Short Paper

The effect of antioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa

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Summary

The aim of this study was to determine the effects of the antioxidants superoxide dismutase (SOD), glutathione peroxidase (GPx) and butylated hydroxytoluene (BHT) on microscopic semen parameters and lipid peroxidation following the freeze-thawing of bull semen. Ejaculates were collected from five Holstein bulls and pooled at 37°C. The semen samples were diluted with a CEY extender containing additives including 100 U and 200 U SOD/ml, 50 U or 100 U GPx/ml and an extender containing no antioxidants (control) and stored in liquid nitrogen. The pooled ejaculates were then diluted with CEY or a Tris (hydroxymethyl) aminomethane (TRIS)-based extender (TEY) alone or with added 0.5, 1.0, 2.0 or 4.0 mM BHT, and the routine semen evaluation was conducted. The lowest production of malondialdehyde (MDA) was obtained by addition of 100 U SOD/ml, 0.5 and 1 mM BHT to CEY extender compared with the other groups. Sperm viability and motility was significantly higher when 0.5, 1 mM BHT and 100 U SOD/ml were added in CEY extender. The highest sperm viability was achieved by addition of 50 U GPx/ml to CEY extender. In addition, sperm motility was significantly higher in samples extended in Tris-egg yolk (TEY) with 0.5 mM BHT compared with the control group. The results suggest that CEY extender can be improved with the addition of SOD and BHT.

Key words: Antioxidant, Cryopreservation, Spermatozoa, Bull

Introduction

Frozen-thawed spermatozoa produce reactive oxygen species (Alvarez and Storey, 1982), and excessive formation of reactive oxygen species during cryopreservation processes has been associated with a decrease in quality of thawed spermatozoa (Stradaioli *et al*., 2007). One way to overcome the detrimental effects of reactive oxygen substances (ROS) on subsequent sperm performance after thawing could be adding antioxidant compounds to the freezing extender to block or prevent oxidative stress (Dandekar *et al*., 2002).

The antioxidant system in the cell is comprised of reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) (Marti *et al*., 2003). Natural antioxidant system and synthetic phenolic antioxidant butylated hydroxytoluence (BHT) have been described as a defense functioning mechanism against lipid peroxidation (LPO) in semen (Shoae and Zamiri, 2008). Thus, supplementation with natural antioxidants or synthetic antioxidants could reduce the impact of oxidative stress during the sperm storage process, and thus improve the quality of chilled semen. In spite of these promising results, the use of SOD, GPx and BHT in freezing extenders is not commonplace, and few results are available which evaluate the potential effect of these antioxidants on frozen-thawed bull sperm (Marti *et al*., 2003). Therefore, the present

study was undertaken to investigate the effects of antioxidants such as SOD and GPx antioxidants and BHT in extenders on semen quality and lipid peroxidation following the freeze-thawing process.

Materials and Methods

Semen samples from 5 mature Holstein bulls, belonging to Sh. Hassan Breeding Center (near Tabriz, Iran), were collected by using an artificial vagina (42°C). Semen samples were pooled in order to obtain sufficient sperm for the experiment and also to eliminate the bull effect. Five pooled ejaculates were included in the study.

A citate-based extender [sodium citrate 2.9 g/dL, 20% egg yolk, 7% glycerol, pencillin G sodium, 1000 IU/ml, streptomycin sulphate, 1000 µg/ml] was used as the base extender (Cryodiluent). Each pooled ejaculate was divided into five equal aliquots and diluted (37°C) with the base extender containing 100 U SOD/ml, 200 U SOD/ml, 50 U GPx/ml or 100 U GPx/ml and no antioxidant (control).

Then pooled ejaculates from the same bulls were divided into 2 aliquots and diluted with either the CEY extender and Tris-egg yolk (TEY) extender [Tris (hydroxymethyl) aminomethane (TRIS) (3.028 g/dL); citric acid monohydrate (1.675 g/dL); fructose (1.25 g/dL); 20% egg yolk; 7% glycerol; penicillin G sodium (1,000 IU/ml); streptomycin sulphate (1,000 µg/ml); and double distilled water to make a volume of 100 ml]. Different concentrations of 0, 0.5, 1.0, 2.0 and 4 mM BHT were prepared in ethanol in pre-warmed (37°C) test tubes. The ethanol was allowed to evaporate so that a thin crystallized layer of BHT was deposited on the inner surface of the tubes. Each of these semen extenders was added into the four experimental groups and incubated at 37°C for 5 min to allow uptake of BHT by sperm. In both experiments, semen extender was equilibrated in an equilibration chamber for 4 h at 4°C before filling in 0.5 ml French straws. The straws were placed on steel racks and held on liquid nitrogen at -196°C for 10 min. Frozen straws were then immediately immersed in liquid nitrogen (-196°C) and stored for 4 weeks until further assessment.

Five straws for each concentration of SOD, GPx and BHT were thawed at 37°C in a water bath for 30 s. A wet mount was made using a 5 µl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each pooled semen sample. The mean of the three successive estimations was recorded as the final motility score.

Biochemical assays

Biochemical assays were performed on sperm samples immediately after thawing, following centrifugation and washing. An aliquot (500 µl) of semen from each semen sample was centrifuged at 800 × g for 10 min. The sperm pellet was separated and washed by resuspension in phosphate buffered saline (PBS) and recentrifuged three times. After the last centrifugation, 1 ml deionized water was added to the sperm sample (Dandekar *et al*., 2002).

MDA concentrations

Malondialdhyde (MDA) concentrations, as indices of the LPO in the semen samples, were measured using the thiobarbituric acid reaction in accordance with the method described by Placer *et al*. (1966).

Statistical analysis

The procedure was replicated three times for each treatment. The results are quoted as mean ± SEM. The Statistical analyses were carried out using the general linear model procedures (GLM) of SAS (2004), following this, the post-hoc was performed by Tukey test to determine significant differences among the mean values. Differences with confidence values of $P<0.05$ were considered to be statistically significant.

Results

Data for influence of SOD and GPx on frozen-thawed bull semen quality and MDA are summarized in Table 1. The lowest production of MDA was obtained by the addition of 100 U SOD/ml to semen

extender compared with the other groups (P<0.05). Sperm viability was significantly higher (P<0.05) in samples extended with 100 U SOD/ml compared with the control group. No significant differences were observed in sperm motility, following supplementation with SOD after thawing of the semen.

No significant differences were observed in LPO following supplementation with GPx. Sperm viability was significantly higher (P<0.05) in samples extended with 100 U GPx/ml compared with the control group. However, there was no significant difference in sperm motility between the treatment groups.

Data for the effect of different concentration of BHT on frozen-thawed bull semen in CEY and TEY extender and MDA are summarized in Tables 2 and 3, respectively. The lowest production MDA, was obtained by addition of 0.5 mM BHT to semen extender compared with the other groups (P<0.05). The highest sperm viability and motility was achieved by the addition of 0.5 mM BHT to CEY semen extender compared with the control group (P<0.05). The highest production MDA was obtained by the addition of 4 mM BHT to semen extender compared with the other groups (P<0.05). The highest sperm motility was seen by the addition of 0.5 mM BHT to TEY extender compared with the control group (P<0.05).

Discussion

In the current study, it was shown that sperm viability was significantly higher in samples extended with 100 U SOD/ml compared with 200 U SOD/ml. The different effects of the two levels of SOD might be explained according to the report of Shoae and Zamiri (2008) who showed that the

Table 1: Effects of different concentrations of superoxide dismutase (SOD) and glutathione peroxidase (GPx) supplementation on the sperm viability, progressive sperm motility and malondialdehyde (MDA) of cryopreserved-thawed bovine sperm in a citrate-egg yolk (CEY) extender (n=5 bulls, mean±SEM)

Parameters	SOD (U/ml)			GPx (U/ml)	
	0	100	200	50	100
MDA (nmol/dL)	0.94 ± 0.10^{a}	1.41 ± 0.5^{b}	2.14 ± 0.98^{a}	1.09 ± 0.22^{a}	1.36 ± 0.33^{a}
Sperm viability (%)	46 ± 1.8^{a}	56 ± 1^{b}	51 ± 1^{a}	56 ± 2^{b}	55 ± 2^{b}
Sperm motility (%)	44 ± 1.9^{a}	46 ± 1^{a}	43 ± 1^{a}	48 ± 2^{a}	44 ± 1^{a}

Means with superscripts ($^{a, b}$) within the same row demonstrate significant differences (P<0.05)

Table 2: Effects of different concentrations of butylated hydroxytoluene (BHT) supplementation on the sperm viability, progressive sperm motility and malondialdehyde (MDA) of cryopreserved-thawed bovine sperm in a citrate-egg yolk (CEY) extender (n=5 bulls, mean±SEM)

Parameters	Concentration of BHT (mM)				
	0	0.5	1	2	4
MDA (nmol/dL)	2.75 ± 0.66^{a}	1.26 ± 0.19^{b}	1.51 ± 0.42^{b}	2.81 ± 0.38^{a}	5.20 ± 0.61^{c}
Sperm viability (%)	$52 \pm 1^{a, b}$	55 ± 1^{a}	55 ± 4^{a}	$46 \pm 1^{b, c}$	41 ± 2^{c}
Sperm motility (%)	38 ± 1^{a}	45 ± 1^{b}	44 ± 1^{b}	$35 \pm 4^{b, c}$	31 ± 2^{c}

Means with superscripts ($^{a, b}$ and ^{c}) within the same row demonstrate significant differences (P<0.05)

Table 3: Effects of different concentrations of butylated hydroxytoluene (BHT) supplementation on the sperm viability, progressive sperm motility and malondialdehyde (MDA) of cryopreserved-thawed bovine sperm in a Tris-egg yolk citrate (TEY) extender (n=5 bulls, mean±SEM)

Parameters	Concentration of BHT (mM)				
	0	0.5	1	2	4
MDA (nmol/dL)	3.90 ± 0.38^{a}	5.4 ± 0.17^{b}	5.54 ± 2.31^{b}	$6.07 \pm 0.44^{a, b}$	6.84 ± 0.29^{c}
Sperm viability (%)	42 ± 1^{a}	40 ± 1^{b}	37 ± 2^{b}	40 ± 1^{b}	35 ± 2^{b}
Sperm motility (%)	35 ± 1^{a}	40 ± 3^{b}	$41 \pm 1^{a, b}$	32 ± 1^{c}	30 ± 1^{c}

Means with superscripts ($^{a, b}$ and ^{c}) within the same row demonstrate significant differences (P<0.05)

excessive amount of antioxidants caused high fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damages. The results of the present study showed that the post-thaw bull semen quality was not improved in terms of sperm motility by SOD inclusion in the CEY extender. Differences in preservation protocols and extender formulations among laboratories, the time of addition/exposure of sperm with antioxidant, concentration of antioxidants and between species may explain, at least in part, this variability.

In the current study addition of GPx to semen extender (50 U/ml and 100 U/ml) did not improve post thaw sperm motility of bull semen; however, sperm motility was not affected by the formation of LPO. This was in contrast with the report of Stradaioli *et al*. (2007), who showed GPx can easily penetrate cell membranes and protect them against oxidative damage in cattle.

In this study, using 0.5 mM and 1 mM BHT in CEY extender led to an increase in post thaw sperm motility and viability of bull sperm, similar results were also documented by Killian *et al*. (1989), who found that addition of 0.5 mM and 0.75 mM BHT in whole milk extender led to an increase in post-thaw motility of bull sperm. It seems that optimal concentration of BHT depends upon the species of animals. Moreover, higher concentrations of BHT can increase the fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damage (Shoae and Zamiri, 2008). These results suggest that considerable difficulties still exist with regard to species differences, extender components and technical abilities when using BHT to preserve sperm motility and viability.

In conclusion, among all the antioxidants tested, SOD and BHT addition in semen CEY extender were of greater benefit to the chilled bull semen together with the best ROS inhibition results.

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