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Can permeable super oxide dismutase mimetic agents improve the quality of frozen–thawed ram semen?

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ABSTRACT

This study was carried out to assess the effects of MnTBAP, a cell permeable antioxidant, on motility, membrane integrity, capacitation status and in vitro fertilization ability of frozen-thawed ram semen. Fresh semen ejaculates were collected with artificial vagina from five rams, mixed and divided into five equal fractions, and diluted (1:20 v/v) with commercial extender, Bioxell®, containing 0 (control), 50, 100, 150 and 200 μM of MnTBAP. All diluted sperm suspensions were cooled to 5 °C for 2 h followed by transfer into 0.5 ml French straws before being stored in liquid nitrogen.

The results showed that MnTBAP supplementation of extender improved ram semen quality in a dose-dependent manner. Accordingly, the extender supplemented with 150 μM MnTBAP resulted in higher sperm motility and improved acrosomal membrane integrity compared to control. However, further supplementation (200 μM) with MnTBAP not only did not improve the results but inversely affected motility and membrane integrity. The results of in vitro fertilization (IVF) indicated that the presence of MnTBAP in semen extender has a marginal beneficial effect on developmental competence of inseminated oocytes, though this improvement was not significant.

In conclusion, this study demonstrated that semen extender supplemented with MnTBAP can reduce the oxidative stress provoked by freeze/thaw processes. Moreover, beneficial effect of 100 μM of MnTBAP on preservation of spermatozoa in a non-capacitated state post freezing, an important criterion for in vitro or in vivo fertilization, was observed. However, at 150 μM of MnTBAP, the harmful effects of cryopreservation on membrane integrity were decreased. Regarding to importance of non-capacitated spermatozoa during IVF or artificial insemination, the optimum MnTBAP concentration appears to be 100 μM for commercial ram semen extender tested here.

Introduction

The theoretical and practical impacts of cryobanked semen in dissemination of genetic progress, improvement selection programs and conservation of genetic resources are numerous. Considering these vast applications of semen cryopreservation in farm animal biotechnology, it is well accepted that even a minor improvement in the quality of frozen/thawed semen may have magnificent effects on the desired outcomes from improved fertilization capacity to economic profit [26,3].

Despite considerable progress in semen cryopreservation process, the massive injuries that occur at cellular and molecular levels are yet remained as the main drawbacks of this technique, resulting in lower fertility rate of frozen-thawed vs. fresh semen [6,12]. Importantly, several lines of evidences that come from in vitro and filed studies indicating species-specific differences in cryo-withstand of semen collected from different mammals. In this sense, rabbit and human sperm have proven high resilient to freezing shock, whereas, ram, boar and bull sperm have tremendously lower resistance to cryopreservation [22]. Therefore, any improvement in freezing of ram semen can enhance the breeding programs in this valuable farm species.

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Structurally, spermatozoa are rich in phospholipids making them prone to lipid peroxidation [23]. On the other hand, due to minute cytoplasmic-to-nuclear ratio, the antioxidant reserve of sperm is very low and may not be sufficient to overcome considerable oxidative stresses generated during cryopreservation [8]. Moreover, the process of sperm handling before freezing prolongs exposure time to light and atmospheric oxygen, which subsequently create an oxidative environment with reduction in natural antioxidant concentration, and these events collectively make spermatozoa more sensitive to oxidative damage [17].Therefore, it seems that the progressive increase in oxidation is one of the main etiologies of deterioration in sperm integrity during freezing.

Different strategies have been used to improve semen cryopreservation efficiency including optimizing the cryoprotective effect of cryodiluents and cooling/warming rates. In addition, antioxidant supplementation of the freezing media has become customary in production of cryodiluents [7,13,31].

Antioxidant supplementation have increased the oxidative tolerance of frozen-thawed spermatozoa in different species using different antioxidants [8,9,20]. Moreover, there is a delicate balance between reactive oxygen species (ROS) and naturally generated antioxidants in cytoplasm of most somatic cells. Despite this, since sperm abandon most of its cytoplasm during maturation, increasing number of evidences show that sperm cells are highly susceptible to oxidative stress [21]. On the other hand, physiological amounts of ROS are necessary for normal sperm functions including capacitation and hyperactivation to acquire final fertilizing capability [2,11,1]. Thus, regardless of antioxidant concentration, the kind of antioxidant also may affect the final fertilizing capabilities of spermatozoa in different species.

Metalloporphyrins are novel class of catalytic antioxidants that scavenge a wide range of ROS such as superoxide, peroxynitrite and lipid proxyl radicas. Manganese (III) meso-tetrakis (4-benzoic acid) porphyrin (MnTBAP) is a synthetic metalloporphyrin-based compound, which readily penetrates into cellular membranes and acts as a potent antioxidant both in cytoplasm and mitochondria [25]. Regarding to its permeability into the cells and solubility in water and subsequently in diluents media, present study was carried out to enhance the quality of ram semen after freezing thawing through the addition of MnTBAP to freezing media. It is important to note that in this study, we used a commercial freezing media, Bioxell®, to see whether supplementation with MnTBAP might have effects on final fertilizing capabilities of ram sperm.

Materials and methods

Chemicals

All chemical reagents, as well as antioxidant (MnTBAP) were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated. Bioxell® as an extender was obtained from IMV (IMV technologies France).

Semen collection and processing

Semen sample from 5 mature Bakhtiari rams (3 and 4 yr of age) with proven fertility were collected by artificial vagina twice a week during the breeding season. Immediately after collection, the ejaculates were immersed in a warm water bath at 35 °C until arrival at laboratory for microscopic assessment (approximately within 30 min).The ejaculates containing spermatozoa with greater than 70% motility, concentration higher than 3×10^9 sperm/ml and volume varying between 0.75 and 2 ml, were accepted for experiment .To eliminate individual differences, semen samples from the five rams in each replicate were pooled. Each pooled sample was split into five equal aliquots and diluted (1:20 v/v) with a Bioxel® extender containing 0 (control group), 50, 100, 150 and 200 μM of MnTBAP. The diluted semen was gradually cooled to 4 °C for 2 h. The cooled samples were drawn into 0. 5 ml French straws (Biovet, L'Agile France), heat sealed and balanced at 4 °C for 1 h. After equilibration, the straws were exposed to liquid nitrogen vapor for 12 min, plunged into liquid nitrogen (LN2), and stored in LN2 until thawed and used for evaluation of sperm parameters and in vitro fertilization. The straws were thawed individually in a water bath (37 °C), for 30 s. Sperm evaluation was performed on all semen samples immediately after thawing by a single trained individual.

Semen evaluation after thawing

Analysis of sperm motility

Percentage of sperm motility was estimated according to Gil et al. [14]. After thawing, at least five straws from different freezing treatments were diluted with fertilization medium (Tyrode's albumin lactate pyruvate medium- Fert-TALP) and loaded onto a sperm counting chamber (Sperm Processor, Aurangabad, India) individually, three different microscopic fields for each semen sample were analyzed using a phase-contrast inverted light microscope (CKX41; Olympus, Tokyo, Japan) equipped with a heating plate maintained at 37 °C. The mean of the three successive estimations were recorded as the final motility score.

Sperm plasma membrane integrity

The hypo-osmotic swelling test (HOST) was used to evaluate the functional sperm membrane integrity according to Revell et al. [29].The test was performed by incubation of 25 μl semen with 200 μl of HOST solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) for 30 min at room temperature(RT). A wet mount was made using a 5 μl drop of homogenized mixture and placed directly on microscopic slide and covered by a cover slip. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of spermatozoa with swollen and curved tails were recorded.

Acrosomal status

Acrosome reaction was assessed with chlortetracycline (CTC) staining as described by Perez et al. [28] and Sharafi et al. [30]. A CTC working solution (750 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D, L-cysteine at a pH of 7.8. Then 5 μl of semen was mixed with 20 μl CTC working solution. After 20 s, the reaction was stopped by the addition of 5 μl 1% (v/v) glutaraldehyde in 1 M Tris–HCl, pH 7.8. Smears were prepared on a clean microscope slide and examined under an epifluorescent microscope (BX51; Olympus; Japan).

In vitro fertilization

Oocyte in vitro maturation and fertilization

The conditions for in vitro oocyte maturation were performed as described by Forouzanfar et al. [12]. In brief, ovaries were collected from slaughtered ewes, the cumulus-oocyte complexes (COCs) were recovered by aspiration, washed in aspiration medium (Hepes-Tissue Culture Media (H-TCM) + 10% Fetal Calf Serum (FCS) + 100 IU/ml heparin) and groups of 8–10 COCs were cultured in maturation medium (TCM-199 + 10% FCS + 5 mg/ml follicle-stimulating hormone + 5 mg/ml luteinizing hormone + 0.1 mM cysteamin) in 5% CO_2 at 39 °C and maximum humidity for 22–24 h. For in vitro fertilization (IVF), matured COCs were washed in fertilization medium [27] and groups of 40–50 COCs were transferred into 200-μl drops of fertilization medium under mineral oil. Five straws of frozen spermatozoa representing one treatment

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were thawed and pooled. The motile spermatozoa were separated from the immotile by centrifugation (700 g/15 min-RT) on Pure Sperm layers of 80 and 40 (Nidacon; Gothenburg, Sweden), before being inseminated (total 2×10^6 spermatozoa/ml) in COC containing droplets. The inseminated COCs were incubated for 22–24 h in 5% CO_2 in humidified air at 39 °C.

Embryo culture

Embryo culture was carried out in modified Synthetic Oviduct Fluid (SOF) medium [16]. Twenty-four hours after insemination, cumulus cells were removed from presumptive zygotes by repeated pipetting. Groups of six embryos then were transferred into 20-ml droplet of culture SOF up to day 3 post insemination (day 0 = day of insemination) before being transferred to SOF medium supplemented with 10% charcoal stripped serum and 1.5 mM glucose up to blastocyst stage. Cleavage, blastocyst and hatched rates in each group were assessed on days 3, 8 and 9 post insemination, respectively.

Statistical analysis

The results are reported as the mean ± standard error (SE) of each experiment. The analysis of variance (ANOVA) was used for treatments comparisons .One-way ANOVA with the Tukey multiple comparison post-test was used to compare the different stimuli. $P \leqslant 0.05$ was considered to be significant.

Results

Motility

The effect of MnTBAP on total motility, membrane integrity and acrosome status of Bakhtiari ram semen following freeze–thawing are presented in Figs. 1 and 2 respectively. While a significant increasing in sperm motility in MnTBAP 150 μM in comparison with control group was observed (37.62 vs. 31.53%), this improvement decreased significantly in MnTBAP 200 μM (27.14%) in comparison with all treatments and control groups. However, in all treatments, except for MnTBAP 200 μM, with increasing the MnTBAP concentration, the percentage of total sperm motility increased so that the best result was related to MnTBAP150 μM group compared to all the other groups.

Membrane integrity

As depicted in Fig. 1, with increasing MnTBAP concentration, an increase in percentage of intact plasma membrane integrity

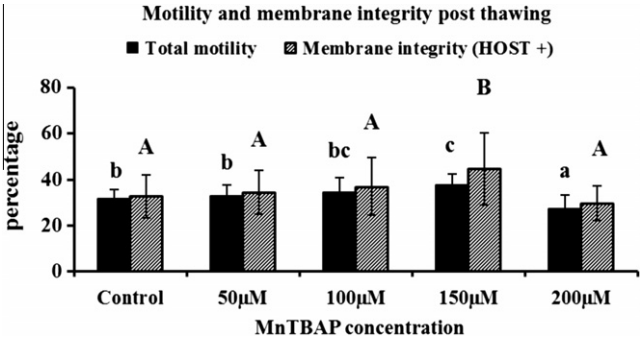

Fig. 1. Post-thawing total motility and membrane integrity of Bakhtiari ram semen diluted with Bioxell® extender containing 0 (control), 50, 100, and 150 and 200 μM of MnTBAP. [a–c]: Different superscripts within the same column demonstrate significant differences ($P < 0.05$).

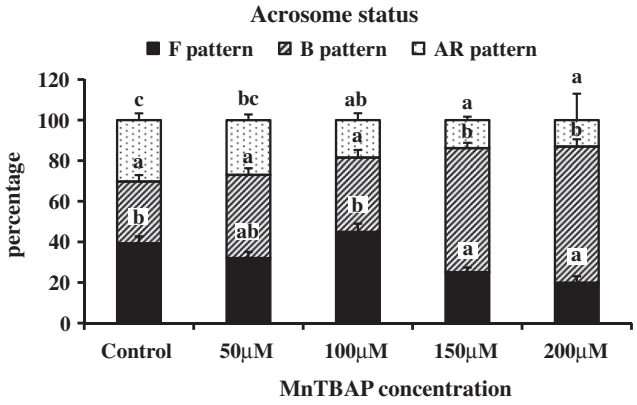

Fig. 2. Post-thawing acrosome status (F, B and AR patterns) of Bakhtiari ram semen diluted with Bioxell® extender containing 0 (control), 50, 100, 150 and 200 μM of MnTBAP. [a–c]: Different superscripts within the same column demonstrate significant differences ($P < 0.05$).

(HOST+) was observed, and in 150 μM of MnTBAP reached to maximum (44.68%) and significant with all other treatments and control groups. However supplementation with 200 μM of MnTBAP (29.62%), didn't resulted in improved membrane integrity and decreased non-significantly in comparison with control(32.69%), MnTBAP 50 (34.44%) and 100 μM (36.93%) groups.

Acrosome status

Fig. 2 shows percentages of three types of sperm head following freezing-thawing for acrosome status assessments after CTC staining: F pattern or uniform fluorescence head (uncapacitated sperm), B pattern or post-acrosomal region without fluorescence (capacitated sperm) and AR pattern or fluorescent-free head or a thin fluorescent band on the equatorial segment (acrosome reacted spermatozoa). The percentages of F pattern were decreased in all treatments groups except those for MnTBAP 100 μM (44.91%) which was non-significantly higher than control (39.25%) and significantly higher than MnTBAP 150 μM (25.1%) and MnTBAP 200 μM (19.85%) groups. A significant decreasing also was seen in MnTBAP 150 and 200 μM in comparison with control and MnTBAP 100 μM groups.

The percentages of B pattern increased with increasing MnTBAP concentration in all treatments groups which was significantly higher in MnTBAP 150 μM (61.13%) and 200 μM (67.12%) to other treatments and control (30.5%) and non-significantly in MnTBAP 50 (40.99%) and 100 μM (36.63) groups in comparison with control group. The AR pattern decreased with increasing concentration of MnTBAP and was significant between MnTBAP 200, 150 and 100 μM with control group.

In vitro fertilization ability and embryo culture

Since MnTBAP treatment marginally improved the motility and membrane integrity in MnTBAP100 and 150 μM, in vitro fertilizing abilities of frozen-thawed spermatozoa only assessed for these two groups and compared with the control group. Fig. 3 shows the cleavage, blastocyst formation and hatched embryo rates, in terms of number of inseminated oocytes, cleaved embryos and blastocysts, respectively. Despite highest and non significant blastocyst rate was observed for MnTBAP 100 μM (40.79%), hatching rate decreased with increasing concentration of MnTBAP and was significant between control and MnTBAP150 μM (18.45 vs. 45.09%).

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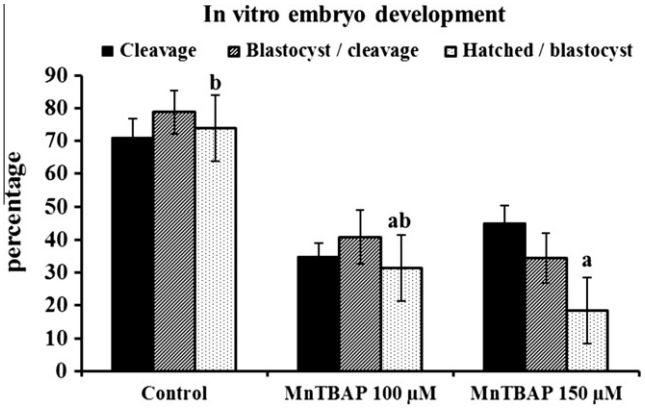

Fig. 3. In vitro development of sheep oocytes fertilized by frozen-thawed Bakhtiari ram semen diluted with Bioxell® extender supplemented with 0 (control), 100, and 150 μM of MnTBAP. [a,b]: Different superscripts within the same column demonstrate significant differences ($P < 0.05$).

Discussion

The mammalian sperm membrane is rich in polyunsaturated fatty acids making it susceptible to lipid peroxidation. In addition, unavoidable prolonged exposure time to the light and oxygen, during sperm handling for assisted reproduction technology (ART) process, collectively make spermatozoa more sensitive to oxidative damage[22,17]. Membrane lipid peroxidation due to ROS, impairs sperm functions including motility, membrane and DNA integrity, and ultimately reduces fertility [18,5]. Therefore, addition of antioxidants during ART process is highly recommended.

The results of this study showed that supplementation of MnTBAP to the extender improves ram semen quality, especially semen parameters. This effect appears to be dose-dependent. Accordingly, the extender supplemented with150 μM of MnTBAP resulted in the higher sperm motility and improved acrosomal membrane integrity compared to control. The current findings were in agreement with previous studies and indicated favorable effects of enzymatic antioxidant on post thaw viability of different mammalian species (19,24,8]. However, further supplementation (200 μM) with MnTBAP not only did not improve the results but inversely affected motility and membrane integrity. This affect may be attributed to toxicity of MnTBAP at this concentration. Similarly, Foote et al. [10] showed that addition of higher than optimal concentration of Tempo, a super oxide dismutase mimetic agent to bovine semen extender is toxic.

Importantly, by looking to Figs. 1 and 2, it is indicated that between the safe dosages of MnTBAP, the increment of MnTBAP concentration resulted in a series of interconnected changes in semen quality parameters. Accordingly, the stepwise increases in sperm motility were correlated with the increased membrane integrity, decreases in non-capacitated and acrosome reacted sperm, and thereby serial decreases in the percentages of capacitated sperm. Although this regular pattern of changes can be considered as a proof of principle for the effectiveness of MnTBAP, this also may provide some further considerations for the choice dose of MnTBAP for in vitro and in vivo studies. Sperm motility is the crucial factor for passage through cervix, cumulus cells and finally penetration of zona pellucida. Thus, sperm motility, despite being the most challenging factor for assessment of frozen-thawed semen samples, further studies have shown that precocious capacitation may preclude the potential applications of frozen/thawed sperm to be used for in vitro, and particularly in vivo, fertilization [15]. This is likely due to precociously acrosome reactions in response to triggers far from the proper site of fertilization near to oocyte zona pellucida. The results of this study reveal that higher percentage of non-capacitated sperm is present in MnTBAP100 μM group.

Among sperm parameters, membrane integrity has particular importance, since it is the frontier shelter to external stresses, in addition to begins susceptible to lipid peroxidation. It also plays fundamental roles in sperm metabolism, capacitation, acrosome reaction and finally fusion of sperm and oocyte [4,15]. Therefore, assessment of membrane integrity by the hypo osmotic swelling test (HOST) revealed that supplementation of 150 μM MnTBAP significantly reduces the harmful effects of cryopreservation process.

Therefore, considering the beneficial effect of 100 μM MnTBAP on preservation of non-capacitated state of semen sample post freezing and reduce harmful effects of cryopreservation on membrane integrity at 150 μM MnTBAP, we assessed the effect of these two concentrations on the potential of cryopreserved samples to participate in fertilization post thawing.

The results of IVF in the present study indicated that the presence of MnTBAP in semen extender have a marginal beneficial effect on developmental competency of inseminated oocytes, though this improvement was not significant. These results in agreement with some related studies in bull and boar that did not found a significant beneficial effect of antioxidant in extender on in vitro and in vivo fertility of the cryopreserved semen [32,33]. This may be due to great compensatory repairing role of oocyte to restore the damages induced during sperm freezing especially at the chromatin level. Regarding to cleavage and blastocyst rates, there was no significant differences between the groups but on general hatching rate appears to be adversely affected by MnTBAP supplementation, wich was insignificant for MnTBAP 100 μM and significant for MnTBAP 150 μM when compared to control group. Whether this phenomenon is related to reduce ROS level possibly required for hatching remains to be evaluated or understood in future.

In conclusion, this study demonstrated that the extender supplemented with MnTBAP could reduce the oxidative stress provoked by freeze/thaw and improved ram sperm motility, acrosomal membrane integrity, and plasma membrane integrity after freeze–thaw process. The optimum MnTBAP concentration appears to be 100 μM for ram semen extender. The effectiveness of extender containing MnTBAP may be related to tilt in the balance between H_2O_2 and $OH^{\cdot}$ production, in favor of H_2O_2 production. $OH^{\cdot}$ is considered as the most damaging type of ROS which is converted to H_2O_2 by super oxide dismutase activity of MnTBAP. However, we did not find significant beneficial effect of this antioxidant on blastocyst formation; this does not preclude its possible beneficial effect on post implantation development, which needs to be assessed. However, if MnTBAP to be added to extenders assisted hatching needs to be complemented.

Authors' role

Conceived and designed the experiments: MF, MHNE. Performed the experiments: MF, SFE, SMH, MH, SOH, AA, MT. Analyzed data: MF. Wrote the paper: MF, MHNE.

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