CRYOPRESERVATION INDUCES CAPACITATION-LIKE CHANGES OF THE SWAMP BUFFALO SPERMATOZOA

D.J. Talukdar¹, K. Ahmed¹, S. Sinha¹, S. Deori²*, G.C. Das¹ and Papori Talukdar³

ABSTRACT

A total of forty ejaculates were collected from eight buffalo bulls by artificial vagina method. Each ejaculate was split into two parts, one part was used for in vitro capacitation by TALP medium, and the rest was frozen in Tris-egg yolk-citrate-glycerol extender. The results revealed that the live acrosome reacted and total acrosome reacted frozen thawed spermatozoa differed significantly (P<0.01) from in vitro capacitated spermatozoa at different hours of incubation. The incidence of hyperactivated motility, total HOST-reacted spermatozoa, sperm membrane protein and cholesterol level of frozen thawed spermatozoa also differed significantly (P<0.01) from in vitro capacitated spermatozoa during different hours of incubation except at 0 h for hyperactivated motility, 6 h for total HOST-reacted spermatozoa, 1 h for sperm membrane protein level and at 2 h for cholesterol level. The study suggests that cryopreservation induces capacitation-like changes of the swamp buffalo spermatozoa in respect of acrosomal status, plasma membrane integrity, membrane protein and cholesterol levels.

Keywords: capacitation, cryopreservation, spermatozoa, swamp buffalo

INTRODUCTION

Artificial insemination (AI) is useful for the improvement of milk productivity in buffaloes by the propagation of animals with high genetic potential (Ciptadi et al., 2012). The best preservation technique to date of post-thaw survival is restricted to about 50% of the sperm population (Watson, 1995). The final cryopreservation goal of semen is not only to maintain the initial motility but also to maintain the necessary metabolism to produce viable sperm to survive in the female reproductive tract at the time of fertilization. The freezing and thawing process provokes morphological or biochemical cryogenic damage resulting in sperm dysfunction and changes in cell’s membrane. The exposure of spermatozoa to low temperatures shortens their capacitation time, changing the membrane lipid architecture, membrane permeability and the reducing efficiency of enzymes extruding calcium ions. These changes resemble capacitation, and are likely to reduce long-term sperm viability and alter their motility. The term “cryocapacitation” have been introduced to emphasize the fact that cryopreservation procedures induce capacitation-like changes in spermatozoa (Watson, 1995; Cormier and Bailey, 2003). These cooling-related capacitation-like changes in spermatozoa, may affect the fertility of

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cryopreserved semen, by rendering the cells less stable in the female reproductive tract, after artificial insemination and therefore relatively short-lived. Such changes cannot easily be distinguished from true capacitation. Keeping in view, the study had been planned with the objective to study the functional characteristics of *in vitro* capacitated and frozen-thawed spermatozoa of swamp buffalo bull.

**MATERIALS AND METHODS**

A total of 40 semen samples were collected by artificial vagina method from each eight swamp buffalo bulls aged five to eight years maintained at College of Veterinary Science, Guwahati, Assam, India. Each ejaculate was evaluated for volume, mass activity, and initial motility immediately after collection. Samples having volume 1.0 ml or more, mass activity 3 or more and initial sperm motility 70% or more were divided into two parts, one part was used for *in vitro* capacitation by TALP medium (NaCl-92.9 mM; KCl-4 mM; NaHCO3-25.9 mM; CaCl2 .2H2O-10 mM; MgCl2.6 H2O-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 sulphate-50 µg/ml and Deionised triple distilled water up to 1000 ml) at concentration of 6×10^9 spermatozoa/ml (Rogers and Yanangimachi, 1975) at 37°C for 6 h and the rest was frozen with Tris-egg yolk-citrate extender [Tris (hydroxy methyl) amino methane-2.422 g; citric acid monohydrate-1.36 g; fructose-1.0 g; penicillin G sodium-1000 IU/ml; streptomycin sulphate-100 mg/ml; double distilled water up to 100ml] with 20% egg yolk and 6.4% glycerol to yield approximately 60 million motile sperm/ml. The extended semen was equilibrated for 4 h at 5°C before filling in 0.25 ml French mini 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 (-196°C) and stored. The frozen semen straws were thawed at 37°C for 30 seconds. Each sample of *in vitro* capacitated sperm was evaluated at one hour interval starting from 0 h and all the samples of frozen thawed and *in vitro* capacitated were evaluated for hyperactivated motility (Marquez and Susan, 2004), total HOST-reacted sperm (Jeyendran *et al.*, 1984), acrosomal status by using FITC labelled *Pisum sativum* agglutinin (Kaul *et al.*, 2001) and live intact acrosome using Eosin-Nigrosin-Giemsasta staining technique (Tamuli and Watson, 1994), sperm membrane protein (Cheema *et al.*, 2011) and cholesterol level (Srivastava *et al.*, 2013) by using quality kit (Siemens Ltd., 589, Sayajipura, Ajwa Road, Vadodara-390 019, Gujarat, India) in a Systronics Spectrophotometer 106. The statistical analysis of the data was done using SAS Enterprise Guide 4.2 version.

**RESULTS AND DISCUSSION**

The results of analysis of variance (ANOVA) are presented in Table 1. ANOVA revealed highly significant effect of source (*in vitro* capacitated vs frozen thawed spermatozoa).

Before fertilizing of the oocyte, mammalian spermatozoa undergo the sequence of membrane alterations associated with accumulation of calcium ion and the increase of tyrosine phosphorylation resulting in sperm hyperactivation (Hewitt and England, 1998; Petrunkina *et al.*, 2003), which is characterized by high-amplitude and asymmetrical
Table 1. Functional integrity of *in vitro* capacitated and frozen thawed swamp buffalo spermatozoa (mean*± se).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Stage</th>
<th>0</th>
<th>1</th>
<th>2</th>
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<th>Frozen Thawed</th>
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<tr>
<td>Hyperactivated Motility (%)</td>
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<td>In vitro capacitation (hours)</td>
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<td>0</td>
<td>14.00±0.80</td>
<td>35.75±1.12</td>
<td>52.87±1.38</td>
<td>60.25±1.05</td>
<td>74.50±1.78</td>
<td>47.50±1.11</td>
<td>41.25±1.30</td>
<td>16.00±0.76</td>
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<tr>
<td>Frozen Thawed</td>
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<td>Total HOST reacted Sperm (%)</td>
<td>82.55±1.00</td>
<td>78.02±1.26</td>
<td>77.22±1.12</td>
<td>75.55±1.21</td>
<td>71.70±1.49</td>
<td>69.25±1.61</td>
<td>61.57±2.20</td>
<td>62.20±1.31</td>
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<td>Total acrosome reacted sperm (%) (Eosin-Nigrosin-Giems stain)</td>
<td>8.62±0.33</td>
<td>45.57±2.97</td>
<td>52.42±2.49</td>
<td>50.92±2.39</td>
<td>56.92±1.88</td>
<td>54.27±1.83</td>
<td>49.37±2.59</td>
<td>27.28±0.81</td>
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<tr>
<td>Total acrosome reacted sperm (%) (FITC-PSA)</td>
<td>7.90±0.59</td>
<td>43.50±2.46</td>
<td>53.20±1.44</td>
<td>68.00±1.87</td>
<td>69.65±1.39</td>
<td>68.47±1.42</td>
<td>75.12±1.24</td>
<td>38.72±1.79</td>
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<tr>
<td>Sperm membrane Protein (mg/ 10^9 sperm)</td>
<td>5.13±0.12</td>
<td>3.92±0.13</td>
<td>3.25±0.12</td>
<td>2.64±0.11</td>
<td>2.55±0.13</td>
<td>1.82±0.06</td>
<td>1.45±0.08</td>
<td>3.95±0.10</td>
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<td>Cholesterol (µg/ 10^8 sperm)</td>
<td>21.95±0.44</td>
<td>19.08±0.51</td>
<td>14.65±0.51</td>
<td>12.94±0.47</td>
<td>11.07±0.59</td>
<td>8.45±0.54</td>
<td>5.64±0.46</td>
<td>14.74±0.60</td>
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</table>

*40 observations.

Means bearing different superscripts in a row differ significantly.
flagellar beating that assists sperm in penetrating the oocyte zona pellucida (Marquez and Susan, 2004). Hyperactivation has been considered part of the capacitation process because sperm have been observed to hyperactivate 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). Some of the proteins that become tyrosine phosphorylated during capacitation have been localized to the flagellum, and therefore it has been proposed that they are involved in hyperactivation (Si and Okuno, 1999). The incidence of hyperactivated motility of frozen thawed spermatozoa in the present study differed significantly (P<0.01) with hyperactivated motility of in vitro capacitated spermatozoa at different hours of incubation and the incidence was slightly higher than 0 h and lower than 1 h which might be due to cryopreservation as cryopreserved spermatozoa have poor calcium efflux mechanisms and are less efficient in extruding calcium ions resulting in rapid accumulation of cytosolic calcium ion (Bailey et al., 1994) and when these intracellular ion concentrations reach threshold levels, capacitation followed by acrosome reaction is triggered without the activation of zona pellucida receptors in the sperm plasma membrane or the associated signal transduction system (Bailey et al., 2000).

The determination of the acrosome status in cryopreserved sperm is of the fundamental importance as cryopreservation directly damages sperm membrane, which could be followed by a loss of the acrosomal matrix contents. The incidence of total live acrosome reacted spermatozoa in the present observation differed significantly (P<0.01) between in vitro capacitated spermatozoa at different hours of incubation and frozen thawed spermatozoa. The incidence of live acrosome reacted spermatozoa in frozen thawed spermatozoa was higher than 0 h and lower than 1 h of incubation, which might be due to partial capacitation of buffalo spermatozoa during freezing and thawing followed by equilibration (Watson, 1995). There is substantial evidence that cryopreservation promotes the premature capacitation of spermatozoa (Bailey et al., 2000; Watson, 2000) and this cryocapacitation is frequently cited as one of the factors associated with the reduced longevity of cryopreserved spermatozoa in the female reproductive tract by a loss of the acrosomal matrix contents. Detection of the viability and stages of acrosomal exocytosis, either spontaneous or induced, was carried out using fluorescent probes. FITC-Pism sativum lectin (FITC-PSA) 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 has been used as a selective acrosomal staining of the spermatozoa of human (Cross et al., 1986), stallion (Casey et al., 1993) and monkey (Cross et al., 1989). 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 (Tesarik et al., 1993). The total acrosome reacted (PSA-ve) spermatozoa in the present observation differed significantly (P<0.01) between frozen thawed spermatozoa and in vitro capacitated spermatozoa at different hours of incubation. The incidence in frozen thawed spermatozoa was slightly higher than in 0 h and lower than in 1 h of incubation. This might be due to cryocapacitation which could be followed by a loss of the acrosomal matrix contents (Cross et al., 1986).

The plasma membrane integrity of sperm is of crucial importance for optimal sperm function and only a sperm with an intact plasma membrane can undergo a series of complex changes in the female reproductive tract and can acquire the ability to fertilize an oocyte (Yanagimachi, 1994). The mean percentage of total HOST reacted spermatozoa of frozen thawed semen in the present study differed significantly (P<0.01) with in vitro capacitated spermatozoa at different hours of incubation except 6 h where the values was in close proximity. This might be due to cryopreservation results partial capacitation that induced membrane phase changes, which are thought to result in lateral phase separation of membrane components and increased membrane permeability for solutes (Hammerstedt et al., 1990). The disruption of plasma membrane integrity caused by disarrangement of lipids within the membrane during cryocapacitation may induce further cellular damage and consequently lead to irreversible damage to its integrity (Jeyendran et al., 1984; Watson, 1995).

Capacitation is a post-testicular developmental and maturational process of mammalian spermatozoa occurring during their transit through the female reproductive tract with modification of sperm surface proteins, added or removed and an array of proteins have been shown to undergo tyrosine phosphorylation in different species (Luconi et al., 1996; Galantino et al., 1997). 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). In the present observations, there was leakage of proteins from the frozen thawed 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 frozen thawed spermatozoa may be because of acrosomal damage and in capacitated, because of acrosomal damage as well as due to capacitation and acrosome reaction. As the mean sperm membrane protein level of spermatozoa in frozen thawed semen differed significantly (P<0.01) from in vitro capacitated spermatozoa at different hours of incubation except 1 h where the values was in close proximity. The similarity of sperm membrane protein level in frozen thawed and in vitro capacitated sperm at 1 h of incubation at 37°C in TALP medium in the present study indicated the initiation of capacitation of spermatozoa which corroborated the findings of earlier workers (Watson, 1995; Cromier and Bailey, 2003; Kadirvel et al., 2009) who reported that the cryopreserved sperm are in a partially capacitated state. 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 (Lessard et al., 2000), segregation of membrane proteins (De Leeuw et al., 1990), inactivation of membrane-bound enzymes and decreased lateral protein diffusion within the membrane (Watson, 1995). The molecular mechanisms of capacitation are not
completely elucidated; however, recent studies have demonstrated involvement of numerous structural and biochemical modifications in spermatozoa, such as changes in membrane composition and membrane fluidity (Harrison et al., 1996; Green and Watson, 2001), increased intracellular calcium (Visconti et al., 1998), activation of ion channels (Florman et al., 1998) and generation of reactive oxygen species (de Lamirande et al., 1997). Various reports suggested an active participation of the sperm plasma membrane in the process of capacitation, mainly through the loss of cholesterol (Cross, 1998; Visconti et al., 1999). The cholesterol efflux during in vitro capacitation increases the disorder of phospholipid packing, and results in increased bilayer permeability (Cross, 1998). 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 Cerolini et al. (2001) and Kadirvel et al. (2009) who observed decreased free cholesterol content and increased phospholipids and triglycerol content after freezing-thawing of boar semen. Furthermore, sperm membranes are known to release phospholipids into surrounding medium during cold shock (Darin-Bennett et al., 1973). 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). Release of cholesterol from the membrane plane, a probable consequence of peroxidative damage to membrane lipids, could lead to the premature capacitation of cryopreserved bull spermatozoa (Cormier et al., 1997). The loss of phospholipids in cryopreserved samples occurs at a more rapid rate when compared to fresh samples and follows the pattern expected for lipid peroxidation (Alvarez and Storey, 1992). The present study observed that the mean cholesterol level of spermatozoa in frozen thawed semen differed significantly (P<0.01) from in vitro capacitated spermatozoa at different hours of incubation except 2 h where the values was in close proximity. Therefore, cholesterol efflux may represent an integral part of the intrinsic regulatory property of sperm to undergo capacitation-like changes during cryopreservation. The reduction in cholesterol content which was similar in post thawed spermatozoa and in vitro capacitated spermatozoa after 2 h of incubation indicated the partial capacitation status of post thawed spermatozoa.

CONCLUSION

In conclusion, this study showed that cryopreservation induces capacitation-like changes of the Swamp buffalo spermatozoa in respect of acrosomal status, plasma membrane integrity, membrane protein and cholesterol levels.

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