Indian Journal of Animal Sciences **86** (11): 1255–1258, November 2016/Article

Effect of cholesterol loaded cyclodextrin on activity of antioxidants during cryopreservation of buffalo (*Bubalus bubalis)* semen

S A LONE[1], J K PRASAD[2], S K GHOSH[3], NARENDRA KUMAR[4], SHOWKAT A BHAT[5] and G K DAS[6]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 23 January 2016; Accepted: 9 August 2016

ABSTRACT

The present study was carried out to investigate the effect of cholesterol loaded cyclodextrin on activity of antioxidants during cryopreservation of buffalo semen. Ejaculates (48) with mass motility ≥3+ from 3 bulls (16 from each bull) were collected as per standard procedure. Each ejaculate was divided into group I (control): diluted with Tris-egg yolk-glycerol dilutor up to 60×10^6 sperm/ml and group II: treated with cholesterol loaded cyclodextrin @ 3 mg/120×10^6 sperm and incubated at 37ºC for 10 min and then finally diluted with Tris-egg yolk-glycerol dilutor up to 60×10^6 sperm/ml. French medium straws (0.5 ml) were filled with the extended semen samples, sealed with polyvinyl alcohol powder and kept for 3 h at 5ºC for equilibration and then kept in automatic programmable freezer till temperature of straws reached –145°C followed by plunging into liquid nitrogen (–196°C). Ejaculates were evaluated freshly, before freezing and after thawing for seminal attributes (progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response) and antioxidants (superoxide dismutase, catalase, glutathione peroxidase and total antioxidants). After thawing, activity of superoxide dismutase, catalase and total antioxidants were significantly higher in group II as compared to group I. It is concluded that cholesterol loaded cyclodextrin enhances seminal antioxidant activity.

Key words: Antioxidants, Buffalo, Cholesterol loaded cyclodextrin, Cryopreservation, Semen

Mammalian spermatozoa have evolved an antioxidant system to protect themselves against oxidative stress. The best known antioxidants include superoxide dismutase, catalase and glutathione peroxidase (Guthrie and Welch 2012). Activity of these antioxidants is higher in seminal plasma as compared to other biological fluids (Lewis *et al*. 1997). During cryopreservation process, sperm plasma membrane structure and function undergoes serious detrimental changes (Purdy 2006). Cholesterol as a component of sperm plasma membrane plays an important role in regulating membrane fluidity and stability and efflux of cholesterol leads to capacitation (Muller *et al.* 2008). Adding cholesterol or its analogues to the medium reduces capacitation process (Serin *et al.* 2011). Due to hydrophobic nature of cholesterol, it is insoluble in aqueous semen diluents. Cyclodextrins, which are obtained by enzymatic degradation of starch, are capable of inserting the cholesterol into synthetic and cell membranes due to presence of an internal hydrophobic core, in addition to an external hydrophilic face (Dodziuk 2006). Addition of cholesterol loaded cyclodextrin (CLC) to semen increases its freezability (Rajoriya *et al.* 2014 and Naseer *et al.* 2015) and protects spermatozoa against oxidative damages during cryopreservation (Naseer *et al.* 2015).

The protective effect of cholesterol against oxidative stress in spermatozoa (Naseer *et al*. 2015) laid down the hypothesis that reduction of oxidative stress is another factor behind the enhanced cryosurvival rate of cholesterol loaded cyclodextrin treated spermatozoa. However, no information is available on the effect of cholesterol loaded cyclodextrin on activity of antioxidants during cryopreservation of semen. Therefore the present study was designed to determine the effect of cholesterol loaded cyclodextrin on activity of antioxidants during cryopreservation of buffalo semen.

MATERIALS AND METHODS

Experimental design: Semen was collected from Murrah buffalo bulls (3), 4–6 year old, maintained at the Germ Plasm Centre of Animal Reproduction Division, ICAR-Indian Veterinary Research Institute, Izatnagar. These bulls were reared under the similar feeding and management conditions during the entire period of the study.

Preparation of cholesterol loaded cyclodextrin (CLC): Methyl-β-cyclodextrin was loaded with cholesterol as described by Purdy and Graham (2004). Briefly, 200 mg of cholesterol was dissolved in 1 ml of chloroform in a glass

Present address: [1]Ph.D Scholar (drloneshabir@gmail.com), Animal Reproduction, Gynecology and Obstetrics. [4,5]Ph.D Scholar, Division of LPM (drshowkatbhat813@gmail.com, narendrakumarvet@gmail.com), ICAR-National Dairy Research Institute, Karnal, Haryana. [2]Senior Scientist (jkprasad2001@yahoo.com), [3,6]Principal Scientist (subratakghosh@yahoo.com, gkdasivri@gmail.com), Division of Animal Reproduction.

tube. In second glass tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml of methanol. A 0.45 ml aliquot of the cholesterol solution was added to the cyclodextrin solution, and the mixture was stirred until the combined solution appeared clear. This was followed by pouring of mixture into a glass petri dish and removing of solvents using a stream of nitrogen gas. The resulting crystals were allowed to dry for an additional 24 h and then were removed from the dish and stored in a glass container at 22°C. A working solution of cholesterol loaded cyclodextrin (CLC) was prepared by adding 50 mg of CLC to 1 ml of Tris diluent at 37°C and mixing the solution briefly using a vortex mixer.

Semen collection: Semen was collected during the morning hours using an artificial vagina as per the standard method. Ejaculates (48) from 3 bulls (16 from each bull) were selected on the basis of mass activity (+3 and above) and individual progressive motility (70% and above).

Semen processing and preservation: Immediately after collection of semen, a fraction of each ejaculate was evaluated for various seminal attributes (progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response) and antioxidants (superoxide dismutase, catalase, glutathione peroxidase and total antioxidants). Each ejaculate was divided into group I: Control (diluted with Tris-egg yolk-glycerol dilutor up to 60×10^6 sperm/ml) and group II: cholesterol loaded cyclodextrin (CLC) was added @ 3 mg/120×10^6 sperm, incubated at 37ºC for 10 min for entry of cholesterol into sperm plasma membrane and finally diluted with Tris-egg yolk-glycerol dilutor up to 60×10^6/ml and loaded in French medium straws. Filled straws were then subjected to a combined cooling with an equilibration period of 3 h at 5°C. The rack along with the straws was transferred to biological cell freezer for automated freezing. The freezing in biological cell freezer was carried @ 5°C/min for 4° to –10°C; 40°C/min for –10° to –100°C and 20°C/min for –100° to –140°C. Straws were then plunged into liquid nitrogen (–196°C) for storage until assayed. Semen was evaluated before freezing and after thawing for seminal attributes (progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response) and antioxidants (superoxide dismutase, catalase, glutathione peroxidase and total antioxidants).

Semen analysis: The concentration and individual progressive motility of spermatozoa (%) in semen samples was determined using sperm quality analyzer (Johnston *et al.* 1995). The live sperm percentage was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.* 1953). Acrosomal intactness was determined by Giemsa stain (Watson 1975). Hypo-osmotic swelling test (HOST) was carried out according to the method described by Jeyendran *et al.* (1984).

Estimation of activity of antioxidants: Superoxide dismutase (SOD) was estimated as per the method of Madesh and Balasubramanian (1997) with some modifications. The reaction mixture consisted of 100 µl of seminal plasma, 60 µl of 1.25 mM MTT, 1280 µl of PBS (pH 7.4) and 15 µl of 1 mM pyrogallol. Pyrogallol solution was freshly prepared every time to prevent auto-oxidation. In blank, seminal plasma was replaced with same amount of PBS. Formazan crystals formation reaction by reduction of MTT was terminated by addition of 1.5 ml of DMSO and reading was taken spectrophotometrically at 570 nm using double beam UV-VIS spectrophotometer. One unit of SOD was defined as the amount of protein required to inhibit MTT reduction by 50%. The total SOD activity was expressed in units/mg of protein present in seminal plasma.

Catalase (CAT) was estimated by spectrophotometric method as described by Bergmeyer and Grabl (1983) and was expressed as mM H_2O_2 utilized/min/mg protein. Activity of Glutathione peroxidase (GP_x) and total antioxidants (TA) in seminal plasma were carried out with GP_x and antioxidant assay kits, respectively.

Statistical analysis: Data were analyzed using unpaired t-test by Statistical Analysis System (SAS 2011) Software Programme, version 9.3 and results were expressed as mean ± SD.

RESULTS AND DISCUSSION

The present study illustrates the effect of cholesterol loaded cyclodextrin on activity of various antioxidants (superoxide dismutase, catalase, glutathione peroxidase and total antioxidants) during cryopreservation of buffalo semen. Mean values of seminal attributes at fresh, at pre-freeze and post-thaw stage are presented in Table 1. Seminal attributes were significantly higher in group II as compared to group I (control) at pre-freeze ($P<0.05$) and post-thaw ($P<0.01$) stage. Average values of antioxidants at fresh, at pre-freeze and post-thaw stage are presented in Table 2. No significant difference in activity of any antioxidant was observed between group I and group II at pre-freeze stage. At post-thaw stage, average activity of superoxide dismutase, catalase and total antioxidants were significantly ($P<0.05$) higher in group II as compared to group I. However, no significant difference in activity of glutathione peroxidase was observed at pre-freeze and post-thaw stage.

Superoxide dismutase is one of the enzymatic antioxidants involved in dismutation of superoxide anion ($O_2^{-\bullet}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2) and also protects the spermatozoa against deleterious effects of oxygen and lipid peroxidation (Sikka 1996). Average fresh seminal superoxide dismutase activity was higher than the levels reported by Mayuri (2006) but lower than the reports of Kumar *et al*. (2015). Percentage reduction in superoxide dismutase activity from fresh to post-thaw was 69.23 and 48.07, in control and treatment groups, respectively. Reduced post-thaw superoxide dismutase activity was in agreement with reports of Rajoriya *et al.* (2013) and Bilodeau *et al.* (2000). Higher post-thaw superoxide dismutase activity in treatment group may be due to lower levels of reactive oxygen species and lipid peroxidation.

Fresh seminal glutathione peroxidase activity was higher than that reported by Waheed *et al.* (2013). Mean seminal glutathione peroxidase activity was reduced during freezing which was in agreement with Kadirvel *et al.* (2014). The

Table 1. Effect of cholesterol loaded cyclodextrin on seminal attributes at fresh, at pre-freeze and post-thaw stage.

Seminal attribute	Fresh stage	Pre-freeze stage		Post-thaw stage	
		Group I	Group II	Group I	Group II
Progressive motility	85.27±5.25	74.58^{B}±5.63	79.47^{A}±5.28	51.35^{b}±3.38	60.83^{a}±3.62
Viability	89.45±4.70	78.33^{B}±5.24	83.27^{A}±4.52	56.89^{b} ±3.03	65.06^{a}±2.91
Acrosomal integrity	88.01±3.20	80.17^{B}±3.26	85.71^{A}±2.48	58.80^{b}±2.53	66.87^{a}±2.99
HOS response	77.37±5.38	70.91^{B}±5.92	75.62^{A}±5.24	51.30^{b} ±4.43	62.48^{a}±4.92

Mean showing different superscripts in upper case letters ($^{A, B}$) and in lower case letters ($^{a, b}$) in row differ significantly at 5% ($P<0.05$) and 1% ($P<0.01$), respectively.

Table 2. Effect of cholesterol loaded cyclodextrin on activity of seminal antioxidants at fresh, at pre-freeze and post-thaw stage.

Antioxidant	Fresh stage	Pre-freeze stage		Post-thaw stage	
		Group I	Group II	Group I	Group II
Superoxide dismutase	0.52±0.01	0.39±0.06	0.41±0.06	0.16^{b} ±0.03	0.27^{a} ±0.05
Glutathione peroxidase	91.60±37.71	111.96±39.85	110.30±38.96	59.82±21.97	68.97±20.65
Catalase	0.31±0.07	0.042±0.00	0.043±0.00	0.001^{b}±0.00	0.02^{a} ±0.01
Total antioxidants	1.82±0.13	1.71±0.11	1.76±0.11	1.34^{b} ±0.09	1.56^{a} ±0.10

Means showing different superscripts in row differ significantly at 5% ($P<0.05$).

percentage reduction in activity of glutathione peroxidase from fresh to post-thaw stage was 34.69 and 24.70, in Group I and II, respectively. No significant difference in glutathione peroxidase activity was observed between control and treatment group.

Average fresh catalase activity was similar to the reports of Mayuri (2006). Catalase activity was reduced 99.67% in group I and 93.54% in group II, from fresh to post-thaw stage. Kadirvel *et al.* (2014) reported no detectable activity of catalase in frozen-thawed spermatozoa. The reduction in activity of antioxidants in frozen-thawed semen was in agreement with the reports of Bilodeau *et al.* (2000).

Fresh seminal total antioxidants were higher than reported by Eghbali *et al*. (2010). There was reduction in total antioxidants during equilibration, the reduction may be due to consumption of antioxidants by the reactive oxygen species during equilibration. The reduction in total antioxidants from fresh to post-thaw stage was 26.37 and 14.28%, respectively, in group I and II. The higher activity of total antioxidants in cholesterol loaded cyclodextrin treated semen may be due to reduced formation of free radicals which in turn lead to reduced consumption of antioxidants. The higher total antioxidant activity may be due to presence of cholesterol which is an antioxidant and also due to reduced production of free radicals. It is well known that treatment of sperms with cholesterol loaded cyclodextrin protects against freezing, thawing induced damages in various animal species (Rajoriya *et al.* 2014 and Yildiz *et al*. 2015).

In conclusion, addition of cholesterol loaded cyclodextrin significantly enhances the post-thaw activity of superoxide dismutase, catalase and total antioxidants which in turn lead to better freezability of buffalo semen. The cryoprotective influence of cholesterol loaded cyclodextrin on buffalo semen might be due to its antioxidant property. These findings may need to be further validated with large number of semen samples.

REFERENCES

Bergmeyer H U J and Grabl M. 1983. Methods of enzymatic analysis. 3rd ed. *Weinheim Verlag Chemie.* pp. 273–302.

Bilodeau J F, Chatterjee S, Sirard M A and Gagnon C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development* **55**: 282–88.

Campbell R G, Hancock J L and Rothschild L. 1953. Counting live and dead bull. *Journal of Experimental Biology* **30**: 44–49.

Dobziuk H. 2006. Molecules with holes– cyclodextrins. Dodziuk H (ed.), Cyclodextrins and their complexes. *Wiley-VCH Verlag GmbH & Co*, Weinheim, Germany. pp. 1–30.

Eghbali M, Alavi S M, Rezaii S A and Ansari M. 2010. Calcium, magnesium and total antioxidant capacity (TAC) in seminal plasma of water buffalo (*Bubalus bubalis*) bulls and their relationships with semen characteristics. *Veterinary Research Forum* **1**:12–20.

Guthrie H D and Welch G R. 2012. Effects of reactive oxygen species on sperm function. *Theriogenology* **78**: 1700–08.

Jeyendran R S, Vander, Ven H H, Parez M, Crabo B G and Zaneweld L J D. 1984. Development of an assay to assess the functional integrity of the human membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70**: 219–28.

Johnston R C, Clarke G N, Liu D Y and Gordon Baker H W.1995.

Assessment of sperm quality analyser. *Fertility Sterility* **63** (5): 1071–76.

Kadirvel G, Kumar S, Ghosh S K and Perumal P. 2014. Activity of antioxidative enzymes in fresh and frozen thawed buffalo (*Bubalus bubalis*) spermatozoa in relation to lipid peroxidation and semen quality. *Asian Pacific Journal of Reproduction* **3** (3): 210–17.

Kumar P, Kumar D, Sikka P and Singh P. 2015. Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. *Animal Reproduction Science* **152**: 26–31.

Lewis S E, Sterling E S and Young I S. 1997. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertility and Sterility* **67**(1): 142–47.

Madesh M and Balasubramanian A K. 1997. Micro titre assay for superoxide dismutase using MTT reduction by superoxide. *Indian Journal of Biochemistry and Biophysics* **35**:184–88.

Mayuri R. 2006. 'Studies on oxidative damages and effects of certain antioxidants on buffalo spermatozoa during cryopreservation'. *M.V.Sc. Thesis* submitted to Deemed University, IVRI, Izatnagar, UP, India.

Muller K, Muller P, Pincemy G, Kurz A and Labbe C. 2008. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa. *Biology of Reproduction* **78**(3): 390–99.

Naseer Z, Ejaz A, Aksoy M, Kucuk N, Serin I, Caylan A, Boyacloglu M and Kum K. 2015. Protective effect of cholesterol-loaded cyclodextrin pretreatment against hydrogen peroxide induced oxidative damage in ram sperm. *Cryobiology* **71**: 18–23.

Purdy P H and Graham J K. 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. *Cryobiology* **48**: 36–45.

Purdy P H. 2006. A review on goat sperm cryopreservation. *Small Ruminant Research* **63** (3): 215–25.

Rajoriya J S, Prasad J K, Ghosh S K, Perumal P, Kumar A, Kaushal S and Singh M. 2013. Effects of seasons on enzymatic changes and cholesterol efflux in relation to freezability in Tharparkar bull semen. *Asian Pacific Journal of Reproduction* **2:** 280–88.

Rajoriya J S, Prasad J K, Ghosh S K, Ramteke S, Barik N C, Das G K and Pande M. 2014. Cholesterol loaded cyclodextrin increases freezability of buffalo bull (*Bubalus bubalis*) spermatozoa by increasing cholesterol to phospholipid ratio. *Veterinary World* **7**: 702–06.

Serin I, Aksoy M and Ceylan A. 2011. Cholesterol loaded cyclodextrin inhibits premature acrosomal reactions in liquid-stored spermatozoa. *Animal Reproduction Science* **123**(1–2): 106–111.

SAS Institute Inc. 2011. SAS® 9.3 System Options: Reference, Second Edition. *Cary NC SAS Institute Inc*.

Sikka S C. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Frontiers in Bioscience* **1**: 78–86.

Waheed M M, Gouda E M and Khalifa T A. 2013. Impact of seminal plasma superoxide dismutase and glutathione peroxidase on cryopreserved buffalo spermatozoa. *Animal Reproduction Science* **142**(3–4): 126–30.

Watson P F. 1975. Use of Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. *Veterinary Record* **97**: 12–15.

Yildiz C, Yavas I, Bozkurt Y and Aksoy M. 2015. Effect of cholesterol-loaded cyclodextrin on cryosurvival and fertility of cryopreserved carp (*Cyprinus carpio*) sperm. *Cryobiology* **70**: 190–94.