.46(11.23)	5.08±0.30(55.12)*	3.92(40.70)**
5	4.40±0.95	0.75(13.89)	0.65±0.28(12.87)*	2.62±0.12(40.5)^	0.55(18.09)	2.0±0.32(43.29)*	1.35(30.04)**
6	5.11±0.94	0.61(14.62)	1.59±0.40(23.73)*	3.03±0.61(40.7)^	0.55(21.15)	3.93±0.87(54.47)*	2.34(30.74)**

Figures in parentheses ( ) represent percentage extraction of proteins.
Figures in parentheses ( )*, ()** represent percentage leakage of proteins into the media during incubation and capacitation and AR respectively. Figures in parentheses ()^ represent percentage decrease in sperm protein due to capacitation.
Correlation of rate of acrosome reaction with protein content of uncapacitated spermatozoa (r=0.82), capacitated-spermatozoa (r=0.91), -SME; (r=-0.52) and –IM (r=0.76); S- Spermatozoa

Changes in proteins during capacitation and acrosome reaction of spermatozoa :

(a) Quantitative changes :

There was high protein content in the uncapacitated- and capacitated- spermatozoa of all the six bulls (Table 1) which decreased from 35.4% to 47.5% during *in vitro*

capacitation of spermatozoa in all the bulls and it was statistically significant (P<0.05, Table 1). There was 8.50 to 18.28% extraction of membrane bound proteins from uncapacitated- and 11.23 to 23.01% from capacitated- spermatozoa. Thus, there was 2.73 to 4.73% increase in extraction of membrane bound proteins in capacitated/acrosome reacted spermatozoa. The percentage extraction of proteins showed a correlation with the rate of acrosome reaction (Table 1).
There was 11.52 to 26.35% and 43.29 to 61.63% leakage of proteins in uncapacitated- and capacitated spermatozoa respectively. Thus, the leakage of proteins only due to capacitation and acrosome reaction was 30.04% to 43.43% (Table 1) which corresponded with the rate of acrosome reaction in respective bulls and showed a significant correlation (r=0.74). In bull # 5 the leakage of proteins into medium under control as well as capacitating conditions was low as compared to that in other bulls. Moreover, there was low rate of acrosome reaction but the number of sperm abnormalities was higher i.e. >40% whereas in case of other bulls it was within a lower range i.e. from 17 to 22%. A higher and positive correlation of rate of acrosome reaction with protein content of uncapacitated- (r=0.82), capacitated- spermatozoa (r=0.91) and incubation media (r=0.76) was found (Table 1).

Table 2
Relative mobilities (mr) of proteins in sperm membrane extract (SME) of uncapacitated- and capacitated-spermatozoa as detected by SDS-PAGE (Relative Mobilität von Protein im Extrakt der Spermienmembran bei nicht- und kapazitierten Spermien ermittelt nach Fraktionierung mit SDS-PAGE)

Mol. Wt (kDa)	SME of uncapacitated spermatozoa						SME of capacitated spermatozoa					
	Bull #											
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
220	-	-	-	-	-	0.20	-	-	-	-	-	-
130	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	0.54	0.57	-	0.57	-	-	-	-	-	-
70	-	-	-	-	0.59	0.59	-	0.69	-	-	-	-
35	-	-	0.72	0.73	-	0.73	0.72	0.74	0.74	-	0.74	0.73
25	-	0.78	-	0.79	-	-	0.78	-	-	0.78	-	-
12.5	0.85	0.84	0.84	-	0.84	-	-	0.85	0.86	0.85	0.86	-
<12.5	0.95, 0.96	0.95	-	-	-	-	-	-	0.91	-	-	0.90

(b) Qualitative changes:

SDS-PAGE showed the presence of 12.5, <12.5 kDa; 25, 12.5, <12.5 kDa; 100, 35, 12.5 kDa; 100, 35, 25 kDa; 70, 12.5 kDa and 220, 100, 70, 35 kDa, proteins in SME of uncapacitated spermatozoa of bull # 1, 2, 3, 4, 5 and 6 respectively. On the other hand, proteins with mol wt of 35, 25 kDa; 70, 35, 12.5 kDa; 35, 12.5, <12.5 kDa; 25, 12.5 kDa; 35, 12.5 kDa and 35, <12.5 kDa were fractionated from the SME of capacitated spermatozoa of bull # 1, 2, 3, 4, 5 and 6 respectively (Table 2). There was leakage of 2-5 proteins (<12.5-240 kDa) from uncapacitated spermatozoa and 4-7 (<12.5-130 kDa) proteins from capacitated/acrosome reacted spermatozoa in all the

bulls (Table, 3). More of high mol wt (160 to >240 kDa) and low mol wt proteins (<12.5 to 25 kDa) were leaked out from the uncapacitated spermatozoa, while only medium mol wt proteins leaked out from the capacitated/acrosome reacted spermatozoa. In bull # 4, there was leakage of a more number of proteins during the capacitation of its spermatozoa as well as the rate of acrosome reaction was also high (87%). It was also observed that 100 kDa protein gets leaked out as such or as a modified protein during capacitation/acrosome reaction of spermatozoa of bulls with higher rate of capacitation/acrosome reaction (bull # 3, 4 and 6 , Table 2 and 3).

Table 3
Relative mobilities (mr) of proteins in incubation media (IM) of uncapacitated- and capacitated-spermatozoa as detected by SDS-PAGE (Relative Mobilität des Proteins im Inkubationsmedium bei nicht- und kapazitierten Spermien ermittelt nach Fraktionierung mit SDS-PAGE)

Mol. Wt (kDa)	Incubation Media of uncapacitated media						Incubation Media of capacitated media					
	Bull #											
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
>240	-	-	0.04, 0.08	0.06	-	0.04, 0.07	-	-	-	-	-	-
220	-	-	0.19	-	-	0.17	-	-	-	-	-	-
160	0.38	-	-	-	0.38	-	-	-	-	-	-	-
130	-	-	-	-	-	-	-	-	-	0.47	-	-
100	-	-	-	-	-	-	-	-	-	0.50	-	-
70	-	0.68	-	-	-	-	0.55, 0.62	-	0.67	-	-	-
35	-	-	-	-	-	-	0.74	0.72, 0.74	-	0.73, 0.72	-	0.73
25	0.77	-	-	-	0.77	-	0.79	0.81	0.77, 0.78, 0.81	-	0.77	0.77
12.5	-	-	-	-	-	-	0.85	0.86	0.85	0.86	0.85	-
<12.5	0.89	0.93	0.91	0.98	0.90, 0.94	0.93	-	0.90	-	0.88, 0.91	0.88,0.91, 0.93	0.87, 0.88

Changes in surface antigens during capacitation and acrosome reaction :
In SME of both uncapacitated- and capacitated-spermatozoa of six bulls, three antigens were detected by immunodiffusion reaction of anti Ig, against SME of bull # 1. Anti Ig against SME of bull # 2 could also detect only 3 antigens in SME of capacitated spermatozoa of all the six bulls but uncapacitated spermatozoa of four bulls (3, 4, 5 and 6), while one additional antigen was detected in SME of bull # 1 and 2. Anti Ig against SME of bull # 3 could detect one antigen in SME of uncapacitated spermatozoa of bull # 6 and two in that of bull # 1, 2, 3, 4 and 5 respectively. On the other hand, same anti Ig could detect one in bull # 1, 2 and 6; two in bull # 4 and three antigens in bull # 3 and 5 in SME of capacitated spermatozoa (Table 4).

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Table 4
Number of antigens in SME of uncapacitated and capacitated spermatozoa, as detected by anti lg against SME of uncapacitated spermatozoa of bull # 1, 2 and 3 (Anzahl Antigene im SME bei nicht- und kapazitierten Spermien ermittelt durch anti lg SME Behandlung von nicht- und kapazitierten Spermien der Bullen Nr. 1, 2 und 3)

Anti lg against SME of bull #	**SME of bull #**											
	1		**2**		**3**		**4**		**5**		**6**	
	UC	**C**	**UC**	**C**	**UC**	**C**	**UC**	**C**	**UC**	**C**	**UC**	**C**
1	3	3	3	3	3	3	3	3	3	3	3	3
2	4	3	4	3	3	3	3	3	3	3	3	3
3	2	1	2	1	2	3	2	2	2	3	1	1

UC – Uncapacitated
C – Capacitated

Discussion

The induction of capacitation *in vitro* was confirmed microscopically by the occurrence of darting and zig-zag movements as also reported in buffalo bull spermatozoa (SIDHU et al., 1984). In the present observations, there was leakage of proteins from the uncapacitated as well as capacitated spermatozoa but the leakage was significantly more in the latter as compared to the former. However, the leakage of proteins in the uncapacitated spermatozoa may be because of acrosomal damage (AD) and in capacitated, because of AD as well as due to capacitation and AR. As the percentage leakage of proteins from the capacitated spermatozoa corresponded with the rate of acrosome reaction in respective bulls (DHANJU et al., 2005) and the two showed a significant correlation, the persent observations suggest that the rate of AR can be predicted from the leakage of proteins into the medium due to capacitation and AR. However, the release of proteins into the control medium may be attributed to the leakage of hyaluronidase enzyme (HAFEZ and HAFEZ, 2000), which is loosely bound to the acrosomal membrane and thus indicates acrosomal damage and low integrity of the sperm membrane in cross-bred cattle bulls. In case of bull # 5, there was less leakage of proteins under control as well as capacitating conditions which reveals higher integrity of the sperm membrane in this bull as compared to that in other bulls. But the low percentage of acrosome reaction in this bull may be because of the presence of more number of sperm abnormalities. From the high and positive correlation of rate of AR with protein content of capacitated spermatozoa and incubation media, it can be inferred that percentage capacitation viz a viz fertility of a bull can be predicted from the protein content of spermatozoa and amount of protein released into the incubation medium during capacitation/acrosome reaction in the presence of heparin in the incubation medium.

Potentiation of acrosomal proteolytic activity (APA) by heparin was observed in general as well as in a bull depending manner by GILLES et al. (2001) and APA was used as an indicator of AR by them. However, it has already been demonstrated that capacitation of bovine sperm by heparin requires at least 4h exposure of sperm to heparin and suggested that the changes in plasma membrane prior to an AR can be detected by exposure of bovine sperm to lysophosphatidylcholine (PARRISH et al., 1988). The leakage of only medium mol wt proteins during capacitation corroborates with the observations of SUNDHEY et al. (1994) in goat spermatozoa. The observation of leakage of a 100 kDa protein as such or as a modified protein during

capacitation in present studies corroborate with a similar observation on a 45 kDa protein in cauda epididymal spermatozoa of guinea pig (NOLAND and OLSON, 1989).

Because spermatozoa are relatively silent in transcription and translation, posttranslational modifications perform the regulatory functions in these cells during capacitation. Capacitation is correlated with activation of a signal transduction pathway leading to protein tyrosine phosphorylation. A large number of proteins in the range of 40 to 110 kDa are tyrosine phosphorylated during *in vitro* capacitation of mammalian spermatozoa including man, mouse, bore and bull (BOUE et al., 1996; MANDAL et al., 1999; FLESCH and GADELLA, 2000; FLESCH et al., 2001; ECROYD et al., 2003). ECROYD et al. (2003) also reported tyrosine phosphorylation of HSP-90 for the first time in human and rat spermatozoa when incubated under conditions that support capacitation. Thus, the present observations involving the change in mol wt of proteins leaked out from capacitated spermatozoa indicating the reorganization/alteration of proteins during capacitation and acrosome reaction may also be because of phosphorylation of this particular group of proteins during this process in cattle bulls.

The removal/exposure of antigens from the sperm surface during capacitation and acrosome reaction is also corroborated with the observations of SUNDHEY et al. (1994) which also showed the removal of 140-160 kDa surface antigens during acrosome reaction of goat spermatozoa and KAUL et al. (2000) in which six antigens were recognized in uncapacitated spermatozoa and four antigens in capacitated spermatozoa and only one antigen in acrosome-reacted spermatozoa with antisera against purified goat sperm plasma membrane. SAXENA and TOSHIMORI (2004) also reported a molecular modification of a sperm specific molecule (MC31/CE9) during capacitation/acrosome reaction. The mol mass of this molecule gets reduced from 28-26 kDa to 23-20 kDa during capacitation and it gets glycosylated indicating minor translational modification of this antigen.

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Corresponding Author
Dr (Mrs) C. K. DHANJU
Department of Zoology & Fisheries
Punjab Agricultural University
LUDHIANA, INDIA

E-Mail: ckdhanju@yahoo.com