ANTIOXIDANT EFFECTS OF *MENTHA PIPERITA* L. EXTRACTION ON KINETICS PARAMETERS OF CRYOPRESERVED MOGHANI RAM SPERMATOZOA

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Abstract

Oxidative stress during freezing-thawing reduces motility, viability, membrane functions, antioxidant capacity and finally sperm fertility. Mentha piperita L. has antioxidant properties due to phenolic compounds. The aim of current study was to evaluate the effect of Mentha piperita extract as a natural antioxidant on dynamic parameters post-thawed Moghani ram sperm. In this study, four Moghani ram were used for semen collection twice a week by an artificial vagina and ejaculates with same condition were pooled. Different levels of ethanol extract of Mentha piperita L. (0, 2, 4, 8, 12 and 16ml in dL diluents solution) were added to Tris based diluents. Following cooling and freezing of semen samples, they were stored in liquid nitrogen until evaluation. After freezing-thawed, the dynamic parameters were evaluated using CASA system. The results showed that the addition of 4 and 8ml/dl extracts resulted in higher ($p < 0.05$) percentages of total motility. Addition of 4 ml/dl extract improved progressive motility compared to the control group and high levels of extract groups ($P <0.05$). The percentages of LIN, STR and VSL were higher ($P<0.05$) in the extender containing 8 mL/dL extract (54.43±4.42; and 94.20±7.93, respectively). Addition of 4 and 8 mL/dL extract of Mentha piperita L. to the extender significantly ($P<0.05$) improved VCL and VAP parameters compared to the control and 16 mL/dL extract groups. In conclusion, supplementation of extender with Mentha piperita L. extract improves post-thawed ram sperm motility and velocity parameters in a dose dependent manner.

Key words: *Antioxidant, Mentha piperita L. extract, Ram semen, Freeze-thawing*

1. INTRODUCTION

Artificial insemination (AI) using frozen–thawed sperm is an important reproductive technique used for livestock farming. The technique is based on cryopreservation that induces partially irreversible damages to sperm (Purdy, 2006), which may result in 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 amounts production of reactive oxygen species (ROS) and lipid peroxidation of the phospholipids in the membrane by free radicals such as $O2^{-}$ and H2O2 (Wang *et al.* 1997; *Sinha et al.* 1996). Based on this information, Curry *et al.* (1994) and Lamirande *et al.* (1997) suggested that the efficient antioxidant systems must be used to avoid lipid peroxidation and sperm dysfunction.

All cells have numerous antioxidant systems employed to scavenge free radicals, thus minimizing cellular damage. The removal of ROS is catalyzed by antioxidant enzymes such as glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and catalase (CAT). Numerous non-enzymatic defences (vitamin C, vitamin E, and glutathione (GSH)) are also employed to provide protection (Youdim and Deans, 1999). It has been reported that the concentrations of a number of these endogenous antioxidant defense systems are adequate to cope with the normal production of free radicals but an imbalance between free radical production and their removal results with ageing allowing progressive damage to occur (Bunker, 1992; Cao *et al.* 1996). Therefore, for protecting the sperm against oxidative damage, numerous researchers have investigated the effect of a various synthetic and natural antioxidants on spermatozoa during cryopreservation processes. Research for a

safer and effective natural antioxidant is underway and several natural sources are being examined 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). For this reason, there is a growing interest in using them as natural antioxidants. Several studies have shown that the use of herbal antioxidants during the freeze-thaw process of sperm had positive effects on sperm quality (Zanganeh *et al.* 2013; Baghshahi *et al.* 2014; Khodaei Motlagh *et al.* 2014; Daghigh Kia *et al.* 2016).

Mentha piperita L., a medicinally important plant belongs to the Family Lamiaceae (African pharmacopoeia, 1985). Nowadays it is widely cultivated throughout all regions of the world. Peppermint (*Mentha piperita* L.) is one of the most widely consumed single ingredient herbal teas, or tisanes. Peppermint tea, brewed from the plant leaves, and the essential oil of peppermint are used in traditional medicines. In vitro, peppermint has significant antimicrobial and antiviral activities, strong antioxidant and antitumor actions, and some antiallergenic potential. The antibacterial and antioxidant activities of peppermint oil were reported by Rasooli et al. (2008). The phenolic constituents of the leaves include rosmarinic acid and several flavonoids, primarily eriocitrin, luteolin and hesperidin. Peppermint yields 0.1–1% of volatile oil composed primarily of menthol (29–48%), menthone (20–31%), menthofuran (6.8%) and menthyl acetate (3–10%). Other pharmacologically active ingredients include bitter substances, caffeic acid, flavonoids (12%), polymerized polyphenols (19%), carotenes, tocopherols, betaine, choline and tannins (Karuza et al., 1996; Sokovic et al., 2009). The antioxidant effects of *Mentha piperita* extract in semen extenders against cryodamage to ram sperm have not yet been studied. Therefore the aim of the current study was to determine the effects of different concentrations of *Mentha piperita* L. as a natural antioxidant on frozen- thawed ram sperm motility; velocity parameters using the sperm class analysis system.

2. MATERIALS AND METHODS

2.1. Preparation of Mentha piperita extract

For preparation of the *Mentha piperita* extract, collected Peppermint plants were dried at room temperature for 10 days. In brief, dried plants of *Mentha piperita* (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 *Mentha piperita* extract. Extract was maintained at 4 °C until used.

2.2. Semen collection, Extender preparation and cryopreservation

This study was performed at the Iranian Moghani sheep Breeding Center located in Jafarabad city, Province Ardebil, Iran. The animals were kept under natural photoperiod and maintained using conventional feeding, housing and lighting conditions. 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 and then evaluated. Only samples containing spermatozoa with greater than 80% motility, concentration and volume higher than 3 ×109 sperm/ml and 0.75 mL respectively, were accepted for experiment. To eliminate individual differences, semen were pooled and processed for extending.

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 ejaculate was divided into 5 equal aliquots, and diluted (37 ◦C) using base extender containing different concentrations of *Mentha piperita* extract (2, 4, 8, 12, and 16 mL/dL), or no extract (control), with a final concentration of 100 $\times 10^6$ 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 evaluation of sperm parameters. The frozen straws were thawed individually in a water bath (37 ◦C) for 30 s for evaluation.

2.3. Sperm motility and velocity evaluation after thawing

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 analysis (CASA, Video Test Sperm 3.1) was used to analyze 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 µL 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, %), VAP (average path velocity, µm/s), VSL (straight linear velocity, µm/s), VCL (curvilinear velocity, µm/s) and ALH (amplitude of lateral head displacement, µm), LIN (linearity index (LIN = (VSL/VCL) × 100) and STR (straightness, %). For each evaluation, 10 fields were assessed to include at least 200 spermatozoa.

2.4. Statistical analyses

All data were analyzed by completely randomized design using the General Linear Models procedure of SAS version 9.1 (SAS Institute, 2004). Differences between Lsmeans were determined by Tukey-Kramer's test and $P < 0.05$ was considered as the significant level. Data were expressed as Lsmean ± SEM.

3. RESULTS

The effects of *Mentha piperita* extract on motility and velocity parameters of frozen -thawed ram semen are presented in Table 1. The results showed that the addition of 4 and 8ml/dl extracts resulted in higher ($p < 0.05$) percentages of total motility (53.31±3.54%; and 56.15±3.32%, respectively). The percentage of progressive motility was higher ($P < 0.05$) in the extender containing 4 mL/dL extract (41.28±2.94%) compared to the control group (30.44±2.53%).

Table 1. Effect of *Mentha piperita* extract on motility and velocity parameters of frozen -thawed ram spermatozoa (Lsmean ±SEM).

Parameters	Levels of *Mentha piperita* 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 (%).

a, b, c: Means on the same row within each parameter bearing common superscripts are not significantly different ($P < 0.05$).

SEM: standard error of the means.

The percentages of LIN and STR were higher (P<0.05) in the extender containing 8 mL/dL extract (54.43±4.42% and 94.20±7.93% respectively). Addition of 4 and 8 mL/dL extract of *Mentha piperita* L. to the extender significantly (P<0.05) improved VSL, VCL and VAP parameters compared to the control and 16 mL/dL extract groups. The percentage of ALH was higher (P<0.05) in the extender containing 4 mL/dL extract compared to the control groups (4.45 ±0.35 and 3.11 ±0.41, respectively).

The lowest ($p < 0.05$) percentage of VSL parameter (38.95 ±3.14) was observed in group containing 16 mL/dL extract as well.

4. DISCUSSION

Because of synthetic antioxidants are effective and cheap than natural antioxidants they are widely used in the food industry (Pin-Der *et al.* 1997). However, their safety has been questioned and some of these antioxidants are banned in European countries because of carcinogenic effects of them (Shahidi, 1997). Therefore, now day researchers are studying on safer and effective natural antioxidant. Various plant products such as fruits, leaves, seeds, and oils contain antioxidants such as flavonoids, tannins, coumarins, curcumanoids, xanthons, phenolics, lignans and terpenoids (Jeong *et al.* 2004). Several synthetic antioxidants such as cysteine (Bucak *et al.* 2007), glutathione (Uysal *et al.* 2007) and butylated hydroxyl toluene (BHT) (Naijian *et al.* 2013), have been applied for semen extender as well. Although the beneficial effect of medicinal plant species extract as an herbal antioxidant has been reported in some studies (Yadegarinia *et al.* 2006). Hence, because of the toxicity problems of synthetic antioxidants, using natural antioxidants is developing. However, very few studies have considered the effect of natural antioxidants on spermatozoa.

Sperm cryopreservation causes some functional and structural damages to sperm. These damages are dependent on several factors which can affect the post thawing outcome (Purdy, 2006). Oxidative stress during semen storage is one of the important factors (Stradaioli *et al.* 2007). Plasma membrane of mammalian spermatozoa contains high polyunsaturated fatty acids and is therefore highly sensitive to lipid peroxidative damage by free radicals such as superoxide ion, hydrogen peroxide, hydroxyl radicals and peroxyl, resulting from reactive oxygen species (ROS) during aerobic incubation (Sinha *et al.* 1996). The damage finally leads to decrease of sperm motility, membrane integrity, fertility, and increase fragmentation of the sperm DNA (Bucak *et al.* 2010). Evans and Maxwell (1990) reported that ram sperm membrane contain higher polyunsaturated/saturated fatty acids ratio, hence the sperm membrane of this species is more susceptible to oxidative stress than other species. Some studies have reported that antioxidants reduce the produced 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 *Mentha piperita* 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 *Mentha piperita* extract to the extender improved the total and progressive Motility, velocity parameters and viability of frozen -thawed ram spermatozoa. It was shown that water extracts of plants contain phenolic substances that have a role in the prevention of decomposition oxidative fat (Radwan Nadiia *et al.* 2008). The attacks of ROS during cryopreservation lead to reduction of oxygen and it is related to lipids peroxidation of the sperm membranes that destroys the structure of the lipid matrix. This damage finally leads to reduction of sperm motility and viability, membrane integrity, leakage of intracellular enzymes and damage of the sperm DNA (Bucak *et al.* 2010). Supplementation of semen extender with antioxidants can protect the sperm against oxidative damage during freezing and thawing processes. Phenolic phytochemicals have been regarded as possible antioxidants. The main volatile components of the essential oil are menthol and menthone. Antioxidant and free radical scavenging effects of Phenolic phytochemicals result in protecting cellular components against free radical. Mimica-Dukic et al. (2003) reported that *Mentha piperita* reduced the radical generator 2,2-diphenyl-1-picrylhydrazyl (DPPH) by 50% and inhibited the generation of the OH radical in the Fenton reaction by 24%.

In the present study, treatment of *Mentha piperita* extract resulted in a significant improvement in motility and velocity parameters and this improvement was doses related. Therefore, *Mentha piperita* extract may play a protective role against oxidative damage and scavenge produced free radicals from cells 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 (Daghigh Kia *et al.* 2016).

5. CONCLUSION

In conclusion our data showed that supplementation of semen extender with 4 and 8mL/dL *Mentha piperita* L. extract significantly improved the quality of frozen-thawed ram semen, probably due to polyphenolic compounds having antioxidant activity.

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