Iranian Journal of Applied Animal Science

Antioxidant Effects of Thyme (*Thymus vulgaris*) Extract on Ram Sperm Quality during Cryopreservation

Research Article

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Received on: 12 Jul 2017
Revised on: 22 Sep 2017
Accepted on: 30 Sep 2017
Online Published on: Jun 2018

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Online version is available on: www.ijas.ir

ABSTRACT

Oxidative stress during freezing-thawing reduces sperm motility, viability, membrane functions and antioxidant capacity. *Thymus vulgaris* has antioxidant properties due to phenolic compounds. The purpose of current study was to evaluate the effect of *Thymus vulgaris* extract as a natural antioxidant on post-thawed Moghani ram sperm quality. Semen samples were collected twice a week from four rams. Samples were then pooled and diluted with Tris based extender supplemented with different concentrations of *Thymus vulgaris* (0, 2, 4, 8, 12 and 16 mL per dL diluent solutions). Following the cooling and equilibration stages for semen samples, the samples were frozen and stored in liquid nitrogen until evaluation. Following this freezing procedure, samples were thawed, and the sperm motility and velocity parameters, viability and plasma membrane integrity were evaluated. Our results showed that the addition of 2 and 4 mL/dL extracts at the extender stage increased the total motility percentages (P<0.05). Addition of 4 mL/dL extract improved progressive motility and percentages of viability compared to the control group and high levels of extract groups (P<0.05). Plasma membrane integrity of sperm in 4 and 8 mL/dL extract groups were greater (P<0.05) than the control and 12 and 16 mL/dL extract groups. Also, addition, 16 mL/dL extract had a significantly negative effect on all evaluated traits (P<0.05). In conclusion, supplementation of extender with intermediate amounts of *Thymus vulgaris* extract improves the quality of ram sperm after freezing and thawing in a dose-dependent manner.

KEY WORDS antioxidant, freeze-thawing, ram semen, *Thymus vulgaris* extract.

INTRODUCTION

Artificial insemination (AI) using frozen-thawed semen is a reproductive technique used for farm animals. The basis for this technique is semen cryopreservation that induces partially irreversible damages to sperm (Purdy, 2006), which may cause loss of sperm motility, viability, plasma membrane integrity, and ultimately male fertility (Baghshahi *et al.* 2014).

Physical and chemical damages during cryopreservation are associated with significant production of reactive oxygen species (ROS) and peroxidation of lipids in the cells membrane by free radicals such as O_2 and H_2O_2 (Wang *et al.* 1997; Sinha *et al.* 1996).

Based on this information, Curry *et al.* (1994) and Lamirande *et al.* (1997) indicated that efficient antioxidant systems should be applied for preventing of lipid peroxidation and sperm dysfunction.

All animal cells have several antioxidant systems that scavenge free radicles in order to reduce cellular damage. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) catalyze the removal of produced ROS in cells. Vitamin C, vitamin E and glutathione (GSH) as non-enzymatic defenses are also employed for protecting of cells (Youdim and Deans, 1999). Endogenous antioxidants are sufficient to prevent free radicals that are produced in the normal state in the cells but an increase in free radical production leads to oxidative damage (Bunker, 1992; Cao *et al.* 1996).

Therefore, for protecting the sperm against oxidative damage, numerous researchers have studied the effects of various synthetic and natural antioxidants on spermatozoa during cryopreservation processes. Research into safer and more effective natural antioxidant is needed because of the toxicity problems of synthetic antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tert-butyl hydroquinone (TBHQ) (Daghigh Kia *et al.* 2016). Various plant products contain antioxidant compounds such as flavonoids, tannins, coumarins, curcumanoids, xanthons, phenolics, lignans and terpenoids (Jeong *et al.* 2004). Therefore, there is increased interest among researchers in using plant products as natural antioxidants. Several studies have shown that during the freeze-thaw process of semen, the use of herbal antioxidants had positive effects on sperm quality (Zanganeh *et al.* 2013; Baghshahi *et al.* 2014; Khodaei Motlagh *et al.* 2014; Daghigh Kia *et al.* 2016).

Thyme (*Thymus vulgaris*), is a species of flowering plant, an aromatic and medicinal plant in the mint family *Lamiaceae* (Stahl-Biskup and Saez, 2004; Pournazari *et al.* 2017; Haselmeyer *et al.* 2015) with a wide distribution throughout Asia and especially in Iran. Aromatic plants have been studied extensively because a rich source of natural antioxidant is available in their essential oils or diverse extracts (Kulisic *et al.* 2005). Thyme extract has some constituents such as thymol, carvacrol, flavanoid, caffeic acid and labiatic acid (Leung and Foster, 1996), showing antibacterial, antimycotic, antioxidative, natural food preservative, and mammalian age-delaying properties (Jackson and Hay, 1994; Letchamo *et al.* 1995; Baranauskiene *et al.* 2003). Schwarz *et al.* (1996) showed that the phenolic compound of thyme extract have a strong antioxidant activity compared to α-tocophenol and butylated hydroxyl anisole. Thymol and carvacrol, the most important component of the extract of Thyme, has antioxidative activities (Schwarz *et al.* 1996; Hudaib *et al.* 2002; El-Nekeety *et al.* 2011). The antioxidant effects of thyme extract in ram semen extenders with a view to protecting against cryo-damage to sperm have not yet been studied. Therefore the purpose of the current study was to determine the effectiveness of different concentrations of thyme (*Thymus vulgaris*) as a natural antioxidant on frozen- thawed ram sperm motility; velocity parameters using the computer-assisted sperm motility analysis (CASA) system, viability and plasma membrane integrity.

MATERIALS AND METHODS

Preparation of *Thymus vulgaris* extract

For preparation of the *Thymus vulgaris* extract, collected Thyme plants were dried at room temperature for 10 days. In brief, dried plants of *Thymus vulgaris* (100 g) were powdered, soaked in 500 mL of 60% ethanol for 24 hours and the mixture was filtered. Soxhlet apparatus was used for the extraction of *Thymus vulgaris* extract. Ethanol was evaporated from the extract on a rotary evaporator at 50 ˚C. The extract was maintained at 4 ˚C until used.

Semen collection, extender preparation and cryopreservation

This study was carried out at the Iranian Moghani sheep Breeding Center located in Jafarabad city, Province Ardebil, Iran. The animals were kept under a natural photoperiod and were fed with high quality conventional feed offered at *ad libitum*. Four mature and fertile rams (3-4 years old, mean live weight of 70±4.2 kg), were used in this study. Ejaculates were collected twice a week for 8 weeks by an artificial vagina (42-43 ˚C). The fresh semen samples were immediately transported to the laboratory and kept in a water bath at 37 ˚C until evaluation. Only samples containing spermatozoa with greater than 80% motility, concentration and volume higher than 3 × 10^9 sperm/mL and 0.75 mL, respectively, were accepted for experiment. Semen samples were pooled to eliminate individual differences between donor animals and then processed using extender.

The Tris-based extender was composed of Tris 3.07 g (hydroxymethyl-aminoethane, Merck 64271, Germany), fructose 1.26 g, citric acid 1.64 g, hen egg yolk 15% (v/v), glycerol 5% (v/v) and double-distilled water (100 mL). The pooled semen was divided into 5 equal aliquots, then base extender containing different concentrations of *Thymus vulgaris* extract (2, 4, 8, 12 and 16 mL/dL), or no extract (control), was used for semen dilution at 37 ˚C. The final concentration of diluted semen was 100 × 10^6 spermatozoa/mL. Diluted semen samples were aspirated into 0.25 mL French straws and sealed with polyvinyl alcohol powder and balanced at 4 ˚C for 1 h. After equilibration, the straws were exposed to liquid nitrogen (LN2) vapor, 5 cm above the LN2 for 12 min, plunged into LN2, and stored in a liquid nitrogen tank until thawed and used for assessing of sperm quality. The frozen straws were thawed individually in a water bath (37 ˚C) for 30 s prior to evaluation.

Semen evaluation after thawing

Sperm motility and velocity parameters

For analyzing the motility parameters, sperm samples were incubated after thawing in a water bath at 37 ˚C for 5 min. A computer-assisted sperm motility analyzer (CASA, Video Test Sperm 3.1) was used to evaluate sperm motility and velocity characteristics. Thawed semen was diluted in a Tris-based extender (without egg yolk and glycerol) and analyzed immediately after dilution. A 5 µ Laliquat of diluted semen was placed directly on a pre-warmed microscope slide and covered by a cover slip and sperm motility characteristics were determined with a 10 × objective at 37 ˚C. The following motility values were recorded: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s) and amplitude of lateral head displacement (ALH, µm), linearity index (LIN) (LIN= (VSL/VCL)×100) and straightness (STR, %). For each evaluation, 10 fields were assessed to include at least 200 spermatozoa.

Sperm viability evaluation

Sperm viability was assessed using a modification of the eosin-nigrosin staining method described by Evans and Maxwell (1990).

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 double distilled water. A mixture of 5 µL of frozen-thawed semen and 5 µL of eosin-nigrosin stain was smeared on a pre-warmed slide and allowed to air dry in a dust-free environment.

Two hundred spermatozoa from different microscopic fields were observed under a phase-contrast microscope using a 400x objective, and the number of unstained heads of spermatozoa (live) and stained / partial stained heads of spermatozoa (dead) were counted.

Sperm plasma membrane integrity

Sperm membrane functionality was evaluated using the hypo-osmotic swelling test (HOST). In brief, firstly 20 µL of thawed diluted semen was added to 200 µL of a 100 mOsM hypo-osmotic solution (9 g fructose+4.9 g sodium citrate per liter of bi-distilled water) in microtubes and incubated at 37 ˚C for 30 min. After 30 min incubation at room temperature, the mixtures were homogenized. Then, a drop of fixed semen was placed on a warm slide and spread with a cover slip.

A total of 200 spermatozoa were analyzed per slide using bright-field microscopy (400×objective) in at least 5 different microscopic fields. Sperm with swollen or coiled tails were considered to have functional membranes (Revell and Mrode, 1994).

Sperm abnormality

Hancock solution (62.5 mL formalin (37%), 150 mL sodium saline solution, 150 mL buffer solution and 500 mL bi-distilled water) (Schafer and Holzmann, 2000) was used for the assessment of the morphological abnormalities. For this test, three drops of each sample were added to eppendorf tubes containing 1 mL of Hancock solution. A drop of the fixed spermatozoa was placed on a slide and covered with a cover glass.

The slides were assessed by phase-contrast microscopy using a 100x oil immersion objective. A total of 200 spermatozoa per slide were counted for determining sperm abnormalities and reported as percentages.

Statistical analyses

Each treatment was replicated five times. Three straws in each replicate were thawed and pooled for assessing of sperm quality. All data were analyzed by complete randomized design using the general linear models procedure of SAS version 9.1 (SAS, 2004). Differences between LS means were determined by Tukey-Kramer's test and $P < 0.05$ was considered as the significant level. Data were expressed as LS means ± SEM.

RESULTS AND DISCUSSION

The effects of *Thymus vulgaris* extract on motility and velocity parameters of frozen -thawed ram semen are presented in Table 1. Samples cryopreserved in 2 and 4 mL/dL *Thymus vulgaris* extract had higher percent total motility (57.92±4.56% and 55.38±4.17%) compared to other groups (P<0.05).

Extended semen supplemented with 4 mL/dL extract exhibited higher (P<0.05) percentages of progressive motility following the freeze–thaw process compared to control, 12 and 16 mL/dL extract groups. The percentage of VSL was higher (P<0.05) in the extender containing 4 and 8 mL/dL extract (68.74±4.34 and 66.61±4.95, respectively) compared to the other groups. The lowest (P<0.05) percentage of progressive motility (26.42±2.12%) and VSL parameter (40.93±3.25) was observed in group containing 16 mL/dL extract as well. As shown in Table 1, 4 mL/dL extract caused a significant increase in VCL parameter in comparison to the control, 12 and 16 mL/dL extract groups (P<0.05).

VAP parameter level was higher (P<0.05) in 4 mL/dL (73.12±5.67) compared to 16 mL/dL extract (56.24±4.60). There was no (P>0.05) difference in ALH parameter between the treatments groups (Table 1). For parameters of LIN and STR the lowest performance (P<0.05) was observed at 16 mL/dL of extract (41.63±3.10% and 73.28±6.31%, respectively).

Table 1 Effect of *Thymus vulgaris* extract on motility and velocity parameters of frozen -thawed ram spermatozoa (LS mean±SE)

Parameters	Levels of *Thymus vulgaris* extract (mL/dL)					
	Control	2	4	8	12	16
TM (%)	43.13±4.32[cd]	57.92±4.56[a]	55.38±4.17[ab]	52.26±4.52[abc]	45.33±4.15[cd]	39.51±3.41[d]
PM (%)	32.24±3.16[bc]	40.21±3.56[ab]	42.79±4.12[a]	38.45±4.32[ab]	34.17±3.40[bc]	26.42±2.12[cd]
VSL (μm/s)	51.61±3.12[b]	58.39±4.81[ab]	68.74±4.34[a]	66.61±4.95[a]	49.32±4.54[bc]	40.93±3.25[c]
VCL (μm/s)	109.32±9.76[bc]	118.33±7.65[ab]	131.36±7.78[a]	128.14±8.14[ab]	112.73±6.34[bc]	102.37±7.56[c]
VAP (μm/s)	63.18±4.12[ab]	74.93±5.32[a]	73.12±5.67[a]	71.26±6.56[a]	60.85±6.24[ab]	56.24±4.60[b]
ALH (μm)	3.31±0.32	3.75±0.41	3.76±0.37	4.03±0.31	3.64±0.34	3.45±0.34
LIN (%)	46.35±3.87[ab]	51.13±3.46[ab]	52.31±3.68[a]	51.93±3.74[a]	46.21±4.61[ab]	41.63±3.10[b]
STR (%)	82.15±6.18[ab]	78.73±5.59[a]	93.24±7.20[a]	93.43±7.75[a]	81.72±6.05[ab]	73.28±6.31[b]

TM: total motility (%); PM: progressive motility (%); VSL: straight linear velocity; VCL: curvilinear velocity (μm/s); VAP: average path velocity (μm/s); ALH: amplitude of lateral head displacement (μm); LIN: linearity (%) and STR: straightness (%).
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SD: standard deviation.

Table 2 shows the results of different concentrations of *Thymus vulgaris* extract supplementation on viability, membrane integrity and acrosome abnormality parameters of frozen-thawed ram spermatozoa. The number of viable sperm was increased (P<0.05) in the extender containing 4 mL/dL extract (55.13±3.35%) compared to control, 12 and 16 mL/dL extract groups (46.08±3.32%, 43.21±3.56% and 39.31±3.23%, respectively). The results showed that plasma membrane integrity was higher (P<0.05) in 4 and 8 mL/dL extract groups (54.21±3.15% and 56.11±4.07%, respectively) compared to control, 12 mL/dL and 16 mL/dL extract groups (43.41±2.63, 40.43±3.31% and 39.93±2.93%, respectively). As represented in Table 2, there were no significant differences among the groups in the percentages of acrosome abnormalities (P>0.05).

Synthetic antioxidants are widely used in the food industry because of they are effective and cheaper than natural antioxidants (Duh and Yen, 1997). However, their safety has been questioned and some of these antioxidants are banned in European countries because of carcinogenic effects (Shahidi, 1997). Therefore, currently researchers are studying safer and potentially effective natural antioxidants. Several plant products contain antioxidantcompounds such as flavonoids, tannins, coumarins, curcumanoids, xanthons, phenolics, lignans and terpenoids (Jeong *et al.* 2004). Various synthetic antioxidants such as cysteine (Bucak *et al.* 2007), glutathione (Uysal and Bucak, 2007) and butylated hydroxyl toluene (BHT) (Naijian *et al.* 2013), have been used for dilution of semen as well. The beneficial effects of medicinal plant species extracts as herbal antioxidants have been reported in some studies (Kulisic *et al.* 2005; El-Nekeety *et al.* 2011; Roby *et al.* 2013). Hence, because of the toxicity problems of synthetic antioxidants, use of natural antioxidants is increasing. However, very few studies have considered the effect of natural antioxidants on spermatozoa. Sperm cryopreservation causes some functional and structural damage to sperm. Several factors lead to this damage which can affect the quality of spermatozoa after thawing (Purdy, 2006).

During semen processing and cryopreservation, oxidative stress is one of the important factors (Stradaioli *et al.* 2007). The plasma membrane of mammalian spermatozoa is highly sensitive to lipid peroxidative damage because of its high polyunsaturated fatty acid composition. During aerobic incubation, some of free radicals such as hydrogen peroxide, superoxide ion, peroxyl and hydroxyl radicals produce reactive oxygen species (ROS) that can damage cells (Sinha *et al.* 1996).
The damage finally leads to decreased sperm motility, membrane integrity, fertility, and increased fragmentation of the sperm DNA (Bucak *et al.* 2010). Evans and Maxwell (1990) reported that ram sperm membrane has a high polyunsaturated to saturated fatty acids ratio, hence the spermatozoa membrane of rams is more susceptible to oxidative stress than that of other species. Some studies have reported that antioxidants reduce the production of free radicals following the freeze–thawing process (Ashrafi *et al.* 2013). Therefore, for protecting sperm from the deleterious effects of cryopreservation, antioxidants have been used in semen diluents (Watson, 2000).

In the current study we have applied ethanol extract of *Thymus vulgaris* as a natural antioxidant in Tris-based semen extender for protecting sperm against oxidants. Our data showed that addition of 2 and 4 mL/dL *Thymus vulgaris* extract to the extender improved the total and progressive motility, velocity parameters and viability of frozen-thawed ram spermatozoa.

These finding were similar to the results in broiler chickens where supplementing with thyme caused an increase in the percentage of living sperm and sperm motility (Shanoon and Mahdi, 2012).

Also, Ahmed *et al.* (2015) showed that the adding thyme leaf powder to the diet of male rats improved sperm count and motility.

In another study, the addition of thyme and fish oil to the diets of stallions affected total and progressive motility, membrane integrity and viability of spermatozoa (Kheradmand Garmsir *et al.* 2014).

Table 2 Effect of *Thymus vulgaris* extract on viability, membrane integrity and acrosome abnormality of post-thawed ram spermatozoa (LS mean±SE)

Parameters	Levels of *Thymus vulgaris* extract (mL/dL)					
	Control	2	4	8	12	16
Viability (%)	46.08±3.32[bcd]	51.45±4.23[abc]	55.13±3.35[a]	53.32±4.12[ab]	43.21±3.56[cd]	39.31±3.23[d]
HOST (%)	43.41±2.63[bc]	50.81±3.28[ab]	54.21±3.15[a]	56.11±4.07[a]	40.43±3.31[c]	39.93±2.93[c]
Acrosome abnormality (%)	28.25±3.12	23.41±2.95	24.50±2.24	26.12±2.88	28.23±3.03	26.93±2.60

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).
SD: standard deviation.

However, Alasadiy (2014) reported that *Thymus vulgaris* alcoholic extract had no significant effect on mouse sperm quality which might be a product of both potent androgenic activities and antioxidant properties of thyme.

It was shown that aqueous extracts of plants such as thyme contain phenolic compounds that can prevent oxidative fat decomposition (Radwan Nadiia *et al.* 2008). The attack of ROS during cryopreservation led to a reduction of oxygen and it is related to lipid peroxidation of the sperm membranes that destroys the structure of the lipid matrix. This damage finally reduces sperm motility and viability, membrane integrity, intracellular enzymes activity and damages to the sperm DNA (Bucak *et al.* 2010). Supplementation of semen extender with antioxidants can protect sperm against oxidative damage during the freezing and thawing processes. Phenolic phytochemicals have been regarded as possible antioxidants. Carvarcrol, thymol and flavanoid are the major phenols in Thyme. Antioxidant effects of phenolic phytochemicals protect cellular components against oxidative free radicals (El-Nekeety *et al.* 2011).

In the present study, treatment using thyme extract resulted in a significant improvement in membrane integrity and this improvement was dose related. Therefore, thyme extract may play a protective role against oxidative damage and scavenge free radicals produced in cells (Loziene *et al.* 2007).

El-Nekeety *et al.* (2011), Abd El Kader and Mohamed (2012) and Hamzawy *et al.* (2012) have also reported the beneficial effect of thyme extracts on oxidative stress in liver in control and hepatic injured rats. The hypo-osmotic swelling test (HOST) is used for determining resistance of the sperm plasma membrane to damage. The loss in membrane permeability under the stress of swelling driven by the hypo-osmotic treatment results in this damage that is a form of a membrane stress test.

HOST test is particularly useful when testing the membrane-stabilizing action of antioxidants (Sariozkan *et al.* 2015). Flavonoids prevent the access of detrimental molecules to the hydrophobic region in the cell membrane and therefore increase membrane integrity (DaghighKia *et al.* 2016).

CONCLUSION

In conclusion, our data showed that the quality of frozen-thawed ram semen is significantly improved by adding 4 mL/dL *Thymus vulgaris* extract to the semen extender, an effect probably due to polyphenolic compounds having antioxidant activity. More studies are recommended to determine the appropriate doses of thyme extract in semen extender.

ACKNOWLEDGEMENT

The authors sincerely appreciate the cooperation of the staff of the Agricultural Organization of Ardabil and the Moghani Sheep Breeding Station, Jafar-Abad, especially Mr. Ing. GH. R. Mardaneh and SH. Mabodi.

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