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Effect of L-Cysteine in Tris-citric Egg Yolk Extender on Post-Thaw Quality of Nili-Ravi Buffalo (*Bubalus bubalis*) Bull Spermatozoa

Muhammad Sajjad Ansari,[1]* Bushra Allah Rakha,[1] Nemat Ullah,[2] Syed Murtaza Hussain Andrabi,[3] Muhammad Khalid[4] and Shamim Akhter[1]

[1]Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan,
[2]Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi
[3]Animal Reproduction Laboratory, Animal Sciences Institute, National Agricultural Research Center, Islamabad, Pakistan
[4]The Royal Veterinary College, Department of Veterinary Clinical Sciences, Hawkshead Lane, North Mymms Hatfield, Hertfordshire AL9 7TA, UK

Abstract.- Present study was designed to investigate the effect of cysteine (0.0, 0.5, 1.0, 2.0 and 3.0 mM) in extender on the post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. Semen was collected from five adult Nili-Ravi buffalo bulls of similar age group with artificial vagina (at 42°C) for three weeks (replicates). Qualifying semen ejaculates were split into five aliquots and diluted in tris-citric egg yolk extender containing either 0.0 (control) or 0.5 or 1.0 or 2.0 or 3.0mM cysteine at 37°C having 50×10^6 spermatozoa ml^{-1}. Diluted semen was cooled to 4°C in 2 hours and equilibrated for 4 hours at the same temperature. Cooled semen was filled in 0.5 ml French straws at 4°C, kept over liquid nitrogen vapours (5cm) for 10 min and then plunged in the liquid nitrogen for storage. Thawing of frozen semen was performed after 24 hours of cryopreservation at 37°C for 30 seconds. Sperm motility, viability, plasma membrane integrity and normal apical ridge were assessed at 0, 2 and 4 hour post thaw. Sperm motility, viability, plasma membrane and acrosomal integrity were highest ($P<0.05$) in extender containing cysteine 1.0mM at 0, 2 and 4 hour post thaw as compared to 0.5, 2.0, 3.0mM and control. In conclusion, L-cysteine (1.0mM) in extender improved the freezability of Nili-Ravi buffalo bull spermatozoa.

Key words: Nili-Ravi buffalo bull; antioxidants; L-cysteine; cryopreservation; post thaw semen quality.

INTRODUCTION

It is well recognized that cryopreservation process decreases the viability of buffalo bull spermatozoa (Andrabi *et al*., 2009). Moreover, buffalo sperm is more sensitive to freezing stress (Raizada *et al*., 1990; Sansone *et al*., 2000). Freeze-thawing of the spermatozoa is associated with oxidative stress (Bilodeau *et al*., 2000; Ball *et al*., 2001; Chatterjee *et al*., 2001) which accelerates the production of reactive oxygen species (ROS) molecules (Baumber *et al*., 2005) due to plasma membrane lipid peroxidation (Cotran *et al*., 1989; Lenzi *et al*., 2002; Kankofer *et al*., 2005). It has also been observed that buffalo sperm are more sensitive to oxidative stress as compared to cattle spermatozoa (Nair *et al*., 2006; Kumaresan *et al*., 2005, 2006) which may be due to higher contents of polyunsaturated phospholipids present in sperm membrane (Sansone *et al*., 2000). Over production of ROS molecules increases the damages to functional and structural integrity of the buffalo sperm during freeze-thawing process (Kumaresan *et al*., 2005, 2006; Garg *et al*., 2008).

Buffalo semen is equipped with defensive system consisting of enzymatic (catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic (vitamin C, E, glutathione, cysteine) antioxidants which protect the sperm from ROS mediated cryo-injuries (Garg *et al*., 2008; Andrabi *et al*., 2009). This indigenous antioxidant system is insufficient (Baumber *et al*., 2005; Sreejith *et al*., 2006; Nichi *et al*., 2006) to protect the sperm from oxidative stress which causes higher lipid peroxidation in buffalo semen (Nair *et al*., 2006). Moreover, freeze-thaw cycle reduces the level of indigenous antioxidant in the mammalian semen (Alvarez and Storey, 1992; Beconi *et al*., 1993; Bilodeau *et al*., 2000; Stradaioli *et al*., 2007). Therefore, to protect the sperm integrity during

* Corresponding author: m.sajjad.ansari@gmail.com
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freeze-thawing of buffalo bull spermatozoa extra antioxidants supplementation is recommended (Andrabi *et al*., 2008; Kumaresan *et al*., 2005, 2006).

Cysteine is an amino acid and precursor molecule of glutathione which is intracellular antioxidant to protect the cell from ROS deleterious effect to the cell organelles (Meister and Anderson, 1983). Freeze-thawing of the semen causes a significant reduction in the glutathione contents in boar (Gadea *et al*., 2004) and bull sperm (Bilodeau *et al*., 2000; Stradaioli *et al*., 2007). Cysteine addition in extender improved the preservability of Indian (Murrah), Egyptian and Italian buffalo bull sperm (Singh *et al*., 1990; Dhami and Sahni, 1993; Dhami *et al*., 1994; El-Sheshtawy *et al*., 2008; Del Sorbo *et al*., 1995) in liquid and/or frozen state. However, information on the use of cysteine to improve post thaw quality of Nili-Ravi buffalo bull spermatozoa is lacking. Therefore, present study was designed to identify the effect of L-cysteine in extender on post-thaw semen characteristics of Nili-Ravi buffalo bull spermatozoa.

MATERIALS AND METHODS

Preparation of extenders

Tris-citric acid was used as a buffer for the experimental extenders. It consisted of 1.56 g citric acid (Fisher Scientific, UK) and 3.0 g tris–(hydroxymethyl)-aminomethane (Research Organics, USA) in 74 ml distilled water. The pH of buffer was 7.0 and the osmotic pressure was 320 mOsmol Kg^{-1}. Fructose (Scharlau, Spain) 0.2% wt/vol; glycerol (Riedel-deHaen, Germany) 7%; egg yolk 20% v/v: antibiotics combination; streptomycin sulphate @ 1mg/ml, procaine penicillin@300iu/ml, benzyl penicillin@ 100IU/ml available as Sinbiotic® (China) were added. Five Experimental extenders were prepared by adding L-cysteine at the rate of 0.0, 0.5, 1.0, 2.0 or 3.0 mM in extender.

Semen collection and initial evaluation

Semen was collected from five adult Nili-Ravi buffalo bulls maintained at Semen Production Unit Qadirabad, Sahiwal, Pakistan. Semen was collected with artificial vagina at 42°C at weekly intervals for 3 weeks (replicates). After collection, semen samples were transferred to the laboratory immediately for initial evaluation (volume, motility, concentration). Sperm progressive motility (%) was assessed (X 200) with phase contrast microscope. Sperm concentration was measured with Neubauer haemocytometer. The neat semen samples qualifying minimum standard of 1 ml volume, 60% motility and 0.5 billion spermatozoa ml^{-1} of sperm concentration were selected for further processing The qualifying ejaculates were pooled in order to have sufficient semen for a replicate and held for 15 min at 37°C in the water bath before dilution. Pooled semen was split into five aliquots for dilution in five different experimental extenders.

Semen processing

Semen aliquots were diluted in a single step at 37°C with one of the five experimental extenders at the rate 50×10^6 motile spermatozoa ml^{-1}. Diluted semen was cooled to 4°C in 2 hours and equilibrated for 4 hours at 4°C. Semen was then filled in 0.5 ml French straws (IMV, France) with suction pump at 4°C in the cold cabinet unit (Minitub, Germany) and kept over liquid nitrogen vapours (at 5 cm height of liquid nitrogen level) for 10 min. Straws were then plunged into liquid nitrogen (-196°C) and stored. After 24 hours of cryopreservation, the semen straws were thawed at 37°C for 30 seconds in water bath and then incubated for 6 hours for assessment of post-thaw semen quality.

Post-thaw sperm functional assays

All the sperm quality assays (motility, viability, plasma membrane integrity and morphology) were performed at 0, 2 and 4 hours post thaw.

Sperm progressive motility

A drop of thawed semen sample was placed on prewarmed glass slide and covered with cover slip. Progressive motility was assessed with phase contrast microscope at X 400 at 37°C.

Sperm viability

Sperm viability was assessed using 0.4% Trypan blue stain. For this purpose, 5µl semen sample and equal amount of trypan blue solution was mixed with cover slip edge on glass slide and

air dried for 10 min for fixation. Air dried slides were examined under phase contrast microscope (X 1000; oil immersion). Spermatozoa stained blue were considered as non viable; while unstained as viable. A total of one hundred spermatozoa were counted for each sample.

Sperm plasma membrane integrity

Sperm plasma membrane integrity (PMI) of buffalo bull spermatozoa was assessed by hyposmotic swelling (HOS) assay (Jeyendran *et al.*, 1984). Solution for HOS assay consisted of sodium citrate 0.73 g and fructose 1.35 g in 100 ml distilled water (osmotic pressure ~190 mOsmol Kg^{-1}). For the assessment of sperm tail plasma membrane integrity, 50 µl of frozen thawed semen was mixed with 500 µl of HOS solution and incubated for 30-40 min at 37°C. A drop of semen sample was placed on glass slide and covered with cover-slip to examine under phase contrast microscope (X 400) for the assessment of sperm PMI. One hundred spermatozoa per experimental extender per replicate were counted for their swelling characterized by coiled tail indicating intact sperm plasma membrane.

Sperm acrosomal integrity

To assess the acrosomal integrity100 µl semen sample was fixed in 500 µl of 1% formal citrate (2.9 g tri-sodium citrate dehydrate, 1 ml of 37% solution of formaldehyde, dissolved in 100 ml of distilled water). Normal acrosome was characterized by normal apical ridge (NAR). One hundred spermatozoa per experimental extender were counted with phase contrast microscope (X 1000) under oil immersion

Statistical analysis

The data are presented as means±SD values of different parameters used to assess the post-thaw quality of the semen samples diluted in extender with different concentrations of cysteine. Data were analyzed using analysis of variance (ANOVA). When F ratio was significant, LSD test was applied to compare the treatment means (MSTAT-C Ver.1.42).

RESULTS

Effect of L-cysteine on post thaw sperm progressive motility (%) of buffalo spermatozoa

The data on the effect of L-cysteine on progressive sperm motility at 0, 2, 4 hours post-thaw of buffalo bull spermatozoa is presented in Figure 1. Percentage of sperm motility was highest (P<0.05) in extender containing L-cysteine 1.0mM (58.3±2.9, 43.3±2.9, 30.0±5.0) as compared to extender containing L-cysteine 0.5, 2.0, 3.0mM and control at 0, 2 and 4 hours post thaw.

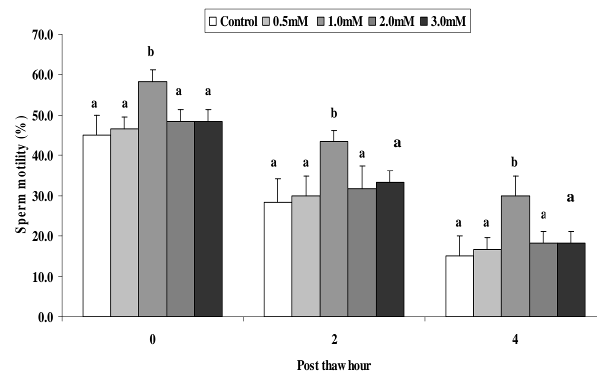

Fig. 1. Effect of L-cysteine addition in extender on the progressive motility of buffalo bull spermatozoa at 0, 2 and 4 hours after thawing. Bars with different letters show significant (P<0.05) differences at a given time.

Effect of L-cysteine on post thaw viability (%) of buffalo bull spermatozoa

The data on the effect of L-cysteine on sperm viability at 0, 2, 4 hours post-thaw of buffalo bull spermatozoa is presented in Figure 2. Sperm viability was highest (P<0.05) in extender containing L-cysteine 1.0mM (89.7±2.1, 75.3±1.2, 61.0±2.0) as compared to extender containing L-cysteine 0.5, 2.0, 3.0mM and control at 0, 2 and 4 hours post thaw.

Effect of L-cysteine on post thaw plasma membrane integrity (%) of buffalo bull spermatozoa

The data on the effect of L-cysteine on plasma membrane integrity at 0, 2, 4 hours post-thaw of buffalo bull spermatozoa are presented in

Figure 3. Sperm with intact plasma membrane was highest (P<0.05) in extender containing cysteine 1.0mM (82.7±3.0, 68.0±2.6, 54.3±0.9) as compared to extender containing L-cysteine 0.5, 2.0, 3.0mM and control at 0, 2 and 4 hours post thaw.

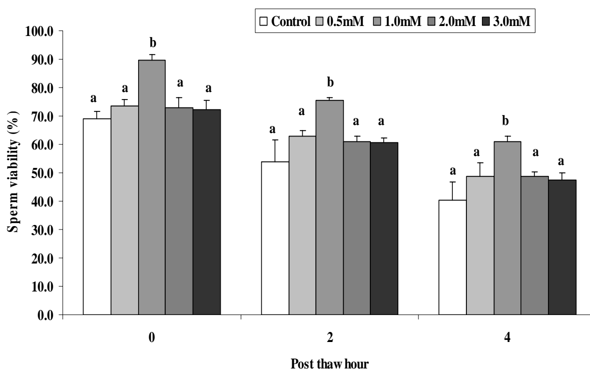

Fig. 2. Effect of L-cysteine addition in extender on viability of buffalo bull spermatozoa at 0, 2 and 4 hours after thawing. Bars with different letters show significant (P < 0.05) differences at given time.

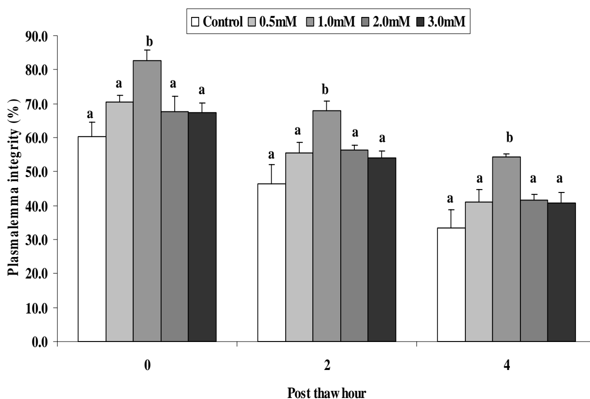

Fig. 3. Effect of L-cysteine addition in extender on plasma membrane integrity of buffalo bull spermatozoa at 0, 2 and 4 hours after thawing. Bars with different letters show significant (P < 0.05) differences at a given time.

Effect of L-cysteine on post thaw normal apical ridge (%) of buffalo bull spermatozoa

The data on the effect of L-cysteine on normal apical ridge at 0, 2, 4 hours post-thaw of buffalo bull spermatozoa are presented in Figure 4. Sperm with intact acrosomes was highest (P<0.05) in extender containing L-cysteine 1.0mM (92.0±1.2, 87.3±1.2, 84.3±0.9) as compared to extender containing L-cysteine 0.5, 2.0, 3.0mM and control at 0, 2 and 4 hours post thaw.

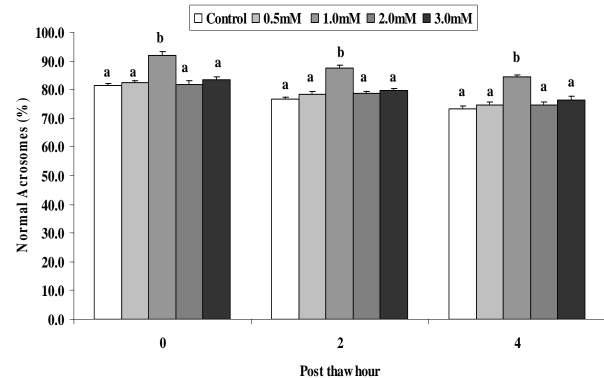

Fig. 4. Effect of L-cysteine addition in extender on the acrosomal integrity of buffalo bull spermatozoa at 0, 2 and 4 hours after thawing. Bars with different letters show significant (P < 0.05) differences at given time.

DISCUSSION

Pattern of sperm motility is affected by physico-chemical properties of the diluents (Akhter *et al*., 2008). In present study, post thaw sperm progressive motility was significantly higher in extender containing L-cysteine 1mM as compared to 0.5, 2.0, 3.0mM and control. Our results are inline with the findings of previous studies in which cysteine addition in extender improved the sperm motility in liquid (Dhami *et al*., 1994) and cryopreserved semen (Singh *et al*., 1990; Dhami and Sahni, 1993) of Indian (Murrah), Egyptian (El-Sheshtawy *et al*., 2008) and Italian buffalo bulls (Del Sorbo *et al*., 1995). It is suggested that cysteine supplementation in extender maintained the sperm motility by scavenging the ROS molecules (Alvarez and Storey, 1983) in the semen-extender complex (Aruoma *et al*., 1989; Bucak *et al*., 2008) which causes lipid peroxidation of the sperm plasma membrane associated with poor sperm motility (Urata *et al*., 2001).

The number of viable sperm per dose after cryopreservation significantly affects the fertility

rates in the field (Andrabi *et al*., 2006). In present study, post thaw sperm progressive motility was significantly higher in extender containing L-cysteine 1mM as compared to 0.5, 2.0, 3.0mM and control. In similar study, Bucak *et al*. (2008) observed a non-significant increase in the percentage of viable spermatozoa after the addition of cysteine in post thawed ovine semen with significantly increased catalase activity. Moreover, Uysal and Bucak (2007) observed a significantly higher percentage of viable sperm after the addition of cysteine in extender in ovine semen.

Structural and functional sperm plasma membrane integrity is essential for the fertilization process and its evaluation is of particular importance. Furthermore, the process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active plasmalemma (Jeyendran *et al*., 1984). In present study, percentage of sperm with intact functional plasma membrane was significantly higher in extender containing L-cysteine 1mM as compared to 0.5, 2.0, 3.0mM and control. In similar studies on ovine semen, addition of cysteine resulted in higher percentage of sperm with functional plasma membrane with significantly increased catalase activity (Uysal and Bucak, 2007; Bucak *et al*. 2008; Özkan *et al*., 2008). Similarly, Atessahin *et al*. (2008) reported an increase in plasma membrane integrity of the buck semen after the addition of cysteine. El-Sheshtawy *et al*. (2008) reported a significantly higher percentage of sperm with intact plasma membrane after the addition of cysteine (5mM) in Egyptian buffalo bull semen. It is suggested that cysteine supplementation in extender protected the membrane integrity by scavenging the ROS molecules (Alvarez and Storey, 1983) directly and/or indirectly in the semen-extender complex (Aruoma *et al*., 1989; Bucak *et al*., 2008) which can destroy the sperm cell membrane (Cotran *et al*., 1989).

The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is required at the proper time to facilitate fertilization (Thomas *et al*., 1997). A high correlation between the percentage of intact acrosome and fertility of frozen bovine spermatozoa was observed after 2 and 4 hours of post-thaw incubation (Saacke and White, 1972). In present study, number of spermatozoa with intact acrosome was significantly higher in extender containing L-cysteine 1mM as compared to 0.5, 2.0, 3.0mM and control. Atessahin *et al*. (2008) reported a non significant effect of cysteine addition on the sperm with normal acrosome of the buck semen. Uysal and Bucak (2007) observed a significantly higher percentage of sperm with intact acrosome after the addition of cysteine in extender in ovine semen (Özkan *et al*., 2008). It is relevant to mention that Bucak *et al*. (2008) observed a non-significant increase in the percentage of sperm with normal acrosomes after the addition of cysteine in diluter in post thawed ovine semen with significant increase in catalase activity. El-Sheshtawy *et al*. (2008) reported a significantly higher percentage of sperm with intact acrosome after the addition of cysteine (5mM) in Egyptian buffalo bull semen. It is suggested that cysteine maintains the sperm motility by scavenging the ROS molecules through GSH mediated protective cycle and/or increase antioxidant activity in the semen-extender complex (Bucak *et al*., 2008).

The results of the present study are inline with the findings of previous studies on buffalo bull semen in which cysteine supplementation in extender improved the quality of liquid (Dhami *et al*., 1994) and cryopreserved semen of Indian (Murrah) (Singh *et al*., 1990; Dhami and Sahni, 1993), Egyptian (El-Sheshtawy *et al*., 2008) and Italian buffalo bull (Del Sorbo *et al*., 1995). However, in these studies a high dose of cysteine (Indian, 0.1%; Egyptian, 5mM; Italian, 6mM) has been identified that improved the semen quality. It is pertinent to mention that higher concentration of cysteine has been found deleterious to ovine (Uysal and Bucak, 2007) and bovine semen (Johnson *et al*., 1954). In present study, cysteine concentration of 2.0 and 3.0mM in extender found non-beneficial for Nili-Ravi buffalo spermatozoa. It is relevant to mention that cysteine at 0.5mM concentration can successfully protect the sperm from motility loss in vitro of bovine spermatozoa (Bilodeau *et al*., 2001) and supplementation of cysteine HCl more than 1.0mg/ml in extender reduced the livability of bovine spermatozoa stored at 4°C (Johnson *et al*., 1954). In contrary to pervious studies on bulls of different buffalo breeds where high dose of cysteine

have beneficial effects, we found no beneficial effect on post thaw quality of Nili-Ravi buffalo bull semen with 2 mM and 3mM of cysteine in extender. This difference may be due to difference in experimental procedures, breeds, season and reproductive health of the bulls which affect the semen quality and/or freezeability of buffalo semen (Andrabi *et al*., 2009).

In conclusion, although L-cysteine supplementation @ 1mM in extender improved the post thaw quality of the Nili-Ravi buffalo bull spermatozoa, however, the routine use of such a supplementation in buffalo semen extender could only be recommended after performing fertility trails.

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