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Effects of various cryoprotectants on bull sperm quality, DNA integrity and oxidative stress parameters ☆

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ABSTRACT

The objectives of this study was to compare the effects of type and concentration of cryoprotectants glycerol (G), ethylene glycol (EG) and dimethyl sulfoxide (DMSO) on the plasma membrane and DNA integrity as well as antioxidant activity of cryopreserved Eastern Anatolian red bull sperm. Ejaculates were collected from the three bulls using an artificial vagina twice a week. The ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the evaluated samples. The pooled ejaculates were also split into seven equal experimental groups and diluted with the modified base extender to a final spermatozoa concentration of 15×10^6 /ml. The extended samples were cooled slowly to 4 °C and equilibrated for 4 h. They were then loaded into 0.25 ml French straws and frozen using a digital freezing machine at 3 programmed rates: −3 °C/min from +4 °C to −10 °C, −40 °C/min from −10 °C to −100 °C, and −20 °C/min from −100 °C to −140 °C. Thereafter, the straws were plunged into liquid nitrogen at −196 °C. Frozen straws were thawed individually at 37 °C for 30 s in a water bath to analyse progressive motility and sperm motion characteristics as well as membrane integrity using hypo-osmotic swelling test. Biochemical assays were performed in a spectrophotometer using commercial kits. DNA damage was evaluated by Comet Assay using Image Analysis System. 6% G exhibited the greatest percentages of CASA (43.7 ± 2.92%) and progressive (26.4 ± 2.64%) motilities when compared to the other groups ($P < 0.001$). 6% G and 6% EG showed the greatest values of preserved membrane integrity ($P < 0.001$). 6% DMSO and 3% EG + 3% DMSO resulted in greater chromatin damage than the other groups ($P < 0.001$). The antioxidant activities of GPx, GSH, and CAT as well as the total antioxidant activity were affected by the type of cryoprotectant; notably, 2% G + 2% EG + 2% DMSO yielded the lowest activities when compared to the other groups ($P < 0.001$).

In conclusion, no advantages were found in using EG or DMSO to replace G in bull sperm cryopreservation. Freezing with cryoprotectant 6% G yielded the best post-thaw sperm characteristics for Eastern Anatolian Red bull spermatozoa.

Introduction

The Eastern Anatolian Red cow breed is a member of the Bos taurus cow subspecies. The breed hails primarily from Turkey and has the ability to adapt to harsh conditions. The population of pure Eastern Anatolian Red cows has decreased dramatically in the last decade, making the breed one of the endangered genetic resources of Turkey [2]. Although spermatozoa cryopreservation is an invaluable technique for helping endangered species [38,37], it can cause mechanical damage to spermatozoal membrane, oxidative damage to membrane phospholipids, DNA and alter spermatozoa metabolism [30]. Despite these negative effects, cryopreservation has enabled the storage of animal genetic materials, helping to preserve allele variation and genome of rare and endangered wildlife species [25]. The success of cryopreservation depends not only on preserving the motility of the spermatozoa

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but also on maintaining their metabolic function [47]. Glycerol (G) is the most widely used cryoprotectant for bull sperm because it reduces the mechanical damage to spermatozoa during the freezing process [16]. The optimum concentration of G for freezing bull sperm is influenced by other components in the overall sperm diluent, or extender [50]. Specifically, when used in high concentrations, G can cause great osmotic damage to spermatozoa because G passes through the sperm membrane much slower than other cryoprotectants [19,21]. However, the literature suggests that other cryoprotectants, such as dimethyl sulfoxide (DMSO) has been shown to protect spermatozoa against cryodamage as well as G [26]. Other researchers believe that a low-molecular-weight cryoprotectant, such as ethylene glycol (EG), may cause less damage to spermatozoa than G because its low molecular weight allows it to cross the plasma membrane more easily [33]. EG has also shown better post-thaw motility results for bull sperm when compared with both G and DMSO, which may be due to a reduction in the osmotic stress [21]. It is generally accepted that a substantial number (50%) of sperm are damaged during cryopreservation [47]. The main cause of damage to cells undergoing cryopreservation is the formation of intracellular ice crystals [29]. This damage may be reduced by the incorporation of cryoprotectants [4]. The composition of the extender and the inclusion of suitable cryoprotectants are important factors in successful sperm cryopreservation [14]. It is therefore necessary to adjust the extender composition, the type of cryoprotectant, the amount of cryoprotectant, and the cryopreservation protocol to meet the specific needs sperm of each species or breed [31]. Thus, the objective of this study was to compare the effects of type and concentration of cryoprotectants glycerol (G), ethylene glycol (EG) and dimethyl sulfoxide (DMSO) on the plasma membrane and DNA integrity as well as antioxidant activity of cryopreserved Eastern Anatolian red bull sperm.

Material and methods

Animals and semen collection

Three Eastern Anatolian Red bulls (2–3 years of age) with good quality semen characteristics (>80% forward progressive motility and concentrations of at least 1.0×10^9 spermatozoa/ml) were selected to be the semen source. The bulls were clinically proven to be free from any general or genital diseases and were maintained at the Livestock Central Research Institute (Ankara, Turkey). Ejaculates were collected from the bulls using an artificial vagina twice a week. The ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the evaluated samples. The pooled semen sample was immersed in a water bath at 35.5 °C until it could be assessed for total and progressive motility as well as sperm concentration. This study was replicated ten times for each group. The experimental procedures were approved by the Animal Care Committee of the Faculty of Veterinary Medicine, Istanbul University.

Semen processing

The total semen volume was determined from the graded collection tube soon after collection, and its concentration was determined using an Accucell photometer (IMV Technologie, L'Aigle, France). Progressive motility was evaluated subjectively using a phase contrast microscope (200×, Olympus BX43, Tokyo, Japan) at 37 °C. A Tris-based extender (T) (30.7 g of Tris, 16.4 g of citric acid, 12.6 g of fructose, 20% v/v egg yolk, and 1000 ml of distilled water at a pH of 6.8 with no cryoprotectant) was used as the base for the experimental extenders. The T was divided into seven parts and modified as follows: 6% (v/v) G was added (G6); 6% EG was added (EG6); 6% DMSO was added (DMSO6); 3% G and 3% EG were added (GEG3); 3% G and 3% DMSO were added (GDMSO3); 3% EG and 3% DMSO were added (EGDMSO3); 2% G, 2% EG, and 2% DMSO were added (GEGDMSO2). The pooled ejaculates were also split into seven equal experimental groups and diluted with the modified base extender to a final spermatozoa concentration of 15×10^6 /ml. The extended samples were cooled slowly to 4 °C and equilibrated for 4 h. They were then loaded into 0.25 ml French straws and frozen using a controlled rate freezer (Digitcool 5300 ZB 250, IMV, France) at 3 programmed rates: −3 °C/min from +4 °C to −10 °C, −40 °C/min from −10 °C to −100 °C, and −20 °C/min from −100 °C to −140 °C.

Assessment of in vitro sperm quality

Subjective motility was assessed using a phase-contrast microscope (100×, Olympus BX43, Tokyo, Japan). A drop of semen was placed on a pre-warmed microscope slide and was subjectively assessed at 37 °C for its percentage of progressive motility. In addition to estimating the subjective sperm motility, a computer-assisted sperm motility analysis program (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) was used to analyse sperm motion characteristics. CASA was pre-adjusted for bovine sperm analysis. A semen sample was diluted 1:4 in Lactated Ringer solution, and the diluted semen sample was placed onto a pre-warmed 20 mm chamber slide (Leja 4, Leja Products BV, The Netherlands). The sperm motility characteristics were determined using a 10× objective microscope lens at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), average path velocity (VAP, μm/s), straight linear velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm/s), and beat cross frequency (BCF, Hz). A minimum of 10 microscopic fields were analysed for each assessment, which included at least 300 cells.

The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoal membranes. The HOS test was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spread on a warm slide with a cover slip and then was examined using a phase-contrast microscope (400×, Olympus BX43, Tokyo, Japan) [40]. The number of swollen spermatozoa out of 200 was counted; swelling is characterised by a coiled tail, indicating that the plasma membrane is intact.

For the evaluation of sperm abnormalities, 10 μl of each sample was added to Eppendorf tubes containing 1 ml of Hancock solution [42]. One drop of this mixture was placed on a slide and covered with a cover slip. The percentages of acrosome, head, tail and total abnormalities out of 200 oil-immersed spermatozoa were determined using a phase-contrast microscope (1000×, Olympus BX43, Tokyo, Japan).

Assessment of DNA damage

Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. Semen samples were immersed in water at 37 °C for 30 s and then centrifuged at 600*g* for 10 min at room temperature. The seminal plasma was removed, and the remaining sperm cells were washed with phosphate buffer solution (PBS; Ca^{2+} and Mg^{2+} free) twice to yield a concentration of 1×10^5 spermatozoa/cm^3. Each microscope slide was pre-coated with a layer of 0.65% high melting-point agarose in distilled water and thoroughly dried at room temperature. Twenty-five microlitres of the sperm cell suspension was mixed with 75 μl of 0.65% low melting-point agarose at 50 °C, and then a drop of the mixture was placed on a

pre-coated slide and covered with a cover slip. The slides were allowed to solidify for 10 min at 4 °C in a moist box. Then, 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 dithiothreitol (pH 10) for 1 h at 4 °C. The slides were then removed from the lysis buffer, drained, and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, which contained 300 mM NaOH and 1 mM EDTA (pH 13). The slides remained in the unit for 20 min to allow the DNA to unwind. Next, electrophoresis was performed at 25 V and an adjusted 300 mA for 20 min at room temperature. Subsequently, the slides were washed with a neutralising solution of 0.4 M Tris (pH 7.5) to remove the alkali and detergents. After neutralisation, the slides were stained with 65 μl of 20 μg/ml ethidium bromide and covered with a coverslip. All of these steps were conducted under dimmed lighting to prevent additional DNA damage. The images of 100 randomly chosen nuclei were analysed using a fluorescent microscope at a magnification of 400× (Zeiss, Germany). Nucleotide DNA extends under electrophoresis to form "comet tails," and the relative intensity of DNA in the tail reflects the frequency of DNA breakage. Thus, the percentage of the total DNA in the comet tail was taken as a direct measure of the DNA break frequency. Tail DNA (%) was assessed in 100 cells using the Comet Assay III image analysis system (Perceptive Instruments, UK). The analysis was performed blindly by one slide reader.

Biochemical assays

Semen samples were centrifuged at 4 °C and 1000*g* for 15 min. to separate out the spermatozoa. The pellet was washed 3 times using 0.5 ml of PBS. This final solution was homogenised 5 times by sonication in cold for 15 s. For the Lipid Peroxidation Analysis (LPO), 120 μl of the homogenate was mixed with 10 μl of 0.5 mM butyl hydroxyl toluene (BHT) and stored at −80 °C until analysed. The rest of the homogenate was centrifuged at 8000*g* for 15 min, and the supernatant was separated and stored at −80 °C for a different enzyme analysis.

Results

As shown in Table 1, using EG and DMSO to replace G as a cryoprotectant did not yield higher percentages of sperm motility assessed subjectively or by CASA after thawing. Likewise, those experimental conditions did not improve the sperm motility characteristics (VAP, VSL, VCL, ALH and BCF). G6 exhibited the greatest percent motility assessed by CASA (43.7 ± 2.92%) and progressive (26.4 ± 2.64%) motilities when compared to the other groups ($P < 0.001$). G6 and EG6 showed the greatest values of preserved membrane integrity ($P < 0.001$). There were no significance differences in the total abnormalities percentages among any of the treatment groups ($P > 0.05$).

As shown in Table 2, DNA damage was affected by the type of cryoprotectant used; DMSO6 and EGDMSO3 resulted in greater chromatin damage than the other groups ($P < 0.001$).

As shown in Table 3, the antioxidant activities of GPx, GSH, and CAT as well as the total antioxidant activity were affected by the type of cryoprotectant; notably, GEGDMSO2 yielded the lowest activities when compared to the other groups ($P < 0.001$).

Discussion

Spermatozoa are very sensitive to alterations in the osmolality of the surrounding solution. If the osmolality difference becomes intolerable, spermatozoa lose their motility, an irreversible effect [20,49]. Motility is one of the most important factors in assessing bull and other mammalian sperm because it gives information about the sperm cell's energy sources [46]. This study showed that using EG and DMSO to replace G as a cryoprotectant did not improve the sperm's motility after thawing. Spermatozoa frozen in T containing 6% G exhibited the greatest percentages of subjective (58 ± 2.13%), CASA (43.7 ± 2.92%) and progressive (26.4 ± 2.64%) motilities when compared to the other groups ($P < 0.001$). In contrast with our findings, it has been reported that EG quickly penetrates the sperm cell during freezing and is easily removed during thawing due to its low molecular weight, causing less osmotic stress [30]. It has been stated in the literature that G causes more osmotic stress than other cryoprotectants [30], and EG can be used successfully for freezing bull [21], stallion [7], buffalo [45] and ram sperm [32]. In a previous study, Guthrie et al. [21] reported that EG had fewer harmful effects on sperm motility than did G; therefore, EG can be used as an alternative cryoprotectant. In a study on the sperm of Holstein breed bulls, El-Harairy et al. [17] shown that bull sperm samples that were cryopreserved in 3.5% G and 3.5% DMSO together exhibited higher motility and less abnormal morphology than those frozen in either 7% G or 7% DMSO alone. In our study, specifically the tests using only EG or DMSO and the tests of combined cryoprotectants, motility values were found to be lower however, the frequencies of abnormal sperm were greater than those described by Guthrie et al. and El-Harairy et al. In a bull sperm freezing study, Swelum et al. [45] used EG and G as cryoprotectants in diluents composed of either Tris or skimmed milk powder. They determined that using EG with Tris created superior cryoprotectant characteristics than when using G, in all areas except motility. After being frozen in a Tris solution containing EG as the cryoprotectant, the thawed spermatozoa's viability, acrosome integrity, plasma membrane integrity and abnormal spermatozoa rate were 61.15 ± 0.73%, 9.10 ± 0.81%, 71.75 ± 0.72% and 24.85 ± 0.41%, respectively. When skimmed milk powder was used instead of Tris, the values of those same markers were

Table 1
Mean (±SE) sperm values in frozen thawed bull semen.

Analysis	G6	EG6	DMSO6	GEG3	GDMSO3	EGDMSO3	GEGDMSO2	P
Subjective motility (%)	58 ± 2.13^{d}	50 ± 2.98^{c}	10.5 ± 1.57^{a}	51.5 ± 1.07^{c}	51.5 ± 2.36^{c}	39.5 ± 2.63^{b}	51 ± 1.45^{c}	***
CASA motility (%)	43.7 ± 2.92^{e}	31.7 ± 2.61^{bc}	6.4 ± 1.22^{a}	37.8 ± 1.24^{d}	30.1 ± 1.79^{bc}	27 ± 1.87^{b}	34.1 ± 1.79^{cd}	***
Progressive motility (%)	26.4 ± 2.64^{e}	17.3 ± 1.18^{c}	1.1 ± 0.23^{a}	22.2 ± 1.29^{d}	15.1 ± 0.90^{bc}	12.2 ± 1.35^{b}	18.4 ± 1.53^{cd}	***
VAP (μm/s)	112.6 ± 1.24^{d}	96.9 ± 1.54^{bc}	71.6 ± 2.10^{a}	108.4 ± 2.14^{cd}	$102.7 \pm 1.65b^{bcd}$	95.5 ± 1.53^{b}	94.9 ± 9.56^{b}	***
VSL (μm/s)	89.6 ± 0.78^{d}	80.5 ± 1.16^{b}	52.5 ± 1.42^{a}	89.9 ± 1.23^{d}	85.4 ± 1.09^{c}	77.7 ± 0.71^{b}	85.8 ± 1.35^{c}	***
VCL (μm/s)	209.7 ± 2.47^{d}	173.3 ± 2.99^{b}	155.5 ± 5.45^{a}	193.0 ± 4.86^{c}	186.0 ± 3.54^{c}	174.3 ± 4.05^{b}	188.6 ± 4.26^{c}	***
ALH (μm/s)	8.7 ± 0.06^{c}	7.2 ± 0.11^{a}	7.6 ± 0.31^{ab}	7.9 ± 0.15^{b}	7.7 ± 0.13^{ab}	7.5 ± 0.19^{ab}	7.8 ± 0.16^{b}	***
BCF (Hz)	26.3 ± 0.45^{ab}	30.2 ± 0.46^{e}	25.2 ± 0.76^{a}	28.3 ± 0.42^{cd}	28.5 ± 0.30^{cde}	29.5 ± 0.86^{de}	27.6 ± 0.59^{bc}	***
HOS T (%)	43.8 ± 2.88^{d}	43.8 ± 2.27^{d}	18.7 ± 1.50^{a}	39.2 ± 2.69^{cd}	31.0 ± 2.11^{b}	40.6 ± 2.61^{cd}	35.5 ± 1.59^{bc}	***
Total abnormalities (%)	18.6 ± 0.92	18.9 ± 0.89	19.2 ± 0.80	18.6 ± 1.07	18.8 ± 0.84	17.9 ± 0.60	17.9 ± 0.60	–

$^{a-e}$ Different superscripts within the same row demonstrate significant differences (***$P < 0.001$).
– No significant difference ($P > 0.05$).

Table 2
Mean (±SE) DNA damage values in frozen thawed bull semen.

Analysis	G6	EG6	DMSO6	GEG3	GDMSO3	EGDMSO3	GEGDMSO2	P
Tail length	63.3 ± 5.90	68.1 ± 4.29	73.34 ± 3.37	75.6 ± 4.70	58.9 ± 5.59	63.9 ± 3.81	59.6 ± 2.50	–
Tail intensity (%)	12.5 ± 0.95^{a}	14.9 ± 1.35^{ab}	20.6 ± 1.79^{c}	10.9 ± 0.75^{a}	11.4 ± 1.02^{a}	18.2 ± 2.33^{bc}	12.9 ± 0.79^{a}	***
Tail moment (μm/s)	4.7 ± 0.76^{ab}	6.0 ± 0.88^{bc}	10.1 ± 0.61^{d}	4.8 ± 0.69^{ab}	3.5 ± 0.70^{a}	7.4 ± 1.14^{c}	4.6 ± 0.47^{ab}	***

^{a,b,c} Different superscripts within the same row demonstrate significant differences ($^{***}P < 0.001$).
$^{-}$ No significant difference($P > 0.05$).

Table 3
Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioksidant activities in frozen thawed bull semen.

Analysis	G6	EG6	DMSO6	GEG3	GDMSO3	EGDMSO3	GEGDMSO2	P
GPx (mU/ml−10^{9}cell/ml)	13.2 ± 0.16^{a}	13.2 ± 0.13^{a}	15.4 ± 0.30^{b}	16.0 ± 0.13^{c}	13.3 ± 0.25^{a}	16.1 ± 0.08^{c}	13.0 ± 0.16^{a}	***
LPO (μm/ml−10^{9}cell/ml)	0.94 ± 0.20	1.13 ± 0.31	0.73 ± 0.23	1.26 ± 0.33	0.89 ± 0.29	0.72 ± 0.29	0.66 ± 0.23	–
GSH (μm/ml−10^{9}cell/ml)	19.0 ± 1.32^{a}	27.6 ± 7.22^{ab}	35.7 ± 9.73^{b}	16.6 ± 0.79^{a}	28.5 ± 7.04^{ab}	16.3 ± 1.61^{a}	16.2 ± 0.57^{a}	***
CAT (μm/ml−10^{9}cell/ml)	12.9 ± 1.49^{b}	23.4 ± 4.24^{c}	11.2 ± 2.71^{b}	12.5 ± 3.29^{b}	18.6 ± 4.06^{bc}	10.3 ± 2.77^{ab}	2.0 ± 0.44^{a}	***
Total antioksidant activities (mmol/trilox/ml−10^{9} cell/ml)	11.2 ± 1.00^{b}	18.2 ± 2.83^{c}	10.1 ± 1.81^{b}	10.9 ± 2.19^{b}	15.0 ± 2.71^{bc}	9.4 ± 1.85^{ab}	3.9 ± 0.30^{a}	***

^{a,b,c} different superscripts within the same row demonstrate significant differences ($^{***}P < 0.001$).
$^{-}$ No significant difference ($P > 0.05$).

55.95 ± 0.56%, 64.10 ± 0.61%, 66.45 ± 0.78% and 27.30 ± 0.75%, respectively. These results are higher than our findings, in which the use of 3% and 6% EG showed lower viability and integrities as well as higher levels of abnormal spermatozoa.

Additionally, several studies performed in different species have reported results inconsistent with our findings. The combined use of G and DMSO yielded better viability and motility results in a goat sperm freezing study than was found here [44]. Together, 2% G and 4% DMSO cause less damage to the acrosome structure of rabbit sperm after freezing and thawing [51] than we observed here in bull sperm. Lastly, 3% EG used in stallion sperm freezing showed better cryoprotectant effects than either higher concentrations of EG (6% and 9%) or any amount of G Mantovani et al. [27].

In a dog breed sperm freezing study, Martins-Bessa et al. [28] reported that after freezing and thawing, EG did not produce better motility or acrosome integrity results than either G alone or their combined use. Although the concentrations of G, EG and DMSO used were different in this study, the results obtained were similar to those of Martins-Bessa et al. In a study on bulls, Awad et al. [6] showed that using 3% G as a cryoprotectant yielded better motility (72.38%) and progressive motility (29.50%) than either EG (at concentrations of 3%, 2% and 1%) or methanol. In a buffalo sperm study, Rohilla et al. [41] found that using 6.8% G as a cryoprotectant yielded 46.80 ± 0.17% motility, 58.10 ± 1.20% viability and 57.20 ± 0.58% acrosome integrity, which was greater than the results of their 5% EG group (which were 40.90 ± 0.33% motility, 50.30 ± 0.45% viability, and 47.40 ± 0.50% acrosome integrity). However, their abnormal spermatozoa rates (9.30 ± 0.03% in the 5% EG group and 10.30 ± 0.07% in the 6.8% G group) were lower than those values obtained in our study. On the other hand, Celeghini et al. [10] reported abnormal spermatozoa rates of 18% when freezing bull sperm using two different diluters. Parallel with our findings, they found that neither EG nor DMSO nor the combined use of all three cryoprotectants yielded better results than G alone. Based on our results, we can hypothesize that the difference in the results may be due to the density of the other substances used in the diluent as well as different freezing protocols.

It has been reported that after the process of freezing and thawing, the progressive motility and CASA motility parameter values positively correlate with the resistance of the spermatozoa to cold shock and their fertility [34]. Similar results have been shown in other species [13,15,35,39]. Consistent with our study, Awad et al. [6] suggested that the CASA values (VAP, VSL, VCL and LIN) were affected by the type and concentration of the cryoprotectant. In addition, they reported that the VAP and VCL values were higher in the test groups containing G and EG and that the VSL and LIN values were higher in the high concentration of G group. However, the CASA values did not differ among any of the groups with various concentrations of EG. In this study, the CASA values were different between each group. The greater values of VAP, VSL, VCL and ALH shown in the G6 group post-thaw indicates a hyperactive and rapid spermatozoa presence similar to the findings of Freitas et al. [18] and Muino et al. [34]. Those researchers showed that semen with rapid and progressive sperm had the best post-thaw sperm longevity.

The DNA integrity was also affected by the type of cryoprotectant used. DMSO6 and EGDMSO3 resulted in more sperm with damaged DNA than the other groups. Other studies have reported that, while the temperature difference that occurs during the freezing and thawing process causes little change to sperm morphology, it reduces the spermatozoa acrosome reaction and alters the DNA structure [5,22]. In addition, DNA damage of 1.2–3% is normal in bulls with high fertility [9]. The freezing and thawing process performed on ram [36] and bovine [10,24] sperm was shown to cause permanent structural alterations to DNA that, in turn, reduces fertility. Januskauskas et al. [23] reported that the occurrences of abnormal spermatozoa are positively correlated with DNA damage. Spermatozoa plasma membrane damage also correlates with DNA damage [24]. In our study, the greatest plasma membrane integrity was found in G6, EG6, GEG3 and EGDMSO3; however, motility values were the greatest in G6. Similarly, Cormier et al. [11], Correa and Zavos [12], and Watson and Stewart [48] all reported that bull sperm quality are positively correlated with the frequency of motile spermatozoa. Our study showed that using DMSO as a cryoprotectant, alone or together with EG, caused the greatest amount of DNA damage (20.6 ± 1.79% for DMSO6 and 18.2 ± 2.33% for EGDMSO3); DMSO6 caused the lowest plasma membrane integrity, leading to defects. Thus, DMSO6 and EGDMSO3 may not be successful cryoprotectants, or at least may not be able to protect the DNA and plasma membrane of spermatozoa from heat and osmotic stress.

With regard to antioxidants, the activities of GPx, GSH, and CAT as well as the total antioxidant activity were affected by the type of cryoprotectant used. Notably, group GEGDMSO2 yielded the lowest antioxidant activities of all treatment groups ($P < 0.001$). During the dilution and cooling of sperm, reactive oxygen species (ROS) are generated by aerobic respiration, affecting membrane integrity

and causing fragmentation [1]. At the same time, freezing of the sperm decreases the antioxidant capacity of the spermatozoa [3,8]. When CAT is active in the sperm diluent, ROS damage to spermatozoa is reduced or eliminated [43]. In our study, the CAT and total antioxidant activities were the lowest in the GEGDMSO2 group, but this group did not exhibit many negative effects on the CASA and progressive motility results. This result may indicate that ROS attacks on the spermatozoa were reduced due to contents of the diluents other than CAT, so that ROS did not damage sperm functionality.

In conclusion, no advantages were found in using EG or DMSO to replace G in bull sperm cryopreservation. Freezing with cryoprotectant G6 yielded the best post-thaw sperm characteristics for Eastern Anatolian Red bull spermatozoa.

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