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Anti-oxidative Effects of Ethanol Extract of *Origanum vulgare* on Kinetics, Microscopic and Oxidative Parameters of Cryopreserved Holstein Bull Spermatozoa

Research Article

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ABSTRACT

Origanum vulgare contains high levels of phenolic compounds such as gallic acid and polyphenols such as rosmarinic acid and quercetin. The purpose of this study was to investigate the effects of *Origanum vulgare* extract, as a natural antioxidant, on freezing-thawing semen quality in Holstein bulls. Three Holstein bulls (5-6 years old, mean live weight 800 kg) were used for semen collection twice a week for two months. Ethanol extract of *Origanum vulgare* (2, 4, 8, 12, 16 and 20 mL/dL extender) was added to a citrate-yolk-base extender. After freezing-thawing sperm motility parameters, viability and membrane integrity were determined using a CASA system, eosin-nigrosin staining, and hypo-osmotic swelling test, respectively; malondialdehyde concentration and activity of superoxide dismutase and catalase were also measured. The percentages of motility were higher ($P<0.05$) in the freezing extender containing 4 mL/dL *Origanum vulgare* extract (72.34±7.98). Addition of 2 and 4 mL/dL extract of *Origanum vulgare* significantly improved the motility, viability and plasma membrane integrity of the spermatozoa following freezing-thawing process compared to the control group. Addition of 4 mL/dL extract significantly reduced the concentration of malondialdehyde compared to the control group ($P<0.05$). The activity of superoxide dismutase and catalase increased significantly by inclusion of 4 and 8 mL/dL extract of *Origanum vulgare* to the extender (1.86±0.18 and 1.92±0.28 U/mg protein; 4.54±0.13 and 4.28±0.28 U/mg protein, respectively). The activity of superoxide dismutase and catalase was significantly increased by inclusion of 4 and 8 mL/dL extract to the extender. In conclusion, addition of 2 and 4 mL/dL extract of *Origanum vulgare* to the semen extender improved the post-thawed quality of semen, which may be due to increasing in antioxidant enzyme activity and reduction in lipid peroxidation.

KEY WORDS antioxidant, bull sperm, cryopreservation, *Origanum vulgare*.

INTRODUCTION

The cryopreservation of spermatozoa has provided special opportunities for the preservation of genetic resources and improving breed programs by the artificial insemination technique (Holt, 1996). Nowadays, semen cryopreservation has many applications such as solving problems of infertility, some diseases, conservation semen and DNA conservation of some important species (Barbas and Mascarenhas, 2009). However, sperm cryopreservation stimulates intracellular ice crystals formation, increasing osmotic and chilling injury that causes sperm cell damage (Isachenko *et al.* 2003). Freezing and thawing processes impose physical and chemical insults on the sperm membrane that decrease sperm viability and fertilizing ability (Alvarez and Storey, 1992). Both damages are associated with excessive genera-

tion of reactive oxygen species (ROS) and peroxidation of the phospholipids in the membrane (Wang *et al.* 1997; Lasso *et al.* 1994). The imbalance between ROS production and biological systems that control free radicals is the main cause of damage to the membrane and structural composition of sperm (Bilodeau *et al.* 2000).

Mammalian semen contains antioxidant compounds including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GSH-PX), taurine and hypotaurine that protect the sperm against peroxidative damage (Bilodeau *et al.* 2000; Zini *et al.* 1993). However, the endogenous anti-oxidative capacity may be insufficient to prevent lipid peroxidation (LPO) during cooling storage of sperm; therefore, the use of antioxidants during the freeze-thaw process may be beneficial (Aurich *et al.* 1997). Using natural antioxidants is increasing because of the toxicity problems of synthetic antioxidants such as butylated hydroxyl toluene (BHT), propylgalate (PG), butylatedhyd roxylanisole (BHA) and tertiary butyl hydroquinone (TBHQ) that are commonly used in lipid-containing foods. However, there are limited data regarding the effects of natural antioxidants on spermatozoa (Yanishlieva and Marinova, 1996).

Origanum vulgare, (Lamiaceae) is an aromatic plant with a wide distribution throughout Asia and especially in Iran. *Origanum vulgare* contains some aqueous compounds such as rosmarinic acid, eriocitrin, luteolin-7-oglucoside, apigenin-7-o-glucoside (Kulišić *et al.* 2007), origanol A and B (Matsuura *et al.* 2003) and ursolic acid (Heo *et al.* 2002). Rosmarinic acid and origanol A and B, the most important components of the aqueous extract of *Origanum vulgare* have anti-oxidative activities (Matsuura *et al.* 2003; Kulišić *et al.* 2007).

No study has reported the effects of *Origanum vulgare* extract in semen extenders against cryo-damage to bull sperm. The present study was conducted to determine the effect of an ethanol extract of *Origanum vulgare* in the semen extender on sperm motility parameters, viability, plasma membrane integrity, total antioxidant capacity (TAC) and LPO, as well as antioxidant activities in terms of superoxide dismutase (SOD) and catalase (CAT), in post-thawed bull semen.

MATERIALS AND METHODS

Preparation of *Origanum vulgare* extract

Collected *Origanum vulgare* plants were dried for 10 days at room temperature and powdered by an electric mill. Fifty grams of the plant powder was soaked in 60% ethanol for 24 hours and the mixture was filtered. Soxhlet apparatus was used to evaporate organic solvent and the concentrated material was obtained as extracts and maintained at 4 ˚C until used. Different concentrations of *Origanum vulgare* extract were prepared in accordance with the protocol of Dehghan *et al.* (2007).

Semen source and preparation

This experiment was performed at the Animal Breeding Center located in Tabriz city, northwest of Iran. Ejaculates were collected from three Holstein bulls (5-6 years old, mean live weight of 800±50 kg), regularly used for breeding purpose, based on their fertility estimation through *in vitro* tests, including viability and motility evaluation and field fertility tests. Semen samples were collected twice a week for two months by an artificial vagina (45 ˚C). The ejaculates were immediately transferred to the laboratory and submerged in a water bath (34 ˚C), until semen evaluation was done. The volume of the ejaculate was estimated in a conical tube graduated at 0.1 mL intervals. Sperm concentration was determined by means of an Accucell photometer (IMV Technologies, L'Aigle, France). The ejaculates meeting the following criteria were used: volume between 5 and 10 mL; sperm concentration $\geq 1 \times 10^9$ sperm mL^{-1}; percentage of motile sperm $\geq$ 70%; and $\leq$ 10% abnormal sperm. The ejaculates were pooled to compensate for within-individual variations in seminal quality. A citrate-egg yolk extender containing sodium citrate dihydrate (2.9 g dL^{-1}), penicillin (1000 IU mL^{-1}), streptomycin (1000 µg mL^{-1}), 25% hen egg yolk, 7% glycerol and double-distilled water (to 100 mL) was used (Ashrafi *et al.* 2011).

The extender was divided into two parts (A and B); 3% and 11% (v/v) glycerol were added to part A and part B, respectively. Part A was warmed to 37 ˚C while part B cooled to 5 ˚C. *Origanum vulgare* extract was added to part A at 0, 2, 4, 8, 12, 16 and 20 mL/dL. The pooled ejaculate was divided into seven equal aliquots and diluted in the semen extender containing the extract, at a final concentration of 1×10^8 sperm per mL. This was then cooled slowly to 5 ˚C, mixed with part B, packaged in 0.5 mL straws, sealed and frozen on liquid nitrogen vapor at approximately −15 ˚C min^{-1} from +5 to −150 ˚C. Then straws were stored in a liquid nitrogen tank. Frozen straws were thawed at 37 ˚C for 30 s in a water bath for microscopic evaluation (Ashrafi *et al.* 2011).

Semen evaluation

Viability evaluation

Sperm viability was assessed by means of the nigrosin–eosin staining method. The final composition of the stain was: eosin-Y 1.67 g, nigrosin 10 g and sodium citrate 2.9 g, dissolved in 100 mL of distilled water. A sub-sample (5 µL) of frozen-thawed semen was placed on a pre-warmed slide and mixed with 5 µL nigrosin-eosin stain and spread into a uniform smear (Mahmood and Ijaz, 2006). After air-

drying, the smear was observed under a microscope (Nikon, Japan) at 1000 × magnification, using immersion oil to count the live (unstained) and dead (stained/partial stained) sperm.

Evaluation of motility parameters

For evaluating the motility parameters, sperm samples were incubated after thawing for about 5 min at 37 ˚C. A computer-assisted sperm motility analysis (CASA, Hoshmand Fanavaran, Version 6, Amirkabir Medical Engineering Co., Tehran, Iran) was used to analyze sperm motion characteristics. A 5 µL of diluted semen was transferred onto a pre-warmed microscope slide (37 ˚C) and covered by a cover slip. The following variables were obtained: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN, %). At least 200 spermatozoa were assessed in each CASA analysis.

Assessment of sperm membrane integrity

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails, where 50 µL of thawed semen was mixed with 500 µL of a 100 mOsM hypo-osmotic solution (9 g fructose+4.9 g sodium citrate per liter distilled water) and was incubated at 37 ˚C for 60 min. Then, 5 µL of the mixture was spread with a cover slip on a warm slide at 37 ˚C. A total of 200 sperm were evaluated using bright-field microscopy (400×magnification) and the sperm with swollen or coiled tails were recorded (Revell and Mrode, 1994).

Biochemical assay

Frozen straws were thawed for 60 seconds at 37 ˚C for biochemical measurements. Five-hundred µL of semen sample was centrifuged at 800 × g for 10 min, the supernatant was removed and the pellet washed with phosphate buffered saline (PBS) (pH 7.4). This procedure was repeated for three times. After the last centrifugation, 1mL of deionized water was added to the pellet and was snap-frozen and stored at -70 ˚C until further analysis (Roca *et al.* 2004).

Concentration of MDA (MDA), as an indicator of lipid peroxidation in the semen sample, was measured using the thiobarbituric acid reaction as described by Placer *et al.* (1996).

The absorbance of the supernatant was read using a spectrophotometer (UV-1200, Shimadzu, Japan) at 532 nm.

Superoxide dismutase (SOD) was measured according to the method described by Ukeda *et al.* (1997). The reaction is dependent on the presence of superoxide anions that cause pyrogallol oxidation. The inhibition of pyrogallol oxidation by SOD was monitored using a spectrophotometer at 420 nm and the amount of enzyme producing 50% inhibition was defined as one unit of enzyme activity (U/mg protein). Catalase (CAT) was measured by monitoring the decomposition of hydrogenperoxide (Aebi, 1984).

Statistical analysis

Each treatment was replicated 5 times. The experiment was done by completely randomized design. Data were analyzed by SAS (2001) software using the GLM procedure. The Tukey-Kramer's test was used for mean comparisons. Data were expressed as mean ± SD. The probability values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

The effects of *Origanum vulgare* extract in the freezing extender on post-thaw motility parameters, viability and membrane integrity in bull sperm are presented in Table 1. The percentages of TM were higher (P<0.05) in the freezing extender containing 4 mL/dL *Origanum vulgare* extract (72.34±7.98).

The percentages of PM, LIN and VAP were higher (P<0.05) in the extender containing 4 mL/dL extract (64.91±5.62; 62.25±1.19 and 37.75±3.39, respectively). For parameters of VCL the highest performance (P<0.05) was observed at 2, 4 and 8 mL/dL of extract. Addition of 2 and 4 mL/dL extract of *Origanum vulgare* to the extender significantly improved VSL parameter compared to the control group (37.98±5.77 and 40.11±2.68, respectively). The ALH values were not different (P<0.05) between treatments.

Addition of 2 and 4 mL/dL extract of *Origanum vulgare* to the extender improved (P<0.05) the viability parameter (73.91±4.85 and 79.13±6.55) and addition of 4 mL/dL extract to the extender improved (P<0.05) the sperm plasma membrane integrity compared to the control group. The effects of *Origanum vulgare* extract on oxidative parameters in thawed bull semen are summarized in Table 2. Inclusion of 4 mL/dL extract of *Origanum vulgare* significantly resulted in lower concentration of MDAMDA compared to the control group (15.69±0.76 versus 18.16±1.08 nmol/dL). On the other hand, with increasing extract levels, MDAMDA concentration increased, therefore adding of 20 mL/dL of extract (P<0.05) increased MDAMDA concentrations compared to the control group (20.19±1.61 *vs.* 18.16±1.08 nmol/dL). The activity of SOD and CAT increased significantly by inclusion of 4 and 8 mL/dL extract of *Origanum vulgare* to the extender (1.86±0.18 and 1.92±0.28 U/mg protein; 4.54±0.13 and 4.28±0.28 U/mg protein, respectively). Sperm cooling causes some functional and structural damages to sperm in a time-dependent process in most species.

Table 1 Mean (±SEM) percentages of sperm motion parameters, viability and membrane integrity of frozen–thawed Holstein bull sperm at different levels of *Origanum vulgare* extract

Parameters	Levels of *Origanum vulgare* extract (mL/dL)						
	Control	2	4	8	12	16	20
TM (%)	56.56^{bc}±6.51	68.79^{ab}±4.53	72.34^{a}±7.98	59.03^{ab}±11.62	49.92^{bcd}±11.71	40.69^{cd}±8.66	33.55^{d}±5.51
PM (%)	49.18^{bc}±4.25	57.12^{ab}±5.45	64.91^{a}±5.62	49.78^{bc}±5.78	41.15^{cd}±10.61	33.53^{de}±9.14	24.05^{e}±6.49
VCL (µm/s)	42.34^{b}±0.99	52.32^{a}±3.61	52.63^{a}±4.97	50.02^{a}±7.11	42.83^{b}±9.07	36.97^{bc}±6.39	30.42^{c}±3.59
VSL (µm/s)	29.11^{bc}±1.47	37.98^{a}±5.77	40.11^{a}±2.68	34.28^{ab}±3.74	28.28^{bc}±6.47	23.22^{cd}±4.24	17.81^{d}±3.15
VAP (µm/s)	27.77^{bcd}±3.09	35.39^{ab}±6.81	37.75^{a}±3.39	31.68^{abc}±3.52	25.92^{cde}±3.52	22.91^{de}±5.81	18.04^{e}±2.08
ALH (µm)	1.94^{ab}±0.07	2.34^{a}±0.18	2.31^{a}±0.23	2.15^{ab}±0.38	2.11^{ab}±0.32	1.92^{ab}±0.16	1.86^{b}±0.21
LIN (%)	54.58^{bc}±3.75	58.11^{ab}±6.44	62.25^{a}±1.19	52.48^{bcd}±1.77	49.01^{cd}±4.65	46.68^{d}±0.81	38.94^{e}±2.51
Viability (%)	66.31^{cd}±1.95	73.91^{ab}±4.85	79.13^{a}±6.55	69.13^{bc}±6.65	60.82^{d}±7.74	50.99^{e}±5.09	46.06^{e}±2.66
HOST (%)	63.45^{bc}±3.59	71.92^{ab}±4.15	76.42^{a}±6.65	65.33^{abc}±7.37	57.03^{cd}±5.58	48.25^{de}±6.25	41.97^{e}±4.66

TM: total motility (%); PM: progressive motility (%); LIN: linearity (%); ALH: amplitude of lateral head displacement (µm); VCL: curvilinear velocity (µm/s); VAP: average path velocity (µm/s) and HOST: hypo-osmotic swelling test (%).
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 Mean (±SEM) of the oxidative parameters in frozen-thawed bull semen at different levels of *Origanum vulgare* extract

Parameters	Levels of *Origanum vulgare* extract (mL/dL)						
	Control	2	4	8	12	16	20
MDA (nmol/dL)	18.16^{ab}±1.08	17.31^{bc}±1.69	15.69^{c}±0.76	18.57^{ab}±1.79	18.82^{ab}±2.03	20.04^{a}±1.69	20.19^{a}±1.61
SOD (U/mg protein)	1.46^{bc}±0.13	1.62^{abc}±0.26	1.86^{a}±0.18	1.92^{a}±0.28	1.32^{c}±0.18	1.25^{c}±0.19	1.23^{c}±0.21
CAT (KU/mg protein)	0.12±3.81^{b}	3.88^{b}±0.13	4.54^{a}±0.13	4.28^{a}±0.28	3.72^{b}±0.24	3.61^{b}±0.25	3.53^{b}±0.24

MDA: malondialdehyde; SOD: activity level of superoxide dismutase and CAT: catalase.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

For preventing these damages, the semen needs to be diluted with appropriate extenders for freezing-storage (Buyukleblebici *et al*. 2014). *In vitro* studies have shown that antioxidants remove free radicals such as superoxide ion, hydrogen peroxide, hydroxyl radicals and peroxyl (Ashrafi *et al*. 2013). Due to the ability of phenolic compounds to act as chelating in binding the hydroxyl group and metal ions, they inhibit the activity of free radicals (Osawa, 1994). Antioxidant activity of *Origanum vulgare* extract against lipid peroxidation has been demonstrated by several researchers (Vekiari *et al*. 1993). Several studies have shown that *Origanum vulgare* contains phenolic compounds and flavonoids, especially rosmarinic acid, quercetin, kaempferol, apigenin, rutinand origanol which can cleanse free radicals such as superoxide anion and hydroxyl (Kikuzaki and Nakatani, 1989; Cervato *et al*. 2000). In the current study, sperm motion parameters as well as sperm viability increased with inclusion of 2 and 4 mL/dL *Origanum vulgare* extract in the semen extender. The peroxidation of sperm lipids damages the structure of lipid matrix in the spermatozoa membranes and it is related to quick loss of intracellular ATP leading to axonemal damage and reduced sperm viability (Sanocka and Kurpisz, 2004).

There is a strong correlation between ROS production and decreased sperm motility (Armstrong *et al*. 1999), so it has been determined that peroxide (H_2O_2) radical can be distributed across the sperm membranes and there is an inhibited activity of key enzymes such as glucose-6-phosphate dehydrogenase (Aitken *et al*. 1997).

This enzyme controls glucose concentration through diverting path of hexose monophosphate and NADPH activity which have major role in ATP production and sperm motility (Aitken *et al*. 1997). The results of the present study showed that inclusion of 4 mL/dL *Origanum vulgare* extract in the freezing media increased plasma membrane integrity of frozen-thawed bull sperm. Previous work (Cervato *et al*. 2000) have shown that *Origanum vulgare* extract is effective in prevention of the peroxidative procedure by counteracting different type of free radicals in the first step, then stopping peroxidation catalysis by iron-chelating and iron-oxidizing properties and at last pausing lipid-radical chain reactions. Another advantageous activity was glycosylation of lipoproteins which is directly related to their peroxidation (Cervato *et al*. 2000). Several studies reported a negative correlation between the MDA production and sperm viability (Guthrie and Welch, 2012). The MDA production is usually used to determine LPO in various cell types including sperm cells (Sikka, 1996).

The semen antioxidant system having both enzymatic and non-enzymatic antioxidants prevents or limits peroxides formation. Inadequate amounts of antioxidants, or the prevention of antioxidant enzymes, increases oxidative stress, damaging spermatozoa. One of the byproducts of lipid peroxides decomposition is MDA, which is commonly used in biochemical assays to show the degree of peroxidative damage produced by spermatozoa (Najafi *et al*. 2014).

The results of present study showed that in the freezing extender containing 4 mL/dL *Origanum vulgare* extract

MDA concentration was significantly lower than that of the control group. Phenolic compounds (especially flavonoids) can change peroxidation kinetics by modifying the lipid packing order. They also stabilize membranes by reducing membrane fluidity and inhibit free radicals diffusion and restrict peroxidative reaction (Arora *et al*. 2000; Blokhina *et al*. 2003). Our data are in agreement with the finding Zhao *et al*. (2009), who found a significant correlation between Rhodiola sacra aqueous extract concentrations and MAD in frozen-thawed boar semen.

Membrane permeability increases after cooling and this may be due to the increased membrane leakiness and specific protein channels. Calcium regulation is influenced by cooling and this has harmful effects on cell function, consequently cell death. Calcium absorption during cooling affects capacitation and fusion events between plasma membrane and acrosomal membrane (Purdy, 2006). Peroxidation of polyunsaturated fatty acids in sperm cell membranes is an autocatalytic, self-propagating reaction, which can increase cell dysfunction associated with loss of membrane function and integrity.

The HOST test assessed the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling driven by the hypo-osmotic treatment. Thus, this provided a form of a membrane stress-test, which is particularly useful when testing the membrane-stabilizing action of antioxidants (Sariozkan *et al*. 2015).

Flavonoids increase membranes integrity by preventing the access of deleterious molecules to the hydrophobic region of the bilayer, including those that can affect membrane stability and those that induce oxidative damage to the membrane components (Michalak, 2006).

SOD is an enzymatic biological antioxidant, which scavenges ROS, therefore controls oxidative stress in mammalian sperm. Furthermore, GSH is able to directly react with many ROS and is a co-factor for GSH-Px, catalysing toxic H2O2 and hydroperoxides reduction (Bilodeau *et al*. 2001).

In our study the activity of CAT and SOD increased by inclusion of 4 and 8 mL/dL of extract in the extender. It seems that phenolic compounds stimulate the activities of antioxidant enzymes such as SOD and CAT which reduce the number of free radicals and may also increase the production of molecules protecting sperm cells against oxidative stress (Cervato *et al*. 2000). In the present study, the most effective concentration of *Origanum vulgare* extract in microscopic evaluations of bull sperm freezing extender was 4 mL/dL. On the other hand, almost for all measured parameters, inclusion of 16 and 20 mL/dL extract in the freezing extender can counteract the ROS-induced oxidative stress. So it may impede the ROS-associated functions of sperm (Roca *et al.* 2004) and it can be due to the osmotic changes, pH and disturbing the balance extender compounds.

Previous studies reported that addition of rosemary extract in goat (Zanganeh *et al*. 2013) and boar (Malo *et al*. 2010; Malo *et al*. 2011) semen freezing extender had beneficial effects. So freezing extender improved the post-thaw sperm quality, showing a negative significant correlation between rosemary concentration and MDA concentration and also the number of free radicals, ROS. On the other hand, the use of *Rosmarinus officinalis* in semen extender increased the sperm motility parameters, sperms viability and production of molecules protecting sperm cells against oxidative stress. In addition to the negative effects on motility, viability and LP, ROS can also damage sperm mitochondria and consequently sperm motility. A negative correlation between sperm LPO and sperm motility in cryopreserved semen has also been observed (Amini *et al.* 2015).

In another study, addition of rosemary extract (10 g L^{-1}) to bull semen freezing extender significantly improved the post-thaw quality of bull semen by means of increasing the sperm motility, viability and reducing the amount of LPO (Daghigh Kia *et al*. 2014).

The results of this study showed that rosemary extract significantly increased intracellular defense systems and cell membrane compounds against the ROS production following cryopreservation (Daghigh Kia *et al*. 2014). Effects of the antioxidant properties of rosemary are related to some compounds such as carnosic acid, carnosol, rosmarinic acid and 3,4-dihydroxyphenyllactate (phenolic depside) (Bai *et al.* 2010; Mulinacci *et al.* 2011).

In similar studies, antioxidant properties of *Rodhiola sacra* aqueous, agenus of Chinese herb have been investigated and a correlation between concentrations of this antioxidant and MDA after thawing was observed (Zhao *et al*. 2009).

Recently, the beneficial effect of *Ilex paraguayensis* was exhibited in semen cryopreservation, presenting higher percentages of total and progressive motility (Malo *et al*. 2010).

The excessive production of ROS not only causes LPO and DNA fragmentation, but also influences ATP, NADPH production and proteins phosphorylation in the spermatozoa (Cocchia *et al*. 2011). Adenosine triphosphate is necessary for sperm motility. Also, phosphorylation of proteins plays a main role in capacitation, the acrosome reaction and sperm penetration of the zonapellucida (Cocchia *et al*. 2011). Therefore, increased ROS levels may be reduced fertilizing ability of the spermatozoa (Wishart, 1982).

Our results are in agreement with other studies showing that botanical extracts could benefit mammal semen post-thaw quality. However, this is the first study reporting the use of *Origanum vulgare* as an anti-oxidative supplement in semen extender. Also, it should be noticed that the animal

species, the type of freezing extender and freezing methods of the present study were different from the mentioned studies. There is a need to research which of the different plants evaluated have a better performance, or if the use of mixes of botanical extracts can show a better performance.

CONCLUSION

Addition of *Origanum vulgare* extract to the semen extender at 2 and 4 mL/dL improved the quality of frozen-thawed bull semen, probably due to polyphenolic compounds having antioxidant activity.

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