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The effect of superoxide dismutase mimetic and catalase on the quality of postthawed goat semen

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ABSTRACT

Manganese(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin chloride (MnTE) is a cell-permeable superoxide dismutase mimetic agent which can convert superoxide to hydrogen peroxide (H_2O_2). Supplementation of MnTE to a commercial semen extender can protect sperm from superoxide but not H_2O_2. Therefore, we proposed that addition of catalase (0.0, 200, or 400 IU/mL) in combination with MnTE (0.1 μM) may further improve the cryopreservation efficiency of goat semen in commercially optimized freezing media such as Andromed. Therefore, ejaculates were obtained from three adult bucks twice a week during the breeding season and diluted with Andromed supplemented with or without MnTE and catalase and were frozen in liquid nitrogen. Sperm parameters and reactive oxygen species contents were evaluated 2 hours after dilution (before freezing) and after freezing/thawing. The results revealed that all the treatments significantly ($P \leq 0.05$) improved sperm motility, viability, and membrane integrity after freezing and reduced reactive oxygen species content compared with the control group, but maximum improvement was obtained in MnTE + 400 IU/mL catalase. In addition, supplementation with these antioxidants significantly ($P \leq 0.05$) increases the cleavage rate after IVF. In conclusion, the results of present study suggest that addition of antioxidant MnTE or catalase to commercial optimized media, such as Andromed, improves total motility, membrane integrity, and viability of goat semen samples after thawing. But the degree of improvement for these parameters significantly ($P \leq 0.05$) higher when MnTE and catalase were simultaneously added to the cryopreservation media.

1. Introduction

Despite advances in semen cryopreservation, a variety of injuries occur at cellular and molecular levels during semen cryopreservation which may impair sperm function and fertilization potential [1,2]. Major drawbacks of semen cryopreservation are reduction of sperm viability, motility, plasma membrane integrity, and impaired function of the survived cell population [3]. These phenomena are accounted by osmotic stress, cold shock, intracellular ice crystal, and oxidative stress induced by reactive oxygen species (ROS) [4]. Among the aforementioned factors, excessive production of ROS plays a central role in induction of cryoinjury. Reactive oxygen species acts as a double-edged sword in many physiological phenomena. Basal

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levels of ROS are required for normal sperm motility, capacitation, and acrosome reaction, whereas excess ROS lead to various forms ofcellular damages, including impairment of membrane, DNA damage, and apoptosis [5,6]. Somatic cells have acquired a delicate balance between ROS generation and ROS scavengers but unlike most somatic cells, sperm as one of the smallest cells of the body has little cytoplasm. This makes sperm susceptible to ROS injuries which can affect sperm motility [7], viability [8], DNA integrity, and energy metabolism [9]. Moreover, ROS can also be produced by exogenous sources such as semen handling during cryopreservation process, exposure to light, oxygen, and shearing forces generated by centrifugation. Therefore, supplementation of semen diluents with antioxidants has been advised, and a wide variety of antioxidants have been used for semen cryopreservation in bovine [10], water buffalo [11], stallion [12], ram [13] and goat [14]. Antioxidants used during semen cryopreservation can be divided into two main categories: cell-permeable and impermeable antioxidants. In addition, antioxidants can be categorized as enzymatic, enzyme-mimetic, and nonenzymatic. Recent research has suggested that cell-permeable enzyme-mimetic antioxidants are considered as valuable compounds for reducing intracellular ROS levels [15]. In this regard, we and other researchers have shown that addition of cell-permeable enzyme-mimetic antioxidants to commercially optimized cryopreservation media with antioxidant activity can further improve the quality of these media [13,14]. Manganese(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin chloride (MnTE) is a manganese porphyrin compound with a superoxide dismutase (SOD) mimetic activity which has been shown to successfully rescue animal models for some oxidative stress–related diseases [16]. We previously proposed that 0.1 μM of MnTE can improve sperm membrane and acrosomal integrity. Furthermore, we showed that *in vitro* rate of blastocyst formation after freeze-thawing of goat semen supplemented with MnTE was higher compared with that of its corresponding control [14].

Superoxide dismutase is one of the enzymatic antioxidants, which converts superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2), whereas catalase can convert this H_2O_2 into oxygen and water [17,18]. Therefore, supplementation with MnTE is expected to increase H_2O_2, which is a relatively stable cell-permeable molecule with high oxidant potential. In other words, the association of SOD mimetic (MnTE) and peroxide scavengers (catalase) may promote better sperm freezing performance. Therefore, the aim of this study was to address whether addition of catalase in combination with MnTE or alone into commercially optimized freezing media could further improve the efficiency of cryopreservation of goat semen.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma Chemical CO. (St. Louis, MO, USA) unless stated otherwise. MnTE was a gift from Dr Ines Batinić-Haberle (Department of Radiation Oncology, Duke University Medical School, Durham, NC 27710, USA). Andromed as a semen extender was obtained from Minitube, Germany.

2.2. Semen collection, processing, and freezing–thawing

Semen collection and processing procedures were carried out according to Forouzanfar et al. [14]. In brief, ejaculates were obtained by artificial vagina from three adult Bakhtiari bucks aged 2 to 3 years, twice a week during the breeding season (September/October 2013) in the animal farm of Reproductive Biotechnology Research Center at Royan Institute (Isfahan, Iran latitude 32°39′N). Samples from each male were kept separated and transported at 35 °C to the laboratory for microscopic assessment within 30 minutes. Only ejaculates showing higher than 70% motile and 80% morphologically normal sperm and more than 1 mL in volume were used for experiments. To minimize individual differences, semen samples from the three bucks in each replicate were pooled. A total of six ejaculates from each goat were processed. For freezing, all the treatments were repeated six times with the pooled semen samples. Andromed was used as the base freezing extender. Each pooled semen sample was divided into six equal aliquots and diluted (1:20 [v:v]) with the extender containing no antioxidant (control group), 0.1 μM of MnTE (Mn), 200 IU/mL catalase (CAT 200), 400 IU/mL catalase (CAT 400), 0.1 μM of MnTE plus 200 IU/mL catalase (Mn + CAT 200), and 0.1 μM of MnTE plus 400 IU/mL catalase (Mn + CAT 400). Concentrations of MnTE and catalase were based on our previous report [14] and Roca et al. [19], respectively. The diluted semen was cooled to 4 °C over a period of 2 hours and drawn into 0.5-mL French straws (Biovet, L'Agile, France), heat sealed and stored at 4 °C for 1 hour for more equilibration. The straws were exposed to liquid nitrogen (LN_2) vapor for 12 minutes, plunged into LN_2, and stored in LN_2 until thawed and used for evaluation of sperm parameters and IVF. Thawing was carried out by plunging sealed straws in a 37 °C water bath for 30 seconds. The thawed samples were individually evaluated by a single trained individual.

2.3. Semen evaluation after thawing

2.3.1. Measurement of sperm motility

For assessment of sperm motility after thawing, four or five straws from one treatment in each group were thawed and pooled. The samples were diluted with a fertilization medium (Tyrode's albumin lactate pyruvate medium, Fert-TALP) to a final concentration of 1×10^6 spermatozoa/mL. The percentages of rapid progressive (class A), sperm that swim fast in a straight line; slow progressive (class B), sperm that move forward but tend to travel in a curved motion; and nonprogressive (class C), sperm that do not move forward despite that they move their tails, as well as total motility which refers to the population of sperm that display any type of movement, were assessed according to the software setting of the computer-assisted sperm analysis system (VideoTest Sperm software 2.1; 1990–2004, VideoTest Ltd, Russia)

[20], which was a setup for goat sperm evaluation. For each sample, 5 μL of sperm suspension was loaded on a prewarmed slide and then covered by a 18 × 18-mm coverslip; a minimum of 500 sperm per sample were analyzed in at least three different microscopic fields.

2.3.2. Assessment of sperm plasma membrane integrity

Sperm plasma membrane integrity was estimated by hypoosmotic swelling test (HOST) on the basis of curled and swollen tails [21]. For this purpose, 25 μL of the diluted semen (for prefreezing or thawed specimen) was mixed with 200 μL of HOST solution (100 mOsm/L, 57.6-mM fructose, and 19.2-mM sodium citrate) for 30 minutes at room temperature; subsequently 5 μL of homogenized mixture was mounted and directly investigated using an inverted microscope (CKX41; Olympus, Japan). The percentages of spermatozoa with swollen and curved tails, categorized as intact plasma membrane, were estimated from recording at least 200 spermatozoa in more than five different microscopic fields.

2.3.3. Assessment of sperm viability

Sperm viability was assessed using eosin–nigrosin staining which was done for prefreezing (2 hours after semen dilution) and postthawing experiments with the same protocol. Fifty microliters of diluted or thawed semen was mixed with 100 μL of eosin (1%) in a test tube for 30 seconds; the mixture then was stained with nigrosin (10%) for a further 30 seconds and mixed gently. One drop of stained semen was smeared on a slide, allowed to air dry in a dust-free environment, and examined directly at a magnification of × 1000 under oil immersion using a light microscope. Two hundred sperm were assessed for each replicates. Sperm heads that were unstained were classified as "live sperm," whereas sperm that appeared as pink or red were considered as "dead sperm."

2.3.4. Assessment of oxidative stress in sperm using flow cytometery

We used flow cytometery (FACScan; Becton Dickinson, San Jose, CA, USA) to determine the ROS content in thawed and diluted semen as described previously [22]. In brief, the samples from each treatment/replicate were centrifuged at 700 × *g* for10 minutes, the supernatant was discarded, and the pellet was resuspended in 1 mL of PBS. The percentages of ROS-positive spermatozoa in each treatment were determined after incubating one million sperm per mL with 5 μM of 2′,7′-dichlorofluorescin diacetate (DCF-DA) for 30 minutes at room temperature. Regarding mechanism of ROS reaction with DCF, it is important to note that once DCF-DA enters cells, it loses its ester group, and on interaction with ROS, it produces a fluorescence compound. Live sperm produce a detectable amount of physiological ROS, and therefore, the sperm become DCF positive. To differentiate between the physiological amount of ROS and the pathologic ROS production, we reduced the concentration of DCF-DA, and therefore, sperm producing super physiological concentration of DCF-DA were detectable at this 5-μM concentration of DCF-DA.

2.4. IVF and embryo culture

In vitro fertilization and embryo culture were performed as described by Forouzanfar et al. [14]. Goat ovaries were recovered at the local slaughterhouse, placed in normal saline (0.9% sodium chloride) at a temperature between 25 °C and 35 °C, and then transported to the laboratory within 2 hours. The cumulus–oocyte complexes (COCs) comprised at least four to 10 layers of cumulus cells, and oocytes with a uniform cytoplasm and homogenous distribution of lipid droplets in the cytoplasm were recovered by aspiration from follicles of more than 2 mm diameter on the surface of the ovaries and selected for the IVM. The selected COCs were washed three times in the aspiration medium (HEPES-tissue culture media + 10% fetal calf serum + 100 IU/mL heparin) and then cultured in a maturation medium (tissue culture medium 199 + 10% fetal calf serum + 5 mg/mL FSH + 5 mg/mL LH + 0.1-mM cysteamine) in 5% CO_2 at 39 °C and maximum humidity for 20 to 22 hours. For sperm preparation, five straws representing one freezing operation in each replicate were thawed, pooled, and washed through two gradients (40%–80% solutions) of PureSperm (Nidacon; Gothenburg, Sweden) to separate motile sperm with normal morphology by centrifugation (700 × *g* for 15 minutes at room temperature). Matured COCs (totally 1254 for all treatments and control group) were partially stripped of the cumulus cells, transferred into 100-μL drops of fertilization medium, and fertilized *in vitro* at 39 °C and 5% CO_2, 5% O_2, and 90% N_2 atmosphere. *In vitro* fertilization experiments were repeated three times using pooled frozen-thawed samples in each group. For IVF, sperm at a final concentration of 2×10^5 sperm were coincubated with 20 to 25 matured oocytes/100-μL droplet for 22 hours under the same gas atmosphere condition as for IVM. Presumptive zygotes were mechanically denuded of their cumulus cells *via* pipetting and cultured in the modified synthetic oviduct fluid medium for the first 3 days after insemination (Day 0 = day of insemination). Embryos were then transported to the synthetic oviduct fluid medium supplemented with 10% charcoal-stripped serum and 1.5-mM glucose up to blastocyst stage. Subsequently cleavage, blastocyst, and hatched rates in each group were assessed on Days 3, 8, and 9 after insemination, respectively.

2.5. Statistical analysis

The results are reported as the mean ± standard error for each experiment. The ANOVA was used for treatment comparisons. One-way ANOVA with the Tukey multiple comparison *post hoc* test was used to compare the different stimuli. A probability value of 0.05 or less was considered statistically significant. For IVF treatments, the Student *t* test was used to compare the mean, when the data from the supplemented groups (CAT 200, CAT 400, and Mn + CAT 400) were pooled and compared with the control group.

3. Results

Table 1 shows the effects of antioxidant(s) supplementation in freezing media on the percentage of sperm

Table 1
Effect of antioxidant(s) supplementation on motility of Bakhtiari buck spermatozoa in prefreezing and postthawing conditions.

Groups	Prefreezing motility (%) ± SE					Postthawing motility (%) ± SE				
	Class A	Class B	Class C	Class A + B	Total	Class A	Class B	Class C	Class A + B	Total
Control	8.32 ± 0.41^{ab}	29.02 ± 0.81^{ab}	34.97 ± 0.82	37.33 ± 0.84	72.3 ± 0.86	8.51 ± 0.28^{a}	18.52 ± 0.39^{a}	16.33 ± 0.94^{a}	27.03 ± 0.54^{a}	43.36 ± 1.39^{a}
Mn	7.1 ± 0.33^{a}	29.51 ± 1.11^{ab}	37.26 ± 1.56	36.61 ± 1.14	73.86 ± 1.49	12.11 ± 0.69^{b}	20.33 ± 0.83^{a}	17.11 ± 0.55^{ab}	32.44 ± 1.14^{b}	49.55 ± 1.4^{b}
CAT 200	8.58 ± 0.62^{ab}	26.88 ± 0.98^{a}	39 ± 1.46	35.46 ± 1.23	74.46 ± 1.22	13.51 ± 0.71^{b}	25.66 ± 0.41^{b}	16.55 ± 0.58^{a}	36.49 ± 1.15^{b}	53.04 ± 1.33^{b}
Mn + CAT 200	7.08 ± 0.35^{a}	32.57 ± 0.9^{b}	33.70 ± 1.21	39.65 ± 0.96	73.35 ± 1.27	11.96 ± 0.51^{b}	24.25 ± 0.82^{b}	17.62 ± 0.92^{ab}	36.21 ± 1.13^{b}	53.84 ± 1.78^{b}
CAT 400	8.01 ± 0.71^{ab}	29.26 ± 1.4^{ab}	38.76 ± 1.6	37.27 ± 1.51	76.03 ± 1.18	18.05 ± 0.99^{c}	29.01 ± 0.98^{c}	15.46 ± 0.66^{a}	47.06 ± 1.03^{c}	62.52 ± 1.2^{c}
Mn + CAT 400	9.56 ± 0.38^{b}	28.88 ± 0.57^{ab}	37.85 ± 1.3	38.44 ± 0.57	76.28 ± 1.33	17.53 ± 0.92^{c}	32.16 ± 0.95^{d}	20.34 ± 1.26^{b}	49.70 ± 1.38^{c}	70.03 ± 0.94^{d}

Results are expressed as mean ± standard error (SE) of six separate replicates.
Different superscripts within the same column report significant differences at $P < 0.05$. Groups contain: control, no antioxidant supplemtation; Mn, Andromed supplemented with 0.1 μM of MnTE; CAT 200, Andromed supplemented with 200 IU/mL of catalase; Mn + CAT 200, Andromed supplemented with 0.1 μM of MnTE and 200 IU/mL of catalase; CAT 400, Andromed supplemented with 400 IU/mL of catalase; and Mn + CAT 400, Andromed supplemented with 0.1 μM of MnTE and 400 IU/mL of catalase. Sperm motility characteristics in computer-assisted semen analysis: class A represents goat sperm with progressive and fast motility, class B represents goat sperm with progressive but slow motility, and class C represents goat sperm with nonprogressive motility.
Abbreviation: MnTE, manganese(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin chloride.

motility parameters before freezing (2 hours after dilution) and after freeze thawing. There were no significant ($P \leq 0.05$) differences in total and class A + B motility between the treatments in prefreezing conditions. After thawing, CAT 400 and Mn + CAT 400 groups resulted in better maintenance of total and class A + B motility compared with the control and other treatments. Mn, CAT 200, and Mn + CAT 200 groups better maintained total and class A + B motility after thawing compared with the control. It is of interest to note that Mn + CAT 400 treatment yields higher postthaw motility compared with the control and other treatments ($P < 0.05$). Table 2 presents the percentage of sperm viability, intact membrane integrity, and DCF-positive sperm in semen samples before freezing and after thawing. Before freezing, percentages of sperm viability and intact membrane integrity were significantly ($P \leq 0.05$) higher in CAT 400 and Mn + CAT 400 groups compared with those of the other groups. After freezing, the percentage of sperm viability was significantly ($P \leq 0.05$) higher in Mn + CAT 400 and CAT 400 groups compared with that in all other groups. This parameter in the Mn + CAT 400 group was significantly ($P \leq 0.05$) higher than that in the CAT 400 group. The percentage of sperm intact membrane integrity was significantly ($P \leq 0.05$) higher in the Mn + CAT 400 group compared with that in all the other groups.

Before freezing, percentages of DCF-positive sperm were significantly ($P \leq 0.05$) reduced in the Mn + CAT 400 and CAT 400 groups compared with the other groups, except for the CAT 400 with Mn + CAT 200 and CAT 200 groups. After thawing, the lowest ROS contents were related to CAT 400 and Mn + CAT 400 groups (32% and 35.6%) and the highest ROS content was observed in the control group (51%) which was significantly ($P \leq 0.05$) higher than all treatment groups.

Finally, the analysis of sperm fertilizing ability and developmental competence showed that there was no significant ($P \leq 0.05$) difference between the cleavage rate and the developmental competence to reach the blastocyst and hatching stages between the control, CAT 200, CAT 400, and Mn + CAT 400 groups (Table 3). However, when the data from the supplemented groups (CAT 200, CAT 400, and Mn + CAT 400) were pooled, only a significant ($P \leq 0.05$) increase in the cleavage rate was observed compared with the control group (Fig. 1).

4. Discussion

Empirical evidence reveals that substantial amount of ROS produce during semen cryopreservation impairs sperm physiological functions after thawing. To ameliorate these side effects, supplementation of semen cryopreservation media with ROS scavengers are recommended [23,24]. In line with other researchers, we showed that supplementation of sperm cryopreservation media with superoxide mimetic agents, such as MnTBAP [13], MnTE, and Tempol [25], can improve semen quality after thawing. MnTE is an SOD mimetic agent and is able to convert O_2^- to H_2O_2. H_2O_2 can be converted to H_2O and O_2 by enzymes such as H_2O_2 peroxidase or catalase [26–28]. In accordance with this fact, the aim of this study was to evaluate whether

Table 2
Effect of antioxidant(s) supplementation on viability, membrane integrity, and reactive oxygen species (ROS) contents of Bakhtiari buck spermatozoa in prefreezing and postthawing conditions.

Group	Viability (%) ± SE		Membrane integrity –HOST^{+} (%) ± SE		ROS content (%) ± SE	
	Prefreezing	Postthawing	Prefreezing	Postthawing	Prefreezing	Post thawing
Control	74.87 ± 0.17^{a}	32.22 ± 0.81^{a}	76.93 ± 0.11^{a}	39.44 ± 0.91^{a}	43 ± 0.58^{c}	51 ± 1.15^{d}
Mn	75.45 ± 0.08^{b}	34.72 ± 0.86^{ab}	77.15 ± 0.13^{a}	40.46 ± 1.34^{a}	42 ± 0.58^{c}	48 ± 0.58^{cd}
CAT 200	75.86 ± 0.18^{b}	38.89 ± 1.09^{b}	77.43 ± 0.15^{a}	49.07 ± 0.91^{b}	39 ± 0.58^{b}	42 ± 2.52^{bc}
Mn + CAT 200	75.63 ± 0.11^{b}	39.07 ± 1.03^{b}	77.23 ± 0.15^{a}	48 ± 0.82^{b}	39 ± 0.58^{b}	41 ± 0.58^{b}
CAT 400	76.42 ± 0.08^{c}	45 ± 1.36^{c}	78.06 ± 0.13^{b}	51.67 ± 0.99^{b}	37.33 ± 0.33^{ab}	35.66 ± 0.33^{ab}
Mn + CAT 400	76.92 ± 0.14^{c}	57.13 ± 1.34^{d}	78.23 ± 0.13^{b}	57.96 ± 1.23^{c}	36.33 ± 0.33^{a}	32 ± 1.53^{a}

Results are expressed as mean ± standard error (SE) of six separate replicates.
Different superscripts within the same column report significant differences at $P < 0.05$. Groups contain: control, no antioxidant supplementation; Mn, Andromed supplemented with 0.1 μM of MnTE; CAT 200, Andromed supplemented with 200 IU/mL of catalase; Mn + CAT 200, Andromed supplemented with 0.1 μM of MnTE and 200 IU/mL of catalase; CAT 400, Andromed supplemented with 400 IU/mL of catalase; and Mn + CAT 400, Andromed supplemented with 0.1 μM of MnTE and 400 IU/mL of catalase.
Abbreviations: HOST, hypoosmotic swelling test; MnTE, manganese(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin chloride.

catalase alone or in combination with MnTE can improve semen quality after thawing. As it is expected that co-action of SOD and catalase together may remove the oxidative radical and prevents the formation of highly toxic radicals such as hydroxyl radical.

Therefore, to evaluate the effect of catalase, MnTE, and their combination on sperm quality, we initially assessed the effect of these agents on sperm motility during dilution of sperm with cryopreservation media. Our results showed that addition of catalase, MnTE, and their combination to cryopreservation media for 2 hours did not affect the total motility of sperm and percentage of sperm with rapid and slow (class A + B) progressive motility (Table 1), suggesting that the physiological amount of ROS is not reduced to the extent which can affect sperm motility. Previous study have reported that excessive reduction of ROS may affect sperm motility [29,30], and therefore, our results reveal that the concentration of antioxidants used in this study does not reduce the ROS production to below the physiological level.

Before freezing, in contrary to the Mn group, the Mn + CAT 400 group slightly but significantly ($P \leq 0.05$) increased class A motility. These observations may suggest that reduction of oxygen radical (O_2^-) to hydrogen peroxide (H_2O_2) with MnTE may have led to increase the H_2O_2 level which may have prone sperm to lipid peroxidation, and this effect may have been ameliorated by addition of catalase which may account for improved class A motility, improved viability, and membrane integrity before freezing. Reduced ROS production before freezing further reiterates this conclusion.

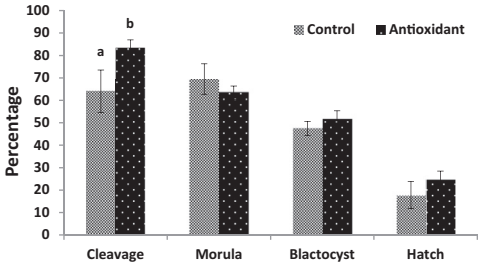
To assess the effect of these compounds on semen freezing performance, sperm motility, viability, and membrane integrity were assessed and all the three parameters were significantly ($P \leq 0.05$) decreased after freezing–thawing. Thus, it can be concluded that cryoinjury during semen cryopreservation induced reduction in overall sperm motility, viability, and membrane integrity. In accordance to literature [14], reduction of these parameters, concomitant with the increase of ROS production after freezing, may partly account for these cryoinjuries. Furthermore, addition of MnTE insignificantly improved sperm viability and membrane integrity, reduced the percentage of DCF-positive sperm, and significantly improved sperm motility after freezing. These results are in agreement with our previous report on MnTE [14]. Similar to Mn, CAT 200 and CAT 400 groups also improved the above parameters, and the degrees of improvement were significant ($P \leq 0.05$) for all the four aforementioned parameters compared with those of the control group. These results suggest that these scavengers can reduce cryoinjuries mediated by ROS production. Furthermore, catalase at both concentrations appears to be more effective than MnTE.

Considering the fact that both MnTE and catalase are effective in ameliorating the cryoinjuries; in the next experiment, we aimed to see whether their combination could be more effective than when they are individually applied. The results of total sperm motility after thawing revealed that Mn + CAT 400 significantly ($P \leq 0.05$) improved sperm motility compared with Mn, CAT 400, and Mn + CAT 200, suggesting that this combination is

Table 3
In vitro development of goat cumulus–oocyte complexes (COCs) fertilized by frozen-thawed goat semen diluted with the Andromed extender supplemented with catalase and/or MnTE.

Group	COCs number	Cleavage number (% ± SEM)	Blastocyst/cleavage number (% ± SEM)	Hatched/blastocyst number (% ± SEM)
Control	344	220 (64.05 ± 9.49)	104 (47.49 ± 3.10)	18 (17.74 ± 6.05)
CAT 200	298	245 (82.31 ± 7.13)	124 (50.89 ± 6)	30 (24.91 ± 5.15)
CAT 400	298	253 (85.21 ± 9.03)	136 (53.83 ± 6.63)	22 (16.50 ± 3.69)
Mn + CAT 400	314	259 (82.58 ± 4.39)	129 (50.14 ± 7.57)	40 (31.29 ± 7.06)

Results are expressed as mean ± standard error of the mean (SEM) of three separate replicates.
Different superscripts within the same column report significant differences at $P < 0.05$. Groups contain: control, no antioxidant supplemtation; CAT 200, Andromed supplemented with 200 IU/mL of catalase; CAT 400, Andromed supplemented with 400 IU/mL of catalase; and Mn + CAT 400, Andromed supplemented with 0.1 μM of MnTE and 400 IU/mL of catalase.
Abbreviation: MnTE, manganese(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin chloride.



Different superscripts within the same column demonstrate significant differences at $P < 0.05$.

Fig. 1. *In vitro* development of goat cumulus–oocyte complexes fertilized by frozen-thawed goat semen diluted with the Andromed extender (control) and Andromed supplemented with antioxidants. Results are expressed as mean ± standard deviation of three separate replicates.

highly effective in protecting semen sample against cryoinjuries. The degree of the reduction in total sperm motility after thawing was around 8%, which to our knowledge is the lowest reduction in sperm motility after thawing so far reported in the literature for goat or related species [31,32]. Concomitant with improvement of motility, a significant ($P \leq 0.05$) reduction in the percentage of DCF-positive sperm was also observed compared with the control. Thereby, suggesting that parallel reduction of O_2^- and H_2O_2 during cryopreservation of semen has improved sperm quality after thawing. Parallel reduction of $O_2^-$and H_2O_2 decreases the chance of OH radical formation, known as the most toxic ROS. It was also interesting to note that, after thawing, the percentage of DCF-positive sperm increased in all the treatments when compared with prefreezing, except in the CAT 400 and CAT 400 + Mn groups. These data suggest that cryoinduced ROS production is reversed by these two treatments. In addition to the improved total motility, a significant ($P \leq 0.05$) improvement was observed in class A + B motility after thawing in the CAT 400 and CAT 400 + Mn groups compared with the control. Considering the fact that these types or classes of motility contribute to fertilization rather than class C, this may provide a proof of principle for cryoprotective effects of these antioxidants. The percentage of viability in prefreezing condition ranged from 74.87 to 76.92. Although there was significant ($P \leq 0.05$) differences between the CAT 400 and Mn + CAT 400 groups compared with the control and other groups, the observed difference, although significant ($P \leq 0.05$), was minute. As expected, the viability of goat spermatozoa after thawing decreased in all treatments and control groups. Nevertheless, Mn + CAT 200, CAT 200, CAT 400, and Mn + CAT 400 groups resulted in better maintenance of viability compared with control. Thus, we suggest the addition of 400 IU/mL of catalase alone or in combination with 0.1 μM MnTE to the extender could protect goat sperm against the harmful effects of ROS and improve sperm motility, viability, and membrane integrity during semen cryopreservation. These results are in line with our hypothesis that addition of Mn as a cell-permeable SOD mimetic reduces the intracellular superoxide to H_2O_2, and H_2O_2 can escape the cell where it is reduced by catalase to nontoxic compounds H_2O and O_2.

It should be noted that, in the present study, the percentage of sperm viability is lower than the percentage of its equivalent motility in all treatments after thawing. We believe that two reasons may account for this difference: (1) Sperm might be initially motile but cryoinjury to membrane may allow leakage of the dye into these cryopreserved sperm and (2) sperm just recovered from the cryoshock injury are introduced to a second shock when exposed to eosin–nigrosin staining, and this may increase leakage of dye into sperm, and therefore, the percentage of motile sperm appears higher than the percentage of viable sperm after thawing.

Integrity of sperm plasma membrane, as the first shelter against external stresses, plays a fundamental role in sperm metabolism, capacitation, acrosome reaction, and finally fusion of sperm and oocyte [33]. Therefore, assessment of sperm plasma membrane integrity by the HOST in combination with other tests could predict the fertilization capacity of cryopreserved semen. In this regard, our results showed no significant ($P \leq 0.05$) differences among treatments in prefreezing condition, except for CAT 400 and Mn + CAT 400 groups, which showed significantly ($P \leq 0.05$) higher membrane integrity. However, after freezing, as expected there was a significant ($P \leq 0.05$) decrease in sperm membrane integrity in all the groups, as a consequence of cryoinjury. However, the degree of reduction in membrane integrity was significantly ($P \leq 0.05$) lower in the entire treatment groups compared with that in the control group, except for Mn. Thus, suggesting that the treatment has to certain degree protected sperm from cryoinjuries. Our results are in concordance with previous work which showed that supplementation with antioxidants such as water-soluble vitamin E analogue, Trolox C, and catalase reduces cryopreservation-induced lipid peroxidation and H_2O_2 generation [34]. It has been reported previously that the high polyunsaturated fatty acid content in mammalian sperm membrane renders the sperm very susceptible to lipid peroxidation, which occurs as a result of the oxidation of the membrane lipids by ROS [35]. Therefore, lipid peroxides can affect fertility potential by reducing sperm motility, membrane integrity and can induce sperm DNA damage. The gold standard test for assessment of treatments during cryopreservation of semen shows the ability of the cryopreserved sample to maintain fertility potential after freezing. In this study, we compared the fertilization and *in vitro* developmental rates of the control group with the CAT 200, CAT 400 and Mn + CAT 400 groups. Because the results of the latter groups were similar, therefore, we pooled the results of the latter groups and compared the results with the control group. The results revealed that addition of antioxidants increases the fertilization potential of cryopreservation sample as we observed a higher cleavage rate in the antioxidant groups compared with the control group. However, as expected and reported in the literature, the rate of embryo development to blastocyst and their ability to hatch were similar between the two groups [13,14].

In conclusion, our results suggest that addition of antioxidant MnTE or catalase to commercial optimized media, such as Andromed, improves the viability and motility of goat semen samples after thawing. But the

degree of improvement in motility was significantly ($P \leq 0.05$) higher when MnTE and catalase were simultaneously added to the cryopreservation media. This improvement resulted in a significantly ($P \leq 0.05$) higher rate of fertilization after thawing compared with the control treatment in the absence of these antioxidants. On the basis of present findings, the supplementation of antioxidants, catalase at 400 IU and MnTE at 0.1 μM in the semen extender, provided great benefit to buck semen during freeze-thawing. Further studies are suggested to evaluate *in vivo* fertility rates of frozen-thawed buck spermatozoa supplemented with catalase and MnTE for goat artificial insemination. To our knowledge, the degree of maintenance of sperm viability and motility, after thawing, obtained in this study is higher than those reported in literature for the goat.

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