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ARTICLE *in* CRYOBIOLOGY · AUGUST 2010

Impact Factor: 1.59 · DOI: 10.1016/j.cryobiol.2010.05.005 · Source: PubMed

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The effect of raffinose and methionine on frozen/thawed Angora buck (Capra hircus ancryrensis) semen quality, lipid peroxidation and antioxidant enzyme activities ☆

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ARTICLE INFO

Article history:
Received 19 January 2010
Accepted 26 May 2010
Available online 31 May 2010

Keywords:
Angora buck semen
Antioxidants
Antioxidant activity
Cryopreservation
Sperm characteristics

ABSTRACT

The aim of the present study was to determine the effects of different doses of raffinose and methionine on post-thawed semen quality, lipid peroxidation and antioxidant enzyme activities of Angora buck (Capra hircus ancryrensis) sperm following cryopreservation.

Ejaculates collected from three Angora bucks were evaluated and pooled at 37 °C. Semen samples, which were diluted with a Tris-based extender containing the additives raffinose (2.5, 5, 10 mM) and methionine (2.5, 5, 10 mM) and an extender containing no antioxidants (control), were cooled to 5 °C and frozen in 0.25 ml French straws. Frozen straws were thawed individually at 37 °C for 20 s in a water bath for evaluation. The freezing extender supplemented with 2.5 and 5 mM methionine led to higher percentages of CASA motility (63.6 ± 7.0; 63.4 ± 3.1%, respectively), in comparison to the controls ($P < 0.01$) following the freeze–thawing process. The addition of antioxidants did not provide any significant effect on the percentages of post-thaw subjective and CASA progressive motilities as well as sperm motion characteristics (VSL and VCL), compared to the control groups ($P > 0.05$). The freezing extender with raffinose (5 and 10 mM) and methionine at three different doses (2.5, 5 and 10 mM) led to lower percentages of acrosome abnormalities, in comparison to the controls ($P < 0.001$). In the comet test, raffinose (5 and 10 mM) and methionine (10 mM) gave scores lower than those of the controls, and thereby reduced DNA damage ($P < 0.05$). Malondialdehyde formation was found to be lower (1.8 ± 0.1 nmol/L) in the group of 5 mM raffinose, compared to the controls following the freeze–thawing process ($P < 0.01$). The additives did not show any effectiveness on the maintenance of SOD, GSH-PX and GSH activities, when compared to the controls ($P > 0.05$). In conclusion, methionine and raffinose play a cryoprotective role against sperm CASA motility, acrosome abnormality and DNA damage. Raffinose 5 mM exhibited antioxidative properties, decreasing MDA levels. Further studies are required to obtain more concrete results on the characterization of microscopic parameters and antioxidant activities in cryopreserved goat sperm with different additives.

Introduction

Cryopreservation and artificial insemination (AI) have been introduced as assisted reproductive tools for the conservation of genetic resources through sperm banks, particularly in conjunction with allowing the widespread dissemination of valuable genetic material in small ruminant breeding. However, the use of AI in small ruminants has important challenges related to sperm parameters, which are affected by cryopreservation [48]. There are important differences in terms of sperm quality and fertilizing ability between fresh and frozen–thawed semen, due to cryopreservation being associated with cold shock, ice crystal formation, membrane alterations and oxidative stress [49]. The latter is also involved in lipid peroxidation (LPO) generating excessive reactive oxygen species (ROS). Therefore, freezing/thawing processes result in loss of motility of spermatozoa, deterioration of acrosomal and plasma membrane integrity, deoxyribonucleic acid (DNA) damage and apoptosis of sperm [4,12,17]. The integrity of sperm DNA has vital importance to the sperm cell. Some authors suggest that sperm DNA integrity

☆ *Statement of funding:* This study was supported by Directorate of Lalahan Livestock Central Research Institute, Ankara, Turkey.

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0011-2240/$ - see front matter Crown Copyright © 2010 Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2010.05.005

is a more objective marker of sperm function, as opposed to sperm parameters such as motility. DNA damage could be a result of free radical-induced damage [29,44]. ROS is physiologically produced in various organs. The presence of ROS in semen demonstrates a balance between the cellular production of these molecules and their destruction by antioxidative compounds. Spermatozoa have a limited antioxidant defense system and high concentration of polyunsaturated fatty acids [3,14,17,37,50]. The inclusion of additives such as cysteine, glutamine, taurine and trehalose in the freezing extender has improved the quality of semen against ROS-induced damage. Antioxidative and cryoprotective effects of additives, which improve sperm function, such as motility, membrane integrity, endogenous antioxidant activities and fertilizing ability, have been demonstrated in various species [5,10,33,34,45,51].

Raffinose, a trisaccharide, like other sugars, plays a cryoprotective role by interacting with membrane lipids and proteins and decreasing the risk of intracellular ice crystal formation, which causes cellular osmotic dehydration during cryopreservation [2,41]. The cryoprotective properties of various sugars against freezing–thawing induced damages have been documented in various cell types [2,8,51]. The effect of raffinose related to oxidative stress has been considered as indirect effects of sugar signalling and triggering the production of specific ROS such as hydroxyl radicals scavengers [46]. Methionine acts as a precursor amino acid for glutathione. The protective effect of methionine against oxidative damage has been documented in various tissues, based on treatment with methionine. In addition, sulphur-containing compounds (e.g. methionine) were shown to chelate lead and remove it from tissues, and thereby to play a vital role in detoxification [27,30,31]. In addition, methionine plays an important role in the antioxidant defense mechanism by reacting readily with oxidants to form methionine sulfoxide [24]. But the underlying mechanisms that could explain the antioxidant effects of methionine is not yet fully understood.

We aimed to assess the influence of methionine and raffinose at different doses on motility, morphology, DNA integrity and antioxidant activities, such as glutathione (GSH), glutathione peroxidise (GSH-PX) and superoxide dismutase (SOD), of cryopreserved-thawed Angora buck sperm.

Materials and methods

Chemicals

The additives (methionine M-5308 and raffinose R7630) and other chemicals used in this study were obtained from Sigma–Aldrich, St. Louis, MO, USA.

Animals and semen collection

Semen samples from 3 mature Angora bucks (3 and 4 years of age), of superior genetic merit and fertility capacity, were used in this study. The Angora bucks, belonging to the Lalahan Livestock Central Research Institute (Ankara, Turkey), were maintained under uniform nutritional conditions. Ejaculates were collected twice a week from the Angora bucks with the aid of an artificial vagina during the breeding season (autumn to early winter). Immediately after collection, the ejaculates were immersed in a warm water bath at 33 °C until their assessment in the laboratory. Semen assessment was performed within approximately 20 min.

Semen extending, freezing and thawing

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals and the sperm concentration was determined by means of a haemocytometer [39], while sperm motility was estimated using phase-contrast microscopy (100× magnification). Only ejaculates between 1 and 2 ml in volume, spermatozoa with >80% progressive motility and a concentration higher than 2.5×10^9 spermatozoa/ml were pooled, balancing the sperm contribution of each male to eliminate individual differences [10]. Six mixed ejaculates were included in the study. A Tris-based extender (Trizma base 254 mM, citric acid monohydrate 78 mM, D (−) fructose 70 mM, egg yolk 15% (v/v), glycerol 6% (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 methionine (2.5, 5, 10 mM), and raffinose (2.5, 5, 10 mM), and no anti-oxidant (control), respectively – for a total of seven experimental groups, 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 (medium-sized) 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 stored for one 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 analyse 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 to 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 characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), VSL (straight linear velocity, μm/s) and VCL (curvilinear velocity, μm/s). For each evaluation, 10 microscopic fields were analysed to include at least 300 cells.

Assessment of sperm acrosome abnormalities

For the assessment of sperm acrosome abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution and 500 ml double-distilled water) [35]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of sperm acrosome abnormalities such as swollen, knobbed, ruffled and disintegrated were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (magnification 1000×, oil immersion).

Assessment of sperm DNA damage

Preparation for assessing sperm DNA damage

Diluted semen samples were centrifuged at 300*g* for 10 min at 4 °C. Seminal plasma was removed and remaining sperm cells were

washed with (Ca^{2+} and Mg^{2+} free) PBS to yield a concentration of 1×10^5 spermatozoa/cm^3 [1].

Determination of sperm DNA damage using comet assay

Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions.

Embedding of sperm in agarose gel: Each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and thoroughly dried at room temperature. Next, 100 μl of 0.7% low melting point agarose at 37 °C was mixed with 10 μl of the cell suspension and dropped on top of the first layer. Slides were allowed to solidify for 5 min at 4 °C in a moist box. The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothereitol (pH 10) for 1 h at 4 °C. Then the slides were incubated overnight at 37 °C in 100 μg/ml proteinase K (Sigma) and added to the lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at room temperature at 25 V and was adjusted to 300 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents. After neutralization the slides were stained with 50 μl of 2 μl/ml ethidium bromide and covered with a coverslip. All steps were performed under dim light to prevent further DNA damage [19,38].

Image analyses

The images of 100 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, Japan). Each image was classified according to the intensity of the fluorescence in the comet tail, and given a value of 0, 1, 2, 3 or 4 (from undamaged class 0 to maximally damaged class 4), so that the total score of the slide would range from 0 to 400 arbitrary units (AU) [47]. Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered damaged.

Biochemical measurements

Biochemical assays were performed on sperm samples immediately after thawing following centrifugation and washing.

Malondialdehyde (MDA) concentrations

Concentrations of MDA, as indices of LPO in sperm samples, were measured using the thiobarbituric acid reaction and according to the method described by Placer et al. [28]. The quantification of thiobarbituric acid reactive substances was performed by comparing the absorption with the standard curve of malondialdehyde equivalents generated by the acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. MDA concentrations were expressed as nmol/ml.

GSH, GSH-PX and SOD activities

The GSH content of sperm was measured using the method of Sedlak and Lindsay [36]. The samples were precipitated with 50% trichloracetic acid and then centrifuged at 1000*g* for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris–EDTA buffer (0.2 mol/l; pH 8.9) and 0.1 ml of 0.01 mol/l 5, 5′-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer. GSH values were expressed as nmol/mg protein.

GSH-PX activity was determined according to the method of Lawrence and Burk [22]. 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 oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM H_2O_2. An enzyme source (0.1 ml) was added to 0.8 ml of the above mixture, and this mixture was incubated at 25 °C for 5 min before the initiation of the reaction induced by the addition of 0.1 ml of peroxide solution. The absorbance at 412 nm was recorded for 5 min on a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value was subtracted from each value. GSH-PX activity was expressed as international unit U/g protein for sperm samples.

SOD activity was analysed with the technique of Sun and Zigman [42]. A volume of 0.4 ml sample, 0.1 ml of 0.9% NaCl and 0.4 ml of chloroform was centrifuged for 10 min at 2000*g*. The reaction mixture contained 20 ml 3 mmol/L xanthine, 10 ml 0.6 mmol/L EDTA, 10 ml 0.15 mmol/L nitroblue tetrazolium (NBT), 6 ml 400 mmol/L sodium carbonate, and 3 ml 1 g/L BSA for 20 tests. An aliquot (50 μl) of xanthine oxidase (20 μmol/2 ml) was added to 2.45 ml of the reaction mixture and this was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by adding 1 ml of 0.8 mmol/L $CuCl_2$ to the test. The absorbance was recorded at 560 nm by a spectrophotometer (Techcomp Spectrophotometer UV 7500, Hong Kong). SOD activity was expressed as U/mg protein.

Statistical analysis

The study was repeated six times. The results were expressed as means ± 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

Microscopic sperm parameters (percentages of motility, acrosome abnormality and DNA damage)

The influence of raffinose and methionine on standard semen parameters following the freeze–thawing process was evaluated in six independent experiments. As shown in Table 1, the freezing extender supplemented with 2.5 and 5 mM methionine led to higher percentages of CASA motility (63.6 ± 7.0%; 63.4 ± 3.1%, respectively), in comparison to the controls ($P < 0.01$) following the freeze–thawing process. The addition of antioxidants did not provide any significant effect on the percentages of post-thaw subjective and CASA progressive motilities as well as sperm motion characteristics (VAP, VSL, LIN and ALH), compared to the control groups ($P > 0.05$). The freezing extender with raffinose (5 and 10 mM) and methionine at three different doses (2.5, 5 and 10 mM) led to lower percentages of acrosome abnormalities, in comparison to the controls ($P < 0.001$). In the comet test, raffinose (5 and 10 mM) and methionine (10 mM) gave scores lower than that of the controls, reducing DNA damage ($P < 0.05$).

MDA levels and activities of SOD, GSH and GSH-PX

As shown in Table 2, MDA formation was found to be lower (1.78 ± 0.07 (nmol/L) in the group of raffinose (5 mM) than the control groups following the freeze/thawing process ($P < 0.01$). The additives did not show any effectiveness on the maintenance

Table 1
Mean (±SEM) sperm parameters in frozen–thawed buck semen.

Groups	Control	Raffinose 2.5 mM	Raffinose 5 mM	Raffinose 10 mM	Methionine 2.5 mM	Methionine 5 mM	Methionine 10 mM	*P*
Subjective motility (%)	50.6 ± 2.9	60.0 ± 2.7	57.0 ± 1.2	57.0 ± 4.6	56.0 ± 2.5	59.0 ± 3.7	56.0 ± 3.3	–
CASA motility (%)	45.1 ± 2.7^{b}	56.2 ± 3.4^{ab}	53.2 ± 3.3^{ab}	55.2 ± 1.8^{ab}	63.6 ± 7.0^{a}	63.4 ± 3.1^{a}	53.6 ± 3.5^{ab}	**
Progressive motility (%)	16.7 ± 2.3	17.6 ± 1.6	19.2 ± 1.8	19.4 ± 1.2	16.2 ± 1.9	15.8 ± 1.4	16.6 ± 1.9	–
VSL (μm/s)	74.8 ± 6.4	78.3 ± 3.5	78.0 ± 4.6	81.7 ± 4.3	61.8 ± 4.3	67.3 ± 5.4	69.7 ± 5.4	–
VCL (μm/s)	177.0 ± 10.5	186.7 ± 4.1	187.3 ± 5.1	192.7 ± 5.8	151.8 ± 13.6	166.2 ± 8.8	170.1 ± 6.4	–
Acrosome abnormality (%)	13.0 ± 1.1^{a}	10.0 ± 0.7^{ab}	6.2 ± 0.6^{cd}	5.2 ± 1.0^{d}	7.8 ± 1.3^{bcd}	7.2 ± 1.3^{bcd}	9.2 ± 1.4^{bc}	***
DNA damage score (AU)	80.4 ± 7.9^{a}	56.4 ± 5.9^{a}	39.2 ± 5.1^{b}	40.8 ± 2.9^{b}	55.0 ± 6.9^{a}	60.0 ± 8.7^{a}	46.2 ± 8.1^{b}	*

a–d: Different superscripts within the same row demonstrate significant differences among groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).
–: No significant difference ($P > 0.05$).

Table 2
Mean (±SEM) malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and glutathione (GSH) levels in frozen–thawed buck semen.

Groups	Control	Raffinose 2.5 mM	Raffinose 5 mM	Raffinose 10 mM	Methionine 2.5 mM	Methionine 5 mM	Methionine 10 mM	*P*
MDA (nmol/ml)	2.5 ± 0.2^{abc}	2.2 ± 0.4^{bcd}	1.8 ± 0.1^{d}	2.1 ± 0.3^{cd}	3.1 ± 0.1^{a}	2.8 ± 0.1^{ab}	3.0 ± 0.2^{a}	**
SOD (U/mg protein)	115.0 ± 19.7	116.1 ± 14.6	120.9 ± 16.9	105.6 ± 17.3	112.7 ± 8.9	90.2 ± 18.8	114.7 ± 13.9	–
GSH-PX (U/g protein)	28.7 ± 2.5	25.3 ± 1.9	25.5 ± 1.8	27.2 ± 3.3	32.7 ± 3.4	24.4 ± 3.1	29.8 ± 2.3	–
GSH (nmol/mg protein)	2.9 ± 0.6	3.6 ± 0.3	3.2 ± 0.2	4.7 ± 0.4	1.9 ± 0.4	2.6 ± 1.2	1.9 ± 0.6	–

a–d: Different superscripts within the same row demonstrate significant differences among groups (**$P < 0.01$).
–: No significant difference ($P > 0.05$).

of SOD, GSH-PX and GSH activities, when compared to the controls ($P > 0.05$).

Discussion

Cryopreservation of spermatozoa enhances oxidative stress, which not only disrupts the motility and fertilizing ability of spermatozoa, but also increases DNA damage; causing high frequencies of single- and double-stranded DNA breaks [4–6]. The supplementation of the freezing extender with additives (cysteine, GSH and lipoic acid) may counteract cryodamage to spermatozoa functions, antioxidant capacities and DNA integrity [7,9,20]. Antioxidant mechanisms such as GSH, GSH-PX and SOD exist to maintain defense against oxidative stress-induced damages in semen [13,15,26]. However, the antioxidant capacity of sperm cells is insufficient in preventing oxidative stress during the freeze–thawing process [40].

Among diluent components, raffinose, which is a non-permeant sugar and trisaccharide, may be important in maintaining osmotic pressure, thereby acting as a cryoprotectant [2,23,41]. It was reported that disaccharides provide superior protection of motility, plasma membrane integrity and fertilizing capability of post-thaw sperm in some species [18,32,41,43,52]. The present study showed that raffinose, especially at doses of 5–10 mM, had cryoprotective effect, and preserved acrosome integrity and DNA integrity of sperm against cryodamage. Raffinose at 5 mM reduced MDA levels, but did not increase SOD, GSH or GSH-PX activities, compared to the controls.

It is well-known that SOD is an enzymatic biological antioxidant, which scavenges ROS, such as superoxide anion and hydroxyl radicals, and thus controls oxidative stress in mammalian sperm [16]. Furthermore, GSH is able to react with many ROS directly and is a co-factor for GSH-PX, which catalyses the reduction of toxic H_2O_2 and hydroperoxides [7]. Methionine and thiol-containing antioxidants, protect spermatozoa from oxidative damage, and play an antioxidative role in detoxification [11,21]. Methionine acts as a precursor aminoacid for glutathione and also conserves membrane functional integrity and sperm motility since it enters the cell membrane easily and increases intracellular glutathione biosynthesis [27,30,31]. In addition, methionine residues have been suggested to scavenge ROS, and are thus known as important antioxidants [24]. However, in the present study, methionine could not maintain GSH levels, when compared to the controls. Furthermore, the effect of the three different doses of methionine on LPO levels and SOD activity did not differ significantly from that of the controls. The results of the present study are in agreement with studies showing that methionine did not have any statistical importance for SOD activity in different organs of the rat [25,27]. However, supplementation with methionine at doses of 2.5 and 5 mM statistically provided better CASA motility rates compared to the controls following the freeze–thawing process. This situation may be due to its protective effect on the functional integrity of the membrane and cytoplasmic components such as the axosome and mitochondria of the sperm cells, which are associated with motility. Acrosome integrity was successfully maintained in the presence of methionine with three different doses, compared to the controls, as a result of the cryoprotective effect. As regards DNA integrity, DNA damage decreased when raffinose (5 and 10 mM) and methionine (10 mM) were added to the extender. According to the results of this study LPO does not seem to be an influential factor on the survival and movement potential of spermatozoa following the freeze–thawing process.

Conclusions

Our results showed that methionine provided cryoprotective effect on sperm acrosome abnormalities, while it was not effective on the elimination of MDA formation and maintenance of antioxidant activities. For CASA, the best post-thaw motility rate was provided when the freezing extender was supplemented with 2.5–5 mM methionine. Besides, raffinose at 5 and 10 mM had a cryoprotective effect on sperm acrosome and DNA integrity and decreased MDA levels, in comparison to the control groups. However, not all of the additives improved post-thaw subjective and progressive sperm motilities and motion characteristics. In order to confirm these contradictory results, further studies are needed

to obtain more concrete results regarding the characterization of various antioxidant systems in cryopreserved goat sperm. Additionally, future research should focus on the molecular mechanisms of the cryoprotective effect of the antioxidants raffinose and methionine during cryopreservation. We suggest that supplementation with these additives prior to the freezing process would facilitate the use of semen cryopreservation systems in the goat breeding industry.

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