

**Short Communication**

**ANTIOXIDANT ENZYMES PROFILE DURING CRYOPRESERVATION OF NILI RAVI BUFFALO BULL SPERMATOZOA (*BUBALUS BUBALIS*)<sup>ϕ</sup>**

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**ABSTRACT**

Present study was designed to ascertain antioxidative enzyme shift during cryopreservation of buffalo bull spermatozoa. Enzymatic profile of three enzymes (catalase, superoxide dismutase and glutathione peroxidase/reductase) was established for fresh, post-dilution, pre-freezing and post-thaw stages. A total of twenty semen samples from four bulls were cryopreserved in French straws by using tris based extender. Catalase (U/mL), superoxide dismutase (U/mL) and total glutathione ( $\mu$ M) profile for fresh semen ( $25.8 \pm 1.6$ ,  $93.1 \pm 1.3$ ,  $77.4 \pm 5.2$ ; respectively), lowered with semen extension ( $6.0 \pm 0.6$ ,  $60.7 \pm 2.4$ ,  $63.0 \pm 0.9$  respectively;  $P < 0.05$ ). During equilibration time, the enzymatic profile for catalase remained stable ( $7.0 \pm 0.7$ ;  $P > 0.05$ ) but superoxide dismutase and glutathione profile deteriorated further ( $47.7 \pm 1.4$ ,  $44.6 \pm 0.6$  respectively;  $P < 0.05$ ). During cryopreservation, major shift was observed in catalase profile ( $2.3 \pm 0.3$ ;  $P < 0.05$ ) while superoxide dismutase and glutathione readings were stable in post-thaw evaluations ( $P > 0.05$ ). It is concluded that cryopreservation process deteriorated catalase enzyme profile while superoxide dismutase and glutathione profiles are declined by metabolic activities of spermatozoa in time dependent manner.

**Keywords:** Buffalo semen, Cryopreservation, Antioxidant enzyme activity

**INTRODUCTION**

Mammalian spermatozoa are naturally equipped with enzymatic and non-enzymatic defense systems to cope with the oxidative stress (Lewis *et al.*, 1995). The enzymatic antioxidant systems generally consist of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase/reductase (GSH). These natural antioxidant enzymes are principally, of cytoplasmic in nature. During spermatogenesis major part of cytoplasm is removed during sperm maturational stages. Therefore, mammalian spermatozoa are deficient of adequate reserves of natural antioxidants against the detrimental effects of cryopreservation (Aitken and Fisher, 1994). Additionally, every stage of cryopreservation (cooling, freezing and thawing) further dilutes antioxidative defense mechanism, which results in poor survivability and fertility (Wang *et al.*, 1997). Buffalo spermatozoa are more vulnerable to lethal effects of cryopreservation as compared to cattle due to higher contents of polyunsaturated fatty acid PUFA in their membrane structure (Andrabi, 2009; Chatterjee and Gagnon, 2001). Consequently, buffalo spermatozoa are unable to endure the damages occurring during cryopreservation due to inadequate intrinsic defense system (Aurich *et al.*, 1997).

Literature regarding antioxidant enzymes shift during cryopreservation of water buffalo bull spermatozoa is not available. Therefore, present study

was designed to establish antioxidant enzymes (Catalase, Superoxide dismutase) and total Glutathione profile during different stages of cryopreservation (fresh, post-dilution, pre-freezing, post-thawing) of Nili Ravi buffalo bull spermatozoa. The results of this study will help to enrich extenders with antioxidants in a rationalized pattern to detoxify the detrimental effects of reactive oxygen species (ROS) produced during sperm cryopreservation.

**MATERIALS AND METHODS**

**Selection of animals:** Present study was conducted at Semen Production Unit, Qadirabad, Sahiwal, Pakistan ( $30^{\circ} 43' 0''$  North,  $73^{\circ} 57' 0''$  East). Four donor buffalo bulls of similar age (6 years) were selected. The study was carried out during the peak breeding season.

**Semen collection, extension and freezing:** Artificial vagina maintained at  $42^{\circ}\text{C}$  was used to collect the semen. Two consecutive ejaculates were collected once a week from each animal for five weeks (replicate). Visual motility was evaluated under phase contrast microscope (BH-2, Olympus, Japan) at 200x. Sperm concentration was estimated spectrophotometrically (IMV, France). At least one ejaculate per bull on the day of collection did qualify (volume  $> 1$  ml; concentration  $> 500 \times 10^6$  sperm  $\text{ml}^{-1}$ ; progressive motility  $> 65\%$ ) for further processing. Tris-citric acid based extender [Tris 199.80 mM, citric acid 69.75 mM, fructose 55.56 mM, egg yolk 20% (v/v),

glycerol 7% (v/v), Penicillin 1000 (IU ml<sup>-1</sup>) and Streptomycin 1000 (µg ml<sup>-1</sup>), pH 6.9, osmolarity 295mOsm Kg<sup>-1</sup>] was used in the present study. Semen samples of individual animals were diluted with the extender to a final concentration of  $50 \times 10^6$  spermatozoa ml<sup>-1</sup> at 37°C. Extended semen was cooled to 4°C within 2 h and equilibrated at 4° C for 4h. Semen samples were packed and sealed in 0.54 ml French straws at 4°C. Semen filled straws were frozen in liquid nitrogen vapors for 10 min. Finally straws were plunged and stored in liquid nitrogen (-196°C). Post thaw sperm antioxidant enzymes profile was evaluated after at least 24 h of storage in liquid nitrogen.

**Sperm antioxidant enzymes profile:** Antioxidant profile [catalase, SOD and total glutathione] was determined at different stages of cryopreservation (fresh, post dilution, pre freezing, and post thawing). Semen samples (120µL) fresh/diluted were centrifuged at (1600g, for 5 min) to separate seminal plasma. After discarding the supernatant, 360 µl of 1% Triton X-100 was added for extraction into the precipitate for 20 min, which was subsequently centrifuged at 25°C for 30 min at 4,000g. The precipitate was re-suspended and finally the supernatant was collected containing crude extract of enzymes in the sperm (Hu *et al.*, 2010). The antioxidant profile (catalase, SOD and total glutathione) was analyzed through ELISA. Catalase enzyme profile was analyzed through the commercial kit OxiSelect™ STA-339, (CELL BIOLABS, USA), SOD with Sigma-Aldrich 19160, kit and total Glutathione with OxiSelect™ STA-312, (CELL BIOLABS, USA)(Tuncer *et al.*, 2013).

**Statistical Analysis:** The experiment was repeated five times and results were presented as mean ± SEM. Means were analyzed by 2 factor factorial (bull and stage) model of ANOVA, followed by DMRT post hoc test to define statistically differences in all parameters among all groups using the SPSS soft-ware package (version 16.0; SPSS/PC, Chicago, IL). Differences within values of P<0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

It is well understood that the production of reactive oxygen species promote sperm cell damages by deteriorating plasma membrane and DNA integrity (Atig *et al.*, 2017; Lewis *et al.*, 1995). Buffalo spermatozoa are more vulnerable to lipid peroxidation (LPO) as compared to cattle due to higher contents of polyunsaturated fatty acid PUFA in their membrane structure during cryopreservation (Andrabi, 2009; Chatterjee and Gagnon,

2001). Consequently, sperm cells are unable to withstand the oxidative consequences of LPO due to inadequate intrinsic defense system (Aurich *et al.*, 1997). These concerns are being addressed by antioxidants addition in semen extenders, and now it is a routine practice in bovine cryobiology industry. To better understand the antioxidants addition in buffalo semen extenders a total of 20 semen samples from four Nili-Ravi buffalo bulls were processed for cryopreservation. In Nili-Ravi buffalo it is for the first time to confidently establish antioxidant enzymes activities during different stages of cryopreservation (fresh, post-dilution, pre-freezing and post-thawing). Catalase (U/mL), superoxide dismutase (U/mL) and total glutathione (µM) profile during fresh (25.8±1.6, 93.1±1.3, 77.4±5.2), post dilution (6.0±0.6, 60.7±2.4, 63.0±0.9), pre freezing (7.0±0.7, 47.7±1.4, 44.6±0.6), and post thawing (2.3±0.3, 47.1±1.2, 41.1±0.7) are presented in Table 1. During semen extension, the enzyme concentration of all three mentioned enzymes reduced significantly. This reduction in enzymatic profile was mainly due to dilution effect. To cope with dilution factor, addition of extracellular antioxidants such as vitamin E and C, Alpha-Lipoic acid (ALA), Taurine, Trehalose, Methionine and Carnitine in semen extenders is routine practice in recent years (Sharafi *et al.*, 2015; Treulen *et al.*, 2019).

During equilibration time the catalase profile remained stable which indicates catalase was mainly contributed by seminal plasma and after its dilution with extender, spermatozoal activity did not impact on its concentration. These results are in line with those reported in human seminal plasma (Zini *et al.*, 1993). In contrast to catalase, the concentrations of both superoxide dismutase and glutathione deteriorated further (P<0.05) during equilibration time (Table 2). These results indicate the effect of metabolic activities on these enzymes. Similar values for superoxide dismutase are also reported in bovine (Bilodeau *et al.*, 2000).

Cryopreservation further deteriorate the activities of catalase but super oxide dismutase and glutathione remained stable. These observations are contrary to findings of cattle semen where it is reported that the cryopreservation reduces the superoxide dismutase and glutathione activities (Bilodeau *et al.*, 2000). For human semen, loss of superoxide dismutase is also reported but glutathione remained stable (Alvarez and Storey, 1992).

It is concluded that process of cryopreservation significantly reduced antioxidant enzymes profile during different stages (post dilution, pre freezing and post thaw).

**Table 1. Antioxidant Enzymes Profile during Cryopreservation of Buffalo spermatozoa.**

Stage of Cryopreservation	Catalase U/mL	Superoxide dismutase U/ml	Total Glutathione $\mu$ M
Fresh	25.8 $\pm$ 1.6 <sup>a</sup>	93.1 $\pm$ 1.3 <sup>a</sup>	77.4 $\pm$ 5.2 <sup>a</sup>
Post Dilution	6.0 $\pm$ 0.6 <sup>b</sup>	60.7 $\pm$ 2.4 <sup>b</sup>	63.0 $\pm$ 0.9 <sup>b</sup>
Pre Freezing	7.0 $\pm$ 0.7 <sup>b</sup>	47.7 $\pm$ 1.4 <sup>c</sup>	44.6 $\pm$ 0.6 <sup>c</sup>
Post Thaw	2.3 $\pm$ 0.3 <sup>c</sup>	47.1 $\pm$ 1.2 <sup>c</sup>	41.1 $\pm$ 0.7 <sup>c</sup>

<sup>a-c</sup>: Denote differences (P<0.05) within columns.

Data are mean $\pm$ SE (n=20)

**Table 2. Decline in antioxidative enzymes activity (%) during buffalo sperm freezing.**

Stage of Cryopreservation	Catalase U/mL	Superoxide dismutase U/ml	Glutathione peroxidase $\mu$ M
Fresh to Post Dilution	82.0%	33.3%	15.8%
Fresh to Pre Freezing	76.1%	39.5%	34.4%
Fresh to Post Thaw	94.2%	48.9%	44.1%

Data are mean (n=20)

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