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Effect of β-mercaptoetanol and cysteine on post-thawing quality and oxidative activity of ram sperm and on the viability of vitrified sheep embryos

[*Efeito do β-mercaptoetanol e da cisteína sobre a qualidade e a atividade oxidativa do sêmen ovino após o descongelamento e sobre a viabilidade de embriões ovinos vitrificados*]

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ABSTRACT

The effects of β-mercaptoethanol (BME) and cysteine on the viability and oxidative activity of ram sperm after thawing and on development *in vitro* and viability of vitrified sheep embryos were evaluated. Ejaculates from four rams were pooled and extended, composing six treatments: no antioxidants; 2mM BME; 5mM BME; 2mM BME and 5mM cysteine; 5mM BME and 5mM cysteine; and 5mM cysteine. Sperm motility, membrane and acrosome integrity, mitochondrial functionality, production of reactive oxygen species and total antioxidant capacity were similar across treatments (P>0.05). A medium with no antioxidant presented cleavage and blastocyst development rates (60.3% and 33.6%, respectively) similar (P>0.05) to those of a medium with 50μM BME and 600μM cysteine (64.3% and 36.6%, respectively). Post-thawing viability of vitrified embryos was similar between media (P>0.05). Cysteine and BME had no influence on the post-thawing viability and oxidative activity of ram sperm and on the viability of vitrified sheep embryos.

Keywords: sheep embryos, antioxidant, reactive oxygen species, frozen ram sperm, vitrification

RESUMO

Foram avaliados os efeitos do β-mercaptoetanol (BME) e da cisteína sobre a viabilidade e a atividade oxidativa após o descongelamento do sêmen ovino e sobre o desenvolvimento in vitro *e a viabilidade de embriões ovinos vitrificados. Ejaculados de quatro carneiros foram agrupados e diluídos, compondo seis tratamentos: sem antioxidantes; com BME 2mM; com BME 5mM; com BME 2mM e cisteína 5mM; com BME 5mM e cisteína 5mM; e com cisteína 5mM. Motilidade, integridade da membrana e do acrossoma, função mitocondrial, produção de espécies reativas de oxigênio e capacidade antioxidante total foram semelhantes entre os tratamentos (P>0,05). Em um meio sem antioxidantes, as taxas de clivagem e de desenvolvimento embrionário até blastocisto (60,3%, e 33,6%, respectivamente) foram semelhantes (P>0,05) às obtidas em um meio comBME 50μM e cisteína 600μM (64,3% e 36,6%, respectivamente). A viabilidade pós-descongelamento dos embriões vitrificados não diferiu entre os meios (P>0,05). O BME e a cisteína não influenciaram a viabilidade e a atividade oxidativa do sêmen ovino após o descongelamento e a viabilidade de embriões ovinos vitrificados.*

Palavras-chave: embriões ovinos, antioxidantes, espécies reativas de oxigênio, sêmen ovino congelado, vitrificação

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INTRODUCTION

Ram spermatozoa are highly sensitive to structural and functional damages during freezing and thawing due to the high content of long-chain polyunsaturated fatty acids in their membrane and to the lack of antioxidants in their cytoplasm. Such injuries may result from oxidative stress and lipid peroxidation, due to the spermatozoa's exposure to reactive oxygen species (ROS) derived from their own metabolism (Sinha *et al*., 1996; Peris *et al*., 2007), which leads to premature capacitation (Bailey *et al*., 2000; Aisen *et al*., 2005). Therefore, the inclusion of substances with antioxidant properties in freezing extenders may prevent cryoinjuries in spermatozoa's membranes and organelles that may impair their post-thawing viability (Chatterjee and Gagnon, 2001). Many antioxidant additives have been tested in protocols for freezing ram sperm (Bucak *et al*., 2008; Maia *et al.*, 2010), but due to their inconsistent results, further research in this field is justified.

Thiol antioxidant composts of low molecular weight, such as β-mercaptoetanol (BME), neutralize ROS production by inducing intracellular synthesis of glutathione (GSH) (Luberda, 2005; Hosseini *et al*., 2011). As GSH has no direct antioxidant effect, an increase in its concentration is stimulated through other pathways (Câmara *et al*., 2011). That occurs because BME promotes oxidation of cysteine, an aminoacid involved in GSH intracellular synthesis, into cystine. Even though cystine cannot be metabolized by the cell, BME reacts with cystine producing a disulfide mixture that allows cysteine to become available (Ishii *et al.*, 1981; Takahashi *et al*., 2002), releasing BME to react with other cystine molecules. Although BME stimulates synthesis of mRNA and of essential mitochondrial proteins required to produce energy for sperm motility (Salem *et al*., 1988), antioxidant effects of BME and cysteine on post-thawing ram sperm viability were not yet reported.

In oocytes, cysteine's oxidation is mediated by cells of the *cumulus oophurus*-oocyte complex (COC) (Matos *et al*., 1997). In media for *in vitro* maturation (IVM) of oocytes, cysteine's availability appears to be limiting for GSH synthesis, especially because its concentration is commonly lower than that of cystine, due to self-oxidation (Matos *et al*., 2002). However, the low cysteine's available concentration during the first hours of IVM appears enough to guarantee GSH synthesis. Thus, the cystine generated by cysteine's oxidation may be reconverted in cysteine by the COC cells and incorporated by the embryo, generating GSH (Matos *et al*., 1997). Nevertheless, the effects of the inclusion of BME and cysteine in IVM media on sheep embryo development are still unknown.

This study included two experiments. Experiment 1 evaluated the effect of adding BME and cysteine to the freezing extender on the post-thawing viability and oxidative activity of ram sperm. Experiment 2 evaluated the effects of adding both BME and cysteine to the IVM medium on the development and viability of vitrified sheep embryos.

MATERIAL AND METHODS

In both experiments, all analyses were conducted by the same trained technician and all chemicals were obtained from Sigma-Aldrich Chemical Company (Saint Louis, MO, USA), unless stated differently. All experimental procedures were approved by UFPel's Ethics for Animal Experimentation Committee (protocol number 5541).

In Experiment 1, ejaculates were collected twice weekly using an artificial vagina from four rams of the Crioula Lanada breed (23 ejaculates per ram). The rams were 4-5 years old and kept under semi-extensive conditions at the Universidade Federal de Pelotas (31° 46′ 3″ South, 52° 26′ 55″ West). Only ejaculates presenting sperm motility of 70% or greater and a score of sperm vigor equal or greater than three (in a scale from 1-5) were further processed. After determining spermatozoa concentration in a Neubauer chamber, ejaculates were pooled into a single sample containing 1 x 10^9 spermatozoa, with equal number of viable spermatozoa (2.5 x 10^8) for each ram. The pooled sample was diluted in Tris-egg yolk-glycerol (Evans and Maxwell, 1987) and split in six treatments including: no antioxidants (control); 2mM BME; 5mM BME; 2mM BME and 5mM cysteine; 5mM BME and 5mM cysteine; and 5mM cysteine.

Sperm samples were stored in 0.25mL straws containing 100 x 10^6 spermatozoa and frozen using a programmable freezer (TK3000®, Tetakon, Nutricell, Campinas-SP, Brazil), following a curve of 0.25°C per min, until reaching 5°C. After stabilization for 2h, samples were cooled at 0.5°C per min until -120°C and then immersed in liquid nitrogen. Subsequently, samples were thawed in a water bath at 37°C during 20s.

All parameters of sperm quality were evaluated before freezing and after thawing. Sperm motility was also evaluated after one and two h post-thawing, by optical microscopy at 200X. The integrity of the sperm membrane and the acrosome and the mitochondrial functionality were evaluated using an epifluorescent microscope (Eclipse 80i, Nikon®, Melville, NY, USA) at 400X, with 450–520nm filter wave length. In all analyses, 200 spermatozoa were counted per slide. After exposure to carboxyfluorescein diacetate and propidium iodide, spermatozoa with intact membrane presented green fluorescence, but fluorescence for those with damaged membrane was either red or simultaneously red and green (Harrison and Vickers, 1990). Acrosome integrity was evaluated using *Arachis hypogaea* FITC-PNA conjugate, considering that spermatozoa with intact acrosomes presented red fluorescence, normal conformation and acrosome with green fluorescence, whereas those without integer acrosome also presented red fluorescence, but their conformation was abnormal and their acrosome was not evident (Jiménez *et al*., 2003). Mitochondrial functionality was evaluated using rhodamine 123 solution: green fluorescence indicated functional mitochondria; no fluorescence indicated nonfunctional mitochondria (Evenson *et al*., 1982).

The ROS were evaluated through the cinetic test (Myhre and Fonnum, 2001). After thawing, sperm samples were placed in 1.5mL tubes and centrifuged twice at 800G for 10min. After discharge of the supernatant, the resulting pellet was resuspended in 1mL PBS without Ca and Mg and a 400µl aliquot was frozen for further evaluation of the total antioxidant capacity (TAC) against peroxide radicals. Thereafter, 600µL of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), a general oxidative stress fluorescent marker for ROS, were added to the solution (40µM in PBS). After a second centrifugation, the pellet was resuspended and homogenized for 5s in vortex, remaining incubated with the marker for 30min at 36°C. The sample was centrifuged again and washed with 600µl PBS to remove the excess of H_2DCF-DA. Then, 160µL of the sample were placed in 96-well plates (Corning® 3912) in triplicate, for evaluation of ROS, using a fluoremeter (Victor 2D, Perkin Elmer®, Santa Clara, CA, USA), in 12 cycles with 4-min intervals, with agitation for 2s before and after each cycle, at 36°C. The ROS production was determined by the integration of the area of the fluorescence time curve, expressed per fluorescence unit x 10^7.

The TAC was generated by the thermal decomposition at 36°C of the 2,2'–azobis (2-metilpropionamidine) dihydrochloride (ABAP) (Amado *et al*., 2009). A 400µL aliquot previously stored at -18ºC, for at most 15d, was thawed at room temperature, sonicated for 5s and centrifuged at 800G for 10min. After discharging the resulting pellet, 125µL PBS and 10µL of each sample were added to 4 wells in a 96-well plate (Corning® 3912). In that plate, 7.5µL of Milli-Q water were added to two wells and 7.5µL of the ABAP solution (20µM) were added to the other two, both in duplicate. Then, 10µL of the H_2DCF-DA marker (16µM) were added to all wells. The evaluation was conducted in fluoremeter Victor 2D, Perkin Elmer®, Santa Clara, CA, USA), during 30min with 5min intervals at 36ºC, with 488-529 wave length. The TAC was obtained by dividing the ROS areas with and without ABAP by the ROS area without ABAP (1/relative area).

In Experiment 2, ovaries collected from pubertal sheep at a local slaughterhouse were transported to the laboratory in a saline/gentamicin solution (40mg/ml) at 30°C. The COC were aspirated from 3-6mm follicles with an 18G needle attached to a 10mL syringe. The COC selected for IVM presented homogenous cytoplasm and compact *cumulus oophurus* cells and were cultured for IVM in TCM 199 containing: 20% inactivated serum of sheep in estrous; 100UI/mL penicillin; 100µg/mL streptomycin; 5µg/mL FSH; 5µg/mL LH; 5µg/mL FSH; and 1µg/mL estradiol. Two media were tested, including: 50µM BME and 600µM cysteine (n = 729); or no antioxidants (n = 676).

The IVM was conducted in four-well plates containing 400µl drops covered with mineral oil, during 24h, in an incubator at 39ºC, with 5% CO_2 and saturated humidity. The COC were inseminated with fresh sperm collected from the same rams as described in Experiment 1, including 1 x 10^9 spermatozoa/mL extended (v/v) in Tris-egg yolk (Evans and Maxwell, 1987). Spermatozoa were selected through swim up, after incubating a 200µL sperm aliquot for 2min in a conic tube containing 2mL of Tris-egg yolk supplemented with 0.06g/mL BSA and 0.022g/mL pyruvate. Then, 500µL of the supernatant was diluted in 500µL of SOF (Holm *et al*., 1991) with no amino acids and supplemented with 2% of inactivated serum of sheep in estrous, 50µg/mL streptomycin and 50UI/mL penicillin. That content was centrifuged at 200G for 5min. The potential zygotes were denuded and cultured for 8d in SOF (Holm *et al*., 1991) supplemented with 0.4% BSA at 39°C, in a bag system with atmosphere including 5% CO_2, 90% N_2 and 5% O_2 (Vajta *et al.*, 1997).

Parthenogenetic activation was done by placing, on average, 15 oocytes in wells containing the IVM medium with no spermatozoa. Cleavage rates were determined on the second day after insemination (D2) and blastocyst development rates were determined on D7. The embryos were vitrified in open pulled straws (Vajta *et al*., 1998). Post-thawing embryo viability was evaluated by re-expansion and hatching rates.

In Experiment 1, the integrity of the sperm membrane and acrosome after thawing were compared across treatments by analyses of variance, with comparisons of means by the Tukey test. The other responses were compared by Kruskal-Wallis analysis of variance for non-parametric data, due to lack of normality. Sperm motility was also compared between two post-thawing periods (1 and 2h). In Experiment 2, rates of cleavage, development to blastocyst, re-expansion and hatching were compared across treatments by qui-square tests. All statistical analyses were conducted with Statistix® (2008).

RESULTS

In Experiment 1, before freezing, sperm motility was 80.2 ± 9.0%, membrane integrity was 74.0 ± 18.3%, acrosome integrity was 51.6 ± 17.5% and mitochondrial functionality was 77.9 ± 15.4%. No differences were observed among treatments ($P > 0.05$) for any of the evaluated parameters of post-thawing sperm quality (Tab. 1). Sperm motility after thawing was similar in the two evaluated periods ($P>0.05$): 28.2 ± 0.6% after 1h; and 19.6 ± 0.7% after 2h. The production of ROS and the TAC against peroxide radicals after thawing did not differ ($P>0.05$) among treatments (Tab. 2).

In Experiment 2 (Tab. 3), similar cleavage rates ($P>0.05$) were observed for the control and for the medium including BME and cysteine (60.3% and 64.3%, respectively).

Blastocyst development rates did not differ ($P>0.05$) between treatments. After both 24 and 48h, post-thawing embryo viability was similar between treatments ($P>0.05$).

Table 1. Post-thawing motility, membrane integrity, acrosome integrity and mitochondrial activity for ram sperm frozen in extenders including β-mercaptoethanol (BME) and cysteine (Experiment 1)

Extenders	Motility (%)	Membrane integrity (%)	Acrosome integrity (%)	Mitochondrial activity (%)
Control	31.3±1.6	42.3±3.7	40.9±1.9	64.5±3.0
2mM BME	25.6±1.4	40.4±3.8	35.9±2.8	58.0±2.9
5mM BME	25.9±1.4	42.1±4.8	38.8±2.2	61.9±2.8
5mM cysteine	23.9±1.6	38.9±4.0	44.1±1.7	62.0±3.4
2mM BME + 5 mM cysteine	24.5±1.6	45.1±3.5	40.8±3.3	55.3±3.6
5mM BME + 5 mM cysteine	26.0±1.6	42.7±2.9	45.6±2.0	56.6±3.8

Means ± SEM did not differ ($P > 0.05$).

Table 2. Reactive oxygen species (ROS) and total antioxidant capacity against peroxide radicals (TAC) after thawing for ram sperm frozen in extenders including β-mercaptoethanol (BME) and cysteine (Experiment 1)

Extenders	ROS (Field fluorescence*10^7)	TAC (1/relative area)
Control	1.3±0.1(1.1)	82.2±7.7(71.7)
2mM BME	1.1±0.1(1.1)	96.2±10.5(88.4)
5mM BME	1.1±0.1(0.9)	113.9±35.5(71.2)
5mM cysteine	1.2±0.1(1.1)	82.2±5.7(75.0)
2mM BME + 5 mM cysteine	1.1±0.5(1.0)	75.3±7.6(71.7)
5mM BME + 5 mM cysteine	1.0±0.1(0.9)	108.6±14.3(96.1)

Means ± SEM did not differ (P > 0.05); value in parentheses is the median.

Table 3. Cleavage, development to the blastocyst stage (D7) and post-thawing viability of vitrified sheep embryos in a medium including 50μM β-mercaptoethanol (BME) and 600μM cysteine (Experiment 2)

Media	Cleavage (%)	Blastocyst development (%)	Post-thawing viability (%)	
			Re-expansion (24 h)	Hatching (48 h)
Control	434/720 (60.3)	146/434 (33.6)	10/13 (76.9)	4/13 (30.8)
BME and cysteine	435/676 (64.3)	161/435 (36.6)	13/24 (54.2)	7/24 (29.2)

Rates did not differ across treatments (P < 0.05).

DISCUSSION

This was the first study to evaluate potential post-thawing antioxidant effects of BME and cysteine considering ram sperm quality and viability of vitrified sheep embryos produced *in vitro* as responses altogether. Acceptable rates of embryo development, re-expansion and hatching were obtained in Experiment 2, with 50μM BME associated to 600μM cysteine. Thus, the tested combination of antioxidants did not impair the development of sheep embryos, contradicting previous reports for cattle embryos (Matos *et al*., 2002). However, even though BME facilitates cysteine's absorption through the transport of cystine, improving the synthesis of GSH (Ishii *et al*., 1981), the tested antioxidants did not benefit sheep embryo development. Supplementing IVM medium with increased BME concentrations to improve embryo development may be questionable, since BME may lead to toxicity at concentrations greater than 50μM, as observed for bovine embryos (Mori *et al*., 2006). Our results suggest that supplementation of IVM medium with BME may be feasible when associated with cysteine, since no apparent toxicity was observed at the tested concentrations, but further research is still necessary to investigate concentrations distinct from those tested in the present study and the role of such association of antioxidants during *in vitro* embryo maturation and culture, separately.

In Experiment 1, BME and cysteine were added to the extender before freezing, in opposition to studies with frozen sperm of bulls (Bilodeau *et al*., 2001), boars (Yamaguchi and Funahashi, 2012) and rams (Salem *et al*., 1988; Bucak *et al.*, 2008), in which antioxidants were added to extenders after thawing. The inclusion of antioxidants prior to freezing was aimed to minimize the production of superoxide radicals that commonly occur during all steps of sperm cryopreservation (Chatterjee and Gagnon, 2001). However, the antioxidants may have been consumed before thawing, due to their prolonged contact with spermatozoa during cooling, freezing and thawing, which may explain their lack of effect on ROS production and on TAC. On the other hand, the prolonged exposure to antioxidants did not harm post-thawing sperm viability, indicating that BME and cysteine

apparently were not cytotoxic at the tested concentrations. It is possible that spermatozoa are incapable of metabolizing those antioxidants as other cells do, in which case BME and cystein might react forming a mixed disulfide cysteine that shuts the cysteine molecule down inside the spermatozoa, returning it to its reduced form (Ishii *et al*., 1981). In such form, cysteine would serve as a substrate for GSH synthesis (Matos *et al*., 1997; Bai *et al*., 2008). Although supplementation of extenders with GSH itself and other antioxidants can protect spermatozoa against lipid peroxidation, their benefits for post-thawing viability are controversial not only for ram sperm (Bucak *et al*., 2008; Câmara e*t al*., 2011), but also for the sperm of bulls (Bilodeau *et al*., 2001) and bucks (Sinha *et al*., 1996). Cryotolerance of boar sperm can be improved with supplementation of freezing extenders with GSH, although such effect may be influenced by the individual freezing ability of specific ejaculates (Yeste *et al*., 2014). Trials previously conducted at our laboratory indicated that BME was detrimental for post-thawing sperm quality at concentrations greater than 5 mM, likely due to toxicity (unpublished data). Also, BME did not benefit ram sperm motility 2 h after thawing, contradicting findings reported for bull sperm (Bilodeau *et al*., 2001). A potential cryoprotectant effect of cysteine at the same tested concentration, as reported elsewhere (Bucak *et al*., 2008), was not confirmed by our results. Future studies may test distinct periods for inclusion of antioxidants in freezing extenders.

CONCLUSIONS

Addition of β-mercaptoethanol (BME) and cysteine to the freezing extender, alone or associated, did not influence the post-thawing quality and oxidative activity of ram sperm. Embryo development was unaffected by inclusion of 50 μM BME and 600 μM cysteine in the *in vitro* maturation medium. The tested antioxidants neither impaired nor improved the viability of vitrified sheep embryos produced *in vitro*.

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