TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2015) 39: 629-633
© TÜBİTAK
doi:10.3906/vet-1501-16

Heparin-induced in vitro capacitation changes of swamp buffalo spermatozoa

Dibyajyoti TALUKDAR*, Kutubuddin AHMED, Sourabh DEORI, Gopal Chandra DAS
Department of Animal Reproduction, Gynecology, and Obstetrics, College of Veterinary Science, Assam Agricultural University, Guwahati, Assam, India

Received: 08.01.2015 • **Accepted/Published Online:** 02.06.2015 • **Printed:** 30.10.2015

Abstract: The aim of this study was to evaluate heparin-induced in vitro capacitation-associated changes in spermatozoa of swamp buffalo. Therefore, freshly ejaculated and washed spermatozoa of 8 swamp buffalo bulls were capacitated in vitro in TALP medium supplemented with BSA, heparin, and HEPES buffer at a concentration of 6×10^9 spermatozoa/mL at 37 °C for 6 h. Capacitation status of spermatozoa in terms of the hyperactivated motility, acrosome membrane integrity, total hypoosmotic swelling test (HOST), activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and sperm membrane protein (SMP) and cholesterol content were estimated for each ejaculate at 1-h intervals from 0 to 6 h of incubation. The results revealed that the highest hyperactivation of spermatozoa (74.50 ± 1.78%) and live acrosome reaction (56.92 ± 1.88%) was recorded at 4 h of incubation while the total HOST-reacted spermatozoa and SMP and cholesterol levels decreased significantly ($P < 0.01$) with increasing period of incubation. The AST and ALT activities increased significantly ($P < 0.01$) as incubation period increased. In conclusion, heparin induces in vitro capacitation changes in swamp buffalo spermatozoa as evidenced by the highest hyperactivation of spermatozoa and live acrosome reaction at 4 h of incubation.

Key words: Swamp buffalo, spermatozoa, capacitation, alanine aminotransferase, aspartate aminotransferase, protein, cholesterol

1. Introduction

Capacitation involves a series of molecular activities that include reorganization of membrane proteins, metabolism of phospholipids, reduction in cholesterol level, and hyperactivation of sperm cells (1,2). These capacitation-associated changes induce acrosome reaction, which is an irreversible exocytotic event and a prerequisite for a sperm to bind and penetrate the zona pellucida to fuse with the oocyte plasma membrane during the process of fertilization in the female reproductive tract (1). Acrosomes are necessary for protecting and releasing the enzymatic contents at the right time and place for effective fertilization. The enzymes stored between the inner and outer membrane of acrosome, when released at the time of acrosomal reaction, act sequentially and specifically on the cumulus, corona radiate, and zona pellucida of the ovum. Furthermore, the plasma membrane undergoes capacitational changes and acrosomal reaction in the uterine environment, which is a prerequisite for successful fertilization (3). However, in vitro capacitation is possible in the absence of reproductive tract fluids with the help of specified media containing various compounds like bicarbonate, calcium, heparin, HEPES, and serum albumin that mimic the oviduct fluid (4). The plasma membrane integrity of sperm is of crucial importance for optimal sperm function and only sperm with intact plasma membranes can undergo a series of complex changes in the female reproductive tract and acquire the ability to fertilize an oocyte (1). The exogenous serum albumin stimulates and initiates the acrosome reaction in mammalian spermatozoa during in vitro capacitation by removing fatty acids and cholesterol from sperm membranes (5). First and Parrish (6) proposed 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 in acrosomal reaction (7). 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 (8,9). 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 (10). During fertilization, mammalian sperm membrane proteins (SMPs) are also involved in the penetration of the

* Correspondence: dibya26@gmail.com

cumulus matrix, recognition of the zona pellucida, and fusion with the oocyte plasma membrane (11).

Hence, overall, 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 (9), changes in protein phosphorylation and protein kinase activity (12,13), and increase in bicarbonate (HCO_3^-) concentration, intracellular pH, Ca^{++}, and cyclic adenosine monophosphate (cAMP) levels (14). So far the substrates of tyrosine phosphorylation, efflux of cholesterol from the plasma membrane, and hyperpolarization of the plasma membrane during in vitro capacitation in swamp buffalo spermatozoa have not been investigated. Therefore, this study was undertaken to understand the events involved during in vitro capacitation in swamp buffaloes.

2. Materials and methods

2.1. Semen collection

A total of 40 semen ejaculates were collected by artificial vagina method, 5 each from 8 swamp buffalo bulls aged 5 to 8 years following a twice a week schedule. Bulls were maintained at an institute farm under "Network Project on Swamp Buffalo" in the College of Veterinary Science, Assam Agricultural University, Guwahati, India, under uniform managerial conditions. Immediately after collection each individual ejaculate (5 replications) was evaluated for ejaculation volume, mass activity, and initial motility of spermatozoa as per Salisbury et al. (15). Samples having volume above 1.0 mL, mass activity 3+, and initial sperm motility of 70% or more were capacitated in vitro in TALP medium at 37 °C for 6 h (16).

2.2. Washing of spermatozoa

The fresh semen samples were washed with phosphate-buffered saline (PBS; pH 7.4) by centrifugation at 3000 rpm for 20 min. The supernatant was discarded and the sperm pellet was washed again with 2 mL of PBS. Finally, the washed pellet was resuspended in PBS to make the desired concentration of sperm cells depending upon the experiment (17).

2.3. Capacitation of spermatozoa

Washed fresh spermatozoa were suspended in TALP medium (NaCl - 92.9 mM; KCl - 4 mM; $NaHCO_3$ - 25.9 mM; $CaCl_2.2H_2O$ - 10 mM; $MgCl_2.6H_2O$ - 0.5 mM; sodium lactate - 7.6 mM; sodium pyruvate - 1.3 mM; HEPES - 20 mM; glucose - 0.25%; heparin - 200 µg/ml, BSA - 0.6%, penicillin G - 40 IU/ml, streptomycin sulfate - 50 µg/ml in deionized triple-distilled water) at concentration of 6×10^9 spermatozoa/mL and subsequently incubated at 37 °C for 6 h (14). To assess the status of capacitation, each sample was evaluated at 1-h intervals of incubation from 0 to 6 h. Capacitation-associated changes like hyperactivated motility (18), acrosomal status (19), hypoosmotic swelling test (HOST) results (20), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (21), and SMP (22) and sperm cholesterol level were analyzed (23).

2.4. Hyperactivated motility

Hyperactivated motility of in vitro capacitated spermatozoa was evaluated by taking a drop of sperm suspension from each sample (i.e. hourly intervals from 0 to 6 h of incubation) on a preheated slide covered with a coverslip. The slide was then placed on the warm BioTherm stage and examined under phase contrast optics at a magnification of 40×. The percentage of spermatozoa with hyperactivated and nonactivated motility was recorded accordingly. A spermatozoon was considered to be hyperactivated when it swam in a vigorous figure-eight pattern as described by Marquez and Suarez (18).

2.5. Acrosome membrane integrity

Eosin-nigrosin-Giemsa staining was done as per Tamuli and Watson (19). Briefly, a drop of semen was placed on a warm (35 °C) glass slide, mixed with two drops of prewarmed (35 °C) eosin-nigrosin stain, and left for 30 s. A thin uniform smear out of the mixture was made on a clean grease-free glass slide and air-dried. The dried smear was fixed in a tartrate phosphate buffer (77 mM potassium sodium tartrate, 50 mM sodium dihydrogen orthophosphate, 25 mM potassium dihydrogen orthophosphate in distilled water, pH 7.0) with 10% formaldehyde solution for 10 min. The fixed smear was washed under slow-running tap water for 10 min and then rinsed with distilled water. The smear was then stained with Giemsa working solution for 60 min, rinsed with distilled water, and dried in air. The stained slide was examined under oil immersion objective of the microscope at 1000× magnification. A minimum of 200 spermatozoa were counted and categorized into the following groups: live acrosome intact, live acrosome reacted, dead acrosome intact, and dead acrosome reacted.

2.6. Total HOST-reacted spermatozoa

The functional integrity of the sperm membrane was studied using hypoosmotic solution maintained at 150 mOsm/L (20). A total of 200 spermatozoa were examined in different fields at a magnification of 400× using a phase contrast microscope and total types of swellings were recorded.

2.7. Estimation of ALT and AST activities

The in vitro capacitated semen samples from each bull were evaluated for ALT and AST activity as per Nath et al. (21) using commercial kits (Siemens Ltd., Gujarat, India) and a Systronics Spectrophotometer 106. The enzyme activity was expressed in units/10^8 spermatozoa.

2.8. Extraction and estimation of SMP

Protein extraction was done as per Cheema et al. (22). Aliquots of in vitro capacitated semen were washed two times in PBS (pH 7.4) by centrifugation at 4000 rpm for 10 min. Sperm membrane proteins were extracted by incubating 1.0×10^9 spermatozoa in 1.0 mL of 1% deoxycholate (DOC) in 0.02 M Tris-HCl buffer (pH 6.8) in a boiling water bath (5 min). Sperm suspension was centrifuged at 6000 rpm for 30 min at room temperature. To get sperm membrane extract, 5% mercaptoethanol was added to the supernatant, kept in a boiling water bath for 5 min, and subsequently centrifuged at 6000 rpm for 30 min. The pellet was discarded and supernatant was stored at –20 °C for protein analysis. SMP estimation was done by the Burette method using a Siemens kit (Siemens Ltd., Gujarat, India) in a Systronics Spectrophotometer 106 and expressed as mg/10^9 spermatozoa.

2.9. Estimation of cholesterol content

Cholesterol was estimated according to Srivastava et al. (23). Aliquots of in vitro capacitated semen were washed three times in PBS (pH 7.4) by centrifugation at 800 rpm for 10 min. The pellet of approximately 100 million washed spermatozoa was taken in a 10-mL vial. The sperm pellet was extracted with 20 volumes of chloroform:methanol (1:1, v/v) solution and vortexed for 20 s. Subsequently, it was centrifuged at 800 rpm for 5 min followed by evaporation to dryness under liquid nitrogen. At the time of estimation, 0.5 mL of chloroform was added to each vial and cholesterol was estimated by enzymatic method using a cholesterol assay kit (Siemens Ltd., Gujarat, India) in a Systronics Spectrophotometer 106 and expressed as μg/10^8 spermatozoa.

2.10. Statistical analysis

Data are presented as mean ± standard error (SE). Two-way ANOVA using SPSS for Windows was performed in an electronic environment, and the statistical difference was determined with an inside-group Tukey test. Differences were considered significant at $P < 0.05$ (24).

3. Results

The mean ± SE of different parameters studied in the swamp buffalo spermatozoa at different hours of incubation during in vitro capacitation are presented in the Table.

The hyperactive motility of sperm cells significantly ($P < 0.01$) increased from 0 to 4 h of incubation and then decreased significantly. The highest hyperactive motility was recorded at 4 h of incubation. Live acrosome reacted spermatozoa also gradually increased with the hours of incubation and then decreased from 4 h of incubation. The total HOST reacted spermatozoa significantly ($P < 0.01$) decreased with the hours of incubation. It was 82.55 ± 1.00% at 0 h, which decreased significantly ($P < 0.01$) to 61.57 ± 2.20% at 6 h of incubation. The enzyme activity

Table. Mean ± SE of hyperactivated motility, acrosomal integrity, total HOST reacted sperm, ALT and AST activity, SMP, and cholesterol level of swamp buffalo spermatozoa at different hours of incubation in TALP medium. Means bearing different subscripts in a row differ significantly.

Parameter \ Hour	0	1	2	3	4	5	6	Effect
Hyperactive motility (%)	14.00^{a} ± 0.80	35.75^{b} ± 1.12	52.87^{e} ± 1.38	60.25^{f} ± 1.05	74.50^{g} ± 1.78	47.50^{d} ± 1.11	41.25^{c} ± 1.30	$P < 0.01$
Acrosomal integrity (%)	8.62^{a} ± 0.33	45.57^{b} ± 2.97	52.42^{ec} ± 2.49	50.92^{d} ± 2.39	56.92^{de} ± 1.88	54.27^{ef} ± 1.83	49.37^{c} ± 2.59	$P < 0.01$
HOST reacted sperm (%)	82.55^{g} ± 1.00	78.02^{f} ± 1.26	77.22^{ef} ± 1.12	75.55^{d} ± 1.21	71.70^{c} ± 1.49	69.25^{b} ± 1.61	61.57^{a} ± 2.20	$P < 0.01$
ALT (U/10^8 spermatozoa)	0.89^{a} ± 0.11	1.77^{b} ± 0.15	3.65^{c} ± 0.18	4.91^{d} ± 0.12	6.17^{e} ± 0.20	7.64^{f} ± 0.31	13.20^{g} ± 0.40	$P < 0.01$
AST (U/10^8 spermatozoa)	12.63^{a} ± 1.14	31.28^{b} ± 1.11	39.67^{c} ± 4.52	53.96^{d} ± 1.37	70.39^{e} ± 1.53	88.98^{f} ± 2.28	90.40^{g} ± 2.87	$P < 0.01$
SMP (mg/10^9 spermatozoa)	5.13^{g} ± 0.12	3.92^{f} ± 0.13	3.25^{e} ± 0.12	2.64^{d} ± 0.11	2.55^{c} ± 0.13	1.82^{b} ± 0.06	1.45^{a} ± 0.08	$P < 0.01$
Cholesterol (μg/10^8 spermatozoa)	21.95^{g} ± 0.44	19.08^{f} ± 0.51	14.65^{e} ± 0.51	12.94^{d} ± 0.47	11.07^{c} ± 0.59	8.45^{b} ± 0.54	5.64^{a} ± 0.46	$P < 0.01$

for ALT and AST (U/10^8 spermatozoa) showed significant ($P < 0.01$) increase as the hours of incubation prolonged. SMP (mg/10^9 spermatozoa) dropped significantly ($P < 0.01$) with the hours of incubation. It declined from 5.13 ± 0.12 at 0 h to 1.45 ± 0.08 at 6 h of incubation. Similarly, the cholesterol values also showed a significant ($P < 0.01$) decline from 0 to 6 h of incubation.

4. Discussion

Hyperactivated sperm motility is characterized by high-amplitude and asymmetrical flagellar beating that assists sperm in penetrating the zona pellucida of oocytes (18). 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). Some of the proteins that become tyrosine phosphorylated during capacitation have been localized in the flagellum, and therefore it has been proposed that they are involved in hyperactivation (14). In the present study, the highest hyperactivated motility of the spermatozoa was achieved at 4 h of incubation during in vitro capacitation and thereafter declined until 6 h, which is in agreement with the findings of Bansal (25). This might be due to molecular changes related to the sperm capacitation beginning after 1 h of incubation and, furthermore, a significant ($P < 0.01$) decrease in percentage of hyperactivity from 4 to 6 h showed the occurrence of acrosome reaction, during which many metabolic and ionic changes occurred in sperm membrane leading to decreased hyperactivity. The highest rate of live acrosome reacted spermatozoa was recorded at 4 h of incubation due to capacitation and acrosome reaction followed by a loss of the acrosomal matrix contents (3).

Our findings revealed that the total HOST reacted spermatozoa were significantly ($P < 0.01$) decreased as the incubation period increased. It was suggested that the ability of spermatozoa to swell in the presence of hypoosmotic medium reflects normal water transport across the sperm membrane, a sign of normal membrane integrity and functional activity (20). The absence of sperm tail swelling might be indicative of functional sperm head membranes that react during capacitation and acrosome reaction. Thundathil et al. (26) reported that there was a statistically significant positive correlation between the percentage of uncapacitated spermatozoa and the percentage of HOST positive spermatozoa, and a similarly positive correlation was also obtained between the proportion of spermatozoa with a negative HOST response and the proportion of acrosome reacted spermatozoa.

In our study, it was observed that the ALT and AST activities increased significantly ($P < 0.01$) while incubation period increased. A similar finding was reported by Nath et al. (21), which might be due to acrosome reaction leading to changes in the mitochondrial sheath with loss of protein from the midpiece (27) and increase in cell membrane permeability with or without rupture of cell membrane (28).

It was observed that the sperm membrane protein levels decreased significantly ($P < 0.01$) while incubation period increased, which is in agreement with the findings of Dhanju et al. (29) and showed a correlation with the rate of acrosome reaction. The present observations suggested that the rate of capacitation and acrosome reaction can be predicted from the leakage of proteins from the spermatozoa. Bansal (25) also reported that the protein content of incubation medium (TALP + sperm suspension) increased nonsignificantly ($P < 0.05$) from 0 to 4 h and it was maximum at 4 h. 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.

The spermatozoa acquire a layer of cholesterol sulfate and desmosterol sulfate to stabilize the sperm plasma membrane while passing through the epididymis (1). During in vitro capacitation in the presence of serum and other proteins, there occurs a loss of membrane-stabilizing substance from the surface of sperm facilitating the efflux of the cholesterol. In our study, it was observed that the sperm cholesterol levels decreased significantly ($P < 0.01$) while incubation period increased, which is in agreement with the findings of Sharma et al. (30). Present findings also corroborate with the findings of Visconti et al. (9), who reported that less cholesterol initiates the signal transduction pathway that promotes capacitation by altering the sperm membrane permeability. These membrane alterations increased permeability to ions such as Ca^{2+} and HCO_3^-, which enter the cytoplasm and stimulate the adenylyl cyclase to promote cAMP production, leading to the stimulation of PKA and ultimately initiation of capacitation associated with hyperactivation. Langlais and Roberts (7) reported that, during capacitation, the sperm enzymes get inactivated, which ultimately causes efflux of the cholesterol and influx of Ca^{2+} through the plasma membrane and outer acrosomal membrane, thus resulting in acrosomal reaction. They further postulated the process of sperm capacitation to be associated with membrane cholesterol depletion.

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

References

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, editors. The Physiology of Reproduction. New York, NY, USA: Raven Press; 1994. pp. 189–317.
2. Dhanju CK, Toor G, Cheema RS. Effect of seminal plasma on quantity and quality of proteins leaked during heparin-induced in vitro capacitation of bull sperm. Indian J Anim Res 2009; 43: 136–138.
3. Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 1986; 15: 213–226.
4. De Laminrande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997; 3: 175–194.
5. Meizel S. Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. Am J Anat 1985; 174: 285–302.
6. First NL, Parrish JJ. In vitro fertilization of ruminants. J Reprod Fert Suppl 1987; 34:151–165.
7. Langlais J, Robert KD. A molecular membrane model of sperm capacitation and the acrosomal reaction of mammalian spermatozoa. Gamete Res 1985; 12: 198–224.
8. Cross NL. Role of cholesterol in sperm capacitation. Biol Reprod 1998; 59: 7–11.
9. Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, Kopf GS. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Dev Biol 1999; 214: 429–443.
10. Luconi M, Krausz C, Forti G, Baldi E. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. Biol Reprod 1996; 55: 207–216.
11. Myles DG, Primakoff P. Why did the sperm cross the cumulus? To get to the oocyte. Functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with, the egg. Biol Reprod 1997; 56: 320–327.
12. Baldi E, Luconi M, Bonaccorsi L, Muratori M, Forti G. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. Front Biosci 2000; 5: 110–123.
13. Visconti PE. Understanding the molecular basis of sperm capacitation through kinase design. P Natl Acad Sci 2009; 106: 667–668.
14. Si Y, Okuno M. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. Biol Reprod 1999; 61: 240–246.
15. Salisbury GW, VanDemark NL, Lodge JR. Physiology of Reproduction and Artificial Insemination of cattle. 2nd ed. San Francisco, CA, USA: W. H. Freeman and Co.; 1978.
16. Rogers BJ, Yanagimachi R. Retardation of guinea-pig sperm acrosome reaction by glucose: the possible importance of pyruvate and lactate metabolism in capacitation and the acrosome reaction. Biol Reprod 1975; 13: 568–575.
17. Esteves SC, Sharma RK, Thomas AJ, Agarwal TA. Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test. Int Braz J Urol 2007; 33: 364–376.
18. Marquez B, Suarez SS. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. Biol Reprod 2004; 70: 1626–1633.
19. Tamuli MK, Watson PF. Use of a simple staining technique to distinguish acrosomal changes in the live sperm sub-population. Anim Reprod Sci 1994; 35: 247–250.
20. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Carbo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984; 70: 219–228.
21. Nath KC, Ahmed K, Dutta G, Barthakur T, Borgohain BN. Semen quality and release of certain enzymes during the course of freezing bull spermatozoa. Indian J Anim Reprod 1996; 17: 130–131.
22. Cheema R, Bansal AK, Bilaspuri GC, Gandotra VK. Correlation between the proteins and protein profile(s) of different regions of epididymis and their contents in goat buck. Anim Sci Pap Rep 2011; 29: 75–84.
23. Srivastava N, Srivastava SK, Ghosh SK, Amit K, Perumal P, Jerome A. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. Asian Pac J Reprod 2013; 2: 126–131.
24. SPSS Inc. SPSS for Windows (Release 11.5). Chicago, IL, USA: SPSS Inc.; 2002.
25. Bansal A. In vitro capacitation of bull spermatozoa: role of vitamin E. Webmed Central Reproduction 2010; 1: 1–10.
26. Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. Plasma membrane and acrosomal integrity in bovine spermatozoa with the knobbed acrosome defect. Theriogenology 2002; 58: 87–102.
27. Graham EF, Crabo BG, Schmel MKL. Utilization of enzyme assay in developing techniques for freezing semen. In: Proceedings of the 8th International Zootechnology Symposium. Milan, Italy; 1974. pp. 95–111.
28. Roychoudhary PN, Pareek PK, Gowda HC. Effect of cold shock on GOT and GPT release from bull and ram spermatozoa. Andrologia 1974; 6: 315–319.
29. Dhanju CK, Kaur R, Cheema RS. Protein-leakage during heparin-induced in-vitro capacitation of bull sperm. Arch Tierz Dummerstorf 2006; 49: 426–433.
30. Sharma IJ, Sarkhel BC, Thomas M, Guha M, Singh HS, Parekh HKB. Lipid changes during in vitro capacitation of the buffalo spermatozoa. Indian J Anim Sci 1998; 68: 1030–1031.