Short Communication

The Journal of Animal & Plant Sciences, 24(4): 2014, Page: 1256-1259
ISSN: 1018-7081

THIOGLYCOL IN EXTENDER IMPROVES THE POST-THAW QUALITY OF BUFFALO (*BUBALUS BUBALIS*) BULL SPERMATOZOA

M. S. Ansari, B. A. Rakha[*], S. M. H. Andrabi[**], N. Ullah[**], R. Iqbal, W. V. Holt[***] and S. Akhter[**]

Department of Zoology, University of Gujrat – Gujrat, Pakistan
[*]Department of Wildlife Management, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, 46300, Pakistan
[**]Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, 46300, Pakistan,
[***]Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK
Corresponding Author: Email: sashraf1993@gmail.com

ABSTRACT

This study was designed to evaluate the effect of thioglycol in extender on post-thaw quality of Nili-Ravi buffalo (*Bubalus bubalis*) bull spermatozoa. Semen from three buffalo bulls was collected at weekly intervals (three replicates; two ejaculates per bull per replicate) and qualifying ejaculates (n=18) were cryopreserved in extenders containing thioglycol at 0.0 or 0.5 or 1.0mM. Sperm motility (%; visually), plasma membrane integrity, (%;supravital hypo-osmotic swelling test), viability (%;dual staining technique using Trypan blue and Giemsa stains) and DNA integrity (%;acridine orange test) was assessed at 0, 2 and 4 hour post-thaw. Sperm motility, plasma membrane integrity and viability of buffalo bull spermatozoa were improved in a dose dependent manner with the supplementation of thioglycol at 0.5 mM and 1.0mM compared control. Sperm DNA integrity was similar (P>0.05) in extenders containing 0.5 mM and 1 mM thioglycol that remained higher ($P < 0.05$) than the values of DNA integrity in control. It is concluded that 1.0mM thioglycol in extender improved the motility, plasma membrane integrity, viability and DNA integrity of buffalo bull spermatozoa.

Keywords: Buffalo bull spermatozoa; thioglycol; cryopreservation

INTRODUCTION

Excessive generation of reactive oxygen species (ROS) molecules are evident during cryopreservation of mammalian semen that reduces the viability and fertilization capacity of the spermatozoa (Bilodeau *et al.,* 2000). ROS molecules can cause damage to sperm motility, plasma membrane, acrosomal and DNA integrity (Aitken *et al.,* 1998; Bilodeau *et al.,* 2001; Lenzi *et al.,* 2002; Kumar *et al.,* 2011).

Buffalo sperm plasma membrane have high content of polyunsaturated fatty acids than cattle bull spermatozoa that makes it highly susceptible to the oxidative stress during freeze/thawing process due to the presence of double bonds (Parks *et al*. 1987; Cheshmedjieva and Dimov 1994; Lenzi *et al.,* 2002; Andrabi, 2009). Buffalo semen is equipped with endogenous antioxidant system consisted of enzymatic and non-enzymatic antioxidative agents but this is not sufficient for sperm protection during cryopreservation (Kumar *et al.,* 2011). It suggests that antioxidant supplementation is necessary for protection against ROS-mediated damage/stress in buffalo semen.

Thioglycol, a low molecular weight thiol compound with a reducing power can interact directly with oxidized radicals. It protects cysteine, a precursor of glutathione, from oxidation into cystine and increases its entry into the sperm cells, which are known to trigger glutathione synthesis. Increased concentration of glutathione decreases the occurrence of reactive oxygen species and increases sperm motility and viability (de Matos *et al*., 2002; Feugang *et al*., 2004). It was hypothesized that thioglycol addition in semen extender may improve the post-thaw quality of buffalo bull semen. Therefore, this study was designed to evaluate the effect of thioglycol in extender on quality (motility, plasma membrane integrity, viability and DNA integrity) of buffalo bull spermatozoa at 0, 2 and 4 h post thaw.

MATERIALS AND METHODS

Preparation of extenders: The stock extender was consisted of 1.56 g citric acid, 3.0 g *tris*–(hydroxymethyl)-aminomethane, 0.2 g fructose, 7.0 mL glycerol and 20 mL egg yolk in 73 mL distilled water. Three experimental extenders were prepared by adding thioglycol 0.0, 0.5 or 1.0mM in stock extender.

Collection and initial evaluation of semen: Semen was collected from three Nili-Ravi buffalo bulls (*Bubalus bubalis*) of known fertility and similar age (7–8 years) at weekly intervals (three replicates; two ejaculates per bull per replicate) with artificial vagina (42°C) during summer 2010. Sperm progressive motility (%) was assessed (200X) with phase contrast microscope. Sperm

concentration was measured with bovine photometer ACCUCELL (IMV, France). Qualifying semen ejaculates (n=18) [motility >60%, volume >1.0 mL, concentration >0.5 billion/mL] were processed separately and split into three aliquots for dilution in different experimental extenders.

Processing of semen: Semen aliquots were diluted with one of the three experimental extenders at the rate 50×10^6 motile spermatozoa/mL approximately at 37°C, ~~and~~ cooled to 4°C in 2 hours and equilibrated for 4 h at 4°C. Cooled semen was filled in 0.5 mL French straws with suction pump at 4°C in the cold cabinet unit and kept on liquid nitrogen vapors for 10 minutes. Straws were then plunged into liquid nitrogen (-196°C) for storage. After 24 hours of storage, straws were thawed at 37°C for 30 seconds in water bath and then incubated for 4 hours at 37°C for post thaw semen quality assays. The duration of motility and other sperm characteristics during the post-thaw incubation in an environment that mimic the conditions within the female reproductive tract is an indication of the usability of the semen (Saacke and White, 1972; Kumar *et al*., 2009). The experiment was repeated three times for each bull.

Post-thaw semen quality assays: Sperm motility was assessed as described earlier. **Sperm plasma membrane integrity (**Functional and structural**)** was assessed with supra-vital hypo-osmotic swelling test (HOST). The HOS solution consisted of ~~(~~sodium citrate 0.735 g and fructose 1.351 g/ 100 ml distilled water~~)~~. An aliquot (50µL) of semen was added to the HOS (500µL) solution and incubated for 30 min in water bath at 37 °C (Ansari *et al.,* 2010). A droplet (5µL) of Eosin [0.5% (w/v) sodium citrate 2.9%] was mixed with a drop (5µL) of semen on slide for 10 seconds. A cover slip was placed on the mixture and evaluated using phase contrast microscopy (400x). A total of 100 spermatozoa per preparation were observed in at least five different fields. Clear heads and tails and swollen tails indicated intact, biochemically active sperm membrane~~s~~, while pink heads and unswollen tails indicated disrupted, inactive sperm membrane~~s~~ (Tartaglione and Ritta, 2004). **Sperm viability** was assessed by dual staining procedure with Trypan blue-Giemsa stain as described by (Kovacs and Foote, 1992). Smears were made by taking equal drops of Trypan-blue and semen sample, quickly mixed and air dried. Slides were fixed with formaldehyde-neutral red for 5 minutes, rinsed with distilled water and kept in 7.5% Giemsa stain for 4 hours. After rinsing and air-drying, cover slips were mounted with Balsam of Canada and two hundred spermatozoa were evaluated in each smear with phase contrast microscope at 1000x. Trypan-blue penetrates non-viable, dead spermatozoa with disrupted membrane, which appeared stained in blue, while live, intact spermatozoa appeared unstained. Giemsa accumulates in spermatozoa with an intact acrosome, staining the acrosome region in purple. **Sperm DNA integrity** was assessed using acridine orange assay (Martins *et al.,* 2007; Tejada *et al.,* 1984). Smears of semen were prepared on glass slides, air-dried and fixed for overnight in Carnoy's solution (methanol and glacial acetic acid in a 3:1 proportion). The slides were air-dried and incubated in tampon solution (80 mmol/L citric acid and 15 mmol/L Na_2HPO_4, pH 2.5) at 75°C for 5 minutes to test DNA integrity. Then slides were stained with acridine orange stain (0.2 mg/mL). Stained slides were washed with water to remove background staining; while still wet, covered with cover slips and evaluated with a epifluorescence microscope (480/550 nm excitation/barrier filter). One hundred cells were analyzed of each semen sample. Sperm~~s~~ with normal DNA content presented green whereas those with an abnormal DNA content presented fluorescence that vary from yellow-green to red in spectrum.

Statistical analysis: The data (Mean ± SE) on the effect of thioglycol in extender on semen quality parameters were analyzed by the analysis of variance (ANOVA) using two factor (time x treatment) factorial design in RCBD. When the F–ratio found significant ($P < 0.05$), Duncan's multiple range test was used to compare different treatment means.

RESULTS

The data on the effect of thioglycol supplementation in extender on motility, plasma membrane integrity, viability and DNA integrity of buffalo bull spermatozoa at 0, 2 and 4 hours post thaw are presented in Table 1. The analysis in two factor factorial design under RCBD revealed non-significant interaction (Incubation time x thioglycol levels) for sperm motility, plasma membrane integrity, viability and DNA integrity of buffalo bull spermatozoa. The bull effect on motility, plasma membrane integrity and viability was found non significant (P>0.05) while it remained significant (P<0.05) in terms of DNA integrity. Bull 1 (91.33) was better to maintain DNA integrity compared to bull 2 (90.11) and bull 3 (90.11). Post thaw sperm motility, plasma membrane integrity and viability (live sperm with intact acrosome) of buffalo semen was improved in dose dependent manner ($P < 0.05$) with the supplementation of thioglycol in extender at 0.5 mM and 1.0 mM compared to control at ~~at~~ 0, 2 and 4 hour post-thaw. The DNA integrity of buffalo bull spermatozoa was better maintained ($P < 0.05$) in extenders containing thioglycol at 0.5 mM and 1.0 mM compared to control at 0, 2 and 4 hour post-thaw.

Table No. 1: Effect of thioglycol addition in semen extender on percentage motility, ~~intact~~ plasma membrane integrity, viability and DNA integrity of buffalo bull spermatozoa at 0, 2 and 4 hours after thawing.

Post-thaw Hours	Thioglycol (mM)	Motility	Plasma Membrane Integrity	Viability	DNA Integrity
0	0.0	41.67 ± 2.9^{c}	37.3 ± 4.5^{c}	59.7 ± 0.6^{c}	85.3 ± 1.5^{d}
	0.5	51.67 ± 2.9^{b}	47.7 ± 3.1^{b}	69.3 ± 1.5^{b}	97.0 ± 1.0^{a}
	1.0	63.33 ± 2.9^{a}	61.0 ± 2.6^{a}	82.0 ± 1.0^{a}	97.3 ± 1.5^{a}
2	0.0	26.7 ± 2.9^{e}	23.7 ± 3.8^{d}	44.7 ± 2.9^{e}	82.3 ± 1.5^{e}
	0.5	36.7 ± 2.9^{d}	34.7 ± 3.8^{c}	55.7 ± 3.5^{cd}	94.3 ± 0.6^{b}
	1.0	48.3 ± 2.9^{b}	45.3 ± 2.5^{b}	66.3 ± 3.8^{b}	94.3 ± 0.6^{b}
4	0.0	13.33 ± 2.9^{f}	11.00 ± 2.0^{e}	30.33 ± 3.8^{f}	80.00 ± 1.0^{f}
	0.5	25.00 ± 0.0^{e}	24.33 ± 5.1^{d}	42.67 ± 0.6^{e}	92.00 ± 0.0^{c}
	1.0	33.33 ± 2.9^{d}	33.67 ± 6.0^{c}	52.33 ± 2.9^{d}	92.00 ± 1.0^{c}

Means with different letters show significant ($P < 0.05$) differences down the column throughout the incubation period.

DISCUSSION

Chilling/cryopreservation of buffalo semen resulted in decreased antioxidant activity and higher ROS molecules, decrease in motility, plasma membrane integrity and Viability (El-Sissy *et al.,* 2007; Anzar *et al.,* 2010; Kumar *et al.,* 2011). The buffalo semen is naturally equipped with endogenous antioxidant defence system against ROS, however this system becomes insufficient for cryopreserved semen. Thioglycol is one of the naturally occurring antioxidant which in turn play role in the regulation of this defence system within the cell (de Matos *et al*., 2002; Feugang *et al*., 2004). The results of present study suggest that post thaw sperm motility, plasma membrane integrity and viability of buffalo semen was improved in dose dependent manner ($P < 0.05$) with the supplementation of thioglycol in extender at 0.5 mM and 1.0 mM compared to control at at 0, 2 and 4 hour post-thaw. This is evident from the various studies that thioglycol in maturation media enhances the ATP metabolism in the oocyte, protect the oocyte/embryo viability by directly interacting with oxidized radical, increasing intracellular glutathione levels in oocytes and improved the blastocyst quality (Takahashi *et al.,* 2002; Tsuzuki *et al.,* 2005). Thiogylcol has been reported to protect the ~~sperm~~ motility of bovine spermatozoa, ~~sperm~~ bio-membrane system by reducing lipid peroxidation ~~system~~, sperm viability with similar mechanism by increasing the levels of glutathione in semen, that is reported to reduce the acrosomal damage due to ROS production (Bilodeau *et al.,* 2001; Feugang *et al.,* 2004; Ansari *et al.,* 2010; Ansari *et al.,* 2011).

DNA damage during cryopreservation can lead to reduced fertilization potential of the mammalian spermatozoa (Aitken *et al.,* 1998; Andrabi, 2009; Bucak *et al.,* 2010). In present study, DNA integrity of buffalo bull spermatozoa was better maintained ($P < 0.05$) in extenders containing thioglycol at 0.5 mM and 1.0 mM compared to control at 0, 2 and 4 hour post-thaw. Cryopreservation reduces the DNA integrity of buffalo spermatozoa by reducing total antioxidant potential of semen (Kumar *et al.,* 2011). Thioglycol supplementation improved the development of cloned embryo by inhibiting the apoptosis process (Park *et al.,* 2004a; Park *et al.,* 2004b). It is suggested that thioglycol protect the DNA integrity of the buffalo spermatozoa by reducing oxidative stress. In conclusion, thioglycol 1.0mM in extender improved the motility, plasma membrane integrity, viability (live sperm with intact acrosome) and DNA integrity of buffalo bull spermatozoa.

Acknowledgment: Financial support was provided by Higher Education Commission (HEC) of Pakistan under “HEC Indigenous 5000 PhD Fellowship Program”.

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