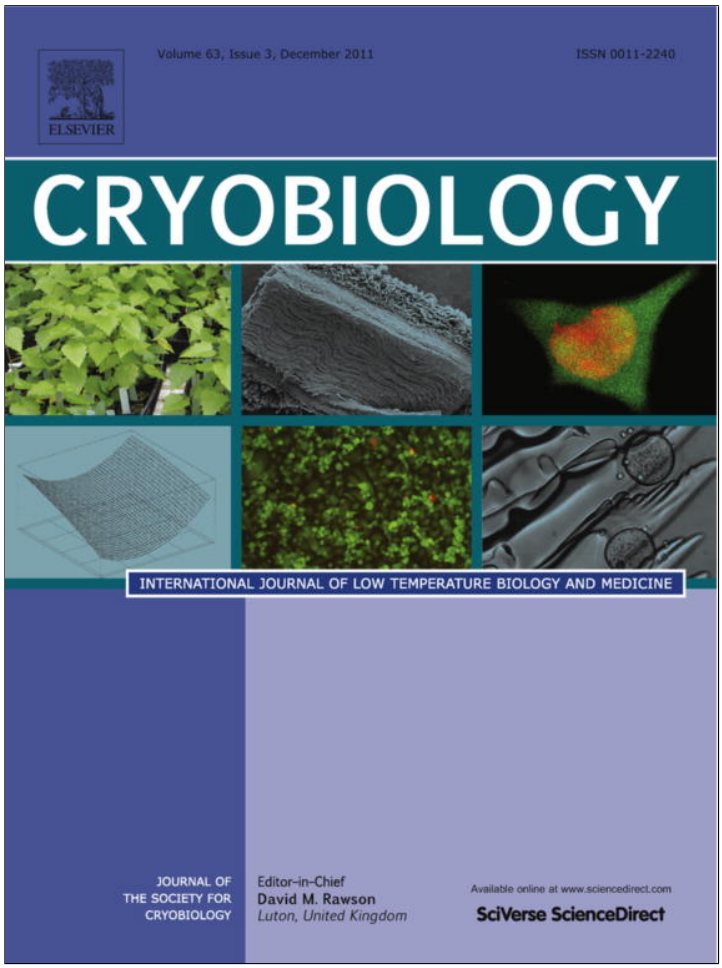

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Effects of dithioerythritol on ram semen after the freeze–thawing process ☆

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ABSTRACT

The aim of this study was to evaluate the effects of dithioerythritol added to cryopreservation extender on the post-thawed sperm parameters, lipid peroxidation and antioxidant activities of Merino ram sperm. Semen samples from 5 mature Merino rams (1 and 2 years of age) were used in the study. Semen samples, which were diluted with a Tris-based extender containing 0.5, 1, and 2 mM dithiothreitol and no antioxidant (control), were cooled to 5 °C and frozen in 0.25 ml French straws. Frozen straws were then thawed individually at 37 °C for 20 s in a water bath for evaluation.

The addition of dithioerythritol at 0.5 and 2 mM doses led to higher percentages of subjective motility (62.9 ± 4.2% and 63.6 ± 1.8%) compared to control (52.0 ± 4.9%, $P < 0.05$). As regards CASA motility, dithioerythritol 0.25 and 2 mM (60.2 ± 4.5% and 59.6 ± 1.2%) groups were higher from that of control (44.2 ± 8.7%, $P < 0.05$). For the CASA progressive motility, 0.25, 0.5 and 2 mM doses of dithioerythritol (22.0 ± 2.1%, 21.7 ± 2.5% and 24.0 ± 1.2%) had increasing effect in comparison to control (15.0 ± 2.5%). Dithioerythritol at 1 and 2 mM doses for ALH provided higher values compared to the control ($P < 0.001$) following the freeze–thawing process. Supplementation with dithiothreitol did not significantly affect the integrities of sperm membrane and acrosome, and mitochondrial activities. No significant differences were observed in biochemical parameters among the groups ($P > 0.05$). Findings of this study showed that dithioerythritol supplementation in semen extenders, was of greater benefit to sperm motility of frozen–thawed ram sperm.

Introduction

Cryopreservation of ram semen has been routinely applied as a valid tool of the small ruminant industry, particularly in conjunction with the dissemination of genetic material and the banking of genome resources to preserve superior breed lines [18]. However, cryopreservation also induces sublethal damage to the spermatozoa, which may result in loss of in vitro sperm functions and in vivo fertilizing capacity. The damage taking place during cryopreservation has been attributed to the cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid–protein reorganizations within the cell membranes [6,26,31,35].

Mammalian spermatozoon contains a high concentration of polyunsaturated fatty acids linked to phospholipids, asymmetrically distributed over the lipid bilayer of the plasma membrane [3,5,19]. It is well known that ram spermatozoa are more sensitive to cold shock than those of other species such as the bull, rabbit and human [33]. The different responses to temperature stress are mainly due to differences in membrane lipid composition. Thus, ram sperm have a higher polyunsaturated/saturated fatty acids ratio and a lower cholesterol/phospholipid molar ratio than other species [18]. This can account for membrane disarrangement, making the sperm more susceptible to peroxidative damage in the presence of reactive oxygen species (ROS, e.g. superoxide anion radical, hydrogen peroxide) with subsequent loss of cell functions of sperm for the fertilization process [4,7,36].

Mammalian semen possess an antioxidant system comprising reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) to prevent oxidative damage. The protective antioxidant systems in spermatozoa are primarily of cytoplasmic origin. Spermatozoa discard most of their cytoplasm during the terminal stages of differentiation, thus lack the significant cytoplasmic component containing antioxidants that counteract the damaging effects of ROS and lipid peroxidation (LPO). Correspondingly, spermatozoa are susceptible to LPO during cryopreservation and thawing, leading to subsequent sperm dysfunctions. Additionally, the concentration of anti-oxidants may decrease considerably by the dilution of the semen. Thus, mammalian sperm may be incapable in preventing LPO during the freeze–thawing process [4,7,8,24,30].

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In recent studies, the supplementation of a freezing extender with antioxidants has been shown to provide a cryoprotective effect on bull [10], ram [11], goat [12], canine [27] and human [7] sperm quality parameters. Dithiothreitol, an antioxidant, is known as a protamine disulfide bond reducing agent [23,33]. It prevents the oxidation of sulfhydryl groups, but also has a mucolytic effect on mucoprotein disulfide bonds, which might possibly damage the frozen membranes [29]. It was stated that dithiothreitol provided a protective effect against toxin-induced apoptosis and oxidative damage in lymphocytes [17]. The addition of dithiothreitol seemed to improve bull and human sperm motility during liquid storage or in the frozen state [9,15,23].

To our knowledge, the role of dithioerythritol in the cryopreservation extender, against cryodamage of ram sperm, have not been investigated. Therefore, the novelty of this study was to determine the influence of dithioerythritol, added to the Tris extender, on Merino ram sperm (motility, integrities of membrane and acrosome, and mitochondrial activity) and biochemical parameters (LPO level and activities of SOD, GPx and CAT) following freeze–thawing process.

Materials and methods

Animals and semen collection

Semen samples from 5 mature Merino rams (1 and 2 years of age) were used in the study. The rams, belonging to the University of Selcuk, Faculty of Veterinary Medicine, Experimental and Education Farm, were maintained under uniform feeding, housing and lighting conditions. A total number of 50 ejaculates were collected twice a week from the rams using an artificial vagina, during the breeding season (autumn to early winter) and then the semen were pooled to minimize individual variation. Ejaculates which met the following criteria were evaluated: volume of 0.5–2 ml; minimum sperm concentration of 3×10^9 sperm/ml; motility of 80%. Seven pooled ejaculates were included in the study.

Semen extending, freezing and thawing

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals, sperm concentration was measured using a haemocytometer while sperm motility was estimated using phase-contrast microscopy (100× magnification). A Tris-based extender (Tris 297.58 mM, citric acid 96.32 mM, fructose 82.66 mM, egg yolk 15% (v/v), glycerol 5% (v/v), pH 6.8) was used as the base extender (freezing extender). Each mixed ejaculate was split into seven equal aliquots and diluted at 37 °C with the base extender containing dithioerythritol at 0.25, 0.5, 1, 2 mM, and no antioxidant (control), with a final concentration of approximately 4×10^8 spermatozoa/ml (in a single step), in a 15 ml-plastic centrifuge tube. Diluted semen samples were aspirated into 0.25 ml French straws, sealed with polyvinyl alcohol powder and equilibrated at 5 °C for 3 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen, for 15 min and plunged into liquid nitrogen for storage. After storage for 1 month, the frozen straws were thawed individually at 37 °C for 20 s in a water bath for microscopic evaluation.

Evaluation of microscopic sperm parameters

Analysis of subjective and CASA motilities

Subjective motility was assessed using a phase-contrast microscope (100× magnification), with a warm stage maintained at 37 °C. A wet mount was made using a 5-μl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score. Besides estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyze sperm motion characteristics. CASA was set up as follows: phase contrast; frame rate – 60 Hz; minimum contrast – 70; low and high static size gates – 0.6 to 4.32; low and high intensity gates – 0.20 to 1.92; low and high elongation gates 7–91; default cell size – 10 pixels; default cell intensity – 80. Thawed semen was diluted (5 μl semen + 95 μl extender) in a Tris-based extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4-μl sample of diluted semen was put onto a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and sperm motility and motion characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s) and ALH (amplitude of lateral head displacement, μm). For each evaluation, 10 microscopic fields were analyzed to include at least 300 cells.

Assessment of sperm plasma membrane integrity

This assessment was performed by staining with the Sperm Viability Kit (SYBR-14/PI Molecular Probe: L 7011 Invitrogen). This staining protocol was modified from Garner and Johnson [21]. A working solution of SYBR-14 was diluted 1:10 with DMSO (Applichem A3006), then divided into equal aliquots (30 μl) after filtering with 0.22 μm Millipore Millex GV filter, and stored at −20 °C. The propidium iodide (PI) was dissolved in distilled water at 2 mg/ml, dividing into equal aliquots (30 μl) after filtering with 0.22 μm Millipore Millex GV filter, and storing at −20 °C. The thawed straw was diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 30 μl of diluted semen was mixed with 6 μl of SYBR-14 and 2.5 μl of PI. The sample was gently mixed, being incubated at 37 °C in the dark for 20 min., then added 10 μl of Hancock solution for stopping the sperm movement. A wet mount was made using a 2.5-μl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 spermatozoa were examined at 400× magnification under a fluorescence microscope (Leica DM 3000 Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany; excitation at 450–490 nm, emission at 520 nm) to assess the sperm membrane integrity. Sperm displaying green–red or red colorization was considered as membrane damaged, while displaying green colorization was considered to be intact membrane.

Assessment of sperm acrosome integrity

Sperm acrosome status was assessed by fluorescein isothiocyanate conjugated to Arachis hypogaea (peanut) (L7381 FITC-PNA, Invitrogen) and by PI staining as described by Nagy et al. [28], with modifications. About 120 μg of FITC-PNA was added to 1 ml of PBS (15630056, Invitrogen) for staining solution, then divided into equal aliquots (100 μl) after filtering and stored at −20 °C. The thawed straw was diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 60 μl of diluted semen was mixed with 10 μl of FITC-PNA and 2.5 μl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min., then was added 10 μl of Hancock solution for stopping the sperm movement. A wet mount was made using a 2.5-μl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400× magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission

at 520 nm) to assess the sperm acrosome integrity. Spermatozoa displaying bright green or patchy green fluorescence were considered as acrosome non-intact or damaged, whereas cells which did not stain green fluorescence in acrosome cap were regarded as acrosome intact.

Assessment of sperm mitochondrial activity

Sperm mitochondrial activity was assessed using staining protocol with the JC-1/PI modified from Garner et al. [22]. A stock solution of 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide (T3168 JC-1, Invitrogen, 1.53 mM) was prepared in DMSO solution and then divided into equal aliquots (100 μl) after filtering, and stored at −20 °C. The thawed straw was diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 300 μl of diluted semen was mixed with 2.5 μl of JC-1 and 2.5 μl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min., then was added 10 μl of Hancock solution to stop the sperm movement. A wet mount was made using a 2.5-μl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400× magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm) to assess the activity. A high level of yellow/orange fluorescence associated with the sperm midpiece (where mitochondria located) indicated high mitochondrial activity. Mitochondria showing low mitochondrial activity was stained green.

Biochemical assays

Briefly, diluted semen samples were centrifuged at 800*g* for 20 min at 4 °C in order to separate the cells from the diluted seminal plasma, and spermatozoa were washed twice with PBS at 800*g* for 20 min. After centrifugation, supernatant was discharged and pellet was completed to 500 μl with PBS. Then the sperm suspension was transferred into a 2-ml beaker in ice water and sonicated with a probe (Bandelin Sonopuls, Bandelın Electronic Heinrichstr-aBe, D-12207, Gerate-Typ UW 2070, Pro-Nr. 51900037369.004, Berlin) for 10 s on ice repeated six times at intervals of 30 s. This treatment rendered all the spermatozoa had their tails destroyed. For LPO analysis, 10 μl, 0,5 mM butyl-hydroxitoluen (B1378 BHT, Sigma–Aldrich Co., St. Louis, USA) was added into 120 μl homogenate samples and stored at −86 °C until analysis. The remaining homogenate was centrifuged at 8000*g* for 15 min at +4 °C and supernatant was collected and stored at −86 °C for GPx, CAT and SOD analysis.

Determination of lipid peroxidation levels

LPO levels were determined using commercial kits by LPO-586™ Oxis Research (Bioxytech, CA, USA) by spectrophotometry (UV 2100 UV–VIS Recording Spectrophotometer Shimadzu, Japan). The assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole with MDA and 4-hydroxyalkenals (LPO) at 45 °C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of *N*-methyl-2-phenylindole in acetonitrile, to yield a stable chromophore with maximal absorbance at 586 nm. The results are expressed as μmol for 10^9 cells/ml.

Determination of superoxide dismutase activity

SOD activity was determined using commercial kit by Oxiselect (Cell Biolabs, Inc., San Diego, CA) by spectrophotometry. Superoxide anions generated by xantin/xantin oxidase system is determined by a chromogen solution. Chromogen is reduced with superoxide anion. This reduction is inhibited by SOD. SOD activity is inversely proportional with decreasing absorbance at 490 nm. The results are expressed as U/ml for 10^9 cells/ml.

Determination of glutathione peroxidase activity

GPx activity was determined using commercial kit by GPx-340™ Oxis Research (Bioxytech, CA, USA) by spectrophotometry (UV 2100 UV–VIS Recording Spectrophotometer Shimadzu, Japan). Organic peroxides produced via GPx, are reduced by GSH-reductase. During this reaction, the conversion of NADPH into $NADP^+$ leads to decrease in absorbance at 340 nm. GPx activity is inversely proportional with decreasing absorbance at 340 nm. The results are expressed as mU/ml for 10^9 cells/ml.

Determination of catalase activity

CAT activity was determined using commercial kits by CAT-520™ Oxis Research (Bioxytech, CA, USA) by spectrophotometry. Conversion of hydrogen peroxide into molecular oxygen and water is directly proportional with CAT activity. Samples reacting with a known concentration of hydrogen peroxide, are incubated 1 min, and the reaction is stopped by sodium azide. Remaining hydrogen peroxide is determined by its reaction with 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulfonic acid. CAT activity is inversely proportional with decreasing absorbance at 520 nm. The results are expressed as mU/ml for 10^9 cells/ml.

Statistical analysis

The study was repeated seven times. The results were expressed as mean ± SEM. Means were analyzed using a one-way analysis of variance, followed by Duncan's post hoc test to determine significant differences in all the parameters between all groups using the SPSS/PC computer program (version 12.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered to be statistically significant.

Results

The addition of dithioerythritol at 0.5 and 2 mM doses led to higher percentages of subjective motility (62.9 ± 4.2% and 63.6 ± 1.8%) compared to control (52.0 ± 4.9%, $P < 0.05$). As regards CASA motility, dithioerythritol 0.25 and 2 mM (60.2 ± 4.5% and 59.6 ± 1.2%) groups were higher from that of control (44.2 ± 8.7%, $P < 0.05$). For the CASA progressive motility, 0.25, 0.5 and 2 mM doses of dithioerythritol (22.0 ± 2.1%, 21.7 ± 2.5% and 24.0 ± 1.2%) had increasing effect in comparison to the control group (15.0 ± 2.5%). Dithioerythritol at 1 and 2 mM doses for ALH provided higher values compared to control ($P < 0.001$) following the freeze–thawing process (Table 1). Supplementation with dithioerythritol did not significantly affect the integrities of sperm membrane and acrosome, and mitochondrial activities (Table 2). No significant differences were observed in biochemical parameters among the groups (Table 3, $P > 0.05$).

Discussion

Reporting the useful effects of dithiothreitol on post-thawed bull semen [23], liquid storage human [9] sperm quality and pronuclear formation and embryonic development following intracytoplasmic sperm injection of boar [33] has given rise to use of dithioerythritol (showing a similar property of dithiothreitol) as an antioxidant. For this study, a hypothesis has also been established that dithioerythritol can show the cryoprotective effects when added to the ram freezing extender.

Table 1
Mean (±S.E.) sperm motility parameters in Merino ram semen supplemented with dithioerythritol following freeze–thawing.

Groups	Subjective motility (%)	CASA motility (%)	Progressive motility %	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)
Control	52.0 ± 4.9 bc	44.2 ± 8.7 b	15.0 ± 2.5 b	114.9 ± 1.8	104.3 ± 2.2 a	184.8 ± 3.3	6.4 ± 0.2 c
Dithioerythritol 0.25 mM	61.7 ± 2.8 ab	60.2 ± 4.5 a	22.0 ± 2.1 a	109.0 ± 3.2	98.0 ± 2.8 abc	191.7 ± 8.2	7.2 ± 0.3 bc
Dithioerythritol 0.5 mM	62.9 ± 4.2 a	51.0 ± 4.6 ab	21.7 ± 2.5 a	110.3 ± 6.3	99.5 ± 5.4 ab	192.3 ± 10.5	7.5 ± 0.2 b
Dithioerythritol 1 mM	49.3 ± 3.2 c	40.9 ± 3.3 b	11.3 ± 1.7 b	106.9 ± 1.2	87.7 ± 2.9 c	203.3 ± 5.2	9.2 ± 0.5 a
Dithioerythritol 2 mM	63.6 ± 1.8 a	59.6 ± 1.2 a	24.0 ± 1.2 a	108.7 ± 2.7	89.0 ± 2.2 bc	204.6 ± 6.3	9.1 ± 0.1 a
P	**	*	**	-	*	-	**

a, b, c: Different superscripts within the same column demonstrate significant differences.
* $P < 0.05$.
** $P < 0.001$.

Table 2
Mean (±S.E.) sperm membrane and acrosome integrities and mitochondrial activities in Merino ram semen supplemented with dithioerythritol following freeze–thawing.

Groups	SYBR-14/PI staining %	FITC/PI staining %	JC-1/PI staining %
Control	54.6 ± 3.4	33.7 ± 5.1	30.7 ± 5.5
Dithioerythritol 0.25 mM	54.9 ± 2.9	32.6 ± 7.8	40.3 ± 6.6
Dithioerythritol 0.5 mM	56.9 ± 1.7	31.0 ± 2.7	21.9 ± 8.7
Dithioerythritol 1 mM	53.8 ± 5.9	39.1 ± 4.9	31.8 ± 11.6
Dithioerythritol 2 mM	46.7 ± 3.4	46.9 ± 3.8	31.5 ± 3.7
P	-	-	-

- : No significant difference ($P > 0.05$).

Table 3
Mean (±S.E.) LPO levels, SOD, GPx and CAT activities in Merino ram semen supplemented with dithioerythritol following freeze–thawing.

Groups	LPO (μmol-10^9 cells/ml)	SOD activity (%inh-10^9 cells/ml)	GPx activity (mU/ml-10^9 cells/ml)	CAT activity (mU/ml-10^9 cells/ml)
Control	24.8 ± 2.7	1.7 ± 0.1	12.9 ± 2.6	220.0 ± 26.7
Dithioerythritol 0.25 mM	29.5 ± 3.0	1.7 ± 0.0	20.2 ± 2.2	233.7 ± 43.5
Dithioerythritol 0.5 mM	30.8 ± 3.2	1.7 ± 0.1	16.5 ± 5.0	201.9 ± 50.5
Dithioerythritol 1 mm	28.6 ± 2.5	1.7 ± 0.1	25.0 ± 9.3	201.7 ± 34.9
Dithioerythritol 2 mM	33.8 ± 5.4	1.8 ± 0.1	21.2 ± 6.6	318.1 ± 43.4
P	-	-	-	-

- : No significant difference ($P > 0.05$).

Cryopreservation of spermatozoa including the decrease in temperature, enhances oxidative stress due to ice crystallization and LPO, and cold shock which irreversibly lead to damage to the sperm organelles, associated with a reduction in sperm functions [1,16,25,34]. The supplementation of freezing extender with antioxidants may counteract cryodamage to sperm parameters and oxidative stress [13,32]. This study was undertaken to ascertain whether dithioerythritol would provide the most effective protection against sperm cryopreservation damage. Mammalian spermatozoa are highly sensitive to LPO, which occurs as a result of the oxidation of membrane lipids by partially reduced oxygen molecules, e.g. superoxide, hydrogen peroxide and hydroxyl radicals. Spontaneous peroxidation of the membranes of mammalian spermatozoa destroys the structure of the lipid matrix, due to the attacks of ROS, formed through the univalent reduction of oxygen, during cryopreservation. The damage ultimately leads to loss of membrane integrity, membrane deterioration, decreased sperm motility, loss in fertility, leakage of intracellular enzymes and damage of the sperm DNA, through the oxidative stress and the production of cytotoxic aldehydes [1,2,7]. In this study, LPO releasing could not be prevented when the samples were cryopreserved with dithioerythritol. As related to the antioxidant activities, dithioerythritol did not elevate SOD, GPx and CAT activities after the freeze–thawing. The current findings relating to LPO and antioxidant activities are also in agreement with the results performed on the bull spermatozoa in which a decrease was not recorded in the level of LPO and mentioned activities in the presence of dithioerythritol for the frozen state [14]. We can state that oxidative stress parameters do not seem an influential factor on the sperm quality following freeze–thawing process.

The axonema and associated fibers of the middle pieces of sperm are covered by mitochondria that generate energy from intracellular stores of ATP which are responsible for sperm motility. Cryopreservation and large amounts of ROS can induce axonemal damage, resulting in the deterioration of sperm motility and morphological functional integrity as a result of ATP depletion. Sperm motility becomes crucial in facilitating the actual penetration of the cumulus cells and zona pellucida of the ovum, and eventually fertilization process [16,20]. In the present investigation, all doses of dithioerythritol led to higher motility percentages when compared to control groups, except for subjective and CASA motilities in 1 mM dithioerythritol. This result was contrast to that previously reported for frozen–thawed sperm of Holstein bulls, where dithioerythritol addition resulted in insignificant increases in subjective and CASA sperm motilities [14].

In conclusion, it is stated that, compared to the control groups, the supplementation of dithioerythritol in the extender did not eliminate LPO production and improve antioxidant activities during the cryopreservation process. Sperm motilities were increased by adding of dithioerythritol at 0.25, 0.5 and 2 mM. These indicate that LPO and antioxidant activities are apparently not a major factor influencing sperm motilities after the thawing of sperm. Further studies are required to obtain more information on the determination of LPO and antioxidant capacities, and to determine the most effective additives (e.g. antioxidants) in the freezing of ram semen. In the future, a success of ram semen freezing will contribute to the widespread use of AI, and increased performance of studies on improvement in small ruminant breeding.

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