Indian Journal of Animal Sciences **83** (3): 247–250, March 2013/Article

Cryoprotective effect of cysteine on cryopreservation associated protein tyrosine phosphorylation in buffalo spermatozoa

G JAGAN MOHANARAO[1] and S K ATREJA[2]

National Dairy Research Institute, Karnal, Haryana 132001 India

Received: 19 April 2012; Accepted: 10 September 2012

ABSTRACT

In the present study the effect of cysteine, an antioxidant, on cryopreservation associated cryodamage in the buffalo spermatozoa was studied. Ejaculated buffalo semen was divided into 2 aliquots. One aliquot was used to analyze fresh semen quality parameters and the other aliquot was cryopreserved in tris based egg-yolk extender with or without cysteine (5 mM) and sperm quality parameters like post-thaw motility, viability and membrane integrity were assessed. All the sperm quality parameters were significantly improved upon the addition of cysteine. Interestingly the number of tyrosine phosphorylated proteins detected was more (p32, p49, p55, p72, p86, p95 and p100) in cryopreserved sperm than the fresh spermatozoa. However the degree of protein tyrosine phosphorylation with respect to p49, p55, p72, p86, p95 and p100 proteins was significantly decreased in the cryopreserved spermatozoa supplemented with cysteine. Hence cysteine acts as a potential antioxidant that reduces the protein tyrosine phosphorylation probably caused by ROS induced signaling pathways by maintaining other sperm quality parameters. The addition of cysteine to the freezing extender helps in minimizing the cryodamage associated with sperm cryopreservation.

Key words: Buffalo, Cryopreservation, Cysteine, Sperm, Tyrosine phosphorylation

Cryopreservation procedures induce changes in spermatozoa such as plasma membrane reorganization and fluidization, calcium influx, protein tyrosine phosphorylation which are collectively termed as cryocapacitation. These are thought to be partly responsible for the reduced fertility of frozen–thawed semen (Bailey *et al.* 2000, Cormier and Bailey 2003). The reduced lifespan of cryopreserved spermatozoa in the female genital tract is particularly problematic in buffalo (Dhami and Kodagali 1990). The success rate of *in vitro* fertilization with cryopreserved buffalo sperm is only 10–20% as compared to cattle which is 30–35% (Totey *et al.* 1992, Nandi *et al.* 2006) and 8 times more cryopreserved bovine spermatozoa compared to fresh, were required to achieve equivalent fertilization rates *in vivo* (Shannon and Vishwanath 1995). The premature capacitation like changes associated with cryopreservation causes ATP utilization, pH change and acrosome reaction leading to reduced fertile life of sperm. Cysteine was used in the cryopreservation of mammalian spermatozoa (Funahashi and Sano 2005, Bucak *et al.* 2007, Uysal *et al.* 2007, Atessahin *et al.* 2008) to improve sperm quality parameters. Our laboratory clearly demonstrated cryocapacitation like changes in buffalo spermatozoa and the cryopreserved sperm quality parameters could be improved upon the addition of cryoprotectants like taurine and trehalose (Reddy *et al.* 2010). The aim of the present study was to determine the effect of cysteine, a known antioxidant, on cryopreserved buffalo sperm quality parameters and importantly on the protein tyrosine phosphorylation which is considered as a biochemical marker for capacitation.

Present address: [1]Research Associate, Animal Genomics Lab,Animal Biotechnology Centre (biochemjagan@gmail.com). [2]Principal Scientist, Reproductive Biochemistry Lab (skatreja @rediffmail.com).

MATERIALS AND METHODS

Semen collection and cryopreservation: The Murrah buffalo bulls (3–5 years of age) were housed at Artificial Breeding Research Complex, National Dairy Research Institute, Karnal, India, under uniform nutritional conditions. Ejaculates (32) from 4 buffalo bulls (8 ejaculates from 1 bull) were collected twice a week by artificial vagina (IMV) in winter. Immediately after collection, ejaculates were immersed in a warm water bath at 38.5 °C and mass motility was assessed by light microscopy. Tris based egg yolk extender (tris 33.2 g/l, citric acid 18.3 g/l, dextrose 7.8 g/l, egg yolk 20% (v/v), glycerol 6.4%, benzyl penicillin 10,00,000 iu/l, streptomycin 1 g/l) was used as the freezing extender. Each ejaculate was divided into 2 aliquots. One aliquot was used for fresh semen analysis and the other

aliquot was diluted with the freezing extender containing without (control) and with cysteine (5 mM) to a final concentration of approximately 80×10^6 cells/mL. Diluted samples were aspirated into 0.25 mL (medium-sized) French straws, sealed with polyvinyl alcohol powder and equilibrated at 4 °C for 4 h. After equilibration, the straws were frozen in liquid nitrogen vapour, 5 cm above liquid nitrogen, for 10 min and then the straws were plunged into liquid nitrogen for storage. After storage for 6–8 weeks, frozen straws were thawed at 37 °C for 30 s in a water bath and used for the study of different semen parameters. Standard semen characteristics like progressive motility, viability and membrane integrity in the fresh semen ejaculates were studied.

Post-thaw sperm motility, viability and membrane integrity: For the motility analysis, five straws per treatment were thawed and 10 µL of semen aliquots were transferred into glass slides and cover-slips were applied. Sperm motility was assessed by determining the percentage of spermatozoa showing any movement of the flagellum. The percentage of linear motile sperm was estimated at 37°C by light microscope at 400´. At least 300 spermatozoa were counted per slide. The mean of the three estimations was used as the final motility score. The sperm viability was assessed using eosin B and nigrosin stains. To determine the membrane integrity hypoosmotic swelling test (HOST) was performed (Revell and Mrode 1994).

Detection of tyrosine phosphoproteins by immunoblotting: Immediately after thawing, semen samples without (control) and with cysteine were collected separately and centrifuged at 275 × g for 6 min each. The extended seminal plasma was discarded and pellet was subjected to three washes by resuspension with 3 mL of sp-TALP (composed of 100 mM NaCl, 10 mM HEPES, 3.1 mM KCl, 0.4 mM EDTA, 0.4 mM $MgCl_2{\cdot}6H_2O$, 0.3 mM $NaH_2PO_4{\cdot}2H_2O$, 21.6 mM Na lactate, 2 mM $CaCl_2{\cdot}2H_2O$, 1 mM Na pyruvate, 25 mM $NaHCO_3$, 1 mg/mL BSA, pH 7.4, osmolarity: 265–270 mOsmol/kg) and centrifugation at 275 × g for 6 min each (Galantino-Homer *et al.* 1997). Sperm proteins were extracted according to the method of Galantino-Homer *et al.* (1997) and total proteins were resolved by SDS-PAGE on a 10% (w/v) uniform gel at constant voltage of 40 V (Laemmli 1970). Cryopreservation associated tyrosine phosphoproteins were detected by an enhanced chemiluminescence-based immunoblotting technique using monoclonal anti-phosphotyrosine antibody (Roy and Atreja 2008a). After chemiluminescence detection of tyrosine phosphoproteins, the X-ray films were photographed by an image analyser (USA) and densitometric analysis of four blot images representing each animal was performed with quantity one image analysis software. To determine the relative changes in the intensities of protein bands, the intensity obtained with the cryopreserved spermatozoa without cysteine was assigned a base value of 100.

Statistical analysis: Data were analysed by analysis of variance and statistical differences between the various treatment group means were determined by Duncan's multiple range test (DMRT) using the Statistical Product and Service Solutions, Version 17.0.1 software. Densitometric data were analysed by students' two-tailed *t*-test.

RESULTS AND DISCUSSION

Effect of cysteine on sperm quality characteristics: Sperm motility, viability and membrane integrity of fresh and cryopreserved (post-thaw) sperm were assessed and the results were shown in Fig. 1. The cryopreservation of semen significantly reduced ($P < 0.01$) the motility of spermatozoa (31.66±1.66%) as compared to their fresh co-ordinates (81.66±1.2%) where as the addition of cysteine to the freezing extender led to higher post-thaw motility (56.66±1.66%). The viability and membrane integrity (percentage of spermatozoa with coiled tails) of the cryopreserved sperm were significantly declined (62.33±1.45% vs 85.66±0.88%; 38.1±1.49% vs 78.33±0.88% respectively; $P< 0.01$) due to freeze–thaw procedures when compared to fresh spermatozoa. However, significant differences were observed in viability (72.66±0.88% vs 62.33±1.45%; $P < 0.01$) and HOST (54.66±0.88% vs 38.1±1.49%; $P < 0.01$) upon the addition of 5 mM cysteine during freeze-thawing process compared to semen without cysteine (Fig. 1).

The cryoprotective action of cysteine, in the present study, in terms of better post-thaw motility, viability and membrane integrity is in close agreement with earlier finding in boar and goat semen where cysteine has been shown to prevent loss of motility, viability and membrane integrity during sperm liquid storage or in the frozen state (Szczesniak-Fabianczyk *et al.* 2003, Bucak and Uysal 2008). Similar to

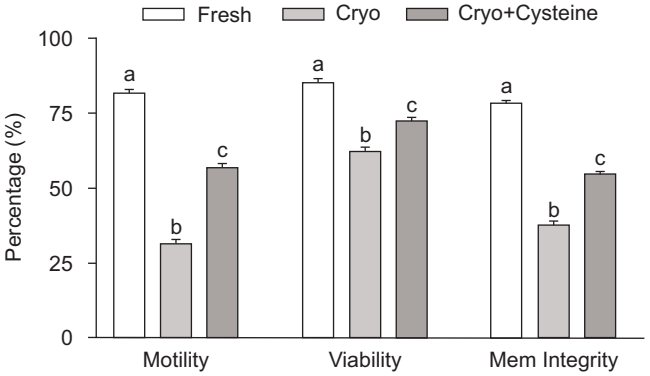

Fig. 1. Effect of cysteine on cryopreserved buffalo sperm quality parameters. The percentages of sperm motility, viability and membrane integrity in frozen-thawed buffalo spermatozoa assessed after freezing in tris-based egg yolk extender supplemented with cysteine (5 mM) and compared to freshly ejaculated semen and semen cryopreserved in tris-based egg yolk extender only. Values are the means±S.E.M. of three experiments performed with semen samples from three different bulls. Within each parameter means with different letters significantly different ($P < 0.01$).

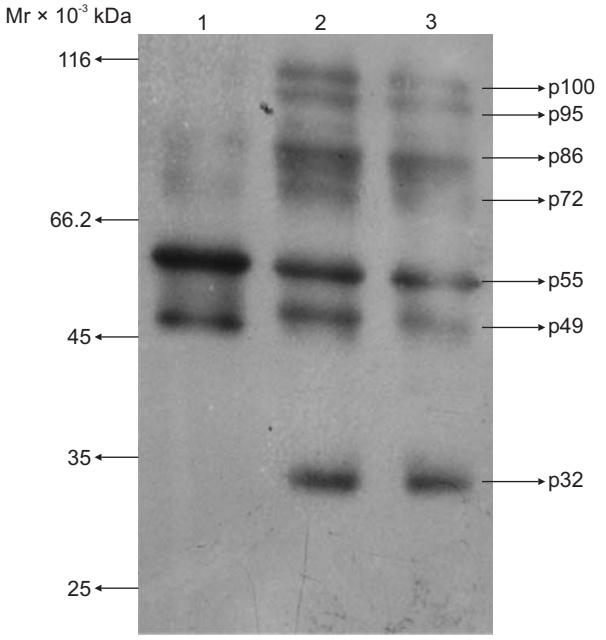

Fig. 2. Effect of cysteine on cryopreservation associated protein tyrosine phosphorylation in buffalo spermatozoa. Sperm proteins were extracted, resolved by 10% SDS-PAGE, electrotransferred and immunoblotted using a monoclonal antiphosphotyrosine antibody. Arrows on the right-hand side of the figure denotes the tyrosine phosphoproteins detected. Lane 1 represents the tyrosine phosphoproteins detected in the fresh spermatozoa. Lane 2 and 3 represents the tyrosine phosphoproteins detected in the cryopreserved spermatozoa without (control) and with cysteine respectively.

this study, the optimal concentration of cysteine needed to improve the quality of frozen thawed boar semen was 5 mM (Kaeoket *et al.* 2010). Mammalian cells can utilize cysteine, which has been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both *in vitro* and *in vivo* (Sagara *et al.* 1993). Similarly in sperm, the generation of glutathione from cysteine might improve the antioxidant status, inhibit lipid peroxidation and protects the cells against the accumulation of reactive oxygen species thereby enhances sperm quality parameters. Cysteine acts as an antioxidant and its supplementation to extender improves post thaw motility, viability and membrane integrity in bull (Uysal *et al.* 2007), boar (Funahashi and Sano 2005), ram (Bucak *et al.* 2007) and goat (Atessahin *et al.* 2008).

Effect of cysteine on protein tyrosine phosphorylation: The effect of cysteine hydrochloride on cryopreservation associated protein tyrosine phosphorylation in buffalo spermatozoa was investigated by immunoblotting and compared with the cryopreserved semen without cysteine and fresh spermatozoa. The number of tyrosine phosphorylated proteins in the cryopreserved spermatozoa was more as compared to the fresh spermatozoa (Fig. 2). In cryopreserved spermatozoa seven proteins of molecular weight 100, 95, 86, 72, 55, 49 and 32 kDa (designated as

Table 1. Relative band intensities (mean ± SEM) of the tyrosine phosphorylated proteins detected in cryopreserved buffalo spermatozoa without or with cysteine

Mass of the detected proteins	Fresh sperm	Cryopreserved sperm	Cryopreserved sperm (5 mM) + Cysteine
p100	absent	100	82.78 ± 2.00[a]
p95	absent	100	86.47 ± 3.14[a]
p86	absent	100	83.72 ± 5.65[a]
p72	absent	100	80.48 ± 3.36[a]
p55	116.71 ± 8.84	100	82.77 ± 3.20[a]
p49	absent	100	78.34 ± 4.26[a]
p45	absent	-	-
p32	absent	100	93.02 ± 4.72

p100, p95, p86, p72, p55, p49 and p32) were tyrosine phosphorylated whereas in fresh spermatozoa only p55 and p45 were detected (Fig. 2). The addition of cysteine to the freezing extender has shown cryoprotective action in terms of protein tyrosine phosphorylation. The extent of tyrosine phosphorylation with respect to p100, p95, p86, p72, p55, p49 proteins was significantly lesser ($P < 0.05$) in presence of cysteine as compared to the cryopreserved semen without cysteine (Fig. 2; Table 1). This clearly demonstrated the cryoprotective ability of the cysteine hydrochloride, an antioxidant against the cryodamage that occurs during cryopreservation of semen. Furthermore, it is of interest to note that the tyrosine phosphorylation of p55 was constitutive in fresh as well as cryopreserved spermatozoa.

In the present study, cryopreservation induced tyrosine phosphorylation was observed with number of proteins (p100, p95, p86, p72, p55, p49 and p32) in buffalo spermatozoa whereas the presence of cysteine, an antioxidant reduced the extent of tyrosine phosphorylation of these proteins. The buffalo sperm undergoes cryocapacitation and the total antioxidant status of the extended seminal plasma and spermatozoa was significantly reduced upon cryopreservation (Reddy *et al.* 2010). Moreover the detection of p95, p49 and p32 proteins in the cryopreserved spermatozoa was consistent with the earlier findings in the fresh buffalo spermatozoa incubated for capacitation in presence of $NO^{\cdot}$, $O_2^{\cdot-}$ and H_2O_2 releasing compounds (Roy and Atreja 2008a, b). These results strongly demonstrated that the generation of reactive oxygen species during cryopreservation might be responsible for the tyrosine phosphorylation of number of proteins in the cryopreserved spermatozoa whereas supplementation of cysteine could have reduced the excess generation of ROS and decreased the protein tyrosine phosphorylation. Protein tyrosine phosphorylation associated with cryopreservation was also observed in bovine (Cormier and Bailey 2003), equine (Thomas *et al.* 2006) and porcine (Vadnais and Roberts 2007) spermatozoa. Satorre *et al.* (2007) observed a decrease in

tyrosine phosphorylation of p32 protein, in presence of an antioxidant α-tocopherol, which is associated with cryopreservation of boar spermatozoa. In our study, similarly, we have observed the protein tyrosine phosphorylation of p32 protein and its extent of tyrosine phosphorylation was decreased in presence of cysteine.

In summary the supplementation of cysteine, an antioxidant, to the freezing extender could confer a greater cryoprotective capacity and improved the buffalo sperm quality characteristics like post-thaw motility, viability and membrane integrity. The main finding emerged from this study was the beneficial effect of cysteine in decreasing the extent of cryopreservation induced protein tyrosine phosphorylation.

ACKNOWLEDGEMENTS

This study was partly supported by World Bank funded National Agricultural Innovation Project (C4/C30014). The authors also thank the Director, National Dairy Research Institute, Karnal 132001, India, for providing the necessary facilities during the course of the study. The authors also thank Amit Kumar Singh, Senior Research Fellow, for providing assistance in collecting semen samples.

REFERENCES

Atessahin A, Bucak M N, Tuncer P B and Kizil M. 2008. Effects of anti-oxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze–thawing process. *Small Ruminant Research* **77**: 38–44.

Bailey J L, Bilodeau J F and Cormier N. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *Journal of Andrology* **21**: 1–7.

Bucak M N, Atessahin A, Varisli O, Yuce A, Tekin N and Akcay A. 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stress parameters after the freeze–thawing process. *Theriogenology* **67**: 1060–67.

Bucak M N and Uysal O. 2008. The role of antioxidants in freezing of Saanen goat semen. *Indian Veterinary Journal* **85**: 148–50.

Cormier N and Bailey J L. 2003. A differential mechanism is involved during heparin-and cryopreservation-induced capacitation of bovine spermatozoa. *Biology of Reproduction* **69**: 177–85.

Dhami A J and Kodagali S B. 1990. Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. *Theriogenology* **34**: 853–63.

Funahashi H and Sano T. 2005. Selected antioxidants improve the function of extended boar semen stored at 10°C. *Theriogenology* **63**: 1605–16.

Galantino-Homer H L, Visconti P E and Kopf G S. 1997. Regulation of protein tyrosine phosphorylation during bovinesperm capacitation by a cyclic adenosine 3', 5'-monophosphate-dependent pathway. *Biology of Reproduction* **56**: 707ˆ19.

Kaeoket K, Chanapiwat P, Tummaruk P and Techakumphu M. 2010. Supplemental effect of varying L-cysteine concentrations on the quality of cryopreserved boar semen. *Asian Journal of Andrology* **12 (5)** : 760–5.

Laemmli U K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–85.

Nandi S, Kumar G V and Chauhan M S. 2006. In vitro production of bovine embryos, we need to stop or proceed-a review. *Agricultural Reviews* **27**: 122–29.

Revell S G and Mrode R A. 1994. Anosmotic resistance test for bovine semen. *Animal Reproduction Science* **36**: 77–86.

Roy S C and Atreja S K. 2008a. Tyrosine phosphorylation of a 38-kDa capacitation-associated buffalo *(Bubalus bubalis)* sperm protein is induced by L-arginine and regulated through a cAMP/PKA-independent pathway. *International Journal of Andrology* **31**: 12–24.

Roy S C and Atreja S K. 2008b. Effect of reactive oxygen species (ROS) on capacitation associated protein tyrosine phosphorylation in buffalo *(Bubalus bubalis*) spermatozoa. *Animal Reproduction Science* **107**: 68–84.

Sagara J, Miura K and Bannai S. 1993. Cysteine uptake and glutathione level in fetal brain cells in primary culture and in suspension. *Journal of Neurochemistry* **61**: 1667–71.

Shannon P and Vishwanath R. 1995. The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. *Animal Reproduction Science* **39**: 1–10.

Reddy N S S, Jagan Mohanarao G and Atreja S K. 2010. Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo *(Bubalus bubalis*) sperm quality following cryopreservation. *Animal Reproduction Science* **119 (3–4)** : 183–90.

Szczesniak-Fabianczyk B, Bochenek M, Smorag Z and Ryszka F. 2003. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. *Reproductive Biology* **3**: 81–87.

Thomas A D, Meyers S A and Ball B A. 2006. Capacitation-like changes in equine spermatozoa following cryopreservation. *Theriogenology* **65**: 1531–50.

Totey S M, Singh G, Taneja M, Pawshe C H and Talwar G P. 1992. In vitro maturation, fertilization and development of follicular oocytes from buffalo *(Bubalus bubalis). Journal of Reproduction and Fertility* **95**: 597ˆ07.

Vadnais M L and Roberts K L. 2007. Effects of seminal plasma on cooling-induced capacitative changes in boar sperm. *Jorunal of Andrology* **28**: 416–22.

Uysal O, Bucak M N, Yavas I and Varisli O. 2007. Effect of various antioxidants on the quality of frozen–thawed bull semen. *Journal of Animal and Veterinary Advances* **6**:1362–66.