Research Article

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The influence of dietary sunflower oil, rich in n-6 polyunsaturated fatty acids, in combination with vitamin C on ram semen parameters, sperm lipids and fertility

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Abstract

BACKGROUND: Dietary fats, fed to ruminant animals, have been observed to improve reproductive function. Sunflower oil is one of the richest sources of polyunsaturated fatty acids (PUFAs), over 60% of which is linoleic acid (C18:2). With regard to recommendations for increased consumption of PUFAs, special emphasis has been given to the importance of consuming more antioxidants to prevent lipid peroxide formation. This study evaluated the effects of dietary n-6 PUFAs and vitamin C (VC) in rams – in particular, on semen quality, blood metabolites, sperm lipids and overall fertility.

RESULTS: Diets supplemented with SFO and VC increased the proportion of motile sperms and their progressive motility improved ($P < 0.01$). Rams fed a diet containing SFO had an increased proportion of sperms with normal acrosomes in their semen samples ($P < 0.01$). The highest levels of lactate dehydrogenase activity (LDH) enzyme were found in control rams (2.60 U mL^{-1}). Feeding SFO significantly affected blood triglyceride and cholesterol concentrations ($P < 0.05$). Diets containing SFO increased the proportion of C18:2c, C20:4, C20:3 and C22 in the sperm lipid composition ($P < 0.05$). Semen of SFO and VC-fed rams was more fertile than that of control rams when it was artificially inseminated to ewes.

CONCLUSION: Feeding rams with supplemental SFO and VC increased semen quality and improved fertility rates.

Keywords: lipid composition; fertility; rams; semen; sunflower oil; vitamin C

INTRODUCTION

Dietary regimes can beneficially affect the fertility of male animals by improving semen quality. Optimal feeding can improve both the quality and quantity of semen.[1] Different dietary fats, fed to ruminant animals, have been observed to improve reproductive function.[2] There is evidence that the lipid composition of the sperm membrane is a determinant of the motility, cold sensitivity and overall viability of sperm.[3] Polyunsaturated fatty acids (PUFAs) act as mediators in various processes in some reproductive tissues, including fluidity of cell membranes, intracellular signaling and susceptibility to oxidative damage.[4]

Fatty acids (FAs) derived from oil seeds (such as sunflower, linseed, cottonseed, rapeseed and soybean) have a beneficial impact on reproductive performance.[5–7] Mammals are unable to synthesize C18:2 and C18:3 FAs. Since they are necessary precursors for other long-chain FAs, these are essential FAs that must be sourced from the diet.[8] Sunflower oil (SFO) is one of the richest sources of PUFAs. It contains omega-6 FAs, over 60% of which is linoleic acid (C18:2).[1,6,7] It had been thought that, in ruminants, the transfer of dietary PUFAs to the semen could be impaired by rumen microorganisms because they hydrogenate those FAs.[9] However, recent research has shown that feeding extra linoleic acid leads to a significant increase in the concentration of this FA in the blood plasma of goats, thereby suggesting that unsaturated FAs have the capacity

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to resist bio-hydrogenation in the rumen.[10] It is possible that the fertility of stored ram sperm can be beneficially affected by dietary linoleic acid and can be used to enhance fertility success.[11]

The PUFAs are vulnerable to oxidation, and these oxidation products can have adverse effects once absorbed from the intestine.[12] Dietary lipids contribute to free radical loads in animals.[13] PUFAs are also associated with increased oxidative stress; this can reduce semen quality.[4] Therefore, when examining the overall effects of PUFAs, the mechanisms that can be used to alleviate their associated oxidation need to be considered.[14] In addition, when the consumption of PUFAs is recommended, the importance of consuming more antioxidants has to be emphasized to prevent lipid peroxide formation.[15]

Dietary antioxidants are scavengers that inhibit the formation of reactive oxygen species such as O_2^- and H_2O_2.[16] At high concentrations, vitamin C (ascorbic acid, VC) has antioxidant properties; it is able to scavenge oxygen and lipid free radicals.[16] In our previous work,[17] we reported the positive effects on semen characteristics and its post-thawed fertility of adding VC to diets containing fish oil n-3 PUFAs. In addition, positive effects of n-6 PUFAs (from seed oils) have been observed on the reproductive performance of rams and on the characteristics of their semen.[7] Mammalian spermatozoa have a high level of PUFAs. However, there is little reliable data on the effect of particular dietary FAs on ram sperm or the persistence of FAs assimilated from the diet.[7] To our knowledge, there are no reports concerning the characteristics of ram semen that focus on the effects of n-6 PUFAs and VC (a dietary antioxidant). The aim of this study, on fat-tailed Moghani rams, was to determine what effects dietary SFO and VC supplementation had on semen characteristics, blood metabolites, lipid composition of sperm and the fertility of semen.

MATERIALS AND METHODS

Animals and location

The experiment was conducted at the Moghani Sheep Breeding Station, located in Jafar-Abad, Ardabil province, Iran (39° 26′ 20.6″ N latitude and 48° 5′ 26.4″ E longitude). Sixteen fertile rams (mean live weight 60 ± 2 kg, 3–4 years old) were randomly allocated to four dietary groups and housed in individual pens. Rams were cared for in accordance with the guidelines of the Iranian Council of Animal Care.[18] Data collection was performed over 12 weeks, from 25 May to 31 August (outside of the physiological breeding season in Iran).

Experimental diets

Diets were formulated to comply with maintenance standards.[19] The experimental diets were: (i) control diet without SFO and VC; (ii) diet containing 25 g kg^{-1} dry matter (DM) SFO; (iii) diet containing 300 mg kg^{-1} DM VC (Rovimix C, Hoffmann-La Roche Ltd); and (iv) diet containing 25 g kg^{-1} DM SFO and 300 mg kg^{-1} DM VC (SFO-VC). Rams had free access to water and mineral blocks. All diets were isoenergetic (2.25 Mcal kg^{-1} DM metabolizable energy) and isonitrogenous (120 g kg^{-1} DM crude protein). The ingredients, chemical composition and FA profile of the diets are shown in Table 1.

Table 1. Ingredients and chemical composition of diets[a]

	Diets without SFO	Diets with SFO
Ingredients (g kg^{-1})		
Alfalfa hay	18.2	56.8
Wheat straw	54.5	22.7
Barley grain	15.2	5.5
Beet pulp (dry)	3.0	3.1
Canola meal	4.2	4.4
Wheat bran	3.0	3.1
SFO	–	2.5
Salt	0.6	0.6
Min. premix[b]	1.2	1.2
Chemical composition		
Metabolizable energy (Mcal kg^{-1} DM)	12.1	12.0
Crude protein (g kg^{-1} DM)	2.24	2.30
Calcium (g kg^{-1} DM)	0.89	0.88
Phosphorus (g kg^{-1} DM)	0.35	0.34
Fatty acids (g kg^{-1} FA)[c]		
Myristic 14:0	32	24
Palmitic 16:0	281	194
Palmitoleic 16:1	46	25
Stearic 18:0	191	104
Oleic 18:c^5 n-9	224	211
Linoleic 18:2c (n-6)	141	413
Linolenic 18:3 (n-3)	43	29
C20:0	–	–
C20:1	–	–
Myristic 14:0	–	–

[a] 300 mg kg^{-1} DM of VC was added to diets with VC.
[b] Contained 195.0 g kg^{-1} calcium, 21.0 g kg^{-1} magnesium, 1000.0 mg kg^{-1} cobalt, 300.0 mg kg^{-1} copper, 120.0 mg kg^{-1} iodine, 3000.0 mg kg^{-1} iron, 2200.0 mg kg^{-1} manganese, 3000.0 mg kg^{-1} zinc, 1.1 mg kg^{-1} selenium.
[c] Fatty acid profile of SFO was (g kg^{-1} FA): 1 g kg^{-1} myristic 14:0, 82 g kg^{-1} palmitic 16:0, 2 g kg^{-1} palmitoleic 16:1, 43 g kg^{-1} stearic 18:0, 256 g kg^{-1} oleic 18:1 n-9, 604 g kg^{-1} linoleic 18:2 n-6, 3 g kg^{-1} linolenic 18:3 n-3, 3 g kg^{-1} C20:0, 2 g kg^{-1} C20:1 and 4 g kg^{-1} C22:0.

Semen collection and evaluation

Semen was collected in an artificial vagina, at 12-day intervals and on six occasions. Prior to collection, the prepuce was cleaned to prevent contamination of the semen sample. Semen was collected in the morning, and transported to the laboratory (at 37 °C) within 10–15 min, then placed in a water bath at 37 °C. All ejaculates were evaluated for volume (mL), pH, sperm concentration (×10^9 sperm mL^{-1}), progressive sperm motility and the proportion of live and normal sperms. Semen volumes were measured using calibrated semen collection tubes. The concentration of sperm was determined with the aid of a Neubauer hemocytometer (after a 1:200 dilution of the semen sample with 2% eosin solution). The concentration of sperm per milliliter of semen was simply calculated by multiplying the total number of sperms in five large squares of the central counting area of the hemocytometer by five (the central counting area of the hemocytometer has 25 large squares), by dilution rate and by 10 000 (the volume over the central counting area of the hemocytometer is 0.1 mm^3 or 0.1 μL). To evaluate progressive sperm motility, a sample of the diluted semen was placed under a cover slip on a pre-warmed (37 °C) microscope slide and assessed subjectively using a phase-contrast microscope (×400 magnification). The proportions of live sperms from total sperms (live and dead) and their morphology were

determined by evaluating 200 sperms in each semen sample (following eosin–nigrosin staining), under a light microscope (×400 magnification).[20] Semen pH was measured directly using a digital pH meter (model pH 211 microprocessor, Hanna, Italy).

Acrosomal abnormalities were assessed by evaluating diluted sperm in buffered formalin-citrate solution.[16] A drop of semen was placed on a slide and examined by phase-contrast microscopy (×100 magnification). Sperms ($n = 200$ per slide) were evaluated visually; the proportion of sperms with normal acrosomes was determined. The hypo-osmotic swelling test (HOS test) was used to evaluate the functional integrity of the sperm membranes. This was performed by incubating 20 µL semen with 200 µL of a 100 mOsm hypo-osmotic solution (9 g fructose and 4.9 g sodium citrate in 1 L distilled water) at 37 °C, for 60 min. After incubation, 100 µL of the mixture was spread on a warm slide under a cover slip. A total of 200 sperms were evaluated in at least five different microscopic fields. The percentage of sperms with swollen and curled tails was then recorded.[21]

Lipid extraction and analysis

The compositions of sperm lipids were determined at the end of the trial (i.e., after 12 weeks). Semen samples were washed twice, following a sixfold dilution of semen, with 0.85% (w/v) NaCl solution, and then centrifuged at 700 × *g* for 20 min.[22] The lipids were extracted – after homogenizing the sperm – with a chloroform–methanol mixture (2:1, v/v).[23] The lipids were transmethylated by refluxing for 30 min with a mixture of methanol–toluene–sulfuric acid (20: 1: 1, v/v/v).[24] The resulting FA methyl esters were analyzed by gas chromatography (Unicam 4600, Cambridge, UK), equipped with a capillary BPX70 column, 30 m × 0.25 mm i.d., 0.25 mm film thickness (SGE, USA) and flame ionization detector. The FA profile of the experimental diets and the SFO were also analyzed.

Seminal fluid and chemical analyses

After evaluation, the ejaculates were centrifuged at 8944 × *g* for 30 min and the seminal fluid was aspirated. LDH activity in the seminal fluid was determined immediately after centrifugation, using a commercial kit (Pars Azmoon Co., Tehran, Iran; LDH analyzer, Kone Co., Finland).[25] The remaining seminal fluid was frozen (−20 °C) until analysis for Na^+ and K^+ concentrations (flame photometer model FP20; SEAC, Italy).

Blood sampling and hormonal assay

Blood was taken from the jugular vein at 12-day intervals and on six occasions. It was collected in heparinized tubes that were immediately placed on ice. The samples were then transferred to the laboratory and centrifuged at 1096 × *g* for 10 min to recover the plasma. Blood plasma was stored at −20 °C until analysis. On analysis day, the samples were thawed at room temperature and analyzed for triglycerides and cholesterol using commercial kits (Pars Azmoon) with an auto-analyzer (Hitachi 917). Testosterone concentrations were determined using a commercial radioimmunoassay kit (Radim Co., Italy), for which the minimum detectable plasma testosterone concentration was 0.35 nmol L^{-1}. The intra- and inter-assay coefficients of variation were 5.5% and 7.7%, respectively.

Synchronization and artificial insemination of ewes

Two of the experimental treatments with better semen features were examined for fertility rate in comparison to the control. Moghani ewes (172 in number, 3–4 years of age; 54–64 kg body weight and without any reproductive problems) were used to determine the effect of dietary SFO and VC supplementation on their fertility rates. Before being used in the experiment, the ewes grazed natural pastures and had free access to mineral block and water. During the experiment, the ewes were housed in pens. They had free access to water and feed, in the form of hay and wheat bran. An estrus synchronization program was performed shortly before the start of the mating season over 12 days, using a controlled intravaginal drug-releasing device (CIDR, containing 0.3 g progesterone; A5439, InterAg, New Zealand); on removal of the CIDR, they received an intramuscular injection of 300 IU eCG (Pregnecol®, Vetoquinol, Australia). Semen was diluted in a Tris–citrate–fructose–yolk extender (TCFY) to provide an insemination dose of 250×10^6 spermatozoa (0.25 mL mini tube straws) and held at 15 °C until insemination. Artificial insemination (AI) of ewes was performed intracervically, approximately 52–53 h after sponge withdrawal,[26] using a speculum with an attached light source and an ovine–caprine AI catheter. Fertilization success was calculated as the number of ewes lambing per number of ewes inseminated. No abortions or stillbirths occurred among the ewes.

Statistical analyses

Arcsine transformation was used for transformation of percentage data. Data on sperm, blood and seminal parameters were analyzed for the effects of SFO, VC, two-way interaction of SFO and VC, time and main effects by time interaction using the MIXED procedure of SAS as a repeated-measurement analysis.[27] Differences among least square means were declared by the Tukey–Kramer test and $P < 0.05$ was considered as the significance level. Data on sperm FA composition was analyzed as a completely randomized design to declare significant differences among experimental treatments by the GLM procedure of SAS.[27] Fertility rates were analyzed using the heterogeneity chi-square test. $P < 0.05$ was considered significant.

RESULTS

Semen parameters

A significant variation was observed over time for all semen parameters ($P < 0.01$; Table 2). Semen parameters improved as the experiment advanced. Semen volume, the proportion of motile sperms and progressive motility were improved by supplementation of SFO and VC (Table 3). The interaction between SFO and VC was only significant for sperm motility and progressive motility ($P < 0.05$). Mass motility was only affected ($P < 0.01$) by SFO feeding. The HOS test improved ($P < 0.05$) in samples from rams fed with SFO and VC, whereas the proportion of sperms with normal acrosomes was only improved ($P < 0.01$) by SFO diets. The highest HOS tests (83.5%) were observed in the SFO-VC rams. Feeding SFO and VC increased ($P < 0.01$) the proportion of live and normal sperms in SFO-VC rams, which had superior proportion *versus* other diets. Sperm concentration was increased ($P < 0.01$) by SFO and VC feeding, whereas the number of total live and normal sperm was affected only by SFO diets. The lowest (14.8%) proportion of abnormal sperm was encountered in the SFO-VC rams.

FA profile of sperm

After 12 weeks, diets containing SFO (SFO and SFO-VC) increased ($P < 0.05$) the proportion of sperm 18:2c (n-6), 20:4 (n-6), 20:3 (n-6)

Table 2. Effect of sampling time on semen characteristics in weeks of experiment

	Week							
	0	2	4	6	8	10	12	*P*-value
Semen volume (mL)	1.10 ± 018b	1.18 ± 0.5b	1.33 ± 0.1ab	1.35 ± 0.2ab	1.55 ± 0.4a	1.65 ± 0.2a	1.64 ± 0.2a	0.001
Mass motility (0–5)	3.41 ± 0.5c	3.84 ± 0.1bc	3.91 ± 0.5b	3.83 ± 0.5b	4.06 ± 0.4ab	4.47 ± 0.1a	4.44 ± 0.3a	0.001
Motility (%)	82.62 ± 1.7d	85.37 ± 2.1cd	86.50 ± 2.9bcd	86.87 ± 2.2bc	89.00 ± 3.9ab	91.19 ± 3.3a	91.87 ± 3.3a	0.001
Progressive motility (%)	79.44 ± 1.8c	80.35 ± 1.9c	81.81 ± 2.7bc	82.19 ± 2.5bc	84.31 ± 3.3ab	86.81 ± 2.9a	86.75 ± 2.9a	0.001
HOST (%)	81.24 ± 1.8b	81.05 ± 2.5b	81.80 ± 2.5b	82.38 ± 2.4ab	82.39 ± 1.6ab	83.55 ± 1.3a	83.74 ± 1.4a	0.001
Normal acrosome (%)	87.87 ± 2.5b	86.71 ± 2.0b	92.26 ± 3.9a	92.44 ± 4.3a	93.01 ± 3.5a	93.13 ± 0.9a	93.27 ± 0.9a	0.001
Sperm content ($\times 10^9$ mL^{-1})	4.42 ± 0.5bc	4.32 ± 0.2c	4.30 ± 0.2bc	4.29 ± 0.6b	4.48 ± 0.6ab	4.62 ± 0.5a	4.69 ± 0.6a	0.001
Live sperm (%)	75.00 ± 3.5bc	70.19 ± 1.9c	71.93 ± 2.1c	73.75 ± 1.6bc	77.90 ± 1.9ab	79.33 ± 3.6ab	80.87 ± 3.6a	0.001
Live and normal sperm (%)	77.16 ± 2.8bc	72.89 ± 2.2c	75.54 ± 1.3bc	79.70 ± 5.2b	82.66 ± 4.2ab	86.05 ± 2.3a	86.97 ± 2.4a	0.001
Total live and normal sperm ($\times 10^9$ mL^{-1})	3.70 ± 0.7c	4.34 ± 1.8c	5.06 ± 0.5bc	5.07 ± 1.2bc	6.26 ± 2.1ab	7.13 ± 1.3a	7.22 ± 1.7a	0.001
Abnormal sperm (%)	22.84 ± 2.8bc	27.10 ± 2.2c	24.45 ± 1.3bc	20.29 ± 5.3b	17.34 ± 4.2ab	13.95 ± 2.3a	13.03 ± 2.4a	0.001

Least square means in a row with differing letters (a, b, c, d) differ significantly ($P < 0.05$).
Data are mean ± SE of all rams over the experimental period.

Table 3. Effect of SFO and VC supplementation on semen characteristics

	Treatments[a]				Effects[b]		
	CON	SFO	VC	SFO-VC	SFO	VC	I
Semen volume (mL)	1.16 ± 0.3	1.48 ± 0.4	1.30 ± 0.4	1.65 ± 0.5	0.001	0.019	0.844
Mass motility (0–5)	3.75 ± 0.4	4.12 ± 0.7	3.94 ± 0.6	4.16 ± 0.6	0.001	0.208	0.389
Motility (%)	82.86 ± 2.8	88.96 ± 5.3	88.86 ± 4.3	89.86 ± 4.4	0.001	0.001	0.001
Progressive motility (%)	77.96 ± 2.6	85.11 ± 3.6	84.57 ± 4.1	84.75 ± 3.4	0.001	0.001	0.001
HOST (%)	80.94 ± 1.9	82.06 ± 1.1	82.73 ± 1.3	83.50 ± 1.6	0.006	0.001	0.605
Normal acrosome (%)	88.53 ± 4.7	93.06 ± 1.8	89.84 ± 4.9	93.54 ± 1.7	0.001	0.134	0.479
Sperm content ($\times 10^9$ mL^{-1})	4.07 ± 0.5	4.65 ± 0.4	4.24 ± 0.6	4.83 ± 0.5	0.001	0.057	0.957
Live sperm (%)	70.58 ± 2.9	76.01 ± 3.9	76.26 ± 3.9	79.42 ± 5.5	0.001	0.001	0.215
Live and normal sperm (%)	73.69 ± 3.0	78.32 ± 7.2	83.37 ± 5.7	85.19 ± 5.3	0.001	0.002	0.171
Total live and normal sperm ($\times 10^9$ mL^{-1})	3.69 ± 1.3	6.48 ± 2.8	5.14 ± 1.7	6.86 ± 2.3	0.001	0.005	0.093
Abnormal sperm (%)	26.31 ± 3.0	21.68 ± 5.7	16.63 ± 7.2	14.81 ± 5.3	0.002	0.001	0.171

[a] CON, control group; SFO, Sunflower oil group; VC, vitamin C group; SFO-VC, Sunflower oil and vitamin C group.
[b] SFO, effect of Sunflower oil; VC, effect of vitamin C; I, interaction of sunflower oil and vitamin C.

and 22:0 FAs, but the SFO-containing diets had no effect on other sperm FAs. VC supplementation did not affect the FA composition of sperm (Table 4).

Chemical and biochemical parameters of seminal fluid

The lowest semen pH was recorded in SFO-VC rams. The individual diets of SFO and VC did not alter sperm Na^+ concentrations but altered ($P < 0.05$) K^+ concentrations. The highest ($P < 0.05$) sperm LDH activity was recorded in control rams and the lowest was in SFO and VC rams (2.60 *vs*. 1.70 and 1.52 U mL^{-1}, respectively; Table 5).

Blood lipid metabolites and testosterone

Plasma triglycerides were not affected by VC supplementation. However, significant changes were recorded in the concentration of blood triglycerides and cholesterol in the SFO rams. The highest values of triglyceride and cholesterol were recorded in the SFO-VC group (22.68 and 67.64 mg dL^{-1}, respectively). Plasma testosterone concentrations were not affected by SFO feeding alone but increased ($P < 0.05$) when rams were fed VC-containing diets (Table 5).

AI and fertility rates

Table 6 presents the fertility rates of ewes artificially inseminated with fresh semen of control, SFO and SFO-VC rams (61.54%, 70.69% and 72.58%, respectively). Although the difference among the treatments was not statistically significant, the fertility rate of ewes inseminated with semen of SFO or SFO-VC rams improved numerically.

DISCUSSION

Dietary fats enhance reproductive vigor by supplying energy and also by actions on reproductive processes not related

Table 4. Effect of experimental diets (after 12 weeks) on FA composition (g kg^{-1}) of sperm lipid

	Treatments[a]				
	CON	SFO	VC	SFO-VC	*P*-value
Myristic 14:0	10.01 ± 0.54	8.40 ± 0.56	9.42 ± 0.62	8.54 ± 0.58	0.421
Palmitic 16:0	27.40 ± 1.52	25.4 ± 1.22	26.53 ± 1.18	24.50 ± 0.98	0.574
Palmitoleic 16:1	0.82 ± 0.04	0.50 ± 0.04	0.52 ± 0.04	0.46 ± 0.05	0.586
Stearic 18:0	14.81 ± 0.22	13.20 ± 0.23	13.80 ± 0.22	13.18 ± 0.22	0.686
Oleic 18:2c n-9	22.45 ± 1.43	21.60 ± 1.22	21.86 ± 1.22	21.00 ± 1.02	0.455
Linoleic 18:2c n-6	11.20 ± 0.98b	14.65 ± 0.86a	12.31 ± 0.22b	15.16 ± 0.22a	0.001
C20:0	0.43 ± 0.02	0.45 ± 0.04	0.46 ± 0.02	0.42 ± 0.03	0.089
C20:1	0.27 ± 0.04	0.30 ± 0.02	0.34 ± 0.04	0.31 ± 0.03	0.102
C22:0	2.02 ± 0.42b	3.60 ± 0.22a	2.20 ± 0.56b	4.08 ± 0.42a	0.001
C20:3 n-6	0.42 ± 0.03b	1.30 ± 0.20a	0.45 ± 0.02b	1.81 ± 0.24a	0.021
C20:4 n-6	1.45 ± 0.01b	2.50 ± 0.02a	1.20 ± 0.01b	2.70 ± 0.02a	0.001
C22:6 n-3	8.53 ± 0.34	7.50 ± 0.42	8.16 ± 042	7.41 ± 0.44	0.199
C24:1	0.40 ± 0.02	0.51 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.099

[a] CON, control group; SFO, sunflower oil group; VC, vitamin C group; SFO-VC, sunflower oil and vitamin C group.
Least square means in a row with differing letters (a, b, c) differ significantly ($P < 0.05$).
$^{*}P \leq 0.05$; $^{**}P \leq 0.01$.
ns, not significant.

Table 5. Effect of SFO and VC supplementation on pH, Na^+, K^+ and LDH concentration in seminal fluid and triglyceride, cholesterol and testosterone concentrations in blood

	Treatments[a]				Effects[b]		
	CON	SFO	VC	SFO-VC	SFO	VC	I
Seminal fluid							
pH	6.32 ± 0.51a	6.37 ± 0.40a	6.41 ± 0.42a	5.39 ± 0.30b	0.099	0.082	0.025
Na^+ (mg dL^{-1})	61.20 ± 4.02b	57.40 ± 5.11b	57.80 ± 5.95b	69.40 ± 3.72a	0.366	0.321	0.001
K^+ (mg dL^{-1})	64.60 ± 3.95b	69.80 ± 5.02a	66.50 ± 4.21ab	67.40 ± 3.84ab	0.013	0.015	0.022
LDH (U mL^{-1})	2.60 ± 0.20a	1.70 ± 0.12b	1.52 ± 0.15b	2.10 ± 0.18ab	0.001	0.011	0.287
Blood							
TG (mg dL^{-1})	17.05 ± 7.6c	19.97 ± 2.6ab	17.76 ± 5.8b	22.68 ± 2.5a	0.001	0.072	0.292
Cholesterol (mg dL^{-1})	45.57 ± 10.2c	57.57 ± 9.3b	50.32 ± 7.2c	67.64 ± 9.6a	0.001	0.001	0.124
Testosterone (nmol L^{-1})	3.74 ± 0.3b	3.79 ± 0.3ab	3.91 ± 0.5ab	4.00 ± 0.4a	0.313	0.010	0.799

[a] CON, control group; SFO, Sunflower oil group; VC, vitamin C group; SFO-VC, Sunflower oil and vitamin C group.
[b] SFO, effect of sunflower oil; VC, effect of vitamin C; I, interaction of sunflower oil and vitamin C.
[c] Least square means in a row with differing letters (a, b) differ significantly ($P < 0.05$).

Table 6. Conception rates ± SD in ewes intracervically inseminated with ram semen

Treatments	Lambed/total inseminated ewes	Lambing rate (%)
Con	32/52	61.54 ± 17.65
SFO	41/58	70.69 ± 17.35
SFO-VC	45/62	72.58 ± 17.00

CON, control group; SFO, sunflower oil group; SFO-VC, sunflower oil and vitamin C group.

to energy. For example, the increased availability of FA precursors favors an increase in steroid and eicosanoid secretion. This can alter ovarian and uterine functions and affect pregnancy rates. Within cells, FAs may have a direct effect on the transcription of genes that encode proteins essential to reproductive events.[5] Therefore, different kinds of this nutrient can be used to enhance reproductive vigor in both male and female animals. This study was conducted between 25 May and 31 August – outside Iran's natural breeding season. When outside of the natural season, managing conditions such as nutrition should prove valuable in improving semen quality. Improvements in semen quality have been noted in rams receiving PUFAs such as fish oil,[17,28,29] soybean oil,[30] olive oil or SFO.[6,7] de Graaf *et al.*[6] did not find any beneficial effect from feeding monounsaturated (e.g., from oleic or linoleic acid) or polyunsaturated (e.g., from olive oil or SFO) FA. However, in the present study, feeding PUFAs such as SFO improved all semen characteristics, including spermatozoal motility, progressive motility and sperm concentration, which is in agreement with the results of Esmaeili *et al.*[7] This is probably due to the high concentration of PUFAs in SFO – particularly linoleic acid – that can be incorporated into sperm lipids[31,32] and/or cause alterations in the fluidity and flexibility of the sperm membrane.[33] Mammals cannot synthesize n-3 and n-6 PUFAs *de novo* because they do not have relevant FA desaturase and elongase enzymes,[33] and

dietary PUFAs have been shown to be incorporated into semen in humans,[33] boars,[34] stallions,[8] rabbits[35] and rams.[7,17] Similarly, some alterations in sperm lipid composition, including increased *cis*-linoleic acid and arachidonic acids, were observed in this study by feeding SFO as a source of PUFAs.

Furthermore, it has been proposed that PUFAs in the testis may affect the packing of membrane-bound receptors, the activity of membrane-bound enzymes such as those associated with secondary messengers and with the resistance of membranes to physical and chemical stress.[36] There is evidence that feeding PUFAs can also affect the biosynthetic pathways involved in prostaglandin synthesis and steroidogenesis. The latter have multiple roles in the regulation of reproductive functions.[4] In addition, high levels of unsaturated FAs in sperm may have an effect on the regulation of cell movement and lipid metabolism.[37]

Long-chain PUFAs, including eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6), are precursors of eicosanoids. These include prostaglandins (PG), prostacyclins (PGI), thromboxanes (TX) and leukotrienes (LT).[38] The removal of two double bonds from AA by cyclooxygenase enzyme leads to the formation of series 2 eicosanoids; the removal of two double bonds from EPA leads to the formation of series 3 eicosanoids. AA is synthesized in the body from n-6 linoleic acid (LA, C18:2n-6) through a number of steps involving desaturation and elongation.[14] LA cannot be synthesized by animals[39] and must, therefore, be obtained through the diet. The n-6 LA is found in a number of sources, including soybean oil, safflower oil and SFO.[14]

In the present study, feeding SFO improved acrosomal integrity and favorably affected the HOS test. The HOS test is a good indicator of sperm membrane integrity; a positive correlation has been reported between the results of this test and non-return rates in female animals.[21]

Among the semen-quality parameters, motility, progressive motility, live and normal spermatozoa and HOS test were all significantly affected by VC supplementation of diets when fed to Moghani rams. When SFO and VC supplements were combined, they augmented the motility and progressive motility of spermatozoa. Although it has been shown that semen quality can be improved by dietary supplementation with PUFAs,[7,14,17] they are vulnerable to oxidation, which can also lead to increased oxidative stress, and this can reduce semen quality. VC is a water-soluble reactive-oxygen scavenger of high potency.[40] In addition, VC can regenerate small antioxidant molecules, such as α-tocopherol, glutathione (GSH) and β-carotene, from their respective radical species.[41] It has been observed that orally administrated ascorbic acid may degrade within the rumen of adult ruminants.[42] Hidiroglou and co-workers[43,44] reported that feeding finely powdered VC increased plasma ascorbic acid concentration in sheep and cattle. These observations support the notion that unprotected VC is partially destroyed in the rumen. The positive effect of VC on seminal parameters could be attributed to the antioxidant effects of VC on food before ingestion as well as its post-prandial effects.

In our Moghani rams, the pH of their seminal fluid was not significantly affected by SFO or VC diets; the lowest pH value was recorded in the SFO-VC group. A negative relationship has been reported, in sheep, between seminal fluid pH and motility parameters.[45,46] The cations Na^+ and K^+ are involved in the establishment of osmotic balance, and the osmolality of seminal plasma plays an important role in the activation of the sperm cell. Potassium ions also have a role to play in maintaining sperm in a quiescent state.[47] Low levels of Na^+ and K^+ ions were associated with low percentages of motile spermatozoa in rams.[48] In the present study, seminal concentrations of K^+ ion were increased by SFO and VC supplementation effects and their interactions. The levels of LDH enzyme in seminal fluid can be considered as a good indicator of a loss of plasma membrane integrity,[49] and a negative relationship has been reported between LDH levels in seminal fluid and the proportion of live spermatozoa.[48] Control rams had the highest LDH activity and the lowest proportion of live sperm.

Plasma cholesterol and TG increased in SFO fed animals, in agreement with the results of Gonthier *et al.*,[50] who observed increased plasma cholesterol by feeding n-3 PUFAs of flaxseed to dairy cows. Petit *et al.*[51] observed that feeding Megalac (a rumen-protected fat supplement rich in saturated FAs), flaxseed or sunflower seed resulted in increased plasma concentration of cholesterol in dairy cows. Feeding SFO had no effect on plasma testosterone levels; adding VC significantly increased the testosterone level. The positive effect of VC may stem from its antioxidant action by protecting the absorbed PUFAs (the precursors in eicosanoid synthesis). Eicosanoids are signaling molecules with important functions in the body. These include inflammation and various aspects of reproduction (i.e., ovulation, estrus, embryo survival and parturition).[14] Also, it has been established that increased dietary n-6 FAs are associated with higher plasma progesterone levels in ewes[52] and bovine corpora luteal cells.[53] Similar effects of PUFAs on steroid hormones may be anticipated in male animals.

The fertility rates we observed using fresh semen administered by cervical AI were similar to those reported previously.[54] As far as we know, there are no prior reports that have studied the feeding of n-6 PUFAs to rams and the effect this has on their semen quality or the fertility of their sperm. In the current study, the fertility rates obtained following cervical inseminations were 61.54%, 70.69% and 72.58%, when using fresh semen in the control, SFO and SFO-VC groups, respectively. Furthermore, the better fertility rate in the SFO and SFO-VC group was consistent with the improved semen quality of those parameters recorded (*in vitro*) within these groups.

CONCLUSION

Feeding SFO as a source of PUFAs compensated for the decreased semen quality encountered outside the physiological breeding season. The composition of PUFAs in sperm changed in response to ingested SFO, so the proportion of 18:2c (n-6), 20:4 (n-6), 20:3 (n-6) and 22:0 FAs increased. Although ascorbic acid may be partially destroyed in the rumen, dietary supplementation of finely powdered VC (a dietary antioxidant) improved semen quality and increased the response to dietary SFO. The results showed that dietary supplementation of SFO and VC had beneficial effects on the fertility of ram semen. Higher fertility rates were recorded for ewes inseminated with fresh semen from rams fed diets supplemented with VC and SFO. However, more research is needed to determine the effects of other sources of PUFAs on domestic animal reproduction. Research is also needed to determine the mode of this action and the capacity of differing methodologies to protect these PUFAs from biohydrogenation in the rumen.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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