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Research Article

Correlative morphological and biochemical changes during *in vitro* capacitation of spermatozoa in Cross bred bulls.

C. K. DHANJU*, RANJNA S CHEEMA AND RAVINDER KAUR

Department of Zoology &Fisheries, College of Basic Sciences & Humanities
Punjab Agricultural University, Ludhiana-141004.

ABSTRACT

The pattern of motility of spermatozoa at an interval of 2hr, percentage of acrosome reaction (AR) and changes in biochemical constituents of spermatozoa and their membrane extracts (SME) i.e. proteins, lipids, phospholipids and cholesterol and their leakage in incubation medium (IM) were studied during *in vitro* capacitation of spermatozoa in freshly ejaculated semen of cross bred bulls after incubation in TALP medium at 37°C for 6hr. Initial motility of spermatozoa was 76 to 80% which declined by 15 to 21% after 6hr incubation in TALP medium as compared to 55 to 62% under control conditions. The motility of spermatozoa was mostly forward and they started showing darting and zigzag movements after 3-4 hr of incubation which confirmed the occurrence of capacitation. vesiculation and acrosome shedding of spermatozoa was observed and their number counted in stained smears of spermatozoa and expressed as percentage of capacitation and AR which was found to be 62-87%. Statistically significant ($p<0.05$) decrease in proteins, lipids, phospholipids and cholesterol contents of spermatozoa was observed during capacitation and these biochemical changes revealed that a remodeling of the sperm membrane occurs during capacitation which prepares it for the exocytotic event of the acrosome reaction.

Key words: Spermatozoa, Capacitation, Biochemical changes, Bulls.

INTRODUCTION

Capacitation is a collective term for the changes that a spermatozoan undergoes when it comes in contact with the female reproductive tract. These changes include reorganization of membrane proteins, metabolism of membrane phospholipids, a reduction in membrane cholesterol levels and hyperactivation (Yanagimachi *et al.,* 1994). These changes together with the subsequently induced acrosome reaction- an irreversible exocytotic events are essential pre-requisites if a sperm is to bind to and penetrate the zona pellucida and thereafter fuse with the oocyte plasma membrane (Yanagimachi *et al.,* 1994). Although capacitation is believed to be a multistep process, the molecular changes that take place during it, are not completely understood.

The declining reproductive performance of cross bred bulls- an important dairy animal, is a cause of great concern and thus the molecular mechanisms for key processes like capacitation need to be worked out to find out the reasons yet unknown for declining male fertility of the cross bred bulls and such an information would be of great use in disciplines like animal breeding. The present studies was planned to asses changes in the biochemical constituents i.e. proteins, lipids, phospholipids and cholesterol of spermatozoa, their membranes as well as the leakage of these biomolecules into the incubation medium (IM) during *in vitro* capacitating.

* Corresponding Author: Dr. (Mrs.) C.K. Dhanju, Department of Zoology &Fisheries,
Punjab Agricultural University, Ludhiana-141004. Tel. –09814903065, Fax : 2400945, Email. ckdhanju@gmail.com

MATERIALS AND METHODS

Procurement and processing of semen: Freshly ejaculated semen of six cross bred bulls was procured from dairy farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Semen samples with >80% motility were centrifuged at 1000 rpm for 5 min and seminal plasma was discarded. Loose pellet of spermatozoa was washed twice in TALP medium (without BSA, Ca^{++} and heparin).

Capacitation and acrosome reaction (AR) : Washed spermatozoa were suspended in TALP medium (Tyrode's modified solution : NaCl 92.9mM; KCl 4mM; $NaHCO_3$ 25.9mM ; $CaCl_2 2H_2O$ 10mM; $MgCl_2 6H_2O$ 0.5mM; Sodium lactate 7.6mM; Sodium pyruvate 1.3 mM; Hepes buffer 20 mM ; Glucose 0.25% ; Heparin 200µg/ml and BSA 0.6%) at a concentration of 6 x 10^9s/ml and was incubated at 37°C for 6h. Similarly, a control was also run in 0.85% saline. Percent motility was observed at 0h, 2h, 4h and 6h of incubation. Smears were prepared at 2h interval, which were air dried, fixed in 5% formalin and stained with giemsa (3.0 ml of stock giemsa+2ml of Sorenson phosphate buffer, pH 7.0 and 35 ml of distilled water). Stained slides were examined under oil emulsion using binocular microscope. The number of vesiculated and acrosome shedded spermatozoa were counted that represented the percentage AR of spermatozoa of six bulls.

Processing of spermatozoa for biochemical analysis : Capacitated (suspended in TALP medium) and uncapacitated spermatozoa (suspended in 0.85% saline) were centrifuged at 6000 rpm for 10 min. Washed spermatozoa in PBS, pH 7.4 and IM were stored at 4°C and -12°C respectively for biochemical analysis.

Detergent extraction of sperm membrane proteins (Kinger *et al.*, 1988): Spermatozoa suspended in 2ml of deoxycholate detergent (DOC) in 0.02M Tris-HCl buffer, pH 8.0 were incubated for 1h at 37°C in a metabolic shaker, at concentration of 2 x 10^9s and centrifuged at 6000 rpm for 30 min at room temperature. The pellet was discarded and supernatant containing sperm membrane proteins, analysed for proteins, total lipids, phospholipids and cholesterol.

Extraction of total lipids (Folch *et al.*, 1957): Total lipids were extracted from capacitated, uncapacitated-spermatozoa, SME and IM using chloroform: methanol (2:1v/v). Total lipids were further analyzed for phospholipids & cholesterol.

Quantitative estimation of proteins, phospholipids and cholesterol: The proteins, phospholipids and cholesterol were estimated by standard methods (Lowry *et al.*, 1951; Ames 1966 and Zlatkis and Zak, 1968).

RESULTS AND DISCUSSION

Capacitation and acrosome reaction: The spermatozoa of all the six cross bred bulls showed 76 to 80% initial motility and TALP medium could sustain the 55 to 65% motility up to 6 h of incubation (Table I). Thus there was 15 to 21 % decline in the percentage motility after 6 h of incubation in capacitating medium as compared to 55 to 62% decline under control conditions. Initially the spermatozoa mostly showed forward motility and they started showing darting and zig-zag movements after 3-4 h of incubation in capacitating medium and this confirmed the occurrence of capacitation process. Similar observations were made in buffalo spermatozoa but the motility decreased to less than 20% by 6h of incubation in BWW medium (Sidhu *et al.*, 1984). Percentage of vesiculated and acrosome shedded spermatozoa (Fig 1.) counted in stained smears was considered as total percentage of capacitation and acrosome reaction which was found to be 62-87% in all the six bulls.

Changes in biochemical components of spermatozoa during capacitation and AR : The concentration of proteins, lipids, phospholipids and cholesterol significantly decreased ($p<0.05$) in capacitated spermatozoa and their membrane extracts as compared to that in uncapacitated spermatozoa and their membranes (Table II). However the concentration of all these biochemical constituents was significantly higher ($p<0.05$) in IM of capacitated as compared to that of uncapacitated spermatozoa which indicates that there is more leakage of

proteins, lipids, phospholipids and cholesterol during capacitation as compared to that under control conditions. The leakage of membrane components during incubation in saline may be due to acrosomal damage which further shows the poor membrane integrity of spermatozoa of the cross-bred bulls. The leakage of proteins in the control medium may be attributed to the leakage of hyaluronidase enzyme, which is loosely bound to the acrosomal apparatus and the leakage of this enzyme into the extracellular fluid also indicates acrosomal damage (Sharma, 1987).

Because spermatozoa are relatively silent in transcription and translation, post-translational modifications perform the regulatory functions in these cells during capacitation. Moreover, the process of sperm capacitation is correlated with activation of a signal transduction pathway leading to protein tyrosine phosphorylation, a key intracellular event and the sperm motility is also regulated by protein phosphorylation as it has been shown that a serine/threonine phosphatase system is involved in motility regulation. Actin polymerization may represent an important regulatory pathway in sperm capacitation and F-actin breakdown occurs before acrosome reaction and they have also demonstrated that *in vitro* capacitation of bull, ram, mouse and human sperm was accompanied by time-dependent increase in actin polymerization (Brener *et al.*, 2003). Such a process may be responsible for maintenance of higher sperm motility in the capacitating than in control medium even in present studies. A large number of proteins in the range of 40 to 110 k Da 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 and Ecroyd *et al.*, 2003). Some changes in protein profile of sperm membrane proteins have also been reported in cross-bred bulls (Dhanju *et al.*, 2004). Although phosphotyrosine expression is an essential prerequisite for fertilization, but the proteins that are phosphorylated during capacitation have not been identified. Thus the decrease in protein content of spermatozoa and their membrane during capacitation in present studies may be because of such molecular changes in them.

A decrease in the lipids, phospholipids and cholesterol content of capacitated spermatozoa as compared to that of uncapacitated ones (Table II) indicates their significant role in the process of Capacitation (Davis *et al.*, 1979). However the percentage extraction of lipids, phospholipids and cholesterol was higher from the sperm membrane of capacitated spermatozoa as compared to that of uncapacitated (Table II) which reveals the increased fluidity of the membranes of capacitated/ acrosome reacted spermatozoa resulting from the mobilization of lipid sub-classes as concluded in stallion sperm (Colenbrander *et al.*, 2002). Decreased cholesterol contents of capacitated spermatozoa and their increased leakage in IM in present studies reveals that cholesterol content of mammalian spermatozoa affect the acrosomal responsiveness and fertilizing ability *in vitro* (Cross, 1998). The C/PL ratio of sperm plasma membrane determines the capacitation state of the sperm as freshly ejaculated sperm has high C:PL ratio and during capacitation cholesterol moves from the sperm membrane to the soluble protein receptors and/or phospholipids move into the sperm membrane (Davis *et al.*, 1979). They suggested that a lower C:PL ratio decreases the membrane microviscosity, relax the packing of phospholipids in the membrane and perhaps permit the greater calcium efflux all leading to unspecified steps of fusion of plasma and outer acrosomal membrane. However, C:PL ratio of IM of capacitated spermatozoa was significantly higher as compared to that of uncapacitated spermatozoa as well as their membrane extracts and this further indicates strong efflux of cholesterol into the medium during incubation in present studies which thus corroborate with the findings showing that C:PL ratio gets decreased during the capacitation process of cross bred bull spermatozoa.

Table I: Motility and acrosome reaction during capacitation of bull spermatozoa

S. No.	Bull #	Percentage motility (mean ± SE) Incubation period (h)				Rate of acrosome reaction after 6 h of incubation (%)
		0	2	4	6	
1	1F 1123	80±0(80±0)	73±1.37(55±3.50)	66±2.74(40±3.54)	64±2.74(22±6.52)	62
2	HHS1129	80±0(78.75±1.25)	75±2.04(52±4.75)	68.75±3.15(30±9.13)	65±2.89(16.25±5.55)	68
3	4F 1130	78±2.24(78±2.24)	71±3.26(55±6.62)	63±4.88(34±9.60)	58±4.19(21±8.73)	79
4	FC 1152	80±0(78±2.24)	67±5.48(44±9.75)	64±4.47(30±9.36)	60±3.54(16±7.59)	87
5	FC 1154	76.25±2.40(76.25±2.40)	70±3.54(42.50±8.54)	61.25±4.27(25±1.50)	55±5.00(20±2.25)	66
6	FC 1168	80±0(80±0)	70±2.74(58.33±20.42)	60.83±6.79(36.67±10.80)	59.17±6.01(25.83±9.80)	70

Figures in parentheses ( ) represent percentage motility of uncapacitated spermatozoa.

Table II: Changes in various biochemical components of uncapacitated-, capacitated- spermatozoa(mg/10^9 s), SME and incubation media (IM)

S. No.	Biochemical components	Uncapacitated			Capacitated		
		Spermatozoa	SME	IM	Spermatozoa	SME	IM
1.	Proteins	5.66±0.62	0.71±0.04(8.3-18.3)	1.19±0.15	3.27±0.32*	0.55±0.03*(11.23-23.01)	3.88±0.49*
2.	Total lipids	9.68±0.67	4.13±0.38(31.4-57.14)	0.60±0.12	7.25±0.52*	4.10±0.87(31.4-82.5)	1.47±0.19*
3.	Phospholipids	5.45±0.43	0.34±0.03(4.8-8.6)	0.07±0.02	4.26±0.34	0.26±0.05(2.8-9.1)	0.66±0.03*
4.	Cholesterol	0.11±0.008	0.05±0.008(16.7-50.0)	0.03±0.004	0.05±0.008*	0.02±0.004(14.3-66.7)	0.14±0.00*
5.	C/PL ratio	0.02±0.003	0.12±0.02	0.29±0.04	0.02±0.01	0.07±0.01	0.18±0.02

$P < 0.05$

*Values in parenthesis represent percentage extraction from the sperm membrane.
Values represented in Mean ± SE

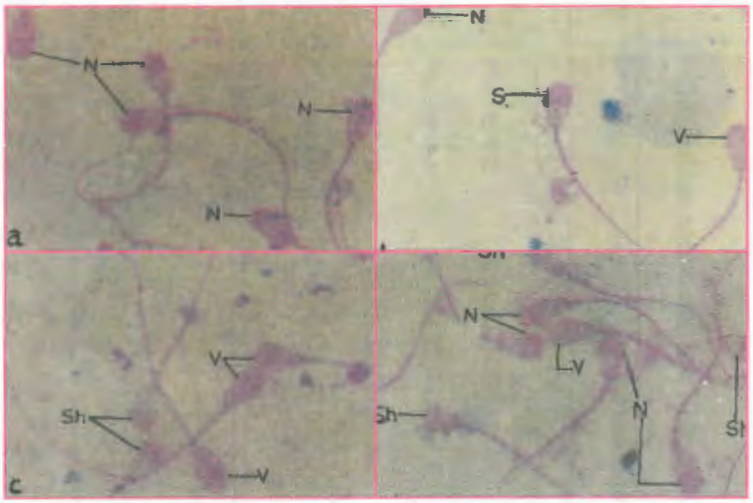

Fig 1: Showing (a) normal (N) spermatozoa and stages of capacitation and crosome reaction like (b) swelling (S), (c) Vesiculation (V) and (d) Shedding (Sh).

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