Review Article

Factors Affecting the Quality of Cryopreserved Buffalo (Bubalus bubalis) Bull Spermatozoa

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Contents
Storage of buffalo (Bubalus bubalis) bull semen in the cryopreserved state is discussed in this article. Fertility rate in buffalo following artificial insemination with frozen–thawed semen is reviewed. To better understand the freezability of bubaline spermatozoa, the available data on biochemical components and the activity of specific enzymes of semen/spermatozoa are given. Moreover, the major factors that may influence the post-thaw viability and fertility of buffalo spermatozoa are examined in detail. In addition, suggestions for improvement in cryogenic procedures for buffalo spermatozoa are also given.

Introduction
The domestic buffalo, Bubalus bubalis, is a distinct species within the Bovidae family. The buffalo population is continuously increasing, and is estimated at over 170 million head (Food and Agricultural Organization (FAO) 2004). More than 95% of the population is located in Asia, where buffaloes play a prominent role in rural livestock production providing the milk, meat and work draft force. In recent decades, buffalo farming has also expanded widely in Mediterranean areas and in Latin America.

Only in India and Pakistan are there well-defined buffalo breeds (Drost 2007). There are 18 river buffalo breeds in South Asia, which are further classified into five major groups designated as the Murrah, Gujarat, Uttar Pradesh, Central Indian and South Indian breeds. The Nili-Ravi buffalo, belonging to the Murrah group, is recognized as the highest milk-producing breeds of buffalo (Cockrill 1974). The swamp buffalo found in Southeast and Far East Asia has low milk production, and is mostly used as a draft animal by small farm holder or is utilized for meat purpose.

The production potential of livestock can be increased by genetic improvement using one of the modern ways of breed improvement, e.g., artificial insemination (AI). Moreover, the quality of frozen–thawed semen is one of the most influential factors affecting the likelihood of conception (Saacke 1984). Application of AI with frozen–thawed semen has been reported on a limited scale in buffalo, because of poor freezability and fertility of buffalo spermatozoa when compared with cattle spermatozoa (Kakar and Anand 1981; Muer et al. 1988; Raizada et al. 1990; Singh and Pant 2000; Andrabi et al. 2001, 2008; Ahmad et al. 2003; Senatore et al. 2004; Kumaresan et al. 2005). Hence, successful cryopreservation of bubaline semen would aid in the creation of long-term storage of male gametes and the maintenance of genetic stock that could improve milk and beef production and its associated economic value internationally.

This article deals with the storage of bubaline spermatozoa in deep-frozen (−196°C) state and reviews the major factors affecting the viability and fertility of cryopreserved buffalo spermatozoa.

Cryopreservation of Spermatozoa

Cryopreservation is a non-physiological method that involves a high level of adaptation of biological cells to the osmotic and thermic shocks that occur both during the dilution, cooling–freezing and during the thawing procedures (Watson et al. 1992; Holt 2000a,b). Damage occurring during the freezing–thawing procedures affect mainly cellular membranes (plasma and mitochondrial) and in the worst case, the nucleus (Blesbois 2007). This damage to membranes has consequences on viability and different metabolic factors including adenosine triphosphate (ATP) concentration in spermatozoa. Therefore, such changes in the integrity of spermatozoa affect the viability and fertility. Table 1 summarizes different stresses encountered by the cell and the effect on the cell of each stressor during the cryogenic processes.

The first successful freezing of buffalo semen was reported by Roy et al. (1956). Basirov (1964) was the first to report the pregnancy with frozen–thawed buffalo bull spermatozoa. Since then, AI has been adopted in buffaloes; however, it remains unpopular because of poor fertility rate with frozen–thawed semen (Muer et al. 1988; Andrabi et al. 2001; Ahmad et al. 2003; Senatore et al. 2004; Kumaresan et al. 2005, 2006; Shukla and Misra 2007).

A summary of available studies on fertility of frozen buffalo spermatozoa with AI is presented in Table 2. A critical assessment in term of first service conception rate of the reports given in Table 2 is difficult, as in most of the studies; the number of inseminations was low. Details such as number of spermatozoa per dose of AI and freezing protocol were not provided for some of the studies. Few studies even lacked the basic information, like on the type of extender used for cryopreservation or the total number of animals inseminated. However, despite the shortcomings in the published reports (Table 2), it can be suggested that the pregnancy rate in buffalo with AI using frozen–thawed semen is not comparable with that of cattle.
Conception rate in bufaloes inseminated with frozen–thawed semen under field condition is approximately 30% (Chohan et al. 1992; Anzar et al. 2003). Published reliable studies on the fertility of liquid stored buffalo semen seem not to be available (Sansone et al. 2000). However, few scattered reports indicate a pregnancy rate of approximately 60% with liquid semen AI in bufaloes (Tomar and Singh 1970; Akhter et al. 2007). Therefore, a pregnancy rate higher than 50% is regarded as a good result after AI with frozen–thawed sperma-
toza in buffalo (Vale 1997). It is relevant to mention that the same pregnancy rates i.e., near 50% under normal circumstances are considered poor in cattle with frozen–thawed spermatozoa.

From above, it is suggested that cryopreservation adversely affects the viability and the fertilizing potential of buffalo bull spermatozoa. Therefore, there is a need to discuss in depth the major factors influencing the successful cryopreservation of buffalo spermatozoa.

Factors Affecting Freezability
Biochemical characteristics of semen
It is reported that buffalo spermatozoa are more susceptible to hazards during freezing and thawing than cattle spermatozoa, thus resulting in lower fertilizing potential (Raizada et al. 1990; Andrabi et al. 2008). Moreover, there are specific biochemical factors that affect the ability of spermatozoa to prevent damages caused by the cryogenic procedures. One of the many possible causes of lower freezability of buffalo bull semen compared to cattle bull can be due to the differences in the lipid ratio of the spermatozoa (Jain and Anand 1976; Tatham 2000; European Regional Focal Point on Animal Genetic Resources, 2003). For example, phosphatidyl choline makes up approximately 66% of all phospholipids found in buffalo sperm plasma membrane (Cheshmedjieva and Dimov 1994) but approximately 50% in case of cattle bull sperm membrane (Parks et al. 1987). Similarly, phosphatidyl ethanolamine makes up approximately 23% of all phospholipids present in buffalo sperm plasma membrane (Cheshmedjieva and Dimov 1994) but almost 10% in case of cattle bull sperm membrane (Parks et al. 1987).

To better understand the nature of bubaline sperma-
toza the available data on biochemical components and the activity of specific enzymes of semen are given in Tables 3 and 4. The values of different constituents given in Tables 3 and 4 show that buffalo whole semen/semenal plasma/spermatozoa/plasma membrane compared to cattle have distinct characteristics, particularly the membrane lipid ratio. Therefore, there is a need to develop biochemically defined extenders and cryogenic procedures that are species specific, and may result in the improvement of viability and fertility of frozen–thawed buffalo spermatozoa.

Buffer
Dilution of semen in a suitable buffer is one of the important factors affecting sperm survival during cryo-
preservation (Rasul et al. 2000). An ideal buffer should have: (i) pH between 6 and 8, preferably 7; (ii) maximum water solubility and minimum solubility in all other solvents; (iii) minimum salt effects; (iv) minimum buffer concentration; (v) least temperature effect; (vi) well-behaved cation interactions; (vii) greater ionic strengths and (viii) chemical stability (Bates 1961; Good et al. 1966; Good and Izawa 1972; Keith and Morrison 1981).

Development of a suitable buffering system for the cryopreservation of buffalo spermatozoa has been in progress for sometime (Rasul et al. 2000). Several studies have concentrated on the use of chemically defined buffers for buffalo semen. In this regard, Matharoor and Singh (1980) tested citrate, Tris or citric acid as buffers for deep-freezing of buffalo spermatozoa. They found that freezing loss was least with Tris-based extender as judged by post-thaw motility. Similarly, Chinnaiya and Ganguli (1980a) found better post-thaw sperm motility with Tris-based extender than citrate or citric acid-based extenders. In another study, Chinnaiya and Ganguli (1980b) found that spermatozoa frozen in citric acid, citrate or Tris-based extender showed similar degree of acrosomal damage and similar recovery rates. However, acrosin activity was greatest in citrate-based diluent and least in Tris buffer.

Ahmad et al. (1986) found that Tris–citric acid based extender is suitable for the cryopreservation of buffalo spermatozoa in terms of post-thaw motility and survival. Later on, Dhami and Kodagali (1990) studied the effects of semen extenders based on Tris or citrate buffer. It was reported that Tris-based extender improved the freezability of buffalo spermatozoa as judged by the extracellular release of spermzoal enzymes and in vivo fertility. Similarly, Singh et al. (1990, 1991) studied semen diluents based on citrate or Tris or citric acid for freezing of buffalo spermatozoa. They found that with Tris-based extender there was least release of lactic dehydrogenase and sorbitol dehydrogenase in buffalo spermatozoa during cryopreservation followed by citrate and citric acid-based extenders. In addition, Tris provided the highest protection against acrosomal damage compared to other buffers tested.

Dhami et al. (1994) studied the effects of semen extenders based on Tris or citrate. It was found that Tris-based extender yielded higher post-thaw spermatozoal motility. Singh et al. (2000) compared Tris-buffer with Laiciphos (IMV, L’Aigle, France; containing laiciphos, egg yolk, distilled water and unknown buffer) and Biociphos (IMV, France; containing biociphos with
Table 2. Fertility rate in buffalo following AI with frozen-thawed semen

<table>
<thead>
<tr>
<th>Reference</th>
<th>Extender</th>
<th>Total number of first AI</th>
<th>Over all first service CR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhoitrekar and Nagareennkar 1971</td>
<td>Skim milk powder-yolk-glycine-citrate-fructose-glycerol</td>
<td>109</td>
<td>45.0</td>
</tr>
<tr>
<td>Bandopadhyay and Roy 1975</td>
<td>Yolk-citrate-glycerol</td>
<td>Information not available</td>
<td>40.6</td>
</tr>
<tr>
<td>Chinnaya et al. 1979</td>
<td>Yolk-citrate-glycerol, Citric acid-whey-glycerol and Tris-yolk-glycerol</td>
<td>315</td>
<td>53.93</td>
</tr>
<tr>
<td>Singh et al. 1980</td>
<td>Tris-yolk-glycerol</td>
<td>72</td>
<td>45.8</td>
</tr>
<tr>
<td>Tuli et al. 1981a</td>
<td>Tris-yolk-glycerol</td>
<td>159</td>
<td>35.22</td>
</tr>
<tr>
<td>Matharoo and Garcha 1986</td>
<td>Tris-yolk-glycerol</td>
<td>825</td>
<td>39.30</td>
</tr>
<tr>
<td>Heuer and Bajwa 1986</td>
<td>Information not provided</td>
<td>61 952</td>
<td>51.78</td>
</tr>
<tr>
<td>Heuer et al. 1987</td>
<td>Lactose-fructose-yolk-glycerol, Skim milk-fructose-yolk-glycerol and Tris-fructose-yolk-glycerol</td>
<td>3220</td>
<td>37.4</td>
</tr>
<tr>
<td>Singh and Singh 1988</td>
<td>Information not available</td>
<td>218</td>
<td>41.0</td>
</tr>
<tr>
<td>Ahmad et al. 1988</td>
<td>Yolk-glycerol with milk or lactose or fructose and lactose</td>
<td>2745</td>
<td>44.7</td>
</tr>
<tr>
<td>Bhavsar et al. 1988</td>
<td>Yolk-lactose-fructose-glycerol</td>
<td>1908</td>
<td>39.2</td>
</tr>
<tr>
<td>Bhavsar et al. 1989a</td>
<td>Tris-fructose-yolk-glycerol</td>
<td>Information not available</td>
<td>45.85</td>
</tr>
<tr>
<td>Bhavsar et al. 1989b</td>
<td>Tris-fructose-yolk-glycerol with or without additives (L-cysteine HC1 H2O, sheep hyaluronidase, beta-amylase or acetylcholine chloride)</td>
<td>3791</td>
<td>46.0</td>
</tr>
<tr>
<td>Singh 1990</td>
<td>Information not provided</td>
<td>Information not provided</td>
<td>39.7</td>
</tr>
<tr>
<td>Haranath et al. 1990</td>
<td>Tris-egg yolk-glycerol</td>
<td>Information not available</td>
<td>51.53</td>
</tr>
<tr>
<td>Dhami and Kodagali 1990</td>
<td>Tris-fructose-yolk-glycerol, Yolk-citrate-glycerol and Lactose-yolk-glycerol</td>
<td>3412</td>
<td>39.9</td>
</tr>
<tr>
<td>Dhami and Kodagali 1991</td>
<td>Information not provided</td>
<td>2995</td>
<td>40.1</td>
</tr>
<tr>
<td>Hassan and Zia Ur 1994</td>
<td>Information not provided</td>
<td>1110</td>
<td>65.26</td>
</tr>
<tr>
<td>Dhami et al. 1994</td>
<td>Tris-yolk-glycerol-, Citrate-yolk-glycerol- and Lactose-yolk-glycerol, with or without (control) cysteine, EDTA and raffinose</td>
<td>853</td>
<td>57.95</td>
</tr>
<tr>
<td>Barnabe et al. 1994</td>
<td>Tris-TES and Tris-yolk</td>
<td>Information not available</td>
<td>53.14</td>
</tr>
<tr>
<td>Dhami et al. 1996</td>
<td>Tris-citric acid-fructose-yolk-glycerol and Whole cow’s milk-yolk-glycerol</td>
<td>806</td>
<td>63.98</td>
</tr>
<tr>
<td>Younis et al. 1999</td>
<td>Lactose-fructose-yolk-glycerol</td>
<td>971</td>
<td>41.8</td>
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<tr>
<td>Barile et al. 1999a</td>
<td>Information not available</td>
<td>217</td>
<td>42.5</td>
</tr>
<tr>
<td>Gokhale and Bhagat 2000</td>
<td>Information not provided</td>
<td>6762</td>
<td>52.0</td>
</tr>
<tr>
<td>Sukhato et al. 2001a</td>
<td>Tris-fructose-yolk-glycerol</td>
<td>178</td>
<td>37.0</td>
</tr>
<tr>
<td>Pant et al. 2001</td>
<td>Information not provided</td>
<td>202</td>
<td>34.95</td>
</tr>
<tr>
<td>Prabhakar et al. 2002</td>
<td>Information not available</td>
<td>1941</td>
<td>59.15</td>
</tr>
<tr>
<td>Taraphder et al. 2003</td>
<td>Information not provided</td>
<td>Information not available</td>
<td>40.75</td>
</tr>
<tr>
<td>Sosa et al. 2003</td>
<td>Milk-, Laiciphos- and Tris- with or without glycerol, DMSO and propylene glycol</td>
<td>Information not available</td>
<td>50.6</td>
</tr>
<tr>
<td>Presicce et al. 2004a</td>
<td>Information not available</td>
<td>67</td>
<td>48.0</td>
</tr>
<tr>
<td>Kanchan and Singh 2005</td>
<td>Information not available</td>
<td>Information not available</td>
<td>29.87</td>
</tr>
<tr>
<td>Anzar et al. 2003</td>
<td>Information not available</td>
<td>Information not available</td>
<td>29.0</td>
</tr>
<tr>
<td>Andrabi et al. 2006</td>
<td>Tris-citric acid-fructose-yolk-glycerol</td>
<td>432</td>
<td>53.0</td>
</tr>
</tbody>
</table>

*Pregnancies were confirmed through rectal palpations.

Buffalo were synchronized with a progesterone-releasing intravaginal device (PRID) containing progesterone and oestradiol benzoate, for 10 days. Seven days after insertion of PRID the buffalo received an injection of pregnant mare serum gonadotropin (PMSG) and an injection of cloprostenol. AI was performed 48, 72 or 96 h after removal of the device.

Oestrus synchronization was performed by inserting a progesterone-releasing intravaginal device (CIDR-B®) into the vagina. Each buffalo was injected intramuscularly with 1 mg of oestradiol benzoate (CIDIROL®) on the day of CIDR-B insertion and 150 IU of ECG upon CIDR-B removal (12 days after insertion). AI was performed between 48 and 50 h after the CIDR-B was removed.

From the results of the above mentioned studies, it is suggested that zwitterion buffers particularly, Tris-citric acid may provide the most satisfactory buffering system to improve the post-thaw freezability and consequently may also improve the fertility of buffalo spermatozoa. It is believed that zwitterion buffers have pH nearer to the pKₐ (acid dissociation constant). Also there pKₐ is least influenced by temperature as compared to other buffers (Graham et al. 1972).
Table 3. Biochemical composition of buffalo semen

<table>
<thead>
<tr>
<th>Characteristic of component</th>
<th>Reference</th>
<th>Whole semen</th>
<th>Seminal plasma</th>
<th>Spermatozoa</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid</td>
<td>Rattan et al. 1980</td>
<td>23.47 mg/100 ml</td>
<td></td>
<td></td>
<td>Amount of lactic acid in cattle bull seminal plasma is 72 ± 5 mg/100 ml (Dabas et al. 1984)</td>
</tr>
<tr>
<td></td>
<td>Dabas et al. 1984</td>
<td>82 ± 6 mg/100 ml</td>
<td>167 ± 9 μg/10^11 cells</td>
<td></td>
<td>Amount of lactic acid in cattle bull spermatozoa is 352 ± 16 μg/10^11 cells (Dabas et al. 1984)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Jain 1987</td>
<td>0.091 ± 0.011 μmol/ml</td>
<td>0.024 ± 0.003 μmol/ml</td>
<td>0.066 ± 0.014 μmol/10^9 cells</td>
<td>Amount of ascorbic acid in whole semen, seminal plasma and spermatozoa of cattle bull is 0.131 ± 0.030, 0.505 ± 0.0185 μmol/ml and 0.0832 ± 0.0337 μmol/10^9 cells, respectively (Jain 1987)</td>
</tr>
<tr>
<td></td>
<td>Banerjee and Ganguli 1973</td>
<td>6.2 ± 0.8 mg/100 ml</td>
<td>3.9 ± 0.5 mg/100 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>Banerjee and Ganguli 1973</td>
<td>441.8 ± 31.9 mg/100 ml</td>
<td>444.9 ± 17.4 mg/100 ml</td>
<td></td>
<td>Amount of citric acid in whole semen and seminal plasma of cattle bull is 531.3 ± 73.4 and 576.9 ± 58.6 mg/100 ml, respectively (Banerjee and Ganguli 1973)</td>
</tr>
<tr>
<td>Fructose</td>
<td>Salem and Osman 1972</td>
<td>368.12–430.92 mg/100 ml</td>
<td></td>
<td></td>
<td>Amount of fructose in seminal plasma of cattle bull is 519.07–618.93 mg/100 ml (Salem and Osman 1972)</td>
</tr>
<tr>
<td></td>
<td>Banerjee and Ganguli 1973</td>
<td>623.8 ± 83.6 mg/100 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rattan et al. 1980</td>
<td>815.71 mg/100 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipids</td>
<td>Jain and Anand 1976</td>
<td>1.500 mg/ml</td>
<td>1.147 mg/10^9 cells</td>
<td></td>
<td>Amount of lipids in seminal plasma and spermatozoa of cattle bull is 2.900 mg/ml and 0.703 mg/10^9 cells, respectively (Jain and Anand 1976)</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>1.750 ± 0.030 mg/ml</td>
<td>1.320 ± 0.030 mg/10^9 cells</td>
<td></td>
<td>Amount of lipids in seminal plasma and spermatozoa of cattle bull is 1.04 ± 0.2 mg/ml and 2.18 ± 0.22 mg/10^9 cells, respectively (Pursel and Graham 1967)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Mohan et al. 1979</td>
<td>91.84 ± 3.91–141.88 ± 3.12 mg/100 ml</td>
<td></td>
<td></td>
<td>Amount of cholesterol in whole semen of cattle bull is 104.412 mg/100 ml (RoyChoudhury 1970)</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Jain and Anand 1976</td>
<td>0.594 mg/ml</td>
<td>0.548 mg/10^9 cells</td>
<td></td>
<td>Amount of phospholipids in seminal plasma of cattle bull is 1.491 mg/ml (Jain and Anand 1976)</td>
</tr>
<tr>
<td></td>
<td>Sidhu and Guraya 1979</td>
<td>0.1735 ± 0.0256 mg/ml</td>
<td>0.3074 ± 0.0923 mg/10^9 cells</td>
<td></td>
<td>Amount of phospholipids in seminal plasma of cattle bull is 0.416 mg/10^9 cells (Jain and Anand 1976)</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>0.069 ± 0.02 mg/ml</td>
<td>0.064 ± 0.02 mg/10^9 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>Jain and Anand 1976</td>
<td>21.7 ± 1.0% of total phospholipids</td>
<td>30.4 ± 1.4% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl choline in seminal plasma of cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 30.0, 26.3 and 24.5 ± 2.2% of total phospholipids, respectively</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>34.1 ± 1.8% of total phospholipids</td>
<td>28.0 ± 1.2% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl choline in spermatozoa of cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 35.6, 30.1 and 17.9 ± 0.8% of total phospholipids, respectively</td>
</tr>
<tr>
<td>Phosphatidyl choline (choline plasmogen)</td>
<td>Jain and Anand 1976</td>
<td>17.3 ± 0.9% of total phospholipids</td>
<td>19.4 ± 1.7% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl choline in seminal plasma of cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 23.6%, 17.6% and 32.9 ± 2.0% of total phospholipids, respectively</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Amount of phosphatidyl choline in spermatozoa obtained from cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 28.0%, 31.8% and 36.8 ± 1.4% of total phospholipids, respectively</td>
</tr>
<tr>
<td>Characteristic of component</td>
<td>Reference</td>
<td>Whole semen</td>
<td>Seminal plasma</td>
<td>Spermatozoa</td>
<td>Comment</td>
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</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>Jain and Anand 1976</td>
<td>11.7 ± 1.5% of total phospholipids</td>
<td>10.8 ± 2.0% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl ethanolamine in seminal plasma of cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 10.5, 5.4 and 5.6 ± 0.4% of total phospholipids, respectively</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>10.8 ± 1.4% of total phospholipids</td>
<td>9.3 ± 1.2% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl ethanolamine in spermatozoa of cattle bull which according to Pursel and Graham (1967), and Jain and Anand (1976) is 20.0%, 9.7% and 5.3 ± 0.4% of total phospholipids, respectively</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine (ethanolamine plasmogen)</td>
<td>Jain and Anand 1976</td>
<td>4.1 ± 0.3% of total phospholipids</td>
<td>3.4 ± 0.5% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl ethanolamine in seminal plasma of cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 16.3%, 5.0% and 9.0 ± 0.9% of total phospholipids, respectively</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>4.9 ± 0.7% of total phospholipids</td>
<td>5.7 ± 0.7% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl ethanolamine in spermatozoa of cattle bull which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 7.2%, 4.1% and 9.0 ± 0.4% of total phospholipids, respectively</td>
</tr>
<tr>
<td>Sphinogomyelin</td>
<td>Jain and Anand 1976</td>
<td>13.1 ± 0.7% of total phospholipids</td>
<td>11.3 ± 0.7% of total phospholipids</td>
<td></td>
<td>Amount of sphinogomyelin in seminal plasma of cattle bull which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 16.3%, 13.2% and 11.6 ± 1.0% of total phospholipids, respectively</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>13.8 ± 0.8% of total phospholipids</td>
<td>17.4 ± 1.3% of total phospholipids</td>
<td></td>
<td>Amount of sphinogomyelin in cattle bull spermatozoa which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 9.1%, 11.5% and 12.2 ± 1.2% of total phospholipids, respectively</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>Jain and Anand 1976</td>
<td>2.8 ± 0.4% of total phospholipids</td>
<td>1.5 ± 0.3% of total phospholipids</td>
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<td>Amount of phosphatidyl serine in seminal plasma of cattle bull is 1.3 ± 0.3% of total phospholipids (Jain and Anand 1976)</td>
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<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>6.1 ± 0.7% of total phospholipids</td>
<td>8.1 ± 0.3% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl serine + Phosphatidyl inositol in seminal plasma of cattle bull is 3.6% of total phospholipids (Clegg and Foote 1973)</td>
</tr>
<tr>
<td>Phosphatidyl serine + phosphatidyl inositol</td>
<td>Sarmah et al. 1983</td>
<td>6.1 ± 0.7% of total phospholipids</td>
<td>8.1 ± 0.3% of total phospholipids</td>
<td></td>
<td>Amount of phosphatidyl serine + Phosphatidyl inositol in spermatozoa of cattle bull is 0.7% of total phospholipids (Clegg and Foote 1973)</td>
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<tr>
<td>Phosphatidyl inositol</td>
<td>Jain and Anand 1976</td>
<td>2.9 ± 0.5% of total phospholipids</td>
<td>0.6 ± 0.1% of total phospholipids</td>
<td></td>
<td>The value of phosphatidyl inositol in seminal plasma obtained in this study differ from that of cattle bull which according to Jain and Anand (1976) is 0.8 ± 0.2% of total phospholipids. The value of phosphatidyl inositol in spermatozoa obtained in this study differ from that of cattle bull which according to Jain and Anand (1976) is 1.0 ± 0.2% of total phospholipids</td>
</tr>
<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>3.1 ± 0.2% of total phospholipids</td>
<td>8.3 ± 0.1% of total phospholipids</td>
<td></td>
<td>Amount of lysophosphatidyl choline in seminal plasma of cattle bull, which according to Clegg and Foote (1973), and Jain and Anand (1976) is 2.2% and 1.2 ± 0.3% of total phospholipids, respectively</td>
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Table 3. Continued

<table>
<thead>
<tr>
<th>Characteristic of component</th>
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<th>Seminal plasma</th>
<th>Spermatozoa</th>
<th>Comment</th>
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</thead>
<tbody>
<tr>
<td>Lysophosphatidyl ethanolamine</td>
<td>Jain and Anand 1976</td>
<td>5.6 ± 1.5% of total phospholipids</td>
<td>4.4 ± 1.0% of total phospholipids</td>
<td>Amount of lysophosphatidyl ethanolamine in seminal plasma of cattle bull, which according to Clegg and Foote (1973), and Jain and Anand (1976) is 2.2% and 1.2 ± 0.3% of total phospholipids, respectively</td>
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<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>6.6 ± 1.0% of total phospholipids</td>
<td>4.4 ± 1.0% of total phospholipids</td>
<td>Amount of lysophosphatidyl ethanolamine in spermatozoa of cattle bull which according to Jain and Anand (1976) is 3.2 ± 0.6% of total phospholipids</td>
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<tr>
<td>Lysophosphatidyl serine</td>
<td>Jain and Anand 1976</td>
<td>1.0 ± 0.3% of total phospholipids</td>
<td>0.7 ± 0.1% of total phospholipids</td>
<td>Amount of lysophosphatidyl serine in seminal plasma of cattle bull is 0.4 ± 0.1% of total phospholipids (Jain and Anand 1976)</td>
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<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>6.6 ± 1.0% of total phospholipids</td>
<td>4.4 ± 1.0% of total phospholipids</td>
<td>Amount of lysophosphatidyl serine in spermatozoa of cattle bull is 3.2 ± 0.6% of total phospholipids (Jain and Anand 1976)</td>
<td></td>
</tr>
<tr>
<td>Diphosphatidyl glycerol (cardiolipin)</td>
<td>Jain and Anand 1976</td>
<td>7.4 ± 1.3% of total phospholipids</td>
<td>5.5 ± 0.7% of total phospholipids</td>
<td>Amount of cardiolipin in seminal plasma of cattle bull, which according to Pursel and Graham (1967), Clegg and Foote (1973), and Jain and Anand (1976) is 5.4%, 8.8% and 5.0 ± 0.5% of total phospholipids, respectively</td>
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<td></td>
<td>Sarmah et al. 1983</td>
<td>3.5 ± 0.5% of total phospholipids</td>
<td>4.9 ± 0.4% of total phospholipids</td>
<td>Amount of cardiolipin in spermatozoa of cattle bull, which according to Clegg and Foote (1973), and Jain and Anand (1976) is 6.3% and 5.9 ± 1.0% of total phospholipids, respectively</td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>Jain and Anand 1976</td>
<td>0.5 ± 0.2% of total phospholipids</td>
<td>0.3 ± 0.1% of total phospholipids</td>
<td>Amount of phosphatidic acid in seminal plasma of cattle bull is 0.4 ± 0.1% of total phospholipids (Jain and Anand 1976)</td>
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<tr>
<td></td>
<td>Sarmah et al. 1983</td>
<td>0.5 ± 0.2% of total phospholipids</td>
<td>0.3 ± 0.1% of total phospholipids</td>
<td>Amount of phosphatidic acid in spermatozoa of cattle bull is 0.2 ± 0.1% of total phospholipids (Jain and Anand 1976)</td>
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<tr>
<td>Neutral lipids</td>
<td>Jain and Anand 1976</td>
<td>0.439 mg/ml</td>
<td>0.286 mg/10^9 cells</td>
<td>Amount of neutral lipids in seminal plasma of cattle bull is 0.896 mg/ml (Jain and Anand 1976)</td>
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<tr>
<td>Glycolipids</td>
<td>Jain and Anand 1976</td>
<td>0.581 mg/ml</td>
<td>0.397 mg/10^9 cells</td>
<td>Amount of glycolipids in seminal plasma of cattle bull is 0.713 mg/ml (Jain and Anand 1976)</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>Jain et al. 1990</td>
<td>32.49 ± 5.10 l mol/ml</td>
<td></td>
<td>Amount of glutathione obtained in whole semen of cattle bull is 45.35 ± 5.07 l mol/ml (Jain and Anand 1976)</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Chaudhary and Gangwar 1977</td>
<td>0.395 mM</td>
<td></td>
<td>Amount of aspartic acid in seminal plasma of cattle bull is 0.369 ± 0.025 l mole/10^9 cells (Al-Hakim et al. 1970)</td>
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<tr>
<td>Glutamic acid</td>
<td>Chaudhary and Gangwar 1977</td>
<td>4.28 mM</td>
<td></td>
<td>Amount of glutamic acid in seminal plasma of cattle bull is 4.352 ± 0.257 l mole/10^9 cells (Al-Hakim et al. 1970)</td>
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<tr>
<td>Serine</td>
<td>Chaudhary and Gangwar 1977</td>
<td>0.60 mM</td>
<td></td>
<td>Amount of serine in seminal plasma of cattle bull is 0.586 ± 0.03 l mole/10^9 cells (Al-Hakim et al. 1970)</td>
<td></td>
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<tr>
<td>Alanine</td>
<td>Chaudhary and Gangwar 1977</td>
<td>0.413 mM</td>
<td></td>
<td>Amount of alanine in seminal plasma of cattle bull is 1.078 ± 0.07 l mole/10^9 cells (Al-Hakim et al. 1970)</td>
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<tr>
<td>Glycine</td>
<td>Chaudhary and Gangwar 1977</td>
<td>1.34 mM</td>
<td></td>
<td>Amount of glycine in seminal plasma of cattle bull is 0.564 ± 0.03 l mole/10^9 cells (Al-Hakim et al. 1970)</td>
<td></td>
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<tr>
<td>Lysine</td>
<td>Chaudhary and Gangwar 1977</td>
<td>0.133 mM</td>
<td></td>
<td>Amount of lysine in seminal plasma of cattle bull is 0.177 ± 0.01 l mole/10^9 cells (Al-Hakim et al. 1970)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Continued

<table>
<thead>
<tr>
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<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyribo-nuclease</td>
<td>Chauhan et al. 1975</td>
<td>315.3 ± 22.66 BU/100 ml</td>
<td>53.7 ± 3.4 BU/100 ml</td>
<td>2007.33 ± 112.01 KU/ml</td>
<td>Amount of deoxyribonuclease in spermatozoa of bull is 2357.4 ± 126.36 KU/ml (Chauhan et al. 1975)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Chauhan and Srivastava 1973</td>
<td>194 ± 10 BU/100 ml</td>
<td>39 ± 6 BU/100 ml</td>
<td>1843.4 ± 126.36 KU/ml (Chauhan et al. 1975)</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Chauhan and Srivastava 1973</td>
<td>312.5 ± 24.04 BU/100 ml</td>
<td>63 ± 6 BU/100 ml</td>
<td>246.8 ± 8 BU/100 ml and 54 ± 3 BU/100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE.

Table 4. Phospholipid composition (% of total phospholipids) of plasma membrane of buffalo and cattle bull spermatozoa

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Buffalo (Cheshmejadieva and Dimov 1994)</th>
<th>Cattle (Parks et al. 1987)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>22.9 ± 1.6*</td>
<td>9.9</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>66.0 ± 3.5</td>
<td>50.3</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>3.5 ± 0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>2.5 ± 0.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>8.0 ± 1.9</td>
<td>12.6</td>
</tr>
<tr>
<td>Lyso phosphatidyl choline</td>
<td>~</td>
<td>1.8</td>
</tr>
<tr>
<td>Phosphatidyl glycerol</td>
<td>4.7 ± 0.6</td>
<td>~</td>
</tr>
<tr>
<td>Diphosphatidyl glycerol</td>
<td>5.0 ± 0.9</td>
<td>~</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

Additionally, the differences regarding efficacy of different buffers suggest that buffalo spermatozoa are more prone to freezing stress as compared to cattle bull spermatozoa possibly because of biochemical factors that influence membrane fluidity during cryogenic preservation (refer to Table 4). Therefore, there is a need to study the influence of selected buffers on pre- and post-cryogenic membrane stability i.e., in terms of biochemical/molecular level changes in lipid bilayer and phase transition.

**Permeable cryoprotectant**

Glycerol is often poly-hydroxylated and capable of hydrogen bonding with water, capable of permeating across the cell membrane, and non-toxic during exposure to cells in the concentration between approximately 1–5 mol/l, depending on cell type and conditions of exposure (Fuller and Paynter 2004). More specifically, the physiological actions of glycerol during the cryopreservation of spermatozoa take place by replacing intracellular water necessary for the maintenance of cellular volume, interaction with ions and macromolecules, and depressing the freezing point of water and the consequent lowering of electrolyte concentrations in the unfrozen fraction so that less ice forms at any given temperature (Holt 2000b; Medeiros et al. 2002).

For cryopreservation of buffalo semen, several studies have been carried out in an attempt to find the optimum levels of glycerol and glycerolization. In this context, Jainudeen and Das (1982) studied the effect of two glycerolization procedures (one step vs two steps) and the influence of glycerol level in the extender (3%, 5% or 7%). They found that glycerolization procedure had no significant effect on sperm survival traits like motility and acrosomal integrity. They also found that post-thaw motility of spermatozoa was significantly better in a 5% glycerol extender, whereas the percentage of intact acrosomes was greater in spermatozoa extended in 3% or 5% glycerol than in spermatozoa extended in 7% glycerol.

In another study, Kumar et al. (1992) found that the best level of glycerol was 6% for Tris- and milk-based diluents, and 9% glycerol for the sodium citrate diluent to obtain better post-thaw motility for buffalo spermatozoa. Ramakrishnan and Ariff (1994), and Nastri et al. (1994) also tried to reduce the glycerol concentrations from 8% to 2% or 3%, but they found that the reduction in glycerol below 5% decreased the post-thaw motility and/or acrosomal integrity of spermatozoa in the extenders tested. Abbas and Andrabi (2002) studied the effects of different concentrations of glycerol (2%, 3%, 4%, 5%, 6%, 7%, 8%, 10% or 12%) on post-thaw sperm quality. They reported that the spermatozoa frozen in 7% were significantly better to those in other concentrations of glycerol as judged by post-thaw motility, survivability and plasma membrane integrity. Regarding glycerolization, Singh et al. (2006) have confirmed that single step is more suitable for the cryopreservation of buffalo spermatozoa in terms of post-thaw forward motility.

Ethylene glycol could be another option for the cryopreservation of buffalo spermatozoa. Permeability of ethylene glycol was found to be higher than glycerol.
in spermatozoa of different species (Gilmore et al. 1995, 1998; Phelps et al. 1999), resulting in lower hydraulic conductivity and then in a reduction in the osmotic stress to which cells are exposed during cooling and freezing (Gilmore et al. 1995). Propylene glycol also has the basic properties of a cryoprotectant i.e., it is miscible with water in all proportions, its solutions in water have profoundly depressed freezing points, and presumably, it has a low intrinsic toxicity as it is widely used in the food and pharmaceutical industries (Arnaud and Pegg 1990). Recently, Valdez et al. (2003) and Rohilla et al. (2005) have tested ethylene glycol or propylene glycol as substitute for glycerol. Their preliminary results suggest that ethylene glycol may be used for freezing bubaline spermatozoa. Therefore, there is a need to study in detail the factors that may affect the viability of frozen buffalo spermatozoa with ethylene glycol as a cryoprotectant. Further studies, are also suggested for testing propylene glycol as a cryoprotectant for buffalo spermatozoa.

Dimethyl sulfoxide (DMSO) is a rapid penetrating cryoprotectants having lower molecular weight than glycerol. Also DMSO may inhibit harmful effect of hydroxyl radicals (Yu and Quinn 1994), as these radicals appear during cell respiration and are detrimental to cell (Johnson and Nasr-Esfahani 1994). More recently, Rasul et al. (2007) studied glycerol and/or DMSO, added either at 37°C or at 4°C as a cryoprotectant for buffalo spermatozoa. The concentrations (%) of glycerol and DMSO adjusted were 0 : 0, 0 : 1.5, 0 : 3, 3 : 0, 3 : 1.5, 3 : 3; and 6 : 0, 6 : 1.5, 6 : 3 respectively. It was concluded that addition of DMSO at the levels investigated did not improve the post-thaw quality of spermatozoa. However, glycerol at a concentration of 6%, when added at 37°C, provided the maximum cryoprotection to the motility apparatus, and plasma membrane integrity of buffalo spermatozoa in Tris-citric acid based extender. The exact mechanism involved in the antagonist effect of DMSO on the cryoprotection ability of glycerol is not understood. Moreover, the lethal effect of DMSO is attributed to its toxic effect rather than osmotic (Rasul et al. 2007). It is believed that because of the lower molecular weight of DMSO, its penetrating ability into the cell is higher than glycerol.

From the available studies, it is therefore, suggested that a glycerol concentration of 5–7% added initially in the extender may be suitable for the cryopreservation of buffalo bull spermatozoa. On the other hand, development of less toxic cryoprotectant could make a significant contribution in improving the quality of frozen–thawed buffalo spermatozoa.

Non-permeable cryoprotectant

Egg yolk is a common component of semen freezing extenders for most of the livestock species, including the buffalo (Sansone et al. 2000). It is widely believed that low density lipoproteins (LDL) contained in egg yolk is largely responsible for sperm protection during cryopreservation (Pace and Graham 1974; Watson 1976). It is suggested that LDL adheres to sperm membrane and provides protection to sperm by stabilizing the membrane. A second hypothesis suggests that phospholipids present in LDL protect sperm by forming a protective film on the sperm surface or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process (Foulkes et al. 1980; Quinn et al. 1980; Graham and Foote 1987). A third mechanism of protection suggests that LDL seizes the deleterious proteins present in seminal plasma thus improving the freezability of spermatozoa (Bergeron and Manjunath 2006). The exact mechanism by which EY preserves the spermatozoa during freeze-thaw process is unknown (Buthgate et al. 2006).

Review of literature reveals that little attention has been paid to the level of egg yolk necessary for freezing buffalo semen, and generally it is used at a concentration of 20% in semen extender (Sansone et al. 2000; Andrabi et al. 2008). Furthermore, the use of egg yolk in higher concentration may have deleterious effects combined with toxicity (amino acid oxidase activity) of dead spermatozoa resulting in lower post-thaw spermatozoa quality (Shannon 1972). The enhanced toxicity associated with increased egg yolk is probably due to the elevated substrate available for hydrogen peroxide formation (Tosis and Walton 1950).

In this regard, Saini and Mohan (1990) studied different levels of egg yolk in extender as a non-permeable cryoprotectant for buffalo semen. The concentration of egg yolk used was 0%, 2%, 5%, 10% or 20%. They concluded that the concentration of egg yolk in the extender could be reduced from 20% to 5% without any compromise in post-thaw motility of spermatozoa. Kumar et al. (1994) studied the effect of different levels of egg yolk (0%, 1%, 5%, 10% and 20%) in Tris-based extender on sperm motility and survival before and after freezing in buffalo. They found that the best post-thaw motility and survivability was with 5% yolk. Singh et al. (1999) studied the effect of different levels of egg yolk on freezability of buffalo semen. They found that egg yolk at 10% was superior for freezability with regards to pre-freeze and post-thaw sperm motility. It was, also suggested that 10% egg yolk is better in a Tris-based extender for freezing buffalo semen compared to at lower concentration (5%).

Recently, Andrabi et al. (2008) investigated the use of duck egg yolk, Guinea fowl egg yolk and Indian indigenous hen (Desi) egg yolk in extender for improving the post-thaw quality of buffalo bull spermatozoa, and compared it with commercial hen egg yolk. It was concluded that duck egg yolk compared to other avian yolk in extender improves the freezability of buffalo bull spermatozoa as judged by motility, survivability, plasma membrane integrity, intactness of acrosome and head, mid-piece and tail abnormalities. In this regard, it is suggested that the improvement or decline in post-thaw quality of mammalian spermatozoa with egg yolk of different avian species in freezing extender is attributed to the differences in biochemical composition of the yolks (Trimeche et al. 1997; Buthgate et al. 2006). Studies investigating the influence of egg yolk from different avian species on Jackass sperm during freeze-thawing have found that the ratio of phosphatidyl ethanolamine : phosphatidyl choline appears to play a role in the level of protection afforded to the sperm.
(Trimeche et al. 1997). This is of interest to mention that Bathgate et al. (2006) reported a significant difference in ratio of phosphatidyl ethanolamine : phosphatidyl choline in chicken egg yolk and duck yolk with a higher ratio in the later. Therefore, it can be put forward that higher ratio of phosphatidyl ethanolamine : phosphatidyl choline in duck egg yolk may have improved the freezeability of buffalo spermatozoa in the study by Andrabi et al. (2008). It is, also, proposed that supplementation of cryodiluent with quail egg yolk for buffalo bull semen needs to be investigated as the ratio of phosphatidyl ethanolamine : phosphatidyl choline in quail yolk is even higher than duck yolk as reported by Bathgate et al. (2006). Finally, as the findings of Andrabi et al. (2008) are preliminary, therefore, it is suggested that further studies are required to establish the source and levels of egg yolk in freezing medium for buffalo spermatozoa.

Polyethylene glycol (PEG) is a non-permeable cryoprotectant that may slow down the process of ice nucleation during cryogenic process, thus protecting the cellular membrane. Other protective mechanism by PEG may be due to its coupling with hydrophobic molecules to produce non-ionic surfactants. Cheshmedjieva et al. (1996) studied the effect of addition of PEG 20 to egg yolk based freezing medium on the cholesterol : phospholipid, sphingomyelin : phosphatidyl choline and unsaturated : saturated fatty acids ratios in buffalo spermatozoa. They concluded that PEG 20 added to extender preserved the lipids of frozen buffalo spermatozoa. Further studies are required to find out that PEG 20 may be a better option for the cryopreservation of buffalo spermatozoa.

Sugars that are not capable of diffusing across a plasma membrane, such as lactose, sucrose, raffinose, trehalose or dextran are also added to the extender as non-permeable cryoprotectant. In these instances, the sugars create an osmotic pressure, inducing cell dehydration and therefore, a lower incidence of intracellular ice formation. These sugars also interact with the phospholipids in the plasma membrane, reorganizing the membrane which results in sperm that is better suited to surviving the cryopreservation process (Molina et al. 1994; Aisen et al. 2002). In early studies, Ahmad and Chaudhry (1980) investigated the lactose or fructose based extenders for cryopreservation of buffalo semen. It was found that the diluent comprising 11% lactose and 6% fructose achieved the best results as tested by post-thaw motility and survivability. Ala Ud et al. (1981) tested the post-thaw motility and survivability of buffalo spermatozoa frozen in homogenized whole milk, Laiciphos (IMV), lactose or citrate-based extender. They found that lactose-based extender gave a better protection to sperm during the cryogenic procedure. Dhami and Sahni (1993) studied the effect of 1% raffinose in semen dilluents (Tris–fructose–yolk–glycerol, egg yolk-citrate-glycerol or lactose-egg-yolk-glycerol) on enzyme leakage (lactate dehydrogenase) from buffalo spermatozoa during freezing. They found that the post-thaw quality of spermatozoa was better with raffinose in Tris-based extender compared to other extenders in terms of release of lactate dehydrogenase.

Keeping in view the current international trends in disease control, it is possible that extenders having ingredients of animal origin (egg yolk) can be the source of microbes/bacteria, consequently resulting in the contamination of semen (Bousseau et al. 1998; Marco Jimenez et al. 2004; de Ruigh et al. 2006). In this regard, LDL extracted from egg yolk (indirect use) or lecithin from non-animal source like soya need to be tested as a non-permeable cryoprotectant in extender for deep-freezing of buffalo spermatozoa.

Antibiotic

It is documented that bacteria in semen and their control via addition of antibiotics in freezing diluents may affect the viability or fertility of cryopreserved bovine spermatozoa (Thibier and Guerin 2000; Morrell 2006). Presence of bacteria in the ejaculates can affect fertilization directly (Morrell 2006), by adhering to spermatozoa (Bolton et al. 1986; Wolff et al. 1993; Diemer et al. 1996), impairing their motility (Panangala et al. 1981; Kaur et al. 1986) and inducing acrosome reaction (El-Mulla et al. 1996). Microbes can also have an indirect effect by producing toxins (Morrell 2006).

Thus, in the use of AI, it is important to control efficiently the population of microorganisms in the semen. Conventionally, benzyl penicillin 1000 IU/ml and streptomycin sulphate 1000 μg/ml alone or in combination is commonly added to the freezing diluents of buffalo bull semen (Sansone et al. 2000; Akhter et al. 2008). Regarding control of bacteriospermia in buffalo bull semen with streptomycin and penicillin (SP), it was found that it is not an effective combination (Gangadhar et al. 1986; Aleem et al. 1990; Hussain et al. 1990; Ali et al. 1994; Amin et al. 1999). More recently, Ahmed and Greesh (2001) and Ahmed et al. (2001a,b) found that bacteria isolated from buffalo bull semen were resistant to penicillin. Also SP was deleterious to post-thaw quality of spermatozoa. They concluded that gentamicin (500 μg/ml) or amikacin (500 μg/ml) or norfloxacin (200 μg/ml) are the antibiotics of choice to be added in extender for efficient preservation of buffalo spermatozoa.

Recently, Hasan et al. (2001) and Akhter et al. (2008) investigated the effects of a relatively new antibiotic combination (gentamicin tylosin and linco-spectin, GTLS) in extender on bacterial and spermatozoal quality of preserved spermatozoa. They concluded that GTLS is more capable than SP for bacterial control of buffalo bull semen as judged by total aerobic bacterial count and/or in vitro antibiotic sensitivity. Moreover, GTLS is not detrimental to spermatozoal viability of buffalo bull. It is relevant to mention that Andrabi et al. (2001) have reported a better conception rate with frozen–thawed semen having GTLS compared to SP (55.2% vs 41.66%). It is therefore, suggested that GTLS extender is more efficient for the preservation of buffalo spermatozoa. Further, that testing of wider range of new antibiotic is recommended in cryodiluents for improvement in quality of frozen–thawed buffalo spermatozoa.
Other additives

Keeping in view the poor freezability of bubaline semen attempts have been made to improve the basic buffers developed to minimize the deleterious effects of cryogenic procedures. There are few scattered studies that have used additives such as antioxidants, chelating agents, metabolic stimulants, detergents etc. for improvement in post-thaw quality of buffalo spermatozoa.

In this regard, Bhosrekar et al. (1990) studied the effect of addition of caffeine or triethanolamine lauryl sulphate to Tris–citric acid-based extender. They reported that the addition of the detergent improved the post-thaw spermatozoa motility. However, inclusion of caffeine to extender did not made any improvement in motility. It is believed that the protective effect of detergents may be exerted directly on the sperm membrane or is mediated through a change in the extending medium such as emulsifying the egg yolk lipids to make them more readily available to the plasmalemma during cryopreservation (Graham et al. 1971; Arriola and Foote 1987; Buhr and Pettitt 1996). On other hand, the failure of caffeine to make any improvement is not understood.

Dhami and Sahni (1993) studied the effect of 0.1% cysteine or 0.1% EDTA (sperm membrane stabilizer and capacitation inhibitor) in semen diluents (Tris–fructose–yolk–glycerol, egg yolk–citrate–glycerol or lactose–egg yolk–glycerol) on enzyme leakage (lactate dehydrogenase) from buffalo spermatozoa during freezing. They found that the addition of cysteine or EDTA to the experimental extenders did not improve the post-thaw quality of spermatozoa in terms of release of lactate dehydrogenase.

Singh et al. (1996) studied the effect of addition of ascorbic acid in the diluent on the quality of deep frozen buffalo bull semen. They found that inclusion of ascorbic acid (2.5 mm) in the semen diluent yielded a significantly higher post-thaw motility and survivability. The antioxidant effect of ascorbate is related to direct vitamin E regeneration by reducing the tocopheroxyl radical in the one-electron redox cycle (Packer et al. 1979; Dalvit et al. 1998). Later on, Kolev (1997) studied the effect of vitamin A, D and E in extender on motility, survivability and acrosomal integrity of cryopreserved buffalo bull spermatozoa. It was suggested that vitamin E at 0.3 mg/ml exhibited the best effects. It is well-known that 2-tocopherol inhibits lipid peroxidation (LPO) in biological membranes, acting as a scavenger of lipid peroxyl and alkoxyl radicals, thus preventing oxidative damage in cryopreserved bovine semen (Beconi et al. 1991).

Fabbrocini et al. (2000) suggested that for freezing buffalo spermatozoa, addition of sodium pyruvate (1.25 mm) to the extender resulted in significantly better post-thaw progressive motility and viability. The beneficial effect of pyruvate and $\alpha$-ketoacids is attributed to its antioxidant property.

Shukla and Misra (2005) studied different antioxidants ($\alpha$-tocoopherol, ascorbic acid or $n$-propyl gallate) added to Tris-based dilutor for improving freezability of bubaline spermatozoa. They found that addition of $n$-propyl gallate (15 $\mu$m) helped in retaining significantly high post-thaw motility and viability of spermatozoa. It is noteworthy that propyl gallate is also an antioxidant. It protects against oxidation by hydrogen peroxide and oxygen-free radicals, in a catalytic manner by converting hydrogen peroxide into water and oxygen.

Kumaresan et al. (2006) studied the effects of addition of oviductal proteins obtained from various stages of the oestrous cycle to Tris-based extenders on spermatozoa characteristics in buffaloes. They found that oviductal proteins differentially affected post-thaw sperm motility, viability, acrosomal integrity, bovine cervical mucus penetration test, hypo-osmotic sperm swelling test and LPO level depending on the region of oviduct and the stage of oestrous cycle at which the proteins were obtained. Overall, it was implied that incorporation of oviductal proteins in extender before freezing improved functions and reduced the LPO levels in buffalo spermatozoa during cryopreservation. The beneficial actions conveyed by oviductal fluid are presently unknown; however, the identification of catalase in cow oviductal fluid by Lapointe et al. (1998) suggests that it may be a mechanism by which the oviductal fluid reduces the damage caused by reactive oxygen species to the spermatozoa.

Recently, Shukla and Misra (2007) conducted a study to improve buffalo semen cryopreservation with the incorporation of Bradykinin (0.5, 1.0 and 2.0 ng/ml) in routinely used extender. They found that incorporation of Bradykinin (2 ng/ml) in Tris-based extender might be useful in improving the quality of frozen-thawed bubaline spermatozoa as determined by live percentage, motility and plasma membrane integrity. The exact mechanism of action of Bradykinin is not yet fully understood.

From the literature cited in this section, it appears that there are some additives, which have some useful effects in terms of improvement in the quality of frozen–thawed buffalo spermatozoa. It is relevant to mention that most of these studies are preliminary. Therefore, it is suggested that further research is required to establish their beneficial effects on cryopreservation of buffalo spermatozoa.

Semen processing

It is generally accepted that the cryopreservation process itself reduces more than 50% of the sperm viability (Watson 1979). During this process, the spermatozoa are subjected to chemical/toxic, osmotic, thermal, and mechanical stresses, which are conspicuous at dilution, cooling, equilibration, or freezing and thawing stage.

The success of semen cryopreservation depends to a notable degree on dilution rate. Originally, semen was diluted to protect spermatozoa during cooling, freezing and thawing, but the rate of dilution was often changed for technical reasons, like to increase the number of females, which could be inseminated with each ejaculate, or to standardize the number of spermatozoa in each dose of frozen–thawed semen (Salamon and Maxwell 2000). In farm animals, the semen has been diluted with specific volumes of extenders or by diluting semen to a specific spermatozoa concentration. Dilution rates of
1 : 1 to 1 : 12 have been successfully used for buffalo semen. Perhaps a better of diluting semen, for comparison purposes, is based on the sperm concentration (Purdy 2006). Reports of buffalo spermatozoa with acceptable fertility, was with frozen samples ranging from 120 × 10^6 to 30 × 10^6 cells/ml (Tahir et al. 1981; Andrabi et al. 2006).

After dilution, the semen is cooled to a temperature close to 4°C or 5°C. Cooling is a period of adaptation of spermatozoa to reduced metabolism. Extended semen is cooled slowly to avoid potential of cold shock. Cold shock is believed to impair function of membrane proteins that are necessary for structural integrity or ion metabolism (Watson 2000). Major changes in bovine spermatozoa during this phase occur near 15 to 5°C, and do not happen below 0°C (Watson 2000). Rapid cooling reduces the rate of fructose breakdown, oxygen uptake, and ATP synthesis by the sperm, which results in the loss of energy supply and motility (Blackshaw and Salisbury 1957; Wales and White 1959). Furthermore, cold shock may increase calcium uptake by sperm (White, 1993). However, some think that a faster cool will not create problem if the semen is extended in an ideal buffering system (Marshall 1984). It has been empirically determined that cooling cattle bull spermatozoa from body temperature to 5°C performed at a rate of 10°C/h has minimum deleterious effects (Parks 1997). In this regard, Dhami et al. (1992) studied the effect of cooling rates (5, 30, 60 and 120 min from 10 to 5°C vs 120 min from 28 to 5°C) on the deep freezing of buffalo semen diluted in Tris-based extender. Their results suggest that buffalo semen can be frozen successfully after 30 min of cooling at 10°C as judged by motility and survivability.

Regarding equilibration, it is traditionally taken as the total time during which, spermatozoa remain in contact with glycerol before freezing. At this stage, glycerol penetrates into the sperm cell to establish a balanced intracellular and extracellular concentration. It should not be overlooked that the equilibrium includes the concentration balance not only of glycerol, but also of the other osmotically active extender components (Salamon and Maxwell 2000). Therefore, this phenomenon interacts with the type of extender (buffer and cryoprotectant) used and could easily interact with other cryogenic procedures (Marshall 1984). In this regard, Tuli et al. (1981b) examined equilibration of buffalo semen diluted in Tris or citric acid-based extender for 2, 4 or 6 h. They found that post-thaw sperm survivability was better after 4 h equilibration than after 2 or 6 h.

Talevi et al. (1994) cooled buffalo semen from 28°C to 5°C in 15 min and then equilibrated at 5°C for 1 h 45 min (fast cooling) or cooled it in 1 h and equilibrated for 1 h (slow cooling). They found that post-thaw sperm motility was significantly higher using the slow than the rapid cooling method. Conversely, the rate of cooling had no significant effect on acrosome integrity. Dhami et al. (1996) conducted a study to determine the relative efficacy of four cooling rates (10/30°C to 5°C; 1 and 2 h each) and two equilibration periods at 5°C (0 and 2 h) for cryopreservation of buffalo ejaculates. They concluded that slow cooling of straws from 30 to 5°C for 2 h compared with faster cooling (1 h) or lower initial temperature (10°C) and 2 h of equilibration at 5°C appeared necessary for successful cryopreservation of buffalo semen as determined by survivability and fertility.

Of considerable importance for the cryopreservation is the cooling/freezing rate in the critical temperature range (~5 to ~50°C) that determines whether the spermatozoa will remain in equilibrium with their extracellular environment or become progressively supercooled with the increasing possibility of intracellular ice formation (Kumar et al. 2003). During slow cooling, the dehydration of the spermatozoa can proceed to the point of osmotic equilibrium between intracellular and extracellular space i.e., cellular dehydration will be maximal. While raising the cooling rate too much, the dehydration is not fast enough to prevent occurrence of intracellular ice nucleation. However, if the cooling rate is within the required values (50–100°C) this results in less excessive intracellular dehydration, less excessive intracellular solute concentrations and less shrinkage of the cells (Mazur 1984; Woelders 1997). Moreover, at optimum cooling/freezing rates, the spermatozoa remain vulnerable to the unfavourable conditions for a shorter period of time (Woelders 1997). It is worth mentioning that for cattle bull semen currently a freezing rate of ~240°C is practiced in general for cryopreservation during the critical temperature zone (Anzar et al. 2002).

Sukhato et al. (2001) determined the effects of freezing rate and intermediate plunge temperature (cooling at 10, 20 or 30°C/min each to ~40, ~80 or ~120°C before being plunged into liquid nitrogen) on post-thaw quality and fertility of buffalo spermatozoa. They found that cooling/freezing spermatozoa from 4 to ~120°C, either at 20 or 30°C/min yielded better progressive motility and fertility rate. Bhosrekar et al. (1994) compared the conventional (over liquid nitrogen in static vapour for 10 min) and control (programmable) freezing methods for buffalo bull semen. It was concluded that freezing at the rate of 17.32°C/min between +4°C and ~40°C with programmable freezer produced better quality frozen semen than the conventional method of freezing. More recently, Rasul (2000) examined the effect of freezing rates on post-thaw viability of buffalo spermatozoa extended in Tris-citric acid-based extender. The freezing rates examined between 4 and ~15°C were 3 or 10°C/min, whereas the freezing rates investigated between ~15 to ~80°C were 10, 20 or 30°C/min. It was concluded that the different freezing rates tested gave similar results in terms of post-thaw spermatozoal viability as judged by visual and computerized motilities, motion characteristics, plasma membrane integrity and intactness of acrosomal ridge.

In the freeze–thaw procedure, the warming phase is just as important to the survival of spermatozoa as the freezing phase. Spermatozoa that have survived cooling to ~196°C still face the challenge of warming and thawing, and thus must traverse twice the critical temperature zone i.e., from ~5 to ~50°C (Marshall 1984). The thawing effect depends on whether the rate of cooling has been sufficiently high to induce intracellular freezing, or low enough to produce cell dehydration. In the former case, fast thawing is required to prevent

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recrystallization of any intracellular ice present in the spermatozoa. Spermatozoa thawed at a fast rate may also be exposed for a shorter time to the concentrated solute and cryoprotectant-glycerol, and the restoration of the intracellular and extracellular equilibrium is more rapid than for slow thawing (Salamon and Maxwell 2000). Also leaving straws in high temperatures for too long time may result in pH fluctuation and subsequently protein denaturation and cell death. A practical thaw for cattle bull spermatozoa, recommended by most AI organizations, is as 35°C water bath for at least 30 s (Marshall 1984). For cryopreservation of buffalo spermatozoa in Tris-based extender, Rao et al. (1986) tested two thawing rates (37°C for 30 s and 75°C for 9 s). They concluded that the best value for post-thaw motility was observed for semen thawed at 37°C for 30 s. Dhami et al. (1992) studied the effect of thawing rates (40°C for 60 s, 60°C for 15 s and 80°C for 5 s) on post-thaw motility of buffalo spermatozoa cryopreserved in Tris-based extender. They reported that thawing at 60°C for 15 s yielded a higher sperm motility compared to other rates. In another study, Dhami et al. (1996) determined the thawing rates for buffalo semen. The thawing rates investigated were 4°C for 5 min, 40°C for 1 min or 60°C for 15 s. They concluded that thawing of semen at 60°C for 15 s yielded high post-thawing spermatozoal recovery and longevity. Sukhato et al. (2001) determined the effect of thawing rates on motility and acrosome integrity of buffalo spermatozoa. Thawing of spermatozoa was performed at the rate of (rapid) 1000°C or (slow) 200°C/min. They concluded that rapid thawing was superior to slow warming. The above-mentioned studies demonstrate that an effective cryopreservation procedure for buffalo spermatozoa can be derived by the systematic examination of various cryobiological factors. Therefore, from these studies the cryogenic procedures for buffalo semen can be outlined as; cooling from 37 or 39 to 4°C at the rate of 0.2–0.4°C/min, equilibration, at least 2 h at 4°C, freezing of straws approximately 4 cm above liquid nitrogen for 10–20 min, or by the fast freezing rates (programmable freezing), and thawing at 45–60°C for at least 15 s. However, there is still need to improve the processing techniques for cryopreservation of buffalo spermatozoa. It is suggested that to devise efficient cooling/freezing rates for buffalo spermatozoa, studies involving use of a cryomicroscope should be carried out, as this will permit a direct and continuous viewing of spermatozoa, while the temperature is controlled accurately (Medranol et al. 2002).

**Season of semen collection**

Freezability of buffalo semen can also be affected by the season of collection i.e., by environmental factors like temperature, humidity and day length in a particular season. For the first time Tuli and Mehar (1983) studied the seasonal variation in freezability of buffalo semen diluted in Tris-based extender. They found that post-thaw spermatozoa motility, significantly increased in winter than summer season. After that, Heuer et al. (1987) studied the effect of season on in vivo fertility of frozen buffalo semen diluted in chemically defined buffers. They reported that semen collected in November (winter) produced significantly higher conception rate than semen collected in June (summer) over a total of 3220 inseminations in both seasons (40.9 vs 34.0%). They attributed 40% of the observed seasonality of buffalo fertility to the male. Bhavsar et al. (1989a) also studied the monthly variations in freezability and fertility of buffalo bull semen. They found that fertility of semen collected, frozen and inseminated during season from July to January (monsoon or late wet season to autumn and winter) was significantly higher than ejaculates processed and inseminated during the season from February to June (spring to early dry summer).

Sagdeo et al. (1991) studied the seasonal variations in freezability of buffalo bull semen. They found from the data of over a 4-year period that the season significantly affected the post-thaw sperm motility, and values being highest in ejaculates frozen in the winter and lowest in summer. Similarly, Bahga and Khokar (1991) studied the seasonal variations in freezability of buffalo bull semen. They found that post-thaw semen motility was significantly affected by season of collection, being lowest in summer and highest in winter (December–January). Younis et al. (1998) studied the freezability of semen collected during the low breeding season (May–July) and the peak breeding season (September–November) in young (3–4 years), adult (6–8 years) and old (12–15 years) buffalo bulls. They reported that post-thaw motility and liveability of spermatozoa frozen in Tris-based extender were significantly higher in adult bulls during the peak breeding season. In addition, the sperm abnormalities and deleterious enzymatic activity in frozen–thawed semen were significantly higher during the low breeding season than in the peak breeding season.

Recently, Koonjaenak et al. (2007a) studied the seasonal effect on quality of frozen–thawed buffalo spermatozoa diluted in Tris-based buffer. They compared post-thaw sperm quality over three seasons of the year (rainy: July–October; winter: November–February; and summer: March–June), with distinct ambient temperature and humidity. Their conclusion was that post-thaw plasma membrane integrity and stability were significantly better in ejaculates processed during winter than in samples processed during the other seasons of the year. From the above-mentioned studies, it is evident that there is a higher loss of viability and fertility during the process of cryopreservation in summer, thus confirming that vitality of buffalo spermatozoa remain comparatively poorer during this season. Moreover, it is suggested that to increase fertility rate in buffalo, semen should be collected and preserved during cooler months and used for AI all over the year.

In another study, Koonjaenak et al. (2007b) investigated the frozen–thawed buffalo sperm nuclear DNA fragmentation by flowcytometry and head morphology over three seasons in tropical Thailand (the rainy season, July–October; winter, November–February; and summer, March–June). They found that the DNA fragmentation index (DFI) values varied statistically among seasons, being lower in the rainy season than in winter or summer, and were affected by the year of semen collection and processing. The proportion of morphologically
abnormal sperm head shapes was low, with no significant differences between seasons. However, DFI was significantly related to the proportion of loose abnormal sperm heads. It was concluded that frozen–thawed buffalo sperm chromatin is not critically damaged by cryopreservation or affected by the seasonal variations in temperature and humidity seen in tropical Thailand.

There is possibility that a seasonal variation in the biochemical composition of seminal plasma and/or spermatozoa may occur as it does in other farm animals (Cabrera et al. 2005; Argov et al. 2007; Koonjaenak et al. 2007a). Recently, Argov et al. (2007) have reported that cattle semen samples collected during the summer and considered to be of good quality had alterations in lipid concentration, fatty-acid composition and cholesterol level. In addition, they provided the first evidence for the existence of a very-low-density lipoprotein receptor (VLDLr) in bovine sperm, suggesting a mechanism for sperm utilization of extracellular lipids. Interestingly, the expression of VLDLr was threefold greater in samples collected during the winter than in those collected in the summer. Therefore, it is suggested that such modifications may explain, in part, the reduced freezability of buffalo semen collected during the summer.

Few scattered reports are available that describe the differences in chemical composition of buffalo seminal plasma and spermatozoa under different climatic conditions (Singh et al. 1969; Mohan et al. 1979; Sidhu and Guraya 1979). However, the information given in these studies are insufficient to explain the variation in freezability of buffalo spermatozoa during the different seasons. Therefore, detailed studies should be carried out to ascertain the biochemical or structural differences in seminal plasma, spermatozoa, or plasmalemma, which might be influencing the freezability of buffalo spermatozoa during the different months/seasons.

Conclusions
Viability and fertility of frozen–thawed buffalo bull spermatozoa is considerably lower than that of cattle bull. Several buffers, cryoprotectants, antibiotics, other agents and various cooling, freezing and thawing rates initially developed for cattle bull spermatozoa have been used, at times with contrasting results. Therefore, a better understanding of the fundamental principle of cryopreservation of buffalo spermatozoa is necessary according to the specific requirements. Moreover, there is a need to develop biochemically defined extenders and cryogenic procedures that may result in improvement in viability and fertility of frozen–thawed buffalo spermatozoa. Besides this, the season during which the semen is collected should also be considered as a variable affecting quality of cryopreserved buffalo spermatozoa.

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