Effect of Dietary Fish Oil Supplementation on Ram Semen Freeze ability and Fertility Using Soybean Lecithin and Egg Yolk-Based Extenders

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Abstract

Ram semen cryopreservation is not efficient for artificial insemination in commercial herds. Beneficial effects of dietary fish oil have been evaluated for cryopreservation of ram semen in soybean lecithin and egg yolk based extenders. A factorial study (2 diets $\times$ 2 extenders) was used to analyze the effects of two diets supplemented with fish oil (n-3 fatty acid) or palm oil (saturated fatty acids; SFA) to freeze ram semen in two extenders containing soybean lecithin (SL) or Egg yolk (EY). Motility characteristics, membrane integrity, abnormal morphology, mitochondria activity, acrosome integrity, apoptotic status, and fertilizing ability were assessed after freeze-thawing. Although diet had significant ($P \leq 0.05$) effects on the quality parameters of frozen-thawed sperm, effects of extenders on these traits were not significant ($P > 0.05$). The higher significant ($P \leq 0.05$) percentage of total motility and progressive motility were observed in n-3/SL (44.83 ± 1.56 and 28.33 ± 1.4) and n-3/EY (43.33 ± 1.56 and 28.50 ± 1.4) compared to SFA/SL (32.16 ± 1.56 and 14.00 ± 1.4) and SFA/EY (31.66 ± 1.56 and 12.66 ± 1.4) groups. Moreover, n-3/SL and n-3/EY produced the higher significant ($P \leq 0.05$) percentage of membrane integrity of sperm (39.83 ± 1.4 and 37.33 ± 1.4) compared to SFA/SL and SFA/EY (29.83 ± 1.4 and 28.5 ± 1.4). For viability results, the higher significant percentage of live sperm was observed in n-3/SL and n-3/EY (43.16 ± 1.38 and 45.66 ± 1.38) compared to SFA/SL and SFA/EY (28.66 ± 1.38 and 27.5 ± 1.38). For Fertility trials, n-3 based diets (n-3/SL and n-3/EY) improved significantly ($P \leq 0.05$) pregnancy rate (44% and 46%), parturition rate (42% and 42%) and lambing rate (46% and 44%) compared to the SFA based diets (SFA/SL and SFA/EY). No interaction effects have been found between diets and extenders ($P > 0.05$). It seems that dietary fish oil can improve the

semen performance after freezing-thawing process and artificial insemination aside from type of extenders.

Keywords: Ram, Sperm freezing, Mitochondria activity, Apoptosis, Pregnancy rate

1. Introduction

Polyunsaturated fatty acids (PUFAs) in sperm cells play a crucial role for metabolism energy [1], plasma membrane fluidity [2] as well as many related function to fertilization events [3]. Ram sperm cells contain high amount of omega-3 polyunsaturated fatty acids specially docosahexaenoic acid (DHA) and eicosapentaenoic (EPA) which are synthesized in the body from short chain fatty acids (alpha-linolenic acid) [4]. DHA and EPA are provided for body using their precursors (ALA) in the diet [5] or direct inclusion to the diet [2, 4]. Efficacy of dietary fish oil as common source of omega-3 fatty acids has been evaluated to improve sperm quality as well as fertility in human [2, 6, 7], bull [8], boar [9-12], ram [13, 14], goat [15] and avian species [16, 17]. These beneficial effects are mainly related to their impacts on the sperm plasma membrane which leads to protection of sperm against environmental damages [14]. Sperm plasma membrane is strongly affected by various biochemical and anatomical damages in freezing procedure [18]. Therefore, supplementation of diet with sources of DHA and EPA can be an efficient strategy for protection of sperm against cryoinjuries. Moreover, we used a new plant-based extender for cryopreservation of ram semen containing soybean lecithin which was applied for freezing of ram [19], goat [20] and human spermatozoa [21]. Soybean lecithin considerably increased the percentage of motility, viability and cellular parameters of frozen-thawed sperm via formation of a layer around of sperm against reactive oxygen species (ROS) [19]. Up to now, no study has been conducted for evaluation of frozen-thawed sperm quality and

fertility obtained from ram fed diets supplemented with omega-3 and cryopreserved with soybean lecithin based extender. Therefore, we performed this experiment to assess several frozen-thawed sperm parameters such as motion characteristic, membrane integrity, morphology, mitochondria activity, acrosome integrity and viability. Finally, artificial insemination was performed to evaluate the fertility potential of thawed sperm.

2. Materials and methods

Chemicals used in this study were obtained from Sigma Co (St. Louis, MO, USA) and Merck (Darmstadt, Germany) unless otherwise indicated. Approval for the present study was given by the Research Ethics Committees of Royan Institute.

2.1. Animals and diets

Twenty mature fat-tailed Zandi rams with proven fertility (3-4-year-old and 75 ± 2.5 kg body weight) were randomly assigned into two groups in the breeding season as follows: 1) control group received a diet (AFRC, 1995) supplemented with palm oil (3% DM) and 2) fish oil based diet received a diet (AFRC, 1995) supplemented with fish oil (3% DM, Khazar Fish Powder Company, Iran). Both diets were set isocaloric and isonitrogenous (ME: 2.24 Mcal/kg in DM and CP: 12% in DM, Table 1). After 60 days feeding of rams with diets, semen collection was begun and the collected semen from each group divided into two aliquots for cryopreservation in two extenders (Egg yolk based extender and Soybean lecithin based extender). Therefore, this experiment was performed in a factorial based design (2 diets $\times$ 2 extenders) with 6 replicates.

2.2. Semen collection and cryopreservation

Semen samples were collected from each ram using artificial vagina twice a week during 3 weeks (Six replicates). Ejaculation samples were accepted for experiment if the following parameters were observed

: volume ranging between 1 - 2 ml; sperm concentration of 3×10^9 sperm/ml and sperm total motility higher than seventy percent. To eliminate individual differences, semen obtained from each group was pooled and then divided into two groups to be diluted with two extenders (extender supplemented with 1% soybean lecithin vs. extender supplemented with 20% egg yolk). The component of the basic extender was according to our previous study [22]. After dilution of semen with extenders, semen was loaded into 0.25 ml French straws reach up to final concentration of 100×10^6 sperm/ml and sealed with polyvinyl alcohol powder and held at 4 °C for 120 min. Then, straws were placed 7 cm above the liquid nitrogen for 12 min and finally plunged into the liquid nitrogen for storage. For evaluation of frozen semen, straws were melted at water bath (37 °C, 40 s).

2.3.Sperm evaluation after cryopreservation

2.3.1. Motion characteristics

Sperm class analyzer (Animal Version 12.3 CEROS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to evaluate motion characteristics. Evaluated parameters consisted of: Total motility (TM, %); progressive motility (PM, %); average path velocity (VAP, m/s); straight-line velocity (VSL, m/s); curvilinear velocity (VCL, m/s); amplitude of lateral head displacement (ALH, m); beat/cross frequency (BCF, Hz); straightness (STR, %) and linearity (LIN, %)[23].

2.3.2. Membrane integrity and Morphology

Hypo osmotic swelling test (HOST) was performed according to Revell and Mrode with slight corrections to evaluate membrane integrity [24]. Twenty micro liters of semen were added to 200 μ l of the hypo osmotic solution (9.0 g fructose, 4.9 g trisodium citrate, in 1000 ml of H₂O with 100 m osm/kg water). The sperm were evaluated under the invert light microscope after 45 min incubation at room temperature. Two hundred sperm were recorded in 5 different microscopic fields. Then, the number of sperm with swollen and non-swollen tails were recorded as membrane intact and damaged, respectively. Hancock solution was also used to assess the frozen-thawed sperm morphology. Three drops of each sample were added to eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution and 500 ml double-distilled water). One drop of this mixture was put on a slide and covered with a cover slip. The percentage of sperm with abnormal acrosome (acrosome and cap abnormalities, detached heads, abnormal mid-pieces and tail defects) was recorded by counting a total of 200 spermatozoa under phase-contrast microscope (magnification 1000 $\times$, oil immersion).

2.3.3. Mitochondria integrity

Rhodamine-123 (R123; Invitrogen TM, Eugene, OR, USA) and PI was used for assessment of the percentage of sperm with active mitochondria [25]. Ten microliters of Rhodamin-123 solution (0.01 mg/ml) was added to 300 μ l of diluted semen samples by Tris buffer and incubated for 20 min in the dark room. Then, sperm suspension was centrifuged at 500 $\times$ g (3 min), and again was resuspended in 500 μ l Tris buffer. Then, 10 μ l of PI (1 mg/ml) was added to sperm suspension. The sperm was analyzed with a FacsCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer. For each sample, 10000 events were recorded. Sperm with active mitochondria was identified by positive signal for Rh123 and negative signal for PI.

2.3.4. *Acrosome integrity*

Five hundred μl of the sperm (1×10^6 sperm/ml) was placed into a 1.5 ml microtube. Then, sample was centrifuged ($600 \times g$, 10 min) and the resultant sperm pellet was dissolved in 100 μl of 96% ethanol. After 15 min, the sperm was fixed and 10 μl of mixed sperm was placed in a thin line on a glass slide to evaporate the remaining ethanol. Then, 30 μl of fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (PSA-FITC; 50 $\mu\text{l}/\text{ml}$) was added to the sperm which was placed on the top of the sperm. Then, the glass slide was incubated for 20 min and subsequently dripped 10 times in distilled water, allowed to dry and then was mounted with glycerol. Three hundred sperm per sample were assessed by a fluorescence microscope (BX51; Olympus) at 400x magnification. Sperm with a green fluorescent band in the head area was considered as intact, those with no fluorescence in the head area or with a green fluorescent band at the equatorial level was recorded as damaged acrosome [26].

2.3.5. *Phosphatidyl serine externalization*

Annexin-V and PI kit was used to detect phosphatidyl serine externalization as an index of apoptosis changes in the sperm cells. Sperm was prepared according to the study of Sharafi et al [23] with a slight modification. Sperm was washed in calcium buffer and adjusted to the concentration of 1×10^6 sperm/ml followed by the addition of 10 ml Annexin V-FITC (0.01 mg/mL) to 100 ml of the sperm suspension, which was then incubated for 20 minutes on ice. Then, 10 ml of propidium iodide (PI; 1 mg/ ml) was added to the sperm suspension and incubated for at least 10 minutes on ice. Afterwards, suspension was evaluated by flow cytometer. After flow cytometric procedure, sperm subpopulations were classified into four groups: 1) live non-apoptotic cells negative for both Annexin-V and PI (A^-/PI^-); 2) early apoptotic cells positive for Annexin-V but negative for PI (A^+/PI^-); 3) late apoptotic cells

positive for both Annexin-V and PI (A^+/PI^+); and VI) necrotic cells negative for Annexin-V but positive for PI (A^-/PI^+). Finally, the viable sperm and early apoptotic sperm was counted as live sperm and late apoptotic sperm and necrotic sperm was counted as dead sperm (Fig.3).

2.4.Artificial insemination

For evaluation of frozen-thawed sperm fertility potential, 400 fat-tailed Zandi ewes with 55 ± 5 kg weight and 3-4 years old received CIDR (Easy-Breed™, CIDR®, New Zealand) for 12 days and 500 IU intramuscular eCG (Sanofi Animal Health, Libourne Cedex, France) at the time of CIDR removal. Fifty four h after CIDR removal ewes were assigned into four equal groups (100 ewes per group; according to experimental groups) and inseminated via laparoscopic method. Pregnancy diagnosis performed by an ultrasound unit (Falco 100, premedical) that equipped with a 3.5 MHz sector probe in day 50 after insemination. Pregnancy, parturition, lambing and twinning rate were recorded as an index of fertility and reproductive performance.

2.5.Statistical analysis

Sperm data was analyzed via GLM procedure of SAS software (version 9.1; SAS Institute, 2002, Cary, NC, USA). The Tukey test was used to compare the means of sperm parameters. Reproductive parameters were analyzed by GENMOD procedure using Chi-Square.

3. Result

Results of motion characteristics of frozen-thawed sperm using two diets (n-3 and SFA) with two extenders (SL and EY) have been shown in Table 2. Although diet had significant ($P \leq 0.05$) effects on the motion quality of frozen-thawed sperm, effects of extenders on these traits were not significant ($P > 0.05$). Moreover, interaction effects of diets and extenders were not significant

($P>0.05$) on the motility parameters of frozen-thawed sperm. The higher significant ($P\leq 0.05$) percentage of total motility was observed in n-3/SL and n-3/EY compared to SFA/SL and SFA/EY groups. Furthermore, higher significant percentage of progressive motility was produced in n-3/SL and n-3/EY compared to SFA/SL and SFA/EY. For VAP, VSL, VCL, ALH, BCF, STR and LIN, no significant ($P>0.05$) effects were observed between groups.

Figure 1 shows the average of membrane integrity, total abnormality, mitochondrial activity, acrosome integrity, viability and apoptosis status of frozen sperm after thawing in various groups. n-3/SL and n-3/EY produced the higher significant ($P\leq 0.05$) percentage of membrane integrity (39.83 ± 1.4 and 37.33 ± 1.4) compared to SFA/SL and SFA/EY (29.83 ± 1.4 and 28.5 ± 1.4). For morphology parameters, no significant difference was observed in case of total abnormality between groups. For mitochondria activity and acrosome integrity, no significant differences ($P>0.05$) were observed among four groups.

Externalization of phosphatidyl serine (PSE) of sperm after freeze-thawing in groups containing different diets and extenders showed that diets had significant ($P\leq 0.05$) effects on the results of PSE, whereas extender had not significant effects. The higher significant ($P\leq 0.05$) percentage of live sperm was observed in n-3/SL and n-3/EY (43.16 ± 1.38 and 45.66 ± 1.38) compared to SFA/SL and SFA/EY (28.66 ± 1.38 and 27.5 ± 1.38).

For dead spermatozoa, n-3/SL and n-3/EY (56.83 ± 1.69 and 54.33 ± 1.69) produced the lower significant ($P\leq 0.05$) percentage compared to SFA/SL and SFA/EY (71.34 ± 1.69 and 72.5 ± 1.69).

Table 3 has shown the results of fertility potential of frozen-thawed sperm after artificial insemination. n-3 based diets (n-3/SL and n-3/EY) significantly ($P\leq 0.05$) increased pregnancy

rate, parturition rate, and lambing rate compared to the SFA based diets (SFA/SL and SFA/EY). For twinning rate, no significant difference was observed among groups.

4. Discussion

In this trial, fish oil inclusion to the diet significantly improved the quality indices of sperm such as motility, membrane integrity, viability and fertilizing ability after cryopreservation. The reason of this improvement may be increment of PUFA in the sperm membrane especially in the head and tail by feeding of rams with n-3 supplemented diet. In mammalian, presence of long chain fatty acids is crucial for many activities of sperm from spermatogenesis up to fertilization [27, 28]. Dietary DHA increased the motility, membrane fluidity and flexibility of sperm tail [3]. Therefore, increasing in the percentage of motile and viable sperm in the present study could be justified due to presence of n-3 source (fish oil) in the diet of rams. Our findings are in agreement with those reported beneficial effects of fish oil for fresh storage of goat [15] and ram [14] semen. For bull, although motion characteristics of fresh sperm were improved by fish oil supplement, these indices were not modified in frozen state [8].

Presence of DHA acyl-chain in phospholipids bilayers of sperm plasma membrane led to the increase of the conversion between extended and loop conformation resulted in improvement of flexibility of sperm plasma membrane [28]. In our study, rams which were fed SFA diets produced the higher significant number of frozen-thawed dead sperm which is in compliance with Argov-Argaman, (2013) who reported that increment of saturated fatty acid in the membrane of sperm could reduce membrane fluidity as well as resistance against cryoinjuries [29]. Furthermore, DHA may enhance a series of actions which lead to prevent the early apoptosis in the sperm cell [30].

Morphology of sperm after cryopreservation was not affected by diet in our study which was not in agreement with Strzezek et al. (2004) who reported that n-3 fatty acid of the diet increased the normal morphology of sperm in liquid storage [11]. This discrepancy may be related to the states of storage (fresh vs. frozen).

Flow cytometric procedures showed that n-3 fatty acids are not effective for preservation of mitochondria potential and acrosome integrity of sperm after cryopreservation. In this regard, Revell and Mrode (1994) have reported that PUFAs improved mitochondria activity and acrosome integrity of sperm if the semen sample has poor quality [24]. Therefore, our expectation to increase the mitochondria activity and acrosome integrity of sperm under consumption of n-3 had a little chance because our method for evaluation of mitochondria activity and acrosome integrity just shows the integrity in acrosome and mitochondria of sperm aside from being alive or dead.

Fertility evaluation at the farm was an important trial in this study to verify in vitro results of sperm performance. Fish oil groups (n3/SL and n3/EY) produced higher percentage of reproductive performances including pregnancy rate, parturition rate and lambing rate compared to SFA groups (SFA/SL and SFA/EY). These results are in agreement with a study [3] which reported that infertile men have lower concentration of n-3 in seminal plasma compared to normal men. Although several experiments have been conducted to evaluate the effects of fish oil on sperm quality of different species [1, 8, 31, 32], for the first time we performed artificial insemination for evaluation of cryopreserved semen of rams which were fed dietary fish oil. Due to several physiological aspects of reproductive tracts in sheep, frozen-thawed sperm is not reliable for desire fertility. However, this study had an average of 31.5% pregnancy rate from

frozen-thawed sperm which was comparable with other studies using frozen-thawed ram sperm for artificial insemination [33, 34].

We applied two extenders (soybean lecithin and egg yolk based extender) for cryopreservation of sperm obtained from rams fed two diets (n-3 vs. SFA). In our previous studies [19, 22, 35] soybean lecithin (SL) had shown beneficial effects for protection of ram sperm against cryoinjuries because it can adhere to the sperm plasma membrane and preserve the sperm against ROS [22]. Moreover, several problems of egg yolk such as contamination, agglutination, and standardization of extender containing egg yolk lead to the increase of the request for replacement of egg yolk in cryopreservation media [35]. In some studies, SL had higher protection effects [18] compared with egg yolk and in others SL had equal effects with egg yolk [22]. Therefore, our hypothesis was that which extender (SL or EY) is more efficient for cryopreservation of sperm obtained from rams were fed dietary fish oil. In all of sperm parameters and fertility results, extenders had not significantly effects on the frozen-thawed sperm quality and extenders had equal preservation effects. Also, no interaction effect was observed between extenders and diets. However, regarding to problems related to contamination, agglutination and standardization of extends containing for egg yolk in extenders, we would rather use SL in cryopreservation media for frozen storage of ram semen during long period.

Conclusion

Supplementation of diet with fish oil for two months improves semen quality and fertility after freezing-thawing process aside from type of extenders. It's recommended to use SL containing extender for freezing-thawing process of ram semen.

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Table1. Components of diets

	Control (Palm oil)	Treatment (Fish oil)
Alfalfa (% DM)	41.96	41.96
Corn silage (% DM)	28	28
Straw (% DM)	9.5	9.5
Barley (% DM)	6.04	6.04
Wheat bran (% DM)	10.18	10.18
Palm oil (% DM)	3	0
Fish oil (% DM)	0	3
CaCO ₃ (% DM)	0.5	0.5
NaCl (% DM)	0.32	0.32
Vitamin E (% DM)	0.5	0.5
Metabolizable energy (Mcal/kg DM)	2.24	2.24
CP (% in DM)	12	12
Ether extract (% in DM)	5.28	5.28
NDF (% in DM)	48.6	48.6
Calcium (% in DM)	0.79	0.79
Phosphorus (% in DM)	0.31	0.31

Table2. Motion parameters of frozen-thawed ram sperm using diets (n-3 and SFA) and extenders (SL and EY).

Groups	n-3/SL	n-3/EY	SFA/SL	SFA/EY	SEM
TM (%)	44.83 ^a	43.33 ^a	32.16 ^b	31.66 ^b	1.56
PM (%)	28.33 ^a	28.50 ^a	14.00 ^b	12.66 ^b	1.40
VAP (μm/s)	98.66	97.86	99.85	99.26	2.34
VSL (μm/s)	71.65	70.80	70.41	70.53	0.93
VCL (μm/s)	171.63	168.66	169.80	169.46	1.84
ALH (μm)	9.11	8.96	8.36	8.10	0.33
BCF (Hz)	28.03	28.55	26.66	27.45	0.68
STR (%)	72.67	72.61	70.83	71.17	1.73
LIN (%)	41.75	41.97	41.50	46.66	2.71

Sperm samples were collected from 20 rams (10 rams per each group) fed two diets (fish oil & palm oil) for cryopreservation in two extenders (soybean lecithin & egg yolk extenders). Means $\pm$ SEM of total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat/cross frequency (BCF), straightness (STR) and linearity (LIN) were assessed after thawing. Different letters showed significant differences at $P \leq 0.05$ between groups. Fish oil omega-3 (n-3); palm oil saturated fatty acid (SFA); soybean lecithin (SL); egg yolk (EY).

Table 3. Effect of diets (n-3 and SFA) and freezing extenders (SL and EY) on reproductive performance of fat-tailed Zandi ewe.

Diet	n-3		SFA	
Extender	SL	EY	SL	EY
Pregnancy rate (%)	44 ^a (44/100)	46 ^a (46/100)	20 ^b (20/100)	16 ^b (16/100)
Parturition Rate (%)	42 ^a (42/100)	42 ^a (42/100)	16 ^b (16/100)	16 ^b (16/100)
Lambing Rate (%)	46 ^a (46/100)	44 ^a (45/100)	18 ^b (18/100)	17 ^b (17/100)
Twining Rate (%)	9.52 (4/42)	7.14 (3/42)	12.5 (2/16)	6.25 (1/16)

Fertility potential of frozen-thawed sperm were assessed after cryopreservation of sperm samples obtained from 20 rams (10 rams per each group) fed two diets (fish oil & palm oil). Frequency of pregnancy rate, parturition rate, lambing rate and twining rate were counted after artificial insemination. Different letters showed significant differences at $P \leq 0.05$ in the same rows between groups. Fish oil omega-3 (n-3); palm oil saturated fatty acid (SFA); soybean lecithin (SL); egg yolk (EY).

Fig. 1. Sperm samples were collected from 20 rams (10 rams per each group) fed two diets (fish oil & palm oil) for cryopreservation in two extenders (soybean lecithin & egg yolk extenders). Means $\pm$ SEM of Membrane integrity, total abnormality, mitochondrial activity, acrosome integrity, viability and apoptosis status were assessed after thawing. Different letters showed significant differences at $P \leq 0.05$ between groups. Fish oil omega-3 (n-3); palm oil saturated fatty acid (SFA); soybean lecithin (SL); egg yolk (EY).