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SPERM FATTY ACID COMPOSITION, SEMEN QUALITY, AND REPRODUCTIVE PERFORMANCE OF ROOSTERS FED DIETS SUPPLEMENTED WITH N-3 FATTY ACIDS AND GINGER (*ZINGIBER OFFICINALE*)*

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Abstract

This study aimed to investigate the effect of dietary n-3 fatty acids and ginger (*Zingiber officinale*) supplementation on semen quality, sperm fatty acids, and reproductive performance of roosters. Seventy-two roosters (30 weeks old) were randomly allocated into 4 dietary treatments including 1) basal diet as the control group (F0G0), 2) basal diet supplemented with 20 g/kg fish oil (F2G0), 3) basal diet supplemented with 30 g/kg ginger powder (PG) (F0G3), and 4) basal diet supplemented with 20 g/kg fish oil and 30 g/kg ginger powder (F2G3) for 10 consecutive weeks. The levels of sperm linolenic acid, EPA, and DHA were higher in fish oil-fed roosters (P<0.05). A lower percentage of sperm linoleic and arachidonic acids were recorded in F2G0 and F2G3 groups (P<0.05). At 38 weeks of age, a higher sperm volume was found in F0G3 roosters than in the F0G0 and F2G0 groups. From week 34 to the end of the experiment, sperm viability, sperm abnormality, and sperm motility were significantly improved in the GP-fed roosters (F0G3 and F2G3 groups) (P<0.05). Significantly lowest sperm concentration overall the experiment was observed in the F2G0 group (P<0.05). Higher testosterone levels and lower malondialdehyde (MDA) content were recorded in F0G3 and F2G3 groups in comparison to the F2G0 (P<0.05). Also, the fertility rate of collected eggs from F0G3 and F2G3 groups was higher compared to F2G0 group (P<0.05). In conclusion, although the use of fish oil in roosters' diets alone had a negative effect on some parameters related to reproductive performance, the use of ginger powder alone or along with fish oil improved semen quality and fertility potential.

Key words: antioxidant, ginger powder, male broiler breeder, n-3 fatty acids, semen quality

Despite the small number of males in breeder flocks compared to hens (ratio 1:10), they have a significant effect on flock fertility (Qi et al., 2019). In the broiler breeder lines, genetic selection is based on producing chickens with improved growth performance traits such as high slaughter weight and low feed conversion ratio. However, these parameters may be negatively correlated with reproductive traits, especially in roosters (Teymouri Zadeh et al., 2020). Since fatty acids desaturase enzymes are lacking in the animal's body, adding polyunsaturated fatty acids (PUFAs) to their diets is needed (Esmaeili et al., 2015). It has been demonstrated that different types of dietary lipids can alter the profile of fatty acids in poultry products (Fouad et al., 2020). For example, Blesbois et al. (1997) reported that the use of fish oil (FO) in the diet of Sasso roosters affected the fatty acid profiles of seminal plasma and spermatozoa, leading to increasing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels and decreasing arachidonic acid and docosatetraenoic acid. Additionally, it has been reported that the inclusion of fatty acids in the diets improved rooster's semen quality (Safari Asl et al., 2018). As α-linolenic acid is a precursor of EPA and DHA in the body and FO is one of the common sources of this substance, the dietary inclusion of FO may improve semen quality by increasing the efficiency of DHA and EPA synthesis (Golzar Adabi et al., 2022; Brinsko et al., 2005). It appears that dietary n-3 fatty acids may improve sperm function by changing the fatty acid profile of sperm, changing the susceptibilities of membrane PUFAs to lipid peroxidation, and modifying the integrity and function of the membrane (Golzar Adabi et al., 2022; Teymouri Zadeh et al., 2020). Although several studies have reported positive effects of FO supplementation on sperm progressive motility, it is well known that unsaturated fatty acids such as EPA and DHA readily undergo peroxidation (Teymouri Zadeh et al., 2020; Cerolini et al., 2006). Also, avian sperm due to having high amounts of PUFAs can easily undergo lipid peroxidation in the presence of reactive oxygen species (ROS) (Raei et al., 2021). Therefore, to reduce oxidative

stress and improve sperm fertility potential, antioxidant compounds should be included in diets supplemented with fatty acids (Gholami-Ahangaran et al., 2021).

Ginger (*Zingiber officinale* Roscoe*, Zingiberaceae*) contains biologically active substances such as gingerol, shogaols, gingerdiol, and gingerdione (Banihani, 2019). In addition, ginger rhizome has been found to contain a wide range of antioxidant compounds, such as vitamin E, ascorbic acid, pyridoxine, beta-carotenes, quercetin, lutein, lycopene, tannin, and genistein (de Lima et al., 2018). Medicinal characteristics of ginger are attributable to antioxidant (Danwilai et al., 2017), immunomodulatory (Ali et al., 2008), anti-inflammatory (Jeena et al., 2013), antimicrobial (Park et al., 2008), antitumorigenic (Akhlaghi et al., 2014), and antiapoptotic properties (Ali et al., 2008). The use of ginger (in different ways), for improving semen quality, has been previously investigated in several experiments on male humans and animals (Hosseini et al., 2016; Akhlaghi et al., 2014; Saeid et al., 2011). Amin and Hamza (2006) and Amin et al. (2008) reported improvements in sperm viability, sperm motility, and sperm abnormality in male albino rats fed orally 1 g/kg/day with ethanol extract of ginger for 26 days. It has been demonstrated that the oral consumption of 250 mg ginger powder (GP) in infertile men at twice a day decreased sperm DNA fragmentation, however, it had no effect on sperm count and sperm mortality (Hosseini et al., 2016). In male birds, dietary supplementation of aqueous extract of ginger (Saeid et al., 2011) and GP (Akhlaghi et al., 2014; Shanoon, 2011) was shown to improve semen quality including sperm concentration, sperm abnormalities, sperm motility, and sperm viability. Although the effects of FO as an n-3 fatty acid source and GP as an exogenous antioxidant source on semen quality and reproductive performance have been studied in several investigations, their simultaneous effect is lacking in the literature. Therefore, the current study aimed to evaluate semen quality, sperm fatty acid profile, testosterone concentration, malondialdehyde (MDA) content, and reproductive performance in roosters fed FO and GP.

Material and methods

Birds' management and study design

Seventy-two 27-week-old breeder roosters (Ross 308) were kept in individual wire cages (70 × 60 × 75 cm) under the same environmental conditions (15 L: 9D light schedule at 21–23°C ambient temperature). The basal and experimental diets (Table 1) were applied according to a standard commercial mash for Ross broiler breeder roosters, in which all diets were balanced as isocaloric and isonitrogenous diets (2700 kcal/kg and 11.5% crude protein). The sand was replaced by FO and GP in the experimental diets to meet or slightly exceed instructions from the Ross 308 parent stock manual (Aviagen, 2016). Feeding, which was performed at the beginning of lighting period (6:00 am), started with 125 g/day/bird and gradually increased to 136 g/day/bird along with the growth of the roosters.

Table 1. Ingredients and chemical composition of experimental diets fed to breeder roosters

Ingredients	Treatments[1]			
	F0G0	F0G3	F2G0	F2G3
Corn	62.24	62.93	52.72	53.26
Wheat bran	23.87	22.91	33.14	32.74
Soybean meal (44%)	5.69	3.00	4.52	2.51
Sunflower meal	2.14	5.03	1.51	3.31
Sand	3.0	0.0	3.0	0.0
Ginger powder	0.0	3.0	0.0	3.0
Fish oil	0.0	0.0	2.0	2.0
Dicalcium phosphate	1.19	1.19	1.14	1.15
Limestone	0.92	0.92	0.96	0.95
Vitamin premix[2]	0.1	0.1	0.1	0.1
Trace-mineral premix[3]	0.15	0.15	0.15	0.15
Common salt	0.25	0.24	0.24	0.23
$NaHCO_3$	0.20	0.22	0.22	0.25
L-lysine HCL	0.1	0.15	0.13	0.16
DL-methionine	0.1	0.09	0.1	0.1
Choline chloride 70	0.03	0.04	0.04	0.04
L-threonine	0.02	0.03	0.03	0.05
Total	100	100	100	100
Composition				
Metabolizable energy (kcal/kg)	2700.00	2700.00	2700.00	2700.00
Crude protein (%)	11.50	11.50	11.50	11.50
Ca (%)	0.70	0.70	0.70	0.70
avP (%)	0.35	0.35	0.35	0.35
Lysine (%)	0.44	0.44	0.44	0.44
Methionine (%)	0.28	0.28	0.28	0.28
Threonine (%)	0.33	0.33	0.33	0.33

[1]F0G0, control diet with no ginger powder and/or fish oil; F0G3, containing only 30 g/kg ginger powder; F2G0, containing only 20 g/kg fish oil; F2G3, containing 20 g/kg fish oil and 30 g/kg ginger powder.

[2]Supplied per kg of diet: retinol, 14,000 IU; cholecalciferol, 3,000 IU; niacin, 50 mg; α-tocopherol, 30 mg; calcium pantothenate, 20 mg; menadione, 4 mg; riboflavin, 7.0 mg; pyridoxine, 5.5 mg; folic acid, 1.5 mg; and biotin, 50 mg.

[3]Supplied per kg of diet: Fe ($FeSO_4 \cdot H_2O$), 85 mg; Mn ($MnSO_4 \cdot H_2O$), 90 mg; Zn (ZnO), 67.3 mg; Cu ($CuSO_4 \cdot 5H_2O$), 11 mg; iodine (KI), 2 mg; and Se (Na_2SeO_3), 0.18 mg.

According to Zhao et al. (2011), fresh ginger rhizomes were obtained from a local market and after cleaning and slicing, were dried at 40°C. The dried rhizomes were milled (Skiold A/S, Jutland, Denmark), screened (300 μm), and stored at room temperature (18 to 22°C) in airtight plastic bags away from sunlight before dietary mixing. The GP supplied 91.7, 8.98, 5.21, and 7.78% of dry matter, CP, ether extract, and crude ash, respectively, which were analyzed according to AOAC (2000) methods 930.15, 990.03, 920.39, and 942.05. The identifica-

tion of 6-gingerol, 6-shogaol, and galanolactone as major components of ginger was performed according to Rong et al. (2009). Firstly, 0.05 g ginger powder was ultrasonicated in ethanol for 30 min and extracted. The extract was filtrated with 0.45 µm membrane filters and the filtrate was characterized by HPLC. HPLC with photodiode array (PDA) detection (Shimadzu Class VP system) analysis was carried out on an Inertsil ODS-3 (GL-Science, 5 µm, 4.6 × 250 mm) column with oven temperature held at 40°C. The extract was eluted at a flow rate of 1 ml/min with a mobile phase gradient consisting of a mixture of solvent A (water: methanol:1,4- dioxane, 50:15:35) and solvent B (acetonitrile: methanol:1,4 dioxane, 50:15:35) (0 min, 80:20; 20 min, 60:40; 25 min, 15:85; 25–30 min, 0:100; >30 min, 80:20). The peaks of 6-gingerol, 6-shogaol and galanolactone were identified by comparing their retention times and coincidence of UV–visible spectra with corresponding standard solutions, respectively. Spectral data for the authentic standard: 6-gingerol, retention time 8.6 min λ_{max} at 210, 280 nm; 6-shogaol, retention time 17.2 min, λ_{max} at 212, 278 nm; galanolactone, retention time 22.6 min, λ_{max} at 215 nm. The content of 6-gingerol, 6-shogaol, and galanolactone in the GP was 9.24, 5.89, and 6.25 g per kg, respectively.

A 2 × 2 factorial design in a complete randomized design, with 18 replications of individual roosters in each group was used, in which the first factor was fish oil, either 0 or 20 g FO/kg of diet, and the second factor was ginger powder, either 0 or 30 g GP/kg of diet (Akhlaghi et al., 2014; Golzar Adabi et al., 2022). Birds were allocated into 4 groups including: 1) F0G0 group received a basal diet; 2) F0G3 group received the basal diet supplemented with 30 g GP/kg of diet; 3) F2G0 group received the basal diet supplemented with 20 g FO/kg of diet, and 4) F2G3 group received the basal diet supplemented with 30 g GP/kg of diet and 20 g FO/kg of diet. The composition of fatty acids in FO and experimental diets is shown in Table 2. The composition of fatty acids in FO and experimental diets was determined by gas chromatography (Kamran Azad et al., 2009) (Unicam 4600, USA) equipped with a BPX70 fused silica capillary column and a flame ionization detector. For this purpose, the initial temperature was adjusted to 160°C and was increased at the rate of 20°C/min to 190°C. The temperature of the injector was 240°C and the detector remained stable at 280°C. The column head pressure of the carrier gas (helium) was 20 psi and the sample volume was 0.2 µl. Pentadecanoic acid (Sigma, St. Louis, MO, USA) was used as an internal standard. Fatty acids were identified by matching their retention times with those of their respective standards.

Semen collection and sampling days

Before applying treatments, roosters were accustomed to semen collection for two weeks (27–29 weeks of age) using the abdominal massage method, according to Burrows and Quinn (1937). Then, roosters were fed the treatments for 10 weeks (30–40 weeks of age) and semen was collected every two weeks from each bird between 32 and 40 weeks of age.

Table 2. Fatty acids composition of experimental diets and fish oil (FO) (% of total FA)

Fatty acid	Molecular formula	Treatments[1]				
		F0G0	F0G3	F2G0	F2G3	Fish oil
Myristic	14:00	1.22	1.31	2.11	2.09	3.39
Palmitic	16:00	26.49	27.43	23.99	23.68	22.1
Heptadecanoic	17:00	0.2	0.31	0.39	0.51	0.92
Stearic	18:00	3.48	3.32	3.71	3.11	4.61
Arachidic	20:00	0.48	0.39	0.41	0.35	0.21
Behenic	22:00	0.29	0.33	0.39	0.41	0.32
Lignoceric	24:00	1.11	1.09	0.18	0.14	0.5
Palmitoleic	16:01	1.22	1.14	1.78	2.01	4.59
Heptadecenoic	17:01	0.11	0.19	0.16	0.23	0.59
Oleic	18:1 n-9	31.53	30.02	31.88	31.29	33.14
cis-11-eicosenoic	20:01	0.32	0.28	0.38	0.26	1.62
Erucic	22:1 n-9	0.11	0.12	0.18	0.15	0.1
Nervonic	24:1	0.19	0.29	0.35	0.32	0.84
Linoleic	18:2 n-6	30.97	31.53	25.82	26.98	2.71
cis-11,14 eicosadienoic	20:2 n-6	0.005	0.007	0.006	0.003	0.004
cis-8,11,14 eicosatrienoic	20:3 n-6	0.06	0.02	0.07	0.08	0.15
Linolenic	18:3 n-3	1.53	1.62	1.49	1.72	1.03
EPA	20:5 n-3	0.41	0.39	1.69	1.48	5.8
DHA	22:6 n-3	0.08	0.05	4.79	5.03	17.26
Others		0.195	0.16	0.22	0.16	0.12
ΣSFA[2]		33.27	34.18	31.18	30.29	32.05
ΣMUFA[2]		33.48	32.04	34.73	34.26	40.88
ΣPUFA[2]		33.06	33.62	33.87	35.29	26.95
n-6		31.04	31.56	25.90	27.06	2.86
n-3		2.02	2.06	7.97	8.23	24.09
n-6/n-3		15.36	15.32	3.25	3.29	0.12

[1]F0G0, control diet with no ginger powder and/or fish oil; F0G3, containing only 30 g/kg ginger powder; F2G0, containing only 20 g/kg fish oil; F2G3, containing 20 g/kg fish oil and 30 g/kg ginger powder.

[2]ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids.

Sperm fatty acid profile

The sperm fatty acid composition of all roosters was measured at the end of the experiment, according to Folch et al. (1957) and Golzar Adabi et al. (2011). The lipid extraction was performed by dissolving chloroform: methanol (2:1 vol/vol) in a methanolic NaOH solution (2%). The lipid was trans-methylated by refluxing for 30 min with a mixture of n-hexane and saturated NaCl solutions. 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). The gas chromatography conditions were as follows: injector temperature, 240°C; detector temperature, 300°C; carrier gas, helium; split ratio, 1:30; temperature program, 180°C for 2 min followed by an increase to 230°C at 5°C/min, which was maintained at 24°C. The fatty acid percentage was integrated and then calculated by means of direct normalization of the peak areas. Each fatty acid was identified in the form of a methyl ester by comparing the retention times with the standard acquired from Sigma.

Semen evaluation

Semen volume was measured by a graduated test tube. After dilution of the semen (1:500 with saline), a droplet of semen was placed on the Neubauer hemocytometer (American Optical Company, New York, USA) to determine semen concentration (Raei et al., 2021).

For evaluation of sperm viability, 10 µl of diluted semen (1:100 in saline) was placed on a pre-warmed microscope glass slide and mixed for 30 s with 20 µl of eosin-nigrosin staining, then a smear was spread by another slide. After drying, viability was examined by counting 250 spermatozoa under phase contrast at × 1000 magnification (CKX41, Olympus, Tokyo, Japan). Sperm cells with stained heads are dead, while those with unstained heads are considered to be viable (Golzar Adabi et al., 2011). To evaluate sperm abnormalities the same preparations were done, and the percentages of sperms with detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails, and protoplasmic droplets were calculated by counting a total of 300 sperm under a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan, magnification: × 1000) (Golzar Adabi et al., 2007).

Sperm forward motility was assessed by placing a 3 µl of diluted ejaculate with 2.9% sodium citrate (1:100) on a pre-warmed (40°C) slide covered with a coverslip on a compound Olympus-BX50 light microscope (Olympus Optical) at × 400 magnification (Golzar Adabi et al., 2022).

Body weight and testes weight changes

The roosters were weighed individually every week from 30 to 40 weeks of age. Left, right, and combined testes were dissected and subsequently weighed using an electronic analytical scale immediately after euthanizing the roosters.

Measurement of plasma testosterone

At the end of the experiment, blood samples were collected from the brachial vein, and were centrifuged at 1000 × *g* for 10 min at 4°C to separate the plasma. Testosterone concentrations were detected using an enzyme linked immune sorbent assay (ELISA), according to the guide provided by the manufacturer (Cat no. MBS9424390, MyBioSource). This assay was performed in 96-well plates and absorbance was measured at 450 nm.

Measurement of seminal MDA concentration

The thiobarbituric acid reaction (TBA) was used for measuring malondialdehyde (MDA) content as an indicator of lipid peroxidation in semen (Botsoglou et al., 1994). In brief, after mixing 1 ml of semen plasma with 1 ml of 20% (wt/vol) trichloroacetic acid and centrifugation at 960 × *g* for 10 mins, 1 ml of the obtained supernatant was mixed with 1 ml of 0.67% (wt/vol) TBA. After incubation of the mixture in a water bath at 70°C for 30 mins and cooling, the absorbance of the product formed by MDA and TBA was determined at 532 nm using a spectrophotometer (UV-1200, Shimadzu, Japan).

Assessment of reproductive performance

In examining reproductive performance, artificial insemination (AI) was performed according to the method described by Akhlaghi et al. (2014) with a slight modification at the end of the trial. Semen samples from roosters in each group were obtained, pooled, and diluted in the Lake extender (containing 1.92 g sodium L-glutamate monohydrate, 0.5 g potassium acetate, 0.08 g magnesium acetate tetrahydrate, 0.8 g glucose, 0.3 g polyvinylpyrrolidone [Mr 10000], and 100 mL of water [343 mOsm/kg, pH 7.08]). To perform AI, 20 individual hens per experimental group were inseminated (300 × 10^6 spermatozoa/hen) at 02:00 pm on specific days for approximately two weeks (two times per week). Produced eggs were collected daily, numbered, and stored at 13°C and 75% relative humidity up to 5 days after the last AI. In each group, 300 settable eggs were placed in a common incubator. Eggs candled on day 7 and the number of hatched chicks on day 21 were used to calculate the fertility and hatchability rate, respectively. The embryonic mortality was determined within the incubation period in 3 stages of early (0–6 d), mid (7–17 d), and late (18–21 d plus pipped). After hatching the percentage of first grade hatchlings was also determined (Borghei-Rad et al., 2017).

Statistical analysis

All data were evaluated for normality and homogeneity of variances using the Shapiro-Wilk and Levene's tests, respectively. PROC GLM and PROC MIXED were used to analyze single and repeated measurement data, respectively (SAS, 2001). The chi-square test was used to analyze the effects of treatments on fertility and hatchability. Tukey's test was used to examine statistical differences among treatments at a level of significance set at $P \leq 0.05$.

Results

Sperm fatty acid composition

The effect of experimental diets on the fatty acid composition of sperm at the end of the study is presented in

Table 3. The sperm of roosters fed on diets containing FO (F2G0 and F2G3 groups) included a higher proportion of linolenic acid, EPA, and DHA, and lower levels of linoleic and arachidonic acids compared to F0G0 and F0G3 groups (P<0.05). In addition, due to the higher amount of n-3 and lower content of n-6, the ratio of n-6/n-3 was lower in the sperm of roosters fed on diets containing FO (P<0.05). Comparing the data presented in Tables 2 and 3 revealed that the profile of dietary fatty acids, especially in EPA and DPA, was like the profile of sperm fatty acids in experimental roosters.

Semen characteristics

The effect of dietary treatments and their interaction on seminal attributes including semen volume, sperm concentration, percentage of live sperm, percentage of abnormal sperm, and percentage of sperm motility are presented in Table 4. Semen volume significantly increased in birds fed on F0G3 compared to F0G0 and F2G0 by 4.34% (P<0.05). The lowest sperm concentration was significantly recorded in F2G0 birds (4.27×10^9 cells/ml; P<0.05). Roosters fed on diet containing GP (F0G3 and F2G3) had a higher percentage of live sperm than those fed on F0G0 and F2G0 (P<0.05). In addition, sperm motility significantly increased by 3.27 and 3.66% in roosters fed on F0G3 and F2G3 diets compared to birds fed on F0G0 (P<0.05). Adding only FO to the diet caused significant depression in sperm motility (P<0.05). The FO × GP interaction was found significant for all seminal parameters (P<0.01). Sperm concentration and sperm motility were significantly affected by dietary FO so that adding 20 g/kg of FO significantly decreased sperm concentration and sperm motility compared to the diet without FO (P<0.05). On the other hand, GP as a main effect significantly increased all semen quality parameters (P<0.01).

Table 3. Fatty acids composition of sperm in rooster fed diets containing the different levels of fish oil (FO) and ginger powder (GP)

Fatty acids	Molecular formula	Treatments[1]					
		F0G0	F0G3	F2G0	F2G3	SEM	P-value
Palmitic	16:00	13.14	12.98	13.14	12.71	0.19	0.08
Stearic	18:00	22.18	21.91	21.94	21.56	0.27	0.11
Arachidic	20:00	0.89	0.91	0.95	0.97	0.05	0.17
Lignoceric	24:00:00	0.16	0.11	0.14	0.18	0.03	0.09
Heptadecenoic	17:01	1.32	1.21	1.19	1.25	0.08	0.12
Oleic	18:1 n-9	13.65	13.23	12.84	13.05	0.17	0.08
cis-11-eicosenoic	20:01	3.92	3.81	4.09	3.98	0.09	0.15
Erucic	22:1 n-9	0.04	0.08	0.07	0.09	0.008	0.18
Nervonic	24:01:00	0.28	0.21	0.33	0.29	0.07	0.11
Linoleic	18:2 n-6	6.11 a	6.01a	4.92b	4.85b	0.25	0.05
cis-11,14 eicosadienoic	20:2 n-6	2.05	2.12	2.46	2.39	0.17	0.24
cis-8,11,14 eicosatrienoic	20:3 n-6	2.11	1.93	1.79	1.85	0.12	0.13
Arachidonic	20:4 n-6	12.05 a	12.73 a	9.58 b	9.69 b	0.15	0.004
DTA	22:4 n-6	18.71	19.0 8	19.93	18.85	0.13	0.16
Linolenic	18:3 n-3	0.79 b	0.83 b	1.15 a	1.47 a	0.16	0.05
EPA	20:5 n-3	0.59 b	0.73 b	1.13 a	1.64 a	0.14	0.008
DHA	22:6 n-3	1.84 b	2.01 b	4.21 a	5.03 a	0.11	0.03
Others		0.17	0.11	0.14	0.15	0.07	0.11
ΣSFA[2]		36.37	35.91	36.17	35.42	0.68	0.21
ΣMUFA[2]		19.21	18.54	18.52	18.66	0.32	0.12
ΣPUFA[2]		44.25	45.44	45.17	45.77	1.04	0.14
n-6		41.03 a	41.87 a	38.68 b	37.63 b	0.79	0.001
n-3		3.22 b	3.57 b	6.49 a	8.14 a	0.25	0.0009
n-6/n-3		12.74 a	11.73 a	5.96 b	4.62 b	0.17	0.004

Means within the same column without common letters are significantly different (P<0.05).

[1]F0G0, control diet with no ginger powder and/or fish oil; F0G3, containing only 30 g/kg ginger powder; F2G0, containing only 20 g/kg fish oil; F2G3, containing 20 g/kg fish oil and 30 g/kg ginger powder.

[2]ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids.

Table 4. Seminal attributes in rooster fed diets containing the different levels of fish oil (FO) and ginger powder (GP) as average between 32 and 40 weeks[1]

Item	F0G0	F0G3	F2G0	F2G3	SEM	Effect (P-value)		
						fish oil	ginger	interaction
Semen volume (ml)	0.46 b	0.48 ab	0.46 b	0.50 a	0.006	0.23	<.0001	<.0001
Sperm concentration (× 10^9 cells/ml)	4.58 a	4.61 a	4.27 b	4.65 a	0.070	0.0002	<.0001	<.0001
Live sperm (%)	79.02 b	82.84 a	77.34 b	84.91 a	0.50	0.69	<.0001	<.0001
Abnormal sperm (%)	9.86 ab	9.25 bc	10.54 a	8.12 c	0.26	0.40	<.0001	<.0001
Sperm motility (%)	81.20 b	83.85 a	75.95 c	84.18 a	0.51	<.0001	<.0001	<.0001

Means within a row with letters are significantly different (P<0.05).

[1]F0G0, control diet with no ginger powder and/or fish oil; F0G3, containing only 30 g/kg ginger powder; F2G0, containing only 20 g/kg fish oil; F2G3, containing 20 g/kg fish oil and 30 g/kg ginger powder.

Table 5. Body weight (BW), testes weight, plasma testosterone concentration, and semen malondialdehyde (MDA) level in rooster fed diets containing different levels of fish oil (FO) and ginger powder (GP)[1]

Item	F0G0	F0G3	F2G0	F2G3	SEM	Effect (P-value)		
						fish oil	ginger	interaction
Initial BW (kg)	4.17	4.01	4.04	3.99	0.12	0.54	0.38	0.71
Final BW (kg)	4.52	4.41	4.47	4.39	0.08	0.69	0.26	0.69
Testes weight (gr)	40.56	40.99	40.26	41.01	0.99	0.89	0.56	0.94
Testosterone (ng/ml)	3.96 ab	5.09 a	3.42 b	4.65 a	0.27	0.14	<.0001	<.0001
MDA (nM/ml)	1.46 b	0.55 d	2.89 a	0.84 c	0.02	<.0001	<.0001	<.0001

Means within a row with letters are significantly different (P<0.05).

[1]F0G0, control diet with no ginger powder and/or fish oil; F0G3, containing only 30 g/kg ginger powder; F2G0, containing only 20 g/kg fish oil; F2G3, containing 20 g/kg fish oil and 30 g/kg ginger powder.

Table 6. Fertility, hatchability, and embryonic mortality rates in rooster fed diets containing different levels of fish oil (FO) and ginger powder (GP)[1]

Item	F0G0	F0G3	F2G0	F2G3	SEM	Effect (P-value)		
						fish oil	ginger	interaction
Fertility (%)[2]	87.33 ab	91.33 a	83.67 b	91.67 a	1.63	0.32	0.002	0.009
Hatchability (%)[3]	89.30	90.87	88.06	91.26	1.30	0.75	0.09	0.32
First-grade chicks	90.68	92.75	90.54	92.86	1.21	0.99	0.09	0.38
Embryonic mortality (%)[4]								
Early stage (1–6 d)	7.44 ab	5.20 c	9.16 a	5.59 bc	0.40	0.02	<.0001	<.0001
Mid stage (7–15 d)	2.92	3.22	3.20	2.80	0.94	0.93	0.95	0.98
Late stage (18–21 d plus pipped)	1.77	1.66	1.38	1.22	0.79	0.60	0.86	0.96

Means within a row with letters are significantly different (P<0.05).

[1]F0G0, control diet with no ginger powder and/or fish oil; F0G3, containing only 30 g/kg ginger powder; F2G0, containing only 20 g/kg fish oil; F2G3, containing 20 g/kg fish oil and 30 g/kg ginger powder.

[2]Number of fertile eggs divided by the total number of eggs set.

[3]Chicks number divided by fertile eggs.

[4]As a percentage of fertile eggs.

Body weight and testes weights

As it can be seen in Table 5, the effect of dietary FO and GP as main effect and FO × GP interaction were not significant for initial and final body weights, and testes weights parameters (P>0.05). Based on the results, all the mentioned parameters were not affected by treatments (Table 5).

Testosterone concentration

The main and interaction effects of dietary FO and GP on rooster's plasma testosterone concentration were significant (P<0.05, Table 5). The effect of dietary GP and FO × GP interaction were significant for testosterone so that roosters fed on the diet containing 30 g/kg GP had higher testosterone levels compared to the diet without

GP (P<0.05). But FO as a main effect had no significant effect on plasma testosterone levels (P>0.05). According to Table 5, the testosterone level increased from 3.42 (F2G0) to 4.65 ng/ml (35.97%) by adding 30 g/kg GP to the diet containing 20 g/kg FO (F2G3).

Lipid peroxidation

As it can be seen in Table 5, FO and GP as main effects and their interaction on MDA content were significant (P<0.01). Inclusion of 20 g/kg FO to F0G0 diet significantly increased MDA value (P<0.01) while adding 30 g/kg GP significantly lowered MDA level (P<0.01). Semen MDA level was influenced by the FO × GP interaction. Adding 30 g/kg GP to the diet containing FO significantly decreased the MDA content from 2.89 to 0.84 nM/ml (P<0.01; Table 5). Also, adding GP to control (F0G0) diet decreased the MDA level from 1.46 to 0.55 nM/ml (P<0.01).

Reproductive performance

Table 6 contains the main and FO × GP interaction results on fertility, hatchability, chick quality, and embryonic mortality. Fish oil as a main effect had no significant influence on reproductive performance parameters (P>0.05) except for early stage of embryonic mortality (P<0.01). Feeding roosters with 20 g/kg FO increased embryonic mortality at the early stage of incubation period (P<0.05). Adding 30 g/kg GP to the diet with or without FO significantly increased the fertility rate and decreased early stage embryonic mortality (P<0.01). The effects of dietary treatments and the respective P-values for fertility, hatchability, chick quality, and embryonic mortality are summarized in Table 6. Hatchability, chick quality, and embryonic mortality at mid and late stages of incubation were not affected by dietary treatments (P>0.05). Fertility rate was higher in birds fed on diets F0G3 and F2G3 compared with F0G0 and F2G0 birds (P<0.01). For example, fertility in F2G3 was higher than F2G0 by 9.53% (P<0.01). Also, lower embryonic mortality in the early stage of incubation period was recorded for F0G3 and F2G3 birds. Fish oil inclusion to F0G0 diet led to higher embryonic mortality from 7.44 to 9.16 % (P<0.01).

Discussion

Since nutrition impacts semen quality, nutritional approach is one of the strategies to improve semen quality in different species (Bilcik et al., 2005). In some studies, the positive effects of the application of FO as a source of n-3 fatty acids on the semen quality in men (Safarinejad, 2011), bulls (Samadian et al., 2010), rams (Masoudi and Dadashpour Davachi, 2021), and roosters (Safari Asl et al., 2018; Cerolini et al., 2000) have been reported. It has been demonstrated that dietary supplementation with 20 to 50 g/kg FO increases semen volume, spermatozoa number, and forward progressive motility in roosters (Cerolini et al., 2006). Hudson and Wilson (2003) reported that roosters fed on a diet supplemented with 20 g/kg menhaden oil as an n-3 PUFA-rich source have a higher fertility rate than other birds. It is well known that FO affects energy production by promoting the activity of mitochondria and beta-oxidation in birds (Fouad et al., 2020; Safari Asl et al., 2018). It is hypothesized that dietary FO may enhance the forward progressive motility of sperm by increasing energy production (Fouad et al., 2020; Kamali Sangani et al., 2017). Despite the beneficial effects of inclusion of dietary FO, some studies have shown that semen quality is declined in diets enriched with PUFA that lack an antioxidant source (Golzar Adabi et al., 2022; Teymouri Zadeh et al., 2020; Hlungwani et al., 2015). In sperm, the presence of high concentrations of PUFAs increases susceptibility to ROS attacks, accordingly, an imbalance between the ROS production and the available defense antioxidants results in lipid peroxidation (Raei et al., 2022 a, 2021). Therefore, such methods as the addition of a natural antioxidant to PUFA sources to improve semen quality will be valuable (Teymouri Zadeh et al., 2020).

In the current study, the content of linolenic, EPA, and DHA was higher in the sperm of roosters fed on diet containing FO (F2G0 and F2G3) compared to other groups, which confirms the successful transfer of dietary PUFAs to sperm. In concert with this study, a positive association of fatty acid composition between rooster sperm and their diet has been reported in some studies (Safari Asl et al., 2018). It has been shown that feeding roosters with 20 g/kg of flaxseed oil as an n-3 PUFA source causes higher DPA and DHA content in sperm compared to the control group (Zanussi et al., 2019). Also, Golzar Adabi et al. (2022) indicated a clear impact of dietary FO on sperm fatty acid profile; roosters fed 20 g/kg of FO had a higher proportion of n-3 fatty acid than the control group. Contrary to our results, Akhlaghi et al. (2014) reported that the administration of dietary GP (30 g/kg) increased the content of DHA and DTA and decreased arachidonic and linoleic acids in the sperm of roosters. In one study, it was shown that the addition of GP levels to the diet significantly reduced saturated fatty acids and increased unsaturated fatty acids (Ezzat et al., 2018).

In the current study, results of semen quality including volume, concentration, sperm viability, sperm motility, and sperm abnormality showed that feeding roosters with diets supplemented with FO and GP had a better response than feeding with FO alone. Although arachidonic and DTA may be crucial for sperm motility and its fusion with the oocyte due to their role in contributing to the membrane's high fluidity and flexibility (Hosseini et al., 2016), this hypothesis was not observed in our study, at least in the F2G0 group. The beneficial effect of dietary administration of herbal antioxidants on male reproduction, especially semen quality, has been reported in many investigations (Golzar Adabi et al., 2022; Raei et al., 2021; Zanussi et al., 2019; Akhlaghi et al., 2014). In different species, the positive effects of ginger

or its extracts/fraction on semen characteristics (volume, concentration, viability, morphology, DNA integrity, and membrane integrity) have been introduced (Akhlaghi et al., 2014; Amin and Hamza 2006; Amin et al., 2008). Our results showed high semen volume in roosters receiving GP (F0G3) and FO with GP (F2G3). The cause for this effect is unknown; however, it might be inferred that an increased secretory and/or a decreased absorptive activity in the rete testis or efferent ducts might have contributed to enhancing the semen volume (Ommati et al., 2013). Similarly, semen concentrations decrease with age, which was slower in F0G3 and F2G3 groups. The decrease in semen concentration in male broiler breeders is related to the age-related increase in ROS production. It is possible that the antioxidant effects of GP keep the semen concentration trend constant (Borghei-Rad et al., 2017). Semen quality parameters such as sperm motility, sperm concentration, and other sperm motion characteristics were negatively affected by high ROS levels in semen (Behnamifar et al., 2021). In addition, ROS production is positively correlated with sperm defects such as amorphous heads, damaged acrosomes, mid-piece defects, cytoplasmic droplets, and tail defects (Raei et al., 2021; Behnamifar et al., 2021; Borghei-Rad et al., 2017). This is in concert with our findings, which showed higher sperm abnormality in group fed on FO alone (F2G0). It has been reported that ginger and its extracts have a positive effect on semen quality mainly by enhancing the level of testicular cholesterol and testicular blood flow, stimulating the synthesis of LH and the activity of molecular defense mechanism in the testes, and reducing blood glucose concentration (Banihani, 2018). Also, improving semen quality by ginger administration may be attributable to increasing testicular weight, recycling of testosterone receptors, and enhancing the LH and testosterone levels (Banihani, 2019).

The evaluation of testicular weight, as the main location of the male reproductive system, is valuable for assessing the reproductive status. In the present study, the testicular weight was not affected by the experimental groups. However, it is demonstrated that roosters fed on a diet supplemented with antioxidants present high testicular weights which is positively correlated with semen quality (Raei et al., 2022 b; Golzar Adabi et al., 2022). Reportedly, testicular weight affects sperm's health and production (Sun et al., 2019). Additionally, a positive correlation between testicular size and sperm quality has been reported (Talebi et al., 2018). Like our findings, Khaki et al. (2009) reported that adding 50 and 100 mg of GP/kg in rats' diet did not cause changes in testicular weight and semen quality.

We observed that the diets supplemented with GP (F0G3 and F2G3 groups) exhibited a lower concentration of MDA content when compared to the diets without GP. These findings show that FO needs to be protected against lipid peroxidation, and PUFAs of FO without any protection can cause damage to sperm. MDA is the main by-product of lipid peroxidation, and its assessment can provide information regarding the peroxidative damage in sperm (Raei et al., 2022 b, 2021). Because spermatozoa cells are unable to synthesize PUFA or repair damages, protection against peroxidative damage would be an important mechanism for maintaining PUFA in sperm (Raei et al., 2022 b; Behnamifar et al., 2021). At the end of the experiment, a 5.25-fold decrease in MDA content of F2G3 group was found in comparison to F2G0 group. It seems that active compounds of GP including gingerol, shogaols, gingerdiol, gingerdione, and some related phenolic ketone derivatives can improve the antioxidant capacity of semen (Zhao et al., 2011). Similar findings have been reported in aged male broiler breeder fed on different ginger levels (Akhlaghi et al., 2014). It is stated that ginger and its bioactive substances, by enhancing the activity of the antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase in the male reproductive system (Ghlissi et al., 2013) and reducing the testes damage (detected as decreasing levels of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase) significantly increase the antioxidant capacity (Banihani, 2019; Ghlissi et al., 2013).

It is believed that using the antioxidant compounds in the rooster diet enhanced testosterone levels (Golzar Adabi et al., 2022; Raei et al., 2021; Zanussi et al., 2019). Similarly, we observed that the roosters fed the FO along with GP (F0G3 and F2G3) had higher testosterone levels. It has been reported that testosterone levels led to enhancing sperm concentration and population in epididymis and improving the spermatogenesis process in the testes (Gholami-Ahangaran et al., 2021), which is consistent with our results. Considering the presence of notable amounts of certain nutrients such as manganese in ginger, its consumption may change the concentration of reproductive hormones (Khaki et al., 2009; Lee et al., 2006). Accordingly, it is known that manganese supplementation may increase testosterone synthesis and its concentration by enhancing LH secretion (Lapointe et al., 1996). The decreased testosterone level in the birds fed on FO (F2G0) has coincided with an increased MDA content. It is well known that increasing oxidative stress by lowering testosterone production, degeneration of Sertoli cells, and rupture of the blood-testicular barrier disrupts the process of spermatogenesis and ultimately leads to a reduction in epidermal sperm counts and fertility (Sakr and Badawy, 2011; El-Shahat et al., 2009; Jorsaraei et al., 2008).

As a critical finding in the current study, the fertility rate in roosters fed on GP increased by approximately 8% compared to the F2G0 group. Although in the present study, feeding roosters with FO decreased fertility and increased embryonic mortality at 1–6 d, it has been demonstrated that in other species the use of FO in diet increased the fertility potential (Masoudi and Dadashpour Davachi, 2021). However, in line with our study, it has been reported that the dietary supplementation of FO alone decreased fertility in aged roosters (Golzar

Adabi et al., 2022). In the same study, it was indicated that fertility improved in roosters fed on a supplemented diet with FO and rooibos (as a source of antioxidants) (Golzar Adabi et al., 2022). The reason for this phenomenon is not clear, but oxidation of unsaturated fatty acids in FO may cause induced oxidative damage in related organs and consequently may negatively affect reproductive parameters (Golzar Adabi et al., 2022). According to the reports, ginger's antioxidant and androgenic properties were responsible for increasing fertility potential following its administration (Gholami-Ahangaran et al., 2021; Akhlaghi et al., 2014). Also, it has been demonstrated that the high sperm count in the sperm storage tubules in inseminated hens was the result of feeding aged roosters with an antioxidant source (Borghei-Rad et al., 2017). Akhlaghi et al. (2014) reported that adding GP significantly increased the fertility rate in roosters, which is in contrast with our study. It is probable that antioxidant compounds enhance fertility rate by increasing the persistency of sperm in sperm storage tubules (Raei et al., 2021). Also, a high correlation was shown between semen quality parameters and reproductive performance in different species (Borghei-Rad et al., 2017). It has been demonstrated that lipid peroxidation leads to embryonic mortality which is the result of a high damage to sperm membrane (Triques et al., 2019). The reason behind higher early embryonic mortality rates in the F2G0 group may be attributed to high lipid peroxidation and percentage of abnormal sperm, as well as lack of antioxidant activity which accordingly may negatively affect the embryonic development of chick at early stage (Triques et al., 2019).

Conclusions

In general, based on the results of the present study, GP as a natural antioxidant can improve semen quality parameters including sperm concentration and sperm motility in roosters. Also, the beneficial effects of dietary ginger supplementation on testosterone levels, MDA content, fertility rate, and embryonic mortality rate was observed in the current study. However, adding FO alone in roosters' diet did not have similar positive effects as ginger. Considering the presence of high levels of PUFA in avian sperm membrane, it seems that the application of antioxidant compounds is necessary to improve the reproductive performance of roosters.

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