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Effect of Superoxide Dismutase and Catalase on Viability of Cryopreserved Buffalo Spermatozoa

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Abstract: This study aimed to assess the effects of antioxidants Enzymes Superoxide Dismutase (SOD) and catalase (CAT) or their combinations in reducing the causes of oxidative damage in buffalo bull spermatozoa during cryopreservation. Semen was collected at weekly intervals from 5 mature buffalo bulls and was diluted with Tris-citrate-fructose yolk extender supplemented with different concentrations and combinations of SOD (50, 100 and 200 U/ml) and CAT (50, 100 and 200 U/ml) then processed for cooling and freezing. Semen characteristics (sperm motility, viability, abnormalities, acrosome integrity and membrane integrity) were investigated after cooling to 5°C and freeze-thawing. Results showed that CAT addition (50 and 100 U alone or in different combinations with 50 and 100 U SOD) to extender had beneficial effects on semen chrematistics after cooling and freeze-thawing. SOD addition at concentration 50 and 100 U resulted in significant increase ($p<0.01$) in sperm motility%, membrane integrity%, intact acrosome%, viable sperm% and decrease in sperm abnormalities%. In conclusion addition of SOD and CAT in the semen extender could be useful to preserve buffalo bull spermatozoa against the oxidative stress generated by cryopreservation and may be useful in improving the quality of cryopreserved buffalo bull semen.

Key words: Buffalo-bull · semen · cryopreservation · antioxidant · catalase · superoxide dismutase

INTRODUCTION

Functional sperm parameters are reportedly affected by Reactive Oxygen Species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}) and hydroxyl radical (-OH) [1]. These substances are very toxic, strong oxidants and physiologically produced, in living cells during respiration [2], phagocytic cells present in an ejaculate and female reproductive tract [3, 4]. During various processing procedures for *in vitro* fertilization programs or for AI, ROS seems to be produced and result in reduction of sperm cell motility and damage of the genomic integrity [5, 6]. In addition, defective and dead spermatozoa have been identified as major imperfections of ROS generation during cryopreservation [7]. Generally, cryopreservation is accepted as an important inducer of ROS production [8]. Sperm cells are susceptible to lipid peroxidation as they contain a high content of unsaturated fatty acids in their membranes, while, they lack a significant cytoplasmic component containing antioxidants [5, 9]. Seminal plasma is a powerful source of antioxidants [10] such as SOD and CAT that counteract the toxic effect of O^{2-} and H_2O_2. The SOD converts the O_2^- to H_2O_2 then the CAT converts H_2O_2 into H_2O and O_2, eliminating the potential ROS toxicity [11]. Natural antioxidants exert a protective effect on the plasma membrane in cryopreserved bovine spermatozoa, preserving both metabolic activity and cellular viability [12]. Supplementation with various antioxidants improved the viability and motility of liquid-or cryo-preserved mammalian spermatozoa. Adding glutathione peroxidase with or without SOD to a whole milk glycerol extender improved the percentage of motile bull spermatozoa after freezing and thawing [13]. A combination of SOD and CAT in tris-glucose-yolk diluent had an additive effect to improve the survival of ram spermatozoa at 5°C [14] and post-thaw sperm survival in boar [15].

In a previous study, [16] noticed a significant decrease in antioxidant enzyme activities and increase in lipid peroxidation during chilling and freezing of buffalo bull spermatozoa. [6] concluded that bulls with lower lipid peroxidation in frozen semen had higher chances of siring calves and this was attributed to the deleterious effect of lipid peroxidation on the plasma membrane integrity and sperm DNA, which may have decreased fertilization potential. So far, inadequate information is available on

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the application of antioxidant to prevent cryodamage of buffalo-bull spermatozoa. Thus, the aim of this study was to investigate the role of antioxidant enzymes (SOD, CAT or their combinations) on the viability of cryopreserved buffalo spermatozoa following their addition to the used extender, as a preliminary step for designing improved cryoprotectants for buffalo semen.

MATERIALS AND METHODS

Source of semen and evaluation: Five mature buffalo bulls, maintained at Buffalo Semen Freezing Center, General Organization for Veterinary Services, Ministry of Agriculture, Abbasia, Egypt, kept under optimal and identical condition of feeding and management, were used as semen donor in this study. Semen was collected by artificial vagina at weekly intervals (at 9.00 AM). Samples were pooled and held in a water bath at 37°C, while the volume, sperm concentration and initial percentage of motile sperm were estimated. The ejaculate from different bulls possessing at least 70% visual motility and 80% normal morphology were pooled to avoid bull to bull differences. Visual motility was assessed microscopically with closed circuit television [17]. The sperm cell concentration was determined using the Thoma rulling of the Neubaur haemocytometer.

Semen processing: The control extender for semen cryopreservation was Tris-citric Acid-fructose-egg Yolk (TCFY) diluents, prepared according to [18]. Semen samples were diluted with control extender containing either no antioxidant (control) or different concentrations or combinations of antioxidant enzymes SOD and CAT (treatments). Two separate experiments were performed to evaluate the effect of adding different concentrations of SOD and/or CAT to TCFY extender on post-thaw buffalo-bull sperm chrematistics.

Experiment 1: Adding 50, 100 or 200 U SOD/ml and 50, 100 or 200 U CAT/ml to the control extender.

Experiment 2: According to the result of experiment 1, the effect of addition of different combinations of SOD and CAT (50 or 100 U SOD/ml and 50 or 100 U CAT/ml) to the extender on post-thaw sperm parameters was tested.

Soon after the neat semen evaluation, aliquots of semen samples were diluted at 37°C with each supplemented extender in order to provide approximately a dilution rate of $60x10^6$ spermatozoa/ml. Extended semen was cooled slowly approximately in 2 hrs. to 4°C and equilibrated for 4 hrs. Semen was packed into 0.5 ml polyvinyl French straws and after equilibrium periods, the straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen for 10 minutes and were then dipped in liquid nitrogen. Semen-filled straws were plunged into liquid nitrogen (-196°C) for storage. After 24 hrs storage in liquid nitrogen, semen straws were thawed at 37°C for 1 minute for post-thaw semen quality assessment.

Semen quality assessment: The assessment was undertaken on neat semen, after cooling and freeze-thawing of buffalo bull spermatozoa. Frozen straws were thawed at 37°C for 1 minute. The parameters studied were sperm motility, sperm viability, sperm membrane integrity, total abnormalities and percent of normal intact acrosome.

Sperm motility: Using phase contrast microscope, set at magnification of 400 and equipped with a heating plate (37°C) and attached to closed circuit television, sperm motility was subjectively evaluated [17].

Sperm membrane integrity: Sperm membrane integrity was assessed using the Hyposmotic Swelling (HOS) test [19]. 200 spermatozoa were assessed and the percentage of spermatozoa with curled tails (swollen/intact plasma membrane) was calculated.

Acrosomal integrity: The acrosomal integrity percentage was evaluated under X 1000, by Giemsa staining method adopted by [20].

Sperm abnormalities and viability: This was established by Eiosin/Nigrosin staining [21]. All the semen evaluation was done by single person to avoid individual variations.

Statistical analysis: Data were analyzed statistically for various parameter by 1-way analyses of variance using statistical analysis program [22] user guide, 6.04. In the statistical model the effect of antioxidant addition is the main source of variance. In addition, differences between means were compared with LSD procedure ($P<0.05$).

RESULTS

Experiment 1: Data in Table 1 and 2 showed the effects of adding SOD and CAT to semen extender on some sperm parameters. Sperm motility (%) after cooling (5°C) was significantly ($P<0.05$) improved when CAT was added to the extender at a concentration of 50 U/ml

Table 1: Effect of the addition of superoxide dismutase (SOD) and Catalase (CAT) to the extender on the buffalo bull semen quality parameters after cooling to 5°C

	Semen characteristics				
Treatment	Motility (%)	Membrane integrity (%)	Live sperm (%)	Abnormal sperm (%)	Intact acrosome (%)
Control	63.5±2.24	65.8±1.70	69.3±1.81	9.2±080	74.9±1.18
SOD 50 U/ml	61.0±1.00	67.3±1.99	71.3±1.27	9.3±0.54	78.8±1.83
SOD 100 U/ml	58.0±1.11	71.4±1.65*	73.0±2.03	10.7±0.59	75.4±1.43
SOD 200 U/ml	56.0±1.24	66.4±1.69	70.9±1.68	11.0±1.03	69.9±1.57*
CAT 50 U/ml	69.5±1.57*	73.9±2.45*	72.5±1.62	8.4±0.37	79.2±1.91
CAT 100 U/ml	63.7±1.61	74.4±1.96*	71.5±3.37	9.6±0.73	76.8±0.99
CAT 200 U/ml	60.5±0.91	70.5±1.16*	73.5±1.69	10.1±0.64	74.1±1.11

Within column, (*) indicates significant difference at least at P<0.05 of a given element from control

Table 2: Effect of the addition of superoxide dismutase (SOD) and Catalase (CAT) in the extender on the post-thaw buffalo bull semen quality parameters

	Semen characteristics				
Treatment	Motility (%)	Membrane integrity (%)	Live sperm (%)	Abnormal sperm (%)	Intact acrosome (%)
Control	35.9±1.44	48.9±1.55	49.0±1.86	17.7±1.41	46.4±1.87
SOD 50 U/ml	40.0±1.66	52.6±1.57	53.1±1.72	13.3±80*	50.8±1.28
SOD 100 U/ml	36.6±1.46	54.6±2.07*	55.2±2.13*	17.3±1.03	51.4±2.38*
SOD 200 U/ml	32.0±1.33	48.6±1.42	50.5±1.32	19.1±1.33	44.1±1.89
CAT 50 U/ml	43.5±1.07*	57.0±1.35*	56.9±1.45*	11.3±0.95*	55.0±0.99*
CAT 100 U/ml	42.2±1.08*	58.9±1.36*	55.2±1.96*	14.6±0.87*	53.4±1.68*
CAT 200 U/ml	41.0±2.45*	52.4±1.21	53.9±1.15	16.6±1.23	48.2±1.45

Within column, (*) indicates significant difference at least at P<0.05 of a given element from control

(69.5±1.57) as compared to the control value (63.5±2.24). Meanwhile, the post-thawing sperm motility significantly (P<0.01) improved in 50, 100 and 200 U/ml CAT supplemented groups (43.5±1.07, 42.2±1.08 and 41.00±2.45%, respectively) as compared to the control group (35.9±1.44) as shown in Table 2. No significant effect, for SOD addition to extender, on sperm motility could be detected.

Membrane integrity (HOS) was significantly (P<0.05) higher after cooling in case of 50, 100 and 200 U/ml CAT supplemented groups (73.9±2.45, 74.4±1.96 and 70.5±1.1, respectively) and in case of addition of 100 U/ml SOD supplemented group (71.4±1.65) as compared to the control (65.8±1.70), (Table 1). Also, post-thaw membrane integrity was significantly higher (P<0.01) in case of 50 and 100 U/ml CAT supplemented groups (57.00±1.35 and 58.9±1.36%, respectively) and in 100 U SOD supplemented group (54.6±2.07%) in comparison to the control (48.9±1.55%) one (Table 2).

No significant difference in live sperm% and sperm abnormalities% could be detected between groups after cooling, however, there was significant (P<0.05) improvement in post-thawing live sperm% for the 50 and 100 U/ml CAT addition (56.9±1.45 and 55.2±1.96, respectively) and for the 100 U/ml SOD supplemented (55.2±2.13) compared to control (49.00±1.86) group. An inversely proportion pattern was observed in total sperm abnormalities as in Table 2, whereas, the 50 and 100 U/ml CAT (11.3±0.95 and 14.6±0.87%) and 50 U/ml SOD (13.3±80%) supplemented groups had significantly lower post-thawing sperm abnormalities as compared to non supplemented control group (17.7±1.41).

Percent of post thawing intact acrosome was significantly (P<0.01) improved in case of 50 and 100 U/ml CAT (55.00±0.99% and 53.4±1.68, respectively) and in case of 100 U/ml SOD (51.4±2.38%) supplemented groups (Table 2) compared to the control (46.4±1.87).

Experiment 2: The effects of addition of different combinations of SOD and CAT on buffalo bull semen quality after cooling and freeze thawing were presented in Table 3 and 4.

As shown in Table 3 and 4, addition of combination of SOD and CAT with different concentrations did not significantly improve semen quality (sperm motility, viability and intact acrosome sperm abnormalities and membrane integrity percents).

Table 3: Effect of the addition of different combinations of superoxide dismutase (SOD) and Catalase (CAT) to the extender on the buffalo bull semen quality parameters after cooling to 5°C

	Semen characteristics				
Treatment	Motility (%)	Membrane integrity (%)	Live sperm (%)	Abnormal sperm (%)	Intact acrosome (%)
Control	62.2±2.31	62.4±1.58	66.7±2.14	11.1±0.76	73.7±2.06
50 U SOD+50 U CAT/ml	65.5±1.57	70.4±2.14*	73.5±1.57*	9.4±0.77	76.6±2.28
50 USOD+100 U CAT/ml	66.0±1.79	75.3±2.26*	71.5±2.59	9.7±0.50	75.5±1.56
100 U SOD+50 U CAT/ml	65.0±1.82	71.1±2.01*	72.7±2.33	10.2±0.87	72.6±1.19
100 U SOD+100 CAT/ml	67.5±2.00	75.3±1.80*	70.1±2.01	10.4±1.30	79.1±2.23

Within column, (*) indicates significant difference at least at P<0.05 of a given element from control

Table 4: Effect of the addition of different combinations of superoxide dismutase (SOD) and Catalase (CAT) to the extender on the post-thaw buffalo bull semen quality parameters

	Semen characteristics				
Treatment	Motility (%)	Membrane integrity (%)	Live sperm (%)	Abnormal sperm (%)	Intact acrosome (%)
Control	36.0±1.63	49.8±1.94	48.5±2.77	17.5±1.25	45.1±2.03
50 U SOD +50 U CAT/ml	44.0±4.13*	57.6±1.70*	57.7±1.73*	10.9±0.75*	53.4±1.93*
50 USOD +100 U CAT/ml	40.5±2.52	56.5±2.56*	54.7±2.05*	15.2±1.08	50.4±2.22
100 U SOD +50 U CAT/ml	40.0±2.69	54.1±2.50	54.5±1.95*	17.1±1.63	49.0±1.93
100 U SOD +100 U CAT/ml	43.5±2.98*	59.0±2.37*	56.1±2.5*	14.9±1.02	52.3±2.66*

Within column, (*) indicates significant difference at least at P<0.05 of a given element from control

No significant difference could be detected between the control group and any of combination groups in sperm motility, abnormal sperm and intact acrosome after cooling (Table 3). However, there were significant (P<0.05) improvements in membrane integrity in all supplemented groups compared to the control group (Table 3).

As presented in Table 4, the post-thawing sperm motility was significantly (P<0.05) higher in 50 SOD +50 CAT U/ml (44.00±4.13) and 100 SOD +100 CATU/ml (43.5±2.98) compared to the control group (36.00±1.63).

Membrane integrity was significantly(P<0.05) improved in 50 SOD +50 CAT U/ml (57.6±1.70), 50 SOD +100 CAT U/ml (56.5±2.56) and 100 SOD +100 CAT U/ml (59.00±2.37) compared to the control group (49.8±1.94). The same pattern was noticed for live sperm however, there was significant (P<0.05) decrease in sperm abnormalities only in 50 SOD +50 CAT U/ml (10.9±0.75) compared to the control group (17.5±1.25). Also, intact acrosome% was significantly (P<0.05) higher in case of 50 SOD +50 CAT U/ml (53.4±1.93) and 100 SOD +100 CAT U/ml (52.3±2.66) in comparison with the control group (45.1±2.03).

DISCUSSION

Improvement of bovine semen cryopreservation requires better understanding of the properties of the currently used extenders. Processing of buffalo sperm for cryopreservation often induce an additional source for ROS attack on sperm due to decreased activities of antioxidant enzymes and during these procedures the sperm membrane become more susceptible to lipid peroxidation [16]. In physiological conditions, there are intra-and extra-cellular scavenger systems (enzymatic and non-enzymatic) which prevent the potential toxic effect of ROS. Of these, SOD and CAT activities have been reported in the seminal fluid of a number of mammals. These two enzymes are the most important elements in the protective system, since they are respectively the scavengers of superoxide anion and hydrogen peroxide, which are the first compounds in ROS chain formation [1]. Buffalo spermatozoa are more susceptible to peroxidative damage than that of cattle since buffalo spermatozoa is rich in polyunsaturated fatty acids like arachidonic acids and Decosahexaenoic acids [23, 24].

It was attempted in this study to unravel whether addition of antioxidant enzymes SOD and CAT to extender could improve cryopreservation of buffalo sperm and improving post-thawing semen quality. The results of the present study indicate significant increase of post-thaw sperm motility, membrane integrity, viability and acrosome integrity in case of addition of 50 and 100 CAT or 100 SOD to buffalo bull semen freezing extender. These results were in harmony with those for ram spermatozoa where the addition of 100 or 200 U/mL of bovine liver catalase improved the maintenance of motility, acrosomal

integrity and fertility during storage at 5°C [14]. Also, the results were similar to those in horse [25, 26] and in ram [14], where the addition of 100 and 200 U/ml of catalase improved the maintenance of motility, acrosomal integrity and fertility during storage at 5°C. Also, It was found that catalase also improved oocyte penetration of bull sperm [27]. [28] reported no significant variation in recovery of progressive motility in human semen with added SOD and CAT alone and observed improvement in sperm parameter recovery when they used both SOD and CAT combined, perhaps because of their combined and simultaneous action on superoxide anion and hydrogen peroxide. It was shown that the addition of catalase to bovine semen extender was beneficial to motility of frozen thawed spermatozoa [29]. In contrast to our results, [30] recorded that addition of catalase to equine semen did not affect the maintenance of motility of equine spermatozoa during storage at 5°C.

In conclusion, combined SOD and CAT enzyme supplementation to extender can contribute greatly to the reduction of sperm membrane lipid peroxidation by ROS during buffalo semen cryopreservation and thus allow good semen quality after freezing.

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