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SUPPLEMENTATION OF ROSMARINIC ACID HAS REDUCED OXIDATIVE STRESS ON BULL SPERMATOZOA FOLLOWİNG THE FREEZE THAWING PROCESS

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Abstract

BACKGROUND: Cryopreservation has a side effect on the motility, chromatin integrity and viability of sperm cells. **OBJECTIVE:** The present study investigated the effects of supplementation with rosmarinic acid (RA) Tris extender on sperm quality parameters, plasma and acrosome membrane damage, antioxidant enzyme activity and chromatin integrity following the freeze thawing process on bull spermatozoa. **MATERIALS AND METHODS:** Ejaculates were split into five aliquots and diluted to a final concentration of $15x10^6$ spermatozoa/ml with the Tris extender containing RA (25, 50, 100 and 200 µg/ml) and (control) and then frozen at a controlled rate. **RESULT:** Treatments did not give better results on the percentages of sperm progressive, total motility and sperm motion characters (*P>0.05*); however, RA25 and RA50 exhibited favourable chromatin integrity. In conclusion, RA25 and RA50 increased total antioxidant activity. As a consequence, the amount of MDA and chromatin damage were reduced in sperm cells.

Keywords: Antioxidant, bull semen cryopreservation, oxidative stress, rosmarinic acid

INTRODUCTION

Successful artificial insemination (AI) and pregnancy proportion depend on the capability of cryopreserved spermatozoa to fertilize the oocyte in cattle. For this reason, over the last decades, a wide range of research has been undertaken to improve semen freezing protocols (30). However, exposure to cooling, freezing and rewarming can still result in the loss of up to 50% of the structural integrity and functional viability of the spermatozoa (41). During the cryopreservation process, various factors are known to decrease the viability, motility, fertilizing talent (3,

5) and DNA integrity of sperm cells (37). One of the reactive oxygen species (ROS) generations is normal as a result of the oxidative metabolism (2). However, excess production of ROS induces irreversible lipid peroxidation, and it leads to fertility loss and cell death (1). Flavonoids are able to interact with the lipid bilayer, but their antioxidant activity specific mechanism is still not well explained (28) due to their permeability across membranes and their membrane affinity, which depend on the degree of hydroxylation, the molecular configuration and the length of the side chain (36). Rosemary, which contains rosmarinic acid (RA), is a perennial plant with antioxidant properties due to its bioactive substances, flavonoids and polyphenols (31). RA is notably a lipophilic component. Its bond to the cell membrane depends on the proportion of saturation and unsaturation in the lipid chains (14). Due to sperm cell membranes having a great number of polyunsaturated fatty acids (35), RA could be able to defend and maintain the integrity and fluidity of sperm cell membranes against the adverse effects of ROS (26). A great many studies have been performed to determine the possible favourable effects of antioxidant supplementation (38). Supplementation of semen extender with rosemary extract has been reported to have beneficial antioxidant properties on the post-thaw characteristics of boar (26, 27), buck (42) and bull sperm cells (10). Moreover, antioxidant supplementation's favourable effects can be expected in semen extenders which suffer from motility decrease, plasma membrane injury and chromatin damage and viability loss, all of which are associated with increased levels of oxidative stress. Although most antioxidant studies demonstrate positive effects (12, 32), some do not or even indicate adverse effects (39). Clearly, more research is needed to optimize the use of specific antioxidants in ROS associated with oxidative stress (38). In respect to the antioxidant properties of RA, there are no data regarding its influence on chromatin integrity during the cryopreservation of bull semen, although there are some reports concerning the relationship between semen quality and oxidative stress. Consequently, very few studies have been performed to advance our understanding of the effects of RA. Hence, the objective of the present study was to investigate the effects of supplementation with RA Tris base extender on sperm quality parameters, plasma and acrosome membrane damage, antioxidant enzyme activity and chromatin integrity following the thawing process on bull spermatozoa.

MATERIALS AND METHODS

Animals and study design

Semen was obtained from three Holstein breed bulls. The bulls were free from any genital or other diseases and were raised at Sultansuyu Agribusiness (Sultansuyu, Ankara, Turkey). Specimens had >80% progressive motility, and concentrations up to $1.0x10^9$ spermatozoa/ml were used as semen resources. Ejaculates were collected using an artificial vagina once per week. Semen specimens were immersed at 37°C in a water bath until they could be assessed for progressive motility and total sperm concentration. The study was replicated nine times for each test. After semen collection, the total volume was determined via graded collection tubes and its concentration was determined using a photometer (Minitube GmbH, Tiefenbach, Germany). A Tris-based extender (30.7 g Tris, 16.4 g citric acid, 12.6 g fructose, 20% v/v egg yolk, glycerol 6% (v/v) and 1,000 ml distilled water at pH 6.8) was used as the base extender for the test.

Extracted RA (10 mg) was diluted with 1 ml of 99% ethanol (Merck, KGaA, Darmstadt, Germany), thereby an RA stock solution was prepared. Ejaculates were allocated to five portions and diluted to a final concentration of $15x10^6$ spermatozoa/ml with the base extender containing RA (25, 50, 100 and 200 µg/ml) and additive-free (control: C). Prior to being loaded into 0.25 ml French straws, all samples were cooled slowly to 4°C and equilibrated for 4 h. They were then frozen using a controlled rate freezer (SY LAB Gerate GmbH, Neupurkersdorf, Austria) at 5 programmed rates: 0°C/min from +4°C to +4°C for 3 min; -3°C/min from +4°C to -7°C for 3.67 min; 0°C/min from -7 °C to -7°C for 1 min; -37.67°C/min from -7°C to -120°C for 3 min; 0°C/min from -120°C to -120°C for 5 min. After freezing, the straws were submerged in liquid nitrogen at -196°C**.** The study's experimental design was approved by the Animal Care Committee of Afyon Kocatepe University Veterinary Medicine Faculty for ethics compliance. The authorization number is 49533702/29.

Assessment of spermatozoa motility and motion characters

Sperm motility and motion characters were determined using a sperm class analyzer (SCA) system (CASA; Microptic S.L., Barcelona, Spain) with a 10× objective at 37°C. A 5 µl diluted semen specimen was put onto a pre-warmed slide, covered with a coverslip and then evaluated. Immediately after this, motility values were recorded: nonprogressive motility (%), progressive motility (%) and total motility (%). Average path velocity (VAP, µm s^{-1}), straight linear velocity (VSL, µm s^{-1}), curvilinear velocity (VCL, µm s^{-1}), amplitude of lateral head displacement (ALH, µm), [VSL/VCL] x100 (LIN), [VAP/VCL] x100 (WOB), [VSL/VAP] x100 (STR) and beat cross frequency (BCF) were determined by measuring the frequency with which the sperm track crossed the cell path in either direction and the changeable tracks. Also, spermatozoa that had high velocities, namely hyperactivated spermatozoa, were determined. The spermatozoa motilities were calculated with the speed standards set as fast >80µm/s, medium >60µm/s, slow >20µm/s and static. For each evaluation, a minimum of 220 and a maximum of 370 spermatozoa were analysed in six microscopic fields.

Assessment of functional membrane integrity

Spermatozoa membrane integrity was assessed using the hypoosmotic swelling test (HOST), based on swollen and curled tails. The HOST was performed by incubating 30 µl of semen with the addition of 300 µl of a 100 mOsm hypoosmotic solution (9 g fructose plus 4.9 g trisodium citrate in a litre of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mix was spread on a warm slide with a cover slip. Two hundred sperm cells were evaluated at 400× using a phase-contrast microscope (Model CX41; Olympus, Tokyo, Japan). Sperm with swollen or coiled tails indicated that the plasma membrane integrity was intact (29).

Assessment of abnormal spermatozoa

Sperm abnormalities were evaluated following protocols described previously [33]. Hancock solution (62.5 ml formalin (37%), 150 ml buffer solution, 150 ml saline solution and 500 ml double-distilled water) was used for the evaluation. Three drops of each specimen were added to Eppendorf tubes containing 1 ml of Hancock solution for the assessment of sperm abnormalities. One drop of this mix was put on a slide and covered with a cover slip. The sperm abnormality proportions (head, acrosome, tail and total abnormalities)

were recorded under phase-contrast microscopy at 1000× (Model CX41; Olympus, Tokyo, Japan) by counting a total of 200 oil immersed sperm cells.

Assessment of chromatin integrity

Sperm chromatin integrity was investigated using a single cell gel electrophoresis/comet assay with the CometAssay Kit (Trevigen, Gaithersburg, MD, USA). Before the semen samples were centrifuged at 300×g for 10 min at 4°C, they were thawed at 37°C for 30 s in a water bath. In order to insure that the semen samples had a final concentration of 1×10^5 sperm cell/ml, the upper part of the supernatant was removed, and the remaining sperm cells were washed with phosphate buffer solution (Mg^{2+} and Ca^{2+} free). Each microscope slide was pre-coated with a layer of 1% high melting point agarose in PBS and thoroughly dried at room temperature. Next, 100 µl of 0.7 % low melting point agarose was mixed with 10 µl of the cell suspension at 37°C and dropped on top of the first layer. Slides were allowed to solidify in a moist chamber at 4°C for 5 min. The coverslips were removed, and the slides were immersed in a freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothreitol (pH: 10) at 4°C for 1 h. The slides were further incubated overnight in 100 µg/ml proteinase K (Sigma-Aldrich, St. Louis, MO, USA) and added to the lysis buffer at 37°C. After cell lysis, all slides were washed with deionized water 3 times at 10 min intervals to remove salt and detergent from the microgels. They were then 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 at 25 V for 20 min at room temperature and was adjusted to 300 mA. In order to suspend the alkali and detergents, the slides were washed with a neutralizing solution of 0.4 M Tris (pH: 7.5). Following neutralization, the slides were stained with 50 µl of 2.5 µl/ml ethidium bromide (Sigma, Ankara, Turkey) and covered with a coverslip. Slides were observed under epifluorescent microscopy (Model CX31; Olympus, Tokyo, Japan) and digital images were captured for subsequent analyses/scoring with a comet assay using TriTek CometScore software version 1.5 (TriTek Corp., Sumerduck, VA, USA) (Fig. 1). In each slide, a total of 100 sperm cells were counted for analysis in the five fields. All stages were performed under dark room conditions to guard against further chromatin damage (34).

Assessment of oxidative stress

Total antioxidant capacities were performed colorimetrically using a RelAssay® kit (Rel Assay Diagnostics, Gaziantep, Turkey). Antioxidants in the specimens reduced the dark blue-green coloured 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals to a colourless reduced ABTS form. The change of absorbance at 660 nm was related to the total antioxidant level of the sample. The results were indicated in mmolTroloxequivalents/L. Glutathione peroxidase (GPx) activity was measured using a GPx assay (Bioxytech® GPx-340™; Percipio Biosciences, Inc., Burlington, CA, USA). The assay is based on a nicotinamide adenine dinucleotide phosphate (NADPH) coupled reaction where oxidized glutathione, produced on the reduction of organic peroxide (*tert*-butylhydroperoxide) by GPx, is recycled to its reduced state by utilizing enzyme glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is associated with a decrease in absorbance at 340 nm (using spectrophotometry to

follow GPx activity). The rate of decline in absorbance at 340 nm is directly proportional to the GPx activity. The GPx activity is determined from the correlation of the rate of change of absorbance in the sample to the rate of change in absorbance of known concentrations of GPx. Lipid peroxide, derived from polyunsaturated fatty acids, is unstable and decomposes to form a complex series of compounds, of which the most abundant is malondialdehyde (MDA). The lipid peroxidation level, which depends on MDA, was identified using an MDA commercial kit (MDA-586; OxisResearch™, Portland, OR, USA). One molecule of MDA reacts with two molecules of N-methyl-2-phenylindole (NMPI) to yield a stable with maximum absorption at 586 nm. The MDA was determined from the sample's absorbance at 586 in the sample nm and the standard curve was prepared using the MDA standard provided in the kit. The results were indicated in µmol/mL.

Statistical analysis

Parameters were normally distributed using the Shapiro Wilk normality test. Homogeneities of variance determined with Levene's test groups were compared, using the SPSS Statistics programme version 14.1 (IBM, Armonk, NY, USA). The test ascertained that the variance was homogeneous. Next, comparisons between the groups were made using analysis of variance with the Duncan post hoc test. The differences were considered significant at $P<0.05$. The results were expressed as means or proportions (±standard error).

RESULTS

As presented in Table 1, using RA as an antioxidant did not give better results on the proportion of sperm progressive and total motility assessed by CASA after thawing. As regards sperm motion characters, these treatment conditions did not enhance the sperm motion characteristics (VAP, VSL, VCL, LIN, STR, WOB, ALH, BCF and hyperactivity). Particularly, RA100 and RA200 exhibited unfavourable effects apart from R100's VCL, WOB, ALH and BCF, when compared to the other groups ($P<0.05$). Moreover, as presented in Table 2, middle piece abnormality was different among the treatment groups ($P<0.05$), but were no significance differences in the percentages of head, tail and total abnormalities ($P>0.05$). On the other hand, the chromatin integrity was significantly different among the treatment groups ($P<0.05$). RA25 and RA50 exhibited favourable tail length, tail DNA and tail movement (15.97±2.07 µm/s, 20.02±1.72 µm/s; 10.56±0.72%, 13.38±1.18%; 3.60±0.24 µm/s, 3.37±0.20 µm/s, respectively) when compared to the other groups ($P<0.05$) as shown Table 3. As presented in Table 2, there were no significance differences in the plasma membrane integrity proportion among any of the treatments ($P>0.05$). However, the total antioxidant (TA), MDA and GPx activities were affected by the treatment; notably, RA25 and RA50 yielded the lowest MDA levels and the greatest TA activities when compared to the other groups ($P< 0.05$) as shown in Table 3.

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

Analysis	Control	RA25	RA50	RA100	RA200	P
Nonprogressive motility (%)	42.03±2.37^{a}	44.76±1.79^{a}	40.70±3.34^{a}	37.62±2.81^{a}	18.58±2.39^{b}	*
Progressive motility (%)	31.38±1.86^{a}	30.55±2.63^{a}	27.61±4.05^{a}	12.35±0.99^{b}	1.47±0.62^{c}	*
Total motility (%)	73.42±3.27^{a}	75.31±3.42^{a}	68.31±60.36^{a}	49.97±3.21^{b}	20.07±2.56^{c}	*
VAP (µm/s)	54.38±1.48^{a}	50.86±2.05^{a}	48.65±2.66^{ab}	44.33±1.89^{b}	21.02±2.22^{c}	*
VSL (µm/s)	39.60±1.39^{a}	36.50±1.44^{a}	34.93±2.29^{a}	28.98±1.12^{b}	12.08±1.74^{c}	*
VCL (µm/s)	90.35±2.37^{a}	87.16±3.88^{a}	83.87±3.62^{a}	81.72±4.10^{a}	39.37±3.64^{b}	*
ALH (µm/s)	4.00±0.08^{a}	3.97±0.07^{a}	4.00±0.13^{a}	4.41±0.10^{a}	2.42±0.58^{b}	*
BCF (Hz)	12.70±0.45^{a}	12.16±0.39^{a}	12.35±0.35^{a}	11.40±0.36^{a}	4.31±1.10^{b}	*
LIN (%)	43.88±1.31^{a}	41.97±0.75^{a}	41.36±1.12^{ab}	35.77±1.15^{bc}	30.78±3.84^{c}	*
STR (%)	72.77±1.16^{a}	71.81±0.61^{a}	71.47±0.97^{a}	65.52±0.98^{b}	56.21±3.86^{c}	*
WOB µm s^{-1}	60.20±0.94^{a}	58.43±0.63^{ab}	57.81±0.99^{ab}	54.48±0.99^{b}	53.57±3.40^{b}	*
Hyperactivity µm s-1	27.93±1.62^{a}	27.62±1.96^{a}	25.71±3.96^{a}	18.55±2.24^{b}	4.51±1.23^{c}	*

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

Table 2. Mean (±SE) sperm plasma membrane integrity and abnormality values in frozen thawed bull semen

Analysis	Control	RA25	RA50	RA100	RA200	P
HOS T (%)	34.37±1.56^{b}	37.00±2.27^{b}	36.87±2.65^{b}	38.87±1.38^{b}	48.12±2.10^{a}	*
Head abnormalities (%)	3.45±0.93	3.32±0.39	2.15±0.72	3.84±0.90	2.01±0.70	-
Middle piece abnormalities (%)	3.74±0.67^{a}	2.32±0.50^{ab}	2.78±0.76^{ab}	1.68±0.56^{b}	2.21±0.50^{ab}	*
Tail abnormalities (%)	1.77±0.46	1.97±0.49	1.64±0.62	1.43±0.58	1.07±0.62	-
Total abnormalities (%)	8.96±1.62	7.62±0.53	6.59±1.61	6.96±0.96	5.30±1.38	-

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

Table 3. Mean (±SE) chromatin integrity values, glutathione peroxidase (GPx), malondialdehyde (MDA) and total antioxidant (TA) activities in frozen thawed bull semen

Analysis	Control	RA25	RA50	RA100	RA200	P
Tail lenght (µm/s)	29.43±5.37^{ab}	15.97±2.07^{c}	20.02±1.72^{c}	33.96±2.97^{a}	32.71±2.59^{ab}	*
Tail DNA (%)	29.93±3.86^{a}	10.56±0.72^{c}	13.38±1.18^{c}	20.55±1.68^{b}	24.42±1.80^{ab}	*
Tail moment (µm/s)	13.95±3.24^{a}	3.60±0.24^{c}	3.37±0.20^{c}	8.66±0.75^{b}	7.42±0.60^{bc}	*
GPx (mU/ml)	7.36±0.23^{b}	7.12±0.31^{b}	12.06±2.35^{a}	7.10±2.41^{b}	6.66±0.37^{b}	*
MDA (µmol/mL)	8.52±0.63^{b}	4.98±0.56^{c}	6.92±0.59^{bc}	18.03±0.51^{b}	27.55±1.73^{a}	*
Total antioxidant activities (mmol/trilox/ml-10^{9} cell/ml)	0.59±0.13^{c}	150±0.10^{a}	1.03±0.13^{b}	0.54±0.11^{c}	0.52±0.10^{c}	*

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

DISCUSSION

Oxidative stress can be formed either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant defence system, in respect to the oxidative damage that may occur (8). A sperm cell has limited antioxidants, so it is affected because of semen dilution for AI tool. An exogenous antioxidant supply is most probably an important factor in fertilising ability (15). Sperm cell plasma membrane integrity and motility are commonly believed to be important characteristics for evaluating semen quality. Moreover, the CASA is a useful plasma membrane integrity, the present results are inconsistent with findings of Malo et al. (27) which indicated that rosemary supplementation added to the freezing extender had a beneficial effect and also improved the motility of boar spermatozoa. Zanganeh et al. (42) showed that using rosemary extract (at the level of 4%) improved sperm motility and plasma membrane integrity in buck semen extender after thawing. The addition of 4.5 mg/ml of ascorbic acid to the cryoprotective extender increased bull sperm motility, motion characteristics and plasma membrane integrity (19). Moreover, our progressive and total motility results (control, RA25 and RA50) were greater than those of some previous studies, so the present findings are in disagreement with these previous reports. Daghigh-Kia et al. (10) explained that the supplementation of rosemary extract slightly advanced motility based on the improvement in mitochondrial function. Chhillar et al. (9) reported that trehalose and taurine supplemented semen extender led to greater post-thaw sperm motility (51±1.52, 47±1.15 respectively) compared to sperm extender in their absence (36±.05). On the basis of our findings, it is obvious that the highest concentration of RA (100 and 200 µg/ml) produced inhibition of motility and motion characteristics. This might have been induced either by the excessive removal of intracellular free radicals or by a direct toxic effect. However, our results are in accordance with the results of a recent study by our group which evaluated a different antioxidant supplementation (5 mM dithiothreitol or 25 mM sucrose) on bull spermatozoa in Tris extender. We found that the supplementation of antioxidants did not improve any curative effect on sperm motility, sperm motion characteristic, total abnormality and plasma membrane integrity (6).

Chromatin damage arises from oxidative stress during cryopreservation (38) and has a negative effect on embryo quality and pregnancy outcome (13). Spermatozoa diluting, freezing and thawing are associated with increased ROS production, reduced antioxidant level and increased DNA damage (17). In this study, RA25 and RA50 in semen extender exhibited favourable chromatin integrity and lower MDA production as method, and it is used to determine sperm motion characters (24). In the present study, when the groups were compared with the control group, RA supplementation did not advance the proportion of sperm progressive and total motility or sperm motion characters. This may be responsible for the beneficial effect of the egg-yolk based for optimal results. Specifically, increasing the amount of antioxidant supplementation resulted in adverse effects. RA100 and RA200 exhibited an unfavourable effect on the extender by impairing sperm motility functions ($P<0.05$). Also, in respect to the progressive and total motility, as well as

compared to the control. The greatest chromatin integrity was supported by the lowest oxidative stress. These results are in consensus with the earlier reports by Lewis et al. (25) who demonstrated that there is strong argument indicating that the freeze thawing process causes oxidative stress associated with free radical-mediated damage to sperm cell chromatin. Also, Gürler et al. (18) postulated that excessive ROS can affect chromatin integrity in bull sperm. In another bull sperm study, supplementation of butylated hydroxytoluene at lower concentrations (0.5-1.5 mM/ml) decreased MDA concentrations that protected against DNA damage by reducing oxidative stress (23). Contrary to the present study, Wnuk et al. (40) found a negative correlation between total antioxidant capacity and sperm chromatin damage. Also, Johinke et al. (21) indicated that quercetin supplementation did not have a beneficial effect on the proportion of sperm cells with DNA fragmentation values, and Khalifa et al. (22) reported chromatin instability was associated with high percentages of sperm with damaged plasma membranes and morphological abnormalities. These findings demonstrate that the antioxidant capacity of RA could protect the lipid peroxidation freeze thawing process against these consequences by encouraging chromatin integrity.

In the present study, there were no differences in the proportion of head, tail and total abnormalities and plasma membrane integrity. Our findings are in contrast to the results of studies performed on buck and boar semen by Zanganeh et al. (42) and Malo et al. (27), respectively, in which the addition of RA improved plasma membrane integrity, functionality and viability of post-thawed spermatozoa. Fadel et al. (14) showed that RA is created, antioxidant supplement exhibited cryoprotective activity on sperm plasma membranes in favourable amounts. In bull semen studies, 1 and 2 mM melatonin supplementations caused a significant decrease in total abnormality (8.1±1.3 and 6.9±1.3, respectively); however, none of the treatments affected acrosome abnormality (4). Also, it was shown that plasma membrane integrity was enhanced by supplementation of combinations of 2.5 mM of vitamin C, 100 IU/ml of catalase (1) and melatonin in the extender (4).

GP_X is one of the important antioxidant enzyme defence systems in sperm cells that transforms peroxide (H_2O_2) and superoxide (O^{2-}) radicals into H_2O and O^{2-} (20). MDA is a structural reagent resulting from lipid peroxidation in sperm membranes that causes a toxic effect on the sperm cell. MDA is used for measurement of sperm oxidative stress (11). As a result, the end product reduces the energy source necessary for the sperm motility (16). In the present study, antioxidant activities were affected by the treatment. RA25 and RA50 yielded the lowest MDA levels and the greatest TA activities when compared to the other groups ($P<0.05$). These findings are in agreement with those reported by Daghigh-Kia et al. (10) who demonstrated that the addition of rosemary extract alone (10 g L^{-1}) and in a combination with glutathione (5 mM glutathion+10 g L^{-1} rosemary extract) significantly increased intracellular defence systems against ROS production in bulls. Also, Malo et al. (27) indicated that production of MDA was affected by the presence of rosemary extract (10 g 100 L^{-1}) in the extender following the freeze thawing process in boar semen. However, our findings are inconsistent with the results found in a study on bovine semen by Büyükleblebici et al. (7), in which the addition of α-lipoic acids did not produce any significant effect on GPx activity among treatment groups, while, conversely, the MDA and TA activities were affected. These

reports and our findings indicate that antioxidant level is one of the major factors improving sperm survival following the freeze thawing process. This means that treatment groups may not advance the plasma membrane structure and motility functions; however, RA25 and RA50 can reduce chromatin damage.

In conclusion, our findings show that using RA did not enhance the sperm progressive and total motility or the sperm motion characters of any of the treatment groups after thawing. However, RA25 and RA50 did increase total antioxidant activities and as a consequence of this, the amount of MDA and chromatin damage were reduced in sperm cells.

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