Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender

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Abstract

The aim of this study was to evaluate the effects of ergothioneine and cysteamine as antioxidant supplements in a soybean lecithin extender for freezing ram semen. Twenty-four ejaculates were collected from four rams and diluted with extenders (1.5% soybean lecithin, 7% glycerol) containing no supplements (control) and cysteamine or ergothioneine (2, 4, 6 or 8 mM). Motility by CASA, viability, plasma membrane functionality (HOS test), total abnormality, lipid peroxidation, glutathione peroxidase (GPx) activity and capacitation status (CTC staining) were assessed after thawing. Using 6mM of either antioxidant improved total motility. Cysteamine at 6 mM and ergothioneine at 4 and 6 mM improved viability and reduced lipid peroxidation (malondialdehyde concentration). Both antioxidants improved membrane functionality significantly, except at 8 mM. Progressive motility, kinematic parameters, GPx activity, capacitation status and sperm abnormalities were not influenced by the antioxidant supplements. In conclusion, cysteamine at 6 mM and ergothioneine at 4 or 6 mM seem to improve the post-thawing quality of ram semen cryopreserved in a soybean lecithin extender.

Keywords: Ram semen, antioxidant, cysteamine, ergothioneine, cryopreservation

Introduction

The cryopreservation of spermatozoa has allowed the conservation of genetic resources in sperm banks, the guarantee of a constant commercial supply of semen, and the development of breed improvement programs by means of the artificial insemination (AI) technique [9, 25, 30].

However, the use of AI in small ruminants faces important challenges. Application of semen by AI is less efficient because of the female size. Therefore, sperm quality, which is negatively affected by cryopreservation [55], has a critical impact. There are important differences in terms of sperm quality and fertilizing ability between fresh and frozen–thawed semen due to cold shock, ice crystal formation, membrane alterations and oxidative stress [44, 54].

Membrane integrity and sperm structure can also be compromised by oxidative stress, resulting in lower sperm quality and fertilization rates [25, 26]. Damage of spermatozoa involves the oxidative attack on the methylene groups of unsaturated phospholipids, leading to lipid peroxidation (LPO) [31, 42]. Although semen possesses an antioxidant system, which includes glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and other antioxidants, their activity is affected by cryopreservation, which increases the intensity of LPO [13, 42]. Therefore, naturally occurring antioxidants may be insufficient to prevent LPO on sperm cells during the freezing–thawing process. Therefore, the addition of antioxidants to the extender may have positive effects [34, 43, 50, 56]. There is a great variety of antioxidant substances, including vitamins, enzymes and other free radical scavengers, and their mechanism of action, toxicity and effectiveness vary enormously. Moreover, the effect of antioxidants may change depending on species, medium and protocols [37], and in some cases their presence could be detrimental [38].

Cysteamine is a thiol compound that is known to be an efficient scavenger of the hydroxyl radical, and may contribute to the maintenance of the redox status in oocytes [29]. For instance, the addition of cysteamine to a maturation medium increased GSH synthesis in bovine oocytes [20]. In some reports, cysteamine improved the cryopreservation of frozen ram sperm [9], higher embryo development rates when added to the maturation medium of goat oocytes [49] and

higher rates of motility, lower rates of abnormal spermatozoa and decreased SOD activity on post-thaw Angora goat semen [11].

Ergothioneine, an important low-molecular-mass thiol, is present in millimolar concentrations in some tissues [35]. It scavenges singlet oxygen [18], hydroxyl radicals [3] and peroxyl radicals [6]. In some studies, it protected spermatozoa from oxidized and peroxidized chemicals from fructolysis [35], and improved the post-thaw motility [16] and DNA integrity [17] of frozen-thawed ram sperm.

To our knowledge, the use of cysteamine and ergothioneine, in a soy lecithin-based extender (SL) for cryopreservation of ram semen has not been reported, and the concentrations tested previously [9,16] are limited and possibly suboptimal. Therefore, this study was conducted in order to determine the influence of four concentrations in the 2-8 mM range of cysteamine and ergothioneine in ram spermatozoa frozen in a soy lecithin-based extender, in an attempt to improve post-thawing semen quality. Moreover, there is no information on the effect of these antioxidants in the capacitation status of frozen-thawed spermatozoa, and therefore we have included such an analysis in our experimental design.

Materials and methods

Chemicals

Unless otherwise specified, all chemicals used in this study were obtained from Sigma (St. Louis, MO, USA), and Merck (Darmstadt, Germany).

Semen collection, processing and extender preparation

Semen samples from 4 mature Mehraban rams (3 and 4 years of age), of superior genetic merit and proven fertility, were used in this study. The rams were maintained under uniform feeding, housing and lighting conditions. A total of 6 ejaculates were collected from each rams (two collections per week; total: 24 ejaculates) using artificial vagina, during the breeding season (autumn). In every session, the six ejaculates were pooled to minimize the individual variation. Ejaculates which fit the following criteria were used in the experiment: Volume of 0.75–2 mL, minimum sperm concentration of 3×10^9 sperm/mL, motility of 80% (subjectively assessed) and less than 10% abnormal sperm. Immediately following collection, the ejaculates were placed in a water bath (37 °C), until evaluation in the laboratory. Semen assessment was performed within approximately 10 min.

The basic extender used in this study was composed of 27.1 g/L Tris, 10 g/L fructose, and 14 g/L citric acid. Soybean lecithin was added to the basic extender at 1.5% (wt/vol) and glycerol at 7% (v/v). The osmolarity and pH of this base extender were set at 320 mOsm/kg and 7.2, respectively. The base extenders were supplemented with the antioxidant cysteamine (2, 4, 6 and 8 mM), or ergothioneine (2, 4, 6 and 8 mM). An aliquot did not receive antioxidants, being left as a control. Spermatozoa were extended to a final concentration of 4×10^8 mL^{-1}.

Extended samples were loaded into 0.25 mL French straws (IMV, L'Aigle, France) and equilibrated at 4 °C for 2 h. After equilibration, the straws were horizontally frozen in liquid nitrogen vapours (5 cm above liquid nitrogen) for 12 min, and then plunged into liquid nitrogen for storage. For sperm evaluation, straws were thawed individually at 37 °C for 30 s in a water bath. Sperm evaluation was performed on all semen samples immediately after thawing.

Evaluation of spermatozoa after thawing

Sperm Motility

A computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to evaluate sperm motility and motion parameters. Samples were diluted 1/10 in PBS (pH 7.4) right after analysis. The following variables were analyzed: total motility (MOT, %), progressive motility (PROG, %), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat/cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %). At least 200 spermatozoa were assessed in each CASA analysis.

Sperm viability

Viability of the sperm samples was assessed by means of a nigrosin–eosin staining [25]. The stain was prepared as eosin-Y 1.67 g, nigrosin 10 g, sodium citrate 2.9 g, dissolved in 100 mL of distilled water. Sperm suspension smears were prepared by mixing a drop of the semen sample with two drops of the stain on a warm slide, and immediately spreading the stain with a second slide. The viability was assessed by counting 200 cells under phase-contrast at ×1000 (CKX41, Olympus, Tokyo, Japan). Spermatozoa showing partial or complete purple stain were considered non-viable and only spermatozoa showing strict exclusion of the stain were considered to be alive.

Sperm abnormalities

For the evaluation of total abnormalities in the semen samples, at least three drops of semen were pipetted into 1.5 mL tubes, containing 1 mL Hancock's solution [51]. One drop of this mixture was placed on a microscope slide and covered with a coverslip. The percentage of sperm abnormalities was recorded by counting a total of 200 sperm under a phase-contrast microscope at ×1000 (CKX41, Olympus, Tokyo, Japan). Hancock's solution was prepared by mixing 62.5

mL formalin (37% formaldehyde), 150 mL of sodium saline solution, 150 mL of buffer solution and 500 mL of double-distilled water. Sodium saline solution: 9.01 g NaCl in 500 mL of double-distilled water, buffer solution: (1) 21.7 g $Na_2HPO_4 \times H_2O$ in 500 mL of double-distilled water, (2) 22.254 g KH_2PO_4 in 500 mL of double-distilled water, 100 mL of (1) and 50 mL of (2) were mixed to obtain 150 mL of buffer solution.

Functional membrane integrity

The evaluation of functional membrane integrity of sperm was performed by using the hypo-osmotic swelling test (HOST), considering positive those spermatozoa with curled or swollen tails [48]. In brief, 25 mL semen was added to 200 mL of hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 min incubation, the mixture was homogenized and evaluated under a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan). A total of 200 spermatozoa were counted in at least five different microscopic fields at×400. The percentage of spermatozoa with swollen and curved tails was recorded.

Malondialdehyde (MDA) concentration

Malondialdehyde concentrations, as an index LPO in the semen samples, were measured using the thiobarbituric-acid reaction [24]. Briefly, 1 mL of the diluted semen (250×10^6 spermatozoa/mL) was mixed with 1 mL of cold 20% (w/v) trichloroacetic acid to precipitate proteins. The precipitate was pelleted by centrifugation (960×g for 15 min), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 °C for 10 min. After cooling, the absorbance was determined by a spectrophotometer (UV-1200, Shimadzu, Japan) at 532 nm. All MDA concentrations were expressed as nmol/mL.

Glutathione peroxidase activity

The GSH-PX activity was determined according to the method proposed by [33]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 EU/mL glutathione reductase and 1 mM GSH. The semen sample (0.1 mL) was added to 0.8 mL of the reaction mixture, incubating at 25 °C for 5 min before the initiation of the reaction, induced by the addition of 0.1 mL of a H_2O_2 solution (final H_2O_2 concentration: 0.25 mM). The absorbance at 412 nm was recorded for 5 min using a spectrophotometer (UV-1200, Shimadzu, Japan). The activity was calculated from the slope of the regression line as micromoles of NADPH oxidized per minute. The blank value was subtracted from each value and the GSH-PX activity was expressed as international units (IU)/g protein for the sperm samples (Bradford method).

Capacitation and acrosome reaction status

Capacitation and acrosome status were evaluated by chlortetracycline (CTC) staining [28, 47]. Semen was first centrifuged (1000×g for 10 min). Three µL of semen were mixed with 20 µL CTC working solution (2.42 g Tris, 7.58 g NaCl, 0.0604 g cysteine and 0.0386 g chlortetracycline at 100 mL distilled water), 5 µL of fixing solution (4 mL of 25% glutaraldehyde in 96 mL of 1 M Tris buffer), and 5 µL of mounting solution (10 mL of 1 M Tris buffer, 90 mL glycerol and 2.46 g triethylenediamine). Slides were assessed under an epifluorescence microscope (BX51, Olympus, Tokyo, Japan). Two hundred spermatozoa were evaluated and assigned to one of the following categories: F-pattern (intact), when fluorescence was detected over the whole region of the sperm head, B-pattern (capacitated), when fluorescence was detected in the sperm head except in the post acrosomal region, and AR-pattern (acrosome reacted), with no head fluorescence except for a bright band in the equatorial segment.

Statistical analysis

Data were analyzed in the R statistical environment [53]. To analyze the effects of antioxidant supplementation on sperm parameters, we used linear mixed-effects models, with antioxidant and antioxidant concentration in the fixed part of the models and including the replicates as the grouping factor in the random part of the models. Results are presented as mean±SEM or effect sizes (estimate±SEM of the respective coefficients).

Results

Improvement of motility of frozen-thawed sperm exposed to cysteamine or ergothioneine

The influence of certain additives on the standard semen parameters of frozen ram semen was evaluated in nine independent experiments. As shown in Table 1, a freezing extender supplemented with 6 mM cysteamine and 6 mM ergothioneine led to significantly higher total motility percentages after thawing, in comparison with control levels ($P < 0.05$ and $P < 0.01$, respectively). However, the increase of cysteamine levels to 8 mM resulted in negative effects on motion parameters. Progressive motility and sperm kinetic parameters were not affected by any antioxidant supplementation.

Protective effects of cysteamine or ergothioneine on viability and membrane functionality of frozen-thawed sperm

The effects of different levels of cysteamine and ergothioneine on sperm viability, plasma membrane functionality and the percentage of abnormal spermatozoa are shown in table 2. Cysteamine at 6 mM and ergothioneine at 4 and 6 mM resulted in an increase in the percentage of viable spermatozoa comparing to the control ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively).

The percentage of spermatozoa positive to the HOS test (functional plasma membrane) improved significantly in 2, 4 ($P < 0.05$) and 6 mM ($P < 0.001$) cysteamine and 2, 4 ($P < 0.01$) and 6 mM ($P < 0.001$) ergothioneine respect to the control extender. Nevertheless, there were no significant effects in the percentage of abnormal spermatozoa comparing to the control (overall 21.1%±0.6). As noted previously, levels above 6 mM resulted in negative effects.

Reduction of lipid peroxidation in frozen-thawed sperm by cysteamine or ergothioneine

The results for lipid peroxidation level and the levels of GPx activity after cryopreservation in the different extenders are displayed in table 3. The analysis of treatment effects showed that only 6 mM of cysteamine ($P < 0.001$) and 4 and 6 mM of ergothioneine ($P < 0.01$ and $P < 0.001$, respectively) could reduce MDA production. GPx activity after thawing was not significantly affected by any of the supplements.

Influence of cysteamine or ergothioneine in the proportion of capacitated sperm after freezing and thawing

CTC staining (Table 4) showed that most spermatozoa presented the B pattern (capacitated, 60.4%±0.4), followed by the AR pattern (acrosome reacted, 24.1%±0.5), and the F pattern (uncapacitated, 15.5%±0.3). This pattern distribution was not affected by the supplementation with antioxidants.

Discussion

Cryopreservation produces oxidative stress on the sperm membrane, causing irreversible damage to the spermatozoon structure and changes in the membrane fluidity and enzymatic activity. These changes cause a reduction in sperm motility, viability and fertilizing ability [1, 2, 19]. This

study was performed to investigate if cysteamine and ergothioneine, as antioxidant supplements, could provide an effective protection against oxidative damage during the cryopreservation of ram sperm. There are several studies regarding the effects of these antioxidants on ram semen cryopreservation, but our study contributes by: Testing a range of concentrations in which, according to previous studies [9,16], we might find an optimal ergothioneine and cysteamine concentration; using these antioxidants in a soybean lecithin extender, comparing with the positive effects found in egg yolk based extenders; and confirming previous findings on the suitability of these antioxidants for supplementing ram semen extenders, considering their practical use. We have found that cysteamine and ergothioneine at 6 mM yielded an overall higher quality of the thawed samples, with 4 mM ergothioneine showing also significantly positive effects for some parameters. Çoyan et al. [16], studied the effect of adding different levels of ergothioneine (1, 2 and 4 mM) to a ram semen extender containing egg yolk, finding that increasing levels of ergothioneine led to positive effects on the total and progressive motility and several kinetic parameters. Ergothioneine has also been used for freezing stallion semen, with mixed results. Coutinho da Silva et al. [15], obtained no improvement of sperm motility, whereas Metcalf et al. [41], found that ergothioneine enhanced post-thaw semen quality in some instances. Bucak et al. [9], added cysteamine to a goat extender also containing egg yolk, reporting that cysteamine at 5 mM improved goat sperm motility post-thawing, but at 10 mM it decreased motility. These antioxidants might improve not only post-thawing quality, but also the resilience of spermatozoa during their preparation and after artificial insemination, within the female genital tract.

In contrast to our results, Cirit et al. [14] found no improvement of ram semen quality (obtained by electroejaculation) before or after cryopreservation in the presence of 2.5 or 5 mM

cysteamine. These authors also performed a thermal stress test (TST), finding that the addition of cysteamine to the freezing extender decreased the quality of thawed semen after the incubation. However, Cirit et al. [14] used semen obtained by electroejaculation and cysteamine up to 5 mM, whereas we obtained a significant improvement at 6 mM. These factors could explain why they did not obtain an improvement when adding cysteamine to the freezing extender. Moreover, cryoprotectants such as egg yolk and soy lecithin provide some antioxidant capacity [4], resulting in an apparent lack of effect of supplemented antioxidants. Our results support a positive effect of cysteamine on the cryopreservation of ram semen, encouraging the practical testing of this antioxidant.

Excessive ROS might cause ultrastructural changes in membranes and impair sperm function. If the antioxidant capacity of the extender is insufficient, excessive ROS could attack sperm membranes, which are very susceptible to oxidants [57]. The results of the present study demonstrate that supplementation with cysteamine and ergothioneine can efficiently improve the plasma membrane integrity (viability) and functionality (HOS test). This improvement on membrane integrity and functionality would result in a better protection of the sperm function, which we detected as an improvement of sperm motility. In agreement with our results, Bucak et al. [9] showed that adding 5 mM of cysteamine to a ram freezing extender yielded a higher percentage of viable spermatozoa after thawing, although they did not found any improvement of plasma membrane functional integrity. However, Çoyan et al. [16], found that 4 mM ergothioneine, despite the good motility results, reduced membrane integrity and mitochondrial activity. In these cases either the use of potentially suboptimal antioxidant concentrations or the interaction of the antioxidants with the egg yolk could explain these results. In contrast, we noted

an improvement at 6 mM in motility, viability and membrane functionality, while using an extender with lecithin.

The semen antioxidant system contains both enzymatic and non-enzymatic antioxidants preventing or restricting the formation and propagation of peroxides. Fewer amounts of antioxidants, or the inhibition of antioxidant enzymes, increases the risk of oxidative stress, damaging or killing spermatozoa [45]. One of the by-products of lipid peroxides decomposition is malondialdehyde, which is commonly used in biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa [46]. The results of the present study showed that cysteamine at 6% and ergothioneine at 4% and 6% reduced malondialdehyde levels, which might explain the improvement in membrane status and motility. In contrast, Çoyan et al. [16], reported that ergothioneine (1 to 4 mM) was unsuccessful reducing lipoperoxidation, had no effect on SOD and GPx activities and even decreased catalase activity. Similarly, Bucak et al. tested cysteamine at 5 and 10 mM in ram [9] and at 5 mM in goat [11], and only found an increase in vitamin E concentration when using this antioxidant at 5 mM in ram, with no effects in malondialdehyde and GSH concentrations, and GPx and catalase activities. In fact, cysteamine 5 mM reduced SOD activity in goat [11].

Likewise, GPx activity was not modified by any antioxidant treatment in our samples. Glutathione peroxidase plays a major role in maintaining the peroxidation balance, because it reverts lipid peroxidation, converting peroxides into non-reactive products [27]. Interestingly, Kasimanickam et al. [32], reported a negative correlation between GPx activity in spermatozoa from young rams and the percentage of normal spermatozoa with progressive movement. In addition, the group of animals with reduced semen quality had a greater activity of GPx, suggesting that the overexpression of this enzyme could be an unsuccessful attempt in order to

counteract excessive oxidative stress in the semen. In our case, GPx activity (overall, 25.9±0.5 IU/g protein) was higher than the value reported by Bucak et al. [9] in semen cryopreserved with egg yolk (control, 7.9±1.8 IU/g protein). Our values are in the same range and even higher than the results found for fresh semen [36]. Therefore, it is possible that the lack of improvement in GPx activity could be due to the good initial semen quality and the efficiency of our cryopreservation extender preserving this enzyme, and thus we cannot infer if the presence of supplemented antioxidants could benefit the intrinsic antioxidant system of semen. Bucak et al. [10] showed that adding GSSG or GSH to ram semen recovered GPx activity from control (7.00±1.78 UI/g protein, semen cryopreserved in extender with 10% egg yolk) to levels comparable to our study (GSH 5 mM: 22.02±1.27 and GSSG 5 mM: 20.17±3.38). Atessahin et al. [7] obtained similar results freezing goat semen with 9% egg yolk and using different concentrations of cysteine, taurine and trehalose, with control values of GPx of 6.56±0.17 IU/g protein, increasing to values up to 19.11±3.21 (trehalose 50 mM, higher average value reported).

Finally, we found no improvement in the CTC patterns by adding antioxidants. Many studies have reported that cryopreservation procedures induce capacitation-like changes in spermatozoa such as plasma membrane reorganization and fluidization, calcium influx and protein tyrosine phosphorylation [39, 52]. These changes are collectively termed as cryocapacitation. These capacitation-like changes are thought to be partly responsible for the reduced fertility of frozen–thawed semen [8, 23], and it would be desirable to reduce them. In our study we found that only a minority of spermatozoa presented a CTC pattern compatible with uncapacitated spermatozoa. Moreover, the antioxidant treatments failed in preventing the alterations in sperm physiology leading to cryocapacitation, contrarily to other antioxidants such as melatonin in ram [12] or

trehalose in buffalo [52]. Cryocapacitation is clearly a multifactorial event and could progress by multiple pathways, whereas antioxidants alone might not be able to prevent it.

Interestingly, the 8 mM concentrations did not yield any positive effects, reverting the quality parameters to values comparable to the control. This decrease broke the increasing trend from 2 to 6 mM in most variables, possibly due to the toxic activity of these compounds at concentrations at least equal or higher than 8 mM, which would counteract their positive effects. Antioxidants might have negative effects due to excessive scavenging of free radicals, possibly by altering their physiological levels. Other studies have found negative effects for several antioxidants in the sperm quality of small ruminants, despite of being highly efficient removing free radicals and preventing lipoperoxidation [37, 38]. Indeed, some antioxidants such as Trolox seem to be beneficial if applied after thawing [21, 22], but they could be detrimental if used in the freezing extender [5]. Whereas higher concentrations of cysteamine or ergothionine could be useful if added post-thawing or during liquid storage, it seems undesirable to use these concentrations during cryopreservation, at least when the extender contains lecithin.

Conclusion

In this study, we achieved a higher quality in post-thawed ram semen when the freezing extender was supplemented by 6 mM cysteamine or ergothionine. This concentration increased motility, viability and membrane functionality, while decreased lipid peroxidation. Taking into account previous studies using egg yolk as an extender supplement, it is possible that these antioxidants could interact positively with the soybean lecithin used in our study. Future studies should aim at confirming the usefulness of the supplementation with these antioxidants regarding field fertility.

Acknowledgements

Felipe Martínez-Pastor was supported by the Ramón y Cajal program (RYC-2008-02560, MICINN, Spain). The authors thank M. Mata-Campuzano and L. Ordás for their assistance correcting the manuscript.

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489 Table 1: Effect of cysteamine or ergothioneine on motile parameters of frozen-thawed sperm (The
490 experiment was repeated six times).

Antioxidant	mM	MOT (%)	PROG (%)	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	LIN (%)	STR (%)	ALH (µm)	BCF (Hz)
control		52.5±1.5	24.3±1.5	173±1.9	96.8±1.9	72.8±1.2	42±1	75±1.4	8.1±0.3	26.9±1.2
cysteamine	2	53.7±1.6	25.7±2.5	170.7±1.3	96.5±1.9	74±1.8	43.2±1.2	76.3±2.2	8±0.3	27.2±0.7
	4	54.7±2.1	26.2±2.2	174±1.6	97.8±2.1	75.5±1.4	43.5±1	77.2±2.1	7.9±0.3	28.1±1.1
	6	59.5±2.3*	27.8±1.6	176±1.7	98.3±2.2	77±1.3	44.5±1	77.8±1.5	7.7±0.3	28.1±0.8
	8	49.2±1.9	24.5±1.6	171.2±1.5	95.3±1.7	72.8±1.4	42.2±0.8	76.2±1.9	8.3±0.3	26.3±1.1
ergothioneine	2	54.5±2.2	23.5±1.6	171.3±1.8	95.3±2.2	72±1.8	41.5±1.1	75.2±1.5	7.9±0.3	28.4±0.6
	4	57.2±2.8	25.7±1.4	172.5±1.6	97±2.9	73.2±1.7	42.3±0.8	75.2±1.8	8.2±0.3	27.9±0.9
	6	60.2±2.3**	28.2±2	172.7±1.1	98.8±2.1	75.5±1.9	43.3±1	76.7±0.7	7.8±0.3	27.8±1.1
	8	49.8±1.2	22.8±1.8	169.7±1.3	92.7±1.6	71.3±1.5	41.5±0.8	75.7±1.3	8.2±0.3	27.7±0.8

491 MOT: Total motility; PROG: Progressive motility; VSL: Curvilinear velocity; VAP: Average path velocity; VSL:
492 Straight-line velocity; LIN: Linearity; STR: straightness; ALH: Amplitude of lateral head displacement: BCF:
493 Beat/cross frequency.

494 Asterisks show a significant effect of the treatment using the control as a reference: * P<0.05; ** P<0.01; ***
495 P<0.001.

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Table 2: Effect of cysteamine or ergothioneine on membrane integrity and morphological appearance of frozen-thawed sperm (The experiment was repeated six times).

Antioxidant	mM	Viability (%)	Membrane functionality (%)	Abnormal forms (%)
control		55.8±1.7	44.5±1.4	21.7±1.5
cysteamine	2	57.3±1.6	49.7±2.2*	21.8±1.4
	4	59±1.8	50.3±2.2*	20.2±2
	6	63.3±1.6**	54.3±1.9***	19.7±1.7
	8	52.5±2.1	45.7±1.8	23±2
ergothioneine	2	57.5±2.1	50.8±2**	21±2.5
	4	61.5±2.2*	51.8±2.1**	20.8±2.7
	6	65.3±2.7***	55.3±1.8***	19.3±1.8
	8	53.7±1.1	45.5±1.6	22±2.2

Asterisks show a significant effect of the treatment using the control as a reference: * P<0.05; ** P<0.01; *** P<0.001.

513 Table 3: Effect of cysteamine or ergothioneine on oxidative stress of frozen-thawed sperm (The
514 experiment was repeated six times).

Antioxidant	mM	MDA (nmol/mL)	GPx (IU/g protein)
control		3.5±0.4	26.7±1.5
cysteamine	2	3.1±0.2	25.8±1.3
	4	3±0.2	25.4±1.6
	6	2.3±0.2***	24.9±1.6
	8	3.3±0.2	26.6±1.6
ergothioneine	2	2.9±0.1	26.1±1.1
	4	2.8±0.2*	25.8±1.3
	6	2±0.1***	24.7±1.7
	8	3.1±0.1	26.9±1.6

515 Asterisks show a significant effect of the treatment using the control as a reference: * P<0.05; ** P<0.01; ***
516 P<0.001.

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Table 4: Effect of cysteamine or ergothioneine on capacitation status of frozen-thawed sperm (The experiment was repeated six times).

Antioxidant	mM	F (%)	B (%)	AR (%)
control		15.3±0.8	59.6±1.3	25.1±1.5
cysteamine	2	15±0.9	61±1.3	24±1.4
	4	16±0.8	60.6±1.3	23.4±1.6
	6	16.3±1.1	61.4±1.1	22.2±1
	8	15±0.8	59.5±1.1	25.5±1.1
ergothioneine	2	15.2±0.9	60.5±1.1	24.3±1.2
	4	15.4±0.6	60.5±1	24.1±1.3
	6	16.4±1	61.1±1.2	22.5±1.1
	8	14.8±1	59.3±1.3	26±2

F: Uncapacitated; B: Capacitated; AR: Acrosome reacted.

No significant effect of the treatments using the control as a reference.