

ORIGINAL ARTICLE

## Effect of various levels of catalase antioxidant in semen extenders on lipid peroxidation and semen quality after the freeze-thawing bull semen

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### Abstract

The objective of this study was to evaluate effect of different concentrations of catalase in two extenders on motility, viability and lipid peroxidation bull spermatozoa during semen freezing process. Thirty ejaculates collected from ten Holstein bulls were pooled and evaluated at 37 °C. Pool ejaculated was split into two main experimental groups, 1 and 2. In experiment 1, specimen was diluted to a final concentration of  $30 \times 10^6$  spermatozoa with citrate-egg yolk and in experiment 2; specimen was diluted with tris-egg yolk extender to the same concentration. In both experiments diluted semen was divided into three aliquots, including a control and two test groups. Each aliquot was rediluted with an equal volume of extender either without (control) or with one of the antioxidants contained one of the following antioxidants: catalase (CAT; 100 IU mL<sup>-1</sup>) catalase (CAT; 200 IU mL<sup>-1</sup>) and control group. No significant differences were observed in sperm viability and motility following addition of catalase enzyme at concentration of 100 IU mL<sup>-1</sup> and 200 IU mL<sup>-1</sup> to citrate-egg yolk extender. But the highest sperm viability was achieved by addition of 100 IU mL<sup>-1</sup> and 200 IU mL<sup>-1</sup> catalase to tris-egg yolk semen extender compared with the control group ( $P < 0.05$ ). Malondialdehyde levels did not change with addition of catalase in both extenders compared with the control group. The obtained results provide a new approach to the cryopreservation of bull semen, and could positively contribute to intensive cattle production.

### Introduction

During the freezing process, increase in susceptibility of spermatozoa to lipid peroxidation, as affected by cold shock, plays an important role in ageing of spermatozoa, shortening their life span and affecting the preservation of semen. The major susceptibility is related, in part, to the content of polyunsaturated fatty acids,<sup>1</sup> which are susceptible to undergoing lipid peroxidation in the presence of the reactive oxygen species (ROS). The lipid peroxidation cascade is initiated when spermatozoa are attacked by ROS. It results in a loss of unsaturated fatty acids from the plasma membrane and a corresponding decline in the survival and fertilizing ability of these spermatozoa occurs.<sup>2</sup>

One way to overcome this cascade could be incorporation of antioxidants into freezing extenders. A variety of antioxidants have been tested to either scavenge ROS directly

or effects of counter ROS toxicity in semen of a variety of mammalian and avian species. Among them catalase (CAT) is one of the enzymatic systems that are physiologically contained in ejaculates for protection of spermatozoa against ROS damage, allowing degradation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into oxygen and water.<sup>3</sup> The use of CAT for protecting spermatozoa has been previously tested with varying results.<sup>4-8</sup> In spite of these promising results, the use of CAT in different freezing extenders is not common, and a few results are available that evaluate the potential effect of CAT on frozen-thawed bull spermatozoa in different extenders. The aim of this study was to evaluate effect of different concentrations of CAT in two extenders on motility, viability and lipid peroxidation bull spermatozoa during semen freezing process.

## Materials and Methods

**Animals and semen collection.** Ten fertile Holstein bulls were included to the study. At the beginning of the experiment the animals were 3-6 years old and weighed 300 -900 kg.

Forty ejaculates were collected using artificial vagina method from ten Holstein bulls with known fertility. All the ten bulls utilized in the study were routinely used for breeding purpose based on their fertility assessment through *in vitro* and *in vivo* fertility trials. Semen was collected from the bulls twice a week using artificial vagina (45 °C). The ejaculates were transferred to the laboratory and immersed in a water bath (34 °C), until semen evaluation.

**Semen processing.** Fresh semen was assessed for volume, concentration, motility and viability of spermatozoa. The volume of the semen ejaculates was measured in a conical tube graduated at 0.1 mL intervals and the sperm concentration was determined using hemocytometer. Sperm motility was evaluated at 400x magnification based on the visual estimation. Spermatozoa viability was determined by eosin-nigrosin stain.<sup>9</sup> only ejaculates with motility >70% and sperm concentration of  $1 \times 10^9$  spermatozoa/mL were included in this study. Among the 40 ejaculates collected, 10 were rejected because of low sperm motility. Thus thirty quality ejaculates (> 70% motility), three from each bull, were utilized for the study. This study included two experiments.

In experiment 1, semen diluted with citrate-egg yolk extender [Sodium citrate dihydrate (2.9 g dL<sup>-1</sup>); penicillin (1000 IU mL<sup>-1</sup>); streptomycin (1000 µg mL<sup>-1</sup>); 20% egg yolk ;7% glycerol and double distilled water to make the volume 100 mL] to yield  $30 \times 10^6$  spermatozoa/mL. The diluted semen was divided in three aliquots, including a control and two test groups. Each aliquot was rediluted with an equal volume of extender either without (control) or with one of the antioxidants contained one of the following antioxidants: CAT (100 IU mL<sup>-1</sup>) CAT (200 IU mL<sup>-1</sup>).

In experiment 2, semen diluted with tris-egg yolk extender (Tris 3.028g; Citric acid monohydrate 1.675g; Fructose 1.25g; Penicillin G sodium 1000 IU mL<sup>-1</sup>; Streptomycine sulphate 1000 ug mL<sup>-1</sup>; 20% egg yolk; 7% glycerol and double distilled water to make the volume 100 mL) to yield  $30 \times 10^6$  spermatozoa/mL. The diluted semen was divided into three aliquots, including one control and two test groups. Each aliquot was rediluted with an equal volume of extender either without (control) or with one of the antioxidants contained one of the following antioxidants: CAT (100 IU mL<sup>-1</sup>) CAT (200 IU mL<sup>-1</sup>).

In both experiments, semen extended was equilibrated in an equilibration chamber for 4 h at 5 °C before filling in 0.5 mL French straws.

The straws were placed on steel racks and held in liquid nitrogen at -140 °C for 10 min. Frozen straws were then immediately immersed in liquid nitrogen (-196 °C) and stored for 4 weeks until further assessment. At the time of analysis, three straws of semen from each treatment were thawed at 37 °C for 30s to perform the following semen quality parameters. Three (n=3) replicates were used in each treatment.

### Semen evaluation

**Post-thawed sperm motility.** Progressive motility was assessed using a phase contrast microscope (200 x magnification), with a warm stage maintained at 37 °C. A wet mount was made using a 5 µL drop of semen placed directly on a microscope slide and covered with a coverslip. Sperm motility estimations were performed in three microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score.

**Live and dead spermatozoa.** This was estimated as per standard staining procedure as described by Sidhu and Guraya (1985).<sup>10</sup> The staining solution contains eosin (0.67 g/100 mL), nigrosin (5 g/100 mL) and water to make the volume 100 mL. A drop of diluted semen mixed with eight drops of stain was incubated at 30 °C for 5 min. Then smears made on pre-warmed slides were allowed to dry at 30 °C. The excess stain was washed off in running tap water. The slide was then immersed briefly in ethanol to remove water. Then mounted smear was observed under 400 x objective of the light microscope. Approximately 400 sperms were counted. The dead sperms were stained red and could be easily seen against blue background of Nigrosin stain whereas live sperms were appeared obviously.

### Biochemical assay

Lipid peroxidation (LPO). An aliquot (500 µL) of semen from each sample was centrifuged at  $800 \times g$  for 10 min, sperm pellets were separated and washed by resuspending in PBS and recentrifuging (three times). After last centrifugation, 1 mL of deionized water was added to spermatozoa<sup>8</sup> and they were snap-frozen and stored at -70 °C until further analysis. The samples were thawed before the lipid peroxidation assay. The concentrations of Malondialdehyde (MDA), as indices of the LPO in the sperm samples, were measured using the thiobarbituric acid reaction and according to the method of Placer *et al.* (1966).<sup>12</sup> The quantification of thiobarbituric acid reactive substances was determined comparing the absorption with the standard curve of MDA equivalents generated by the acid catalyzed hydrolysis of 1, 1, 3, 3-tetramethoxypropane. The MDA concentrations were expressed in nmol/10<sup>9</sup>.

### Statistical analysis

The procedure was replicated three times for each treatment. Results are quoted as mean  $\pm$  SEM. Statistical analyses were carried out using the General Linear Model

procedures (GLM) of SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), followed the post-hoc was performed by Tukey test to determine the level of significance among mean values. The P-values less than 0.05 were considered to be significant.

## Results

Data for effect of different concentration CAT enzyme on citrate-egg yolk extender are showed in table 1. In this study reduction of MDA levels were not observed by the concentration 100 IU mL<sup>-1</sup> and 200 IU mL<sup>-1</sup> compared with the control. No significant differences were observed in viability and motility of frozen semen in different concentration of CAT.

Data for influence of CAT in tris-egg yolk semen extender are summarized in Table 2. This study showed no significant difference of MDA levels within different concentrations of CAT. However, significant differences were observed in viability of frozen semen following supplementation with CAT. In this study supplementation of tris-egg yolk extender by different concentrations of CAT (100 IU mL<sup>-1</sup> and 200 IU mL<sup>-1</sup>) increased semen viability compared with control groups ( $P < 0.05$ ). Also addition of different concentration of CAT enzyme on semen egg yolk-tris extender did not improve motility of frozen thawed semen (Table 2).

**Table 1.** Effect of catalase addition on post-thawed bull semen quality cryopreserved in citrate-egg yolk extender and lipid MDA (mean  $\pm$  SEM)

Parameters	Control	CAT 100 IU	CAT 200 IU
MDA (nmol/10 <sup>9</sup> )	2.63 $\pm$ 0.70	1.57 $\pm$ 0.75	1.82 $\pm$ 0.67
Viability (%)	48.30 $\pm$ 2.90	55.00 $\pm$ 5.00	51.70 $\pm$ 5.80
Motility (%)	40.70 $\pm$ 1.15	46.70 $\pm$ 2.89	43.30 $\pm$ 3.00

**Table 2.** Effect of catalase addition on post-thawed bull semen quality cryopreserved in tris-egg yolk extender and lipid peroxidation (mean  $\pm$  SEM)

Parameters	Control	CAT 100 IU	CAT 200 IU
MDA (nmol/10 <sup>9</sup> )	5.62 $\pm$ 1.05	5.32 $\pm$ 1.32	4.42 $\pm$ 0.18
Viability (%)	46.00 $\pm$ 2.00 <sup>a</sup>	51.70 $\pm$ 2.90 <sup>b</sup>	51.70 $\pm$ 2.90 <sup>b</sup>
Motility (%)	44.00 $\pm$ 1.73	46.70 $\pm$ 2.90	44.00 $\pm$ 2.00

Different superscripts (a and b) within the same row demonstrate significant differences ( $P < 0.05$ ).

## Discussion

The process of cryopreservation is known to cause more production and/or accumulation of reactive oxygen species, as antioxidant defenses are reduced in the process.<sup>4</sup> Frozen-thawed bull spermatozoa are more easily peroxidized than fresh spermatozoa.<sup>13</sup> Malonaldehyde produced from the breakdown of polyunsaturated fatty acids serves as a reliable index for determining the extent of the

peroxidation reaction and lipid damage.<sup>14</sup> Lipid peroxidation appears to be the major process of membrane damage as evident from increased MDA concentration in the frozen-spermatozoa. Hence, addition of antioxidants may have beneficial effects on spermatozoa function during the cryopreservation.

Our results showed that addition of CAT to the freezing citrate-egg yolk extender were ineffective on the post thawing viability of spermatozoa. These results are in agreement with works of Lopes *et al.* (1998), that showed CAT was ineffective in preventing human sperm DNA damage.<sup>15</sup> Thus, it has been suggested that H<sub>2</sub>O<sub>2</sub> has little effect on sperm chromatin, however, most research have shown an opposite conclusion. However, Roca *et al.* (2005) indicated that addition of CAT enzyme to semen extender improves post thaw sperm viability and fertility of boars.<sup>8</sup> In other experiment we have shown that adding CAT at a concentration of (100 IU mL<sup>-1</sup>, 200 IU mL<sup>-1</sup>) to tris-egg yolk extended bull semen greatly improved the viability and protected the sperm function, while concurrently reduced the amount of ROS in frozen-thawed spermatozoa. These results are in agreement with works of Maxwell and Stojanov (1996) that indicated addition of CAT to the extender improved survival of liquid stored ram spermatozoa.<sup>6</sup> Therefore, it is evident that CAT supplementation in cryoprotective medium reduces the production of ROS during cryopreservation, and thus protects human spermatozoa from freezing damages.<sup>16</sup> Moreover, the present study showed that effect of addition of CAT enzyme at a concentration of 100 IU mL<sup>-1</sup> and 200 IU mL<sup>-1</sup> to both extended bull semen could not increase the percentage of sperm motility. Similar results were found in ram spermatozoa where the addition of CAT in a chemically defined ram semen diluent was ineffective in improving sperm motility.<sup>17</sup> In equine semen addition of bovine liver CAT did not affect maintenance of motility,<sup>18</sup> perhaps because of the high endogenous CAT activity in this species. These are in contrast with the findings of Bilodeau *et al.* (2001) who indicated that addition of antioxidants to extender was beneficial for motility of frozen-thawed bull spermatozoa, even in absence of an external source of oxidative stress.<sup>4</sup> Ferná'ndez-Santos *et al.* (2007) reported that CAT supplementation to the freezing extender improves the post-thawing motility of red deer spermatozoa.<sup>5</sup> The other study, Ferná'ndez-Santos *et al.* (2008) reported that CAT supplementation after thawing prevented both the decrease of sperm motility and the increase of DNA damage under oxidant stress.<sup>19</sup> In this study the concentration of MDA was not changed significantly with the addition of the CAT to tris and citrate-egg yolk extenders compared to the control group. In spite of the above results, it is necessary to point out that the addition of CAT to extenders for preservation of mammalian semen did not result in variable success

regarding the improvement of sperm survival. Differences between preservation protocols, concentration of antioxidant enzyme, extender formulations among laboratories and species differences in the susceptibility of their spermatozoa to cooling, freezing, and thawing process may explain, at least in part, this variability.

On the basis of the present results it is concluded that catalase is inefficient antioxidant in citrate-egg yolk extender, however, addition of CAT to tris-egg yolk semen extender could be used as an agent to increase viability of bull sperm during semen freezing process. Moreover, obtained results provide a new approach to the cryopreservation of bull semen, and could positively contribute intensive cattle production.

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