CRYOPRESERVATION OF BUFFALO SEMEN

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CERTIFICATE

This thesis by Zahid Rasul, is accepted in its present form by the Department of Biological Sciences as satisfying the thesis requirements for the degree of Doctor of Philosophy in Biology (Reproductive Physiology/Animal Reproduction).

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DEDICATED TO MY PARENTS

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THESIS ABSTRACT

This study was designed to establish the best cryopreservation procedures for buffalo semen. Therefore, the experiments were conducted 1) to identify the suitable buffer among *tri*-sodium citrate (CITRATE), Tris-citric acid (TCA), Tris-Tes (TEST) or Tris-Hepes (HEPEST), 2) to examine the effects of stages of cryopreservation i.e. dilution (D), cooling to 4°C (C), equilibration at 4°C for 4 h (E), and freezing and thawing (FT), 3) if faster freeze rates either during initial and subsequent freezing would improve the post-thaw semen quality and where the intermediate zone of damage to spermatozoa lies, and 4) whether different concentrations of glycerol and/or dimethyl sulfoxide (DMSO), either added at 37 or 4°C, would alter sperm motion characteristics, plasma membrane integrity, and acrosome morphology in buffaloes while using computer-assisted semen analyzer (CASA), hypo-osmotic swelling (HOS) or flow cytometry, and phase-contrast microscope, as assays.

In experiment 1, post-thaw visual motility (%) of spermatozoa tended ($P = 0.07$) to be higher in HEPEST (61.0 ± 2.9) and lower in CITRATE (48.0 ± 2.5). However, computer-assisted motility did not differ due to buffers. Post-thaw linear motility (%) of spermatozoa tended ($P = 0.09$) to be higher in TCA (78.2 ± 5.5) and lower in TEST (52.0 ± 6.9), whereas circular motility (%) was lower ($P < 0.05$) in TCA (11.6 ± 2.8) and higher in TEST (29.8 ± 5.6). Post-thaw sperm curvilinear velocity ($\mu m\ s^{-1}$) was lower ($P < 0.05$) in TCA (69.4 ± 2.0) than CITRATE (79.0 ± 5.8), TEST (87.2 ± 1.6) and HEPEST (82.6 ± 3.0). Post-thaw sperm lateral head displacement (μm) was lowest ($P < 0.05$) in TCA (1.7 ± 0.2) and highest in TEST (3.7 ± 0.6). Nonetheless, plasma membrane integrity and normal acrosomes of buffalo spermatozoa did not vary due to buffering systems.

In experiment 2, visual and computer-assisted motility (%) of spermatozoa did not differ due to dilution, cooling, or equilibration (77.3 ± 2.3 and 90.5 ± 1.2, respectively), whereas these were declined ($P < 0.05$) after freezing and thawing (53.0 ± 4.6 and 48.6 ± 6.5, respectively). Linear motility (%) of spermatozoa was lower ($P < 0.05$) after dilution or equilibration (56.2 ± 2.4) compared to that after cooling and freezing and thawing (79.6 ± 1.4). Sperm curvilinear velocity ($\mu m\ s^{-1}$) was reduced ($P < 0.05$) from 112.4 ±

5.3 at dilution to 96.0 ± 5.8 at cooling, and from 87.6 ± 4.1 at equilibration to 69.4 ± 2.0 at freezing and thawing. Sperm lateral head displacement (μm) varied ($P < 0.05$) at each stage, i.e. dilution, 3.9 ± 0.2; cooling, 2.3 ± 0.2; equilibration, 3.1 ± 0.3 and freezing and thawing, 1.7 ± 0.2. Plasma membrane integrity (%) of spermatozoa was 80.2 ± 3.9 at dilution. It reduced ($P < 0.05$) to 60.4 ± 5.6 at equilibration and then to 32.6 ± 3.8 at freezing and thawing. Normal acrosomes (%) of spermatozoa remained higher after dilution, cooling or equilibration (73.2 ± 2.4) while decreased ($P < 0.05$) after freezing and thawing (61.8 ± 2.4).

Experiment 3 consisted of freezing rates, a) slow or moderate, and b) moderate, fast, or very fast, changed between 4 to –15°C and –15 to –80°C, respectively. Freezing rates either during initial and subsequent freezing did not improve the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of spermatozoa. In the experiment of lethal intermediate zone, visual motility, computer-assisted motility and lateral head displacement reduced ($P < 0.05$) at –40°C, curvilinear velocity at –50°C, whereas plasma membrane integrity and normal acrosomes were adversely affected at –30°C. In the subsequent experiment of lethal intermediate zone, plasma membrane integrity also reduced ($P < 0.05$) at –30°C, either assessed by HOS or flow cytometric assay. The correlation between these assays at various temperatures was significant ($r = 0.90$; $P < 0.05$), that suggested the precision of flow cytometry for the evaluation of spermatozoa in buffaloes.

In experiment 4, post-thaw sperm motilities (visual and computer-assisted), velocities (straight-line, average path, and curvilinear) lateral head displacement, and plasma membrane integrity were higher ($P < 0.05$) in extenders containing 6% glycerol than in extenders with 3 or 0% glycerol. However, post-thaw visual motility, computer-assisted motility and plasma membrane integrity of spermatozoa decreased ($P < 0.01$) in the extenders either having 1.5 or 3% DMSO than that of 0% DMSO. The average values of these variables were maximum ($P < 0.01$) in extenders containing 6% glycerol and 0% DMSO than other combinations of glycerol and DMSO. Post-thaw visual motility, computer-assisted motility, straight-line velocity, and average path velocity of spermatozoa were maximum ($P < 0.05$) in extenders having 6% glycerol being added at 37°C than the other adding temperatures of glycerol. Post-thaw computer-assisted motility of spermatozoa was highest ($P = 0.04$) in those extenders where 6% glycerol and 0% DMSO were added at 37°C than other combinations of glycerol and DMSO additions (37 or 4°C).

Collectively, these experiments suggest that in buffaloes, 1) post-thaw quality of semen can be improved using the Tris-citric acid buffering system, 2) considerable damage to motility apparatus, plasma membrane and acrosomal cap of spermatozoa occurs during freezing and thawing followed by equilibration processes, 3) fast freezing either during initial or subsequent freezing did not improve the post-thaw sperm viability, whereas the intermediate zone of damage to sperm motility apparatus and membrane integrity lies somewhere between –20 to –40°C, and 4) DMSO at the levels investigated did not improve the post-thaw quality of spermatozoa, however, glycerol in 6%, when added at 37°C, provided the maximum cryoprotection to the motility apparatus, and plasma membrane integrity of spermatozoa.

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GENERAL INTRODUCTION

The Nili-Ravi buffalo, classified as river type, is recognized as the highest milk producing breed of buffalo (Cockrill, 1974). Being the principle breed in Pakistan she plays a pivotal role in the livestock industry of the country. Artificial insemination (A.I.) is the only means by which extensive breeding of buffaloes with proven bulls is possible. However, there are many constraints concerned in the development of preservation of buffalo semen. A successful cryopreservation of semen of this breed would aid in the creation of long-term storage of male germplasm and maintenance of genetic stock that could improve milk/beef production and its associated economic value internationally.

First successful cryopreservation of mammalian spermatozoa was reported in 1949 by Polge and coworkers. Furthermore, Smith and Polge (1950) found glycerol to be an outstanding polyhydric alcohol in protecting spermatozoa at low temperatures. These findings were utilized for long-term storage of spermatozoa in A.I. industry and the first live calf produced by A.I. with frozen-thawed bull semen was born in 1951 (Stewart, 1951). Since then, offspring of many mammalian species have been produced by A.I. with frozen semen (for review see Watson, 1990).

In buffalo bulls, Roy and coworkers froze the semen earlier in 1956. Basirov (1964) reported the fertility with frozen-thawed buffalo bull semen for the first time. Since then, A.I. has been adopted in buffaloes but its use is very limited (Ahmad, 1997). The use of frozen semen perhaps reduces the fertility in buffaloes compared to cattle (Radhakrishna et al., 1984; Ala-ud-Din et al., 1990). The quality of frozen semen is one of the factors resulting in low fertility rates. Therefore, a species-specific requirements for the cryopreservation of buffalo semen are very important to ensure high fertility in buffaloes using A.I.

The empirical studies have indicated that many factors are known to affect the viability of cryopreserved spermatozoa (Hammerstedt et al., 1990, Curry et al., 1994). These factors include, primarily, the composition of buffers and cryoprotectants, and secondly, the cryopreservation process itself.

Buffers for Bovine Semen

Dilution of semen in the suitable buffer is one of the important factors affecting sperm survival during cryopreservation. An ideal buffer should have: a) a pK_a around 7, b) greater water solubility, c) difficulty in passing through biological membranes, d) minimum salt effects, e) minimum concentration, and ionic composition, f) solubility of complexes formed with cations, g) greater ionic strengths, and h) stability against enzymatic and nonenzymatic degradation (Good et al., 1966).

Sodium sulfate (Na_2SO_4), glucose and peptone were the constituents of first short-term storage extender for bull spermatozoa (Milovanov and Selivanova, 1933). Subsequently, the phosphate buffer in combination with egg yolk was found to be satisfactory for storage of refrigerated bull spermatozoa (Phillips, 1939). This was replaced by sodium citrate (Salisbury et al., 1941) due to its properties of being a chelating agent and preventing peroxide formation in mammalian spermatozoa. Later, Tris-buffered egg yolk was developed and was shown to be superior to sodium citrate for the cryopreservation of bull spermatozoa (Davis et al., 1963), probably due to its better buffering capacity. In 1966, Good and coworkers found the zwitterion buffers (BES, HEPES, MES, MOPS, PIPES, TES and TRICINE) more stable than Tris in the temperature shift and the pK_a values of these buffers were closer to the optimal pH (7.0) of semen. This finding promoted many workers to use zwitterion buffers for the cryopreservation of bull semen. Among the zwitterions used, Tes buffer titrated to Tris (pH = 7.0) was found the most satisfactory buffering system for diluting bull spermatozoa for freezing (Graham et al., 1972). Since this and early work, little development of other buffers for preservation of bull spermatozoa has been reported (Graham, 1978).

In buffalo bulls, it is generally believed that the buffers used for preservation of semen do not seem to be that effective as in bulls. The causes of ineffectiveness of various buffers used for buffalo semen are not known. This may be due to some biochemical differences between buffalo and bull semen (Banerjee and Ganguli, 1971 and 1973). Alternatively, the difference lies in the lipid composition of plasma membrane of spermatozoa between these species (Guraya and Sidhu, 1975). However, the development of a suitable buffering system for cryopreservation of buffalo spermatozoa has been in progress for sometime. In

earlier studies, sodium citrate buffer was used for the cryopreservation of buffalo sperm (Roy et al., 1956). Later, the use of citric acid whey (Ganguli et al., 1973) and lactose (Shafi and Wierzbowski, 1979) was found satisfactory for the freezing of buffalo semen. In the subsequent studies in buffaloes (Heuer, 1980; Hultnaes; 1982), the successful utilization of TRIS and TES buffers for the cryopreservation of buffalo semen have encouraged their use for A.I. industry.

In buffaloes, semen extended in Tris showed higher post-thaw motility of spermatozoa when compared to citrate, citric acid whey or lactose (Chinnaiya and Ganguli, 1980; Heuer, 1980; Matharoo and Singh, 1980; Tuli et al., 1981). In contrast, post-thaw motility of spermatozoa did not vary when semen was extended in Tris compared to lactose and citrate (Dhami and Kodagali, 1990), milk (Dhami et al., 1996), or skim milk and citrate (Kakar and Anand, 1981). Likewise, the results for the release of enzymes, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), were equivocal. Post-thaw release of these enzymes was less due to Tris compared to citric acid whey or sodium bicarbonate (Tuli et al., 1982). Whereas, the release of these enzymes was greater in Tris compared to citric acid whey (Chinnaiya et al., 1979). However, fertility of buffaloes inseminated with frozen-thawed semen was significantly higher in Tris compared to lactose or citrate (Vasanth, 1979; Dhami and Kodagali, 1990) but did not differ compared to milk extender (Dhami et al., 1996). These controversial findings suggested to identify the suitable buffering system for cryopreservation of buffalo spermatozoa.

Cryoprotectants for Bovine Semen

Glycerol

Glycerol has been used extensively for the cryopreservation of many types of cell, including sperm, since the discovery of its cryoprotective properties by Polge et al. (1949). It acts through 'salt-buffering' mechanism (Lovelock, 1953). Presumably, glycerol dehydrates the cell (Berndtson and Foote, 1969) and form complexes with metallic ions (Lohmann et al., 1964). Furthermore, it reduces the concentration of extracellular media (Meryman, 1971), thermal stress and prevents fracture in the frozen solutions by decreasing the total ice volume expansion during water solidification (Gao, et al., 1995).

Glycerol, in addition to its cryoprotective effects, may have detrimental effects on the structure of plasma membrane and on the metabolism of mammalian cell due to slow

penetrating activity (Parks and Graham, 1992). It has been shown that the molar concentrations of glycerol can affect a) structures in cytoplasm, b) permeability and stability of the plasma membrane, and c) noncovalent attachment of proteins to the sperm surface (Hammerstedt and Graham, 1992). These effects, differing among species, reduce the fertility of boar, chicken and turkey spermatozoa. Thus glycerol is considered as a contraceptive agent (Polge, 1956; see review by Hammerstedt and Graham, 1992). This may be one of the possible reasons of lower fertility in buffaloes as compared to cattle. Glycerol, despite its toxic effects, still continues to be used as a cryoprotectant for the preservation of bovine spermatozoa. Thus, the development of a less toxic cryopreservative could make a significant contribution in the improvement of frozen semen quality of buffalo bulls.

Dimethyl sulfoxide

The other potential cryoprotectants include dimethyl sulfoxide, ethylene glycol and propanediol. These are rapid penetrating cryoprotectants having lower molecular weight than glycerol. Dimethyl sulfoxide (DMSO), since its discovery as a cryoprotective agent (Lovelock and Bishop, 1959), has been used extensively for the freezing of mammalian gametes. DMSO may inhibit the harmful effect of hydroxy radicals (Yu and Quinn, 1994; Johnson and Nasr-Esfahani, 1994) as they appear during cell respiration and are detrimental to cell. DMSO has found to improve post-thaw quality of spermatozoa when used in combination with glycerol in bulls (Page, 1968; Snedeker and Gaunya, 1970) and buffaloes (Ansar et al., 1989). These findings prompted us to investigate the use of DMSO alone or in combination with glycerol for the cryopreservation of buffalo spermatozoa.

Cryopreservation of Semen

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

The dilution of spermatozoa in buffers can chelate metal ions, alter ion-lipid interactions, change the proteins bound to the plasma membrane, immobilize the sugars bind to head groups and modify head group spacing (Hammerstedt et al., 1990). The dilution in cryoprotectant creates hyperosmotic conditions that results in shrinkage of suspended

spermatozoa and they retain their isotonic volume after the penetration of cryoprotectant buffer (Gilmore et al., 1998). This osmotic stress can be potentially lethal to the spermatozoa if it causes sperm to swell or shrink beyond their osmotic tolerance limits (Willoughby et al., 1996). The addition of polyglycols may alter the pore structures, ion transport, and bioenergetic demand of spermatozoa (Hammerstedt et al., 1990).

Cooling of spermatozoa changes the lipid composition and organization of the membrane bilayer (Buhr et al., 1994) thus restricts the mobility of fatty acyl side chains and the lateral movement of the membrane (Watson, 1996). Concomitantly, it changes the permeability of sperm plasma membrane (Ortman and Rodriguez-Martinez, 1994). Cooling may alter bioenergetic balance due to change in flagellar motion and catabolic processes (Hammerstedt et al., 1990). 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, a cold shock may increase the calcium uptake by sperm (White, 1993).

During freezing, ice crystals are formed extracellularly (at about –5°C), thereby raising the solute concentration. In response, the cells are dehydrated, preventing ice crystals formation intracellularly (at about –15°C or lesser) (Mazur, 1977). During extracellular ice formation, the residual unfrozen medium form channels of small size where cells become trapped in the increasing solute concentration (Mazur, 1984). This osmotic assault probably cause changes in pH as buffer salts reach their solubilities (Fishbein and Winkert, 1978), increases cellular dehydration resulting in a cross-linking of the intracellular structures (Levitt, 1962), weakens protein-lipid complexes in the cell membrane and increases phospholipid losses (Lovelock, 1957). These possible effects may decrease sperm viability and have been characterized collectively by Mazur et al. (1972) as "solution effects." It has been suggested that these effects are greatly enhanced in a slow freezing process, whereas intracellular ice is most likely to occur during rapid freezing (Mazur, 1984). It means freezing rates that are either "too high" or "too low" can kill cells. Therefore, an "optimal" freezing rate should and does exist between the high and low, as demonstrated previously in a variety of cells (Mazur et al., 1972).

Thawing causes recrystallization of water, decreases the solute concentration of the surrounding medium, and restoration of the intracellular water content and cell volume (Mazur, 1984 and 1985).

Freezing Rates

In early studies, bull semen was frozen in ampules in dry ice-alcohol bath at the rate of 1-2°C min^{-1} from +5 to –15°C and 4-5°C min^{-1} from –15 to –79°C (Smith and Polge, 1950; Polge, 1953). This became the standard rate in later studies (Polge, 1957; O'Dell et al., 1958). The freezing of semen in straws in liquid nitrogen vapour either conventionally (Adler, 1960) or using controlled rate cell freezer (Almquist and Wiggin, 1973) did not have the detrimental effects on post-thaw sperm survival. Availability of controlled rate freezing prompted the workers to carry out freezing of bull spermatozoa in different cycles (Gilbert and Almquist, 1978; Almquist et al., 1982; Liu et al., 1998). These were based upon the phenomenon of dehydrating the cell first with slow freezing and, secondly to avoid the formation of intracellular ice with fast freezing (Mazur, 1980). The results from most of these studies have indicated that bull spermatozoa could resist wide range of freezing rates when frozen in straws. However, very slow or fast freezing rates were detrimental to post-thaw survival of bull spermatozoa (Rodriguez et al., 1975; Mortimer et al., 1976; Chen et al., 1993).

In buffalo bulls, the semen was frozen earlier with CO_2 by Roy et al. (1956). Later, the technique of freezing of buffalo semen was modified and the straw freezing with static liquid nitrogen vapours became common (Shafi and Wierzbowski, 1979; Ahmad and Chaudhry, 1980). Hultnaes (1982) did not find any difference in post-thaw survival of spermatozoa when freezing of buffalo semen in straws was carried out either 1, 4 or 8 cm above the liquid nitrogen surface. In contrast, semen frozen 2 cm above the liquid nitrogen surface was found superior in post-thaw motility of spermatozoa than that of 4, 6 or 8 cm (Nazir, 1986). Based upon this and earlier work, it is anticipated that the use of controlled rate cell freezer would improve the post-thaw semen quality in buffaloes.

Cryoinjuries to Spermatozoa

It is generally believed that injury to spermatozoa due to cryoprocessing is of two types; a) direct chilling injury and b) indirect or latent chilling injury. Direct injury results in alteration of the plasma membrane and subsequent biochemical and metabolic changes that are immediately evidenced by altered spermatozoal motion or biochemical tests (Varner et al., 1988). Latent damage is detectable until the cells have reached 0 to 5°C. The effects of

cryoinjuries to plasma membrane, acrosomal membrane, nuclear membrane, mitochondrial membrane, and axoneme of the spermatozoa has been reviewed by Watson (1995).

The cryoinjury to sperm plasma membrane causes a breakdown in asymmetry and rushing of more fluid in the outer leaflet, results in change in the physiology of spermatozoa (Watson, 1996). This is evinced by swelling and breakage (Pace et al., 1981), loss of membrane selective permeability (Buhr et al., 1989), leakage and aggregation of phospholipids and proteins, reduction of motility, enzyme activity and viability (Graham and Pace, 1967; Watson, 1981), and inability to control the intracellular calcium (Ca^{2+}) storage (Zhao and Buhr, 1995).

The cryoinjury to sperm acrosomal membrane liberates acrosomal enzymes, required for the penetration of zona pellucida and fusion with the oocyte (Akhtar and Chaudhry, 1989; Palencia et al., 1996), results in low fertility (McLaughlin et al., 1993). The cryopreservation process may causes denaturing of DNA and decreases the fertilizing ability of spermatozoa (Karabinus et al., 1991).

The cryoinjury to sperm mitochondria reduces the ATP production and thus sperm motility (Graham et al., 1984) and velocity (McLaughlin et al., 1992) are adversely affected. Furthermore, the cryoinjury to the microtubules (Courtens and Paquignon, 1985) and radial spokes (Courtens et al., 1989) of the axoneme has been shown to decrease the motility of spermatozoa.

Semen Quality

High quality semen is a key for success to both high production and reproduction in bovines. However, inadequate accuracy in measuring the semen quality assays in laboratory (Graham and Crabo, 1978), and inability to measure fertility with acceptable precision (Saacke et al., 1980) are the two major problems related to the association of semen quality and fertility.

Semen Quality Assays

The assessment of sperm quality is one of the major concerns in semen research and artificial insemination. Presently, no single precise laboratory assay is available to predict the fertility of frozen-thawed semen accurately (Graham et al., 1984). However, the combination of several assays may better predict fertility (Linford et al., 1976; Graham et

al., 1990). The common viability related assays used for the assessment of semen quality include motility, velocity, penetration of cervical mucus, metabolic activity, cell contents, ability to agglutinate (head to head) in the presence of blood serum, ability to pass through sephadex-glass wool filter and structural integrity of cell membrane and acrosome (Saacke, 1984). However, the combination of these assays has provided the equivocal results with fertility. The assays of visual motility, computer-assisted motility and velocity, track motility, and acrosomal integrity have been correlated with fertility in bulls (O'Connor et al., 1981). Whereas, in an other study (Papa, 1982), sperm motility, acrosome integrity, thermoresistence test, cold shock test or Sephadex filter test did not show any relationship with the fertility of frozen bull semen. Recently, sperm penetration assay (IVF) has been strongly correlated ($P < 0.01$, $r = 0.723$) with fertility, whereas motile sperm, viable sperm, and sperm with intact acrosomes were not significantly correlated with the conception rate in bulls (Brahmkshtri et al., 1999).

Semen assays used in the present studies on buffalo bull will be discussed in this part of thesis.

Sperm Motility

Sperm motility relationship with fertility

The results for sperm motility relationship with fertility in bulls are equivocal. Sperm motility was significantly correlated with bull fertility (Linford et al. 1976; Saacke et al. 1980; Pace et al., 1981). In contrast, post-thaw sperm motility was not correlated with fertility in bulls (Soderquist et al., 1991).

Sperm motility assessment methods

Sperm motility is an important criterion for determining the quality of sperm population, both before artificial insemination, and in the laboratory to evaluate the effect of experimental procedures. Sperm motility is routinely estimated visually, by the use of closed circuit television (Graham et al., 1970). Unfortunately, visual determination is highly a subjective assessment method, depends on observer's accuracy, and semen handling techniques (Graham, et al., 1980). Furthermore, it is not a reliable assay for perdicting fertility in bulls (Saacke and White, 1972; Linford, et al., 1976; Brahmkshtri et al., 1999).

For the last several decades, different approaches have been used, to develop the objective methods for the evaluation of sperm motion characteristics. The objective assessments of sperm motility are reasonably accurate and repeatable, based either upon massive movement of spermatozoa (Rothschild, 1948; Glover, 1968) or the measurement of proportion of motile spermatozoa and individual velocities of those spermatozoa (Harvey, 1945; Rothschild, 1953; Katz and Dott, 1975). The details of sperm motion characteristics can provide insights into the physiology of cells, the mechanisms of their transport, and fertilization (Yanagimachi, 1978; Katz and Overstreet, 1980).

The techniques used for the objective assessment of spermatozoa included, microcinematography (David et al., 1981), videomicrography (Katz and Overstreet, 1981; Johnson et al., 1981), time-exposure photomicrography (Rothschild, 1953; Overstreet et al., 1979; Aitken et al., 1985), multi-exposure photography (Makler, 1978), and laser-doppler spectroscopy (Cooke and Hallett, 1976; Hartmann et al., 1983). However, the use of computer-assisted sperm motility was carried out almost two decades earlier (Liu and Warme, 1977; Amann, 1979). This system was found inaccurate when the sample contain less than 25 percent motile spermatozoa (Amann and Hammerstedt, 1980). Concomitantly, O'Connor et al. (1981) concluded that with that system no advantage over conventional laboratory tests for the prediction of fertility of cryopreserved bull spermatozoa.

With the advancement in computer technology, software has been developed that capture a series of video images in digital form, in real time, and provides comprehensive data on sperm motion. This system is highly dependent on its settings (Knuth et al., 1987) and has been used successfully in different species (Budworth et al., 1988; Jasko et al., 1990; Ellington et al., 1993; Berger et al., 1994) due to its more precision.

In human, Holt et al. (1985) recorded, with computer, a very well correlation of sperm speed measurements with those of the in vitro fertilization rate. Badenoch et al. (1990) compared four different methods for evaluating human sperm motility. Those included subjective assessment of a wet film preparation, sperm velocity measured by time-lapse photography, sperm velocity measured by computer analysis and sperm migration across a nucleopore membrane. The subjective assessment of motility was found to be inaccurate, within single observer and between two observers. Both methods of measuring mean sperm velocity were accurate, particularly that using the computer analysis system; a high technical failure rate was found using time-lapse photography.

Sperm migration across a nucleopore membrane was found to be highly inaccurate. Whereas, computer analyzed sperm velocity could predict with overall 91% accuracy. There are several reports demonstrating a good correlation between sperm motion parameters and fertilization outcome (Mathur et at., 1986; Fetterolf and Rogers, 1990; Liu et al., 1991; Parinaud et al., 1996).

In bulls, Budworth et al. (1988) reported, by the use of CellSoft computer system, a good correlation ($r \geq 0.68$; $P < 0.05$) between the competetive fertility index and the percentage of motile spermatozoa, linear velocity and straight-line velocity. Likewise, Amann (1989) reported an R^2 value of 0.86 between the percentage of motile sperm and a competitive fertility index. With the development of modified forms of computerized semen analysis significant correlation with fertility have shown to predict fertilization rates (Check et al., 1990; Fetterolf and Rogers, 1990). Computer analysis of sperm velocity offers a rapid, objective and predictive assessment of sperm function.

Sperm Motion Characteristics

Progressive velocity and the actual movement of the spermatozoa have been shown to be closely related with sperm penetration into the cervical mucus (Aitken et al., 1985), heterologus zona free hamster egg penetration test (Aitken et al., 1982a and 1982b), and in vitro fertilization (IVF; Jeulin et al., 1986).

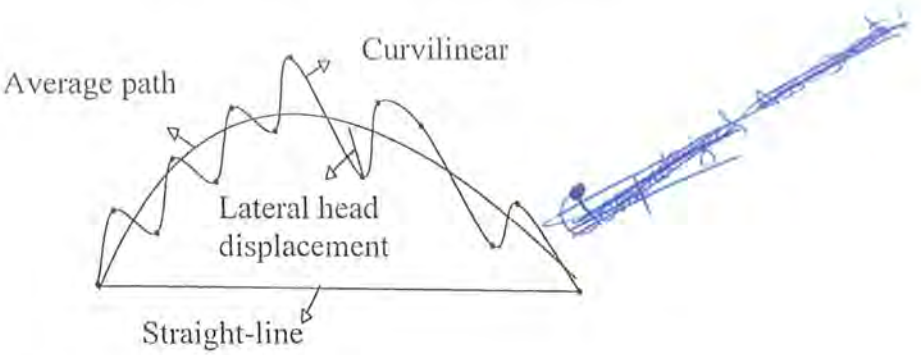
Sperm velocity and lateral head displacement (LHD), as measured by Computer-Assisted Semen Analyzer (CASA), appear to be better predictors of an individual's fertility, than progressive motility of bull and human sperm (Ellington et al., 1993). LHD may indirectly reflects the efficiency of the flagellar beat pattern, appears to be a critical factor whenever the generation of shearing forces is involved, as in sperm penetration of cervical mucus or their ability to penetrate the cumulus oophorus and the zona pellucida (Jeulin et al., 1986). It was poorly correlated with their ability to fuse with zona-free hamster oocytes (Aitken et al., 1985).

Hyperactivation of spermatozoa

Before the achievement of fertilization, the spermatozoa must undergo a series of membrane and metabolic changes called capacitation. During capacitation, sperm hyperactivation is an essential phenomenon (Chang, 1984; Yanagimachi, 1994). The

hyperactive motility of spermatozoa was described as being vigorous and showing high-curvature flagellar movements. The measurement of hyperactive motility of spermatozoa has always been carried out subjectively based on criteria being established by Yanagimachi (1970). To standardize the patterns of hyperactive motility, computer-assisted sperm motility analysis has been used (Mortimer and Mortimer, 1990; Burkman, 1991). The pattern of hyperactivation have been examined in bulls (Iqbal and Hunter, 1995), hamster (Si, 1999), human (Burkman, 1991), and mouse (Aoki et al., 1994) spermatozoa.

The computerized measurements were either used to determine the relationship between hyperactive motility and other fertility assays (Wang et al., 1991) or between sperm motility patterns and fertility per se (Budworth et al., 1988). The schematic model of sperm velocities and lateral head displacement is presented in Fig. 1. The special attributes of sperm movement like VCL (curvilinear velocity), LHD, and LIN (linearity) were used to calculate the hyperactive motility of sperm (Mortimer and Mortimer, 1990; Mbizvo et al., 1993), and the population of sperm showing VCL $\geq$ 100 μm s^{-1}, LHD $\geq$ 5 μm and LIN $\leq$ 60% at 4 h incubation was considered to be hyperactivated sperm in man (Mortimer and Mortimer, 1990). The spermatozoa moving with a speed of more than 100 μm s^{-1} of VCL were graded as hyperactive in human and Holstein bulls (Burkman, 1991; Iqbal and Hunter, 1995).



(adopted from Aman, 1988)

Fig. 1. The schematic model of spermatozoal velocities and lateral head displacement.

Sperm Plasma Membrane Morphology

The sperm plasma membrane plays a pivotal role in controlling sperm fertilizing ability. It directly mediates the contact interactions between the spermatozoa and the oocyte itself

and acts as a receiver of the environmental signals to achieve fertilization. Therefore, the integrity and functional activity of the sperm plasma membrane is of fundamental importance to determine the fertilizing ability of spermatozoa.

Sperm plasma membrane assessment methods

The evaluation of plasma membrane integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium. Furthermore, the process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active membrane (Jeyendran et al., 1984). Several assays have been developed to assess the functional integrity of spermatozoa besides the standard evaluating methods including adenosine triphosphate of sperm, cervical mucus penetration, hemizona attachment assay, zona-free hamster oocyte (ZFHO) penetration assay, triple staining, and sperm acrosin activity (Toda et al., 1992). The assessment of live and dead spermatozoa by supravital staining is mainly carried out with the help of eosin-nigrosin stain (Swanson and Bearden, 1951).

The use of HOS-assay has frequently been reported for different species (Bredderman and Foote, 1969; Jeyendran et al., 1984; Kumi-Diaka, 1993; Correa et al., 1997) including buffalo (Azam et al., 1998). The HOS-assay is a simple, quick and inexpensive method to assess functional integrity of plasma membrane (Jeyendran et al., 1984). The hypo-osmotic swelling test assesses the functional integrity of the sperm membrane and indirectly provides information about membrane associated cell functions (permeability, ion-exchange, O_2 transport, function of membrane associated enzymes). Anyhow, Zavos (1990) has suggested HOS-test as a valuable assay for the assessment of plasma membrane damage during the cryopreservation cycle. The exposure of spermatozoa to a hypo-osmotic solution causes an influx of water through the plasmalemma until an osmotic equilibrium is reached, resulting in a swollen cell, mainly manifesting itself as a curled sperm tail (Fig. 2 A). The plasma membrane surrounding the tail fibers appear to be more loosely attached than the membrane surrounding the head, so that the tail region shows the swelling more clear (Jeyendran et al., 1984). Sperm tail swelling generally indicates that the transport of water across the plasma membrane has occurred normally, an indicative of normal membrane integrity and function as the ability of the spermatozoa to undergo capacitation and fusion with zona free hamster oocyte (Jayandran et al., 1984). The use of HOS-assay

has a good correlation to fertility ($r = 0.90$; Jayandran et al., 1984) and sperm morphology (Van der Ven et al., 1986) in human, and to sperm motility ($r = 0.73$) in bull (Correa and Zavos, 1995). A significant correlation has been reported between the HOS-assay and bovine mucus penetration test ($r = 0.77$), the progressive motility ($r = 0.70$), and sperm mophology ($r = 0.51$) in human (Gehring, 1987).

Sperm Acrosome Morphology

During mammalian fertilization, sperm undergo acrosome reaction which involves the fusion of sperm plasma membrane with the underlying outer acrosomal membrane inducing membrane vesiculation at various sites of the sperm head (Barros et al., 1967). Disruption of the plasma membrane and outer acrosomal membranes releases acrosomal enzymes and exposes the inner acrosomal membrane. The release of acrosomal enzymes may facilitate the penetration of sperm through zona pellucida, and to fertilize the egg (Yanagimachi, 1994). Therefore, the presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is being required at the time of fertilization (Yanagimachi, 1994). The change in acrosomal cap is mainly due to sperm aging or injury (Saacke and Marshall, 1968), which can be effectively determined by fixing the specimen using phase-contrast microcsopy in bulls (Anzar and Graham, 1993). Different acrosomal abnormalities have been reported in semen from certain subfertile bulls (Saacke et al., 1968). Therefore, the assessment of acrosomal integrity is an effective tool to monitor the sperm ability to undergo acrosome reaction and subsequent fertilization.

Sperm acrosome morphology relationship with fertility

The relationship of intact acrosomes with fertility in bovine has been reviewed by Graham et al. (1980), Saacke (1984) and Coulter (1992). The good correlation ($r = 0.21$; $P < 0.05$) between the percentage of intact acrosomes and fertility of frozen bovine spermatozoa was observed after 2 and 4 h of post-thaw incubation (Saacke and White, 1972). The acrosomal integrity has significant relationship with fertility ($r = 0.60$; $P < 0.01$) than with abnormal sperm morphology ($r = -0.27$ to -0.37; $P < 0.01$) of cryopreserved bull semen (Saacke and White, 1972). In a heterospermic study, Saacke et al. (1980) showed that the percentage of intact acrosomes was highly correlated with the competitive fertility index ($r = 0.90$, $P < 0.01$). Sperm acrosomal integrity was positively correlated with fertility in bulls

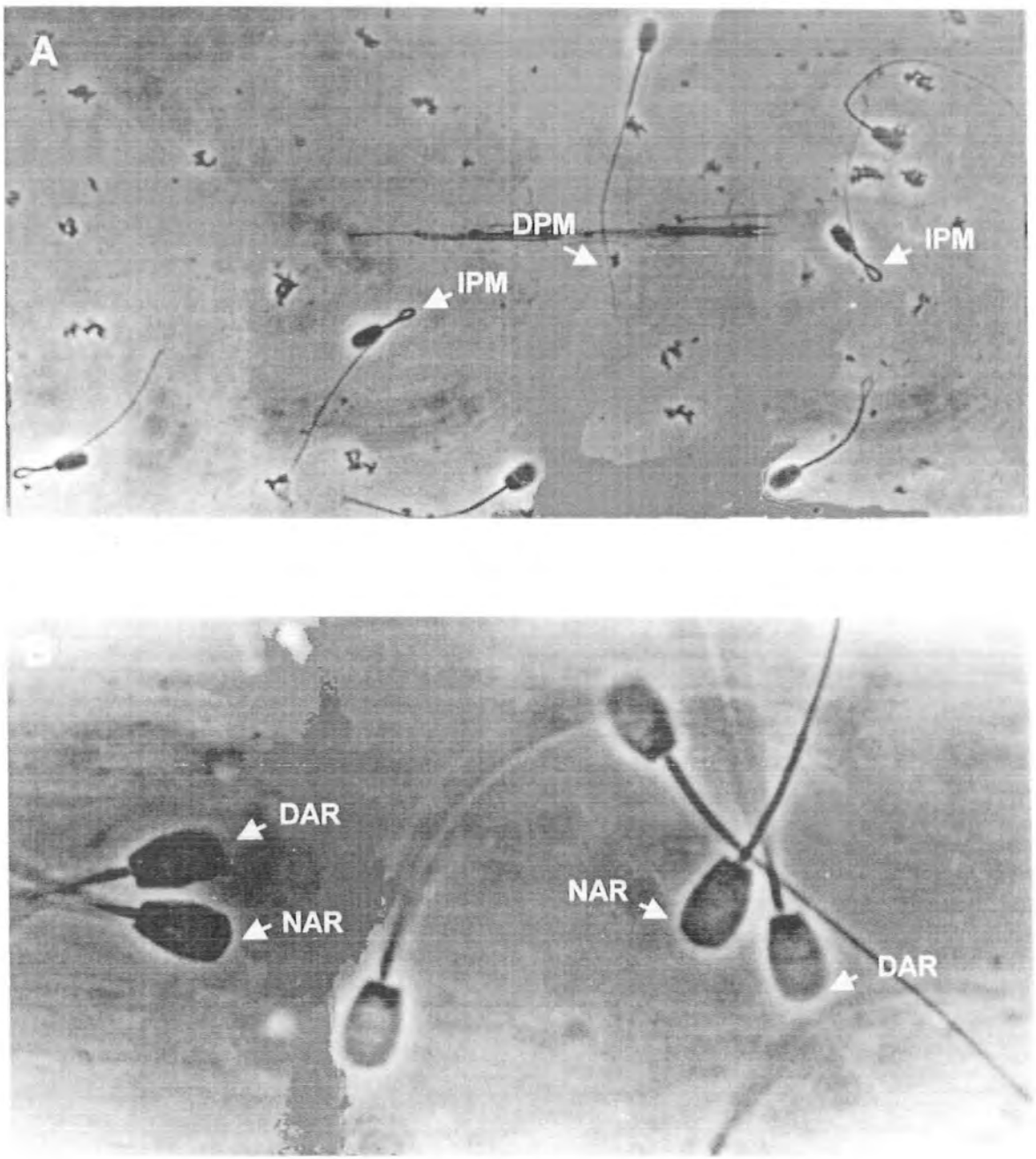


Fig. 2. A) Plasma membrane integrity of buffalo bull spermatozoa subjected to hypo-osmotic swelling test. Curling tail of sperm indicates intact plasma membrane (IPM), whereas straight tail indicates damaged plasma membrane (DPM). (x 224). **B**) Acrosome morphology of buffalo bull spermatozoa. The presence of apical ridge indicates the normal acrosome (NAR), whereas the absent apical ridge indicates the damaged acrosome (DAR). (x 560).

(Berndtson et al., 1981; Pace et al., 1981; Karabinus et al., 1990). Whereas, in a recent study, percent sperm with intact acrosomes was not significantly correlated with the conception rate in bulls (Brahmkshtri et al., 1999).

Sperm acrosome morphology assessment methods

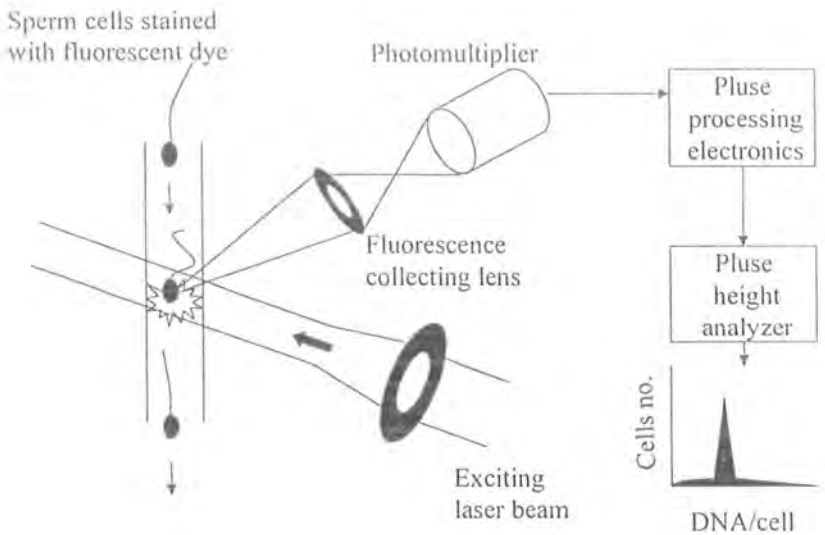
Several methods are available to determine the percentage of acrosome-reacted sperm in a semen sample. Most of them are complicated or require expensive reagents and equipment. Indirect immunofluorescence technique using a monoclonal antibody to acrosomal proteins/enzymes has been developed to determine the acrosomal integrity (Byrd and Wolf, 1986; Rajamahendran et al., 1994). Alternatively, histochemical stains (naphthol yellow S, erythrosin B, and chondroitin sulfates) have been used (Bryan and Akruk, 1977; Lenz et al., 1988). Lectins labeled with fluorochromes have proven useful for sperm from many species (Cross et al., 1985; Cummins et al., 1986). Chlorotetracycline fluorescence patterns and electron microscopy also indicate acrosomal status (Saling et al., 1979; Lee et al., 1987; Perez et al., 1996). Recently, the use of Coomassie Blue G-250 staining method has been shown to be a reliable method for the assessment of acrosomal status in a variety of species (Larson and Miller, 1999). In bovines, the acrosomes are large enough to be identified by simple light microscopy (Sanchez et al., 1995), by differential interference contrast microscopy (Johnson et al., 1976), or by phase-contrast microscopy (Bamba and Cran, 1988). The morphological determination by differential interference contrast microscopy or by phase-contrast microscopy has provided a valuable criterion for evaluating sperm quality in bulls (Saacke and Marshall, 1968; Saacke, 1970). However, the use of phase-contrast microscopy is less expensive and easier to operate over differential interference contrast microscopy (Galli et al., 1989) and will be used in the present studies in buffaloes (Fig. 2 B).

Other Quality Assays

Flow cytometric assay

During the last two decades, the flow cytometry has been introduced to study of mammalian spermatozoa. It is a powerful analytical tool for studying cell structure and function, and thousands of spermatozoa can be analyzed per minute regardless of

preservation medium (Parks, 1992). Flow cytometry offers high precision and sensitivity for evaluating functional aspects of large number of spermatozoa and more than one characteristic of a spermatozoon can be measured simultaneously. When fluorescence stained sample is placed into a sample chamber, the positive air pressure forces the sample into a laminar flowing liquid stream passing through a quartz flow chamber. This sample stream is intersected by laser beam. The laser beam excites the fluorescent dye and the resulting fluorescent signal is detected for each cell and converted to an electrical signal which passes through a multichannel analyzer quantitating the amount of fluorescence per cell (Evenson and Ballachey, 1988). The schematic of flow cytometry is presented in Fig. 3. The flow cytometric assessment has shown to be significant correlated with the microscopic analyses in bovine spermatozoa (Dobrinski et al., 1994; Garner et al., 1997). Fluorescent dyes available for the analyses of several cellular compartments of the spermatozoa are used either singularly or in combination to discriminate between functional or nonfunctional bovine spermatozoa (Thomas et al., 1998). Thus, flow cytometry is a rapid developing



(adopted from Van Dilla and Mendelsohn, 1979)

Fig. 3. The schematic diagram of flow cytometry.

field and its use for buffalo sperm will certainly contribute to the evaluation of semen quality.

This work is based upon a thorough investigation to establish the best cryopreservation procedures for buffalo sperm. The main objectives of my study were:

1. To identify the suitable buffer among *tri*-sodium citrate (CITRATE), Tris-citric acid (TCA), Tris-Tes (TEST) or Tris-Hepes (HEPEST),
2. to examine the effects of stages of cryopreservation i.e. dilution (D), cooling to 4°C (C), equilibration at 4°C for 4 h (E), and freezing and thawing (FT),
3. to determine the intermediate (critical) temperature zone for buffalo sperm and to study various cooling and freezing rates to improve the post-thaw semen quality, and
4. to investigate if different concentrations of glycerol and/or dimethyl sulfoxide (DMSO) either added at 37 or 4°C would alter sperm motion characteristics, plasma membrane integrity, and acrosome morphology in buffaloes while using computer-assisted semen analyzer (CASA), hypo-osmotic swelling (HOS) assay, flow cytometry, and phase-contrast microscope, respectively.

CHAPTER 1

Effect of Buffering Systems on Post-Thaw Motion Characteristics, Plasma Membrane Integrity, and Acrosome Morphology of Buffalo Spermatozoa

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ABSTRACT

This study was carried out to identify the suitable buffer for cryopreservation of buffalo semen. Semen was collected with artificial vagina (42°C) from four buffalo bulls. Split pooled ejaculates (n = 5), possessing more than 60% visual sperm motility, were extended at 37°C either in *tri*-sodium citrate (CITRATE), Tris-citric acid (TCA), Tris-Tes (TEST) or Tris-Hepes (HEPEST). Semen was cooled to 4°C in 2 h, equilibrated at 4°C for 4 h, filled in 0.5 ml straws and frozen in a programmable cell freezer before plunging into liquid nitrogen. Thawing of frozen semen was performed after 24 h at 37°C for 15 s. Sperm motion characteristics, plasma membrane integrity, and acrosome morphology of each semen sample were assessed by using computer-assisted semen analyzer (CASA), hypo-osmotic swelling (HOS) assay, and phase-contrast microscope, respectively. Analysis of variance revealed that percent post-thaw visual motility tended (P = 0.07) to be higher in HEPEST (61.0 ± 2.9) and lower in CITRATE (48.0 ± 2.5). Computerized motility did not vary due to buffering system. Percent post-thaw linear motility tended (P = 0.09) to be higher in TCA (78.2 ± 5.5) and lower in TEST (52.0 ± 6.9). Circular motility (%) was significantly lower ($P < 0.05$) in TCA (11.6 ± 2.8) and higher in TEST (29.8 ± 5.6). Curvilinear velocity ($\mu m\ s^{-1}$) was lower ($P < 0.05$) in TCA (69.4 ± 2.0) than in CITRATE (79.0 ± 5.8), TEST (87.2 ± 1.6) and HEPEST (82.6 ± 3.0). Lateral head displacement (μm) was lowest ($P < 0.05$) in TCA (1.7 ± 0.2) and highest in TEST (3.7 ± 0.6). Plasma membrane integrity and normal acrosomes of buffalo spermatozoa did not differ due to buffering system and averaged 40.0 ± 2.7% and 61.4 ± 4.6%, respectively. Based upon lower circular motility, curvilinear velocity, and lateral head displacement, it is concluded that post-thaw quality of buffalo semen can be improved using the Tris-citric acid buffering system.

Keywords: Buffalo—spermatozoa; Buffers

INTRODUCTION

The first short-term storage extender for bull spermatozoa consisted of sodium sulfate (Na_2SO_4), glucose and peptone (Milovanov and Selivanova, 1933). This was replaced by sodium citrate (Salisbury et al., 1941) due to its properties of being a chelating agent and

preventing peroxide formation in mammalian spermatozoa. Later, Tris based extender was shown to be superior to sodium citrate for the cryopreservation of bull spermatozoa (Davis et al., 1963), presumably due to its better buffering capacity. Buffers containing zwitterions (Bes, Hepes, Mes, Mops, Pipes, Tes and Tricine) had pK_a values closer to the optimal freezing pH of 7.0 and were more stable than Tris in the temperature shift (Good et al., 1966). The bull spermatozoa diluted in zwitterions when titrated to physiological pH with either Tris, NaOH or KOH were more motile but released more glutamic oxaloacetic transaminase (GOT) than sperm diluted in phosphate or citrate (Graham et al., 1972). In the same study, Tes-Tris (Tes buffer titrated to Tris; pH = 7.0) yielded the most satisfactory buffering system for diluting bull spermatozoa for freezing. Since then, little development of other buffers for preservation of bull spermatozoa has been reported (Graham, 1978).

Development of a suitable buffering system for cryopreservation of buffalo spermatozoa has been in progress for sometime and the results are equivocal. Post-thaw motility was higher when buffalo spermatozoa were extended in Tris compared to citrate, citric acid whey or lactose (Chinnaiya and Ganguli, 1980; Heuer, 1980; Matharoo and Singh, 1980; Tuli et al., 1981). However, motility of frozen-thawed spermatozoa did not vary when they were extended in Tris compared to lactose and citrate (Dhami and Kodagali, 1990), milk (Dhami et al., 1996), or skim milk and citrate (Kakar and Anand, 1981). The release of enzymes, GOT and glutamic pyruvic transaminase (GPT), was less due to Tris compared to citric acid whey or sodium bicarbonate after freezing (Tuli et al., 1982). In contrast, Chinnaiya et al. (1979) reported higher release of these enzymes in Tris compared to citric acid whey. The release of enzymes has been shown to be associated with sperm cell injury (Pace and Graham, 1970). Fertility of buffaloes inseminated with frozen-thawed semen was significantly higher when Tris was used compared to lactose or citrate (Dhami and Kodagali, 1990) but did not differ when compared with milk extender (Dhami et al., 1996). The introduction of Computer-Assisted Semen Analyzer (CASA) has provided a more precise quantification of sperm characters than subjective measures in bulls (Budworth et al., 1988; Anzar et al., 1991). The present study was designed to compare the post-thaw motion characteristics, plasma membrane integrity and acrosome morphology between the conventional (Na-citrate) and zwitterion buffers [Tris-citric acid (TCA), Tris-Tes (TEST) and Tris-Hepes (HEPEST)] in

buffalo spermatozoa to identify the suitable buffering system.

MATERIALS AND METHODS

Preparation of buffers

The following four buffers for cryopreservation of buffalo spermatozoa were used.

1. CITRATE. *tri*-Sodium citrate dihydrate (Merck, Darmstadt, Germany) 6.37 g in 200 ml distilled H_20, pH 6.8, 301 mOsm kg^{-1}.

2. TCA. Citric acid (Fluka, Switzerland) 12.50 g in 200 ml distilled H_2O (325 mOsm kg^{-1}) was titrated to Tris-(hydroxymethyl)-aminomethane (Fluka, Switzerland) (Tris; 7.87 g in 200 ml distilled H_2O, 325 mOsm kg^{-1}) to pH 7.0. Osmotic pressure was 320 mOsm kg^{-1}.

3. TEST. Tes (*N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid) (Sigma, St. Louis, MO) 14.89 g in 200 ml distilled H_2O (325 mOsm kg^{-1}) and was titrated to Tris (7.87 g in 200 ml distilled H_2O) to pH 7.0. Osmotic pressure was 303 mOsm kg^{-1}.

4. HEPEST. Hepes (*N*-[2-Hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid]) (Sigma) 15.49 g in 200 ml distilled H_2O (325 mOsm kg^{-1}) was titrated to Tris (7.87 g in 200 ml distilled H_2O) to pH 7.0. Osmotic pressure was 301 mOsm kg^{-1}.

Egg yolk (20%; v/v), fructose (0.2%; w/v), glycerol (6%; v/v), penicillin (1000 I.U. ml^{-1}) and streptomycin (100 μg ml^{-1}) were added to each of the formulated buffer. The extenders were centrifuged at 12000 × *g* for 15 min, and the supernatant was frozen and stored at –20°C. The extenders were thawed at 37°C for experimental use.

Semen collection and initial evaluation

Four mature Nili-Ravi buffalo bulls, maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad, Pakistan, were used in the study. Two consecutive ejaculates were collected with the help of artificial vagina (42°C) at weekly intervals for 5 weeks (replicates) during the months of May and June. The semen was transferred to laboratory within a minute. Visual motility was assessed microscopically (x 400) with closed circuit television (Graham et al., 1970). Sperm concentration was assessed by digital-photometer (Dr. Lange LP 300 SDM; Germany) at 560 nm. The neat semen samples with more than 60% motile spermatozoa were used. The qualifying ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time of 15 min at 37°C in the water bath before dilution.

Semen processing

The schematic diagram of cryopreservation of buffalo spermatozoa are presented in Fig. 1. Four aliquots of semen were diluted at 37°C with each extender in order to provide approximately dilution rate of 50 x 10^6 spermatozoa ml^{-1}. Extended semen was cooled slowly (approximately in 2 h) to 4°C, and equilibrated for 4 h. Semen was packed into 0.5 ml polyvinyl French straws and frozen in a programmable cell freezer (KRYO 10 Series III, Planer, Sunbury-on-Thames, Middlesex, UK) from 4°C to –15°C at the rate of 3°C min^{-1} and from –15°C to –80°C at the rate of 10°C min^{-1}. Semen-filled straws were plunged into liquid nitrogen (–196°C) for storage. After 24 h storage in liquid nitrogen, semen straws were thawed at 37°C for at least 15 s for post-thaw semen quality assessment.

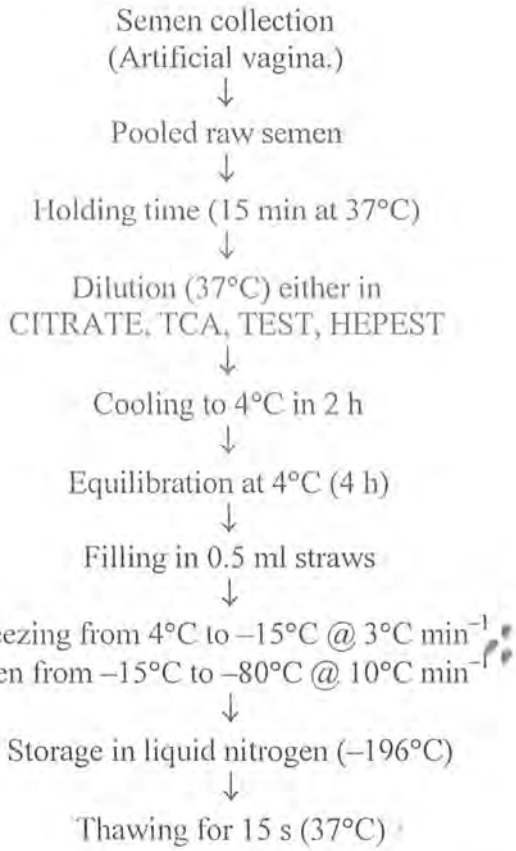

Fig. 1. The schematic diagram of cryopreservation of buffalo semen.

Semen assays

Visual assessment

Visual motility (VMOT, %) was assessed as described earlier.

Sperm motion characteristics

The settings of CASA

Sperm motion characteristics were determined using the Cell Motion Analyzer (SM-CMA version 4.4; Mika Medical, Germany) as previously reported in bulls (Engelmann et al., 1992). The system included an IBM-compatible PC computer with a monitor, a Panasonic KX-P1121 multimode printer, and an Olympus BH-2 microscope equipped with a thermostage, video camera and Panasonic BT-H1450Y digital image video monitor. The video images were fed into IBM-compatible PC through video camera and monitor, that analysed the data according to the programmed algorithms in SM-CMA software. The settings of computer were adjusted to acquire digitized video scan data at the rate of 32 frames s^{-1}. Lower area detection limit was 35 pixels and upper area limit was 300 pixels. Upper velocity limit for immotile spermatozoa was 20 $\mu m\ s^{-1}$. Lower velocity limit for the motile spermatozoa was 30 $\mu m\ s^{-1}$. The spermatozoa moving with velocity between 20 and 30 $\mu m\ s^{-1}$ were considered as locally motile and were not included in the motile category. The spermatozoa moving with a radius of 10 μm were designated as circular.

Validation of CASA

The system was validated before use for percentage of motile spermatozoa to evaluate the accuracy for the absolute standard in buffalo. Semen of four buffalo bulls were pooled ($n = 5$) and diluted at 37°C with sodium citrate in order to provide approximately 50 x 10^6 spermatozoa ml^{-1}. Extended semen was cooled slowly in 2 h to 4°C. For the assessment of sperm motion characteristics cooled semen was divided into two parts. One part of semen remained as such (live) and, other part was repeatedly plunged into liquid nitrogen and thawed (killed). Five mixtures of live and killed spermatozoa were prepared with 100:0, 75:25, 50:50, 25:75 and 0:100 ratio (live:killed) by volume. Correlation between the percentage of motile and the addition of killed spermatozoa was significant ($r = 0.98$; $P < 0.05$) [Fig.2]. The straight-line velocities of these mixtures were 52.6 ± 4.0,

55.6 ± 2.8, 54.0 ± 2.4, 52.0 ± 3.1, and 0.0 ± 0.0 (mean ± SEM), respectively.

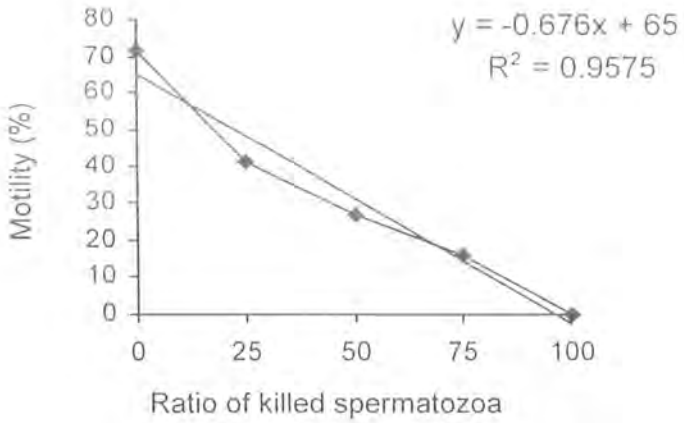

Fig. 2. Percentage decline in sperm motility after addition of killed spermatozoa in buffalo bulls.

Variables determined using CASA

Frozen semen was thawed at 37°C for 15 s and maintained at the same temperature for 5 min before evaluation of motion characteristics. After thoroughly mixing, 5 μl semen sample was placed on a prewarmed (37°C) Makler Chamber (depth 10 μm; Sefi-Medical Industries, Haifa, Israel). The chamber was then placed on the prewarmed stage (37°C) of microscope connected with CASA. Sperm motion characteristics included computer-assisted motility (CMOT, %), linear motility (LMOT, %), circular motility (CIRMOT, %), straight-line velocity (VSL, μm s^{-1}; the straight-line distance from beginning to end of track divided by time taken), average path velocity (VAP, μm s^{-1}; the spatially averaged path that eliminated the wobble of the sperm head), curvilinear velocity (VCL, μm s^{-1}; a measurement of the total distance traveled by a sperm during the acquisition divided by the time taken), lateral head displacement (LHD, μm; deviation of the sperm head about the axis of average path), linearity (LIN = VSL/VCL × 100), and straightness (STR = VSL/VAP × 100). Each observation was based upon the average of three different fields (Anzar et al., 1991).

Hypo-osmotic swelling (HOS)

Plasma membrane integrity (PMI) of spermatozoa was assessed using the HOS assay as described earlier (Jeyendran et al., 1984). HOS solution was prepared by dissolving 0.735 g

sodium citrate and 1.351 g fructose in 100 ml distilled H_2O (osmotic pressure – 190 mOsm kg^{-1}), according to World Health Organization (1992) manual. The HOS assay was performed by mixing 50 μl of the semen samples to 500 μl of the prewarmed (37°C) HOS solution and incubated at 37°C for 30-45 min. After incubation, a drop of semen sample was examined under phase-contrast microscope (x 400). Two hundred spermatozoa were counted for their swelling, characterized by coiled tail, indicating intact plasma membrane.

Normal acrosomes

Semen sample (500 μl) was fixed by 50 μl of 1% formal citrate (Hancock, 1957) which was prepared by adding 1ml of 37% commercial formaldehyde (Merck) in 99 ml of 2.9% (w/v) sodium citrate. Acrosomal integrity characterized by normal apical ridge (NAR) was assessed under oil immersion (x 1000) using phase-contrast microscope (Leica, Leitz Wetzlar, Germany). Two hundred spermatozoa were counted.

Statistical analysis

Results in text are presented as mean ± SEM. Data for each variable were analysed by the analysis of variance (ANOVA). When the F-ratio was significant ($P < 0.05$), Tukey's Honestly Significant Difference Test was used, to compare the treatment means (SYSTAT, 1996).

significant difference

RESULTS

Effect of buffers on post-thaw motility of buffalo spermatozoa

Data pertaining to the effect of buffers on motility after thawing in buffalo spermatozoa is presented in Table 1. The buffers tended to be different ($P = 0.07$), amongst each other in visual motility of spermatozoa. The average percent visual motility of spermatozoa was highest in TEST and HEPEST (60.0 ± 1.0), intermediate in TCA (53.0 ± 4.6), and lowest in CITRATE (48.0 ± 2.5). Percentage of motility assessed using CASA, did not differ due to the buffering systems and the overall average was 54.5 ± 4.4. Percentage of linear motility of spermatozoa showed the tendency to be different ($P = 0.09$) due to buffers. It was highest in CITRATE and TCA (75.0 ± 3.2), intermediate in HEPEST (63.2 ± 7.4), and lower in TEST (52.0 ± 6.9). There was a significant ($P < 0.05$) variation due to the buffers on the

percentage of circular motility of spermatozoa. This was lowest in TCA (11.6 ± 2.8), intermediate in CITRATE and HEPEST (16.0 ± 3.0), and highest in spermatozoa extended with TEST (29.8 ± 5.6).

Table 1
Effect of buffers on post-thaw motility (%) of buffalo spermatozoa (mean ± SEM; *n* = 5).

Variable	CITRATE	TCA	TEST	HEPEST	*P*
VMOT	48.0 ± 2.5	53.0 ± 4.6	59.0 ± 3.7	61.0 ± 2.9	0.07
CMOT	48.0 ± 5.7	48.6 ± 6.5	67.0 ± 6.4	54.4 ± 6.5	0.16
LMOT	71.8 ± 8.3	78.2 ± 5.5	52.0 ± 6.9	63.2 ± 7.4	0.09
CIRMOT	13.0 ± 5.1^{a,b}	11.6 ± 2.8^{b}	29.8 ± 5.6^{a}	19.0 ± 4.6^{a,b}	0.05

^{a,b} Values in the same row with different superscripts differ significantly ($P < 0.05$).
VMOT = Visual motility, CMOT = computer-assisted motility, LMOT = linear motility, and CIRMOT = circular motility.

Effect of buffers on post-thaw velocities, lateral head displacement, linearity, and straightness of buffalo spermatozoa

Data on the effect of buffers on velocities, lateral head displacement, linearity and straightness after thawing in buffalo spermatozoa are presented in Table 2. The buffers did not vary the straight-line and average path velocities of spermatozoa, and the overall averages were 48.6 ± 1.3 (μm s^{-1}) and 55.8 ± 1.6 (μm s^{-1}), respectively. There was a significant effect ($P < 0.02$) of buffers on curvilinear velocity of spermatozoa. This was minimal in TCA, maximal in TEST and intermediate in CITRATE and HEPEST. Similarly, lateral head displacement (μm) of spermatozoa was recorded as the lowest ($P < 0.03$) in TCA (1.7 ± 0.2), highest in TEST (3.7 ± 0.6), and intermediate in CITRATE and HEPEST (2.5 ± 0.3). The percentage of linearity and straightness of spermatozoa did not differ due to treatments and the overall averages were 62.0 ± 3.4 and 87.2 ± 2.3, respectively.

Effect of buffers on post-thaw membrane integrity of buffalo spermatozoa

Average plasma membrane integrity and the normal acrosomes of buffalo spermatozoa did not vary due to buffering systems (Table 3). Their overall values (%) were averaged 40.0 ± 2.7 and 61.4 ± 4.6, respectively.

Table 2
Effect of buffers on post-thaw velocities, lateral head displacement, linearity, and straightness of buffalo spermatozoa (mean ± SEM; $n = 5$).

Variable	CITRATE	TCA	TEST	HEPEST	*P*
VSL ($\mu m\ s^{-1}$)	51.4 ± 2.2	47.0 ± 3.3	46.0 ± 3.7	50.0 ± 2.2	0.54
VAP ($\mu m\ s^{-1}$)	58.4 ± 2.9	51.0 ± 2.7	56.4 ± 2.5	57.2 ± 1.3	0.18
VCL ($\mu m\ s^{-1}$)	79.0 ± 5.8^{a,b}	69.4 ± 2.0^{b}	87.2 ± 1.6^{a}	82.6 ± 3.0^{a,b}	0.02
LHD (μm)	2.2 ± 0.5^{a,b}	1.7 ± 0.2^{b}	3.7 ± 0.6^{a}	2.8 ± 0.3^{a,b}	0.03
LIN (%)	66.6 ± 6.1	67.6 ± 3.5	52.9 ± 4.8	61.0 ± 3.9	0.15
STR (%)	88.4 ± 3.4	91.9 ± 2.4	81.0 ± 3.3	87.3 ± 2.6	0.11

^{a,b} Values in the same row with different superscripts differ significantly ($P < 0.05$).
VSL = Straight-line velocity, VAP = average path velocity, VCL = curvilinear velocity, LHD = lateral head displacement, LIN = linearity, and STR = straightness.

Table 3
Effect of buffers on post-thaw membrane integrity (%) of buffalo spermatozoa (mean ± SEM; $n = 5$).

Variable	CITRATE	TCA	TEST	HEPEST	*P*
PMI	45.2 ± 1.2	32.6 ± 3.8	41.4 ± 5.2	40.8 ± 3.9	0.16
NAR	69.2 ± 0.8	61.8 ± 2.4	53.2 ± 8.3	65.8 ± 4.6	0.16

Values in the same row did not differ significantly ($P > 0.05$).
PMI = Plasma membrane integrity, and NAR = normal apical ridge.

DISCUSSION

The present study for the first time describes systematically the effect of conventional and zwitterionic buffers on sperm motilities and velocities using CASA and membrane integrity in buffalo spermatozoa. Spermatozoa extended in zwitterion based buffers in the present study (TCA, TEST and HEPEST) were visually more motile after thawing than the conventional buffer (CITRATE). Superiority of zwitterion buffers when titrated to physiological pH either with Tris, NaOH or KOH provided more motility than sperm diluted in phosphate or citrate in bulls (Graham et al., 1972). Perhaps zwitterions, which are soluble in water but impermeable to biological membranes (Good et al., 1966),

dehydrated the sperm cells relatively better than CITRATE. Alternatively, the zwitterions might have resisted the change in pH during cooling. It will be interesting to observe the changes in pH of semen diluted in zwitterionic and conventional buffers during the process of freezing in buffalo. The post-thaw motility of spermatozoa was consistently higher in Tris, compared to citrate in several studies in buffalo (Chinnaiya and Ganguli, 1980; Matharoo and Singh, 1980; Tuli et al., 1981). Subsequently, the GOT, GPT release in semen frozen with Tris compared to citrate was shown to be lower and associated with higher fertility in buffalo bulls (Dhami and Kodagali, 1990). In all these studies, sperm motility was assessed visually which is considered as a highly subjective assay with low repeatability (Diebel et al., 1976; Graham et al., 1980). In our preliminary study, a strong relationship (r = 0.98) was found between computerized motility and the known amount of killed spermatozoa in buffalo semen. The present study did not show any effect of buffers on computer-assisted motility. Probably, the use of controlled rate freezing and CASA was responsible for minimizing the source of variation. Likewise, Anzar and Graham (1995) were unable to detect differences in post-thaw computer-assisted motility of Holstein bull spermatozoa diluted either in TEST or TCA.

The pattern of sperm cell movement is sensitive to the chemical and physical properties of the medium in which they are suspended. Sperm motion characteristics, in this study, demonstrated that the percent post-thaw linear motility of spermatozoa was highest in TCA and lowest in TEST, whereas percent post-thaw circular motility of the spermatozoa was lowest in TCA and highest in TEST. Higher number of circular cells in TEST might have decreased the linear movement of the spermatozoa. Presumably, the cells suspended in TEST became partially capacitated due to the presence of two molecules of Tris that have been observed previously (Ijaz et al., 1989) leading to higher curvilinear velocity in Holstein bull spermatozoa (Iqbal and Hunter, 1995). It appears as if linear motility and circular motility among different sperm motilities are the most sensitive variables subject to a change due to buffer in buffalo spermatozoa.

The measurement of velocity has been considered as an indirect indicator of mitochondrial function of a spermatozoon (Graham et al., 1984) and is associated with fertility (Budworth et al., 1988). Curvilinear velocity of post-thaw spermatozoa observed in this investigation differed significantly among buffers, being highest in TEST, intermediate in CITRATE and HEPEST, and lowest in TCA. Indeed cyclic adenosine 3',5'-

monophosphate (cAMP) content in frozen-thawed spermatozoa was variable due to extenders in buffalo (Kakar and Anand, 1981). Higher levels of cAMP are essential for the initiation of hyperactive motility (Yanagimachi, 1994) and increase in curvilinear velocity (Leclerc et al., 1996), possibly through the activation of its dependent pathways. These pathways might have been activated more and may cause early exhaustion of spermatozoa diluted in TEST buffer of this study.

Lateral head displacement, an indicator of flagellar beat pattern of spermatozoa, in the buffalo bulls was lowest in TCA and highest in TEST. The decrease in lateral head movement in hamster spermatozoa has been shown to be associated with the decrease in intracellular calcium (Suarez et al., 1993). Thus lowest lateral head displacement in TCA may be due to the marked decrease in intracellular calcium that negates the hyperactive movement of the spermatozoa. The lateral head displacement in TCA in this study was found to be lower than that reported in Holstein bulls spermatozoa frozen either in CITRATE, TCA, or TEST and being thawed in citrate (Budworth et al., 1988). The straight-line and average path velocities, linearity and straightness of buffalo frozen spermatozoa in this experiment did not differ due to buffering systems. This may be due to limited number of sample size. It may thus be proposed that curvilinear velocity and lateral head displacement are more sensitive kinematics to identify the biochemical and physical changes to the buffalo spermatozoa during freezing and thawing.

Plasma membrane functional integrity of buffalo spermatozoa was evaluated using HOS assay. The swelling ability of buffalo frozen spermatozoa in this experiment did not vary due to the buffers and the overall mean (40.0 ± 2.7 %) was higher compared to those frozen in lactose (24%; Azam et al., 1998). Release of greater amount of alkaline phosphatase, an indicator of cell injury, in the extracellular medium during freezing has been earlier reported due to lactose compared to Tris or CITRATE (Dhami and Kodagali, 1990). Integrity of acrosomal cap has been positively correlated with fertility in bovine (Saacke and White, 1972). In the present study, damage to acrosomal cap of post-thawed buffalo spermatozoa was insignificant due to buffers. Similar observations for CITRATE and TEST have been recorded for frozen spermatozoa of Holstein bulls (Schenk et al., 1987). In contrast, TEST buffer, possibly due to its capacitating activity (Ijaz et al., 1989), resulted in more damage to acrosomal cap compared to CITRATE or TCA (Anzar and Graham, 1995). These differences may be due to the variation in species.

In summary, post-thaw visual motility tended to be higher in zwitterion buffers compared to CITRATE. Linear motility was higher and circular motility, curvilinear velocity, and lateral head displacement were lower in TCA compared to other buffers. It is concluded that Tris-citric acid can be the most satisfactory buffering system to improve the post-thaw semen quality in buffalo.

CHAPTER 2

Changes in Motion Characteristics, Plasma Membrane Integrity, and Acrosome Morphology during Cryopreservation of Buffalo Spermatozoa

A part of this study was presented at 31st Annual Meeting of the Society for the Study of Reproduction in College Station, Texas in August 1998.

ABSTRACT

This study examined motion characteristics, plasma membrane integrity and acrosome morphology of buffalo spermatozoa at different stages of cryopreservation, i.e. dilution, cooling to 4°C, equilibration at 4°C, and freezing and thawing. Semen ejaculates from four buffalo bulls were pooled ($n = 5$) and diluted in Tris-citric acid extender, cooled to 4°C in 2 h, equilibrated at 4°C for 4 h, filled in 0.5 ml straws and frozen in a programmable cell freezer before plunging them into liquid nitrogen. Frozen semen was thawed at 37°C for 15 s. After completion of each stage, sperm motion characteristics, plasma membrane integrity, and acrosome morphology were determined using computer-assisted semen analysis, hypo-osmotic swelling assay and phase-contrast microscopy, respectively. The visual and computerized motility (%) did not differ due to dilution, cooling, or equilibration (77.3 ± 2.3 and 90.5 ± 1.2, respectively) but was reduced ($P < 0.05$) after freezing and thawing (53.0 ± 4.6 and 48.6 ± 6.5, respectively). Linear motility (%) of spermatozoa was lower ($P < 0.05$) after dilution or equilibration (56.2 ± 2.4) than that after cooling or freezing and thawing (79.6 ± 1.4). Sperm curvilinear velocity ($\mu m\ s^{-1}$) was reduced ($P < 0.05$) from 112.4 ± 5.3 at dilution to 96.0 ± 5.8 at cooling, and from 87.6 ± 4.1 at equilibration to 69.4 ± 2.0 at freezing and thawing. Sperm lateral head displacement (μm) differed ($P < 0.05$) at each stage, i.e. dilution, 3.9 ± 0.2; cooling, 2.3 ± 0.2; equilibration, 3.1 ± 0.3 and freezing and thawing, 1.7 ± 0.2. Spermatozoa with intact plasma membrane (%) were 80.2 ± 3.9 at dilution, reduced ($P < 0.05$) to 60.4 ± 5.6 at equilibration and then to 32.6 ± 3.8 at freezing and thawing. Spermatozoa with normal acrosomes (%) remained higher after dilution, cooling or equilibration (73.2 ± 2.4) than after freezing and thawing (61.8 ± 2.4; $P < 0.05$). In conclusion, maximum damage to motility apparatus, plasma membrane and acrosomal cap of buffalo spermatozoa occurs during freezing and thawing followed by equilibration processes.

Keywords: Semen, deep-freezing, quality, cryodamage

INTRODUCTION

It is generally accepted that as much as fifty percent of the sperm viability is reduced during cryopreservation of semen (Watson, 1995). During this process, the spermatozoa are

subjected to chemical, osmotic, thermal, and mechanical stresses. These are manifested at dilution, cooling, equilibration, or freezing and thawing stage. Freezing and thawing has been reported to cause comparatively more damage to spermatozoa. This damage is evinced by loss in motility, acrosomal cap (O'Connor et al., 1981), extracellular glutamic oxaloacetic transaminase (GOT) enzyme (Graham et al., 1972), acrosin (Palencia et al., 1996) and fertility (Shannon and Vishwanath, 1995) in bulls.

The damage to buffalo spermatozoa at different stages of cryopreservation is not clear. Loss of motility (Ahmad and Chaudhry, 1980; Matharoo and Singh, 1980; Kakar and Anand, 1981), release of extracellular GOT, glutamic pyruvic transaminase (GPT) (Chinnaiya et al., 1979; Tuli et al., 1982; Iqbal, 1987; Dhami and Kodagali, 1990), and aspartate amino transferase (Bhosrekar et al., 1991), and reduction in ATP contents (Kakar and Anand, 1981) has been reported in buffalo spermatozoa after freezing and thawing. Similarly, damage to the acrosomal cap of buffalo spermatozoa is higher after freezing and thawing than before freezing (Chinnaiya and Ganguli, 1980) and is associated with the greater release of proteolytic (acrosomal) enzymes , i.e. hyaluronidase (Akhtar and Chaudhry, 1989) and acrosin (Chinnaiya and Ganguli, 1980). In contrast, there are reports indicating a considerable loss in motility of spermatozoa after equilibration stage (Tuli et al., 1981). Concomitantly, there is an increase in the release of lactic dehydrogenase and sorbitol dehydrogenase in buffalo bull semen (Singh et al., 1990). Fertility results in buffaloes either inseminated with liquid or frozen semen remain equivocal (Shabbir et al., 1982; Chohan et al., 1992).

This is a first detailed report on computerized motion characteristics and plasma membrane integrity of buffalo spermatozoa during cryopreservation. Computer-Assisted Semen Analyzer (CASA) provides detailed and objective quantification of sperm motion characteristics than subjective (visual) assessment in Holstein (Budworth et al., 1988) and more recently in buffalo (Rasul et al., 2000) bulls. The hypo-osmotic swelling (HOS) assay has been described as a useful test to assess the functional integrity of plasma membrane in human spermatozoa (Jeyendran et al., 1984). The present study was, therefore, primarily designed to determine the motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa after dilution at 37°C (D), cooling to 4°C in 2 h (C), equilibration at 4°C for 4 h (E), and freezing and thawing (FT).

MATERIALS AND METHODS

Preparation of extender

Tris-citric acid (TCA) was used as buffer system and was prepared by titrating 6.25% (w/v) citric acid (Fluka, Switzerland) with 3.93% (w/v) N-Tris-(hydroxymethyl)-aminomethane (Fluka, Switzerland) to pH 7.0. Final osmotic pressure was 320 mOsm kg^{-1}. TCA was used in combination with 20% (v/v) egg yolk, 0.2% (w/v) fructose, 6% (v/v) glycerol, 1000 I.U. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The extender was centrifuged at 12000 × *g* for 15 min and the supernatant was stored at –20°C until used for semen dilution after thawing at 37°C.

Source of semen and evaluation

Semen was collected from four mature Nili-Ravi buffalo bulls maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad, Pakistan. Two ejaculates from each bull were collected weekly by an artificial vagina at 42°C during the months of May and June. Each ejaculate was transferred to the evaluation room within a minute and kept in a water bath at 37°C and evaluated for sperm motility. The ejaculates from different bulls possessing at least 65% visual motility were pooled (n = 5), to avoid bull to bull difference, and used in this experiment. Sperm concentration was assessed using digital-photometer (Dr. Lange LP 300 SDM; Germany) at 560 nm. The semen was retained at 37°C for 15 min (holding time) before dilution.

Semen processing

Pooled semen was diluted with TCA at 37°C to provide approximately 50 x 10^6 spermatozoa ml^{-1}. The diluted semen was cooled from 37 to 4°C in 2 h, in cold cabinet. Spermatozoa were then allowed to equilibrate for 4 h at 4°C before freezing. Semen was filled in polyvinyl French straws (0.5 ml), just before freezing, at 4°C in the cold cabinet. Semen was frozen from 4 to –15°C at the rate of 3°C min^{-1} and from –15 to –80°C at the rate of 10°C min^{-1}, using programmable cell freezer (KRYO 10 Series III, Planer, Sunbury-on-Thames, Middlesex, UK). Semen straws were plunged into liquid nitrogen and packaged in plastic goblets for 24 h storage in the liquid nitrogen container. Thawing of straws was carried out at 37C for 5 min.

Semen assays

Following semen assays were conducted immediately after dilution, cooling, equilibration, and freezing and thawing.

Visual assessment

Visual motility (VMOT, %) of spermatozoa was assessed under microscope (x 400) attached with a closed circuit television.

Computerized assessment

A drop of semen sample (5 μl) was placed on a prewarmed (37°C) Makler chamber (Sefi-Medical Industries, Haifa, Israel) and analyzed for sperm kinematics using a computerized Cell Motion Analyzer (SM-CMA version 4.4; Mika Medical, Germany), recently validated for buffalo spermatozoa in our laboratory (Rasul et al., 2000). Briefly, the system included an IBM-compatible PC computer with a monitor, a Panasonic KX-P1121 multimode printer, and an Olympus BH-2 microscope equipped with a thermostage, video camera and Panasonic BT-H1450Y digital image video monitor. The video images were fed into IBM-compatible through video camera and monitor that analysed the data according to the programmed algorithms in SM-CMA software.

The settings of computer were adjusted to acquire digitize video scan data at the rate of 32 frames s^{-1}. Lower area detection limit was 35 pixels and upper area limit was 300 pixels. Upper velocity limit for immotile spermatozoa was 20 μm s^{-1}. Lower velocity limit for the motile spermatozoa was 30 μm s^{-1}. The spermatozoa moving with velocity between 20 and 30 μm s^{-1} were considered as locally motile and were not included in motile category. The spermatozoa moving in a radius of 10 μm were designated as circular, otherwise linear.

Each semen sample was evaluated after dilution, cooling, equilibration, and freezing and thawing by CASA. After thawing, frozen semen was incubated at 37°C for 5 min before evaluation. Sperm motion characteristics recorded by CASA included overall motility (CMOT, %), linear motility (LMOT, %), circular motility (CIRMOT, %), straight-line velocity (VSL, μm s^{-1}; the straight-line distance from beginning to end of track divided by time taken), average path velocity (VAP, μm s^{-1}; the spatially averaged path that eliminated the wobble of the sperm head), curvilinear velocity (VCL, μm s^{-1}; total distance traveled by a sperm during the acquisition divided by the time taken), lateral head displacement (LHD,

μm; deviation of sperm head from the average path), linearity (LIN, %; VSL/VCL × 100) and straightness (STR, %; VSL/VAP × 100).

Plasma membrane integrity

Plasma membrane integrity (PMI) was assessed using hypo-osmotic swelling (HOS) assay (Jeyendran et al., 1984). Sodium citrate (0.735 g) and fructose (1.351 g) were dissolved in 100 ml distilled H_2O to prepare HOS solution (osmotic pressure was ~190 mOsm kg^{-1}), as recommended in World Health Organization (1992) manual, and maintained at 37°C for 5 min before use. A 50 μl of each semen sample was mixed with 500 μl of HOS solution and incubated at 37°C for 30 min. After incubation, a drop of 5 μl semen sample was examined under phase-contrast microscope (x 400). Two hundred spermatozoa were assessed for their swelling ability in hypo-osmotic solution. The swollen spermatozoa characterized by coiling of tail were considered to have intact plasma membrane.

Normal acrosomes

Semen sample (500 μl) was fixed in 50 μl of 1% solution of formal citrate cointaining 2.9% (w/v) *tri*-sodium citrate dihydrate and 1% (v/v) commercial formaldehyde (37%). Acrosomal integrity of two hundred spermatozoa having either normal or abnormal apical ridges was assessed using phase-contrast microscope (Leica, Leitz Wetzlar, Germany) at x 1000.

Statistical analysis

Data is presented as mean ± SEM. Analysis of variance (ANOVA) was used to assess differences among stages of cryopreservation on motion characteristics, plasma membrane integrity, and normal acrosome morphology. When the F-ratio was significant ($P < 0.05$), Tukey's HSD test was used to compare treatment means (SYSTAT, 1996).

RESULTS

Motility of buffalo spermatozoa at different stages of cryopreservation

The data on visual, computer-assisted, linear and circular motility of buffalo spermatozoa at different stages of cryopreservation is presented in Fig. 1. The visual sperm motility (%) did not change due to dilution, cooling or equilibration and averaged 77.3 ± 2.3.

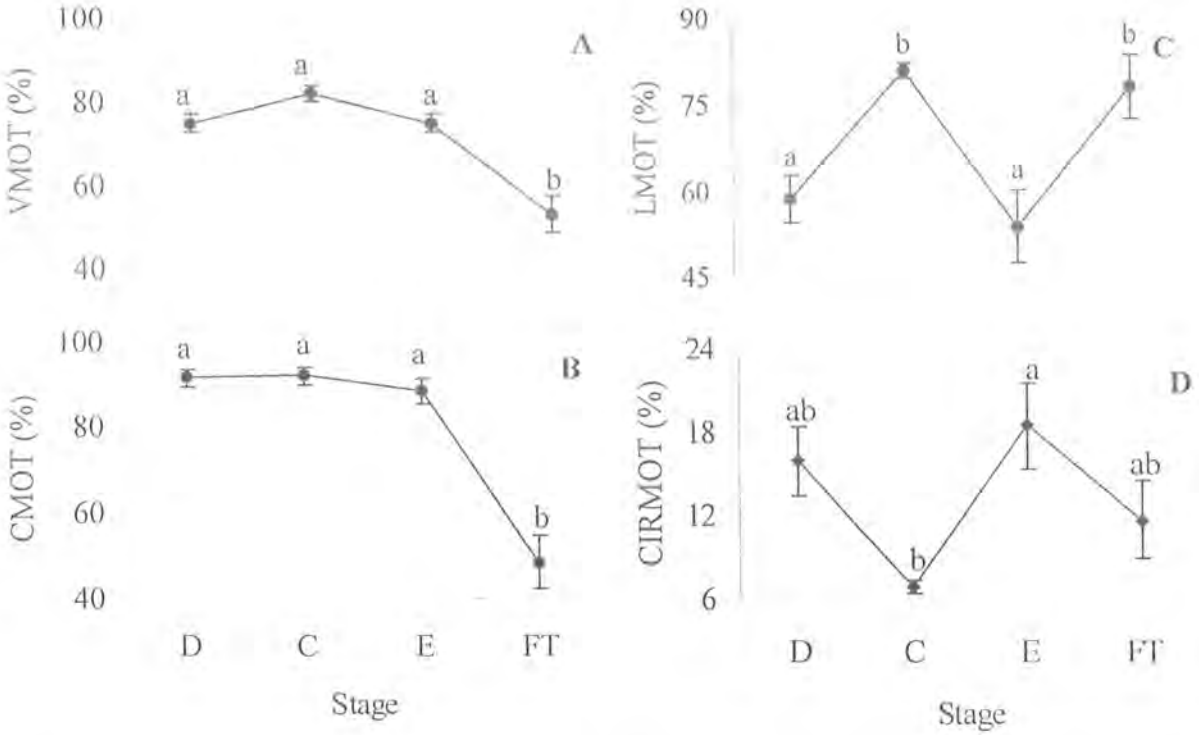

Fig. 1. Changes in (mean ± SEM; $n = 5$) visual (VMOT), computer-assisted (CMOT), linear (LMOT), and circular (CIRMOT) motilities (%) of buffalo spermatozoa after dilution (D), after cooling (C), after equilibration (E), and after freezing and thawing (FT). Percent VMOT (**A**) and CMOT (**B**) reduced ($P < 0.05$) after FT. LMOT (**C**) improved ($P < 0.05$) after C, decreased ($P < 0.05$) after E and again increased ($P < 0.05$) after FT, whereas the reverse was true for CIRMOT (**D**). a,b, Means sharing the same letter are not significantly different ($P > 0.05$).

This was reduced ($P < 0.05$) to 53.0 ± 4.6 after freezing and thawing. Likewise, the percent computerized motility of spermatozoa did not differ due to dilution, cooling, and equilibration and averaged 90.5 ± 1.2. However, it declined ($P < 0.05$) to 48.6 ± 6.5 after freezing and thawing. Linear motility (%) of spermatozoa followed a zigzag pattern in relation to the stages of cryopreservation. It was lower at dilution, improved ($P < 0.05$) transiently soon after cooling, decreased ($P < 0.05$) at completion of equilibration, and again increased ($P < 0.05$) after freezing and thawing. The pattern of circular motility (%) was opposite to that of linear motility.

Velocities, lateral head displacement, linearity, and straightness of buffalo spermatozoa at different stages of cryopreservation

The means (± SE) for sperm velocities, lateral head displacement, linearity, and straightness of buffalo spermatozoa observed after each stage of cryopreservation are presented in Fig. 2.

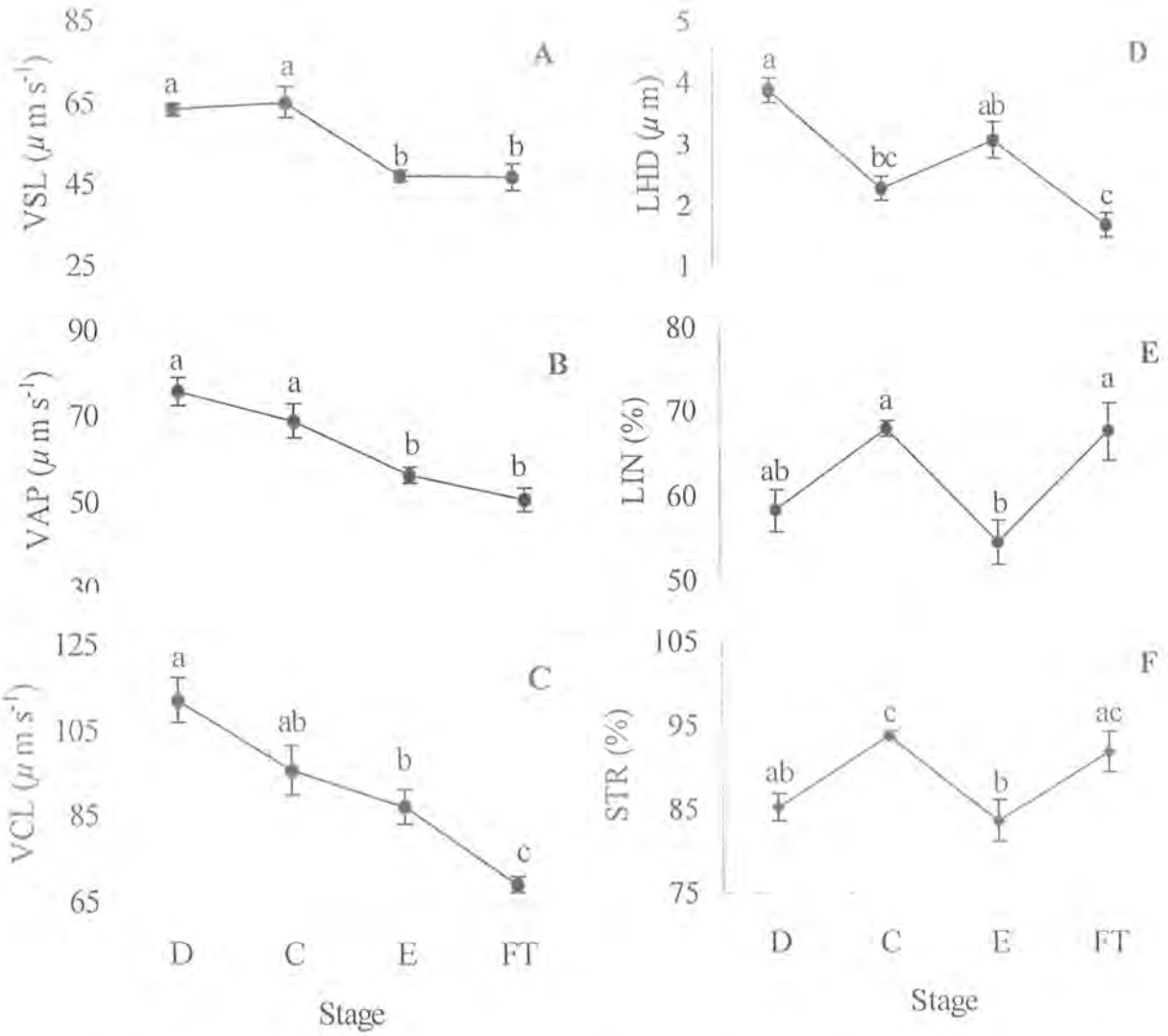

Fig. 2. Changes in (mean ± SEM; $n = 5$) velocities (straight-line, VSL; average path, VAP; and curvilinear, VCL), lateral head displacement (LHD), linearity (LIN), and straightness (STR) of buffalo spermatozoa after dilution (D), cooling (C), equilibration (E), and freezing and thawing (FT). Sperm VSL (**A**) and VAP (**B**) were reduced ($P < 0.05$) after E and FT. Sperm VCL (**C**) was declined linearly ($P < 0.05$) from D to FT. LHD (**D**) of spermatozoa decreased ($P < 0.05$) due to C, increased after E and again decreased after FT. LIN (**E**) and STR (**F**) of spermatozoa increased ($P < 0.05$) after dilution, decreased ($P < 0.05$) due to E and again increased ($P < 0.05$) after FT. a,c, Means sharing the same letter are not significantly different ($P > 0.05$).

The straight-line velocity and average path velocity of spermatozoa remained unaffected due to dilution or cooling but was reduced ($P < 0.05$) after equilibration and freezing and thawing. The curvilinear velocity of spermatozoa almost decreased linearly ($P < 0.05$) from dilution to freezing and thawing. Lateral head displacement of spermatozoa was decreased ($P < 0.05$) due to cooling, increased somewhat after equilibration, before declining ($P < 0.05$) due to freezing and thawing. Linearity and straightness of spermatozoa increased after cooling, decreased ($P < 0.05$) after equilibration, and again increased ($P < 0.05$) after freezing and thawing.

Plasma membrane integrity and acrosome morphology of buffalo spermatozoa at different stages of cryopreservation

Average plasma membrane integrity and the normal acrosomes of buffalo spermatozoa varied ($P < 0.05$) due to stages of cryopreservation [Fig. 3].

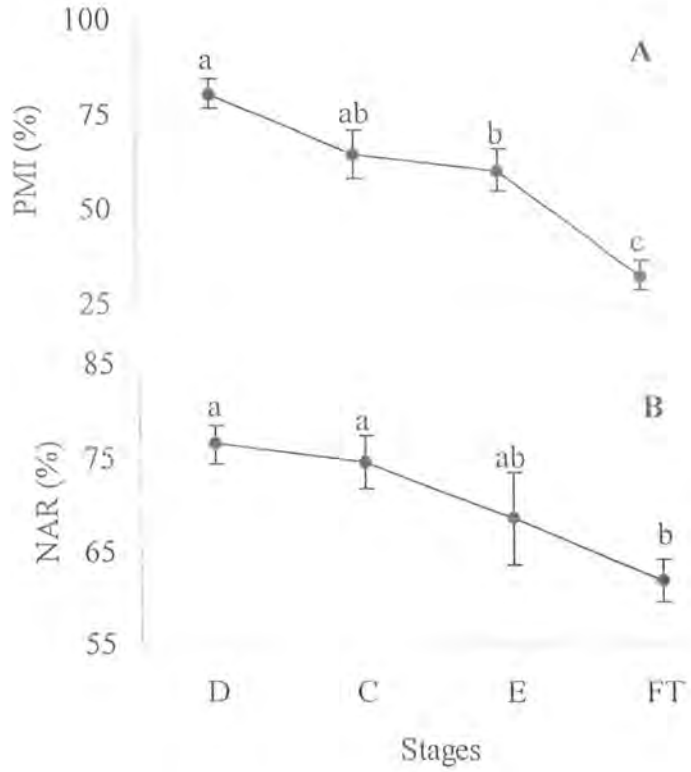

Fig. 3. Changes in (mean ± SEM; $n = 5$) plasma membrane integrity (PMI) and normal acrosomes (NAR) of buffalo spermatozoa after dilution (D), cooling (C), equilibration (E), and freezing and thawing (FT). Spermatozoa with intact plasma membrane (**A**) were decreased ($P < 0.05$) from D to E and further at FT, whereas normal acrosomes (NAR) were reduced ($P < 0.05$) after FT (**B**). a,c, Means sharing the same letter are not significantly different ($P > 0.05$).

Sperm plasma membrane integrity (%) was 80.2 ± 3.9 at dilution, reduced ($P < 0.05$) to 60.4 ± 5.6 at equilibration and then to 32.6 ± 3.8 at freezing and thawing. Spermatozoa with normal acrosomes (%) did not change due to dilution, cooling or equilibration (73.2 ± 2.4) and were higher ($P < 0.05$) than those observed after freezing and thawing (61.8 ± 2.4).

DISCUSSION

Sperm flagella, like somatic cilia, contain an axoneme comprising 9+2 microtubule. The microtubule sliding, responsible for sperm motility (flagellar beat), depends upon the energy produced due to hydrolysis of ATP in mitochondria, located at mid-piece, by dynein-ATPase (Gibbons et al., 1976; Bouchard and Cosson, 1981). The mechanism of energy production and its dissipation to microtubule are highly conserved in mammalian sperm flagella (Cardullo and Baltz, 1991). In present study, the visual motility reduced significantly upto 53% and computerized motility by 49% after freezing and thawing. These motilities were not affected by other stages for example dilution, cooling or equilibration. Similar findings for decline in motilities due to freezing and thawing have been reported in buffalo (Matharoo and Singh, 1980; Kakar and Anand, 1981; Tuli et al., 1981) and bull (Coulter and Foote, 1973; Budworth et al., 1987) spermatozoa. More than 50% of the spermatozoa are usually injured by the cryopreservation process (reviewed by Watson, 1995). These injuries are most likely due to the formation and dissolution of ice crystals in the extra and intracellular environment of the cells and increasing solute concentration (Mazur, 1984). Ice crystal formation in mitochondria and axoneme, during cryopreservation, causes lack of sperm motility (Courtens et al., 1989). Contrary to the usual impression that greater damage to sperm cells occurs due to freezing, in fact, is due to combination of the two stages i.e., freezing and thawing. The sperm cell has to traverse the lethal intermediate zone (-10°C to -50°C) once during freezing and once during warming (Mazur, 1980).

The pattern of sperm motion reflects the biochemical environment and physical conditions imposed on spermatozoa. Although, the early stages of cryopreservation (dilution, cooling and equilibration) did not affect the overall motility, however, these stages change the sophisticated or fine parameters of sperm motion. In the present study, linear motility of spermatozoa significantly increased by cooling (from 37°C to 4°C) and then by freezing and thawing compared to that after dilution or equilibration. In contrast,

circular motility was decreased due to cooling and increased due to equilibration. In this study, the transient decrease in linear motility and increase in circular motility immediately after dilution is due to the glycerol-related osmotic shock to spermatozoa as suggested in ram spermatozoa (Fiser and Fairfull, 1989). Cooling has shown an adverse effect on the plasma membrane permeability of boar spermatozoa (Ortman and Rodriguez-Martinez, 1994). Similarly, the prolonged cooling during equilibration (4 h at 4°C) might change the permeability of buffalo (like boar) sperm as seen by the significant decrease in plasma membrane integrity after equilibration, and freezing and thawing in this study. In addition, the premature capacitation like changes might occur, similar to bull spermatozoa (Cormier et al., 1997). Such changes increase the levels of intracellular calcium resulting in increased circular motility (Suarez et al., 1993), and lateral head displacement (Bailey et al., 1994) of spermatozoa. Indeed, circular motility and lateral head displacement in this study were increased after equilibration. Whether or not, these motion characteristics improve by decreasing the interval of equilibration, will be interesting to study in buffalo spermatozoa.

Sperm velocity reflects their mitochondrial function indirectly (Graham et al., 1984) and has been associated with fertility in bulls (Budworth et al., 1988; Kjaestad et al., 1993). In this study, the sperm velocities were significantly reduced after equilibration. This may be due to increased cell permeability, as discussed earlier, and/or increased viscosity of extracellular medium (Amann and Hammerstedt, 1980). Further decrease in the curvilinear velocity after freezing and thawing, observed in this experiment, could be due to cryoinjuries to the mitochondrial apparatus (Jones and Stewart, 1979) and axoneme (Courtens et al., 1989) of spermatozoa. ATP and cAMP are associated with the initiation and maintenance of sperm motility and velocity (Bouchard and Cosson, 1981). Reduction in the production of ATP contents after freezing and thawing in buffalo spermatozoa has been observed (Kakar and Anand, 1981). Moreover, in ram, the decrease in sperm velocity has been associated with decline in temperature of cryoprotective media (Holt et al., 1988). It appears that equilibration, and freezing and thawing are the most stressful stages to cause a change in the pattern of velocities in buffalo sperm.

The functional integrity of the sperm plasma membrane is of primary importance for the fertilizing ability of a spermatozoon. The hypo-osmotic swelling test assesses the

functional integrity of the sperm membrane (Jeyendran et al., 1984). In the current study, the plasma membrane integrity of spermatozoa was reduced due to equilibration and further deteriorated after freezing and thawing. Partial injuries to the spermatozoa, which might have occurred during equilibration, became more prominent after freezing and thawing. During cryopreservation, sperm plasma membranes are destabilized due to low temperature and high salt concentration (Holt and North, 1994). Lipid contents of plasma membrane of ram (Holt and North, 1984), boar (Buhr et al., 1989) and bulls (Shannon and Vishwanath, 1995) spermatozoa were significantly reduced due to freezing. These membrane changes due to freezing and thawing lead to the release of lactate dehydrogenase, sorbitol dehydrogenase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, hyaluronidase, acid phosphatase and alkaline phosphatase from buffalo spermatozoa (Iqbal et al., 1987; Singh et al., 1990; Chauhan et al., 1994) resulting into loss of motility and acrosomal cap.

The presence of acrosomal cap is important in fertilization process and has highly been related with fertility of the cryopreserved bull semen (Saacke and White, 1972). In this study, the percentage of sperm with a normal acrosome was significantly damaged due to freezing and thawing. Since acrosomes are adversely affected by thawing (Courtens et al., 1989), it is speculated that acrosomal caps might have been 'knobbed out' during thawing of buffalo spermatozoa as demonstrated by the earlier work in bull and rabbit spermatozoa (Bamba and Cran, 1988). Greater release of acrosomal enzymes, hyaluronidase (Akhtar and Chaudhry, 1989) and acrosin (Chinnaiya and Ganguli, 1980; Palencia et al., 1996) was noticed due to freezing and thawing in buffalo and bovine bulls. In the current study, the overall loss in the percent normal acrosome during cryopreservation was about 20%. This was lower than reported previously for buffalo bulls (Chinnaiya and Ganguli, 1980; Azam et al., 1998). It might be due to the use of different extender and/or programmable freezing instead of conventional one.

It seems that semen possesses two kinds of sperm population, i.e. sensitive and resistant, in terms of their ability to withstand cooling and freezing stresses. During prolonged equilibration, the sensitive sperm undergo membrane and axonemal changes, loose their ability to move in straight line resulting into decrease in linear motility, linearity, and straightness, and undergo death during freezing and thawing stresses. On the other hand, resistant sperm population survives cooling, equilibration, and freezing

and thawing stresses. Therefore, linear motility, linearity, and straightness of survived spermatozoa were higher after freezing and thawing than after equilibration, as immotile spermatozoa are excluded while calculating these parameters.

To our knowledge, such a clear effect of stages of cryopreservation on motion characteristics, plasma membrane integrity, and acrosome morphology, collectively, has not been shown previously in buffalo spermatozoa. Briefly, 1) cooling of semen from 37°C to 4°C in 2 h has beneficial effect on linear and circular motility, 2) equilibration of spermatozoa at 4°C for 4 h has adverse effect on linear motility, circular motility, velocities and lateral head displacement, and 3) freezing and thawing causes a considerable damage to motion characteristics (visual or computerized motility, and curvilinear velocity), plasma membrane integrity and acrosomal cap of buffalo spermatozoa. In conclusion, freezing and thawing followed by equilibration cause maximum damage to motility apparatus, plasma membrane and acrosomal cap of buffalo spermatozoa. Further studies are in progress to minimize these damages by altering the equilibration intervals and the rate of freezing in buffalo spermatozoa.

CHAPTER 3

Effect of Rates and Temperatures of Freezing on Motion Characteristics, Plasma Membrane Integrity, and Acrosome Morphology of Buffalo Spermatozoa

ABSTRACT

This study examined if fast freezing would improve the post-thaw semen quality and where the intermediate zone of damage to spermatozoa lies in buffaloes. Pooled semen ejaculates ($n = 5$) from four Nili-Ravi bulls were diluted at 37°C in Tris-citric acid extender. Semen was cooled to 4°C in 2.5 h, equilibrated at 4°C for 4 h, packaged into 0.5 ml semen straws and frozen in a programmable cell freezer from 4 to –80°C. In experiment 1a, the freezing rates between 4 to –15°C examined were either slow (3°C min^{-1}) or moderate (10°C min^{-1}), whereas in experiment 1b, the freezing rates between –15 to –80°C investigated were either moderate (10°C min^{-1}), fast (20°C min^{-1}), or very fast (30°C min^{-1}). After freezing, straws were plunged into liquid nitrogen. These were thawed at 37°C for evaluation. In experiment 2a and b, semen filled straws were collected during freezing at +4, 0, –5, –10, –20, –30, –40, –50 or –80°C. After collection, straws were directly thawed at 37°C for 5 min except a group of straws collected at –80°C were plunged into liquid nitrogen at –196°C before thawing which served as control. Sperm motion characteristics, plasma membrane integrity, and acrosome morphology were determined using computer-assisted semen analysis, hypo-osmotic swelling (HOS) assay or flow cytometric assessment, and phase-contrast microscopy, respectively. Statistical analyses revealed that faster freeze rates either tested for initial (experiment 1a) or subsequent (experiment 1b) freezing did not improve the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. In experiment 2a of lethal intermediate zone, the visual motility, computer-assisted motility, and lateral head displacement reduced ($P < 0.05$) at –40°C, curvilinear velocity at –50°C, whereas plasma membrane integrity and acrosome morphology were adversely affected at –30°C. In the subsequent experiment of lethal intermediate zone (2b), plasma membrane integrity, either assessed by HOS or flow cytometry analyses, reduced ($P < 0.05$) at –30°C. High correlation coefficient ($r = 0.90$; $P < 0.05$) between the two assessment procedures among various temperatures has suggested the flow cytometry a precise method to evaluate the viability of buffalo spermatozoa. In conclusion, fast freezing did not improve the post-thaw sperm viability and the intermediate zone of damage to motility apparatus and membrane integrity of spermatozoa lies somewhere between –20 to –40°C in buffaloes.

Keywords: Buffalo—spermatozoa; Freeze Rates and Temperatures

INTRODUCTION

First successful cryopreservation of mammalian spermatozoa was reported in 1949 by Polge and coworkers. This discovery was utilized by the artificial insemination (A.I.) industry for freezing of bovine semen. It was packed in glass ampules and frozen in dry ice (O'Dell and Almquist, 1957). This method of freezing was changed with the introduction of liquid nitrogen vapour (Adler, 1960) in which straws were used with greater efficiency due to higher surface-to-volume ratio (Rajamannan, 1970). The use of controlled rate cell freezer was demonstrated by Almquist and Wiggin (1973), without having detrimental affects on post-thaw sperm survival. This technique was more suitable for the freezing of large number of straws compared to static liquid nitrogen vapour technique (Landa and Almquist, 1979).

Availability of controlled rate freezing and the finding that extracellular ice formation starts at about –5°C and intracellular at about –15°C or greater (Mazur, 1977) prompted the workers to carried out freezing either in one (Chen et al., 1993; Liu et al., 1998), two (Landa and Almquist, 1979; Almquist et al., 1982) or three freezing cycles (Gilbert and Almquist, 1978). The basis for splitting the freezing in two or three cycles was first to dehydrate the cell with slow freezing and, secondly to avoid the formation of intracellular ice with fast freezing (Mazur, 1980). Results from several studies reviewed by Saacke (1993), indicated that bull spermatozoa could resist wide range of freezing rates when frozen in straws. Extremely slow or fast freezing rates were detrimental to post-thaw survival of bull spermatozoa (Rodriguez et al., 1975; Mortimer et al., 1976; Chen et al., 1993).

The empirical studies have indicated that fifty percent of the sperm viability is reduced due to freezing and thawing process (Watson, 1979). Maximum survival of cells is generally achieved with the optimal freezing rate, that is the rate which is slow enough to prevent the intracellular ice formation and is fast enough to minimize "solution effects". The "solution effects" to the mammalian cell are more conspicuous between the lethal intermediate zone, –10 to –50°C, once during freezing and once during warming (Mazur, 1980). This critical zone has been reported to decrease the motility (in bulls: Luyet and

Keane, 1955; Liu et al., 1998) and membrane integrity (in boar: Mazur, 1985; in ram: Holt et al., 1992) of spermatozoa.

In buffalo bulls, earlier, the semen was frozen with CO_2 (Roy et al., 1956). Later, this method was modified and the straw freezing with static liquid nitrogen vapours became common (Ahmad and Chaudhry, 1980). Post-thaw sperm motility was not significantly affected by the distance of straws above the liquid nitrogen surface (1, 4 or 8 cm) in buffaloes (Hultnaes, 1982). The use of controlled rate cell freezer in two cycles, 3°C min^{-1} from +4 to –15°C and 10°C min^{-1} from –15 to –80°C, yielded acceptable post-thaw survival of spermatozoa in buffalo (Rasul et al., 2000). Based upon this and earlier work we hypothesized that a) faster freeze rates either during initial and subsequent freezing would improve the post-thaw semen quality and b) the lethal intermediate zone, where maximum damage to spermatozoa lies some where in between –10 to –50°C in buffaloes.

Some of the modern semen assays to assess semen quality include the use of computer-assisted semen analyzer (CASA) to evaluate sperm motion characteristics (Budworth et al., 1988), hypo-osmotic swelling (HOS) assay (Jeyendran et al., 1984) and flow cytometric (Garner et al., 1986) determination of plasma membrane integrity, and acrosomal morphology (Saacke and White, 1972). The objective of the present study was to determine the effect of freezing rates on post-thaw motion characteristics, plasma membrane integrity and acrosome morphology and to identify the lethal intermediate zone as reflected by these variables in buffalo spermatozoa.

MATERIALS AND METHODS

Preparation of extenders

Tris-citric acid (TCA) extender was used in these experiments. It was prepared by titrating citric acid monohydrate (Fluka, Switzerland) 12.50 g in 200 ml distilled water (OP = 325 mOsm kg^{-1}) against Tris-(hydroxymethyl)-aminomethane (Fluka, Switzerland) (Tris; 7.87 g in 200 ml distilled water, OP = 325 mOsm kg^{-1}) to pH 7.0. Final osmotic pressure was 280 mOsm kg^{-1}. Egg yolk (20%; v/v), fructose (0.2%; w/v), glycerol (6%; v/v), penicillin (1000 I.U. ml^{-1}) and streptomycin (100 μg ml^{-1}) were added to the formulated extender. The extender was centrifuged at 12000 × *g* for 15 min, and the aliquots of supernatant were frozen and stored at –20°C. Extender was thawed at 37°C before use.

Semen collection and initial evaluation

Four mature Nili-Ravi buffalo bulls, maintained at Livestock Research Station, National Agricultural Research Center (NARC), Islamabad, Pakistan, were used in this study. Two consecutive semen ejaculates were obtained by the use of artificial vagina (42°C) at weekly intervals for five weeks during the months of September to February. The semen was brought to laboratory within a minute. Initial motility was visually assessed microscopically (x 400) attached with closed circuit television. The neat semen samples with at least 65% motile spermatozoa were used. Sperm concentration was assessed by digital-photometer (Dr. Lange LP 300 SDM; Germany) at 560 nm. The qualifying ejaculates were pooled, to have sufficient semen for a replicate and to eliminate the bull effect, for each experiment. The semen was given a holding time of 15 min at 37°C in the water bath before dilution.

Semen processing

Semen was diluted (at 37°C) with TCA extender to provide approximately 50 x 10^6 spermatozoa ml^{-1}. Diluted semen was cooled slowly (approximately in 2.5 h) to 4°C, and equilibrated for 4 h. Semen was packed into 0.5 ml polyvinyl French straws and frozen in a programmable cell freezer (KRYO 10 Series III, Planer, Sunbury-on-Thames, Middlesex, UK) from 4 to –80°C.

Experiment 1a and 1b: Rates of freezing

In these experiments, the freezing rates from the initiation of freezing procedure (starting at 4°C) to –80°C were examined with varying rates. In experiment 1a, the freezing rates between 4 to –15°C examined were either slow (3°C min^{-1}) or moderate (10°C min^{-1}). In experiment 1b, the freezing rates between –15 to –80°C investigated were either moderate (10°C min^{-1}), fast (20°C min^{-1}), or very fast (30°C min^{-1}) (Table 1). After freezing, semen straws were plunged into liquid nitrogen. Thawing was performed at 37°C for 5 min. Post-thaw sperm motion characteristics, plasma membrane integrity, and acrosome morphology were determined in each sample. Each experiment was replicated for five times.

Table 1
Time and rates for different methods for freezing of buffalo spermatozoa.

Freezing method and temperature range	Freezing carried out in experiment	Freezing time (min)	Freezing rate (°C min^{-1})
Slow			
+4 to –15°C	1a	6.33	3.0
–15 to –80°C	1a	6.50	10.0
Total		12.83	
Moderate			
+4 to –15°C	1 and 2	1.90	10.0
–15 to –80°C	1 and 2	6.50	10.0
Total		8.40	
Fast			
+4 to –15°C	1b	1.90	10.0
–15 to –80°C	1b	3.25	20.0
Total		5.15	
Very Fast			
+4 to –15°C	1b	1.90	10.0
–15 to –80°C	1b	2.17	30.0
Total		4.07	

Experiment 2a and 2b: Temperatures of freezing

In these experiments, the freezing rates from the initiation of freezing procedure (starting at 4°C) to –80°C was 10°C min^{-1} and the straws were collected at different temperatures in order to investigate the lethal intermediate zone of damage to buffalo spermatozoa. These were +4, 0, –5, –10, –20, –30, –40, –50 or –80°C. After collection, straws were directly thawed at 37°C for 5 min except a group of straws collected at –80°C were plunged into liquid nitrogen at –196°C before thawing which served as control. In experiment 2a, post-thaw sperm motion characteristics, plasma membrane integrity, and acrosome morphology were analyzed. In experiment 2b, post-thaw plasma membrane integrity of spermatozoa was examined using HOS assay and flow cytometric assessment as well. Each experiment was replicated for five times.

Semen assays

The semen assays were conducted immediately after thawing to assess the viability of spermatozoa.

Visual assessment

Visual motility (VMOT, %) of spermatozoa was assessed by using microscope (x 400) attached to a closed circuit TV.

Computerized assessment

The semen sample of 5 μl was placed on a prewarmed (37°C) Makler chamber (depth 10 μm; Sefi-Medical Industries, Haifa, Israel) and analyzed at 37°C for sperm motion characteristics using a computerized Cell Motion Analyzer (SM-CMA version 4.4; Mika Medical, Germany), recently validated for buffalo spermatozoa in our laboratory (Rasul et al., 2000).

Each semen sample was assessed for motility (CMOT, %); linear motility (LMOT, %); circular motility (CIRMOT, %); straight-line velocity (VSL, μm s^{-1}); average path velocity (VAP, μm s^{-1}); curvilinear velocity (VCL, μm s^{-1}) and lateral head displacement (LHD, μm) of spermatozoa.

Hypo-osmotic swelling (HOS)

Plasma membrane integrity (PMI) was assessed using hypo-osmotic swelling (HOS) assay (Jeyendran et al., 1984). The HOS solution was prepared by dissolving sodium citrate (0.735 g) and fructose (1.351 g) in distilled water (100 ml), according to the guidelines of the World Health Organization (1992). Approximate osmotic pressure was 200 mOsm kg^{-1}. The solution was maintained at 37°C for 5 min before use. The semen sample of 50 μl of each treatment was mixed with 500 μl of HOS solution and incubated at 37°C for 30 min. After incubation, 5 μl of semen sample was examined under phase-contrast microscope (x 400). The spermatozoa with curled tail were considered swollen and having intact plasma membrane. Two hundred spermatozoa were assessed to calculate the percentage of swollen cells.

Normal acrosomes

Semen sample of 250 µl was fixed in 25 µl of 1% solution of formal citrate (2.9 g tri-sodium citrate dihydrate [Merck, Darmstadt, Germany] and 1 ml of a 37% solution of formaldehyde [Merck] dissolved in 99 ml of distilled water). Acrosomal integrity of two hundred spermatozoa either normal or abnormal apical ridges was assessed using phase-contrast microscope (Leica, Leitz Wetzlar, Germany) at x 1000. The percentage of acrosomes with normal apical ridges (NAR) was calculated.

Flow cytometric assessment

Validation of flow cytometer

FACS (fluorescent-activating cell sorter) Calibur Analyzer flow cytometer (Becton-Dickinson, San Jose, CA), equipped with an argon laser, a standard fluorescein isothiocyanate (FITC) and phycoerythrin dichroic filter set, was used in this study. Excitation was at 488 nm and emission at 585 nm. The system was validated before use for determining percentage of spermatozoa with intact plasma membrane in buffalo. Semen of four buffalo bulls was pooled ($n = 5$) and diluted at 37°C with TCA extender in order to provide approximately 50 x 10^6 spermatozoa ml^{-1}. Extended semen was cooled slowly in 2 h to 4°C. For the assessment of sperm plasma membrane integrity cooled semen was divided into two parts. One part of semen remained as such (live) and other part of the semen was repeatedly plunged into liquid nitrogen and thawed (killed). Five mixtures of live and killed spermatozoa were prepared with 100:0, 75:25, 50:50, 25:75 and 0:100 ratio (live:killed) by volume. Each mixture was divided into two aliquots, one was processed for HOS assay, whereas the other for flow cytometric analysis in the same way as described in the following. Correlation between the HOS and flow cytometric assessments of plasma membrane integrity with the addition of killed spermatozoa was significant ($r = 0.98$; $P < 0.05$) [Fig. 1].

Fluorogenic staining of spermatozoa for flow cytometric analysis

Semen samples (500 µl) were diluted at 22 ± 2°C with 500 µl of TCA buffer (pH = 7.0; OP = 300 mOsm kg^{-1}). Each semen sample was divided into two aliquots. In one aliquot, the 10 µl of stock solution (0.27 mg ml^{-1} in phosphate buffer; Garner et al., 1986) of

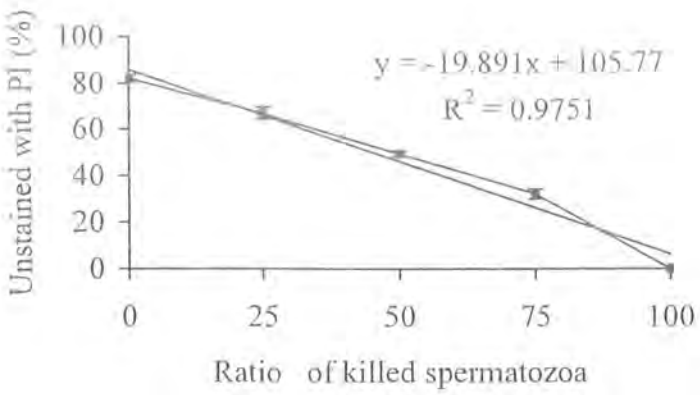

Fig. 1. Percent decline in sperm plasma membrane integrity as determined by flow cytometric (FACS) analysis after addition of killed spermatozoa of buffalo bulls.

propidium iodide (PI; Sigma, St. Louis, MO) was added and kept in dark, whereas the other aliquot was retained as such. The PI stained aliquots, after 15 min, were centrifuged at 500 × *g* for 5 min. Supernatants were aspirated and the pellets were re-suspended in 1000 μl of TCA buffer. Each semen sample (PI stained, and unstained run as a negative control) was then incubated at 37°C for 15 min, fixed in formaldehyde (2.5 mg ml^{-1}; Harrison and Vickers, 1990), and wrapped in aluminium foil to minimize exposure to light. These were stored at 4°C for 24 h before flow cytometric analysis.

Flow cytometry

The parameters measured for each cell were: forward scatter (FSC-H) and side scatter (SSC-H). At least 10,000 cells were evaluated with FACS. The unstained sample of each treatment was run before the evaluation of its stained sample. The dot plot window for unstained cells was gated in order to enable tracing of these cells in the second window being plotted for stained sample. Two cell populations were differentiated, stained and unstained, using the Cell Quest software (Becton-Dickinson, San Jose, CA). Unstained cells for each stained sample were graded as having intact plasma membrane and the stained cells were defined as ones with damaged plasma membrane [Fig. 2].

Statistical analyses

The Statistics (SYSTAT., Version 6.0 for Windows, SPSS, Chicago. IL, U.S.A.,1996) was used to analyze the data. Data are expressed as means ± SEM. In experiment 1a, differences between treatments were determined by a paired Student's *t*-test. In experiment 1b and 2, data were analyzed by the analysis of variance (ANOVA). When the F-ratio was significant ($P < 0.05$), Tukey's Honestly Significant Difference test was used to compare variable means.

RESULTS

Experiment 1a and 1b: Rates of freezing

Effect on post-thaw viability of buffalo spermatozoa

Data of both experiments are summarized in Table 2, 3, and 4, showing insignificant changes in post-thaw motilities (visual, computer-assisted, linear, and circular), velocities (straight-line, average path, and curvilinear), lateral head displacement, plasma membrane integrity and acrosome morphology of buffalo spermatozoa due to freezing rates. However,

Table 2
Effect of freeze rates on post-thaw motilities of buffalo spermatozoa (mean ± SEM; $n = 5$).

	Experiment 1a		Experiment 1b		
Variable	Slow	Moderate	Moderate	Fast	Very Fast
VMOT[†] (%)	51 ± 5	59 ± 5	68 ± 2	71 ± 2	70 ± 3
CMOT (%)	52 ± 6	63 ± 5	71 ± 3	76 ± 3	74 ± 4
LMOT (%)	30 ± 3	37 ± 5	48 ± 6	36 ± 4	47 ± 6
CIRMOT (%)	27 ± 3	28 ± 5	24 ± 4	34 ± 4	23 ± 3

Means within rows and experiments did not differ ($P > 0.05$).
Slow = 3°C min^{-1} from 4 to –15°C, Moderate = 10°C min^{-1} from 4 to –80°C, Fast = 20°C min^{-1} from –15 to –80°C, Very Fast = 30°C min^{-1} from –15 to –80°C, VMOT = visual motility, CMOT = computer-assisted motility, LMOT = linear motility, and CIRMOT = circular motility.
[†]Average visual motility (%) of fresh ejaculates ($n = 5$) in experiment 1a was 66 ± 3, whereas in experiment 1b it was 77 ± 2.

on the overall basis in experiment 1a, the percentages of motilities (visual, computer-assisted, and linear), plasma membrane integrity and acrosome morphology of spermatozoa were slightly improved due to moderate rate of freezing compared to slow rate of freezing. In the subsequent experiment (1b), percent circular motility of spermatozoa decreased ($P = 0.104$) due to very fast freeze rate compared to fast rate of freezing.

Table 3
Effect of freeze rates on post-thaw velocities and lateral head displacement of buffalo spermatozoa (mean ± SEM; $n = 5$).

	Experiment 1a		Experiment 1b		
Variable	Slow	Moderate	Moderate	Fast	Very Fast
VSL ($\mu m\ s^{-1}$)	41 ± 2	42 ± 2	47 ± 3	44 ± 3	47 ± 3
VAP ($\mu m\ s^{-1}$)	55 ± 2	55 ± 1	58 ± 2	59 ± 2	59 ± 2
VCL ($\mu m\ s^{-1}$)	97 ± 4	95 ± 1	94 ± 4	100 ± 3	95 ± 4
LHD (μm)	4.5 ± 0.2	4.7 ± 0.2	4.4 ± 0.3	5.1 ± 0.5	4.3 ± 0.6

Means within rows and experiments did not differ ($P > 0.05$).
Slow = 3°C min^{-1} from 4 to –15°C, Moderate = 10°C min^{-1} from 4 to –80°C, Fast = 20°C min^{-1} from –15 to –80°C, Very Fast = 30°C min^{-1} from –15 to –80°C, VSL = straight-line velocity, VAP = average path velocity, VCL = curvilinear velocity, and LHD = lateral head displacement.

Table 4
Effect of freeze rates on post-thaw membrane integrity of buffalo spermatozoa (mean ± SEM; $n = 5$).

	Experiment 1a		Experiment 1b		
Variable	Slow	Moderate	Moderate	Fast	Very Fast
PMI (%)	42 ± 7	52 ± 6	48 ± 5	51 ± 4	49 ± 7
NAR (%)	31 ± 4	40 ± 6	48 ± 8	56 ± 9	55 ± 11

Means within rows and experiments did not differ ($P > 0.05$).
Slow = 3°C min^{-1} from 4 to –15°C, Moderate = 10°C min^{-1} from 4 to –80°C, Fast = 20°C min^{-1} from –15 to –80°C, Very Fast = 30°C min^{-1} from –15 to –80°C, PMI = plasma membrane integrity, and NAR = normal apical ridge.

Experiment 2a: Temperatures of freezing

Effect on motility of buffalo spermatozoa

Data pertaining to the effect of temperatures of freezing on motilities of buffalo spermatozoa is presented in Table 5. Mean visual and computer-assisted motilities (%) of spermatozoa remained higher after freezing to –20°C. These motilities were declined ($P < 0.05$) after freezing to –40°C. This decline remained consistent upto –80°C. However, these motilities were slightly improved in control. Percent linear motility of spermatozoa tended ($P = 0.09$) to lower at –30°C and maximal at –196°C. Interestingly, percentages of circular motility of spermatozoa remained unaltered by the temperatures of freezing.

Effect on velocities, and lateral head displacement of buffalo spermatozoa

The effect of temperatures of freezing on velocities and lateral head displacement of buffalo spermatozoa is shown in Table 6. Curvilinear velocity ($\mu m\ s^{-1}$) of spermatozoa varied ($P < 0.05$) among treatments. It was maximal at 4°C and declined ($P < 0.05$) to its minimal after freezing to –50 and –80°C. Likewise, sperm lateral head displacement (μm) was higher at 4°C. It reduced ($P < 0.05$) after freezing to –40°C which was consistent upto the end of freezing cycle. However, straight-line and average path velocities of spermatozoa did not differ among treatments.

Effect on membrane integrity of buffalo spermatozoa

The effect of temperatures of freezing on membrane integrity of buffalo spermatozoa is summarized in Table 7. The mean percent plasma membrane integrity and acrosome morphology of spermatozoa was greater at 4°C that decreased ($P < 0.05$) after freezing to –30°C. This decrease was maximum after freezing to –50 and –80°C. The integrity of plasma and acrosomal membranes was restored to some extent in control.

Experiment 2b: Temperatures of freezing

Flow cytometry

The results of FACS analysis are presented in Fig. 2. The window depicts FL1, plotted against FL2. We were able to divide the cells into two major fluorescence levels: the higher level with

Table 5
Effect of temperatures of freezing on motilities (%) of buffalo spermatozoa (mean ± SEM; $n = 5$).

Variable	Experiment 2a: Temperatures of freezing									
	+4°C	0°C	–5°C	–10°C	–20°C	–30°C	–40°C	–50°C	–80°C	–196°C (control)
VMOT	75 ± 0^{a}	$73 \pm 1^{a,b}$	75 ± 0^{a}	$71 \pm 1^{a,b}$	$69 \pm 2^{a,b}$	$63 \pm 4^{a,c}$	$49 \pm 5^{c,d}$	$48 \pm 4^{c,d}$	46 ± 6^{d}	$58 \pm 5^{b,c,d}$
CMOT	86 ± 2^{a}	80 ± 2^{a}	86 ± 2^{a}	79 ± 1^{a}	$74 \pm 4^{a,b}$	$69 \pm 6^{a,c}$	$52 \pm 6^{c,d}$	$51 \pm 4^{c,d}$	47 ± 7^{d}	$59 \pm 5^{b,c,d}$
LMOT	25 ± 6	24 ± 1	24 ± 2	20 ± 2	21 ± 3	18 ± 3	25 ± 6	33 ± 5	27 ± 6	35 ± 3
CIRMOT	35 ± 3	37 ± 3	35 ± 2	39 ± 1	35 ± 2	37 ± 4	33 ± 5	25 ± 4	32 ± 3	30 ± 4

^{a,b,c,d} Means in same row with different superscripts differ ($P < 0.05$).
VMOT = Visual motility, CMOT = computer-assisted motility, LMOT = linear motility, and CIRMOT = circular motility.

Table 6
Effect of temperatures of freezing on velocities ($\mu m\ s^{-1}$) and lateral head displacement (μm) of buffalo spermatozoa (mean ± SEM; $n = 5$).

Variable	Experiment 2a: Temperatures of freezing									
	+4°C	0°C	–5°C	–10°C	–20°C	–30°C	–40°C	–50°C	–80°C	–196°C (control)
VSL	42 ± 2	38 ± 1	39 ± 1	37 ± 1	37 ± 1	38 ± 2	39 ± 1	41 ± 1	37 ± 2	40 ± 1
VAP	58 ± 2	52 ± 1	55 ± 1	52 ± 1	53 ± 2	55 ± 3	53 ± 2	52 ± 1	50 ± 3	54 ± 1
VCL	108 ± 5^{a}	$93 \pm 2^{a,b}$	$101 \pm 2^{a,b}$	$94 \pm 2^{a,b}$	$96 \pm 4^{a,b}$	$100 \pm 4^{a,b}$	$93 \pm 3^{a,b}$	89 ± 4^{b}	86 ± 6^{b}	$93 \pm 2^{a,b}$
LHD	6.1 ± 0.4^{a}	$5.1 \pm 0.2^{a,b,c}$	$5.7 \pm 0.2^{a,b}$	$5.3 \pm 0.1^{a,b,c}$	$5.1 \pm 0.4^{a,b,c}$	$5.6 \pm 0.3^{a,b,c}$	$4.4 \pm 0.2^{b,c}$	$4.4 \pm 0.4^{b,c}$	4.3 ± 0.2^{c}	$4.5 \pm 0.2^{b,c}$

^{a,b,c} Means in same row with different superscripts differ ($P < 0.05$).
VSL = Straight-line velocity, VAP = average path velocity, VCL = curvilinear velocity, and LHD = lateral head displacement.

Table 7
Effect of temperatures of freezing on membrane integrity (%) of buffalo spermatozoa (mean ± SEM; $n = 5$).

Variable	Experiment 2a: Temperatures of freezing									
	+4°C	0°C	–5°C	–10°C	–20°C	–30°C	–40°C	–50°C	–80°C	–196°C (control)
PMI	86 ± 3^{a}	$82 \pm 4^{a,b}$	$74 \pm 4^{a,b,c}$	$74 \pm 4^{a,b,c}$	$66 \pm 7^{a,b,c,d}$	$58 \pm 8^{b,c,d,e}$	$50 \pm 6^{c,d,e}$	41 ± 3^{e}	40 ± 6^{e}	$47 \pm 6^{d,e}$
NAR	74 ± 5^{a}	$67 \pm 6^{a,b}$	$64 \pm 8^{a,b}$	$59 \pm 7^{a,b,c}$	$50 \pm 7^{a,b,c,d}$	$43 \pm 6^{b,c,d}$	$35 \pm 8^{c,d}$	28 ± 3^{d}	28 ± 5^{d}	$40 \pm 7^{b,c,d}$

^{a,b,c,d,e} Means in same row with different superscripts differ ($P < 0.05$).
PMI = Plasma membrane integrity, and NAR = normal apical ridge.

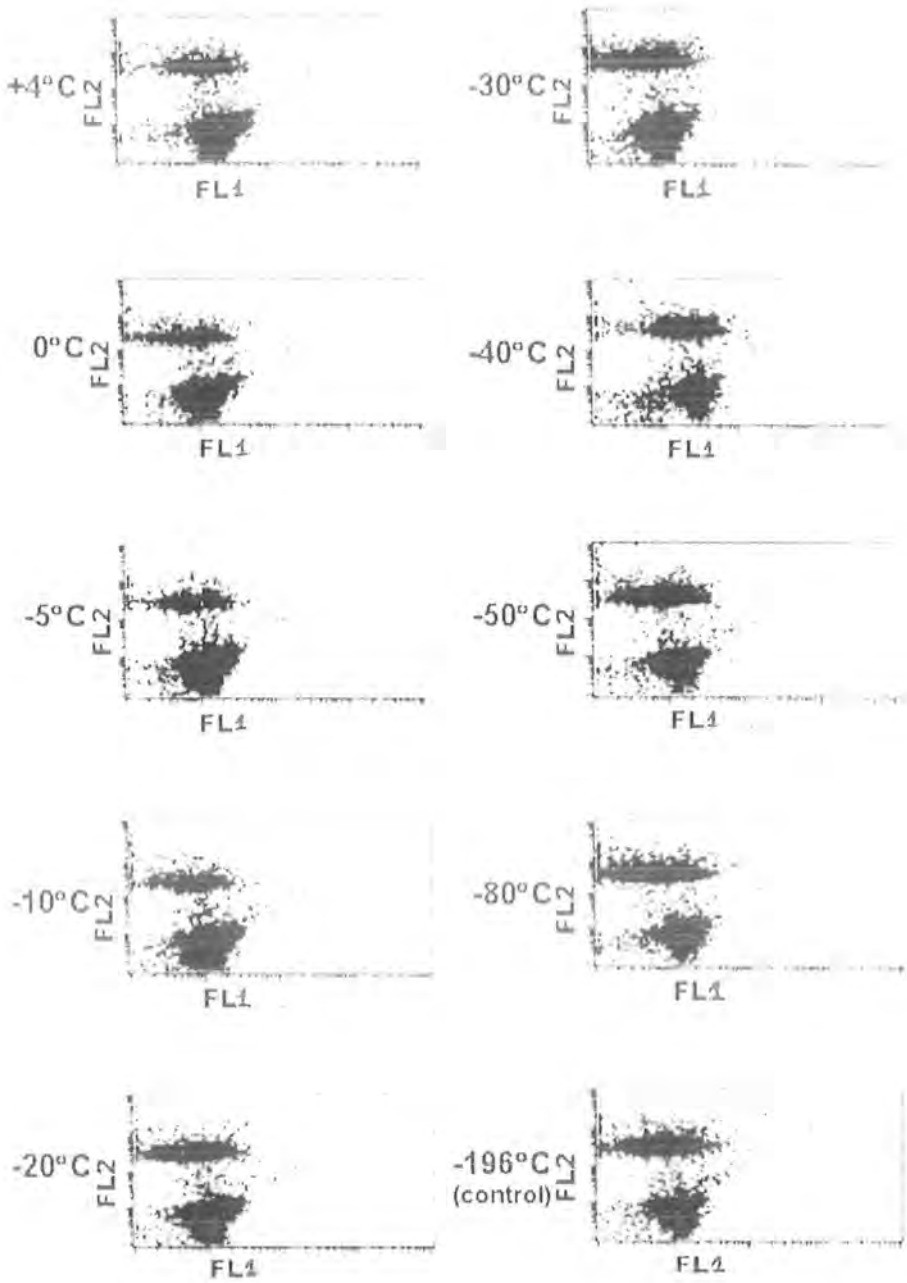

Fig. 2. Dot plots of two populations of buffalo spermatozoa are quantified in each panel. The population of spermatozoa stained with PI (Dead) is illustrated in the upper cluster, whereas that not stained with PI (Live) is gathered in the lower cluster of each panel. Effect of temperature during freezing on flow cytometric analysis of plasma membrane integrity of the spermatozoa was significant at –30°C ($P < 0.05$).

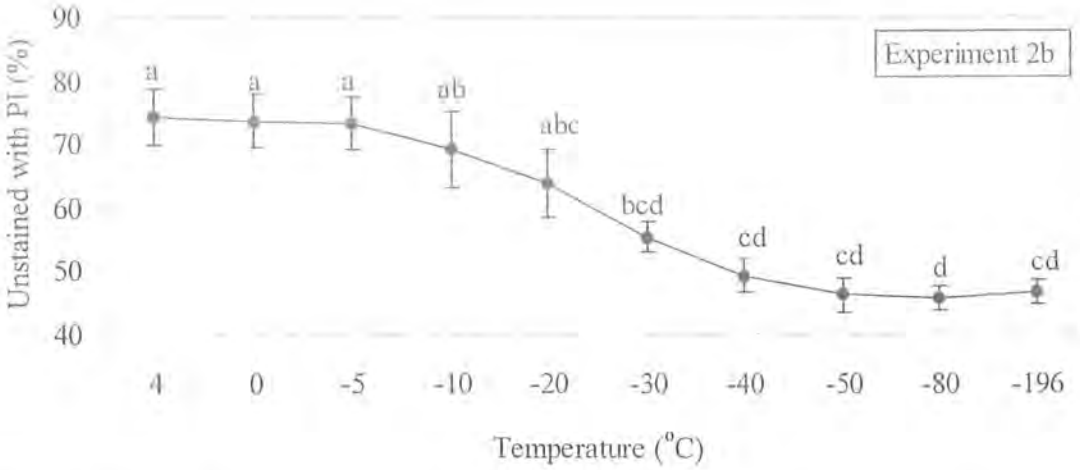

Fig. 3. Effect of temperatures of freezing on plasma membrane integrity of buffalo spermatozoa determined by flow cytometric analysis while staining with PI indicating that it reduced ($P < 0.05$) at –30°C.

fluorescence intensity almost two orders of magnitude higher than the intensity of first level. Dot plot of high level fluorescence intensity represents the cell's population with damaged plasma membrane and low level fluorescence intensity representing the cell's population having intact plasma membrane. The determination of these panels of dot-plots showed plasma membrane integrity remained higher after freezing to –5°C. It reduced significantly ($P < 0.05$) at –30°C that continued upto the end of freezing cycle (Fig. 3). The HOS pattern of plasma membrane damage at various temperatures was similar to that of FACS pattern, being substantiated by indicating a strong correlation ($r = 0.90$; $P < 0.001$) between these evaluations.

DISCUSSION

Effect of rates of freezing on post-thaw viability of buffalo spermatozoa

In an effort to improve the post-thaw viability of buffalo spermatozoa, we compared different freezing rates from 4 to –80°C. The alteration in the freezing rates, from 4 to –15°C in experiment 1a, was an attempt to properly dehydrate the spermatozoa in order to avoid the intracellular ice formation in the subsequent freezing. This study showed that either slow or moderate rates of freezing did not change the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo

spermatozoa. This is in agreement with the previous studies in bulls (Gilbert and Almquist, 1978), which have indicated that freezing of semen from 5 to –15°C either at the rates of 1.2, 5.8 or 9.5°C min^{-1} did not change the progressive motility and acrosome morphology of spermatozoa. However, slightly improved post-thaw motion characteristics, plasma membrane integrity and acrosome morphology of spermatozoa with moderate compared to slow freeze rate probably favoured better osmotic equilibrium across the membranes due to proper dehydration with moderate rate of freezing in buffaloes.

In the subsequent study, the change of freezing rates from –15 to –80°C, either moderate, fast or very fast, to minimize the exposure to spermatozoa to lethal intermediate temperature zone did not alter the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. Probably, the use of narrow range of freezing rates was unable to detect the differences among different variables. Alternatively, buffalo spermatozoa frozen in straws can tolerate a rather wide range of freezing rates without appreciable affect on post-thaw survival (Hultnaes, 1982). This has previously been reported for cattle spermatozoa (Almquist and Wiggin, 1973; Robbins et al., 1976; Gilbert and Almquist, 1978). In the present study, however, circular motility of spermatozoa was comparatively higher ($P = 0.104$) in fast than in very fast freezing rate. This difference was difficult to explain. Nonetheless, based on earlier studies for post-thaw survival of bull (Rodriguez et al., 1975; Mortimer et al., 1976; Chen et al., 1993) and buffalo (Nazir, 1986) spermatozoa, slow freezing rates in buffaloes may be evaded to reduce the "solution effects." Moreover, fertility data are needed to compare among rates of freezing in different cycles in buffaloes.

Effect of temperatures of freezing on post-thaw viability of buffalo spermatozoa

Fifty percent of the sperm viability is decreased due to freezing and thawing process (Watson, 1979). This is true for buffalo spermatozoa (Anzar et al., 1998). To reduce this damage, the present research was conducted to identify the lethal intermediate temperature zone by evaluating the sperm motilities, velocities and membrane integrity at different freezing temperatures in buffalo. Sperm motility, managed by microtubule sliding-induced bends in the flagellar axoneme, is generally initiated by the interactions between various second messengers (principally calcium and cAMP), enzymes,

and phosphoproteins (Lindemann and Kanous, 1989). In this study, the sperm motility either visual or computer-assisted decreased significantly after freezing to –40°C. The decrease in visual motility was consistent with the previous studies in bulls (Luyet and Keane, 1955; Liu et al., 1998), being associated with rapid nucleation and increase in osmotic pressure that occurred due to "solution effects" (Liu et al., 1998). Freezing to subzero temperatures might have damaged the microtubular structure of axoneme that probably decreased the sperm motility in buffaloes. This has been demonstrated in boar (Courtens and Paquignon, 1985) and mouse (Sherman and Liu, 1982) spermatozoa. In current study, little difference in sperm motility (visual and computer-assisted) between –40 and –80°C, suggested the non damaging effect of ice crystals and "solution effects" in this range of temperature. Presumably, the buffalo spermatozoa are less sensitive to temperature changes in the range from –40 to –80°C due to better buffering capacity of Tris, being examined in bulls (Chen et al., 1993). In the present study, linear motility of spermatozoa tended ($P = 0.09$) to decrease at –30°C, whereas circular motility did not change due to freezing temperatures. Conceivable, the freezing rate used herein was responsible to negate the consequential changes in these motilities.

The sperm movement is sensitive to biochemical and physical properties of the surrounding medium. The measurement of velocity has been considered as an indirect indicator of mitochondrial function of a spermatozoon (Graham et al., 1984) and is associated with fertility (Budworth et al., 1988). Curvilinear velocity was maximal at 4°C, whereas it tended ($P = 0.1$) to reduce at 0, –40 and –196°C. However, it increased transiently at –5 and –30°C before declining to its minimal at –50 and –80°C. One interpretation of present information could be that, extracellular ice formation at about –5°C (Hammerstedt et al., 1990) or "solution effects" between the lethal intermediate zone, –10 to –50°C, (Mazur, 1980) would be the source of variation in sperm curvilinear velocity in this study. Alternatively, intracellular concentration of cAMP that decreased under low-temperature conditions, would have resulted in the reduction of curvilinear velocity. Indeed, cyclic adenosine 3′,5′-monophosphate (cAMP) content was lower in the frozen spermatozoa of buffaloes (Kakar and Anand, 1981). Concomitently, mitochondrial apparatus of the mammalian sperm has shown to be altered (Watson, 1979; Courtens and Paquignon, 1985).

Lateral head displacement, an indicator of flagellar beat pattern of spermatozoa, was decreased at –40°C and then remained consistent upto –196°C, suggesting that temperature acts directly on the motor apparatus of buffalo sperm flagella. Clearly, lateral head displacement reduced due to cryopreservation of spermatozoa in human (Fernandes et al., 1990). In another study (Suarez et al., 1993), decrease in lateral head displacement has shown to be associated with the decrease in intracellular calcium in hamster spermatozoa. Thus, the present decrease in sperm lateral head displacement with the decrease in subzero temperatures may be due to decrease in intracellular calcium. It will be of interest to identify such changes in future studies in buffaloes. However, straight-line and average path velocities of buffalo spermatozoa did not change due to temperatures of freezing. It may thus be proposed that curvilinear velocity and lateral head displacement are more sensitive kinematics to identify the biochemical and physical changes occur during freezing of spermatozoa in buffaloes.

The functional integrity of the sperm plasma membrane is of primary importance for the fertilizing ability of a spermatozoon. It was either assessed by HOS or FACS analyses. The use of FACS was an effort to improve the precision and reliability of an additional objective assay to assess the viability of buffalo spermatozoa. A strong relationship (r = 0.90; $P < 0.05$) between HOS and FACS assessments has advocated the use of flow cytomety in semen studies in buffaloes. However, the correlation between fluorometric measure of sperm viability and fertility remains to be established in buffalo spermatozoa. In the present study, plasma membrane integrity of spermatozoa reduced significantly after freezing to –30°C. This damage has reported a little earlier at –20°C in ram (Holt et al., 1992) and bull (Liu, et al., 1998) spermatozoa. In another study (Mazur, 1985), a wider range from –15 to –60°C was shown to be critical in boar spermatozoa. These findings suggest that the damage was either due to cellular dehydration or intracellular ice formation as previously reported in the living cells (Mazur, 1984). It has been demonstrated earlier that nonbilayer lipids associated with the integral membrane proteins, transformed into gel-rich zone due to freezing, whereas the original distribution of these particles did not restore after thawing that may decrease the sperm viability (Holt and North, 1984; Quinn, 1989). Perhaps, these irreversible changes were more conspicuous after freezing of spermatozoa to –30°C in buffaloes. Alternatively, sodium pump expected to become disable as the temperature decreased and the intracellular

sodium load might have increased the damage to buffalo spermatozoa. This has previously been reported for mouse oocyte (Stachecki et al., 1998). To reduce these damages further research on cryopreservation of buffalo spermatozoa should focus on membrane stabilization.

The presence of acrosome has shown good correlation to fertility of cryopreserved bovine spermatozoa after artificial insemination (Saacke and White, 1972). In the present study, the percentage of normal acrosomes reduced significantly after freezing to –30°C, which remained consistent upto –196°C. Based on data reported herein, the pH of the surrounding medium would have dropped rapidly during freezing, that probably altered the acrosome, as previously reported in bovine spermatozoa (Aalseth and Saacke, 1987). Alternatively, thawing of buffalo spermatozoa 'knobbed out' the acrosomal caps, being demonstrated in bull and rabbit spermatozoa (Bamba and Cran, 1988). Greater damage to acrosome (Anzar et al., 1998) and higher release of extracellular hyaluronidase (Akhtar and Chaudhry, 1989) and acrosin (Chinnaiya and Ganguli, 1980) due to freezing and thawing may contribute in the loss of fertility in buffaloes.

In conclusion, various freezing rates tested from 4 to –80°C, did not improve the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. The intermediate zone of damage to sperm motility apparatus and membrane integrity lies somewhere between –20 to –40°C in buffaloes. Further research is required to minimize the damage to buffalo spermatozoa by adding membrane stabilizing agents, and increasing freeze and thaw rates to minimize cell exposure to the lethal intermediate temperature zone.

ABSTRACT

The present study was designed to see if different concentrations of glycerol and/or dimethyl sulfoxide (DMSO), either added at 37 or 4°C, have an effect on changes in post-thaw sperm quality. Semen from four Nili-Ravi bulls of more than 60% of visual motility was pooled at 37°C. Pooled ejaculates (n = 4) were divided into 18 aliquots of 0.5 ml each and extended (1:10) in Tris-citric acid extender. In nine aliquots the concentrations (%) of glycerol and DMSO were adjusted (0:0, 0:1.5, 0:3; 3:0, 3:1.5, 3:3; and 6:0, 6:1.5, 6:3, respectively) in extended semen prior to freezing by one step addition at 37°C, and in the other nine by cooling the semen to 4°C in 2.5 h and addition at 4°C. Semen was equilibrated at 4°C for 4 h, packed in 0.5 ml French straws and frozen in a programmable cell freezer before plunging into liquid nitrogen for deep freezing and storage. Thawing was performed at 37°C for 15 s before evaluation. Post-thaw motion characteristics, plasma membrane integrity and acrosome morphology of buffalo spermatozoa were determined using computer-assisted semen analyzer (CASA), hypo-osmotic swelling (HOS) assay and phase-contrast microscopy, respectively. The statistical analysis revealed that 6% glycerol increased ($P < 0.05$) the motilities (visual and computer-assisted), velocities (straight-line, average path, and curvilinear) lateral head displacement, and plasma membrane integrity of spermatozoa than 3 or 0% glycerol. Whereas, 1.5 or 3% DMSO decreased ($P < 0.01$) the percentages of sperm motilities (visual and computer-assisted) and plasma membrane integrity than 0% DMSO. The combination of 6% glycerol and 0% DMSO provided the highest ($P < 0.01$) visual motility, computer-assisted motility, and plasma membrane integrity of spermatozoa than other combinations of glycerol and DMSO. Likewise, the addition of 6% glycerol at 37°C, responded the maximum ($P < 0.05$) visual motility, computer-assisted motility, straight-line velocity, and average path velocity of spermatozoa than other adding temperature of glycerol. The addition of 6% glycerol and 0% DMSO at 37°C showed the highest (P = 0.04) computer-assisted motility of spermatozoa than other combinations of glycerol and DMSO, added at 37 or 4°C. It is concluded that addition of DMSO at the levels investigated did not improve the post-thaw quality of spermatozoa. However, glycerol in 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 extender.

Keywords: Buffalo—spermatozoa; Cryoprotectants

INTRODUCTION

The empirical studies have indicated that many factors are involved in the retention of viability of cryopreserved spermatozoa (Hammerstedt et al., 1990, Curry et al., 1994). These factors include, primarily, the composition of extender and cryoprotectants, and secondly, the freezing and thawing process. Although, the need for control of ice crystal formation during freezing of cell is of prime importance (Mazur, 1984). Among various cryoprotectants, glycerol, since its' discovery (Polge et al., 1949) has been used extensively for the cryopreservation of many types of cells, including sperm. It reduces thermal stress and prevents fracture in the frozen solutions by reducing the total ice volume expansion during water solidification (Gao, et al., 1995). Furthermore, it acts through 'salt-buffering' mechanism (Lovelock, 1953), binds with the metallic ions (Lohmann et al., 1964) and dehydrates the cell (Berndtson and Foote, 1969) while preventing the concentration of extracellular media (Meryman, 1971).

We have previously shown that freezing and thawing followed by equilibration cause maximum damage to buffalo spermatozoa (Anzar et al., 1998). Moreover, it is generally accepted that 50% of the spermatozoa get damaged during the process of cryopreservation (Watson, 1979) which is mainly due to the hyperosmotic effects, being imposed by glycerol. In fact, glycerol, in addition to its osmotic effects, may have detrimental effects on the structure of the plasma membrane and on the metabolism of the cell (Hammerstedt et al., 1990). These effects, however, differed among species. It has been shown that glycerol has a contraceptive effect upon the fertility of boar, chickens and turkeys spermatozoa (Polge, 1956; see review by Hammerstedt and Graham, 1992). The lower fertility in buffaloes as compared to cattle (Radhakrishna et al., 1984; Ala-ud-Din et al., 1990) may be due to this possible reason.

During freezing, different concentrations of glycerol provided variable kinds of protection to spermatozoa for motility and fertility in bulls (Erickson et al., 1954; Graham et al., 1958). In buffaloes, the 6 and 7% glycerol has been shown to be optimal for better

protection to the sperm motility apparatus (Heuer, 1980; Hultnaes, 1982; Hanif et al., 1999) and the acrosomal cap (Ramakrishnan and Ariff, 1994). The addition of glycerol at various temperatures has been studied in bovines and the results are equivocal. Its' addition at 5°C provided better protection to spermatozoa during freezing compared to addition at temperatures above 5°C both in bovine (Polge, 1953; Choong and Wales, 1964) and buffalo bulls (Kataria and Tuli, 1992). Some workers have, however, reported that the addition of glycerol may be carried out either at 37-32°C, room temperature, or 5°C without any appreciable effects on post-thaw quality of spermatozoa in bovine (Foote, 1970; Arriola and Foote, 1987) and buffalo bulls (Heure, 1980; Hultnaes, 1982). Concomitently, the fertility did not vary in bovine bulls (Graham et al., 1958; Foote, 1970).

Glycerol, despite its' toxic effects to the membranes, still continues to be used as a cryoprotectant for sperm membranes. The development of less toxic cryopreservative could make a significant contribution to the improvement of the quality of frozen semen in buffaloes. It may be achieved by decreasing the optimal concentration of glycerol in the extender and to substitute it with other penetrating cryoprotectant. Dimethyl sulfoxide (DMSO), since its' discovery as a promising cryoprotective agent for living cells (Lovelock and Bishop, 1959), has been used extensively for the freezing of mammalian gametes. Nevertheless, there are studies, indicating the beneficial effect of glycerol in combination with DMSO on post-thaw quality of spermatozoa not only in fish (Lahnsteiner et al., 1996) and rabbit (Bamba and Adams, 1990) but also in bulls (Page, 1968; Snedeker and Gaunya, 1970) and buffaloes (Ansar et al., 1989). Furthermore, it has been suggested that DMSO undergoes rapid penetration in cells than glycerol (Lovelock and Bishop, 1959), with concomitant decrease in osmotic stress. These findings prompted us to investigate if different concentrations of glycerol and/or DMSO, either added at 37 or 4°C, have an effect on changes in post-thaw quality of buffalo spermatozoa.

Recently, the use of computer-assisted sperm analysis (CASA) to evaluate motion characteristics of spermatozoa in buffaloes (Rasul et al., 2000) has provided insight into the physiology and post-thaw quality of cells. Moreover, the combination of computerized determination of motion characteristics, and acrosomal integrity has been correlated with fertility in bulls (O'Connor et al., 1981). Besides, hypo-osmotic swelling (HOS) assay has proven useful to assess the functional integrity of sperm plasma

membrane in buffaloes (Rasul et al., 2000). Thus, both motion characteristics and integrity of plasma and acrosomal membranes were used in the present study to evaluate the effect of glycerol and/or DMSO in buffalo spermatozoa while using CASA, HOS-assay and phase-contrast microscopy, respectively.

MATERIALS AND METHODS

Preparation of extenders

Tris-citric acid (TCA) extender was prepared by titrating the citric acid with Tris-(hydroxymethyl)-aminomethane (Fluka, switzerland) (7.87 g in 200 ml distilled H_20, 325 mOsm kg^{-1}) to pH 7.0. Final osmotic pressure was 275 mOsm kg^{-1}. The TCA was divided into eighteen aliquots. Nine of these consisted of different concentrations (%) of glycerol:DMSO (0:0, 0:1.5, 0:3; 3:0, 3:1.5, 3:3; and 6:0, 6:1.5, 6:3, respectively) for the use at 37°C. Whereas, the other nine were adjusted with the double concentrations of these cryoprotectants for the use at 4°C. All these formulated extenders contained egg yolk (20%, v/v), fructose (0.2%, w/v), pencillin (1000 I.U. ml^{-1}) and streptomycin (100 μg ml^{-1}). The extenders were centrifuged at 12000 × *g* for 15 min, and the supernatants were frozen and stored at –20°C. Extenders were thawed at 37°C before the experimental use.

Semen collection and initial evaluation

Four mature Nili-Ravi buffalo bulls were maintained at Livestock Research Station, National Agricultural Research Center, Islamabad. The semen of these bulls was collected at weekly intervals with the help of artificial vagina at 42°C during the months of November and December. Two consecutive ejaculates were obtained from each bull for four weeks. The semen ejaculates, exhibited more than 60% of fresh visual motility, were pooled ($n = 4$) to have a sufficient volume for one replicate and to eliminate the individual bull effect. Pooled semen was maintained at 37°C for 15 minutes of holding time.

Processing of semen

Each pooled semen sample was divided into eighteen aliquots. The nine aliquots were

extended (1:10) at 37°C with TCA extenders containing different concentrations (%) of glycerol and DMSO (0:0, 0:1.5, 0:3; 3:0, 3:1.5, 3:3; and 6:0, 6:1.5, 6:3) and cooled to 4°C in 2.5 h. The other nine semen aliquots were extended (1:5) at 37°C with TCA extenders without cryoprotectants and cooled to 4°C in 2.5 h. These were further extended (1:5) at 4°C with TCA extenders containing the double concentrations of glycerol and DMSO to achieve the final dilution (1:10) and concentrations of cryoprotectants as were used at 37°C.

The semen was then equilibrated at 4°C for 4 h, packed into 0.5 ml French straws, and immediately loaded into the chamber of a programmable cell freezer (KRYO 10 Series III, Planer, Sunbury-on-Thames, Middlesex, UK). The freezer was programmed to supercool the semen filled straws from 4 to –15°C at the rate of 3°C min^{-1} and then to –80°C at the rate of 10°C min^{-1}. The frozen straws were immediately plunged and stored into liquid nitrogen canes for 24 h before evaluation. Semen filled straws were thawed at 37°C for 15 s. After 5 minutes of incubation at 37°C, the semen quality was evaluated by various semen assays.

Semen assays

Sperm visual assessment

Post-thaw sperm visual motility was assessed microscopically (x 400) attached with a closed circuit television.

Sperm motion characteristics

The 5 μl of semen sample was placed on a prewarmed (37°C) Makler chamber (depth 10 μm; Sefi-Medical Industries, Haifa, Israel) and analyzed for sperm motion characteristics while using a computerized Cell Motion Analyzer (SM-CMA; Mika Medical, Germany), as recently validated for buffalo spermatozoa in our laboratory (Rasul et al., 2000).

The semen sample of each treatment was assessed for motility (CMOT, %); linear motility (LMOT, %); circular motility (CIRMOT, %); straight-line velocity (VSL, $\mu m\ s^{-1}$); average path velocity (VAP, $\mu m\ s^{-1}$); curvilinear velocity (VCL, $\mu m\ s^{-1}$); lateral head displacement (LHD, μ); linearity (LIN; VSL/VCL × 100, %) and straightness (STR; VSL/VAP × 100, %) of spermatozoa.

Hypo-osmotic swelling (HOS)

The HOS-assay was used to determine the functional plasma membrane integrity (PMI) of buffalo spermatozoa. Sodium citrate (0.735 g) and fructose (1.351 g) were dissolved in distilled water (100 ml), as recommended by World Health Organization (1992). Approximate osmotic pressure was 200 mOsm kg^{-1}. This HOS solution was maintained at 37°C for 5 min before use. The 25 μl of the each semen sample was mixed with 250 μl of HOS solution and incubated at 37°C for 30 min. After incubation, a drop of semen sample was examined under phase-contrast microscope (x 400). Two hundred spermatozoa were assessed to calculate the percentage of swollen cells (curled tail/intact plasma membrane).

Normal acrosomes

Semen sample of 250 μl was fixed in 25 μl of 1% solution of formal citrate (2.9 g *tri*-sodium citrate dihydrate [Merck, Darmstadt, Germany] and 1 ml of a 37% solution of formaldehyde [Merck] dissolved in 99 ml of distilled H_2O). Acrosomal integrity of two hundred spermatozoa either normal or abnormal apical ridges was assessed using phase-contrast microscope (Leica, Leitz Wetzlar, Germany) at x 1000. The percentage of intact acrosomes with normal apical ridges (NAR) was calculated.

Statistical analysis

SYSTAT (Version 6.0 for Windows, SPSS, Chicago, IL, U.S.A.,1996) was used to analyze the data. The values were presented as mean ± standard error of mean (SEM). The data were analyzed as a split plot in randomized complete block design (RCBD) using General Linear Models Procedures of the SYSTAT. The model included effect of glycerol, DMSO, temperatures of addition, glycerol × DMSO, glycerol × temperatures of its' addition, DMSO × temperatures of its' addition, and glycerol × DMSO × temperatures of their addition. When the F-ratio was significant ($P < 0.05$), Tukey's HSD test was used to compare treatment means.

RESULTS

The glycerol concentrations affected ($P < 0.05$) the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. The

DMSO concentrations also contributed ($P < 0.01$) to the variation in visual and computer-assisted motility. However, temperature of addition alone did not account for these variations ($P > 0.05$). Glycerol × DMSO interaction contributed to the variation in visual motility ($P < 0.01$), computer-assisted motility ($P < 0.01$) and plasma membrane integrity ($P < 0.01$). Two-way interactions of glycerol with temperature of its' addition influenced the visual motility ($P < 0.01$), computer-assisted motility ($P < 0.01$), straight-line velocity ($P < 0.01$), average path velocity ($P < 0.01$), curvilinear velocity ($P = 0.04$) and plasma membrane integrity ($P = 0.04$). Interaction of DMSO with temperature of its' addition did not cause any variation in any variable tested ($P > 0.05$). Computer-assisted motility, was the only dependent variable which was affected by the three way interaction of glycerol, DMSO and temperature of addition ($P = 0.04$).

The effect of glycerol, DMSO, temperature of addition and their interactions on visual motility and computer-assisted motility is summarized in Table 1. Using pooled data for three DMSO concentrations and two temperatures of addition, visual and computer-assisted motility were observed to be maximum in 6% glycerol (36.5 and 32.5%, respectively), followed by 3% glycerol (11.5 and 10.4%, respectively), and minimum in 0% glycerol (undetectable). When combined over three glycerol concentrations and two temperatures of addition, visual or computer-assisted motility of spermatozoa was higher ($P < 0.05$) in 0% DMSO than in 1.5 or 3% DMSO, indicating the damaging effect of DMSO to buffalo spermatozoa. Furthermore, when the data for temperatures of addition were pooled, the visual and computer-assisted motility of spermatozoa averaged 49.4 and 47.3%, respectively, in 6% glycerol with 0% DMSO, but decreased ($P < 0.05$) to averaged 30.0 and 25.1%, respectively, in 6% glycerol with 1.5 or 3% DMSO. When averaged across three DMSO concentrations, the visual and computer-assisted motility resulted higher ($P < 0.05$) in 6% glycerol when added at 37°C (41 and 38%, respectively), followed by 6% glycerol added at 4°C (34.7 and 27.4%, respectively). The concentrations of glycerol, DMSO and their temperature of addition contributed ($P < 0.04$) to the variation in computer-assisted motility, which was maximum in 6% glycerol with 0% DMSO when added at 37°C.

The effect of glycerol concentrations on post-thaw linear motility, circular motility, lateral head displacement, linearity, straightness, and normal acrosomes of buffalo spermatozoa are presented in Table 2. When combined across all other treatments, the

Table 1

Effect of glycerol and or DMSO, added at 37°C and 4°C, on post-thaw visual and computer-assisted motility (%) of buffalo spermatozoa.

Glycerol (%)	Added at (°C)	Visual motility ($n = 12$)†	Mean ($n = 24$)	Computer-assisted motility ($n = 12$)†	Mean ($n = 24$)
0	37	0.0 ± 0.0^{a}	0.0^{a}	0.0 ± 0.0^{a}	0.0^{a}
	4	0.0 ± 0.0^{a}		0.0 ± 0.0^{a}	
3	37	9.6 ± 2.1^{b}	11.5^{b}	$8.0 \pm 1.2^{a,b}$	10.4^{b}
	4	13.6 ± 1.9^{b}		13.1 ± 1.8^{b}	
6	37	41.3 ± 4.9^{c}	36.5^{c}	37.7 ± 3.5^{c}	32.5^{c}
	4	31.7 ± 3.6^{d}		27.4 ± 3.4^{d}	

Glycerol (%)	DMSO (%)	Visual motility ($n = 8$)††	Mean ($n = 24$)	Computer-assisted motility ($n = 8$)††	Mean ($n = 24$)
0	0	0.0 ± 0.0^{a}	0.0^{a}	0.0 ± 0.0^{a}	0.0^{a}
	1.5	0.0 ± 0.0^{a}		0.0 ± 0.0^{a}	
	3	0.0 ± 0.0^{a}		0.0 ± 0.0^{a}	
3	0	$17.5 \pm 2.8^{b,c}$	11.5^{b}	$15.6 \pm 1.9^{b,c}$	10.4^{b}
	1.5	$9.4 \pm 1.5^{a,b}$		$8.0 \pm 1.5^{a,b}$	
	3	$7.1 \pm 1.0^{a,b}$		$7.3 \pm 1.0^{a,b}$	
6	0	49.4 ± 6.1^{e}	36.5^{c}	47.3 ± 6.9^{e}	32.5^{c}
	1.5	34.4 ± 3.6^{d}		29.1 ± 3.1^{d}	
	3	$25.6 \pm 2.4^{c,d}$		$21.1 \pm 3.1^{c,d}$	

Values (mean ± SEM) in same column with different superscripts differ significantly ($P < 0.05$).

†Data are pooled for three concentrations of DMSO (0, 1.5, 3%) as this treatment had no effect on the mean values.

††Data are pooled for the two temperatures (37°C and 4°C) at which DMSO was added as this treatment had no effect on the mean values.

Table 2

Effect of glycerol, added at 37°C and 4°C, on post-thaw linear motility, circular motility, linearity, straightness, and acrosome morphology of buffalo spermatozoa.

Glycerol (%)	Linear motility (%)	Circular motility (%)	Lateral head displacement (μm)	Linearity (%)	Straightness (%)	Normal acrosome (%)
0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	30.2 ± 4.3^{a}
3	63.2 ± 5.5^{b}	14.5 ± 2.4^{b}	3.2 ± 0.2^{b}	53.6 ± 1.7^{b}	85.2 ± 1.5^{b}	$41.7 \pm 3.6^{a,b}$
6	49.2 ± 2.9^{c}	21.2 ± 1.8^{c}	4.2 ± 0.2^{c}	49.7 ± 1.2^{b}	82.4 ± 1.4^{b}	41.9 ± 3.2^{b}

Values (mean ± SEM; $n = 24$) in same column with different superscripts differ significantly ($P < 0.05$).

Data are pooled for the two temperatures (37°C and 4°C) at which glycerol was added and for three concentrations of DMSO (0, 1.5, 3%) as these treatments had no effect on the mean values.

Table 3

Effect of glycerol, added at 37°C and 4°C, on post-thaw velocities (straight-line, average path, and curvilinear) of buffalo spermatozoa.

Glycerol (%)	Added at (°C)	Straight-line velocity (μm s^{-1}, $n = 12$†)	Mean ($n = 24$)	Average path velocity (μm s^{-1}, $n = 12$†)	Mean ($n = 24$)	Curvilinear velocity (μm s^{-1}, $n = 12$†)	Mean ($n = 24$)
0	37	0.0 ± 0.0^{a}	0.0^{a}	0.0 ± 0.0^{a}	0.0^{a}	0.0 ± 0.0^{a}	0.0^{a}
	4	0.0 ± 0.0^{a}		0.0 ± 0.0^{a}		0.0 ± 0.0^{a}	
3	37	39.2 ± 1.6^{b}	40.0^{b}	46.3 ± 1.3^{b}	47.1^{b}	74.8 ± 3.8^{b}	76.0^{b}
	4	41.0 ± 1.0^{b}		$47.9 \pm 1.4^{b,c}$		$77.3 \pm 3.4^{b,c}$	
6	37	48.3 ± 1.9^{c}	45.0^{c}	57.9 ± 1.6^{d}	54.9^{c}	96.0 ± 2.8^{d}	91.0^{c}
	4	41.7 ± 1.7^{b}		51.9 ± 1.3^{c}		$86.1 \pm 2.5^{c,d}$	

Values (mean ± SEM) in same column with different superscripts differ significantly ($P < 0.05$).

†Data are pooled for three concentrations of DMSO (0, 1.5, 3%) as this treatment had no effect on the mean values.

CHAPTER 4

Effect of Glycerol and or Dimethyl Sulfoxide on Post-Thaw Motion Characteristics, Plasma Membrane Integrity, and Acrosome Morphology of Buffalo Spermatozoa

Table 4
Effect of glycerol and or DMSO, added at 37°C and 4°C, on post-thaw plasma membrane integrity of buffalo spermatozoa.

Glycerol (%)	Added at (°C)	Plasma membrane integrity (%) ($n = 12$†)	Mean ($n = 24$)
0	37	8.2 ± 1.6^{a}	10.0^{a}
	4	11.8 ± 1.6^{a}	
3	37	22.2 ± 2.8^{b}	24.8^{b}
	4	$27.7 \pm 2.1^{b,c}$	
6	37	39.6 ± 3.9^{d}	37.0^{c}
	4	$34.3 \pm 2.5^{c,d}$	

Glycerol (%)	DMSO (%)	Plasma membrane integrity (%) ($n = 8$††)	Mean ($n = 24$)
0	0	6.0 ± 1.2^{a}	10.0^{a}
	1.5	$9.3 \pm 1.2^{a,b}$	
	3	$14.8 \pm 2.3^{a,b,c}$	
3	0	27.4 ± 3.9^{d}	24.8^{b}
	1.5	$25.9 \pm 2.5^{c,d}$	
	3	$20.7 \pm 2.9^{b,c,d}$	
6	0	47.0 ± 4.3^{e}	37.0^{c}
	1.5	31.9 ± 2.9^{d}	
	3	32.0 ± 2.3^{d}	

Values (mean ± SEM) in same column with different superscripts differ significantly ($P < 0.05$).
†Data are pooled for three concentrations of DMSO (0, 1.5, 3%) as this treatment had no effect on the mean values.
††Data are pooled for the two temperatures (37°C and 4°C) at which DMSO was added as this treatment had no effect on the mean values.

sperm did not show any movement and the intact acrosomes averaged only 30% in 0% glycerol. Freezing semen in 3% glycerol increased ($P < 0.05$) the linear motility, and decreased ($P < 0.05$) the circular motility and lateral head displacement of spermatozoa than in 6% glycerol (63% *vs* 49%, 15% *vs* 21%, and 3.2 μm *vs* 4 μm, respectively). Whereas, percent linearity, straightness, and acrosomal retention of spermatozoa did not differ ($P > 0.05$) in 3 or 6% glycerol.

The effect of glycerol concentrations along with their temperatures of addition on different sperm velocities (straight-line, average path, and curvilinear) are summarized in Table 3. When averaged across three DMSO concentrations and two temperatures of addition, the semen frozen without glycerol did not show any sperm velocity. Sperm straight-line velocity, average path velocity, and curvilinear velocity were recorded higher ($P < 0.05$) in 6% glycerol (45, 55 and 91 $\mu m\ s^{-1}$, respectively) than in 3% glycerol (40, 47, and 76 $\mu m\ s^{-1}$, respectively). When combined across three DMSO concentrations, these velocities did not differ ($P > 0.05$) in 3% glycerol either added at 37 or 4°C. Likewise, these velocities did not vary ($P > 0.05$) in 6 or 3% glycerol when added at 4°C. However, straight-line velocity and average path velocity of spermatozoa were maximum in 6% glycerol when added at 37°C than in any other treatment combination.

The effect of glycerol, DMSO, temperatures of addition and their interactions on post-thaw plasma membrane integrity of buffalo spermatozoa is presented in Table 4. When averaged across three DMSO concentrations and temperatures of addition, plasma membrane integrity of spermatozoa frozen in 6% glycerol was superior to 3% glycerol ($P < 0.05$), and plasma membrane integrity for both treatments was better than in 0% glycerol ($P < 0.05$). It averaged 10, 25 and 37% in 0, 3 and 6% glycerol, respectively. When data were pooled for temperatures of addition, plasma membrane integrity (%) was better protected ($P < 0.05$) in 6% glycerol with 0% DMSO than other combinations of glycerol and DMSO. Whereas, when averaged across three DMSO concentrations, the plasma membrane integrity was slightly better in 6% glycerol when added at 37°C than at 4°C.

DISCUSSION

While attempting to reduce the toxicity of glycerol, the recent results do not provide the

evidence of synergistic effect of DMSO with glycerol to maintain the post-thaw viability of buffalo spermatozoa. The interactions between various second messengers (principally calcium and cAMP), enzymes, and phosphoproteins activate and regulate motility via microtubule sliding-induced bends in the flagellar axoneme (Gibbons, 1981; Lindemann and Kanous, 1989). The results of the present study may indicate that these interactions were better reflected in post-thaw visual and computer-assisted motilities in extenders containing 6% glycerol alone than other treatments. Our observations for visual motility are consistent with the earlier studies in bovines (Garcia and Graham, 1987; Kumar et al., 1994), which implicate that freezing of semen in the presence of 6% glycerol resulted in sperm motility significantly higher than that of spermatozoa frozen in 3% glycerol. The buffalo spermatozoa in the current study did not possess any kind of motility frozen without glycerol. This is in contrast to the previous studies in bulls (Gibson and Graham 1969; Pace and Graham, 1974), which indicated considerable motility of spermatozoa frozen without glycerol. These differences may be due to species. Surprisingly, the concentrations of DMSO alone or in combination with glycerol tested in this study did not provide cryoprotection to the motility apparatus of the spermatozoa. The addition of 1.5 or 3% DMSO in combination with 6% glycerol depressed the visual and computer-assisted motilities of buffalo spermatozoa. This negates the early findings in bovine spermatozoa (Page, 1968; Snedeker and Gaunya, 1970; Ansar et al., 1989), which have proposed that 1 or 2% DMSO in combination with 4 or 6% glycerol provide the favourable protection to the motility apparatus. DMSO has been shown to cause changes in microtubule organization of the oocytes (Vincent et al., 1990; Cooper et al., 1996). Perhaps, the use of Tris based extender and programmable freezing for buffalo sperm cryopreservation was responsible for these differences. It could be the fast freezing that would possibly favour the presence of DMSO (Mounib, 1978) in the future studies in buffaloes.

The results for the addition of glycerol and/or DMSO in the present study showed better ($P < 0.05$) post-thaw sperm motility (visual and computer-assisted) by the addition of 6% glycerol at 37°C compared to that at 4°C. The current results of visual motility for the addition of glycerol are in contrast with the previous findings in bulls (Polge, 1953; Choong and Wales, 1964; Arriola and Foote, 1987) and in buffaloes (Hultnaes, 1982; Kataria and Tuli, 1992), which indicated better and/or equal protection to bovine spermatozoa glycerolized either at 37 or 4°C. It is difficult to interpret this discrepancy. Nonetheless, in

the current study, the visual and computer-assisted motilities did not vary enough to account for differences between the addition of DMSO either at 37 or 4°C.

The pattern of sperm cell movement is sensitive to the chemical and physical properties of the medium in which they are suspended. For example, an increase in intracellular calcium, which would result from glycerol-induced leakage of the membranes, probably tend to inhibit local axonemal bending through phosphoprotein pathways that suppress dynein-mediated microtubule sliding (Lindemann and Kanous, 1989). The inhibition of axonemal bending may be reflected in the increase in linear motility, and decrease in circular motility, lateral head displacement and curvilinear velocity of buffalo spermatozoa frozen in 3% glycerol than in 6% glycerol. In contrast, an increase in calcium above some critical concentration would increase the asymmetry of sperm motion which would be reflected as a decrease in linear motility, and increase in circular motility, lateral head displacement and curvilinear velocity of spermatozoa. This probably resulted from a less flexible mid-piece, flagellar bending, and irregular sperm rotation in 6% glycerol than in 3% glycerol, which suggested the toxicity of the optimal concentration of glycerol to the motility apparatus of buffalo spermatozoa. The decrease in linear motility, and increase in circular motility and lateral head displacement in 6% glycerol may be due to glycerol-related osmotic shock to spermatozoa as suggested in ram spermatozoa (Fiser and Fairfall, 1989). These changes manifested the increase in circular motility (Suarez et al., 1993) and lateral head displacement (Bailey et al., 1994) of spermatozoa. Therefore, it can be hypothesized that the concentration of cryoprotectant can dictate the extent of water and ion movement across the membrane. It will be interesting to observe the changes in the concentrations of calcium due to the addition of cryoprotectants in buffalo semen. However, percent post-thaw linear motility, circular motility, lateral head displacement, linearity and straightness of buffalo spermatozoa were not influenced by the concentrations of DMSO alone or in combination with glycerol either added at 37 or 4°C.

The velocity of spermatozoa has been associated with fertility (Budworth et al., 1988) and its' determination indirectly provide the information of the mitochondrial function (Graham et al., 1984). In this study, the sperm post-thaw velocities (straight-line, average path, and curvilinear) were significantly affected due to different concentrations of glycerol added at 37 or 4°C. These velocities were higher in 6% glycerol than in 3% glycerol. In contrast to the previous finding in buffaloes (Rasul et al., 2000), the recent report for

straight-line and average path velocity in 6% glycerol was shown to be lower, whereas the curvilinear velocity was higher. This may be due to differences in the cooling time or season of semen collection. Furthermore, in the present finding, these velocities were recorded higher when 6% glycerol was added at 37°C than 4°C. We believe that these changes in sperm velocities are a consequence of changes associated with cooling, rather than freezing of spermatozoa. Besides increase in the ionic shift across the membrane, as described earlier, the glycerol perhaps reduced the synthesis of ATP at lower temperatures, as reported in mammalian spermatozoa (Hammerstedt et al., 1990). This might have accounted for lower sperm velocities in 6% glycerol being added at 4°C compared to 37°C in buffaloes. Alternatively, the current data suggest that the synergistic effect of glycerol and egg yolk in protecting the cells was comparatively more beneficial during cooling which might have been inflected after freezing and thawing. This effect has been observed during freezing of bull spermatozoa (Pace and Graham, 1974). Neither DMSO nor DMSO in combination with glycerol influenced mitochondrial apparatus of buffalo spermatozoa enough to bring positive changes in the sperm velocities. It appears that the sperm velocities are the most sensitive kinematics to response the addition of glycerol in buffaloes.

HOS-assay has proven useful to assess the functional integrity of spermatozoa in buffaloes (Rasul et al., 2000). In the current study, the increasing concentrations of glycerol increased the integrity of sperm plasma membrane in a linear fashion and the highest cryoprotection was observed with 6% glycerol than with 3 or 0% glycerol. Freezing of semen with lesser concentration of glycerol (0 or 1%) has shown to be associated with the greater release of glutamic oxalacetic transaminase in the surrounding media in Holstein bulls (Pace and Graham, 1970) and reduced quality of buffalo spermatozoa (Heuer, 1980; Hultnaes, 1982). Furthermore, in the present study the percent post-thaw plasma membrane integrity of spermatozoa reduced with 1.5 or 3% DMSO than that of glycerol alone. Perhaps, the affinity of solutes concentration (which protect cells at temperatures below freezing) is greater for glycerol than DMSO. Alternatively, the glycerol developed the strong association with phospholipid head groups during freezing and reduced membrane fluidity (Anchordoguy et al., 1987) better than DMSO in buffaloes. Another possible explanation probably lies in the toxicity of the combinations of penetrating cryoprotectants, as suggested for the freezing of ram spermatozoa (Molinia et al., 1994). Nevertheless, the

concentrations of DMSO and their additions either at 37 or 4°C were unable to draw differences in the plasma membrane integrity of buffalo spermatozoa.

The presence of acrosomal cap is important to assess the viability of a spermatozoon and has been related to fertility of the cryopreserved bull semen (Saacke and White, 1972). The present study showed better protection to the acrosomal cap in the presence of either 3 or 6% glycerol (42%) than without glycerol (30%). Indeed, variation in the post-thaw acrosome morphology among different concentrations of glycerol has been reported to be higher in 5 and 8% glycerol than in 2% glycerol in buffaloes (Ramarkrishnan and Ariff, 1994). In the current study, the spermatozoa frozen without glycerol have suggested that DMSO alone did provide some protection to the acrosomal cap during freezing and thawing. This is in agreement with the earlier work in bulls (Lovelock and Bishop, 1959), where this protection has been reported to be inferior to glycerol alone (Page, 1968; Snedeker and Gaunya, 1970). In this study, the acrosome morphology did not vary enough to account for differences among concentrations of DMSO, and temperatures of additions of glycerol or DMSO either at 37 or 4°C. It appears that acrosomal caps of buffalo spermatozoa are less sensitive to freezing and thawing than that of motility apparatus and plasma membrane. The greater capability of acrosomal cap to bare the stress of freezing and thawing has been observed in bulls (Anzar et al., 1997). Perhaps, thawing in the presence of lesser concentrations of glycerol decreased the protection to the acrosomal cap in buffaloes. The damage to the acrosomal cap due to thawing has been reported in bulls and rabbit spermatozoa (Bamba and Cran, 1988).

In brief, the addition of penetrating cryoprotectants may alter the morphology of spermatozoa. During cryopreservation, cells undergo changes in volume as penetrating cryoprotectants enter and leave the cell. This osmotic response can be lethal to the cells if it is beyond their tolerance limits (Willoughby et al., 1996). The osmotic tolerance limits for buffalo spermatozoa are needed to be established which would minimize cell volume excursion.

In summary, 1) different concentrations of DMSO alone or in combination with glycerol, either added at 37 or 4°C, did not improve the post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa in Tris based extender. 2) 6% glycerol alone when added at 37°C provided the maximum protection to the motility apparatus and membrane integrity of buffalo spermatozoa. 3)

cooling of semen to 4°C prior to the addition of glycerol is not essential for buffalo spermatozoa.

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