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Brief Communication

Supplementation of sperm cryopreservation media with cell permeable superoxide dismutase mimetic agent (MnTE) improves goat blastocyst formation ☆

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ABSTRACT

The aim of this study was to assess whether a cell permeable superoxide dismutase agent such as MnTE, can further improve the quality of frozen/thawed semen sample using a commercially optimized sperm cryopreservation media (Bioxcell). Bioxcell was supplemented with different concentration of MnTE. Sperm membrane integrity, motility, viability and acrosomal status were assessed after freezing. Optimized concentration of MnTE was defined and used to assess fertilization and developmental potential. 0.1 μM MnTE significantly improved membrane integrity while 0.01 μM MnTE significantly improved acrosomal integrity post thawing. Addition of 0.01 μM MnTE also improved blastocyst formation rate. Supplementation of commercially optimized cryopreservation media with MnTE further improves the quality of goat frozen semen sample and may have important consequence of future embryo development. This effect may be attributed to cell permeable behavior of this antioxidant which may protect sperm genome from ROS-induced DNA damage.

Introduction

Semen cryopreservation has been widely used as a method for large scale artificial insemination programs, dissemination of genetic progress, improvement selection programs, conservation of genetic resources in farm animals and fertility preservation in human patients. Despite great progress in technology and methodology of semen cryopreservation, physical and chemical stresses are yet remained as the main drawbacks of mammalian semen cryopreservation [2]. Consequently these injuries may impair in vitro and in vivo fertility potential of the cryopreserved semen sample. Different strategies or approaches have been implemented to overcome these injuries, including usage of different cryoprotectants and the cryodiluents, altering the cooling and/or warming rates and most importantly supplementation of semen freezing media with different antioxidants. It is well demonstrated that generation of reactive oxygen species (ROS) during cooling, freezing and thawing has deleterious effects on biologic materials and has been considered as the main factor in inducing cryoinjuries [3]. Moreover, it has been envisaged that imbalance between the production of ROS and antioxidant defense capacity plays a central role in this process [4]. Unlike somatic cells, due to lack of cytoplasm, spermatozoa possess little antioxidant capacity to prevent oxidative damage and this prone spermatozoa to ROS induce injuries. To overcome this deficiency, semen has a high innate antioxidant capacity. However, during cryopreservation, semen is diluted with extender which makes sperm susceptible to ROS-induced injuries. Furthermore, it has been reported that dead spermatozoa generate considerable amounts of ROS and this effect make sperm more susceptible to ROS-induced cryoinjuries post thawing [4]. These effects can be further potentiated by unenviable in vitro handling procedures including, dilution, centrifugation, and prolongs exposure time to the visible light radiation or atmospheric oxygen [4]. Therefore, supplementation media with antioxidant has received growing interest. Study of literature reveals that the majority of antioxidants supplemented to freezing media are non-cell permeable and at most, they may prevent propagation of lipid radicals at membrane level. We previously proposed that cell permeable antioxidant such as MnTBAP improves cell viability, motility and percentage of uncapacitated spermatozoa in ram following freezing–thawing process [6]. However, it did not improve fertilization or embryo developmental rates, in vitro. Therefore, this study aimed to evaluate the role of manganese (III) meso-tetrakis

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(N-ethylpyridinium-2-yl) porphyrin chloride (MnTE) in cryopreservation of goat semen. MnTE is a cell permeable 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. Furthermore, MnTE has been reported [10] to be 16 time potent than MnTBAP used in our pervious study.

All chemical reagents were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated. Antioxidant (MnTE) was gift from Dr. Ines Batinić-Haberle (Department of Radiation Oncology, Duke University Medical School, Durham, NC 27710, USA). Bioxcell as an extender was obtained from IMV (IMV technologies, France).

Semen samples from four Bakhtiari bucks, aged 2–3 years, with proven fertility were collected with the aid of an artificial vagina twice a week during the breeding season (September/October-2012). Only ejaculates more than 1 ml in volume and showing a minimum of 70% motile and 80% morphologically normal spermatozoa were included in the study. To eliminate individual differences, semen samples from the four bucks in each replicate were combined. Each combined sample was split into four equal aliquots and diluted (1:20 v/v) with extender containing no antioxidant (control group), 0.01, 0.1, and 1 μM of MnTE. The diluted semen was gradually cooled to 4 °C in 2 h and drawn into 0.5 ml French straws (Biovet, L'Agile, France), heat sealed and balanced at 4 °C for one hour. 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 removed from the LN2 and thawed individually by immersion of the sealed straws in a water bath at 37 °C for 30 s.

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 estimates was recorded as the final motility score.

We used the hypo-osmotic swelling test (HOST) to evaluate sperm plasma membrane integrity. Following incubation of 25 μl diluted semen and 200 μl of HOST solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) for 30 min at room temperature, 5 μl drop of homogenized mixture was mounted and directly investigated using an inverted microscope. The percentages of spermatozoa with swollen and curved tails were estimated from recording 200 spermatozoa in more than five different microscopic fields.

We used flow cytometer (FACScan; Becton Dickinson, San Jose, CA) to determine sperm cell viability using propidium iodide (PI) staining (as marker for cell death). After thawing, to exclude debris and aggregates, the samples from each groups were centrifuged twice at 700×*g* for10 min, and resuspended in 1 ml of same medium to final concentration of one million sperm per ml. Samples were stained by 3 μgr/ml of PI for15 min at room temperature before analysis (excitation: 488 nm; emission: 625 nm in the FL3 channel). Approximately 100,000 spermatozoa per sample were examined and the percentages of PI-positive cells (dead spermatozoa) were recorded.

To determine the impact of freezing–thawing process on sperm acrosome, chlortetracycline (CTC) staining was used. Freshly prepared CTC working solution was made by dissolving 750 mM CTC in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D,L-cysteine at a pH of 7.8. CTC solution was kept in the dark at 4 °C until used. For staining, 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. Homogenized mixture was mounted and covered with a cover-slip, and the slide was gently pressed under two folds of a tissue paper to absorb any excess fluid. Slides were examined under an epifluorescent microscope (BX51; Olympus; Japan).

The conditions for in vitro oocyte maturation were performed as described previously [7]. Goat ovaries were obtained from slaughter house. The cumulus–oocyte complexes (COCs) were recovered by aspiration from follicles of more than 2 mm diameter on the surface of the ovaries. Oocytes completely surrounded by cumulus cells were collected and washed in aspiration medium (Hepes-Tissue Culture Media (H-TCM) + 10% Fetal Calf Serum (FCS) + 100 IU/ml heparin). 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.

Matured COCs were washed in fertilization medium and groups of 20–25 COCs were transferred into 100-μl drops of fertilization medium under mineral oil. Five straws of frozen spermatozoa representing one treatment were thawed at 37 °C for 1 min, pooled and washed through Pure Sperm (Nidacon; Gothenburg, Sweden) gradient (40% and 80%) to separate the motile spermatozoa from the immotile by centrifugation (700×*g*/15 min-RT). Matured COC were inseminated with a final concentration of two million sperm per ml. The inseminated COCs were incubated for 22 h in 5% CO_2 in humidified air at 39 °C. Twenty-two hours after insemination, cumulus cells attached to oocytes were mechanically removed via pipetting. The presumptive zygotes were washed 3 times through modified Synthetic Oviduct Fluid (SOF) medium. Groups of six embryos were then transferred into 20-ml droplets of culture SOF for the first 3 days 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. Subsequently cleavage, blastocyst and hatched rates in each group were assessed on days 3, 8 and 9 post insemination, respectively.

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

Table 1 show that MnTE supplementation ranging from 0.01 to 1 μM in pre-freezing condition had no effect on motility and membrane integrity. However, these parameters, in addition to sperm viability, were significantly reduced following freezing and thawing, but MnTE significantly prevented the reduction of membrane integrity at 0.1 μM concentration. It is important to note that supplementation with 1 μM MnTE has potentiated or exaggerate the adverse effect of freezing and thawing on both sperm motility and membrane integrity, despite no negative influence during pre-freezing condition. Furthermore, percentage of sperm with intact acrosome (F-pattern) was significantly higher following freezing and thawing compared to control at 0.01 μM MnTE. Considering the adverse effect of 1 μM MnTE on motility, viability and membrane integrity, to assess the effect of MnTE on fertility potential of cryopreserved semen samples, samples supplemented with 0.01 and 0.1 μM MnTE were used. The results of Table 2 shows with these concentrations of MnTE had no effect of cleavage rate. However, the results for blastocyst formation showed a significant improvement compared to control when semen extender was supplemented with 0.1 μM MnTE during freezing and thawing. This improvement was also observed for hatching rate but the degree of improvement was insignificant.

The quality of frozen-thawed semen is one of the factors that influence the success rate of artificial insemination and is related to degree of oxidative stress induced during freezing and thawing

Table 1
Data on the effect of MnTE supplementation in the freezing media on motility, membrane integrity pre-freezing and motility, membrane integrity, viability and acrosome status of buck spermatozoa post-thawing.

	Pre-freezing		Post thawing					
	Motility	Membrane integrity	Motility	Membrane integrity	Viability	Acrosome status pattern		
						F	B	AR
Control	78.40 ± 0.50	78.09 ± 1.14	35.27 ± 1.96[a]	35.72 ± 0.38[b,c]	36.11 ± 0.77 [a,b]	52.43 ± 2.79[b]	41.73 ± 2.75[a]	5.83 ± 1.37[b]
MnTE 0.01 μM	80.21 ± 0.38	79.36 ± 1.73	36.72 ± 1.04[a]	37.29 ± 0.85[b]	37.55 ± 1.28[a,b]	73.22 ± 2.62[a]	20.98 ± 2.44[c]	5.79 ± 1.51[b]
MnTE 0.1 μM	80 ± 0.67	80.03 ± 1.07	40.21 ± 1.65[a]	43.91 ± 0.60 [a]	43.86 ± 2.98 [a]	52.63 ± 3.00[b]	35.82 ± 2.85[a,b]	11.84 ± 1.66[a]
MnTE 1 μM	78.86 ± 0.45	78.07 ± 2.62	28.44 ± 1.32[b]	32.88 ± 0.90[c]	28.57 ± 2.62 [b]	59.53 ± 2.81[b]	28.42 ± 2.56[b,c]	12.04 ± 1.92[a]

Acrosome status represented as: F pattern; uncapacitated sperm, B pattern; capacitated sperm and AR pattern; acrosome reacted spermatozoa.
[a,b,c] Different superscripts within the same column demonstrate significant differences ($P < 0.05$).

Table 2
In vitro development of goat oocytes fertilized by frozen-thawed goat semen diluted with Bioxell extender supplemented with 0 (control), 0.01, and 0.1 μM of MnTE.

Groups	COCs (number)	Cleavage (% ± SEM)	Blastocyst/cleavage (% ± SEM)	Hatched/blastocyst (% ± SEM)
Control	314	63.00 ± 6.08	23.66 ± 3.17[b]	35.66 ± 7.53
MnTE 0.01 μM	292	67.00 ± 2	29.33 ± 0.66[b]	31.66 ± 1.66
MnTE 0.1 μM	273	69.66 ± 4.05	43.33 ± 1.76[a]	43.00 ± 1.73

[a,b] Different superscripts within the same column demonstrate significant differences ($P < 0.05$).

process, especially in sperm due to scarcity of cytoplasm and thereby reduced antioxidant potential. During natural mating, sperm encountered the anaerobic conditions of vagina and possible oxidative stress is contract by rich antioxidant capacity of semen and substantial concentrations of taurine in the oviduct fluid [1]. In pervious and this study, we stress on the usage of cell permeable antioxidants and we showed that although MnTBAP improved sperm motility and viability post freezing–thawing but it had no significant influence on cleavage rate and blastocyst formation rate but in contrary to our expectation it significantly reduce blastocyst hatching rate [6]. Therefore, in this study we aim to evaluate role of MnTE during sperm cryopreservation, considering more potent antioxidant activity considered for MnTE. Our results revealed that supplementation of MnTE had no adverse effect on sperm motility and membrane integrity in pre-freezing condition in 0.01–1 μM range. However, supplementation with MnTE during freezing and thawing significantly improved sperm membrane integrity at 0.1 μM while supplementation with 1 μM significantly decreased sperm motility and viability, suggesting that it has an adverse effect during freezing and thawing condition which was not observed during pre-freezing condition. The improved effect observed during freezing and thawing is in line with pervious studies showing that semen samples containing high SOD and high proportion of glutathione peroxidase activity before freezing had highest viability following freeze–thaw process [5]. It was interesting to note the both MnTBAP at 100 μM and in this study MnTE at 0.01 μM improved the percentage of sperm with intact acrosome post freezing and thawing. The property of these compounds is important in semen cryopreservation, especially for artificial insemination in which parentage of sperm with intact acrosome have important consequence on pregnancy outcome. The improved acrosome integrity could be ascribed to improved membrane integrity, which plays a fundamental role in sperm metabolism, capacitation, acrosome reaction and finally fusion of sperm and oocyte. It was interesting to note 200 μM MnTBAP reduce the percentage of sperm with intact acrosome by 50% compared to control. However, higher concentration of MnTE had no such an adverse effect. These observations may be explained by the role of ROS, especially superoxide, in the process of capacitation and acrosome reaction. A balance between ratio of superoxide and H_2O_2 may be needed to prevent capacitation and acrosome reaction at the same time preventing formation of hydroxyl radical in the Haber–Weiss reaction which may impair sperm integrity. These observations may in part explain the controversies regarding supplementation of super oxide dismutase mimetic agent during cryopreservation with improving and adverse effect which is likely dependent on the antioxidant concentration and type of species [8,9]. Commonly, fertilization rate is considered as the golden standard for assessment of cryopotential of different semen cryopreservation media. In this study and our previous on MnTBAP, we observed no significant improvement in presumptive fertilization rate assessed by cleavage rate over the control group. Suggesting that despite the improved sperm parameters post freezing and thawing, supplementation with these antioxidants have no significant effect of fertilization potential of thawed samples. However, what was interesting to note in this study was the improved blastocyst rate observed compared to control. This effect may be attributed to adverse effect of ROS production on sperm DNA integrity. It is well established that increased DNA damage reduces blastocyst rate and it is likely that ability of MnTE to convert superoxide to H_2O_2 at extracellular, membrane and intracellular level has led to an improved the quality of semen used for insemination of oocyte and this may have resulted in improved blastocyst formation observed in this study. Our results are in concordance with pervious work [9] which showed that supplementation with antioxidants such as water-soluble vitamin E analogue, Trolox-C and catalase reduce cryopreservation induced lipid per oxidation and H_2O_2 generation. In conclusion the results of present study indicated that the addition of MnTE, a cell permeable superoxide dismutase mimetic agent, to a commercial extender which is already optimized with antioxidant, further improves the quality of frozen thawed goat semen to an extent which improves blastocyst formation rate. We proposed that this beneficial effect of MnTE is likely due to its cell permeable characteristic which in turn my protect sperm genome from ROS damage.

References

[1] I. Batini, I. C-Haberle, C. Spasojevi, P. Hambright, L. Benov, A.L. Crumbliss, I. Fridovich, The relationship between redox potentials, proton dissociation constants of pyrrolic nitrogenes, and in vitro and in vivo superoxide dismutase activities of manganese (III) and iron (III) cationic and anionic porphyrins, Inorg. Chem. 38 (1999) 4011–4022.
[2] A. Bernardini, F. Hozbor, E. Sanchez, M.W. Fornés, R.H. Alberio, A. Cesari, Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage, Theriogenology 76 (2011) 436–447.

[3] M.N. Bucak, P.B. Tuncer, S. Sarıözkan, N. Başpınar, M. Taşpınar, K. Coyan, A. Bilgili, P.P. Akalın, S. Büyükleblebici, S. Aydos, S. Ilgaz, A. Sunguroğlu, D. Oztuna, Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage, Cryobiology 61 (2010) 248–253.
[4] S. Chatterjee, C. Gagnon, Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing, Mol. Reprod. Dev. 59 (2001) 451–458.
[5] N. Cocchia, M.P. Pasolini, R. Mancini, O. Petrazzuolo, I. Cristofaro, I. Rosapane, A. Sica, G. Tortora, R. Lorizio, G. Paraggio, A. Mancini, Effect of sod (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa, Theriogenology 75 (2011) 1201–1210.
[6] M. Forouzanfar, S. Fekri Ershad, S.M. Hosseini, M. Hajian, S. Ostad-Hosseini, A. Abid, M. Tavalaee, A. Shahverdi, A. Vosough Dizaji, M.H. Nasr Esfahani, Can permeable super oxide dismutase mimetic agents improve the quality of frozen–thawed ram semen?, Cryobiology 66 (2013) 126–130
[7] M. Forouzanfar, M. Sharafi, S.M. Hosseini, S. Ostadhosseini, M. Hajian, L. Hosseini, P. Abedi, N. Nili, H.R. Rahmani, M.H. Nasr-Esfahani, In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen, Theriogenology 73 (2010) 480–487.
[8] J. Gil, N. Lundeheim, L. Söderquist, H. Rodriíuez-Martínez, Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen, Theriogenology 59 (2003) 1241–1255.
[9] S. Maia Mda, S.D. Bicudo, C.C. Sicherle, L. Rodello, I.C. Gallego, Lipid peroxidation and generation of hydrogen peroxide in frozen–thawed ram semen cryopreserved in extenders with antioxidants, Anim. Reprod. Sci. 122 (2010) 118–123.
[10] L. Terziev, V. Dancheva, V. Shopova, G. Stavreva, Antioxidant effect of MnTE-2-PyP on lung in asthma mice model, Sci. World J. 2011 (2012) 1–6.