Pakistan J. Zool., vol. 46(1), pp. 17-21, 2014.

Effect of Glutathione in Extender on the Freezability of Sahiwal Bull Spermatozoa

Muhammad Sajjad Ansari,[1,*] Bushra Allah Rakha,[2] Razia Iqbal[1] and Shamim Akhter[3]
[1]*Department of Zoology, University of Gujrat, Gujrat, Pakistan*
[2]*Department of Wildlife Management, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan*
[3]*Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan*

Abstract.- This study was designed to evaluate the effect of glutathione addition in *tris*-citric acid extender on the freezability of Sahiwal bull spermatozoa. Semen was collected from three Sahiwal bulls of similar age group with artificial vagina (42°C) for three weeks (replicates). Qualifying semen ejaculates were pooled, split into three aliquots and diluted in *tris*-citric extender containing 0.0 (control), 0.5 and 1.0mM glutathione. Extended semen was cooled to 4°C in 2 hours and equilibrated for 4 hours at 4°C. After equilibration, semen was filled in French straws (0.5 ml) at 4°C, kept over liquid nitrogen vapours (5cm) for 10 min and then plunged in the liquid nitrogen for storage. Semen was thawed after 24 hours of cryopreservation at 37°C for 30 seconds. Sperm motility, viability, plasma membrane integrity and normal apical ridge were assessed after thawing. Highest ($P < 0.05$) sperm progressive motility, plasma membrane integrity, viability and acrosomal integrity of Sahiwal bull spermatozoa were observed in extender containing glutathione 0.5mM. However, glutathione supplementation greater than 0.5mM was not beneficial for any of semen quality parameters. In conclusion, glutathione addition (0.5mM) in extender improves the post-thaw quality of Sahiwal bull spermatozoa.

Key words: Sahiwal bull spermatozoa, cryopreservation, glutathione.

INTRODUCTION

It is well recognized that cryopreservation reduces the viability of mammalian spermatozoa, which is associated with higher production of reactive oxygen species (ROS) molecules (Baumber *et al.*, 2005). ROS can cause oxidative damage to the spermatozoa affecting their structural and functional integrity (Aitken *et al.*, 1998; Bilodeau *et al.*, 2001; Chatterjee *et al.*, 2001; Lenzi *et al.*, 2002).

In bovine semen, enzymatic (catalase, superoxide, dismutase, glutathione peroxidase/ reductase) and non-enzymatic (vitamin C and E, glutathione, cysteine) antioxidants are present to protect the spermatozoa from the reactive oxygen species molecules. However, the levels of inherent antioxidants are inadequate to protect the sperm integrity against oxidative stress during cryopreservation (Sreejith *et al.*, 2006; Nichi *et al.*, 2006). It was reported that freeze-thawing cycles decrease the level of naturally occurring antioxidants in bovine semen (Bilodeau *et al.*, 2000). The use of exogenous antioxidants in semen extender has been suggested to protect the quality of cryopreserved mammalian semen (Beconi *et al.*, 1993; Bilodeau *et al.*, 2001: Ansari *et al.*, 2010, 2011, 2012).

Glutathione, a naturally occurring tri-peptide, works as an antioxidant in bovine semen to protect the sperm cells against ROS. It was reported that glutathione content decreased in mammalian semen during cryopreservation process (Bilodeau *et al.*, 2000; Gadea *et al.*, 2004). Therefore, it was hypothesized that glutathione supplementation of semen extender might result in improved post thaw quality of Sahiwal bull semen. The present study was conducted to determine the effect of various glutathione concentrations in semen extender on post-thaw quality of the Sahiwal bull spermatozoa.

MATERIALS AND METHODS

Preparation of extenders

Tris-citric acid was used as a buffer (pH 7.0; osmotic pressure 320 mOsmol Kg^{-1}) that consisted of 1.56g citric acid (Fisher Scientific, UK) and 3.0g

* Corresponding author: m.sajjad.ansari@gmail.com
0030-9923/2014/0001-0017 $ 8.00/0

tris–(hydroxymethyl)-aminomethane (Research Organics, USA) dissolved in 73 ml distilled water. Fructose (Scharlau, Spain) 0.2% wt/vol; glycerol (Riedel-deHaen, Germany) 7% v/v; egg yolk 20% v/v and antibiotics *viz*; gentamycin sulphate (Reckitt Benckiser, Pakistan) 500 µg/ml, tylosin tartrate (VMD, Belgium) 100 µg/ml, lincomycin hydrochloride (Pharmacia & Upjohn, Belgium) 300 µg/ml, and spectinomycin hydrochloride (Pharmacia & Upjohn, Belgium) 600 µg/ml) were added to the extender. Glutathione (Merck, Germany) at the rate of 0.0, 0.5, 1.0 mM was added to the extender to prepare three experimental extenders (Ansari *et al*., 2010, 2011).

Semen collection and initial evaluation

Three adult Sahiwal bulls (*Bos indicus*) maintained at the Semen Production Unit Qadirabad, Sahiwal, Pakistan were used in the study. Two consecutive ejaculates from each bull were collected with the help of artificial vagina (42°C) at weekly intervals for a period of 3 weeks (Replicates). Immediately after collection, semen was transferred to the laboratory. Intial percent motility of the spermatozoa was assessed microscopically (at 200x) with closed circuit television while sperm concentration was assessed by Neubauer haemocytometer. The qualifying ejaculates [color (milky white), motility (60%), volume (0.5 mL), concentration (0.5 billion)] were split into three aliquots and held for 15 min at 37 °C in the water bath before dilution.

Semen processing

Semen aliquots were diluted (50×10^6 motile spermatozoa ml^{-1} approximately) in a single step at 37°C. Diluted semen was cooled to 4°C in 2 h at the rate of 0.275°C per min and was equilibrated for 4h 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 (IMV, France), kept on liquid nitrogen vapours for 10 min and stored into liquid nitrogen (-196°C). After 24 h, semen straws were thawed in a water bath at 37°C for 30 seconds. For each extender, semen from three straws were pooled and incubated at 37°C in water bath for assessment of post-thaw semen quality.

Post-thaw sperm functional assays

Sperm quality assays *viz*., motility, viability, plasma membrane integrity and acrosomal integrity were performed at post thawing. All semen quality assays were performed by the same person till the end of experiment.

Sperm progressive motility

A drop of thawed semen sample was placed on pre-warmed glass slide and covered with a cover slip. Progressive motility was assessed with a phase contrast microscope (X 200) at 37°C.

Assessment of sperm viability and acrosomal integrity

Sperm viability and acrosomal integrity were studied by dual staining procedure (Kovacs and Foote, 1992). The supravital stain trypan-blue was used to distinguish live and dead spermatozoa while giemsa stain was used to evaluate the integrity of the acrosome membrane. The trypan blue was prepared by using 0.05g trypan blue and 0.225g NaCl. Briefly, equal drops of trypan-blue and semen were placed on a slide and mixed. Smears were air-dried and slides were fixed with formaldehyde-neutral red (14% formaldehyde and 0.2% neutral red) for 5 min. After rinsing with running distilled water, Giemsa stain (7.5%) was applied for 4 hours. The slides were rinsed, air-dried and mounted with Canada Balsam. 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. One hundred spermatozoa were evaluated in at least five different fields in each smear by phase contrast microscope (1000x, Olympus Bx40, Japan) at 1000x.

Sperm plasma membrane integrity

Plasma membrane integrity of Sahiwal bull spermatozoa was assessed with supravital hypo-osmotic swelling test. Hypo-osmotic swelling (HOS) assay was performed as described by Jeyendran *et al.* (1984). After HOS incubation period, an aliquot (5µl) of the HOS solution was placed on a warm slide and a droplet (5µl) of Eosin

[0.5%, w/v, in 2.92% sodium citrate] was mixed for 10 seconds. A cover slip was placed on the mixture and evaluated with phase contrast microscope (400x). A total of 100 spermatozoa were observed in at least five different fields. Clear heads and tails and swollen tails were indicated intact, biochemically active sperm membranes, while pink heads and tails and unswollen tails were indicated disrupted, inactive sperm membranes.

Statistical analysis

The data on semen quality parameters were analyzed using ANOVA and are presented as mean ± SD. A 5% ($P < 0.05$) level was used to determine statistical significance.

RESULTS

Sperm progressive motility

The data on the effect of glutathione in extender on post thaw motility of Sahiwal bull spermatozoa are presented in Figure 1A. Higher ($P<0.05$) percentage of post thaw sperm motility was observed in extender containing glutathione 0.5mM (55.0 ± 0.0) compared to extender containing glutathione 1.0mM (46.7 ± 3.0) and control (43.3 ± 2.9).

Sperm plasma membrane integrity

The data on the effect of glutathione in extender on the percentage of post thaw sperm plasma membrane integrity of Sahiwal bull spermatozoa is presented in Figure 1B. Higher ($P<0.05$) percentage of sperm with intact plasma membrane was observed in extender containing 0.5mM (52.0±2.0) compared to extender containing glutathione 1.0mM (45.0±2.6) and control (41.0±2.7).

Sperm viability

The data on the effect of glutathione in extender on the post thaw viability of Sahiwal bull spermatozoa is presented in Figure 1C. Post thawed higher ($P<0.05$) percentage of viable spermatozoa was observed in extender containing 0.5mM (79.0 ± 3.0) compared to extender containing glutathione 1.0mM (65.3±3.1) and control (61.7±4.2).

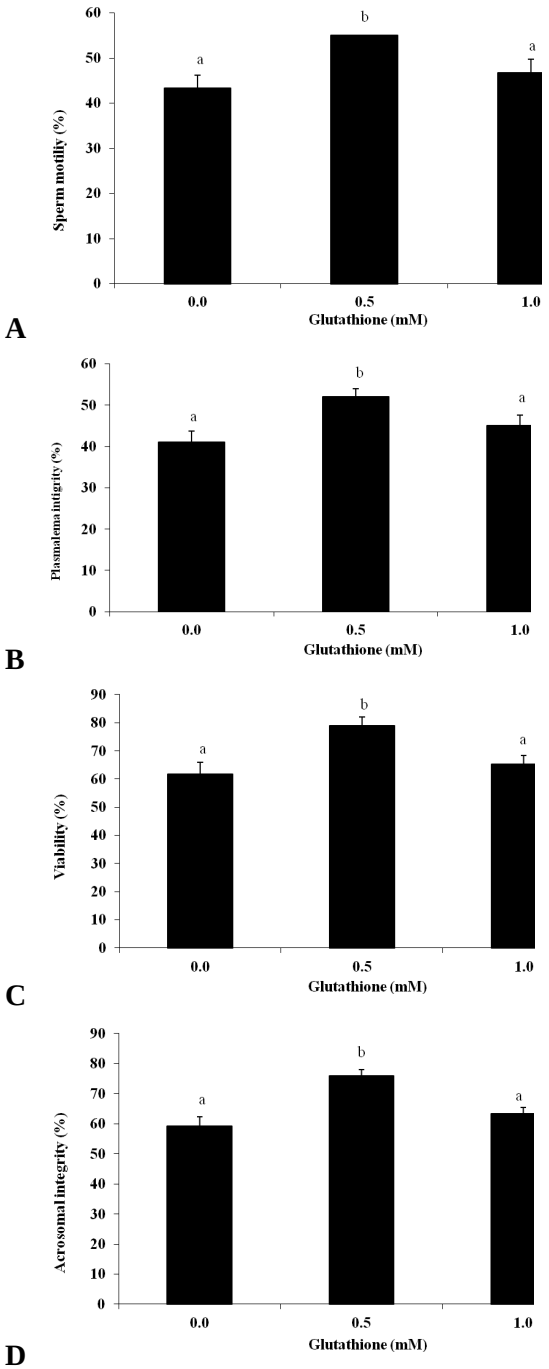

Fig. 1. Effect of glutathione addition in extender on motility (A), plasma membrane integrity (B), viability (C) and acrosomal integrity (D) of Sahiwal bull spermatozoa.

Sperm acrosomal integrity

The data on the effect of glutathione in

extender on percentage of live (with intact acrosomes) Sahiwal bull spermatozoa are illustrated in Figure 1D. Post thawed percentage of viable spermatozoa with intact acrosomes was higher ($P<0.05$) in extender containing 0.5mM (76.0 ± 2.0) compared to extender containing glutathione 1.0mM (63.3 ± 2.1) and control (59.3 ± 3.1).

DISCUSSION

This study was designed to evaluate the effect of glutathione supplementation in semen extender on progressive motility, plasma membrane integrity, viability and acrosomal integrity of Sahiwal bull spermatozoa. The addition of 0.5mM glutathione in semen extender improved the motility, plasma membrane integrity, viability and acrosomal integrity of Sahiwal bull spermatozoa. Although higher levels of ROS molecules in semen become toxic to spermatozoa but it is required for normal functions at physiological levels. It is expected that higher level of glutathione may cause reduction in ROS molecules beyond the normal required physiological level that's why higher dose of glutathione become non-beneficial for the spermatozoa.

It is well recognized that cryopreservation causes the overproduction of ROS molecules due to oxidative stress (Chatterjee *et al.,* 2001). The ROS; hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide are highly transient molecules due to higher reactivity which causes lipid peroxidation of plasma membrane. Higher production of the ROS molecules can damage the bio-membrane system of the mammalian spermatozoa (Bilodeau *et al.,* 2000; Gadea *et al.,* 2004). It has been observed that ROS produced during cryopreservation can deteriorate sperm motility, plasma membrane integrity, viability and acrosomal integrity of bull semen (Baily *et al.,* 2000; Bilodeau *et al.,* 2001; Chatterjee *et al.,* 2001; Ansari *et al*., 2010, 2011, 2012).

Glutathione is principally important naturally occurring antioxidant in semen, having low molecular weight, can easily mobilize the system for the removal of peroxides by a reaction that results in the generation of oxidized glutathione (Meister and Anderson, 1983). As glutathione is present in mM concentrations, this system, as well as being rapid, has a high capacity for the prevention of ROS molecules mediated damage to spermatozoa (Baumber *et al.,* 2005). The product of glutathione oxidation, oxidized glutathione, is known to be toxic and is rapidly converted back to glutathione by the enzyme glutathione reductase (Cotran *et al.,* 1989).

It is suggested that supplementation of the glutathione in semen extender might improve the sperm motility, plasma membrane integrity, viability and acrosomal integrity due to protection against ROS molecules produced from mitochondria, plasma membrane lipid peroxidation and dead/abnormal spermatozoa during freeze-thawing process (Ansari *et al*., 2012; 2010; Cotran *et al.,* 1989). Our findings suggest that glutathione is capable of limiting the extent of cryo-damage during freeze-thawing process inactivating ROS molecules. It is concluded that glutathione addition (0.5mM) in extender improved the post-thaw quality of Sahiwal bull spermatozoa.

REFERENCES

AITKEN, R.J., GORDON, E., HARKISS, D., TWIGG, J.P., MILNE, P., JENNING, Z. AND ANDIRVINE, D.S., 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod.,* **59**: 1037-1046.

ANSARI, M.S., RAKHA B.A., ULLAH, N., ANDRABI, S.M.H., IQBAL, S., KHALID, M. AND AKHTER, S., 2010. Effect of exogenous glutathione in extender on the freezability of Nili-Ravi buffalo (*Bubalus bubalis*) bull spermatozoa. *Anim. Sci. Pap. Rep*., **28**: 235-244.

ANSARI, M.S., RAKHA, B.A., ULLAH, N., ANDRABI, S.M.H. AND AKHTER, S., 2011. Glutathione addition in *tris*-egg yolk extender improves the quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Pakistan J. Zool*., **43**: 46-55.

ANSARI, M.S., RAKHA, B.A., ANDRABI, S.M.H., ULLAH, N., IQBAL, R., HOLT, W.V. AND AKHTER, S., 2012. Glutathione-supplemented tris-citric acid extender improves the post-thaw quality and in vivo fertility of buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Biol.*, **12**: 271-276.

BAILEY, J.L., BILODEAU, J.F. AND CORMIER, N., 2000. Semen cryopreservation in domestic animals: A damaging and capacitating phenomena. *J. Androl.,* **22**: 508-515.

BAUMBER, J., BALL, B.A. AND LINFOR, J.J., 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. *Am. J. Vet. Res.,* **66**: 772-779.

BECONI, M.T., FRANCIA, C.R., MORA, N.G. AND AFFRANCHINO, M.A., 1993. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology*, **40**: 841-851.

BILODEAU, J.F., BLANCHETTE, S., GAGNON, C. AND SIRAD, M.A., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol. Reprod. Develop.,* **55**: 282-288.

BILODEAU, J.F., BLANCHETTE, S., GAGNON, C. AND SIRAD, M.A., 2001. Thiols prevent H_2O_2 – mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*, **56**: 275-286.

CHATTERJEE, S., LAMIRANDE, E.D. AND GAGNON, C., 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: Protection of oxidized glutathione. *Mol. Reprod. Develop.,* **60**: 498-506.

COTRAN, R.S., KUMAR, V. AND ROBBIN, S.L., 1989. *Pathologic basis of diseases*, 4th edn. WB Saunders Co., Philadelphia, P.A., pp. 9–16.

GADEA, J., SELLES, E., MARCO, M.A., COPY, P., MATAS, C., ROMAR, R. AND RUIZ, S., 2004. Decrease in glutathione content in boar sperm cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, **62**: 690-701.

JEYENDRAN, R.S., VAN DER VEN, H.H., PEREZ-PELAEZ, M., CRABO, B.G. AND ZANEVELD, L.J.D., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.,* **70**: 219-228.

KOVACS, A. AND FOOTE, R.H., 1992. Viability and Acrosome staining of Bull, Boar and Rabbit spermatozoa. *Biotech. Histochem.*, **67**: 119-124.

LENZI, A., GANDINI, L., LOMABARDO, F., PICARDO, M., MARESCA, V. AND PANFILI, E., 2002. Polyunsaturated fatty acids of germ cell membranes, glutathione and glutathione-dependant enzyme-PHGPx from basic to clinic. *Contraception,* **65**: 301-304.

MEISTER, A. AND ANDERSON, M.E., 1983. Glutathione. *Annu. Rev. Biochem.*, **52**: 711–760.

NICHI, M., BOLS, P.E.J., ZUGE, R.M., BARNABE, V.H., GOOVAERTS, I.G.F., BARNABE, R.C., SREEJITH, J.N., BRAR, A.S., AHUJA, C.S., SANGHA, S.P.S. AND CHAUDHARY, K.C., 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci.,* **96**: 21-29.

SREEJITH, J.N., BRAR, A.S., AHUJA, C.S., SANGHA, S.P.S. AND CHAUDHARY, K.C., 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci.*, 96: 21-29.

(Received 25 April 2013, revised 26 September 2013)