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Original Research

Effects of Dietary Thyme (*Thymus vulgaris*) and Fish Oil on Semen Quality of Miniature Caspian Horse

Ali Kheradmand Garmsir MSc [a], Ahmad Zareh Shahneh PhD [b], Sayed Mohammad Ali Jalali PhD [a], Houshang Nouri MSc [c,*], Majid Afshar MSc [d]

[a] *Department of Animal Science, Faculty of Agriculture and Natural Resources, Islamic Azad University, Shahrekord, Iran*
[b] *Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran*
[c] *Department of Animal Science, College of Agriculture and Natural Resources, University of Zabol, Zabol, Iran*
[d] *Department of Animal Science, Agriculture and Natural Resources Research Center of Tehran, Khojir Research Station, Tehran, Iran*

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ABSTRACT

Thyme (*Thymus vulgaris*) is a subshrub from the lamiaceae family with plants that are rich in essential oils and antioxidative phenolic substances. The aim of the study was to investigate the effect of dietary thyme and fish oil supplementation on the semen characteristics of miniature Caspian horse. Sixteen stallions were randomly allocated into four groups and received four different diets: unsupplemented control diet, supplemented with fish oil at 2.5% dry matter intake (DMI), supplemented with fish oil (2.5% DMI), and thyme (0.02% DMI), and supplemented with thyme (0.02% DMI). All experimental diets were formulated according to National Research Council (1998). Semen was collected at 0, 30, 60, and 90 days. The semen samples were cooled and preserved at 5°C. Cooled diluted semen samples were evaluated in vitro by microscopic assessments of chilled sperm motility, acrosomal and other abnormalities (head, midpieces, and tail), viability (evaluated by Eosin–nigrosin), and plasma membrane integrity (evaluated by hypo osmolarity swelling test), and the level of malondialdehyde (MDA) was determined during cool storage 0, 24, and 48 hours after collection. The results showed that total and progressive sperm motility and plasma membrane integrity and functionality in all groups were significantly decreased with increasing storage time. On the other hand, the level of MDA in all groups was significantly increased with increasing storage time. Also, the results showed that most sperm quality parameters in this study were significantly higher in fish oil–thyme and fish oil group compared with thyme and control groups after 24 and 48 hours of storage at 5°C. We concluded that dietary supplementation of fish oil and thyme can improve sperm quality in miniature Caspian stallions during storage in cool condition via increasing total and progressive motility and plasma membrane integrity and functionality. More advances in vitro evaluations and artificial insemination are required to reveal the exact effects of thyme on miniature Caspian stallion sperm quality and its fertilizing ability.

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1. Introduction

The use of cooled or frozen shipped semen offers many advantages to breeders. Unfortunately, many stallions produce semen that is unable to provide acceptable fertility after undergoing the rigors of cooling and storage. Cooled

* Corresponding author at: Houshang Nouri, MSc (PhD student), Department of Animal Science, College of Agriculture and Natural Resources, University of Zabol, Karaj, Tehran, Iran.
E-mail address: houshang.nouri@ut.ac.ir (H. Nouri).

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semen can be stored approximately for 1 day without significant change in its fertilizing ability compared with that of fresh semen, but storage for longer time result in decreased sperm quality [1]. The mammalian spermatozoa are very susceptible to oxidative stress because their plasma membranes have a high amount of polyunsaturated fatty acids (PUFA). Along with influencing the response to cooling and freezing, the lipid composition of sperm membranes plays a major role in the physiological changes leading to fertilization [2,3]. Many studies have shown that oxidative stress impairs sperm function, for example sperm motility and plasma membrane integrity [2,3]. On the other hand, it has been found that reactive oxygen species have detrimental effects on spermatozoa [4,5].

Thyme (*Thymus vulgaris*) is a subshrub from the lamiaceae family with plants that are rich in essential oils which contain more than 60 ingredients and antioxidative phenolic substances that have antioxidant properties and antimicrobial activity [6]. Thyme was used in human nutrition as a spice and food flavor and as antioxidant for treatment of many diseases [7]. Also, it has been used in animal feed and in poultry as an antioxidant and growth stimulant [8]. Spermatozoa from all species contain high levels of PUFA, in particular, docosahexaenoic acid (DHA [22:6n−3, an omega-3 fatty acid]). Animals are unable to synthesize PUFA, from saturated or monounsaturated fatty acids; so, they must acquire them from precursor PUFA in their diet. The incorporation of dietary PUFA to sperm has been shown to be effective in a number of species [9–11]. Unfortunately, most proprietary horse feeds are very high in precursors for omega-6 fatty acids, whereas the precursors for omega-3 fatty acids, such as DHA, are very low. Few studies have been done on the effect of antioxidant and PUFA supplementation to improve stallion sperm during cold storage condition. The goal of this research was to determine whether the addition of thyme and fish oil to the diet of miniature Caspian stallions would have a beneficial effect on the motion characteristics of cooled semen.

2. Materials and Methods

2.1. Extraction of Plant Material

The leaves of *T. vulgaris* were ground with an electric grinder, then extracts were made by adding 150 g with 1 L of distilled water, and then left for 48 hours to obtain a final aqueous concentration of 150 mg/mL [12].

2.2. Stallions and Experiment Design

Animals and treatments used in this study were maintained under the approval of the Agriculture and Natural Resources Research Center of Tehran, Khojir Research Station (35°.45′N, 51°.49′E). Miniature Caspian stallions (n = 16) ranging in age from 8 to 13 years were used in a 2 × 2 crossover design study from June to October 2013. Stallions were housed in individual stalls and had ad libitum access to fresh water throughout the study. Each stallion was allowed time for free exercise every other day.

Each stallion were fed daily 1.5% of their body weight (BW) of alfalfa hay and 0.5% of their BW of a standard concentrate (13.0% crude protein, 10.0% crude fiber, 2.9% fat, 0.7% Ca, 0.5% P, 32.5 ppm Cu, 115.1 ppm Zn, 0.4 ppm Se, and 4,586 IU/kg vitamin A). The fatty acid profile of concentrate and fish oil supplements are presented in Table 1. At the beginning of the study, BW ranged from 75 to 119 kg. Stallions were weighed every 2 weeks, and their concentrate intake was adjusted accordingly.

Table 1
The fatty acid profile of concentrate and fish oil supplements

Fatty Acid	Concentrate[a]	Fish Oil[b]
C18:2n−6	4.80 ± 0.80	ND
C20:4n−6	ND	0.10
C18:3n−3	0.41 ± 0.06	ND
C20:5n−3	ND	0.24
C22:5n−3	ND	ND
C22:6n−3	ND	8.06
Total n−6	4.86 ± 0.80	0.10
Total n−3	0.41 ± 0.06	8.30
n−3:n−6	0.41:4.86	8.30:0.10

DM, dry matter; ND, not difference.
[a] Variation attributable to differences in concentrate consumption.
[b] Stallions received their standard diet dressed with 2.5% DM of fish oil.

Stallions were placed into a treatment group based on pretreatment semen collection, depending on the ability of their extended spermatozoa to maintain progressive motility after cooling and storage. Eight stallions produced spermatozoa that were ≥50% progressively motile (PMOT) after 24 hours of cooling and storage, three were 40%–49% PMOT, and five were ≤33% PMOT. Stallions were stratified by pretreatment semen characteristics and then randomly assigned to one of four treatments within strata in an attempt to balance motion characteristics between treatment groups: (1)stallions in control group (control) (n = 4) received their standard diet; (2)stallions in treatment group 2 (fish oil) (n = 4) received their standard diet dressed with 2.5% dry matter (DM) of fish oil; (3)stallions in treatment group 3 (thyme) (n = 4) received their standard diet dressed with 0.02% DM of thyme; and (4)stallions in treatment group 4 (fish oil and thyme) (n = 4) received their standard diet dressed with 2.5% DM of fish oil and 0.02% DM of thyme. Supplemented diets were fed for 90 days to ensure inclusion of one full spermatogenic cycle.

2.3. Semen Collection

Semen was collected from the stallions for three consecutive days every other week. The first two consecutive collections of each sample period were discarded to aid in depletion of extragonadal spermatozoa reserves and to help ensure uniformity of ejaculates. The third consecutive collection was processed for analyses. Semen collection for analyses occurred on day 0, 30, 60, and 90, with day 0 being the onset of dietary antioxidant and fat supplementation. Ejaculates were analyzed as fresh, cooled, and stored for 24 and 48 hours.

2.4. Semen Processing

The gel-free volume of each ejaculate was measured, and concentration was assessed using a densimeter (Animal Reproduction Systems, Chino, CA). A raw semen sample was

preserved in formol-buffered saline for morphologic analysis. The remaining fresh semen was diluted with a commercial semen extender (INRA96; IMV, France) to achieve a desired final concentration of 25×10^6 to 50×10^6 spermatozoa/mL. All the materials used in processing ejaculates were maintained at 37°C in an incubator until the time of use. Three aliquots, each containing 1.5 mL of extended semen, were separated and stored in microcentrifuge tubes. One of the aliquots was kept at 37°C and analyzed for motion characteristics and membrane integrity within 30 minutes of collection. The other two aliquots underwent standard processing for cooled transported semen. These aliquots were stored in separate cooling devices (Equitainer-II; Hamilton Thorne Biosciences, Beverly, MA); one sample was analyzed after 24 hours and the other was analyzed after 48 hours of storage.

2.5. Spermatozoal Analyses

The percentages of total and progressive motile spermatozoa were determined via microscopic assessment; during the analyses of motion characteristics, three different microscopic fields for each semen sample were analyzed using a phase-contrast inverted light microscope (CKX41; 152 Olympus, Tokyo, Japan) equipped with a heating plate maintained at 37°C. The mean of the three successive estimations were recorded as the final motility score a minimum of 500 cells were counted.

Fresh samples were analyzed approximately 15 minutes after dilution with semen extender. After removal from the storage containers, cooled semen samples were placed in an incubator for 15 minutes at 37°C before motility and membrane integrity analyses to ensure maximum reactivation of sperm motility.

Plasma membrane integrity was evaluated by eosin–nigrosin staining [13]. Four microliters of semen samples with 20 μL eosin–nigrosin stain were mixed on a warm slide, and smear was prepared with a second slide. Two hundred spermatozoa were evaluated for plasma membrane integrity under light microscopy (Standard 20; Zeiss, Germany) at ×400.

Plasma membrane functionality was evaluated by HOS test [13]. Twenty-five microliters of semen was diluted to 250 μL of a hypo-osmotic solution (osmolarity, 100 mOsm/kg) and incubated at 37°C for 75 minutes. Afterward, smear was prepared and 200 spermatozoa were determined by counting 200 sperm under phase-contrast microscopy (CKX41; 80 Olympus, Tokyo, Japan) at ×400.

For morphology evaluation, aliquots of semen were fixed in formol-buffered saline solution [14] and were examined under a phase-contrast microscope at a magnification of ×1,000. The morphologic abnormalities were counted as a percentage on at least 200 counted spermatozoa [15].

Malondialdehyde (MDA) concentrations, as lipid peroxidation index, were measured according to the method described by Esterbauer and Cheeseman [16]. To precipitate protein, 1 mL of diluted semen (250×10^6 spermatozoa/mL) was mixed with 1 mL of cold trichloroacetic acid (20% [wt/vol]). The precipitate was pelleted by centrifuging (963 × *g* for 15 minutes), and 1 mL of the supernatant was removed and incubated with 1 mL of thiobarbituric acid (0.67% [wt/vol]) in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was determined by a spectrophotometer (UV-1200; Shimadzu, Japan) at 532 nm.

2.6. Statistical Analysis

Data are presented as mean ± standard deviation. The variability of sperm characteristics between stallions were tested using the Kolmogorov–Smirnov normality test. Data collected in this study were log transformed to stabilize the variances and evaluated using a multivariate general linear model, in which the three collections for each period were considered as repeated measures. Differences were considered significant at a probability level of $P < .05$. The principal component analysis was performed on standardized data.

3. Results

Effects of thyme and fish oil on sperm concentration ($\times 10^6$), ejaculate volume (mL), and percentage of live morphologic abnormal spermatozoa in fresh semen of miniature Caspian horse are presented in Table 2.

3.1. Sperm Concentration ($\times 10^6$)

The results showed a significant differences ($P < .05$) in the sperm concentration between the treatments (Table 2). After 60 days of experiment, fish oil and fish oil–thyme treatment group results a significant increase in sperm concentration (200.1 ± 10.1 and 212.7 ± 8.9 million/mL) compared with thyme (193.3 ± 7.7 million/mL) and control groups (183.1 ± 12.4 million/mL), respectively ($P < .05$). After 90 days, there was a significant difference ($P < .05$) in

Table 2
Values of miniature Caspian stallion semen characteristics in 0, 30, 60, and 90 days after dietary thyme and fish oil supplementation

Day	Control	Fish Oil	Thyme	Fish Oil and Thyme
Sperm concentration (10^6)				
0	169.2 ± 10.4	170.3 ± 8.8	171.7 ± 9.8	176.4 ± 10.4
30	179 ± 21.4	194 ± 17.3	188.6 ± 13.1	204.2 ± 8.8
60	183.1 ± 12.4^{Ab}	200 ± 10.1^{a}	193.2 ± 7.7^{b}	212.1 ± 8.9^{a}
90	185.4 ± 15.2^{b}	210 ± 16.5^{a}	196.5 ± 11.1^{b}	221.7 ± 17.2^{a}
Volume (mL)				
0	18.7 ± 3.41^{Aa}	22.5 ± 2.21^{Aa}	24.5 ± 0.5^{Aa}	21.2 ± 0.89^{Aa}
30	20.3 ± 2.21^{Aa}	22.6 ± 2.35^{Aa}	23.2 ± 1.19^{Aa}	20.4 ± 2.34^{Aa}
60	15.6 ± 6.7^{Ab}	24.2 ± 0.2^{Aa}	19.7 ± 3.7^{Aa}	23.2 ± 0.45^{Aa}
90	14.2 ± 8.4^{Aa}	18.6 ± 4.69^{Aa}	19.8 ± 3.82^{Aa}	19.7 ± 2.90^{Aa}
Abnormal sperm (%)				
0	11.61 ± 1.02^{Aa}	10.37 ± 1.58^{Aa}	10.43 ± 1.48^{Aa}	9.23 ± 1.4^{Aa}
30	10.31 ± 1.14^{Aa}	10.31 ± 1.18^{Aa}	11.32 ± 1.83^{Aa}	9.41 ± 1.64^{Aa}
60	10.6 ± 1.16^{Ab}	9.24 ± 1.91^{Ab}	10.41 ± 1.1^{Aa}	9.38 ± 1.44^{Aa}
90	11.52 ± 1.13^{Aa}	9.20 ± 1.69^{Aa}	10.73 ± 1.83^{Aa}	8.87 ± 1.98^{Ab}
Live sperm (%)				
0	69.37 ± 4.41^{Aa}	70.32 ± 2.20^{Aa}	69.39 ± 4.30^{Aa}	68.61 ± 4.11^{Ba}
30	69.57 ± 5.54^{Aa}	70.34 ± 0.41^{Aa}	70.30 ± 2.61^{Aa}	70.37 ± 4.44^{Ba}
60	71.37 ± 3.31^{Aa}	70.38 ± 4.88^{Aa}	69.87 ± 3.43^{Aa}	71.37 ± 1.11^{Ba}
90	71.12 ± 2.01^{Aa}	72.69 ± 0.09^{Aa}	72.17 ± 2.96^{Aa}	73.37 ± 4.71^{Aa}

^{a,b}Means within a row with different superscript letters are significantly different ($P < .05$).
^{A,B}Means within the same column with different superscript letters are significantly different ($P < .05$).

Table 3
Effects of dietary thyme (*Thymus vulgaris*) and fish oil on total motility of miniature Caspian horse semen

Time of Cooling	Day	Control	Fish	Thyme	Fish and Thyme
Total motility (%)					
Fresh	0	66.00 ± 2.33^{Aa}	66.62 ± 3.13^{Aa}	60.49 ± 5.52^{Aa}	66.12 ± 2.92^{Aa}
	30	60.23 ± 5.88^{Aa}	65.49 ± 2.84^{Aa}	62.41 ± 4.78^{Aa}	67.11 ± 2.22^{Aa}
	60	62.22 ± 4.79^{Aa}	64.22 ± 3.53^{Aa}	60.76 ± 5.43^{Aa}	69 ± 1.52^{Aa}
	90	61.11 ± 6.23^{Aa}	68.30 ± 2.00^{Aa}	59.98 ± 6.71^{Aa}	69.94 ± 1.14^{Aa}
24 h	0	56.63 ± 3.31^{Aa}	57.54 ± 3.09^{Aa}	59.36 ± 2.38^{Aa}	58.33 ± 2.24^{Ba}
	30	56.91 ± 3.11^{Aa}	61.65 ± 1.11^{Aa}	58.58 ± 2.49^{Aa}	63.12 ± 2.33^{Aa}
	60	57.13 ± 2.48^{Ab}	66.06 ± 1.37^{Aa}	58.88 ± 2.55^{Ab}	65.21 ± 1.98^{Aa}
	90	57.41 ± 2.32^{Ab}	62.39 ± 1.17^{Aa}	58.87 ± 2.55^{Ab}	64.19 ± 1.85^{Aa}
48 h	0	46.31 ± 3.31^{Aa}	47.97 ± 2.48^{Ba}	49.97 ± 2.37^{Aa}	49.72 ± 2.68^{Ba}
	30	47.66 ± 3.21^{Aa}	49.09 ± 1.60^{Ba}	48.63 ± 2.30^{Aa}	50.10 ± 2.12^{Ba}
	60	48.58 ± 2.95^{Ab}	53.49 ± 1.50^{Aa}	50.13 ± 1.44^{Ab}	57.02 ± 1.67^{Aa}
	90	48.35 ± 2.96^{Ab}	54.66 ± 1.33^{Aa}	49.28 ± 2.21^{Ab}	56.71 ± 1.64^{Aa}

a,b Means within a row with different superscript letters are significantly different ($P < .05$).
A,B Means within the same column with different superscript letters are significantly different ($P < .05$).

sperm concentration in fish oil and fish oil–thyme groups (210.7 ± 16.5 and 221.7 ± 17.2) compared with thyme and control groups (193.2 ± 7.7 and 183.1 ± 12.4).

3.2. Ejaculate Volume (mL)

Effect of thyme on ejaculate volume of miniature Caspian stallions were shown in Table 2. There was a significant increase in ejaculate volume compared with control, especially the fish oil and fish oil–thyme groups compared with others (thyme and control groups) at 60 days of experiment.

3.3. Abnormal Sperm (%)

There were no significant differences between ($P > .05$) groups in abnormal spermatozoa until 90 days supplementation when the percentage of abnormal sperm in fish oil–thyme group (8.87 ± 1.98) decreased compared with thyme, fish oil and control groups (10.73 ± 1.83, 9.20 ± 1.69, and 11.52 ± 1.13), respectively ($P < .05$). On the other hand, we found significant differences between ($P < .05$) live sperm in fish oil–thyme group with enhanced in percentage, after 90 days of treatment.

Our result confirmed that total and progressive motility of sperms (Tables 3 and 4) and plasma membrane integrity (Table 5) and functionality (Table 6) were significantly decreased with increasing storage time in the presence of the all extenders.

Furthermore, after 24 hours of storage, we could find that total motility was higher in fish oil–thyme group (65.21 ± 1.98 and 64.19 ± 1.85) in comparison with fish oil (62.06 ± 1.37 and 62.39 ± 1.17), thyme (58.88 ± 2.55 and 58.87 ± 2.55), and control groups (57.13 ± 2.48 and 57.41 ± 2.32). In addition, same results were found about total motility in fish oil–thyme group (57.02 ± 1.67 and 56.71 ± 1.64), fish oil group (53.49 ± 1.50 and 54.66 ± 1.33), thyme group (50.13 ± 1.44 and 49.28 ± 2.21), and control group (48.58 ± 2.95, 48.35 ± 2.96) after 48 hours of storage. In day 60, progressive motility was significantly higher in fish oil–thyme (40.03 ± 0.12) and fish oil groups (39.78 ± 2.00) in comparison with thyme (37.74 ± 1.63) and control groups (33.44 ± 1.67) after 24 hours of storage (Table 4). Also, these parameters were in high amount in fish oil–thyme (37.39 ± 0.48) and fish oil group (33.56 ± 0.89) compared with thyme (30.44 ± 1.47) and control groups (28.63 ± 1.93) after 48 hours of storage.

Sixty days after our experiment, we could find that plasma membrane integrity of spermatozoa was higher in fish oil–thyme (43.31 ± 4.77 and 33.37 ± 2.88, respectively) and fish oil groups (42.12 ± 4.02 and 32.92 ± 2.88, respectively) than thyme (40.08 ± 4.80 and 28.31 ± 3.78, respectively) and control groups (38.35 ± 5.74 and 27.11 ± 4.17) (Table 5).

Table 4
Effects of dietary thyme (*Thymus vulgaris*) and fish oil on progressive motility of miniature Caspian horse semen

Time of Cooling	Day	Control	Fish	Thyme	Fish and Thyme
Progressive motility (%)					
Fresh	0	42.00 ± 2.94^{Aa}	43.32 ± 2.89^{Aa}	41.40 ± 2.22^{Aa}	42.66 ± 2.23^{Aa}
	30	42.11 ± 3.30^{Aa}	43.851 ± 4.26^{Aa}	41.66 ± 2.38^{Aa}	43.33 ± 2.23^{Aa}
	60	44.48 ± 2.19^{Aa}	44.88 ± 3.62^{Aa}	42.00 ± 2.44^{Aa}	44.73 ± 2.89^{Aa}
	90	42.11 ± 2.56^{Aa}	42.24 ± 3.30^{Aa}	42.20 ± 2.11^{Aa}	42.11 ± 2.90^{Aa}
24 h	0	36.42 ± 2.23^{Aa}	36.21 ± 1.99^{Ba}	35.84 ± 3.19^{Aa}	37.37 ± 2.44^{Aa}
	30	36.34 ± 2.39^{Aa}	37.48 ± 1.37^{Ba}	37.02 ± 1.49^{Aa}	38.00 ± 0.98^{Aa}
	60	37.44 ± 1.67^{Ab}	39.78 ± 2^{Aa}	37.74 ± 1.63^{Ab}	40.03 ± 0.12^{Aa}
	90	37.71 ± 1.19^{Aa}	39.70 ± 1.09^{Aa}	38.10 ± 1.01^{Aa}	39.62 ± 0.77^{Aa}
48 h	0	26.38 ± 3.77^{Aa}	27.73 ± 3.61^{Ba}	29.13 ± 1.92^{Aa}	29.62 ± 2.07^{Ba}
	30	27.44 ± 2.22^{Aa}	29.93 ± 3.38^{Aa}	28.78 ± 2.58^{Aa}	30.41 ± 1.90^{Ba}
	60	28.5 ± 1.88^{Aa}	33.56 ± 0.89^{Aa}	30.44 ± 1.47^{Ab}	37.39 ± 0.48^{Aa}
	90	28.63 ± 1.93^{Aa}	34.19 ± 0.66^{Aa}	29.86 ± 2.26^{Aa}	36.52 ± 0.69^{Aa}

a,b Means within a row with different superscript letters are significantly different ($P < .05$).
A,B Means within the same column with different superscript letters are significantly different ($P < .05$).

Table 5
Effects of dietary thyme (*Thymus vulgaris*) and fish oil on membrane integrity of miniature Caspian horse semen

Time of Cooling	Day	Control	Fish	Thyme	Fish and Thyme
Membrane integrity (%)					
Fresh	0	45.31 ± 3.71^{Aa}	49.29 ± 2.14^{Aa}	47.71 ± 2.11^{Aa}	48.55 ± 2.83^{Aa}
	30	43.11 ± 3.7^{Ab}	46.00 ± 2.40^{Aa}	44.58 ± 3.80^{Bb}	44.32 ± 3.85^{Bb}
	60	38.54 ± 4.83^{Bb}	47.69 ± 3.30^{Aa}	40.45 ± 4.41^{Cb}	49.26 ± 2.49^{Aa}
	90	35.33 ± 5.58^{Bc}	44.60 ± 3.43^{Bb}	44.14 ± 3.69^{Bb}	48.96 ± 2.88^{Aa}
24 h	0	41.24 ± 4.87^{Aa}	41.88 ± 4.76^{Aa}	41.52 ± 4.53^{Aa}	42.58 ± 4.28^{Ba}
	30	39.21 ± 5.12^{Aa}	41.87 ± 4.71^{Aa}	40.61 ± 4.89^{Aa}	40.39 ± 4.97^{Ba}
	60	38.35 ± 5.74^{Ac}	42.12 ± 4.02^{Aa}	40.08 ± 4.80^{Ab}	43.31 ± 4.77^{Aa}
	90	38.12 ± 5.77^{Ac}	41.52 ± 5.14^{Aa}	39.83 ± 5.33^{Ab}	43.43 ± 4.87^{Aa}
48 h	0	30.72 ± 3.65^{Aa}	33.53 ± 1.80^{Aa}	30.90 ± 3.43^{Aa}	34.53 ± 1.80^{Aa}
	30	30.64 ± 3.77^{Aa}	31.13 ± 2.11^{Aa}	28.87 ± 3.88^{Aa}	31.84 ± 2.76^{Aa}
	60	27.11 ± 4.17^{Ab}	32.95 ± 2.88^{Aa}	28.31 ± 3.78^{Ab}	33.37 ± 2.88^{Aa}
	90	25.90 ± 4.44^{Bc}	31.66 ± 3.07^{Aa}	28.02 ± 3.55^{Ab}	33.47 ± 2.65^{Aa}

^{a,b,c}Means within a row with different superscript letters are significantly different ($P < .05$).
^{A,B,C}Means within the same column with different superscript letters are significantly different ($P < .05$).

In addition, plasma viability of spermatozoa in day 90 was significantly higher in fish oil–thyme (43.84 ± 2.11) and fish oil groups (41.72 ± 3.61) than thyme (40.27 ± 3.80) and control groups (39.92 ± 4.99) after 24 hours of storage (Table 5); whereas, this parameter was significantly higher in fish oil–thyme (34.55 ± 2.11) and fish oil groups (33.46 ± 2.86) than thyme (29.44 ± 3.48) and control groups (27.22 ± 4.07) after 48 hours of storage.

There was no significant difference in MDA production by stallion's spermatozoa on day 90 of experiment in fresh semen and after 24 hours of storage between groups (data are not shown). As shown in Table 7, after 60 -day supplementation, the level of MDA after 48 hours of storage was significantly lower in fish oil–thyme group (4.12 ± 0.13) than thyme (5.02 ± 0.13), fish oil (5.19 ± 0.13), and control groups (5.21 ± 0.11).

4. Discussion

This study was conducted to investigate the effect of dietary thyme and fish oil supplementation on miniature Caspian stallion sperm quality during storage at 5°C. In this study, a significant increase in semen ejaculate volume, number of sperm in the ejaculate and sperm movement, dead sperm percentage, and abnormal sperm morphology was observed with adding fish oil and thyme ($P < .05$), and some in vitro sperm quality parameters (total motility [after 48 hours of storage] and plasma membrane functionality [after 48 hours of storage]) were improved compared with control group.

Previous studies showed that thyme components are flavonoids, terponoids, thymol, carvacol, eugenol, and vitamin E [17–19], which have potential to act as scavengers, superoxidase, and hydroxyl and peroxy radicals released from oxidative phosphorylation [20]. Polyphenol also prevents the oxidation of enzymes that inhibit the formation free radicals [21] and protects the DNA and plasma membrane and mitochondria of sperm. The sperm is more vulnerable and sensitive to free radicals because of the high concentration of unsaturated fatty acids in sperm membrane [22]. On the other hand, fatty acids, n−3 and n−6, are essential for normal physiological functioning and for the health of humans and all domestic species. This explains the low percentage of dead sperm and abnormal sperm morphology with thyme and fish oil. The addition of thyme and fish oil to the diets of stallions was hypothesized to enhance the motion characteristics of spermatozoa, among other factors; data from the Caspian stallions in this study suggest that total and progressive motility and membrane integrity and viability of spermatozoa were affected by

Table 6
Effects of dietary thyme (*Thymus vulgaris*) and fish oil on viability of miniature Caspian horse semen

Time of Cooling	Day	Control	Fish	Thyme	Fish and Thyme
Viability (%)					
Fresh	0	47.13 ± 4.13^{Aa}	49.79 ± 2.99^{Aa}	47.39 ± 3.92^{Aa}	48.32 ± 3.48^{Aa}
	30	45.37 ± 4.45^{Aa}	46.24 ± 3.64^{Ba}	44.67 ± 4.97^{Aa}	45.43 ± 4.31^{Ba}
	60	39.67 ± 5.39^{Bb}	48.28 ± 3.37^{Aa}	41.18 ± 5.17^{Bb}	50.02 ± 2.25^{Aa}
	90	37.36 ± 5.76^{Bb}	47.77 ± 3.74^{Aa}	44.45 ± 5.11^{Aa}	48.53 ± 3.34^{Aa}
24 h	0	43.3 ± 3.13^{Aa}	42.28 ± 3.09^{Aa}	42.82 ± 2.87^{Aa}	44.14 ± 2.17^{Aa}
	30	40.57 ± 4.55^{Aa}	41.73 ± 3.33^{Aa}	40.17 ± 3.60^{Ba}	41.92 ± 3.64^{Aa}
	60	39.77 ± 4.99^{Ba}	41.46 ± 4.01^{Aa}	40.40 ± 3.56^{Ba}	43.38 ± 2.77^{Aa}
	90	39.92 ± 5.32^{Bb}	41.72 ± 3.61^{Aa}	40.27 ± 3.80^{Bb}	43.84 ± 2.11^{Aa}
48 h	0	33.76 ± 2.97^{Aa}	34.67 ± 2.34^{Aa}	32.26 ± 3.13^{Aa}	34.19 ± 2.38^{Aa}
	30	32.96 ± 3.00^{Aa}	33.45 ± 2.94^{Aa}	30.85 ± 3.08^{Ab}	33.38 ± 2.87^{Aa}
	60	30.08 ± 3.23^{Ab}	33.97 ± 2.85^{Aa}	30.73 ± 3.17^{Ab}	34.49 ± 2.84^{Aa}
	90	27.22 ± 4.07^{Bb}	33.46 ± 2.86^{Aa}	29.44 ± 3.48^{Ab}	34.55 ± 2.11^{Aa}

^{a,b}Means within a row with different superscript letters are significantly different ($P < .05$).
^{A,B}Means within the same column with different superscript letters are significantly different ($P < .05$).

Table 7
Effects of dietary thyme (*Thymus vulgaris*) and fish oil on malondialdehyde production by miniature Caspian horse semen after 48-hour cooling

Day	Control	Fish Oil	Thyme	Fish Oil and Thyme
0	5.17^{Aa}	5.19^{Aa}	5.06^{Aa}	5.07^{Aa}
30	5.14^{Aa}	5.22^{Aa}	5.12^{Aa}	5.13^{Aa}
60	5.21^{Aa}	5.18^{Aa}	5.02^{Aa}	4.12^{Bb}
90	5.17^{Aa}	5.17^{Aa}	5.11^{Aa}	5.05^{Aa}

^{a,b}Means within a row with different superscript letters are significantly different ($P < .05$).
^{A,B}Means within the same column with different superscript letters are significantly different ($P < .05$).

thyme and fish oil treatments. The present finding is similar to the results in rats, where adding 100 mg/kg BW per day caused an increase in the percentage of living sperm and movements of sperm, and broiler [12,23]. These findings are consistent with previous studies who emphasized the act of antioxidants in improving the quality of semen and thereby increase the fertility of mice and humans [24,25]. It was found that water extracts of plants that are rich in phenolic substances have a role in the prevention of decomposition oxidative fat as thyme [26,27]. On the other hand, some research from stallions supplemented with long-chain n−3 fatty acids [28] had different reports where motion characteristics of fresh spermatozoa and of cooled and stored spermatozoa were not improved by treatment. In the present study, membrane integrity was significantly affected by fish oil in day 60 of experiment after 48 hours of cooling and there appeared to be proportionate change between the percentage of membrane-intact spermatozoa and the percentage of motile spermatozoa (Table 4). This result is similar to reports from the study concerning cod liver–supplemented diets of boars [29,30].

Lipid peroxidation of sperm plasma membrane can result in increased level of MDA [31,32].

The results of the present study showed that supplementation of fish oil–thyme is more effective for decreasing the level of MDA compared with other groups after 48 hours of cooling. Our result is in agreement with studies on boar [33], cockerel [34], and dog [35] spermatozoa in which a decrease in the level of MDA was observed by the presence of antioxidants in semen extender during storage of spermatozoa.

5. Conclusions

Adding thyme and fish oil to stallion diets significantly enhanced spermatozoa quality or influence prolonged storage, especially in stallions that produce moderate-to-high PMOT spermatozoa without dietary supplementation. More advance in vitro evaluations and artificial insemination are required to reveal the exact effects of fish oil on miniature Caspian stallion sperm quality and its fertilizing ability.

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