EFFECT OF DIFFERENT GLYCEROL CONCENTRATIONS ON MOTILITY BEFORE AND AFTER FREEZING, RECOVERY RATE, LONGEVITY AND PLASMA MEMBRANE INTEGRITY OF NILI-RAVI BUFFALO BULL SPERMATOZOA

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ABSTRACT

The present study as designed to observe the effects of different concentrations of glycerol on sperm motility before/after freezing and thawing, recovery rate, longevity and plasma membrane integrity in Nili-Ravi buffalo bull spermatozoa. Semen was collected and evaluated from four Nili-Ravi buffalo bulls in a standardized procedure. The ejaculates possessing more than 65% visual motility were selected and pooled at 37°C. Pooled samples were divided into eight aliquots of 0.5 ml each and extended (50 x 10^6 sperm/ml) in Tris-citric acid extenders (pH=7.0, OP=320 mOsmol/kg) at 37°C with different (2, 4, 5, 6, 7, 8, 10 or 12%) concentrations of glycerol. Semen was cooled to 4°C in 2 hours, equilibrated for 4 hours, packed in 0.5 ml French straws and frozen from +4°C to -15°C at the rate of 3°C per minute and fro. -15°C to -80°C @ 10°C/ minute in a programmable cell freezer. Semen straws were thawed at 37°C for 30 seconds after 24 hours of storage in liquid nitrogen at -196°C.

The motility before freezing did not differ due to the treatments. The motility after freezing did not differ among the extenders containing glycerol (%) concentrations either 5, 6, or 7. This was higher than that in extender containing glycerol (%) concentrations either 4, 8 or 10. Post thaw motility in extenders having glycerol (%) concentrations either 2 or 12 compared to all other treatments was the lowest. The recovery rate of spermatozoa (%) after freezing and thawing did not differ among the extenders containing glycerol (%) concentrations either 4, 5, 6, 7 or 8. This was higher ($P<0.05$) than that in extenders containing glycerol (%) concentrations either 2 or 10 and was lowest ($P<0.05$) in extender containing 12% glycerol. The mean sperm longevity (37 °C) at 2 hours was highest (32.6 ± 5.2, $P<0.05$) for 6, 7 or 8% glycerol, intermediate (13.9 ± 4.4, $P<0.05$) for 4, 5 or 10% and lowest (3.3 ± 2.5, $P<0.05$) for 2 or 12%. Plasma membrane integrity (%) of spermatozoa frozen in 7% glycerol (42.0 ± 1.3) was superior ($P<0.05$) to 5, 6, 8, 10 or 12% glycerol (29.6 ± 2.9). Plasma membrane integrity of Nili-Ravi buffalo bull spermatozoa was least protected ($P<0.05$), when frozen in 2 or 4% glycerol (10.4 ± 1.8). It is concluded that glycerol (%) concentrations of 6 or 7 in the extender may be suitable for crypreservation of Nili-Ravi buffalo bull spermatozoa.

Key words: Nili-Ravi buffalo-spermatozoa: motility, glycerol

INTRODUCTION

The empirical studies have indicated that many factors including composition of extender, cryoprotectants, and freezing and thawing process are involved in the retention of viability of cryopreserved spermatozoa (Hammersted *et al.*, 1990. Curry *et al.*, 1994). The need for control of ice crystal formation during freezing of the cell is of prime importance (Mazur, 1984). Many cryoprotectants like glycerol, DMSO and sugars are being used for this purpose (Fiser *et al.*, 1982). Among various cryoprotectants, glycerol has been extensively used for the cryopreservation of many types of cells, including the sperm. It reduces the 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 (Rasul, 2000), binds with the metallic ions (Lohmann *et al.*, 1964) and dehydrates the cell while preventing the extracellular media (Meryman, 1971).

It is generally accepted that 50% of the spermatozoa get damaged during the process of cryopreservation (Watson, 1979), which is mainly due to hyperosmotic effects 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 (Hammersted *et al.*, 1990). These effects, however, can differ among species. It has been shown that glycerol has a contraceptive effect upon the fertility of boar,

chicken and turkey spermatozoa (Hammersted and Graham, 1992). The lower fertility in buffaloes as compared to cattle with artificial insemination (Ala-ud-Din *et al*., 1990) may be due to this possible reason. During freezing, different concentrations of glycerol provide variable protection to spermatozoa for motility and fertility in bulls (Graham *et al*., 1958).

The present study was designed to test and optimize the concentration of glycerol added to semen extender as a cryoprotectant, for the freezing of Nili-Ravi buffalo bull spermatozoa. The objective of this study was to compare the motility before and after freezing and thawing, recovery rate, longevity and plasma membrane integrity at eight different concentrations of glycerol.

MATERIALS AND METHODS

Preparation of extender

Tris-citric acid (TCA) was used as a buffer for the experimental extenders, which consisted of 1.56g citric acid (Fluka, Switzerland) and 3.0g Tris-hydroxymethol-aminomethane (Sigma, St. Louis) in 74 ml distilled water. The pH of the buffer was 7.0 and the osmotic pressure was 320 mOsmol/kg. The TCA was divided into eight aliquots. Egg yolk (20%; vol/vol) and glycerol (2, 4, 5, 6, 7, 8, 10 or 12%, vol/vol; Merck, Germany) were added to each of the experimental extenders. Fructose (0.2%; w/v; Merck, Germany), penicillin (1000I.U/ml), and streptomycin (100 µg/ml) were added to each extender. The extenders were centrifuged at 12000 x g for 15 minutes, the supernatant was frozen and stored at –20°C. The extenders were thawed at 37°C before experimental use.

Semen collection and initial evaluation

Four Nili-Ravi bufalo bulls maintained at the Livestock Research Station, National Agricultural Research Center, Islamabad were used for this study. Semen from these bulls was collected once a week with pre-warmed (42°C) artificial vagina for six consecutive weeks during the months of August and September. Visual motility was assessed by using a phase contrast microscope (400X) attached with a closed circuit monitor. The semen ejaculates exhibiting more than 65% fresh visual motility were pooled to have sufficient volume for one replicate and to eliminate the individual bull effect. Sperm concentration was assessed by digital-photometer at 560 nm. Pooled semen was given a holding time of 15 minutes at 37°C in the water bath before dilution.

Semen processing

Pool semen was diluted (50 x 10^6 sperm/ml) at 37°C with each extender (n=8). The diluted semen was cooled to 4°C in 2 hours then allowed to equilibrate at 4 °C for 4 hours before freezing. Filling of semen in 0.5 ml polyvinyl French straws was carried out with suction pump just before freezing at 4°C in the cold cabinet unit (Minitub, Germany). The freezing was performed in a programmable cell freezer (KRYO 10, Series III Planer, UK). The freezer was programmed to supercool the semen filled straws from +4 to –15°C @ 3°C minute^{-1} and then to –80°C at the rate of 10°C minute^{-1}. Frozen semen straws were immediately plunged into liquid nitrogen canes (-196°C) for storage before evaluation. After 24 hours, semen straws were thawed at 37°C for 30 seconds for assessment of post-thaw semen quality.

Assessment of visual motility and longevity

A drop of semen was placed on a pre-warmed glass slide and coverslipped. Visual motility (%) of spermatozoa was assessed by microscope (400X), stage temperature 37°C , with closed circuit television.

Visual motility of the frozen thawed samples was assessed by incubating the semen samples at 37°C. The longevity of samples was checked after every hour till the time when the motility decreased to its lowest.

Plasma membrane integrity

Sperm plasma membrane integrity was assessed by hyposmotic swelling (HOS) assay, as described earlier (Jeyendran *et al*., 1984). The solution of HOS contained sodium citrate 0.73 gm (Merck, Germany) and fructose 1.35 gm (Merck, Germany), dissolved in 100 ml distilled water (osmotic pressure –190 mOsmol/kg). The assay was performed by mixing 50 µl of frozen-thawed semen sample to 500 µl of HOS solution and incubating it at 37 °C for 30 minutes. After incubation, a drop of semen sample was examined under phase contrast microscope (400X). One hundred spermatozoa were counted for their swelling characterized by coiled tail indicating intact plasma membrane.

Statistical analysis

Means (± SEM) were computed for various parameters. Data for each variable was analyzed by the analysis of variance ($P<0.05$). Fisher's LSD test was used to compare variable means (SYSTAT, 1996).

RESULTS AND DISCUSSION

Effects of different glycerol concentrations on the motility before and after freezing and thawing, recovery rate, longevity and plasma membrane integrity of Nili-

Table 1: Effect of different glycerol concentrations on the motility before and after freezing and thawing, recovery rate, longevity, and plasma membrane integrity in Nili-Ravi buffalo spermatozoa.

Glycerol Concentrations (%)	Motility		[1]Recovery rate (%)	[2]Longevity (%)	Plasma membrane integrity (%)
	Before freezing	After freezing and thawing			
2	60.0 ± 2.9^{a}	36.7 ± 3.3 ^{ade}	60.9 ± 3.5^{abe}	3.3 ± 1.7^{a}	6.0 ± 1.2^{a}
4	56.7 ± 1.7^{a}	46.7 ± 4.4 ^{acd}	82.8 ± 9.6^{acd}	15.0 ± 2.9^{b}	14.8 ± 2.3^{ab}
5	61.7 ± 3.3^{a}	50.0 ± 2.9 ^{bcf}	82.0 ± 9.3^{acd}	13.3 ± 4.4^{b}	32.0 ± 2.3^{c}
6	63.3 ± 1.7^{a}	61.7 ± 1.7^{b}	97.4 ± 2.6^{d}	32.4 ± 3.3^{c}	31.9 ± 2.9^{c}
7	60.0 ± 0.0^{a}	56.7 ± 6.0 ^{bc}	91.7 ± 8.3^{cd}	37.0 ± 2.2^{c}	42.0 ± 1.3^{d}
8	60.0 ± 2.9^{a}	48.3 ± 4.4 ^{acd}	80.7 ± 7.3^{acd}	28.3 ± 1.0^{c}	30.8 ± 2.9^{c}
10	60.0 ± 2.9^{a}	43.3 ± 3.3^{df}	72.1 ± 2.9^{ce}	13.3 ± 6.0^{b}	27.4 ± 3.9^{c}
12	53.3 ± 3.7^{a}	25.0 ± 7.6^{a}	47.3 ± 14.6^{b}	3.3 ± 3.3^{a}	25.9 ± .2.5^{bc}

$^{a-f}$ Values (mean ± SEM based on six replicates) within a column with different superscripts differ significantly ($P<0.05$).

1 Recovery rate (%)= (Motility after freezing/Motility before freezing) x 100.

2 Longevity (%) = Sperm longevity (37 °C) at 2 hours.

Ravi buffalo spermatozoa are presented in Table 1. The motility before freezing did not differ due to the treatments (different concentrations of glycerol). The motility after freezing and thawing did not differ among the extenders containing glycerol (%) concentrations either 5, 6, or 7 and averaged 56.1 ± 3.5% ($P<0.05$). This post thaw motility was higher ($P<0.05$) than that in extender containing glycerol (%) concentrations 4, 8 or 10 which averaged 46.1 ± 4.0%. The post thaw motility in extenders having glycerol (%) concentrations either 2 or 12 compared to all other treatments averaged 30.9 ± 5.4% and was the lowest. The recovery rate of spermatozoa (%) after freezing and thawing did not differ among the extenders containing glycerol (%) concentrations either 4, 5, 6, 7 or 8 and averaged 86.9 ± 7.4. This was higher ($P<0.05$) than that in extenders containing glycerol (%) concentrations either 2 or 10 (66.5 ± 3.2) and was lowest ($P<0.05$) in extender containing 12% glycerol (47.3 ± 14.6). The mean sperm longevity (37°C) at 2 hours was highest (32.6 ± 5.2%, $P<0.05$) in extenders containing 6, 7 or 8% glycerol, intermediate (13.9 ± 4.4%, $P<0.05$) in those with 4, 5 or 10% glycerol and lowest (3.3 ± 2.5%, $P<0.05$) in those having 2 or 12% glycerol. Plasma membrane integrity (%) of Nili-Ravi buffalo spermatozoa frozen in 7% glycerol (42.0 ±1.3) was superior ($P<0.05$) to 5, 6, 8 or 10% glycerol (30.5 ± 3.0). The plasma membrane of the spermatozoa was least protected ($P<0.05$), when frozen in 2 or 4% glycerol (10.4 ± 1.8%).

While attempting to optimize the levels of glycerol for cryopreservation of Nili-Ravi buffalo bull spermatoxoa, our results show better post thaw motility (%) and recovery rate (%) of spermatozoa at glycerol (%) concentrations 5, 6 and 7, and are consistent with the earlier studies in bovines (Garcia and Graham, 1987; Kumar *et al.*, 1994). This shows that freezing of semen in the presence of 6% glycerol resulted in sperm motility significantly higher than that of spermatozoa frozen in 3% glycerol. In our study, the motility (longevity) of spermatozoa stored at 37°C after thawing was better in extenders containing glycerol (%) concentrations either 6, 7 or 8 than extenders containing glycerol (%) concentrations either 4, 5 or 10. This was lowest in the extenders having glycerol (%) concentrations either 2 or 10. Longevity (%) has a significant relationship with the conception rate in buffaloes (Rasul, 2000).

In the current study, integrity of plasma membrane of Nili-Ravi buffalo bull spermatozoa was cryoprotected best with 7% glycerol concentration as compared to 5, 6, 8 or 10% glycerol levels, while it was least protected with 2 or 4% glycerol concentrations in the extender. This pattern of cryoprotection of the functional integrity of plasma membrane of buffalo bull spermatozoa is in agreement with the earlier work of Rasul (2000), where this protection to plasma membrane during freezing was highest with 6% glycerol than with 3 or 0% glycerol levels in TCA extender.

In brief, the addition of penetrating cryoprotectants may alter the integrity of plasma membrane of spermatozoa during cryopreservation. 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 Nili-Ravi buffalo bull spermatozoa are needed to be established which would minimize plasma membrane destruction that occurs during freezing.

In summary, glycerol concentrations of 6 or 7% in the TCA extender may be the optimal for cryopreservation of Nili-Ravi buffalo bull spermatozoa.

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