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Effect of Adding Coenzyme Q10 and Ellagic Acid during Cryopreservation on Post-Thaw Quality of Ram Semen

Research Article

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ABSTRACT

The aim of this study was to determine the effects of co-Q10 and ellagic acid on sperm parameters after the freeze–thawing of Ghezel ram sperm. Twenty ejaculates from five Ghezel rams were collected in this study. Semen samples, which were diluted with a soybean lecithin (SL) based extender containing 0.25 m*M* ellagic acid, 0.5 µ*M* co-Q10, 0.25 m*M* ellagic acid + 0.5 µ*M* co-Q10 and no antioxidant (control), were cooled to 4˚C, frozen in 0.25 mL French straws and stored in liquid nitrogen. Sperm motility characteristics, membrane integrity, abnormal morphology, lipid peroxidation and antioxidant activities (glutathione peroxidase, superoxide dismutase and total antioxidant capacity) were evaluated following freeze-thawing. The results showed that 0.5 µ*M* Co-Q10, improved viability, total motility parameters and decreased abnormal sperm and improved linearity (LIN), curvilinear velocity (VCL), straight-line velocity (VSL) and path velocity (VAP) parameters ($P<0.05$). Ellagic acid and the treatment with a combination (0.25 m*M* ellagic acid+0.5 µ*M* co-Q10) improved viability and total motility parameters ($P<0.05$). The additives did not affect the maintenance of superoxide dismutase (SOD) and glutathione peroxidase (GPx), when compared to the control. It can be concluded that addition of 0.5 µ*M* co-Q10 improved the post-thawing quality of ram semen.

KEY WORDS ellagic acid, Q10, ram, sperm.

INTRODUCTION

The potential benefits of artificial insemination are well known which are achieved by semen cryopreservation. Nonetheless the long-term storage of semen decreases sperm metabolic activity and thus fertility (Bailey *et al.* 2000). The highest damage to the sperm is caused by oxidative stress due to the production of reactive oxygen species (ROS). Oxidative stress results in the reduction of sperm activity and performance, that causes loss of motility, plasma membrane integrity and fertility (Najafi *et al.* 2014a). Semen cryopreservation causes some structural, biochemical and functional changes, which leads to various problems in sperm transport, survival and fertility rate in domestic animals (Salamon and Maxwell, 2000). Polyunsaturated fatty acids (PUFA) are sensitive to oxidation and free radicals (Sheweita *et al.* 2005; Eskenazi *et al.* 2005). Ram sperm have a high ratio of unsaturated to saturated fatty acids and cholesterol to phospholipid ratio compared to other species. The large amounts of PUFA make the sperm membrane more vulnerable against oxidative damage especially by ROS. Sperm have multiple mechanisms of defense systems against ROS (Vaseghi-Dodaran *et al.* 2015). These mechanisms include catalase, uric acid, taurine, thiols, ascorbic acid and α-tocopherol but the most important ones are superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GSH) systems (Daghigh Kia *et al.* 2016a). Although these defense

mechanisms are available, mature sperm do not have enough ability to fight against free radicals due to high concentrations of PUFA and failure in their plasma membrane enzyme activity, such as SOD. It has been reported that adding antioxidants to the freezing extender can reduce deteriorative effect of ROS and cold shock (Mata-Campuzano *et al*. 2015; Sariozkan *et al*. 2015) and improves sperm quality rams (Mehdipour *et al*. 2016), goats (Bucak *et al*. 2009), dogs (Funahashi and Sano, 2005) and human (Michael *et al*. 2007). The Co-Q10 is a part of the mitochondrial respiratory chain which has two important roles, first in metabolism and second acts as a fat-soluble antioxidant that protects the membrane and associated lipoproteins (Ernster *et al*. 1993). The Co-Q10 is one of the most significant lipid antioxidants in mitochondrial respiratory chain. In addition, co-Q10 has fat like properties, so releases phospholipids in cell membrane and protects the sperm plasma membrane (Ernster and Dalner, 1995). Ellagic acid is a polyphenol such as Co-Q10 having a wide variety of biological activities, including strong antioxidant properties, anti-cancer, anti-proliferative, anti-mutation and anti-apoptotic (Hassoun *et al*. 2004). Co-Q10 has not been used so far in ram semen freezing and there are only two study on the effects of ellagic acid, one of them was used in freeze-thawing ram semen (Omur and Coyan, 2016) and the other one used it in cooling of ram semen (Omur *et al*. 2014). Therefore, this study investigated the effect of Co-Q10 and ellagic acid in the improvement of ram sperm quality after freeze-thawing process.

MATERIALS AND METHODS

Chemicals

All Chemicals were purchased from Sigma (St.Louis, MO, USA), Merck (Darmstadt, Germany) and Pars Tech Rokh (Mashhad, Iran), unless otherwise indicated.

Animal and semen collection

Semen samples were collected from 5 healthy mature Ghezel rams (3 and 4 years of age) using artificial vagina twice a week (totally twenty samples for experiment) then pooled to avoid an individual variations during experiment and transported at 34 ˚C to the laboratory for initial assessments. Samples were accepted for adding the antioxidant if the following parameters were observed: volume (0.75-2 mL), concentration (greater than 3×10^9 sperm/mL), motility (>70%) and abnormal sperm (<10%). A tris-based extender was used as the freezing extender (Tris 297.58 m*M*, citric acid 96.32 m*M*, fructose 82.66 m*M*, lecithin 1%, glycerol 7% (v/v), pH 6.8). Experimental treatments included four group with 1) ellagic acid 0.25 m*M*, 2) Co-Q10 0.5 µ*M*, 3) ellagic acid 0.25 m*M* + Co-Q10 0.5 µ*M* and 4) a group without antioxidant considering as control group. The samples were placed in the refrigerator for two hours to reach at temperature of 4 ˚C and then loaded into 0.25 mL straws (IMV, L'Aigle, France). The samples were placed 4 cm above liquid nitrogen for 7 minutes and then plunged into liquid nitrogen and stored until thawing. For sperm evaluation, the straws were thawed individually at 37 ˚C for 30 seconds.

Sperm viability

For assessment of live sperm we used eosin-nigrosin staining. After freeze-thawing, 10 µL of the semen sample with 10 µL stain was mixed on a slide and for drying, the slides were held at 37 ˚C. Two hundred sperm were evaluated by microscope at magnification of × 1000. Sperm displaying unstained heads were considered viable, and sperm with stained or partially stained heads were counted as dead sperm (Najafi *et al*. 2014b).

Plasma membrane integrity

The hypo-osmotic swelling test (HOST) was used to assess integrity of plasma membrane. Fifty µL of the semen sample was mixed with 500 µL hypo-osmotic solution (100 mOsmol kg^{-1}) in water bath37 ˚C for 30 minutes. Five µL of the sample was put on a slide and mounted with a coverslip. The phase contrast microscope slides were placed on a heating pad. Two hundred sperm were counted at 400 × magnification and the sperm with swollen tail and spun were considered as sperm with intact plasma membrane (Jeyendran *et al*. 1984).

Assessment of sperm motility

Computer assisted system (CASA; Video Test Sperm 3.1) was used to evaluate the parameters of total motility (TM, %), progressive motility (PM, %), path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat/cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %). To assess the parameters of motility, samples were incubated in warm bath for 5 minutes at 37 ˚C. Then, 5 µL of the sample was placed on a preheated slide, mounted with a cover slip, and then placed on a heating pad microscope. Each sample was randomly selected from at least 10 fields and 200 sperm were analyzed by CASA system. All parameters were photographed at 100 × magnification (Najafi *et al*. 2016).

Sperm morphology assessment

To evaluate sperm morphology, 10 µL of each semen sample was added to 150 µL of Hancock solution (Najafi *et al*. 2013). Then a drop of this mixture was placed on a slide and at least 200 sperm were counted under a microscope

phase contrast with a magnification of × 400 and the percentage of abnormal sperm and abnormal acrosome were estimated.

Lipid peroxidation

The concentrations of malondialdehyde (MDA) were measured by thiobarbituric acid reaction (TBARs). One mL of the diluted semen sample (250×106 sperm/mL) was mixed with 1 ml of cold 20% (w/v) tricholoroacetic acid to precipitate protein.

The precipitate was pelleted by centrifuging (960g for 15 min), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 ˚C for 10 min. After cooling, the absorbance was determined using a wave length of 532 nm spectrophotometer (Placer *et al.* 1996). All MDA concentrations were expressed as nmol/mL.

Total antioxidant capacity (TAC)

Randox kits were used to measure total antioxidant capacity of sperm according to TEAC. TEAC method is based on the inhibition of ABTS cation radical scavenging antioxidants. The ABTS incubated with peroxidase and H_2O_2 to produce a radical cation ABTS and stable blue-green color that has maximum absorbance at 600 nm, which can be measured by a spectrophotometer (Miller *et al*. 1993).

Glutathione peroxidase activity

The activity of GPx was determined using the Ransel Glutathione Peroxidase kit (Randox Laboratories, UK). In this assay, GPx catalyzes the oxidation of GSH with cumene hydroperoxide. In the presence of GR and NADPH GSSG is converted into GSH with concomitant oxidation of NADPH to NADP+. The decrease in absorbance was measured at 340 nm at 37 ˚C (pH 7.2). The GPx activity was normalized to g of protein and expressed in U/g of protein.

Superoxide dismutase activity

Activity SOD in the semen samples was measured using the method and using the Ransod Company Randox kit (RANDOX Laboratories Ltd, UK).

Statistical methods

The experiment was conducted in a completely randomized design with four treatments and four replications. Since the semen samples were pooled, these were not real biological replicates. These are rather technical replicates. The data obtained were analyzed by GLM procedure of SAS (2004). All data were checked for normal distribution by PROC UNIVARIATE and the Shapiro-Wilk test. Mean comparisons were performed using least square means and the significant level was considered $P < 0.05$. Results are shown as Lsmean ± SEM.

RESULTS AND DISCUSSION

The diluents with the addition of Co-Q10 (0.5 µ*M*), ellagic acid (0.25 m*M*) and the treatment with combination of the two compounds ellagic acid (0.25 m*M*) + Co-Q10 (0.5 µ*M*) improved viability and total motility parameters and were significantly different with control group ($P<0.05$).

In addition, Co-Q10 (0.5 µ*M*) decreased abnormal sperm and improved LIN, VCL, VSL and VAP parameters ($P<0.05$). Ellagic acid also improved VAP and VCL compared to the control group (Tables 1 and 2).

The treatment groups did not show any significant difference in GPx and SOD enzymes (Table 3).

Ellagic acid improved total antioxidant capacity compared to the control group ($P<0.05$). The combination of two antioxidants increased MDA levels compared to control groups ($P<0.05$; Figure 1 a, b).

Plasma membrane of ram semen is very sensitive to lipid peroxidation due to ROS because of being rich in unsaturated fatty acids. Ram sperm has high sensitivity against cold shock compared to other species such as cattle, rabbits and human (Mata-Campuzano *et al.* 2015).

Lipid peroxidation of the sperm membrane leads to loss of membrane fluidity and cell activity and infertility (Aitken and Sawyer, 2003). Therefore, additives with antioxidant properties are necessary in order to reduce the adverse effects of sperm cryopreservation. Ellagic acid and Co-Q10 have antioxidant and phenol properties that conserve sperm from adverse damages. Many studies have examined the effects of extracts and phenolic compounds after freeze-thawing rams and bull sperm (Daghigh Kia *et al.* 2015).

Accordingly, comparing the present study with the studies on compound phenols property in ram sperm shows that Co-Q10 improve the motility of ram sperm after freeze-thawing which are the same with the results of Daghigh Kia *et al.* (2016b) and the study on bull sperm by Daghigh Kia *et al.* (2015) and Daghigh Kia *et al.* (2016a) which improved motility of sperm by adding antioxidant to semen extender.

In our study, Co-Q10 (0.5 µ*M*), as a phenolic compound reduced sperm abnormality. But it did not have any effect on membrane integrity, this outcome was contrary with result of Daghigh Kia *et al*. (2016b) on ram sperm freeze-thawing and Daghigh Kia *et al.* (2015) and Daghigh Kia *et al.* (2016a) on bull sperm freeze-thawing.

The results of this study indicate that there is a significant difference in total motility and viability of sperm in Co-Q10 at 0.5 µ*M* level compared to the control group.

Table 1 Effects of different levels of ellagic acid and Co-Q10 on sperm motility parameters after freezing-thawing process

Sperm motility parameters	Antioxidants				SEM	P-value
	Control	EA (0.25)	Co-Q10 (0.5)	EA + Q10 (0.25+0.5)		
TM (%)	48.2^{b}	64.6^{a}	66.3^{a}	63.2^{a}	2.24	0.0001
PM (%)	18.6	27.0	24.6	25.8	3.30	0.0066
VAP (µm/s)	20.5^{c}	34.6^{b}	68.1^{a}	31.8^{bc}	2.83	0.0001
VSL (µm/s)	15.5^{b}	23.9^{b}	46.7^{a}	21.7^{b}	2.20	0.0001
VCL (µm/s)	50.1^{c}	73.8^{b}	91.7^{a}	75.0^{b}	4.38	0.0001
STR (%)	76.8	71.6	68.8	68.8	4.68	0.0001
LIN (%)	30.9^{b}	32.7^{b}	50.9^{a}	29.0^{b}	1.92	0.0001

EA: ellagic acid; TM: total motility; PM: progressive motility; VAP: path velocity; VSL: progressive velocity; VCL: track velocity; STR: straightness and LIN: linearity.
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 The effect of different levels of ellagic acid and Co-Q10 on sperm viability, host and hancock parameters after freezing-thawing process

Sperm assessment parameters	Antioxidant				SEM	P-value
	Control	EA (0.25)	Co-Q10 (0.5)	EA+Q10 (0.25+0.5)		
Viability (%)	55.6^{b}	74.45^{a}	81.31^{a}	77.23^{a}	2.25	0.0001
Membrane integrity (%)	37.61^{b}	54.41^{a}	56.68^{a}	54.76^{a}	4.88	0.0001
Abnormal sperm (%)	25.52^{a}	26.13^{a}	19.53^{b}	25.49^{a}	0.99	0.0001

EA: ellagic acid.
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 3 The effect of different levels of ellagic acid and Co-Q10 on sperm viability, host and hancock parameters after freezing-thawing process

Sperm assessment parameters	Antioxidant				SEM	P-value
	Control	EA (0.25)	Co-Q10 (0.5)	EA + Q10 (0.25+0.5)		
Superoxide dismutase u/mL	101	118.3	105	109.3	6.15	0.0001
Glutathioneperoxydaseu/g protein	10.03	9.37	9.27	8.93	0.26	0.0001

EA: ellagic acid.
SEM: standard error of the means.

These results may be due to the bioenergy role of Co-Q10 in the respiratory chain and the production of ATP (Almeida and Ball, 2005). The important role of recycling of vitamin E by Co-Q10 in the prevention of lipid peroxidation and also in energy production in semen is well-known (Lewin and Lavon, 1997). Co-Q10 is a part of the respiratory chain in mitochondria which plays a role in sperm cell metabolism and fat-soluble antioxidants (Ernstr *et al.* 1993). It is noteworthy that the only lipid-soluble antioxidant producing in our body is Co-Q10, preventing oxidation of proteins, fats and DNA (Ernster, 1993). Co-Q10 is effective on sperm quality and motility, especially in infertile men (Mancini *et al*. 1998; Alleva *et al.* 1997; Lee *et al.* 2006) which are in accordance with the results of our study, due to the improvement of sperm motility. Generally, the better effect of Co-Q10 on sperm motility in comparison ellagic acid may be due to the bioenergetics role of Co-Q10 in the respiratory chain and the production of ATP (Turunen *et al.* 2004; Almeida and Ball, 2005).

Membrane lipid peroxidation leads to disruption of membrane activity, decrease in membrane fluidity and inactivation of receptors and enzymes bands, which ultimately increases non-specific permeability to ions (Esterbauer *et al.* 1990). In the present experiment, TAC was improved with the use of ellagic acid and the combination of two antioxidants.

The MDA level was also higher in the combination treatment group, the difference between results in total antioxidant capacity and MDA justify other results which are based on a mixture of two antioxidants; due to the improved antioxidant capacity and increased levels of MDA, that are likely related to adverse effects or being ineffective on other traits which cause an increase in free radicals and lipid peroxidation.

The two treatment groups were not significantly different from the control group and combination treatments in MDA. Similarly, two antioxidants used in the study had no significant effect on GPx and SOD.

There are few studies on the effects of ellagic acid on ram semen and we compare our results with the studies used extracts that have active ingredient is ellagic acid. In the present study, ellagic acid showed significant effect on viability and total motility that agreed with Reda *et al.* (2016), who observed an improvement in the motility and viability of bull semen using pomegranate juice (PJ) compared to control group and Mansour *et al*. (2013) that used PJ in human semen. Ellagic acid is a polyphenol having variety of biological activities, such as antioxidant (Hassoun *et al.* 2004), anti-mutagenic, anti-apoptosis properties. Although the mechanisms of its action are unknown.

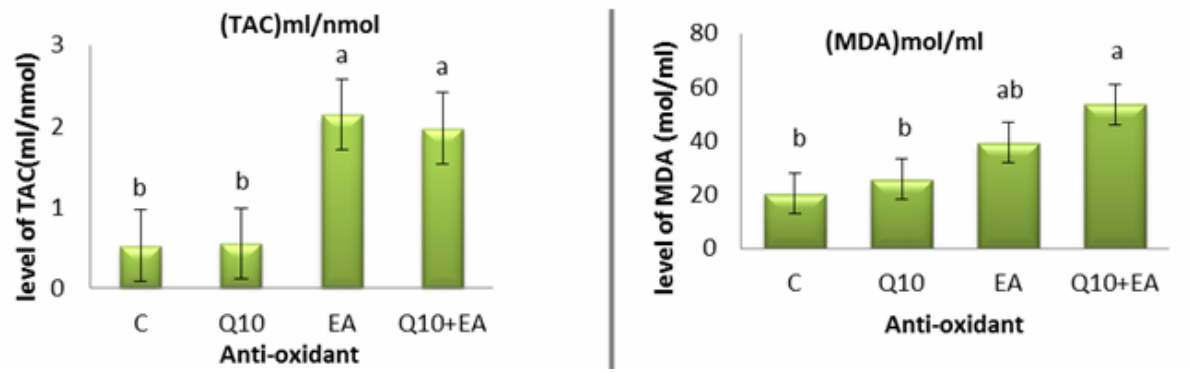

Figure 1 Effects of different levels of ellagic acid and Co-Q10 on TAC and MDA levels after freezing-thawing process

The discussed properties may explain the effect of ellagic acid for increasing sperm motility, viability and capacity to scavenge free radicals (Priyadarsini *et al*. 2002; Seeram *et al*. 2005).

Cisplatin-induced abnormal growth of rat sperm was blocked using ellagic acid (Turk *et al*. 2008). They used different levels of ellagic acid in rat that observed a reduction in MDA level and a significant increase in activity of GSH, GSH-Px and CAT. This result is contrary with our result that ellagic acid did not have significant effect on sperm parameters. They also showed an increase in sperm concentration, sperm motility, sperm cell density, diameter and thickness of the seminiferous tubules of germ cells and decrease in abnormal sperm compared to control group and study on sperm motile and abnormal sperm showed the same result with ours. Similarly, in another study, cyclophosphamide induced lipid per oxidation damage to the structure of the sperm and testicular tissue in rabbits showed the protective effect of eEllagic acid (2 mg/kg) (Ceribasi *et al*. 2010) but in our study ellagic acid did not have any effect on sperm. Ellagic acid has four phenol OH groups with a fused benzofuran structure, therefore has the property of scavenging ROS (reactive oxygen species) and RNS (reactive nitrogen species) which can prevent lipid peroxidation (Bondet *et al*. 1997).

The results of the present study are in contrast to those of Omur *et al*. (2014), who observed an improvement in sperm membrane integrity in the process of cooling ram semen with by using ellagic acid at level of 2 m*M*. However, they showed in another study Omur and Coyan (2016) that all levels of ellagic acid improved acrosome integrity in the process of freezing ram semen.

In a study of Yousefian *et al*. (2014), which was carried out on the Stallion, Co-Q10 at 1 µ*M* improved sperm motility and parameters of sperm membrane. In present study, Co-Q10 at the level of 0.5 µ*M* improved total motility, survival, abnormal sperm, LIN, VSL and VAP parameters, as well as the improvement of sperm membrane integrity.

CONCLUSION

The results showed that Co-Q10 at level of 0.5 µ*M* improved the sperm quality parameters and reduced the oxidative parameters compared with all treatments. We recommend use of Co-Q10 at level of 0.5 µ*M* in order to raise the quality of frozen ram semen.

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