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Effect of rosemary (*Rosmarinus officinalis*) extracts and glutathione antioxidants on bull semen quality after cryopreservation

Hossein Daghigh-Kia[1*], Rastegar Olfati-Karaji[1], Ali Hoseinkhani[1] and Iraj Ashrafi[2]

[1] *Department of Animal Science. Faculty of Agriculture. University of Tabriz. Tabriz (East Azerbaijan), Iran.*
[2] *Young Researchers and Elites Club. Science and Research Branch. Islamic Azad University. Tehran, Iran*

Abstract

The present study determined the effects of the addition of rosemary extract (ROM), glutathione (GSH), and their combination (ROM + GSH) to freezing extender on the quality of bull semen after cryopreservation. Before cryoperservation, the samples were diluted in a tris-egg yolk (TEY) extender containing 5 mM GSH (treatment I), 5 or 10 g L^{-1} ROM (treatments II and III), and ROM with GSH (5 mM GSH with 5 or 10 g L^{-1} of ROM) (treatments IV and V). An extender containing no antioxidants (non-ROM/GSH-treated) served as control group. Kinematic parameters were evaluated by means of a computer-assisted semen analysis (CASA). The viability and membrane integrity of the sperm were assessed using eosin-nigrosin stain and the hypo-osmotic swelling test (HOST) at 0 and 2 h after freeze-thawing. Lipooxidative parameters, superoxide dismutase, and glutathione peroxidase (GPx) activity were assessed after thawing. Treatment III showed positive effects for total motility (TM) ($p < 0.01$), average path velocity (VAP) ($p < 0.001$), viability ($p < 0.01$) and HOST ($p < 0.01$); however, lipid peroxidation (LPO) decreased ($p < 0.05$) and GPx activity increased ($p < 0.05$) immediately after thawing compared to the control. The TM ($p < 0.01$), VAP ($p < 0.01$), viability ($p < 0.01$), HOST ($p < 0.01$) decreased in LPO ($p < 0.01$) and GPx activity ($p < 0.05$) for treatment V and the viability and GPx activity ($p < 0.05$) for treatment I were significantly higher than for the control group at 2 h after thawing. It was concluded that the inclusion of ROM and its combination with GSH improves the post-thaw quality of bull semen.

Additional key words: sperm; freezing extender; reactive oxygen species; herbal antioxidant.

Introduction

Semen cryopreservation has detrimental effects on sperm cell organelles, including cell membranes, mitochondria, and DNA due to the increased level of reactive oxygen species (ROS) (Gil-Guzmán *et al.*, 2001; Meyers, 2005). Oxidation of polyunsaturated fatty acids in the sperm cell membranes is caused by high levels of ROS produced during the freeze/thaw process. This problematic condition causes decreased sperm motility, membrane integrity, increased metabolic changes and, ultimately, decreased fertility of the sperm (White, 1993). The addition of antioxidant compounds to the semen extender before bull semen cryopreservation can decrease ROS levels and their deleterious effects on spermatozoa (Bilodeau *et al.*, 2001). The inclusion of preventive and scavenger antioxidants into the freezing extender has been shown to improve the longevity and quality of thawed spermatozoa for several species (Roca *et al.,* 2005; Shoae & Zamiri, 2008; Bansal & Bilaspuri, 2011).

Decreased levels of glutathione (GHS) as a scavenger antioxidant and an intracellular defensive mechanism can play a decisive role against oxidative stress (Foote *et al.*, 2002; Bucak *et al.*, 2008). The concentration of GSH in semen decreases following cryopreservation (Bilodeau *et al.*, 2000). Studies indicate that supplementation of GSH in semen extender before

* Corresponding author: daghighkia@tabrizu.ac.ir ; hsz6955@yahoo.com
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Abbreviations used: CASA (computer assisted semen analysis); GPX (glutathione peroxidase); GSH (reduced glutathione); HOST (hypo-osmotic swelling test); LPO (lipid peroxidation); MDA (malonaldialdehyde); NADPH (nicotinamide adenine dinucleotide phosphate); PGM (progressive motility); ROM (rosemary extract); ROS (reactive oxygen species); SOD (superoxide dismutase); TBA (thiobarbituric acid); TEY (tris-egg yolk); TM (total motility); VAP (average path velocity); VCL (curvilinear velocity); VSL (straight-line velocity).

freezing (Gadea *et al.*, 2011; Perumal *et al.*, 2011) or after thawing (Gadea *et al.*, 2008) has a positive effect on sperm characteristics.

There is limited information on the effects of the addition of herbal antioxidants to semen freezing extenders. Natural herbs are economical and commonly available. Rosemary (*Rosmarinus officinalis*) is a medicinal plant that grows in many parts of the world. This plant is being increasingly applied as a food additive to improve short- and long-term preservation because of its antioxidative effects. Carnosol, carnosic and rosmanic acids are important antioxidative components of this perennial herb that protect against free radicals and lipid peroxidation (LPO) (Lo *et al.*, 2002).

Studies have shown that herbs can improve the quality of spermatozoa in freezing extender. ROM has been shown to have beneficial effects on boar (Malo *et al.*, 2010, 2011), canine (González *et al.*, 2010), and ovine (Gil *et al.*, 2010) semen freezing. *Rhodiola sacra* aqueous extract (RSAE) obtained from the roots of the Chinese herb Crassulaceae provides strong scavenging activity against superoxide anion radicals (Mook-Jung *et al.,* 2002) and can improve lipooxidative and dynamic parameters of cryopreserved boar spermatozoa (Zhao *et al.*, 2009). At present, no studies have evaluated the effect of ROM on freeze-thawed bull semen. The present study determined the effects of the addition of different concentrations of ROM, a combination of ROM and GSH, and GSH to semen freezing extender on the microscopic and oxidative parameters of freeze-thawed bull spermatozoa.

Material and methods

Experimental animals, sampling, processing and freeze-thaw methods

The experiment was performed at the West and Northwest Iranian Breeding Center in the city of Tabriz, Iran. Four Holstein bulls aged 4 to 5 years were maintained under similar conditions of feeding and housing. The bulls were selected according to their superior genetic merit and their high fertility. Ejaculates were collected twice a week using an artificial vagina. The ejaculates were transferred to the laboratory and submerged in a water bath (34°C) until semen evaluation. Semen samples were evaluated; when achieving normal ranges (volume: 5-12 mL; sperm concentration: $> 1 \times 10^9$ sperm mL^{-1}; motility: > 70%; total morphological abnormalities: ≤ 10%), bull semen was frozen. The ejaculates were then pooled, equalizing the sperm contribution of each male to eliminate individual differences (Gil *et al.*, 2003). The tris-egg-yolk (TEY) extender used in the study comprised 60 mM citric acid, 0.25 M tris-(hydroxymethyl)-aminomethane, 69 mM fructose, 20% egg yolk, 7% glycerol in 80 mL water or ROM, 250 mg L^{-1} gentamycin sulfate, 50 mg L^{-1} tylosin tartrate, 150 mg L^{-1} lincomycin hydrochloride, and 300 mg L^{-1} spectinomycin sulfate antibiotics (Bilodeau *et al.*, 2002; Martínez *et al.*, 2006).

The semen samples were diluted to 50×10^6 motile spermatozoa mL^{-1} and aspirated into 0.5 mL French straws, sealed by machine (IMV Technologies, MRS1, France) and cooled slowly to 5°C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapor in a programmable automatic freezer (Minitube, Germany) and plunged into liquid nitrogen for storage. After one month of storage, the frozen samples were thawed at 37°C for 30 s in a water bath immediately before microscopic evaluation.

Rosemary was collected in January from fresh herbs grown under greenhouse conditions (Golara Greenhouse, Tabriz, Iran). The ROM essence was extracted by soaking 5 or 10 g L^{-1} of fresh leaves in boiling water for 10 min. Once the water had cooled off to 25°C, the solution was filtered to remove the leaves before use (Malo *et al.*, 2011).

The experimental treatments were base extender containing: 5 mM GSH (Sigma-Aldrich, USA) (treatment I); 5 or 10 g L^{-1} ROM (treatments II and III, respectively); 5 mM GSH combined with 5 g L^{-1} or 10 g L^{-1} of ROM) (treatments IV and V, respectively). An extender containing no antioxidants served as control.

Semen evaluation after freeze-thaw

Motility and viability parameters

The kinematic parameters of the frozen-thawed semen samples were evaluated at 0 and 2 h after thawing using computer-assisted semen analysis (CASA). The CASA was equipped with a positive phase-contrast microscope (Nikon, Tokyo, Japan), a hot plate at 37°C, a digital video camera (Samsung, SDC-313B, Korea) and a computer for analysis of spermatozoa motility

(Hoshmand Fanavaran, Tehran, Iran). For this purpose, 5 µL of the sample was placed on a warmed slide (37°C) and covered with a cover slip. Images were recorded using a phase contrast objective microscope at ×100 magnification. CASA captured 50 images per second; 150 images, 8 fields, and 25 sperm cells/field were analyzed. Less than 15 s were spent on analysis of each image.

Using the CASA system default (lowest and highest straight linear velocity, average path velocity, linearity), the sperm cells were divided into three groups: progressive motile sperm, motile sperm, and immotile sperm. The following motility values were recorded: total motility (TM, %), progressive motility (PGM, %), straight linear velocity (VSL, µm s^{-1}), curvilinear velocity (VCL, µm s^{-1}) and average path velocity (VAP, µm s^{-1}). The mean of three successive estimations (> 200 spermatozoa per slide) was recorded as the final data.

Viability (live/dead, %) of the frozen-thawed semen samples were evaluated using eosin-nigrosin stain. The sperm smears were prepared by mixing a drop of semen with two drops of eosin-nigrosin stain on a warm slide and immediately spreading the suspension (< 30s). After air drying, the viability was assessed by counting 100 cells (per slide) under a microscope (×1,000 magnification with emersion oil, Olympus, DP 50, Japan). The final viability was calculated by averaging estimates of three slides. Sperm displaying partial or complete purple staining were considered non-viable; only sperm showing strict absence of stain was considered to be viable (Balestri *et al.*, 2007).

Assessment of membrane integrity

The hypo-osmotic swelling test (HOST) was performed based on curled and swollen tails to evaluate the functional integrity of the sperm membrane. The technique consists of supplementating 30 µL of semen with 300 µL of 100 mOsm hypo-osmotic solution (75 mM fructose and 25 mM sodium citrate in distilled water) and incubating it at 37°C for 60 min. Following incubation, 5 µL of the mixture was placed on a warmed glass slide (37°C) under a cover glass. Swelling sperm and/or sperm with curled tails were recorded as having intact plasma membranes using a bright-field microscope (Nikon, Tokyo, Japan) under ×400 magnification. A total of 400 spermatozoa were measured on the four slides (Buckett *et al.*, 1997).

Biochemical assay

The samples were thawed before the biochemical assays. One aliquot (500 µL) of each semen sample was centrifuged at 800 ×g for 10 min; the sperm pellets were separated and washed with PBS and then resuspended and recentrifuged (3 times). Following the last centrifugation, 1 mL of deionized water was added to the sperm, which was then snap-frozen and stored at –70°C until further analysis.

The LPO of the sperm was estimated by measuring the level of malondialdehyde (MDA) using thiobarbituric acid (TBA). MDA levels were measured after supplementating 100 µL of sperm-cell pellet suspension with TBA-TCA reagent (1.25 mL of 20% w/v trichloroethanoic acid (TCA), 1 mL of 0.2% w/v TBA, and 1.25 mL of 0.05 M H_2SO_4). The mixture was treated in boiling water (95°C) for 60 min. Butanol (4 mL) was added into the mixture and it was centrifuged at 1,500 g for 5 min. The supernatant was removed and the absorbance was measured at 535 nm. The MDA concentration was determined using the specific absorbance coefficient (1.56×10^5 µmol cm^{-3}). The MDA was expressed as pmol mg^{-1} protein of the pelleted sperm cells (Okaha, 1979).

Oxidation of NADPH to $NADP^+$, indicating GPx activity, is associated with a decrease in light absorbance of 340 nm recorded by a spectrophotometer. The rate of decrease in absorbance at 340 nm is directly proportional to GPx activity. The reaction mixture contained 20 µL of sample (3 mg mL^{-1} sperm pellet cells), 1 mL of solution (4 mM glutathione, 5×10^{-5} U mL^{-1} glutathione reductase, 0.34 mM NADPH, 0.05 mM phosphate buffer at pH 7.2, and 4.3 mM EDTA) and 40 µL of cumene hydroperoxide. The light absorbance of the mixture was measured 20 min after using a Biophotometer plus (Eppendorf, Germany) at 340 nm. The GPx value was expressed as mU mg^{-1} protein of the pelleted sperm cells (Paglia & Valentine, 1967).

The superoxide dismutase (SOD) activity was determined using a method where xanthine and xanthine oxidase are employed to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. SOD activity was measured by the degree of inhibition of this reaction. One unit of SOD causes a 50% inhibition in the rate of reduction of INT under the conditions of the assay.

For each standard, the reaction mixture (S) consisted of 30 µL of sample (pelleted sperm cells), 1 mL reagent

(0.05 mM xanthine, 0.025 mM INT, 40 mM CAPS at pH 10.2, and 0.94 mM EDTA) and 150 µL of xanthine oxidase. A cuvette containing a mixture of sample was assayed by bio-photometer at 505 nm wavelength in two steps: (1) the initial absorbance after 30 s; and (2) the second absorbance 3 min after adding xanthine oxidase. The control was also evaluated. The SOD value was expressed as U mg^{-1} protein in the pelleted sperm cells (Misra & Fridovich, 1972).

Statistical analyses

Statistical analyses were performed using SAS software (version 9.1.3). Semen quality data (total motility, progressive motility, viability, membrane integrity) was subjected to arcsine transformation before being analyzed; all the data obtained from this study was analyzed using the GLM procedure. The least square means were calculated to determine the differences between the experimental treatments for the post-thaw evaluation times (0 and 2 h incubation at 37°C).

Results

PGM, TM, VCL, VAP, VSL, viability, and membrane integrity (positive responses to HOST) were evaluated at 0 and 2 h of incubation following freeze-thawing. Biochemical assays were performed immediately after freeze-thawing of semen samples.

The effect of ROM dosage and addition of GSH to the extender on post-thaw TM, PM, VCL, VAP, VSL, viability, and integrity of plasma membrane for bull spermatozoa at 0 and 2 h after thawing are shown in Table 1. PGM did not significantly increase for any treatments at 0 and 2 h after thawing. The highest levels of TM were observed for treatment III compared to treatment I and the control group ($p < 0.05$) at 0 h. Treatment V showed a significantly higher percentage of TM over the control ($p < 0.05$) at 2 h. Supplementation of the medium with 10 g L^{-1} ROM significantly increased VAP over the other treatments ($p < 0.01$) at 0 h. At 2 h, VAP was higher ($p < 0.01$) for treatment V than the control. No significant differences were observed between treatments at 0 h and 2 h for VCL and VSL.

The addition of all treatments (ROM, GSH, ROM + GSH) to the semen extender increased the viability and membrane integrity at both evaluation times. At 0 h, the highest HOST values were obtained for treatments III and V over the control ($p < 0.05$). The HOST values for treatment V were significantly higher at 2 h ($p < 0.05$).

The highest values for viability were obtained for treatment III over the control ($p < 0.05$) at the 0 h; no significant differences were found among the other treatments. Viability was highest for treatments I and

Table 1. Effect of different concentrations of rosemary extracts (ROM) and reduced glutathione (GSH) on some parameters after freeze-thawing at 0 and 2 h incubation in bull spermatozoa

ET	Variables	C	I	II	III	IV	V	RSD	*p*-value
0 h	PGM (%)	32.7	34.3	38.2	39.2	35.9.7	36.3	5.0	0.351
	TM (%)	46.9^{b}	50.1^{b}	51.8^{ab}	59.4^{a}	50.8^{ab}	52.3^{ab}	4.9	0.033
	VAP (µm s^{-1})	73.9^{b}	76.2^{b}	79.6^{ab}	86.2^{a}	74.6^{b}	77.2^{ab}	5.3	0.005
	VCL (µm s^{-1})	99.4	102.8	103.4	105.7	100.4	103.8	4.5	0.192
	VSL (µm s^{-1})s	55.9	57.8	59.3	63.4	56.8	56.4	6.5	0.383
	HOST (%)	48.7^{b}	51.7^{bc}	53.9^{abc}	60.2^{a}	50.9^{abc}	56.0^{ac}	4.9	0.028
	Viability (%)	51.9^{b}	52.2^{b}	56.8^{ab}	59.0^{a}	56.5^{ab}	54.7^{ab}	5.0	0.042
2 h	PGM (%)	17.2	20.3	19.8	21.0	20.4	21.8	2.9	0.192
	TM (%)	28.4^{b}	32.7^{ab}	33.3^{ab}	33.3^{ab}	33.7^{ab}	40.2^{a}	5.0	0.030
	VAP (µm s^{-1})	43.0^{b}	46.0^{ab}	43.9^{ab}	47.1^{ab}	45.7^{ab}	50.7^{a}	3.6	0.018
	VCL (µm s^{-1})	73.2	77.6	75.8	75.9	76.8	78.7	4.8	0.482
	VSL (µm s^{-1})	35.3	37.8	36.2	39.3	37.6	40.8	4.4	0.307
	HOST (%)	33.6^{b}	37.6^{ab}	35.7^{ab}	36.3^{ab}	37.8^{ab}	41.9^{a}	3.5	0.018
	Viability (%)	34.4^{c}	40.8^{ab}	35.9^{bc}	39.2^{abc}	38.7^{abc}	42.1^{a}	4.5	0.031

ET: evaluation time. PGM: progressive motility. TM: total motility. VAP: average path velocity. VCL: curvilinear velocity. VSL: straight linear velocity. HOST: hypo osmotic swelling test. C: control. I: 5 mM GSH. II: 5 g L^{-1} of rosemary extract. III: 10 g L^{-1} of rosemary extract. IV and V: combination of rosemary extract (5 and 10 g L^{-1}, respectively) with 5 mM GSH. RSD: residual standard deviation. * Different superscripts (^{a,b}) in the same row indicate a significant difference ($p < 0.05$).

Table 2. Effect of different concentrations of ROM and GSH on some lipooxidative parameters after freeze-thawing in bull spermatozoa

Variables	C	I	II	III	IV	V	RSD	*p*-value
MDA (pmol mg^{-1} Pro)	12.6^{b}	10.9^{ab}	12.4^{b}	10.0^{a}	11.6^{ab}	9.4^{a}	1.54	0.015
GPX (mU mg^{-1} Pro)	58.6^{b}	64.8^{a}	61.9^{ab}	64.9^{a}	59.2^{ab}	65.5^{a}	4.1	0.020
SOD (U g^{-1} Pro)	14.6	16.2	16.7	16.1	15.9	17.4	2.6	0.550

MDA: malonaldialdehyde. GPX: glutathione peroxidase. SOD: superoxide dismutase. C: control; I: 5 mM GSH. II and III: 5 and 10 g L^{-1} of rosemary extract, respectively. IV and V: combination of rosemary extract (5 and 10 g L^{-1}, respectively) with 5 mM GSH. RSD: residual standard deviation. * Different superscripts (^{a,b}) in the same row indicate a significant difference ($p < 0.05$).

V at 2 h compared to the control group ($p < 0.05$). There was a significant difference between treatments V and II at 2 h ($p < 0.05$), but no significant differences were observed between the other treatments.

The effects of ROM and GSH on LPO and antioxidant activity in freeze-thawed bull semen are summarized in Table 2. The results show that MDA levels were lowest for treatments III and V over the other treatments ($p < 0.05$). The highest GPx activity was observed for treatments I, III, and V ($p < 0.05$) over the control group. SOD did not significantly differ between control and the other treatments.

Discussion

During semen cryopreservation, spermatozoa function is affected by three major factors: temperature change, formation of ice crystals in the extracellular environment, and osmotic and/or toxic stress (Watson, 2000). Biochemical and biophysical changes associated with cryopreservation result in decreased motility and viability rates and, ultimately, impaired fertility of semen are unavoidable.

ROS has a negative effect on cryopreserved spermatozoa function (Wiechetek & Slaweta, 1987; Gadea *et al.*, 2004; Roca *et al.*, 2005; Bucak *et al.*, 2008; Shoae & Zamiri, 2008; SarIözkan *et al.*, 2009; Bansal & Bilaspuri, 2011; Cocchia *et al.*, 2011; Hu *et al.*, 2011; Ashrafi *et al.,* 2013). It has been suggested that the application of antioxidants such as cysteine, taurine, alpha-tocopherol (vitamin E), superoxide dismutase, catalase, glutathione, butylated hydroxytoluene and melatonin into the semen media can have beneficial effects on decreasing ROS production. Recently, researchers have focused on the use of natural antioxidants to avoid the healthy and toxicity problems originating from synthetic antioxidants in the processing and packaging of foods that contain lipids.

Little information is available on the effect of ROM on oxidative parameters, including antioxidant enzyme activity and LPO in cryopreserved bull spermatozoa. However some studies have reported positive effects of ROM and other natural herbs (*Rhodiola sacra*) using aqueous supplementation prior to cryopreservation for ovine, boar, and canine semen (Zhao *et al.*, 2009; Gil *et al.*, 2010; González *et al.*, 2010; Malo *et al.*, 2010, 2011). In the current study, different combinations of ROM and GSH were applied for bull semen based on the data obtained from previous studies (Zhao *et al.*, 2009; Gil *et al.*, 2010; González *et al.*, 2010; Malo *et al.*, 2010, 2011; Gadea *et al.*, 2011; Perumal *et al.*, 2011).

Although different factors cause spermatozoa defects during cryopreservation, the overproduction of ROS is known to play a major role in this regard (Sikka, 1996). Excessive production of ROS may produce high levels of peroxides and free radicals, which affect LPO and the spermatozoa membrane. A high level of ROS may decrease sperm motility, viability, plasmalemma integrity, and protein denaturation (Farber *et al.*, 1990; White, 1993; Agarwal & Said, 2005). Another important effect of LPO is irreversible leakage of intracellular enzymes (White, 1993). The presnt study showed that supplementation of TEY with ROM (treatment III) and in combination with GSH (treatment V) significantly decreased LPO and increased GPX activity.

SOD activity showed no significant change across treatments. Previous studies have reported that H_2O_2 has a more adverse impact on frozen-thawed sperm than O_2^- does. Bilodeau *et al.* (2002) and Roca *et al.* (2005) believe that TEY extender has a limited capacity to neutralize H_2O_2 after thawing for bull semen. It appears that treatments III and V maintained intact membrane lipid composition by increasing GPX activity or decreasing the production of H_2O_2.

Studies have reported that ROM and RSAE significantly decrease the MDA concentration of cryopreser-

ved boar spermatozoa (Zhao *et al.*, 2009; Malo *et al.*, 2011). Zhao *et al.* (2009) reported a direct correlation between the concentration of RSAE and the GSH in sperm cells. They showed that decrease of LPO levels was associated with the increase of the GSH and GPx activity when the freezing medium of boar semen was supplemented with RSAE (Zhao *et al.*, 2009). The main cause of the LPO decrease and GPx increase is unknown, and the active ingredients of ROM and their functional mechanisms on sperm cells organelles are not clear.

Spermatozoa kinematic parameters decreased dramatically in the presence of high levels of ROS following cryopreservation (Bansal & Bilaspuri, 2011). GSH scavenged hydrogen peroxide, which is generated by aerobic energy production systems of sperm cells, and prevented an LPO cascade (Baker & Aitken, 2004).

It has been reported that the addition of GSH to freezing extender prevents the harmful effects of the freeze-thaw process. Moreover, the addition of GSH to semen freezing extender results in a significant improvement in the oocyte penetration rate, *in vitro* embryo production, decrease in DNA fragmentation, conception rate, plasmalemma integrity and motility in bovine spermatozoa (Gadea *et al.*, 2008; Perumal *et al.*, 2011) and in the motility of boar spermatozoa (Gadea *et al.*, 2005). Earlier studies indicate that spermatozoa kinematic variables such as motility, VCL, VAP, and VSL correlate with the bull semen conception rate and fertility (Al-Qarawi *et al.*, 2002; Perumal *et al.*, 2011).

The results of the present study show that ROM supplementation (treatments III and V) helped maintain bull sperm TM and VAP. These treatments showed better results than the GSH treatment, especially treatment III, at 0 h. Since this study differs from others in terms of animal species, type of extender, source and conditional harvesting of ROM, it cannot be directly compared with the findings of other studies. It is believed that a decrease in sperm motility following freeze-thawing is primarily related to damaged mitochondria (Ruiz Pesini *et al.*, 2001); thus, the small increase in motility in this study is probably a result of improvement in mitochondrial function for treatments III and V. Future studies should focus on treatment effects on mitochondrial function.

Acording to previous studies there is an evidence of a negative correlation between motility and the LPO rate (Kasimanickam *et al.,* 2007), which is consistent with the findings of the present study; it may be due to simultaneous improvment of LPO and motility for cryopreserved spermatozoa following treatments III and V.

The freeze-thawing process alters the sperm membrane, changes the transfer of proteins and, subsequently, alters permeability to water, sugars, and electrolytes. These interactions decrease the percentage of viable spermatozoa (Bailey *et al.*, 2003). The present study showed that inclusion of ROM (treatment III) and its combination with GSH (treatment V) improved the viability and membrane integrity rates successfully in frozen-thawed spermatozoa.

Previous reports have indicated that there is a negative correlation between MDA production and sperm viability and sperm function (Aitken *et al.*, 1989; Guthrie & Welch, 2012). The results of the present study showed that the addition of ROM and its combination with GSH probably protected the lipids, proteins and other ultra-structure compounds of the cell membrane against cryo-damage. The increased rate of viability and membrane integrity observed after treatments III and V may have contributed to the decrease in MDA production and electrolyte transportation disorders in sperm cells following cryopreservation.

Carnosic acid and carnosol are phenolic diterpenes of the abietane type, and rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactate (phenolic depside). These substances in rosemary extract contain prominent antioxidant, anti-inflammatory and cytotoxic roles (Cheung & Tai, 2007; Bai *et al.,* 2010; Mulinacci *et al.,* 2011). In the present study, the improvment in some sperm characteristics upon supplementation of ROM may be the result of the presence of these substances in ROM. The inclusion of 10 g L^{-1} of ROM (treatment III) significantly increased TM, VAP, viability and HOST values at 0 h over the control; however, this was not observed at 2 h. Interestingly, supplementation of 5 mM GSH with 10 g L^{-1} of ROM (treatment V) resulted in higher values for these parameters at the 2 h, but not at 0 h. These findings suggest that ROM alone does not improve sperm characteristics.

Based on previous studies for bull sperm, levels of the intracellular antioxidants glutathione (58-78%) and superoxide dismutase (50%) decreased or disappeared during cryopreservation (Bilodeau *et al.*, 2000; Stradaioli *et al.*, 2007). It appears that the limited amount of glutathione in treatment III (ROM) and the depletion of glutathione in the sperm cells produced the different results at 0 h and 2 h. This deficiency at 2 h decreased

with the addition of GSH and ROM (treatment V). Treatment V did not improve sperm characteristics at 0 h of incubation, perhaps because of the limited exposure time of sperm cells to GSH. The interval from antioxidant supplementation to initial evaluation (0 h) may have been too short to demonstrate the effectiveness of rosemary and glutathione. In treatment V, GSH apparently supplements the role of ROM in decreasing or regulating oxidative factors in the semen freezing medium.

The addition of GSH had a limited effect on sperm quality over that of the control. This antioxidant increased viability, membrane integrity and GPx activity; however, it had little protective effect on the maintenance of LPO in sperm cells after thawing. Because intercellular antioxidants decrease during cryopreservation, the supplementation of only one antioxidant may not be sufficient for the regulation of total ROS in the sperm cells. The present study found that ROM (10 g L^{-1}) had a considerably better effect on TM, VAP, viability, and membrane integrity than GSH and that ROM has a high capacity for strengthening the sperm defensive system against cryodamage over that of GSH.

In conclusion, the addition of ROM alone (10 g L^{-1}) and in a combination with GSH (5 mM GSH + 10 g L^{-1} ROM) significantly increased intracellular defense systems and cell membrane compounds against the ROS production following cryopreservation. The addition of ROM (10 g L^{-1}) significantly improved sperm survival rate and motility. Further studies are needed to determinate clinical and *in vitro* applications for these antioxidants.

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