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Cryo-capacitation changes during cryopreservation of swamp buffalo spermatozoa

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ABSTRACT

Cryopreservation of sperm cells induces cryo-capacitation like changes. These changes are associated with reduced survival of sperm cells in the female reproductive tract that ultimately associate with poor fertility. The present study was designed with the objective to investigate the cryo-capacitation changes in relation to acrosomal status, transaminases activities, sperm membrane protein and cholesterol content in swamp buffalo spermatozoa. Results revealed that the total mean incidences of acrosome reacted spermatozoa and the mean activity of ALT and AST were significantly higher after equilibration and freezing. The mean level of sperm membrane protein and cholesterol was significantly lower after equilibration and freezing. It may be concluded from the present study that cryopreservation induces capacitation-like changes in swamp buffalo spermatozoa.

Key words: Cryo-capacitation, Cryopreservation, Spermatozoa, Swamp buffalo

Cryopreservation causes considerable changes in the morphological and functional attributes of spermatozoa, which ultimately lead to poorer fertility of cryopreserved semen compared to fresh. Several mechanisms were attributed to the reduced fertility of cryopreserved semen, and among them are capacitation-like changes in frozen-thawed spermatozoa (Thomas *et al.* 2006, Talukdar *et al.* 2015). Frozen-thawed spermatozoa have an altered membrane state, which is functionally similar to capacitated and/or acrosome reacted sperm (Watson 1995). Earlier studies have demonstrated similarities between the changes associated with capacitation and cryopreservation (Kadirvel *et al.* 2011) and termed the process as cryo-capacitation (Watson 1995). Cryo-capacitation is one of the major factors associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract (Talukdar *et al*. 2015), resulting in reduced fertility of frozen-thawed semen. Although the molecular mechanisms of capacitation are not completely elucidated, various reports have described an active participation of sperm plasma membrane in the process of capacitation, mainly through loss of cholesterol (Kadirvel *et al*. 2009). Cholesterol efflux leads to changes in membrane architecture, increased bilayer permeability and fluidity that give rise to the capacitated state of the sperm cells (Talukdar 2014). There is also alteration in the sperm membrane proteins which might be due to sub lethal damage occurred during cryopreservation leading to loss of sperm surface proteins (Kadirvel *et al*. 2011), segregation of membrane proteins (Srivastava *et al*. 2013a), inactivation of membrane- bound enzymes and decreased lateral protein diffusion within the membrane (Watson 1995). The capacitation – like changes was reported due to freezing thawing in spermatozoa of bull, boar, equine and riverine buffalo by various workers. However, to the best of our knowledge there is no report of such study on the swamp buffaloes. Therefore, the present study was undertaken to assess the cryo-capacitation changes during cryopreservation of swamp buffalo sperm cells in respect of acrosomal status, transaminases activities, sperm membrane protein and cholesterol content.

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MATERIALS AND METHODS

Semen collection and preservation: The study was conducted on 8 swamp buffalo bulls, aged between 5 to 8 years maintained under Network Project on Swamp Buffalo at College of Veterinary Science, Khanapara, Guwahati, India. Ten ejaculates from each bulls were collected twice a week following artificial vagina method. Immediately after collection, each ejaculate was evaluated for volume, mass activity and initial motility, and those having volume 1.0 ml or more, mass activity 3 + or more (in 0 to 4+ scale) and initial sperm motility 70% or more were used for freezing with Tris-egg yolk- citrate glycerol extender. The extended semen was equilibrated in an equilibration

chamber for 4 h at 5ºC before filling in 0.25 ml French straws. After filling and sealing, the semen straws were placed in a rack at 4 cm above liquid nitrogen in the vapour phase for 8 min and finally plunged into liquid nitrogen container (–196ºC). The semen straws were stored in liquid nitrogen for 10 days and thawed at 37ºC for 30 sec before post-thaw evaluation. The semen samples were evaluated at three stages viz., fresh, after equilibration and after freezing for acrosomal status by using FITC labelled *Pisum sativum* agglutinin, ALT and AST activities and quantified for sperm membrane protein and cholesterol levels.

Washing of spermatozoa: Each semen samples before evaluation were washed with PBS (pH 7.4) and centrifuged at 3,000 rpm for 20 minutes. The supernatant was aspirated with the help of a micro pipette. The sperm pellet was washed again by the same procedure after adding 2 ml of PBS to it and resuspended in PBS to make desired concentration of sperm depending upon the experiment.

Evaluation of acrosomal status of spermatozoa by using Fluorescein isothiocyanate labeled Pisum sativum agglutinin (FITC labeled PSA): The semen aliquots were washed three times with PBS by centrifugation at 2,000 rpm for 15 min (Kaul *et al.* 2001). Smears were prepared from the aliquots on microscope slides, air dried and dipped in absolute methanol for 15 min and finally allowed to dry rapidly at room temperature. The methanol-treated smears were incubated for 30 min at room temperature in a moisture chamber with a solution of FITC-conjugated PSA (50 µg/ml) in PBS. The slides were then washed in distilled water to remove unbound probe and rinsed in a stream of water, and finally dipped in excess water for at least 15 min. After drying, smears were examined immediately, without mounting, in an epifluorescence microscope equipped with CFDA filter set (excitation filter BP, 450– 495 nm; emission filter, LP 520 nm). A minimum of 200 spermatozoa were examined for each set of experiments. The strongly labelled sperm showing complete green fluorescence of acrosomal region revealed the presence of the acrosome, whereas, the absence of acrosomal staining or its confinement to the equatorial segment were signs of loss of acrosome (Fig.1).

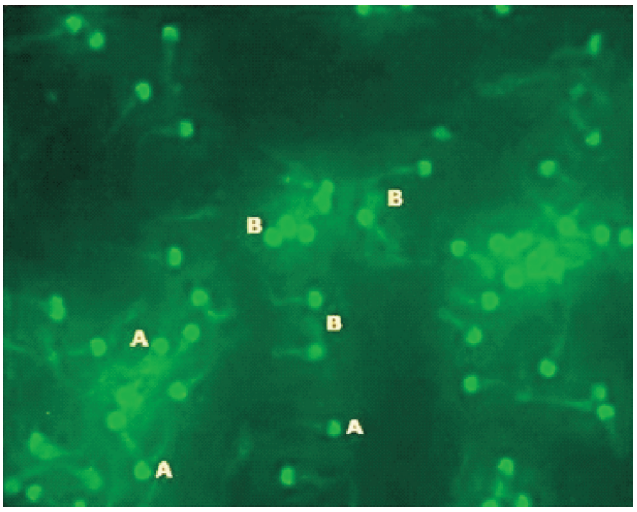

Fig. 1. FITC labeled PSA stain: (A) PSA positive (Acrosome intact); (B) PSA negative (Acrosome reacted).

Estimation of ALT and AST activities: Each semen samples at different stages of cryopreservation from each bull were evaluated for ALT and AST activity using commercial diagnostics kits as per the recommendation of manufacturer in a Systronics Spectrophotometer 106 and expressed in unit/ 10^8 spermatozoa.

Extraction and estimation of sperm membrane protein: Sperm membrane proteins were extracted from the spermatozoa 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 boiling water bath for 5 min (Cheema *et al.* 2011). Sperm suspension was centrifuged at 6,000 rpm for 30 min at room temperature. To get sperm membrane extract, 5 % mercapto-ethanol was added to the supernatant, kept in boiling water bath for 5 min and again centrifuged at 6,000 rpm for 30 min. The pellet was discarded and supernatant was stored at –20ºC for protein analysis. Estimation was done by burette method using commercial diagnostic kit in a spectrophotometer and expressed in mg/ 10^9 spermatozoa.

Cholesterol estimation: Aliquots of semen were washed three times with PBS (pH 7.4) by centrifugation at 800 rpm for 10 min (Srivastava *et al*. 2013b). The pellet of approximately 100 million washed spermatozoa was taken in a 10 ml vial. The sperm pellet was extracted with 20 volume of chloroform: methanol (1:1, V/V) solution and vortexed for 20 seconds. 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 cholesterol assay kit in a spectrophotometer and expressed in µg/10^8 spermatozoa.

Table 1. Per cent acrosomal changes (PSA -ve), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity sperm membrane protein (SMP) and cholesterol level (mean ± SE) of fresh, equilibrated and frozen thawed swamp buffalo spermatozoa

Stage/ Parameters	Fresh	Equilibrated	Frozen thawed	Effect
PSA -ve	7.60^a ±0.58	20.32^b ±1.10	38.72^c ±1.79	P<0.01
AST(unit/10^8 spermatozoa)	12.63^a ±1.14	28.60^b ±1.71	57.20^c ±4.52	P<0.01
ALT(unit/10^8 spermatozoa)	0.89^a ±0.11	1.73^b ±0.21	4.00^c ±0.20	P<0.01
SMP(mg/ 10^9 sperm)	5.13^a ±0.12	3.95^b ±0.10	3.83^c ±0.11	P<0.01
Cholesterol (µg/ 10^8 sperm)	21.95^a ±0.44	18.27^b ±0.40	14.74^c ±0.60	P<0.01

Means bearing different superscripts in a row differ significantly.

Statistical Analysis: The mean and standard error of all the parameters studied were calculated. The mean values were compared using independent sample t-test as per Snedecor and Chochran (1989).

RESULTS AND DISCUSSION

The mean ± SE of different parameters studied in the swamp buffalo spermatozoa in fresh semen, after equilibration and after freezing are presented in Table 1.

FITC-Pisum *satiuum* lectin was used to assess acrosomal status by staining glycoproteins in the acrosome of permeabilised spermatozoa. Fluorescein-conjugated plant lectins like fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC- PSA) from the edible pea were used as a selective acrosomal staining of the spermatozoa of human (Cross *et al.* 1986), monkey (Cross *et al.* 1989) and stallion (Thomas *et al.* 2006). As PSA binds to the acrosomal contents, the progress of the acrosome reaction is indicated by the intensity and distribution of fluorescence over the acrosomal region. The acrosome reaction in buffalo spermatozoa commences just anterior to the equatorial segment and proceeds in an arborizing fashion towards the apical ridge (Watson and Plummer 1986). Therefore, as the reaction progresses, more acrosomal contents will be lost and therefore, less fluorescence will be seen. Staining only in the equatorial segment is also characteristic of a cell that has only recently completed its acrosome reaction whereas, cells devoid of staining in this region have fully completed the acrosome reaction some time previously (Kaul *et al.* 2001). In the present study, the mean incidences of acrosome reacted (PSA-ve) differed significantly ($P<0.01$) between the stages of evaluation and the total mean incidences of acrosome reacted spermatozoa was significantly ($P<0.01$) higher after equilibration and after freezing than fresh which might be due to partial capacitation of buffalo spermatozoa occurs during freezing and thawing followed by equilibration (Watson 1995).

The mean activity of AST and ALT differed significantly ($P<0.01$) between the stages of evaluation i.e. at fresh, after equilibration and freezing. The activity of transaminases (AST and ALT) enzymes are good indicator of semen quality. Good quality semen was characterized by lower AST and ALT activities (Pratap *et al.* 1999). The mean activity of AST and ALT was significantly ($P<0.01$) higher after equilibration and freezing in comparison to fresh semen. This might be due to temperature shock and sperm cell injury associated with freezing (Singh *et al.* 1991), changes in mitochondrial sheath with loss of protein from mid piece (Pratap *et al.* 1999) and increase in cell membrane permeability with or without rupture (Rastegarnia *et al.* 2010).

During capacitation, sperm surface proteins are modified, added or removed and an array of proteins have been shown to undergo tyrosine phosphorylation in different species (Cheema *et al.* 2011, Srivastava *et al.* 2013a) and in fertilization, these mammalian sperm membrane proteins are also involved in the penetration of cumulus matrix, recognition of zona pellucida and fusion with the oocyte plasma membrane (Bansal 2010). In the present study, there was significant ($P<0.01$) leakage of proteins from the equilibrated and frozen thawed spermatozoa. The leakage of proteins in the equilibrated and frozen thawed spermatozoa may be because of acrosomal damage and the alteration in the sperm membrane proteins might be due to sublethal damage which was occurred during cryopreservation leading to loss of sperm surface proteins (Talukdar *et al.* 2015), segregation of membrane proteins (Srivastava *et al*. 2013a), inactivation of membrane- bound enzymes and decreased lateral protein diffusion within the membrane (Watson 1995).

Results revealed that the mean levels of cholesterol differed significantly ($P<0.01$) between the stages of evaluation and was significantly ($P<0.01$) lower after equilibration and freezing than in fresh semen. 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 (Srivastava *et al.* 2013b). Various reports suggested an active participation of the sperm plasma membrane in the process of capacitation, mainly through the loss of cholesterol (Talukdar *et al.* 2015). The cholesterol efflux during *in vitro* capacitation increases the disorder of phospholipid packing, and results in increased bilayer permeability (Kadirvel *et al.* 2009). Similar to physiological and *in vitro* capacitation, a significant reduction of cholesterol content after cryopreservation was observed in the present study. Our results are in agreement with Kadirvel *et al.* 2009 and Bansal 2010 who observed decreased free cholesterol content and increased phospholipids and triglycerol content after freezing–thawing of boar semen. The mechanism of loss of cholesterol during cryopreservation is not completely understood. However, most of the cholesterol loss is due to slow diffusion from cell and a net transfer of cholesterol from rat and bovine sperm to the medium has already been demonstrated (Ehrenwald *et al.*1988). In the present study the mean cholesterol level of spermatozoa in frozen thawed semen differed significantly ($P<0.01$) from equilibrated spermatozoa. Therefore, cholesterol efflux may represent an integral part of the intrinsic regulatory property of sperm to undergo capacitation-like changes during cryopreservation.

This study provides evidence that premature capacitation occurs in partially (extended and cooled) or fully cryopreserved swamp buffalo spermatozoa, which are probably less able to reach the site of fertilization in oviduct than uncapacitated spermatozoa. So once the spermatozoa are capacitated, they exhibit elevated metabolic rates, increased membrane fluidity and permeability and if they do not achieve fertilization, they undergo spontaneous acrosome reactions due to an uncontrolled infux of Ca^{2+}. Hence, the fertilizing lifespan of cryocapacitated spermatozoa is limited, considering that the fertility of

cryopreserved semen *in vivo* is poor compared to fresh semen and premature capacitation may contribute to this reduced fertility.

REFERENCES

Bansal A. 2010. *In vitro* capacitation of bull spermatozoa: Role of vitamin E . *Webmed Central Reproduction* **1**:1–10.

Cheema R, Bansal A K, Bilaspuri G C and Gandotra V K. 2011. Correlation between the proteins and protein profile(s) of different regions of epididymis and their contents in goat buck. *Animal Science Papers and Reports* **29**: 75–84.

Cross N L, Morales P, Fukuda M and Behboodi E. 1989. Determining acrosomal status of the cynomolgus monkey *(Macaca jbscicularis)* sperm by fluorescence microscopy. *American Journal of Primatology* **17**: 157–163.

Cross N L, Morales P, Overstreet J W and Hanson F W. 1986. Two simple methods for detecting acrosome-reacted human sperm. *Gamete Research* **15**: 213–26.

Ehrenwald E, Parks J E and Foote R H. 1988. Cholesterol efflux from bovine sperm. I. Induction of the acrosome reaction with lysophosphatidyl choline after reducing sperm cholesterol. *Gamete Research* **20**: 145–57

Kadirvel G, Kathiravan P and Kumar S. 2011. Protein tyrosine phosphorylation and zona binding ability of *in vitro* capacitated and cryopreserved buffalo spermatozoa. *Theriogenology,* **75** : 1630–39.

Kadirvel G, Kumar S, Kumaresan A and Kathiravan P. 2009. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Animal Reproduction Science* **116**: 244–53.

Kaul G, Sharma G S, Singh B and Gandhi K K. 2001. Capacitation and acrosomal reaction in buffalo bull spermatozoa assessed by chlortetracycline and *Pisum sativum* agglutinin fluorescence assay. *Theriogenology* **55**: 1457–68.

Pratap N, Reddy V N V, Sharma P A, Honnappa T G, Devraj M, Krishnaswamy A and Arrora V K. 1999. Estimation of transaminases (AST and ALT) in cryopreserved Murrah buffalo semen. *Indian J. Animal Reproduction* **20**: 159–60.

Rastegarnia A, Eviaz S Y and Shafepour V. 2010. Studies on seminal plasma enzymes (GOT, GPT and LDH) profile and its relationship with quality of buffalo frozen semen. *Journal of Veterinary Clinical Research* **1**: 189–99

Singh J, Pangaonkar G R, Biswas R K and Shrivastava A K. 1991. Studies on leakage of tansaminases from buffalo spermatozoa in relation to deep freezing in certain extenders. *Indian Journal of Animal Reproduction* **12**: 135–40.

Snedecor G W and Cochran W G. 1989. *Statistical Methods*. 7th edn. The Iowa State University, Iowa (USA).

Srivastava N, Jerome A, Srivastava S K, Ghosh S K and Amit K. 2013a. Bovine seminal PDC–109 protein: A review of biochemical and functional properties. *Animal Reproduction Science* **138**: 1–13.

Srivastava N, Srivastava S K, Ghosh S K, Amit K, Perumal P and Jerome A. 2013b. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. *Asian Pacific Journal of Reproduction* **2**: 126–31.

Talukdar D J, Ahmed K and Talukdar P. 2015. Cryocapacitation and fertility of cryopreserved semen. *International Journal of Livestock Research* **5**: 11–18.

Talukdar D J. 2014. 'Morphological and functional characterization of *in vitro* capacitated and frozen thawed Swamp buffalo spermatozoa'. Ph. D. Thesis, Assam Agricultural University, Guwahati, India.

Thomas A D, Meyers S A and Ball B A. 2006. Capacitation-like changes in equine spermatozoa following cryopreservation. *Theriogenology* **65**: 1531–50.

Watson P F and Plummer J M. 1986. Relationship between calcium binding sites and membrane fusion during the acrosome reaction induced by ionophore in ram spermatozoa. *Journal of Experimental Zoology* **238**: 113–l18

Watson P F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and assessment of their post-thawing function. *Reproduction Fertility and Development* **7**: 871– 91.