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DOCOSAHEXAENOIC ACID AND ALPHA-TOCOPHEROL IMPROVE SPERM CRYOSURVIVAL IN GOAT

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ABSTRACT

The aim of this study was to investigate the effect of adding a polyunsaturated fatty acid source (n-3 fatty acids), accompanied by alpha-tocopherol, to extender on freezing ability and fatty acid (FA) composition of goat sperm. *Mahabadi* bucks were used in this research. In first experiment, the pooled semen was divided into 12 groups, in a 3×4 factorial design including four levels of n-3 FA (0, 0.1, 1 and 10 ng ml^{-1}) and three levels of vitamin E (VE) (0, 0.1 and 0.2 mmol). The percentage of motility, progressive motility and viability of semen were evaluated. The treatment of 0.2 mmol VE and 10 ng ml^{-1} n-3 FA had the best semen quality in comparison to the control and the other treated groups, after thawing. The second trial was conducted to determine fatty acid content of sperm after the treatment with 0.2 mmol VE and 10 ng ml^{-1} n-3 FA and the treatment without VE and FA. Adding FA to the extender led to an increase in Docosahexaenoic acid (n-3) level before freezing ($P \leq 0.01$). The overall proportion of n-3 FA to n-6 FA was significantly higher in the FA group than in the other group ($P \leq 0.01$) before freezing, and the ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA) was higher ($P \leq 0.05$) in the FA group before freezing than that after thawing. Results suggest that the addition of n-3 FA with an antioxidant could improve freezing ability of goat semen via changing the lipid composition of sperm cell.

Key words: goat; sperm; freezing; N-3 fatty acids; vitamin E

INTRODUCTION

Cryopreservation as a technique for storage of goat semen has advantages but thawing and freezing induce the detrimental effects in terms of sperm ultrastructural, biochemical and functional damage (Watson, 2000), resulting in a reduction of motility, membrane integrity and fertilizing ability (Purdy, 2006).

The purpose of a freezing extender is to supply a source of energy for sperm cells, and protect them from temperature-related damage and maintain a suitable environment for the spermatozoa to survive temporarily. Currently, egg yolk is used as a common component of semen cryopreservation extenders in domestic animals. It has been shown to have a beneficial effect on sperm cryopreservation as a protector of the plasma membrane and acrosome against temperature-related injury because of the presence of phospholipids, in association with others components (Purdy, 2006). However, the dilution of goat semen into extenders containing egg yolk or milk can have a detrimental effect on the quality of the sperm cells during freezing and thawing, due to the presence of egg yolk-coagulating enzyme (EYCE) and bulbourethral gland secretion glycoprotein (BUSgp60), respectively (Pellicer-Rubio and Combarnous, 1998; Sias *et al.*, 2005). Moreover, egg yolk can present a major risk of contamination (Bousseau *et al.*, 1998). Hence commercial extenders with soybean lecithin as an egg yolk substitute have recently become available for freezing animal semen (Gil *et al*., 2003; Van Wagtendonk-de Leeuw *et al.*, 2000). Lecithin (phosphatidyl choline) is a major phospholipid in sperm cell membrane that

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plays an important role in sperm viability. The plasma membrane is a highly dynamic structure that regulates not only extracellular exchanges, but the process of fertilization as well (Flesch and Gadella, 2000). Differences in lipid composition of the sperm plasma membrane is a key factor in the differing freezing ability of sperm (Parks and Lynch, 1992). In many mammalian species, up to 60 % of the total fatty acids are long-chain polyunsaturated fatty acids (LCPUFA) of the n-3 series (Poulos *et al*., 1973). This specific lipid composition confers a greater fluidity and flexibility on the plasma membrane due to the presence of the many double bonds. This specific physical change in characteristics may give membranes a better resistance to damages arising from the formation of ice crystals (Maldjian *et al*., 2005). High concen¬tration of Docosahexaenoic acid (DHA) in both semen and sperm has been suggested to be positively associated with sperm motility in humans (Poulos *et al*., 1973; Rooke *et al*., 2001). Docosahexaenoic acid may contribute to the membrane fluidity that is necessary for the motility of sperm tails (Bwanga, 1991). Indeed, it was found that the sperm obtained from asthenozoospermic men had lower levels of DHA when compared with sperm from normozoospermic men (Poulos *et al*., 1973). In regards to cryotolerance, the spermatozoa collected from African elephants (*Loxodontia africana*) had higher levels of membrane docosahexaenoic acid (22:6, n-3) and docosapantaenoic acid (22:5, n-3 and n-6) as compared to spermatozoa collected from Asian male elephants (*Elephas maximus*). Interestingly, African elephant spermatozoa can be cryogenically frozen while the same protocols have failed to cryogenically freeze spermatozoa collected from Asian elephant (Swain and Miller, 2000). Therefore, the objective of current study was to evaluate the effects of adding different levels of n-3 fatty acid and vitamin E to a free egg-yolk extender on freezing ability of goat sperm.

MATERIAL AND METHODS

Animals and location

Six three-year old Mahabadi bucks with average weight of 65±2.52 kg from the goat flock of the department of animal Science, university of Tehran, in Karaj (35°48´N, 51°2´E) were used during autumn 2008.

Semen collection

Semen samples were collected using an artificial vagina from the mentioned six bucks for 4 weeks (total ejaculations = 24). The collected samples of raw semen were transferred to the laboratory of the Animal Breeding Center of Iran, and kept in a water bath at 34°C. Samples were evaluated using a phase-contrast microscope at 400 x and those with motility value ≥70% were chosen. Then, the proper samples were pooled and used in each week (each sample was thawed 48 hours after freezing).

Experimental design

In the first experiment, pooled semen was extended using Bioxcell® extender with different levels of n-3 FA (Viva Pharmaceutical Inc, Canada) (0, 0.1, 1 and 10 ng ml^{-1}) and VE (Sigma Chemical Co., St. Louis, MO, USA) solved in ethanol (% 0.05) (0 , 0.1 and 0.2 mmol). The extended semen samples were placed into tubes and incubated at 37°C for 15 min for uptaking fatty acids and vitamin E by spermatozoa. Five samples of fresh and frozen-thawed semen from each treatment were used for semen quality evaluation.

The treated group with 0.2 mmol VE and 10 ng ml^{-1} n-3 FA showed the best quality after thawing compared to the control and the other treated groups, so this treatment along with control (without FA and VE) were chosen for the third trial. In this experiment, fatty acid composition of sperm in two groups was determined before freezing and after thawing.

Freezing and thawing process

Diluted semen was cooled down to 4–5°C over 2 h and then frozen in straws. A Styrofoam box containing liquid nitrogen was used to cryopreserve the semen samples. The rack containing the samples was placed into the liquid nitrogen vapor at a height of 4 cm above the liquid for 8 min, after that, the straws were plunged in liquid nitrogen. The straws were thawed by placing them in a 37°C water bath for 30s (Purdy, 2006).

Semen evaluation

a) *Post-thawed sperm motility and progressive motility*
A drop of fresh and frozen-thawed semen was placed on a pre-warmed slide and covered with a coverslip. Motility and progressive motility percentages were assessed under a phase-contrast microscope at 200 x magnification. Recovery rate of spermatozoa was also calculated (Hafez and Hafez, 2000).

b) *Sperm viability and abnormality*
A small drop of fresh or frozen-thawed semen was placed on a pre-warmed slide and mixed with a relatively larger drop of the supravital stain [1 % (w/v) eosin B, 5 % (w/v) nigrosin in 3 % tri-sodium. Three hundred and thirty three spermatozoa in ten view fields (thirty three spermatozoa per view field) were counted to detect unstained heads of spermatozoa (live) and/or stained/partially stained heads of spermatozoa (dead) under a magnification 400x. In this slide spermatozoa were examined for the following abnormal morphologies: detached head, abaxial head, malformed head, bent tail and coiled tail (Evans *et al*., 1989).

Fatty acid composition of sperm

Fresh and frozen-thawed samples were diluted with an equal volume of 0.85 % (wt/vol) NaCl followed by centrifugation at 1000 g for 10 min at room temperature to separate the seminal plasma from the cell pellet (Sariozkan *et al.*, 2010). The upper diluted plasma layer was transferred into a fresh test tube and the cell pellet was washed with 1 ml of 0.85 % (wt/vol) NaCl and recentrifuged as described above. The sperm cell pellet was resuspended in 2 ml of 0.85 % (wt/vol) NaCl. The resulting sperm pellet was washed twice with saline (Surai *et al*., 2000). The total lipid was extracted from the sperm after homogenization in a suitable excess of chloroform–methanol (2:1, v/v) (Folch *et al.*, 1957). Trans-methylation of these sample was performed using Metcalf´ method (Metcalf *et al*., 1996). The resultant fatty acid methyl esters were analyzed by gas chromatography (HP6890 with FID detector and autosampler HP7683, Hewlett Packard,Wilmington, DE, USA) using a capillary column system Carbowax, 30 m × 0.25 mm in diameter, 0.25 µm film thickness (Alltech Ltd., Carnforth, Lancashire, UK).

Statistical analysis

The results were expressed as mean± SEM. Data were analyzed using GLM procedure of SAS 9.1 (SAS Institute, Cary, NC, USA). LSMean test was used for treatments´ mean comparisons. Differences with values of $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

First experiment

The mean percentages of post-thawed sperm characteristics of different groups are shown in table 1.

a) Motility

The effect of n-3 FA, VE (main effects) and their interaction (our twelve treatments were shown in table 1) on sperm motility percentage was significant. The MOT percentage of sperm was 44.16±0.16, 45.21±0.16 and 48.54±0.16 for 0, 0.1 and 0.2 mmol VE, respectively and the latter level had significantly highest value ($P\leq0.05$). The MOT percentage was 43.88±0.79, 46.11±0.79, 45.27±0.79 and 48.61±0.79 for 0, 0.1, 1, and 10 ng.ml^{-1} FA, respectively and the latter level had significantly highest value ($P\leq0.05$).

The motility percentage was significantly higher in 0.1 FA and 0.2 VE and 10 ng ml-1 FA, 0.2 mmol VE groups than other treatment groups.

b) Progressive motility

The effect of n-3 FA, VE and their interaction on sperm progressive motility was significant. The PMOT percentage of sperm was 36.45± 0.59, 35.83± 0.59 and 40.21± 0.59 for 0, 0.1 and 0.2 mmol VE, respectively and the latter level had significantly highest value ($P\leq0.001$). The PMOT percentage was 35.83±0.68, 37.77±0.68, 36.94± 0.68 and 39.44±0.68 for 0, 0.1, 1, and 10 ng.ml^{-1} FA, respectively and the latter level had

Table 1: In vitro characteristics of post-thawed sperm for different levels of n-3 FA and vitamin E in goat

Treatments	Semen Characteristics (%)				
	Motility (SEM=1.95)	Progressively motility (SEM =0.84)	Viability (SEM =0.72)	Abnormality (SEM = 0.31)	Recovery rate (SEM = 3.01)
F_0V_0[1] (without Ethanol)	52^{a}	37^{a}	54^{a}	6.26	70.33^{a}
F_0V_0 (with Ethanol)	15^{c}	10^{f}	48.37^{bcd}	5.31	20.79^{d}
$F_{0.1}V_0$	15^{c}	10^{f}	47.97^{cd}	5.55	21.43^{cd}
F_1V_0	16.66^{c}	11.66^{ef}	41.71^{f}	5.24	20.95^{d}
$F_{10}V_0$	21.66^{c}	15de	41.40^{f}	5.85	30.15^{c}
$F_0V_{0.1}$	16.66^{c}	10^{f}	47.37^{de}	6.05	23.33^{cd}
$F_{0.1}V_{0.1}$	16.66^{c}	10^{f}	44.44^{e}	6.36	23.33^{cd}
$F_1V_{0.1}$	20^{c}	11.66^{ef}	43.85^{e}	5.24	27.77^{cd}
$F_{10}V_{0.1}$	21.66^{c}	11.66^{ef}	50.52^{a}	5.25	30^{c}
$F_0V_{0.2}$	18.33^{c}	10^{f}	43.93^{e}	5.55	26.19^{cd}
$F_{0.1}V_{0.2}$	31.66^{b}	21.66^{c}	49.19^{abc}	5.65	44.13^{b}
$F_1V_{0.2}$	21.66^{c}	16.66^{d}	45.45^{de}	6.36	30.95^{c}
$F_{10}V_{0.2}$	33.33^{b}	26.66^{b}	50.42^{ab}	5.35	46.35^{b}

a,b,c,) Values in each column that do not have any common letter are significantly ($P\leq 0.05$) different

1. Fatty acid and vitamin amount in base extender

Table 2: Fatty acid composition of spermatozoa lipid from goat semen in the control group (without FA) and n-3 FA group (with FA).

Fatty acid	Before freezing		After thawing		SEM
	Without FA	With FA	Without FA	With FA	
C14:0[1]	7.48[a]	6.64[b]	7.54[a]	7.74[a]	0.22
C16:0	38.14[a]	36.62[b]	37.36[b]	37.85[b]	0.58
C18:0	22.50[a]	22.28[a]	23.46[a]	23.44[a]	0.72
C18:1	9.02[c]	9.95[a]	9.17b[c]	9.33[b]	0.33
C18:2	16.5[a]	15.84[a]	15.34[b]	15.06[b]	0.72
C18:3	0.37[a]	0.73[b]	0.19[a]	0.22[a]	0.12
EPA[2]	0.29	0.27	minor	minor	0.16
DHA[3]	6.7[b]	8.12[a]	5.95[c]	5.24[c]	0.16
n-3	7.36[b]	9.12[a]	6.14[c]	6.44[c]	0.16
n-6	16.5[a]	15.84[a]	15.34[b]	15.06[b]	0.72
Ratio of n-3/n-6	0.44[b]	0.57[a]	0.40[c]	0.43[c]	0.02
PUFA[4]	22.86[b]	24.96[a]	21.48[b]	20.52[b]	0.84
SFA[5]	68.12[a]	65.54[a]	68.36[a]	69.03[a]	1.27
PUFA/SFA	0.33[b]	0.38[a]	0.31[c]	0.30[c]	0.009
MUFA[6]	9.02[c]	9.95[a]	9.17[bc]	9.33[b]	0.33

abc) Values in each row that do not have any common letter are significantly (P≤ 0.05) different
1) The numbers after C show the number of carbon and number of double bond between two carbons in the structure of fatty acid, respectively.
2) Eicosapantaenoic acid. 3) Docosahexaenic acid. 4) Polyunsaturated fatty acid. 5) Saturated fatty acid. 6) Monounsaturated fatty acid.

significantly highest value (P≤0.05).

The progressive motility percentage was significantly higher in 10 ng.ml-1 FA, 0.2 mmol VE group than in other treatment groups.

c) Abnormality

There were no significant differences between the levels of VE, n-3 FA and their interactions.

d) Viability

The effect of n-3 FA, VE and their interaction on sperm viability percentage was significant. The viability percentage of sperm was 59.88±0.25, 62.22±0.25 and 61.70±0.25 for 0, 0.1 and 0.2 mmol VE, respectively and the former level had significantly lowest value (P≤0.001). The viability percentage was 61.45±0.29, 61.57±0.29, 60.01±0.29 and 62.02±0.29 for 0, 0.1, 1, and 10 ng ml^{-1} FA, respectively and 1 ng ml-1 FA group had significantly lowest value (P≤0.05).

The viability percentage was significantly lower in 1 and 10 ng ml-1 FA with 0 mmol of VE groups than in other treatment groups.

e) Recovery rate

The effect of n-3 FA, VE and their interaction on sperm recovery rate was significant. The recovery rate of sperm 23.33± 1.51, 26.11± 1.51 and 36.9± 1.51 for 0, 0.1 and 0.2 mmol VE, respectively and the latter level had significantly highest value (P≤0.001). The recovery rate was 23.44±1.74, 29.63±1.74, 26.56±1.74 and 35.5±1.74 for 0, 0.1, 1, and 10 ng ml-1 FA, respectively and the latter level had significantly highest value (P≤0.05).

The 0.1 and 10 ng ml-1 FA with 0.2 mmol VE level had significantly higher recovery rates than the other treatment groups.

Second experiment

The percentage of fatty acids of sperm lipids, n-3 and n-6 FA percentages, Monounsaturated Fatty Acids (MUFA) percentage, n-3/n-6 ratio and PUFA/SFA ratio are shown in table 2. The DHA percentage was significantly higher in the FA group before freezing and after thawing with comparison to the group without FA. The ratio of n-3/n-6 decreased after thawing in both groups but it was higher in the FA group before freezing.

In the first experiment, adding n-3 FA and VE as a biological antioxidant improved motility parameters of frozen-thawed sperm. Addition of palmitic acid or linoleic acid into the ram semen´s extender have significantly increased motility and viability of post-thawed sperm, in vitro fertility and in vitro blastocyst production (Badr *et al.*, 2004). In contrast, in the other experiment, vitamin A, cod liver and flaxseed oil as a n-3 fatty acid source

loaded on cyclodextrin could not improve post-thawed sperm quality in bull (Amorim *et al*., 2008).

In the current research, the level of DHA, n-3 FA, ratio of n-3/n-6 and PUFA in group FA was higher than the group without FA that reflects the effective incorporation of n-3 FA into sperm cell membrane. Before freezing, docosahexaenoic acid percentage was higher in the FA group (10 ng ml^{-1} FA, 0.2 mmol VE) than that in the group without FA (0 ng ml^{-1} FA, 0 mmol VE) and it decreased significantly in both groups after thawing. Possible reasons of this decrease could be as follows: lipid peroxidation has been reported as being enhanced during cryopreservation of spermatozoa and this could account to some extent for the decrease in LCPUFA observed in this experiment. Another plausible explanation for the decrease in the proportion of polyunsaturated fatty acids could be an increase in the amount of saturated fatty acids taken up or passively bound to the sperm membranes which would cause a decrease in the proportion of the LCPUFA (Maldjian *et al.*, 2005).

The positive effect of n-3 FA on sperm characteristics is probably related to an increase in DHA proportion in sperm membrane lipids. Spermatozoa from asthenozoospermic, oligozoospermic and oligoasthenozoospermic men had lower levels of docosahexaenoic acid than those from normozoospermic men. In addition, a significant positive correlation has been observed between DHA and sperm motility, sperm concentration and normal sperm morphology (Aksoy *et al*., 2006).

The supplemented n-3 FA probably enhances PUFA proportion in sperm head and tail membrane which improves fluidity that is necessary for sperm motility. Furthermore, analysis of fatty acids from the head and tail of monkey sperm showed that DHA composed 1.1 and 19.6 percent of total fatty acids of head and tail, respectively; consequently, 99 % of sperm DHA is in the tail. This difference between lipid composition of the head and tail may be necessary for specific functions of sperm since fat plays a major role in integrity, fluidity, stability, and permeability of plasma membrane. Therefore, high proportion of DHA in the sperm tail may be necessary because they increase sperm motility via increasing membrane fluidity in sperm tail and thus, improving sperm tail flexibility required for motility (Connor *et al*., 1998). In addition, improved fluidity and flexibility increases a tolerance to freezing and preventing of sperm cell membrane from disintegrating by ice crystal formation during freezing process. It is assumed, that PUFA plays a major role in cell movements, lipid metabolism, and sperm ability to attach and penetrate the oocyte. Decreasing in DHA proportion of sperm phospholipids is accompanied by a decrease in sperm number and motility in aged bull ejaculations (Kelso *et al*., 1997). Comparison of PUFA composition in plasma and spermatozoa from infertile men with idiopathic oligoasthenoteratozoospermia and normal men showed that the n-3 FA concentration was lower in plasma and spermatozoa from infertile men in comparison to normal men (Safarinejad *et al*., 2010).

Experiments with rooster (Cerolini *et al*., 2006), boar (Rooke *et al*., 2001) goat (Dolatpanah *et al.*, 2008) and turkey (Zaniboni *et al*., 2006) showed that inclusion of fish oil in the diet increased the number of progressively motile sperm. These reports certify the results of the current study.

Even though using ethanol as a solvent for adding α-tocopherol and α-tocopherol succinate to equine semen extender had no harmful effect on sperm parameters (Almeida and Ball., 2005) but, in our study sperm parameters in positive control group in comparison with the negative control (ethanol-free) group were significantly decreased. It may be assumed that ethanol can have destructive effect on the sperm samples in this experiment. In fact, the observed loss of motility, progressive motility and viability may be the result of an interaction between damage due to freezing and due to the use of ethanol. Using other soluble substances (e.g. cyclodextrine) instead of ethanol during the semen freezing process is suggested (Brooks, 1990); which is, however in contrast with the oocyte-related experiments. However, it should be mentioned that the use of fatty acid and vitamin E can compensate to some extent for the destructive effects of ethanol.

In the current study, sperm motility, progressive motility and viability were affected by different levels of VE. Results of this study were consistent with the previous observations where supplementation of the extender with α-tocopherol prevented oxidative damage and thus improved sperm motility (Breininger *et al*., 2005; Jeong *et al*., 2009). Extracellular antioxidants are extremely important for the protection of mammalian spermatozoa against oxidative stress because the cytoplasmic extrusion associated with sperm morphogenesis depletes these cells of their internal store of antioxidant enzymes (Jeong *et al*., 2009). Among the antioxidants, especially α-tocopherol can break the covalent links that reactive oxygen species (ROS) have formed between fatty acid side chains in membrane lipids. This result indicates that α-tocopherol plays an important role in reducing membrane damage caused by excessive ROS production during cryopreservation.

Using egg yolk enriched in n-3 fatty acids without any antioxidant in diluents, failed to improve the quality of sperm following cryopreservation (Maldjian *et al*., 2005), but using FA accompanied by vitamin E in the present study increased post-thawed sperm quality. Therefore, for seeing the positive effects of n-3 FA, inclusion of an antioxidant into extender is useful. In conclusion, the present study showed that the adding n-3 FA accompanied by vitamin E to Bioxcell extender

increased post-thaw sperm quality in goat. Improved characteristics of frozen-thawed sperm may be due to effective incorporation of DHA into the cell membrane before freezing and that has protective effects on sperm membrane.

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