Original Research

In Vitro Capacitation of Boar Spermatozoa: Role of Heparin

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Abstract

The aim of this study was to evaluate heparin induced in vitro capacitation associated changes in spermatozoa of crossbred Hampshire boar. Therefore, freshly ejaculated and washed spermatozoa of 4 Hampshire boar were in vitro capacitated in TALP medium supplemented with BSA, heparin and hepes buffer at a concentration of 6 × 10^9 spermatozoa/ml at 37 ºC for 5 hours. Capacitation status of spermatozoa in terms of the hyperactivated motility, acrosome membrane integrity, total hypomostic swelling test (HOST) reacted spermatozoa, sperm membrane protein (SMP) and cholesterol content were estimated for each ejaculate at 1 hour interval starting from 0 to 5 hours of incubation. The results revealed that the highest hyperactivation of spermatozoa (71.86 ± 1.55%) was recorded at 4 hours of incubation while the percentage of detached acrosome, HOST reacted spermatozoa, SMP, cholesterol levels decreased significantly (P < 0.01) with increasing period of incubation. In conclusion, heparin induces in vitro capacitation changes in the boar spermatozoa as evident by highest hyperactivation of spermatozoa at 4 hours of incubation.

Key words: Functional Integrity, Hampshire Boar Spermatozoa, Heparin, In-Vitro Capacitation

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Introduction

Spermatozoa must undergo a preparation period for successful fertilization known as capacitation (O' Flaherty *et al.*, 1997). Capacitation can be termed as the sum of biochemical and biophysical modifications that take place in sperm cell during its transport through the female genital tract that enables them to reach and bind to the zona pellucida, undergo acrosome reaction, penetrate the egg vestments and finally fuse with the oocyte (Parrish *et al.*, 1988). The biochemical changes associated with the capacitation process

include an efflux of cholesterol from the plasma membrane leading to an increase in membrane fluidity and permeability to bicarbonate and calcium ions, hyperpolarization of the plasma membrane (Visconti *et al*., 1999), changes in protein phosphorylation and protein kinase activity (Baldi *et al*., 2000; Visconti, 2009) and increase in bicarbonate (HCO_3) concentration, intracellular pH, Ca^{++} and cyclic adenosine monophosphate (cAMP) levels (Si and Okuno, 1999). The acrosome reaction stimulates and initiates by exogenous serum albumin in mammalian spermatozoa during in vitro capacitation by removing fatty acids and cholesterol from sperm membranes (Meizel, 1985). First and Parrish (1987) stated that heparin-like glycosaminoglycans remove decapacitating factors from the sperm plasmalemma and play a direct role in calcium uptake. During capacitation, the sperm enzymes get inactivated and ultimately cause efflux of the cholesterol and influx of Ca^{2+} through the plasma membrane and outer acrosomal membrane, thereby resulting into acrosomal reaction (Talukdar *et al.,* 2015a). Various reports suggested an active participation of the sperm plasma membrane in the process of capacitation, mainly through the loss of cholesterol and membrane bilayer permeability (Visconti *et al*., 1999). 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 (Luconi *et al.,* 1996). During fertilization, mammalian sperm membrane proteins are also involved in the penetration of cumulus matrix, recognition of zona pellucida and fusion with the oocyte plasma membrane (Myles and Primakoff, 1997).

So far the substrates of tyrosine phosphorylation, efflux of cholesterol from the plasma membrane, hyperpolarization of the plasma membrane during in vitro capacitation in boar spermatozoa has not been investigated. Therefore, this study was undertaken to understand the events involved during in vitro capacitation in boar spermatozoa.

Materials and Methods

A total of 24 ejaculates, six from each four trained healthy crossbred Hampshire boars (Hampshire × Indigenous) of one to two years of age maintained at ICAR-All India Coordinated Research Project (AICRP) on Pig, Assam Agricultural University, Guwahati, Assam, India were selected for the present study. The semen was collected twice weekly by Simple fist method (Tamuli, 1994). Immediately after collection, the semen samples were evaluated for volume, concentration and initial sperm motility. The ejaculates having initial sperm motility 70 per cent or more were used for the present study. For in vitro capacitation a required concentration of 6×10^9 spermatozoa/ml was added to 2 ml of prewarmed phosphate buffered saline (PBS) solution (Talukdar *et al.,* 2015b) and centrifuged at 3000 rpm for 20 minutes. The supernatant was aspirated with the help of micro pipette. The sperm pellet was washed again by the same procedure after adding 2 ml of PBS to it. After second wash the spermatozoa were resuspended with 2 ml of medium TALP (NaCl - 92.9 mM; KCl - 4 mM; $NaHCO_3$ - 25.9 mM; $CaCl_2.2H_2O$ - 10mM; $MgCl_2.6$

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H_2O - 0.5mM; sodium lactate - 7.6mM; sodium pyruvate - 1.3mM; HEPES - 20mM; glucose - 0.25%; heparin - 200µg/ml, BSA - 0.6%, penicillin G - 40 IU/ml, streptomycin sulphate - 50 µg/ml in deionised triple distilled water) which was preheated at 37°C in a water bath taken in a glass stopper centrifuge tubes and gently mixed by inverting the tube. The tube was placed in slanting position in a BOD incubator and incubated at 37°C for 5 hours. To assess the status of capacitation, each sample was evaluated at 1 hour interval of incubation starting from 0 till 5 hours. Capacitation associated changes like hyperactivated motility (Marquez and Susan, 2004), acrosomal status (Watson, 1975), hypo-osmotic swollen test, HOST (Jeyendran *et al*., 1984), sperm membrane protein (Srivastava *et al*., 2013a) and total sperm cholesterol (Srivastava *et al.*, 2013b) were analyzed.

Data are presented as means ± standard error (SE). Two - way - ANOVA using SPSS software for Windows was performed.

Results and Discussion

The mean ± S.E. of different parameters studied in the boar spermatozoa at different hours of incubation during in vitro capacitation are presented in Table 1.

Table 1: Mean ± SE of hyperactivated motility, acrosomal integrity, total HOST reacted sperm, SMP and cholesterol level of boar spermatozoa in different hours of incubation in TALP medium

Hour / Parameter	0	1	2	3	4	5	Effect
Hyperactive motility (%)	18.96^{a}±0.76	30.42^{b}±1.08	42.71^{c}±0.95	58.33^{d}±1.40	71.86^{e}±1.55	54.79^{d}±1.93	P < 0.01
Detached Acrosome (%)	3.24^{a}±0.28	17.95^{b}±0.59	29.86^{c}±1.03	40.00^{d}±0.93	70.02^{e}±0.90	79.17^{f}±2.03	P < 0.01
HOST reacted sperm (%)	76.32^{a}±0.71	72.04^{b}±0.77	69.38^{c}±0.69	66.30^{d}±0.77	65.07^{d}±0.82	60.59^{e}±0.47	P < 0.01
SMP (mg/ 10^9 spermatozoa)	24.9^{a}±0.15	19.2^{b}±0.28	15.4^{c}±0.20	14.6^{d}±0.25	12.9^{e}±0.27	11.4^{f}±0.18	P < 0.01
Cholesterol (µg/ 10^8 spermatozoa)	31.84^{a}±0.29	27.68^{b}±0.22	22.35^{c}±0.33	19.04^{d}±0.26	14.4^{e}±0.25	8.57^{f}±0.31	P < 0.01

Means bearing different superscripts differ significantly

Analysis of variance revealed that the hyperactivated motility increased significantly at each hour and the highest hyperactivation of spermatozoa was recorded at 4 hours of incubation at 37°C in TALP medium (71.86 ± 1.55 per cent) and declined significantly at 5 hours of incubation (Fig. 1). The present finding was in close agreement with the observation reported by Dhanju *et al.* (2006) and Kong *et al*. (2008). Hyperactivation has been considered part of the capacitation process because sperm have been observed to be hyperactivated while undergoing capacitation. During capacitation, several sperm proteins become phosphorylated on tyrosine residues and this phosphorylation has been demonstrated to be regulated by a cAMP pathway through activation of protein kinase A (PKA).

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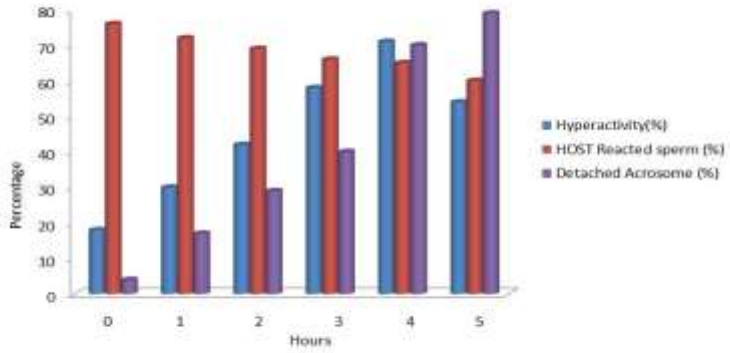

Fig.1 Percentage of hyperactivity, HOST reacted sperm and detached acrosome of in-vitro capacitated boar spermatozoa at different hours of incubation in TALP medium

Some of the proteins that become tyrosine phosphorylated during capacitation have been localized at the flagellum, and therefore it has been proposed that they are involved in hyperactivation (Si and Okuno, 1999). In the present study, the hyperactivated motility of the spermatozoa in TALP medium increased significantly ($P<0.01$) up to 4 hours then it decreased up to 5 hours, which was also reported by Bansal (2010). This might be due to molecular changes related to the sperm capacitation began after 1 hour of incubation and further, a significant ($P < 0.01$) decreased in per cent hyperactivity from 4 h to 5 hours showed the occurrence of acrosome reaction during which many metabolic and ionic changes occurred in sperm membrane leading to the decreased in per cent hyperactivity. The highest incidence of acrosomal changes was recorded on 5 hours of incubation at 37°C in TALP medium (79.17±2.03%). The present findings was in close agreement with the observation reported by Tareq *et al.* (2010). The mean incidence of acrosomal changes recorded in the present study was higher than that reported by Bansal (2010). In the present study, the total acrosomal changes were significantly ($P<0.01$) increases (Fig. 1) while incubation period increased in TALP medium which might be due to acrosome reaction.

The mean HOST reacted spermatozoa observed in the present experiment was higher than previously reported for boar by Matson *et al.* (2009). In the present study, the total HOST reacted spermatozoa were significantly ($P<0.01$) decreased (Fig. 1) while incubation period increased in TALP medium which might be due to acrosome reaction as capacitation significantly reduces the membrane integrity. This process prepares sperm membrane for acrosome reaction and consequently for sperm–oocyte fusion. It has been shown that capacitation exerts a negative effect on membrane integrity (Talukdar *et al.*, 2015a). The process of centrifuging and pellet resuspending had a negative effect on the sperm membrane. Also, Maldjian *et al.* (2005) suggested that washing and sperm pre-incubation may have a detrimental effect on pig sperm viability. The present finding suggested that the porcine spermatozoa capacitated *in vitro* displayed a

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significant reduction in membrane integrity and this phenomenon can be successfully evaluated by the HOST test. The sperm membrane protein levels in the present study decreased significantly (P< 0.01) while incubation period increased in the TALP medium, which was in agreement with Dhanju *et al.* (2006); Talukdar *et al.*, (2015b) who reported that the protein content in capacitated spermatozoa of bulls decreased significantly (P<0.05) during *in vitro* capacitation increased (Fig. 2) and it showed a correlation with the rate of acrosome reaction.

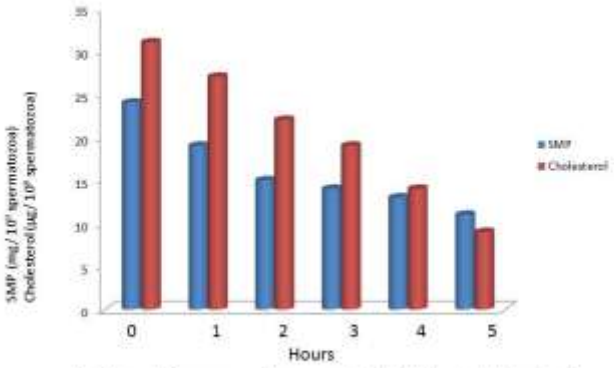

Fig.2 Level of sperm membrane protein (SMP)and cholesterol of in-vitro capacitated boar spermatozoa at different hours of incubation in TALP medium

The present observations suggested that the rate of capacitation and acrosome reaction can be predicted from the leakage of proteins from the spermatozoa. Bansal (2010) also reported that the protein content of incubation medium (TALP + sperm suspension) increased non-significantly (P ≥ 0.05) from 0 hour to 4 hour and it was maximum at 4 hour. This might be due to stimulatory effect of capacitation in terms of protein leakage which was perhaps essential for increasing membrane fluidity leading to acrosome reaction. Dapino *et al.* (2009) observed that there was remodeling of sperm membrane surface protein in presence of heparin on the acrosomal region in capacitated spermatozoa.

In the present study, it was observed that the sperm cholesterol levels decreased significantly (P< 0.01) while incubation period increased in TALP medium (Fig. 2). Similar findings, as reported by Shadan *et al.* (2004); Talukdar *et al.* (2015b) and that during capacitation, changes take place in the partitioning of cholesterol between raft and non-raft regions of the membrane that are responsible for initiating intracellular signal transduction pathways. The present finding also corroborated with the findings of Visconti *et al.* (1999) who reported that less of cholesterol initiate the signal transduction pathway that promotes capacitation by altering the sperm membrane permeability. These membrane alteration increased permeability to ions such as Ca^{2+} and HCO_3^-, which enters the cytoplasm and stimulate adenylyl cyclase to

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promote cyclic Adenosine monophosphate (AMP) production leading to the stimulation of protein kinase A (PKA) and ultimately initiated the capacitation process associated with hyperactivation. Talukdar *et al.* (2015b) reported that during capacitation, the sperm enzymes get inactivated which ultimately cause efflux of the cholesterol and influx of Ca^{2+} through the plasma membrane and outer acrosomal membrane and thus, resulting into acrosomal reaction. They further postulated that the process of sperm capacitation to be associated with membrane cholesterol depletion.

Conclusion

In conclusion, heparin induces in vitro capacitation changes in the boar spermatozoa as evident by highest hyperactivation of spermatozoa at 4 hours of incubation. The total acrosome reacted, HOST reacted spermatozoa, SMP, cholesterol levels decreased significantly with increasing period of incubation, which is related with the rate of capacitation and acrosome reaction. So, the process of sperm capacitation is associated with membrane protein and cholesterol depletion in boar spermatozoa.

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