EFFECT OF REDUCING THE NUMBER OF SPERM CELLS (PER INSEMINATION), INCREASING ENERGY AND CRYOPROTECTING CONCENTRATIONS ON MOTION CHARACTERISTICS AND MEMBRANE INTEGRITY IN FROZEN THAWED BUFFALO SPERMATOZOA

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ABSTRACT

The objective of this study was to find out the minimum number of sperm cells per dose of insemination that will not affect the conception rate, if levels of egg yolk and glycerol are increased. For this purpose, sperm motion characteristics and plasma membrane integrity in three concentrations of sperm cells per dose, each with two levels of egg yolk and glycerol were compared. Semen was collected from buffalo bulls using an artificial vagina. Split pool ejaculates possessing more than 60% visual sperm motility were diluted in Tris-citric acid (TCA) extender at 37°C, either in (1) 30-6-20 (Number of sperm cells in millions/0.5 ml insemination dose–Glycerol%-Egg yolk %), (2) 15-6-20, (3) 7.5-6-20, (4) 30-10-20, (5) 15-10-20, (6) 7.5-10-20, (7) 30-6-30, (8) 15-6-30, (9) 7.5-6-30, (10) 30-10-30, (11) 15-10-30 and (12) 7.5-10-30. Semen was cooled to 4°C in 2 hours, equilibrated at 4°C for 4 hours, filled in 0.5 ml straws and frozen in a programmable cell freezer (+4 to −15°C@-3°C/minute and then to -80°C@-10°C/minute) before plunging them into liquid nitrogen (-196°C). Thawing of frozen semen was performed after 24 hours at 37°C for 15 seconds. Sperm motion characteristics, including motilities (computer-assisted, linear and circular), velocities (straight-line, average path and curvilinear), and lateral head displacement (LHD) were assessed using computer assisted semen analyzer (CASA). Plasma membrane integrity was determined by using Hypo-Osmotic Swelling assay (HOS). Analysis of variance revealed that visual motility, computer–assisted motility, linear motility, circular motility, and plasma membrane integrity did not vary significantly when cell number was reduced from $30x10^6$/dose to $15x10^6$/dose. However, visual motility, computer-assisted motility and plasma membrane integrity were reduced significantly ($P<0.05$) when cell number was decreased to $7.5x10^6$/dose. The velocities (straight-line, average path, curvilinear) and LHD did not vary significantly due to the reduction in number of sperm cells/dose, levels of glycerol and egg yolk. In conclusion, reduction of sperm number to $15x10^6$ cells/dose did not affect sperm motion characteristics and plasma membrane integrity at both levels of glycerol and egg yolk, but when reduced to $7.5x10^6$/dose, although linear and circular motilities, velocities and LHD were not affected, but visual and computer-assisted motilities, and plasma membrane integrity were reduced ($P<0.05$).

Keywords: Buffalo-spermatozoa; sperm numbers, glycerol, egg yolk

INTRODUCTION

The demand for semen from genetically superior sires for improving the dairy potential has made it necessary that a minimum number of frozen sperm cells be established so that the number of doses/bull can be increased to get maximum fertility of each insemination (Jondet, 1972). It is economically and biologically important that only semen with a high probability of success in terms of impregnation be processed and distributed. Besides genetic improvement, fertility is also one of the most important consideration in artificial insemination (AI), and no fewer sperms per insemination unit should be used which are essential (Salisbury *et al.*, 1978).

The influence of number of sperm cells per insemination studied in bulls revealed that $5\text{-}15x10^6$ progressively motile spermatozoa are necessary to reach an acceptable level of fertility (Sullivan, 1970). Usually the cell number varies from $10\text{-}30x10^6$/dose with varying results and in some studies even $5\text{-}20x10^6$ sperms/dose has also been analysed in cattle (Schenk *et al.*, 1987). Recent reports suggest that with un-frozen bull semen, having sperm concentration of $1x10^6$/ml or

$2.5x10^6$/ml can even produce reasonable pregnancy rates (Allen and Seidel, 1996). However, the effect of this level of semen dilution on sperm viability has not been adequately examined.

Many cryoprotectants are being used like glycerol, DMSO and sugars e.g. disaccharides or trisaccharides (Fiser *et al.*, 1982). Among these, glycerol is the most widely used cryoprotectant for cattle and buffalo bull spermatozoa, functioning on the hypothesis of "salt-buffering" mechanism (Rasul, 2000). Presumably, glycerol dehydrates the cell (Berndtson *et al.*, 1981) and forms complexes with metallic ions (Meryman, 1971). Furthermore, it reduces the concentration of extracellular media, thermal stress and prevents fracture in the frozen solutions by decreasing the total ice volume expansion during water solidification (Gao *et al.*, 1995). Thus glycerol prevents the spermatozoa from mechanical destructiveness during the deep freezing process.

Egg yolk is added in bull semen extenders mainly as an energy source as it provides the sperm cells with lecithin, proteins, lipo-proteins and similar complexes (Semenova, 1987). The development of more complete buffers to combine with egg yolk and a reduction in its volume from 50% to 20% improved sperm survivability (Salisbury *et al.*, 1978).

The present study was designed to test the hypothesis that by reducing the number of sperm cells and increasing the supplementation of energy source (egg yolk) and cryoprotectant (glycerol) will not adversely affect motilities and membrane integrity of buffalo bull spermatozoa. The objective of this study was to compare the motion characteristics and plasma membrane integrity in three concentrations of sperm cells per dose, each with two levels of glycerol and egg yolk.

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) and 3.0g Tris-(hydroxymethyl)-aminomethane (Sigma) in 74ml distilled water. The pH of the buffer was 6.8 and the osmotic pressure was 320 mOsmol/kg. The TCA was divided into twelve aliquots. Egg yolk (20% or 30%; vol/vol) and glycerol (6% or 10%; vol/vol; Merck) were added to each of the experimental extenders. Fructose (0.2%; w/v; Merck), penicillin (1000 I.U/ml), and streptomycin (100 µg/ml) were added to each of the formulated extender. The extenders were centrifuged at 12000 x g for 15 minutes, and 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 adult Nili-Ravi buffalo bulls, maintained at the Livestock Research Station, National Agricultural Research Centre, Islamabad, were used in the study. Two consecutive ejaculates were collected with the help of artificial vagina at weekly intervals for 6 weeks during the months of July and August 2000. The semen ejaculates, exhibited more than 60% of 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

Pooled semen was diluted at 37°C with each extender (n=12) in order to provide approximately $30x10^6$, $15x10^6$ and $7.5x10^6$ spermatozoa/0.5ml. Each dilution rate had two levels of egg yolk and two levels of glycerol as mentioned above. The diluted semen was cooled to 4°C in 2 hours and 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. The freezing was performed in a programmable cell freezer. 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}. Frozen semen straws were immediately plunged into liquid nitrogen canes (-196°C) and stored for 24 hours before they were thawed at 37°C for 15 seconds.

Semen assays

After 5 minutes of incubation at 37°C, the semen quality was evaluated by various semen assays for post-thaw semen quality assessment.

Visual motility assessment

A drop of semen was placed on a pre-warmed glass slide, cover slipped and examined under microscope (400X).

Sperm motion characteristics

A computer-assisted semen analyzer was used for precise quantification of sperm motion characters as recently described in buffalo bulls from our laboratory (Rasul *et al.*, 2000). A drop of semen was placed on Makler chamber and analyzed for motility (%), linear motility (%), circular motility (%), straight-line velocity (µm/second), average path velocity (µm/second), curvilinear velocity (µm/second) and lateral head displacement (LHD, µm).

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) and fructose 1.35 gm (Merck) 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 incubated 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

Analysis of variance (ANOVA) was used to assess differences due to treatments on motion characteristics and plasma membrane integrity. Tukey's HSD test was used to compare treatment means where necessary (SYSTAT, 1996).

RSULTS AND DISCUSSION

The effects of number of sperm cells, levels of glycerol and egg yolk on motion characteristics and plasma membrane integrity in frozen thawed buffalo spermatozoa are presented in Tables 1 and 2. The effect of cell number was significant ($P<0.05$) on visual motility (VMOT), computer-assisted motility (CMOT), and plasma membrane integrity (PMI). The overall means of VMOT, CMOT and PMI reduced significantly ($P<0.05$), when the cell number was decreased to $7.5x10^6$/dose. However, cell number did not affect the linear motility (LMOT), circular motility, velocities (straight-line, average path, curvilinear) and LHD of buffalo spermatozoa. The effects of egg yolk and glycerol at different levels on VMOT, CMOT, LMOT, circular motility, PMI, velocities and LHD were non-significant. There was no interaction of cell number x egg yolk, cell number x glycerol, egg yolk x glycerol and cell number x egg yolk x glycerol.

In our study, VMOT and CMOT did not differ when the cell number was reduced from $30x10^6$/dose to $15x10^6$/dose. These results are similar to those of Nadir *et al.* (1993), who examined two sperm concentrations i.e., 20 or $10x10^6$spermatozoa/0.5ml with their effect on post thaw quality and fertility, and concluded that the higher dose had better motility and significantly improved conception rate in cattle. The percentage of post-freeze motility of bull spermatozoa has been reported to decrease when the ejaculate was initially diluted to $250x10^6$ spermatozoa/ml and finally extended to $20x10^6$ spermatozoa/ml in either yolk-citrate or yolk-Tris extender (Benson *et al.*, 1968). However, in the present study the LMOT and circular motility were similar when the cell number was even reduced to $7.5x10^6$spermatozoa/0.5ml. The reason might be that the ejaculate volume presumably affected the post thaw sperm viability due to seminal plasma component, while concentration may affect survivability due to some factor present in spermatozoa themselves, as suggested by Graham and Schmehl (1984). This concept supports the idea that increase in the cryoprotective agent alongwith energy supply may provide similar results even if the sperm number is reduced.

The swelling ability of frozen-thawed spermatozoa was affected in this experiment when sperm concentration was reduced to $7.5x10^6$/dose. This decline in the functional integrity of buffalo spermatozoa can be associated with the greater release of glutamic oxalacetic tranaminase in the surrounding media due to the increased levels of cryoprotectant (Rasul, 2000).

The pattern of sperm cell movement is sensitive to the chemical and physical properties of the medium in which they are suspended (Rasul *et al.*, 2000). The mean velocities (straight-line, average path and curvilinear) and LHD did not differ in this study, either due to reduction in the number of sperm cells or levels of glycerol and egg yolk.

The results for glycerol levels of present study are in accordance with that of Abbas and Andrabi (2001), who concluded that a large number of Nili-Ravi buffalo bull spermatozoa survived during freezing in diluent containing 6 or 7% glycerol. Fiser *et al.* (1982) reported that more than 8% glycerol was toxic to ram spermatozoa, which is in contrast with the present study where glycerol level upto 10% was not detrimental to post freeze quality of buffalo spermatozoa. In one of the recent studies by Rasul (2000), post thaw motility of buffalo spermatozoa was significantly higher in the presence of 6% glycerol as compared with the spermatozoa frozen in 3% glycerol or did not survive freezing in the absence of glycerol. As far as the egg yolk levels are concerned, Salisbury *et al.* (1978) suggested that 10-20% egg yolk concentration is required for optimum post thaw sperm motility in bulls. Semenova (1987) added 15% egg yolk to the buffer and Naz *et al.* (1990) preferred 10% over 15% egg yolk for long term preservation of ram spermatozoa. Ali *et al.* (1994) also reported that 10% egg yolk in TCA extender gave higher lambing rate. These results are in contrast to the present study where no effect of levels of egg yolk was found alongwith reduction in cell number/dose. Moreover, there was no interaction between different levels of egg yolk and glycerol used.

In summary, reduction of sperm number from $30x10^6$ cells/dose to $15x10^6$ cells/dose did not affect sperm motion characteristics and plasma membrane integrity at both levels of glycerol and egg yolk, but

Table 1: Effect of number of sperm cells, levels of glycerol and egg yolk on post thaw visual motility, computer assisted motility, linear motility, circular motility and plasma membrane integrity in buffalo spermatozoa (Mean±SEM).

	Cell No.	30 (x 10^6/dose)				15 (x 10^6/dose)				7.5 (x 10^6/dose)			
	Glycerol (%)	6		10		6		10		6		10	
Variable	Egg Yolk (%)	20	30	20	30	20	30	20	30	20	30	20	30
VMOT(%)		43±3	30±6	40±7	31±5	33±7	32±5	26±5	40±6	22±5	23±5	18±3	31±6
	Mean	36±5a				33±6				24±5 b			
CMOT(%)		41±6	47±10	44±10	29±9	36±9	34±7	35±8	31±9	20±4	24±10	18±6	26±7
	Mean	40±8a				34±8 ab				22±7 b			
LMOT(%)		42±4	54±5	46±7	38±6	39±9	44±8	48±4	39±7	37±5	41±2	35±8	43±3
	Mean	45±6				43±7				39±5			
CiMOT(%)		21±4	19±6	23±9	14±7	29±7	21±9	25±5	27±3	15±8	26±7	24±9	26±4
	Mean	19±7				25±6				23±7			
PMI(%)		40±7	33±2	48±6	43±7	38±8	42±5	39±5	37±6	31±9	30±7	24±4	20±5
	Mean	41±5				39±6 a				26±6 b			

Mean values in same row with different superscripts differ significantly (P<0.05)
VMOT= Visual motility, CMOT= Computer assisted motility; LMOT = Linear motility; CiMOT = Circular motility and PMI = Plasma membrane integrity.

Table 2: Effect of number of sperm cells, levels of glycerol and egg yolk on post-thaw velocities, and lateral head displacement of buffalo spermatozoa (Mean±SEM).

	Cell No.	30 (x 10^6/dose)				15 (x 10^6/dose)				7.5 (x 10^6/dose)			
	Glycerol (%)	6		10		6		10		6		10	
Variable	Egg Yolk (%)	20	30	20	30	20	30	20	30	20	30	20	30
VSL(µM/s)		38±2	35±4	41±4	31±2	43±2	44±6	39±2	40±6	43±4	41±4	33±7	43±4
	Mean	36±3				42±4				40±0			
VAP(µM/s)		59±3	53±4	53±3	47±3	59±4	59±5	52±3	54±3	63±4	54±5	56±12	57±3
	Mean	53±3				56±4				58±6			
VCL(µM/s)		107±5	97±4	94±5	86±7	109±9	94±8	93±7	93±8	109±8	92±8	109±24	96±6
	Mean	96±5				97±8				109±24			
LHD (µM)		6±0	6±1	4±0	5±1	5±0	4±1	4±0	4±1	6±0	4±0	7±2	4±0
	Mean	5±0.5				4±0.5				5±0.5			

Values in same row did not differ signif332icantly (P<0.05)
VSL = Straight-line velocity; VAP = Average path velocity; VCL = Curvilinear velocity and LHD = Lateral head displacement.

when reduced to 7.5x10^6/dose, although linear and circular motilities, velocities and LHD were not affected, visual and computer-assisted motilities, and plasma membrane integrity were reduced (P<0.05).

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