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Effect of *In ovo* Injection of Flaxseed Oil on Broiler Breast Meat in Chicken Embryo Model: Meat Quality, Antioxidant Capacity and Fatty Acid Profile

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Abstract

Using *in ovo* injection method, specific nutrients and antioxidants can be provided in precise doses at certain times for maximum absorption by the embryo. The objective of this study was to examine the effect of *in ovo* injection of flaxseed oil on the quality and antioxidant properties as well as fatty acid profile of breast meat of day-old broilers. 20 one-day-old fertilized eggs were randomly divided into two groups of 10; one control and one FSO (flaxseed oil) which received 100 µL of flaxseed oil by injection into the allantoic sac. The amounts of fat, vitamin E, total phenol, carotenoid, TBARS (thiobarbituric acid reactive substances) value, color, texture and fatty acid profile of the chicken breast meat were measured. *In ovo* injection of flaxseed oil significantly increased the fat content. The mean amounts of vitamin E in control and FSO were 0.54 and 0.71 mg/100g, respectively ($P<0.05$). Carotenoid content, redness and yellowness of the broilers breast meat in FSO increased in comparison to control, while TBARS value and lightness index decreased insignificantly. FSO showed lower saturated fatty acids and higher unsaturated, monounsaturated, polyunsaturated, unsaturated/saturated and n-3 fatty acids, but these differences were not significant. Based on the results of this study, *in ovo* injection of flaxseed oil improved oxidative stability of day-old broiler breast meat through increasing vitamin E content.

Keywords

Antioxidant activity

Breast meat

Chicken embryo

In ovo injection

Linum usitatissimum

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Introduction

Poultry meat contains significant amounts of polyunsaturated fatty acids and is susceptible to fat oxidation. Fat oxidation is among the main problems in meat industry resulting in loss of meat flavor and nutritional value over time. Lipid oxidation in meat is one of the most important causes of poor quality and shorter shelf life (Zhaleh *et al.*, 2019). During poultry embryo development the balance between antioxidants and prooxidants in the tissue leads to the normal growth of the embryo and the postnatal survival of the chickens. Chicken embryo relies on natural antioxidants accumulated in egg yolk to protect tissues against fat oxidation during hatching which seems to be an important determinant of chicken survival during the first days after hatching (Surai, 2000). Vitamin E is a generic name for all tocopherol and tocotrienol derivatives with biological activity of alpha-tocopherol. Vitamin E is an essential nutrient for growth and health of all animal species as it involves in preventing muscle atrophy, synthetizing prostaglandins and improving the immune response. The most well-known function of vitamin E is its antioxidant activity. Its aromatic ring reacts with and destroy reactive forms of oxygen and free radicals protecting the unsaturated fatty acids against oxidation and preventing oxidative damage to membrane lipids (Lee & Han, 2018). Anticancer and antioxidant effects of phenolic compounds have been demonstrated (Kalogianni *et al.*, 2020; Starčević *et al.*, 2015). Phenolic compounds include simple phenols, coumarins, lignins, lignans, concentrated and hydrolyzable tannins, phenolic acids and flavonoids. These substances act as antioxidants to prevent heart diseases, inflammation, cancer, diabetes and human cell mutagenicity (Khoddami *et al.*, 2013). Carotenoids are fat-soluble pigments found in plants, fungi, bacteria and algae as well as in many foods such as fruits, vegetables and fish. They have a wide range of biological effects (provitamin, antioxidant and colorant) and also play role in the immune system. The most important carotenoids are β- and α-carotene, lycopene, lutein and cryptoxanthin. Epidemiological studies have demonstrated that consuming foods rich in carotenoids can reduce the incidence of cardiovascular diseases (CVD), osteoporosis, diabetes, age- related macular degeneration (AMD), cataract as well as infectious diseases such as human immunodeficiency virus (HIV), (Milani *et al.*, 2017).

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N-3 polyunsaturated fatty acids in diets are essential for normal growth and health and may reduce the risk of cardiovascular disease, allergy, diabetes, arthritis and other inflammatory diseases. Consumption of each of n-3 or n-6 alone causes many health problems. A balanced ratio of n-3 and n-6 fatty acids is important for body to function properly and use these fatty acids optimally (Kanakri *et al.*, 2017). The body of living organisms is not able to synthesize essential fatty acids, so these compounds such as n-3 and n-6 are supplied through food consumption. The most important n-3 fatty acids include alpha-linolenic, eicosapentaenoic and docosahexaenoic acids. N-6 fatty acids include linoleic and arachidonic acids. The main precursor of the n-3 family is alpha-linolenic acid found in flaxseed oil, walnuts, canola oil, soybean oil, etc. The main precursor of n-6 fatty acids is linoleic acid which is found in large amounts in the oil of many plants, eggs and meat (Sijben *et al.*, 2001).

Flaxseed (*Linum usitatissimum*) oil also known as linseed oil is widely used in Asian, American and European countries (Oomah, 2001). Flaxseed contains oil (30-40%), protein (20-25%), fiber (20-25%), moisture (4-8%), ash (3-4%), mucilage (5-8%), cyanogenic glycosides (0.05-0.1%), lignins and phenylpropane derivatives (Rebole *et al.*, 2002). The chemical composition of flaxseed is affected by genetics, growth medium, seed preparation steps and analysis method (Coşkuner & Karababa, 2007). Flaxseed oil contains fatty acids such as linolenic, linoleic and oleic as well as a low amount of stearic and palmitic acids. It is one of the richest plant sources of n-3 fatty acids (alpha-linolenic acid) and can be used to enrich poultry products (Goyal *et al.*, 2016; Shafey *et al.*, 2014). The healthful effects of flaxseed oil are resulted from the presence of polyunsaturated fatty acids (PUFA) and dietary fiber preventing cardiovascular diseases effectively (Anjum *et al.*, 2013). Using *in ovo* injection method, specific nutrients can be provided in precise doses at certain times for maximum absorption by the embryo (Surai *et al.*, 1999). Various studies have shown that *in ovo* injection of growth factors, antibodies, aromatase inhibitors and food additives can improve the skeletal muscle growth, gastrointestinal tract development, immune response, growth performance, carcass traits and meat quality of broilers (El-Fakhrany *et al.*, 2021; El-Fakhrany *et al.*, 2022; Gholami *et al.*, 2015). Tavaniello *et al.* (2020) demonstrated that *in ovo* injection of galactooligosaccharide as a prebiotic diminished the negative effects of heat stress on the meat quality of broilers. El-Senousey *et al.* (2018) reported that *in ovo* injection of ascorbic acid reduced plasma malondialdehyde (MDA) content of day-old broilers. So far, no study has been done on the effects of *in ovo* injection of flaxseed oil on the quality of broilers breast meat, although many studies have been done on the effects of dietary supplementation with flaxseed on meat quality. The aim of this study was to investigate the effects of *in ovo* injection of flaxseed oil on the quality, antioxidant properties and fatty acid profile of day-old broiler breast meat.

Materials and methods

Flaxseed oil preparation and fatty acid analysis

Flaxseed oil was prepared from Barij Essence Company. Fat extraction and fatty acids methylation were performed according to Folch *et al.* (1957) and Eratte *et al.* (2017), respectively. Fatty acid profile was determined by gas chromatography (GC-4600, Unicam, Germany), (Table 1).

Table 1. Fatty acid content of flaxseed oil by gas chromatography–mass spectrometry

No	Fatty acid	Quantity (%)	*RI
1	C12:0	0.01	5.14
2	C14:0	0.03	6.78
3	C16:0	6.24	8.19
4	C16:1	0.04	8.66
5	C17:0	0.04	9.02
6	C17:1	0.01	9.14
7	C18:0	4.12	9.38
8	C18:1	15.83	9.56
9	C18:2	13.66	10.10
10	C18:3	58.17	10.29
11	C20:0	0.14	10.47
12	C20:1	0.06	10.55
13	C20:2	0.01	11.00
14	C22:0	0.10	12.19
15	C22:1	0.02	12.36
16	C24:0	0.05	12.53
	Total	98.53	

* RI: retention index

Treatment preparation

First 20 fertilized one-day-old eggs were prepared and candled to ensure the viability of the embryos. Then they were randomly divided into 2 groups of 10 as control and flaxseed oil (FSO) groups. The latter received flaxseed oil (100 µL) on day 5 by injecting into the allantoic sac. To do so, the egg was candled to find the air sac and disinfect it. Next, the oil was injected 2 mm above the air sac and the injection site was covered with paraffin. The incubation program was as follows: from day 1 to day 19, 37.5 °C, 60% relative humidity and rotation sequence 24 times a day and from day 19 temperature of 36.5 °C and 70% humidity (Seifi *et al.*, 2015). After hatching and checking for apparent defects, the chicks were killed in a humane manner. Next, the breast muscles of each chicken were immediately sampled and the samples on ice were quickly transformed to the food hygiene laboratory of the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies.

Fat measurement

Total fat content of breast muscle was measured using guidelines provided by the International Association of Agricultural chemists (AOAC, 2000).

Vitamin E measurement

Vitamin E was extracted from breast muscle using Peng *et al.* (1993) method. 50 mg of breast tissue were placed in a tube containing 280 µL of phosphate buffer saline (PBS) solution to which 35 µL of collagenase (1.5 mg/35 µL PBS) were added, incubated at 37 °C for 60 min and homogenized by a homogenizer for one min. Then 250 µL of this homogenized solution with 250 µL of sodium dodecyl sulfate and ethanol solution (1:9 ratio) were poured into a new tube to which 100 µL of vitamin E acetate as the internal standard and 500 µL of hexane were added. The resulting mixture was stirred for one min and centrifuged for five min at 7000 rpm. Finally, 250 µL of the supernatant was removed and dried with

nitrogen gas. Vitamin E was measured by High-performance liquid chromatography (HPLC) (Model AZURA, KNAUER company, Germany) with the mobile phase and the flow rate being methanol and 1.1 mL/min, respectively. Nova Pak-C18 chromatography column (3.9×300 mm) and ultraviolet detector at a wavelength of 292 nm were used and the results were expressed in mg/100g.

Total phenol content measurement

Total phenol content was measured by Min and Ahn (2009) method. 5 g of the meat sample were homogenized in a test tube using phosphate buffer and glycerol (20%) by a homogenizer. 1 mL of 1% Follin-Ciocalteu solution and 1 mL of sodium carbonate solution (7.5% w/v) were added to 1 mL of the sample solution. The samples then were incubated for 90 min at room temperature in the dark. Next, the light absorbance by the samples against control (methanol) was read by a spectrophotometer (Model JENWAY 6305, JENWAY Company, UK) at 765 nm. Gallic acid was used as standard and total phenol content was expressed in mg gallic acid equivalent per kg of meat (mg GAE/kg).

Total carotenoid content measurement

1 g of minced meat with 10 mL of 80% acetone were mixed and homogenized. Then 1 mL of the homogenized mixture with 9 mL of 80% acetone were mixed and centrifuged at 8000 rpm for 15 min and the supernatant was removed for total carotenoid content measurement. 80% acetone was used as blank. The absorbance of total carotenoid content was read at 480 nm and expressed in μg/g (Thaipong *et al.*, 2005).

TBARS value measurement

TBARS value was measured by Shirahigue *et al.* (2011) method. Briefly, 5 g of meat were homogenized with 15 mL of homogenizing solution in a centrifuge. After filtration, 5 mL of the supernatant was mixed with 5 mL of 0.02 M thiobarbituric acid. The samples were incubated in water bath at 100 ℃ for 40 min and cooled in cold water. Next, the absorbance was measured by a spectrophotometer (Model JENWAY 6305, JENWAY Company, UK) at 532 nm. The results were expressed in mg of malondialdehyde (MDA) per kg of meat (mg MDA/kg).

Color analysis

Color was analyzed by Hunter Lab Ultra Scan VIS (Hunter Associates Laboratory Inc., Reston, VA, USA) and described as L* (lightness), a* (redness) and b* (yellowness). Measurements were made perpendicular to the surface in five different sites and the mean values of L*, a* and b* were analyzed.

Shear force measurement

Shear force of the breast muscles was measured by texture analyzer (Brookfield Texture pro CT V1.2 Build 9, England). Samples (thickness of ~ 1 cm and length of 3 cm) were compressed to 2-50% of the original thickness at room temperature. The force-time deformation curve was obtained with a 25 kg load cell applied at a cross speed of 2 mm/s (Partovi *et al.*, 2019).

Analysis of fatty acids of breast meat

First whole fat of breast muscle was extracted using chloroform and methanol (Folch *et al.*, 1957). Then the fatty acids of the extracted fat were methylated (Dugan *et al.*, 1966) and analyzed by gas chromatography (ThermoQuest 2000, Manchester, UK).

Statistical analysis

The Shapiro-Wilk test was used to confirm the normality of the data, and the independent samples t-test was used to determine the difference between the means in two groups. Data analysis was performed by SPSS software version 25 (SPSS Inc., Chicago, IL, USA). In all analyzes, ($P<0.05$) were considered as statistically significant.

Results and discussion

The effect of *in ovo* injection of flaxseed oil on fat content of breast meat of day-old broilers is shown in Table (2). The mean total fat of breast meat for control and FSO samples were 5.58% and 6.03%, respectively ($P<0.05$). Our results are in agreement with the findings of Anjum *et al.* (2013) who reported that enrichment of the diet with flaxseed increased the total fat content of breast and leg meat. Crespo and Esteve-Garcia (2001) showed that carcass fat and abdominal fat of broilers increased when they fed a diet enriched with 10% flaxseed. However, Olomu and Baracos (1991) and Mridula *et al.* (2011) reported no changes in fat content of broiler muscles by adding flaxseed oil to the diet.

The effect of *in ovo* injection of flaxseed oil on vitamin E content of breast meat of day-old broilers is shown in Table (2). *In ovo* injection of flaxseed oil had a significant ($P<0.05$) effect on vitamin E content while did not affect total phenol content. The newly hatched chicks are protected against oxidation mainly through high concentrations of natural antioxidants primarily vitamin E and in some cases (wild birds) via carotenoids (Surai, 2002). Narciso-Gaytán *et al.* (2011) showed that increased amount of vitamin E in muscles could increase the oxidative stability of breast and leg meat of broilers. The amount of carotenoids of the breast muscle of broilers in FOS group increased insignificantly (Table 2). Sadighara *et al.* (2015) showed that injection of rice bran oil into the chorioallantoic membrane and egg yolk increased the amount of carotenoids of embryo liver and brain. Similary, Seifi *et al.* (2015) reported that *in ovo* injection of rice bran oil into the embryo significantly increased the carotenoids content of breast muscle.

TBARS value for FSO group was lower than that for control group insignificantly (Table 2). Seifi *et al.* (2015) showed that rice bran oil injected into the egg yolk or chorioalantoic membrane decreased TBARS value and increased the oxidative stability of breast muscle. Moghimian *et al.* (2019) reported that flaxseed oil and fish oil reduced MDA content in mice suffering from ischemic renal disease. Prasad (2005) revealed that feeding lignin extracted from flaxseed reduced the progression of atherosclerosis in rabbits as a result of a 35% reduction in serum MDA thereby diminishing the oxidative stress.

The effect of *in ovo* injection of flaxseed oil on the color of day-old broiler breast meat is shown in Table (2). *In ovo*

injection of flaxseed oil reduced L* (lightness) value while increased a* (redness) and b* (yellowness) values insignificantly. In a similar way, Shafey *et al.* (2014) reported that feeding flaxseed meal (80 g/kg ration) reduced L* value and increased a* value of pectoralis major fillet from 3.22 to 4.00. However, this relationship was not demonstrated in the study conducted by Mridula *et al.* (2011). Sirri *et al.* (2009) reported a positive correlation between total hem pigment content and a* value of meat and a negative correlation between total hem pigment content and L* value. Our result is consistent with the results obtained by Samadi *et al.* (2015) who stated that enrichment of diet with antioxidant sources such as artichokes reduced L* value of quail leg and breast meat. Pekel *et al.* (2012) reported that the amount of fat in the diet was inversely related to L* value of broiler meat. Our result is in agreement with their finding.

In ovo injection of flaxseed oil had no significant effect on shear force of day-old broiler breast meat (Table 2). These results have been reported previously by other researchers (Nardoia *et al.*, 2017; Wang *et al.*, 2017). Attia *et al.* (2017) reported that enrichment of diet with turmeric had no effect on texture attributes of broiler meat.

Table 2. Antioxidant capacity and quality of breast meat of day-old broilers after *in ovo* injection of flaxseed oil

	Control	*FSO	P value
Total fat (%)	5.58 ± 0.42^{a**}	6.03 ± 0.20^{b}	0.04
Vit E (mg/100g)	0.54 ± 0.14^{a}	0.71 ± 0.04^{b}	0.03
Total phenol (mg GAE/kg)	235.03 ± 14.42^{a}	227.41 ± 27.86^{a}	0.56
Carotenoid (µg/g)	5.51 ± 0.47^{a}	5.58 ± 0.26^{a}	0.73
TBARS (mg MDA/kg)	3.29 ± 0.26^{a}	3.10 ± 0.33^{a}	0.38
L*	53.04 ± 2.15^{a}	51.56 ± 0.89^{a}	0.15
a*	2.82 ± 0.46^{a}	3.06 ± 0.15^{a}	0.27
b*	12.23 ± 2.20^{a}	13.23 ± 0.35^{a}	0.31
Shear force (N)	28.24 ± 0.61^{a}	27.51 ± 1.70^{a}	0.36

The results were expressed as mean ± SD. *FSO: Flaxseed oil group. **Dissimilar letters in each row are significantly different ($P < 0.05$).

Effect of in o*vo* injection of flaxseed oil on fatty acid profile of day-old broiler breast meat is shown in Table (3). The main fatty acid of broiler breast meat was oleic acid (18:1) followed by palmitic acid (16:0) and linoleic acid (18:2). *In ovo* injection of flaxseed oil decreased saturated fatty acid (SFA) content while increased monounsaturated fatty acid (MUFA), unsaturated fatty acid (UFA), polyunsaturated fatty acid (PUFA), UFA/SFA ratio and n-3 fatty acids content insignificantly. Many studies have been conducted on the effects of enrichment of diet with flaxseed on the components of broiler meat (Shen *et al.*, 2005), however little research has been carried out on the effect of *in ovo* injection of flaxseed oil on the quality and antioxidant properties of meat. The reason for being insignificant may be attributed to the amount of flaxseed oil injected to the eggs and due to the lack of similar researches, the comparison is not possible. Our results are consistent with the result obtained by Narciso-Gaytán *et al.* (2011) who showed that enrichment of diet with flaxseed oil increased the amount of oleic, linoleic, linolenic and arachidonic acids. Mirshekar *et al.* (2015) reported that feeding flaxseed oil decreased SFA content and increased PUFA content of broiler breast meat. Our findings are in consistence with their results. They attributed the decrease in C14:0 and C16:0 to their elongation and unsaturation to C16:1 to be formed, acetyl-coA carboxylase (ACC) activity as well as fatty acid synthase activity. Similarly, Zhaleh *et al.* (2020) reported that diets containing flaxseed decreased the level of SFA (C16:0 and C18:0 in leg muscle) and increased the amount of PUFA especially linolenic acid in breast and leg muscles (Zhaleh *et al.*, 2020).

Table 3. Effect of *in ovo* injection of flaxseed oil on fatty acid profile of day-old broiler breast meat

	Control	*FSO	**P value
C14:0	0.77 ± 0.00	0.77 ± 0.00	0.42
C16:0	24.28 ± 0.29	21.41 ± 2.88	0.16
C16:1	3.19 ± 0.02	4.19 ± 1.01	0.16
C18:0	10.01 ± 1.09	10.02 ± 0.64	0.98
C18:1	29.38 ± 1.27	29.44 ± 1.00	0.95
C18:2	21.97 ± 1.82	22.97 ± 0.24	0.44
C18:3	1.25 ± 0.06	1.26 ± 0.03	0.88
C20:3	0.49 ± 0.00	0.48 ± 0.01	0.11
C20:4	0.72 ± 0.00	0.72 ± 0.00	1.00
C20:5	1.20 ± 0.04	1.20 ± 0.02	0.91
C22:5	3.26 ± 0.53	3.58 ± 0.06	0.41
C22:6	1.09 ± 0.02	1.09 ± 0.02	0.85
Total	97.64 ± 0.25	97.16 ± 0.21	0.06
SFA	35.06 ± 1.39	32.21 ± 2.36	0.14
UFA	62.58 ± 1.24	64.94 ± 2.14	0.17
MUFA	32.57 ± 1.29	33.63 ± 1.94	0.47
PUFA	30.01 ± 1.57	31.31 ± 0.21	0.22
UFA/SFA	1.78 ± 0.11	2.02 ± 0.20	0.15
PUFA/SFA	0.85 ± 0.06	0.97 ± 0.07	0.10
PUFA/MUFA	0.91 ± 0.07	0.92 ± 0.04	0.85
n-3	1.25 ± 0.06	1.26 ± 0.03	0.88
n-6	21.97 ± 1.82	22.97 ± 0.24	0.40
n-6/n-3	17.46 ± 0.66	18.18 ± 0.46	0.19

The results were expressed as mean ± SD. *FSO: Flaxseed oil group, SFA: saturated fatty acid, UFA: unsaturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid. **$P < 0.05$ was considered statistically significant.

Enrichment of diet with flaxseed oil increased n-3 fatty acid content of broiler muscles and the longer the time of feeding flaxseed oil, the higher the amount of n-3 fatty acids in meat. In the case of long time consumption, flaxseed oil could also increase n-6 content of breast (Mirshekar *et al.*, 2015). The positive effects of feeding diets containing flaxseed on increased level of PUFA and n-3 fatty acids especially linolenic acid (C18:3) in leg and breast muscles of broilers and turkeys have been demonstrated (Anjum *et al.*, 2013; Jankowski *et al.*, 2015; Mridula *et al.*, 2011; Olomu & Baracos, 1991; Zhaleh *et al.*, 2020). The ratio between PUFA and SFA is an important parameter for nutritional assessment due to its protective role in human health. Our result is in agreement with the results reported by Cui *et al.* (2018) who showed that enrichment of diet with *Moringa oleifera* leaves increased PUFA content commonly being directly related to fat oxidation, however decreased TBARS value indicated that the oxidative stability of meat improved.

Conclusion

In ovo injection of flaxseed oil increased fat and vit E content of day-old broiler breast meat. Due to the important role of vitamin E in improving the immune responses, preventing different kinds of diseases especially in newly hatched chicks and also increasing the oxidative stability of meat, it could be concluded that *in ovo* injection of flaxseed oil may have beneficial effects on health of day-old broilers and extention of meat shelf life.

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Author contributions

Sara Balvayeh: Data collection, Writing the draft of the manuscript; **Razieh Partovi**: Revising and editing the manuscript, Supervising the study, Approval of the final version; **Behrokh Marzban Abbasabadi**: Presenting the research idea and study design, Data collection; **Shohreh Alian Samakkhah**: Data analysis and interpretation

Conflict of interest

The authors declare that there is no conflict of interest.

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