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Effect of various antioxidants and their combinations on bull semen cryopreservation

Şükrü GÜNGÖR[1,*], Ayhan ATA[1], Muhammed Enes İNANÇ[1], John P. KASTELIC[2]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Turkey

[2]Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

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Abstract: Our objective was to evaluate the effects of adding 50 mM trehalose (TRE), 1 mM cysteine (CYS), or 400 µg catalase (CAT), individually or in combinations, to a Tris-based semen extender and determine the effects on fresh, chilled-equilibrated, and frozen-thawed bull sperm. Ejaculates from three Holstein bulls (1–3 years old) were pooled, divided into six equal portions, and put into a Tris-based extender supplemented with no additive (control), TRE, CYS, CAT, TRE + CYS, or TRE + CYS + CAT. In chilled-equilibrated semen, motility was highest in CAT but lowest in the control ($P < 0.05$). In thawed semen, motility was lower ($P < 0.05$) in CAT than in all other groups, which were not different. Based on a hypoosmotic swelling test (HOS test), TRE, CYS, and their combinations had higher ($P < 0.05$) percentages of sperm with an intact plasma membrane than the control or CAT groups. The TRE group had the least ($P < 0.05$) morphologically abnormal sperm. Plasma membrane and acrosomal integrity (PMAI) and high mitochondrial membrane potential (HMMP) values were highest in TRE and TRE + CYS and were lower ($P < 0.05$) in the control. Furthermore, HMMP was positively correlated with PMAI and HOS tests, but negatively correlated with major + minor abnormalities ($r = 0.66$, 0.242, and –0.349). In conclusion, the addition of TRE, with or without CYS, to a Tris-based semen extender improved several aspects of the frozen-thawed quality of bull semen. However, addition of CAT, by itself or in combination with other additives, did not protect bull sperm against cryopreservation-induced defects.

Key words: Antioxidant, bull semen, flow cytometry, spermatological characteristics, trehalose

1. Introduction

Although bull semen is frequently cryopreserved, there are many deleterious effects on frozen-thawed sperm [1], including decreases in motility, DNA and plasma membrane integrity, and sperm mitochondrial membrane potential, plus increased lipid peroxidation [2–4]. As semen extender substantially affects the quality of frozen-thawed sperm [5], there is a strong impetus to continue to improve extenders and freezing methods [6].

Due to their lipid composition and fatty acid ratios, sperm membranes are very sensitive to temperature changes during cooling and freezing [7], with much potential for oxidative stress and cell damage [8]. Various antioxidants, sugars, proteins, and enzymes [9–11] have improved frozen-thawed sperm by increasing antioxidant capacity, thereby reducing sperm damage due to free radicals and oxidative stress [8]. For example, exogenous catalase (CAT) can confer protection from oxidative damage [12]; CAT supplementation improves the viability of bull [13], red deer [14], swine [15], dog [16], and rooster [10] sperm after chilling or freezing processes. Various carbohydrates, monosaccharides, and disaccharides had protective effects on frozen-thawed sperm [17,18]. In that regard, the disaccharide trehalose (TRE) apparently potentiated activities of catalase, superoxide dismutase, and glutathione, thereby protecting sperm membranes from oxidative damage and lipid peroxidation [11]. In addition, cysteine (CYS) stimulates glutathione synthesis and decreased oxidative stress [19], improving bull sperm membrane integrity [9] and buck sperm motility [20].

The use of TRE, CYS, and CAT for protecting cryopreserved sperm has been tested with varying results [21–23]. However, a simultaneous comparison of these compounds, added alone and in combination to extender for bull semen cryopreservation, has apparently not been reported. Our objective was to evaluate the effects of adding TRE, CYS, and CAT, individually or in combinations, to a Tris-based semen extender and determine the effects on diluted, chilled-equilibrated, and frozen-thawed bull semen characteristics.

* Correspondence: sukrugungor@mehmetakif.edu.tr

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Bulls

Semen was collected by artificial vagina from three Holstein bulls (1–3 years old) and pooled (30 ejaculates and six replicates). All activities involving bulls were reviewed and approved by the Burdur Mehmet Akif Ersoy University Animal Care Committee (Protocol 31/192).

2.3. Semen processing

All ejaculates meet minimum quality standards (mass activity ≥ +++3 [scale of 1 to 5]; volume ≥ 5 mL; sperm concentration ≥ 0.8×10^9 /mL; and initial motility ≥ 75%) [3]. Ejaculates were pooled and semen was extended in a Tris-based extender comprising 3.07 g Tris, 1.64 g citric acid, and 1.26 g fructose per 100 mL distilled water with 7% glycerol and egg yolk 20% (v/v), to a final sperm concentration of 100×10^6/mL.

Based on previous experiments [9,24–26], we used 50 mM TRE, 1 mM CYS, and 400 µg CAT individually or in combinations. Pooled semen was divided into six equal portions and put into a Tris-based extender supplemented with no additive (control), TRE, CYS, CAT, TRE + CYS, or TRE + CYS + CAT. For all groups, semen was diluted and cooled from 37 to 4 °C over 2.5 h, and equilibrated semen was loaded in to 0.25-mL straws (20×10^6 spermatozoa/straw) that were exposed to nitrogen liquid vapor (5 cm above liquid nitrogen) for 12 min before being plunged into liquid nitrogen (–196 °C). Straws were stored for at least 3 months and samples were analyzed after thawing in a water bath at 37 °C for 30 s.

2.4. Sperm evaluation

2.4.1. General

Motility evaluation was done soon after semen was collected and extended (fresh), after cooling and equilibration (equilibrated), and after freezing and thawing (postthaw), although assessment of the hypoosmotic swelling (HOS) test, sperm morphology, high mitochondrial membrane potential (HMMP), and plasma membrane and acrosomal integrity (PMAI) were done only for frozen-thawed sperm.

2.4.2. Motility

A wet mount was prepared by placing 1 drop (~5 µL) of diluted sample on a warmed slide and covering it with a cover slip. Total sperm motility was subjectively estimated using a phase-contrast microscope [400× magnification, Nikon Eclipse E600 (Tokyo, Japan)] at 37 °C. Motility estimates were done by viewing at least five fields for each sample and the mean was recorded.

2.4.3. Flow cytometry

Flow cytometry was done with a Cytoflex Flow Cytometer (Beckman Coulter, USA) with a 50-mW laser output (488-nm laser beam) with emission filters of 610 ± 20, 585 ± 42, and 525 ± 40 nm. For each analysis, ~10×10^3 events were collected.

2.4.3.1. Plasma membrane and acrosome integrity

For thawed sperm, the evaluation of PMAI was done by multiparameter sperm analysis with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC/PNA)-propidium iodide (PI) molecular probes. For this, one straw was thawed (37 °C for 30 s) and semen was extended to 5 × 10^6 sperm (10 µL) in 496 µL of PBS. Then 5 µL of FITC/PNA (L7381, 100 µg/mL) and 3 µL of PI (L7011, 2.99 mM) were added and incubated for 30 min in a water bath (37 °C) in a dark area [27]. Thereafter, debris was gated out and PMAI analyses were performed with CytExpert 2.2 software (Beckman Coulter, USA) [28].

2.4.3.2. Mitochondrial membrane potential

For thawed sperm, HMMP was determined with 5,5',6,6'-tetrachloro-1,1'3,3'- tetramethylbenzimidazolyl-carbocyanine iodide (JC-1)-PI molecular probes. For this, one straw was thawed (37 °C for 30 s) and extended to 5 × 10^6 sperm (10 µL) in 487 µL of PBS. Then 10 µL of JC-1 (T3198, 0.153 mM) and 3 µL of PI (L7011, 2.99 mM) were included and incubated for 30 min in a water bath (37 °C in a dark area). After incubation, debris was gated out and analysis of HMMP was performed with CytExpert 2.2 software (Beckman Coulter, USA) [28].

2.4.4. Membrane integrity (HOS test)

The HOS test was used to evaluate sperm membrane integrity. For this, 10 µL of semen was placed in 1 mL of 100 mOsm buffer (1.35 g fructose, 0.735 g tri-sodium citrate/100 mL distilled water) at 37 °C for 1 h. Then 5 µL was placed on a slide, cover-slipped, and evaluated with bright-field microscopy [400× magnification, Nikon Eclipse E600 (Tokyo, Japan)]. At least 200 sperms were assessed and the percentage with curled and swollen tails was recorded [29].

2.4.5. Sperm morphology

Abnormal spermatozoon rate was determined by fixation liquid method. Spermatozoon were fixed with Hancock solution. At least 200 sperms per slide were examined under phase-contrast microscopy at 1000× magnification (Nikon Eclipse E600, Tokyo, Japan). Sperm abnormalities were defined as major or minor, as previously described [30,31].

2.5. Statistical analyses

Data for sperm motility, PMAI, HMMP, HOS test, and major, minor, and major + minor sperm defects were analyzed to detect differences among groups. First, normality of data distribution was confirmed with a Shapiro–Wilk test; then data were analyzed by ANOVA, with a Tukey test used to locate differences. Independent statistical analyses were conducted for each end point for fresh, equilibrated, and postthaw sperm. Pearson correlations between HMMP and PMAI, motility, HOS tests, and sperm abnormalities were also calculated.

All data analyses were performed with SPSS 22.0 for Windows, with $P < 0.05$ considered significant. All data were reported as arithmetic mean ± standard error of the mean (SEM).

3. Results

There was no significant effect of group on motility of fresh sperm (Table 1). In chilled-equilibrated semen, motility was highest in the CAT and lowest in the control group (mean ± SEM, 75.7 ± 2.3% vs. 68.6 ± 1.4%, respectively). In postthaw semen, motility was lowest ($P < 0.05$) in CAT (27.1%), but not significantly different among the remaining five groups. For PMAI and HMMP, values were highest in TRE and TRE + CYS (no significant difference between these two groups for either end point) and were lowest ($P < 0.05$) in the control group (Table 2). There was a lower ($P < 0.05$) percentage of HOS test-positive sperm in the control and CAT groups compared to the other four groups (Table 3), whereas major, minor, and major + minor defects were lowest ($P < 0.05$) in the TRE group. HMMP was positively correlated with PMAI ($r = 0.66$; $P < 0.01$) and HOS test ($r = 0.342$; $P < 0.05$) and negatively correlated with major + minor abnormalities ($r = –0.349$, $P < 0.05$; Table 4).

4. Discussion

Sperm function and the fertility of frozen-thawed semen can be decreased in a variety of ways, especially due to the actions of free radicals [32]. In the present study, bull sperm was cryopreserved with various antioxidant compounds (TRE, CYS, and CAT) individually or in combination. The addition of TRE or TRE + CYS to the extender was generally favorable with regards to PMAI, HMMP, HOS tests, and sperm morphology. Previous studies showed that TRE had positive effects on sperm morphology during the freezing process and this was similar to our findings [29].

Based on motility, there was no evidence that any of the extenders had deleterious effects on fresh semen. Addition

Table 1. Mean (±SEM) motility (%) of fresh, chilled/equilibrated, and frozen-thawed bull sperm in various extenders.

Motility (%)	Control	TRE	CYS	CAT	TRE + CYS	TRE + CYS + CAT
Fresh	80.0 ± 1.5	77.8 ± 1.8	78.6 ± 2.1	80.7 ± 1.7	82.1 ± 1.5	82.8 ± 1.8
Equilibrated	68.6 ± 1.4^{a}	70.1 ± 1.3^{a}	71.4 ± 1.4^{ab}	75.7 ± 2.3^{b}	72.1 ± 1.0^{ab}	72.8 ± 1.5^{ab}
Postthaw	47.1 ± 1.5^{a}	52.8 ± 2.1^{a}	54.3 ± 2.5^{a}	27.1 ± 3.8^{b}	52.8 ± 1.0^{a}	48.6 ± 1.4^{a}

TRE (50 mM); CYS (1 mM); CAT (400 µg); TRE + CYS (50 mM + 1 mM); TRE + CYS + CAT (50 mM + 1 mM + 400 µg).
$^{a, b}$ Within a row, means without a common superscript significantly differ ($P < 0.05$).

Table 2. Mean (±SEM) PMAI and HMMP of frozen-thawed bull sperm.

End point (%)	Control	TRE	CYS	CAT	TRE + CYS	TRE + CYS + CAT
PMAI	43.4 ± 0.5^{a}	51.1 ± 1.1^{b}	45.8 ± 0.6^{a}	41.0 ± 3.7^{a}	54.6 ± 1.1^{b}	34.6 ± 1.1^{c}
HMMP	27.8 ± 0.8^{a}	48.7 ± 1.1^{b}	39.2 ± 2.0^{c}	33.0 ± 1.2^{d}	47.4 ± 1.9^{b}	31.1 ± 0.4^{a,c}

TRE (50 mM); CYS (1 mM); CAT (400 µg); TRE + CYS (50 mM + 1 mM); TRE + CYS + CAT (50 mM + 1 mM + 400 µg).
$^{a–d}$ Within a row, means without a common superscript significantly differ ($P < 0.05$).

Table 3. Mean (±SEM) percentages of sperm positive in HOS test or with major or minor morphological abnormalities in frozen-thawed bull sperm.

End point (%)	Control	TRE	CYS	CAT	TRE + CYS	TRE + CYS + CAT
HOS test	65.0 ± 2.2^{a}	78.2 ± 1.1^{b}	74.4 ± 2.0^{b}	63.7 ± 2.2^{a}	75.2 ± 1.0^{b}	75.3 ± 0.7^{b}
Major abnormalities	4.4 ± 0.3^{a}	3.1 ± 0.3^{b}	4.3 ± 0.4^{a}	4.4 ± 0.4^{a}	4.0 ± 0.5^{ab}	3.8 ± 0.3^{ab}
Minor abnormalities	18.6 ± 1.3^{a}	12.3 ± 0.5^{b}	18.0 ± 1.1^{a}	25.3 ± 1.9^{c}	16.0 ± 0.8^{a}	19.7 ± 1.0^{a}
Major + minor abnormalities	23.0 ± 1.1^{a}	15.4 ± 0.8^{b}	22.4 ± 1.2^{a}	29.7 ± 1.8^{c}	20.0 ± 1.0^{a}	23.6 ± 1.1^{a}

HOS test; TRE (50 mM); CYS (1 mM); CAT (400 µg); TRE + CYS (50 mM + 1 mM); TRE + CYS + CAT (50 mM + 1 mM + 400 µg).
$^{a–c}$ Within a row, means without a common superscript significantly differ ($P < 0.05$).

Table 4. Correlations between HMMP and various end points in frozen-thawed bull sperm.

End point	Correlation coefficient	P-value
PMAI	0.66	<0.01
Total motility	0.26	0.125
HOS test	0.342	<0.05
Major abnormalities	–0.217	0.204
Minor abnormalities	–0.326	0.053
Major + minor abnormalities	–0.349	<0.05

of 1 mM CYS to semen extender resulted in the highest postthaw total motility, although it was only significantly greater than that of CAT ($P < 0.05$). CYS has a potential positive role on antioxidants that scavenge reactive oxygen species (ROS) by enhancing glutathione synthesis during cryopreservation [33,34].

Although addition of 400 µg/mL CAT to the sperm medium resulted in the highest motility for chilled sperm, it also resulted in the significantly lowest motility after the freezing process. Compared to other groups, CAT decreased postthaw motility, as well as plasma membrane and acrosome integrity and morphological integrity, consistent with previous studies [24,35]. Similarly, 200 or 400 µg CAT also decreased postthawed sperm motility in ram semen [24]. Although 50–100 µg CAT failed to improve motility in fresh extended or cooled monkey semen [36], conversely, 400 µg CAT had positive effects on the postthaw motility of bull [22,37], goat [23], boar [38], and ram [39] sperm.

Despite ample evidence that excessively high ROS concentrations are deleterious, physiological ROS concentrations have critical roles in sperm physiology, acrosome reaction, capacitation, hyperactivation, and signaling to ensure fertilization [40]. Strategic use of antioxidants can mitigate excessive ROS concentrations and enhance sperm quality and function, whereas excessive antioxidant activity could be very detrimental. Although it was suggested that since CAT is an endogenous product, it does not need to be supplemented [36], perhaps apparent differences among studies are related to the species and/or concentrations of CAT used.

In the present study, PMAI was highest with the combination of TRE + CYS, although it was not significantly different than that of TRE. PMAI is important for fertilization, as essential enzymes are stored in the acrosome. Similar to our study, the addition of TRE to the extender of semen from red deer [41] and goats [42] improved postthaw PMAI. Furthermore, TRE significantly increased the viability and acrosome integrity of ram sperm [21] and promoted the integrity of mouse sperm [43].

HMMP was highest in the TRE group (but not significantly different from the TRE + CYS group) and lowest in the control group. Mitochondria are the main source of ROS, with actions of TRE to reduce oxidative stress occurring in the mitochondria [44]. Lee et al. [45] accounted for why TRE, despite having similar cryoprotective effects compared to other sugars, resulted in a higher rate of sperm with high membrane fluidity. Sperm motility is mostly dependent on mitochondria, located in the midpiece and producing energy essential for propagation and generation of flagellar waves [46]. Sperm membrane integrity and viability are critical aspects of sperm quality and function, including maintenance of homeostasis and the ability to move, interact with the environment, and achieve fertilization. A motile spermatozoon should be considered viable and viable spermatozoa must have an intact plasma membrane, as the latter is essential for the sperm's interactions with the environment and other cells [47]. Consequently, there is an association between plasma membrane integrity and HMMP [1]. Dense fibrils in the mitochondrial axoneme produce intracellular ATP [8], required for sperm motility [48]. Furthermore, mitochondrial membrane potential evaluation not only reflects mitochondrial function but is also evidence of early apoptosis, as impaired mitochondrial function may lead to increased apoptosis [49]. In the present study, HMMP was positively correlated with PMAI ($r = 0.66$, $P < 0.01$) and negatively correlated with the HOS test ($r = –0.342$, $P < 0.05$) and total abnormality ($r = –0.349$, $P < 0.05$). This finding was similar to results for frozen-thawed human [50] and bovine [2] sperm.

In conclusion, the addition of 50 mM TRE alone or in combination with 1 mM CYS to Tris-based freezing medium improved the postthaw quality of bull semen. Further studies with a wider range of concentrations, combinations of both compounds, and effects on fertility of frozen-thawed semen are warranted.

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