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Numbers of Follicles, Pregnancy, Expression of Reactive Oxygen Species (ROS), and Ovarian Genes in Sheep Treated with Vitamin E, L-Carnitine, and Fish Oil

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ABSTRACT

This study was performed to evaluate the effects of vitamin E, L-carnitine, and fish oil on the numbers of follicles, pregnancy, Reactive Oxygen Species (ROS) expression, and expression of several ovarian genes in sheep. For this purpose, 256 sheep were randomly divided into eight experimental groups. They were given a diet supplemented with vitamin E, L-carnitine, and fish oil alone and in combination. FBS, estradiol levels, size of the follicles, and pregnancy rate were observed. Expressions of Superoxide Dismutase 1 (*SOD1*), Superoxide Dismutase 2 (*SOD2*), Transforming Growth Factor –β (*TGF-β*), and Peroxisome Proliferator-Activated Gamma Receptor (*PPAR-γ*) genes were measured using Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and ROS was measured using fluorescence microscope. At the time of mating and pregnancy, the highest number of follicles and pregnant ewes were observed in the groups supplemented with fish oil and antioxidants (p<0.01). The lowest numbers of follicles and pregnancy was observed in the group supplemented with fish oil alone (p<0.01). The highest expressions of *SOD1* and *TGF-β* genes (p<0.01) as well as *SOD2* and *PPAR-γ* (p<0.05) were observed in the group of concomitant use of fish oil with antioxidants. The highest amount of ROS was found in fish oil group (p<0.01), and the lowest was found in the groups supplemented with fish oil and antioxidant (p<0.01). The use of fish oil along with vitamin E and L-carnitine improved follicle function and increased pregnancy rate by reducing ROS in ewes' ovaries as well as increasing the expression of *SOD1*, *SOD2*, *TGF-βRI*, and *PPAR-γ* genes. The use of fish oil along with antioxidants increases follicles and improves fertility in sheep.

Keywords: fish oil; follicles; L-carnitine; pregnancy; vitamin E

INTRODUCTION

Reproductive performance is a significant production parameter in sheep, especially when the goal is to produce milk and meat (Ali *et al.*, 2019). Parameters for reproductive function in an animal or herd include lambing rate, fertility rate, and age of the first fertility (Assan, 2020). Nutrition has a vital impact on numerous reproductive functions, including the hypothalamic-pituitary-gonadal (HPG) axis (Mugabe *et al.*, 2017). Flushing or using proper diets before or during the mating season can improve ovulation rate, twinning rate, fertility rate, and lower the risk of miscarriage in early pregnancy (Burritt *et al.*, 2012).

Vitamin E, L-carnitine, and omega-3 have been shown to have effects on fertility. Fish oil has received a lot of attention in recent years because of the abundance of omega-3 polyunsaturated fatty acids (PUFA-ω3) (DURMUŞ, 2018). A diet rich in PUFA-ω3 positively affects fertility by affecting ovule quality and embryo implantation (Nehra *et al.*, 2012). Omega-3 fatty acids have been shown to improve ovule growth (Moallem *et al.*, 2013). PUFAs are essential for cell proliferation during the formation of antral follicles (Ruiz-Sanz *et al.*, 2019). Vitamin E is one of the most effective nutrients known for immune system function. This effect is partly due to its protective effect against PUFA oxidation (Lewis *et al.*, 2019). α-Tocopherol is the most common form of vitamin E in tissues (Torquato *et al.*, 2020). Vitamin E is a major fat-soluble antioxidant that scavenges peroxyl radicals and prevents PUFA oxidation. In the presence of vitamin E, peroxyl radicals react with α-tocopherol instead of lipid hydroperoxide, preventing further oxidation (Lee & Han, 2018; Alemi *et al.*, 2014). L-Carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) is a natural compound that facilitates the transfer of fatty acids to the mitochondria for β-oxidation (Ishikawa *et al.*, 2014). L-carnitine can improve mitochondrial function by maintaining the mitochondrial wall under stress (Li & Yu, 2012). L-Carnitine is an effective inhibitor of free radicals (Surai, 2015). In addition, L-carnitine can reduce liver fat peroxidation since it has a protective

role against reactive oxygen species (ROS) by applying antioxidant properties (Jin *et al.,* 2019).

Some important genes in ova maturation and ova fertility are peroxisome proliferator-activated gamma receptor (*PPAR-γ*), superoxide dismutase 1 (*SOD1*), superoxide dismutase 2 (*SOD2*), and transforming growth factor beta (*TGF-β*). The expression of these genes had previously been established in sheep ovaries (Minge *et al.,* 2008; Kala, 2017; Yang *et al.,* 2019). The activity of *PPAR* nuclear receptors controls steroidogenesis, tissue regeneration, angiogenesis, lipid metabolism, activation of immune cells, and production of proinflammatory mediators. All three *PPAR* isotypes are active in sheep ovaries (Blitek & Szymanska, 2019; Vitti *et al.,* 2016). *PPAR-γ* is expressed in granulosa cells to produce estradiol and regulate follicular fluid content and luteal cells to produce progesterone. *PPAR-γ* expression increases during follicle growth (Vitti *et al.,* 2016). In a study with *PPAR-γ* deletion (in animals), regular ovulation was observed. Still, the level of progesterone secretion and consequently the embryo implantation rate was significantly reduced (Cui *et al.,* 2002). Furthermore, the close relationship between *PPAR* and polycystic ovary syndrome has been reported (Rostamtabar *et al.,* 2021). *TGF-β* plays a vital role in normal follicular growth and fertility. Ewes with mutations in the *TGF-β* family became infertile due to the abnormal development of follicles after the initial stage (Galloway *et al.,* 2000). *TGF-β* has been shown to play an essential role in the process of follicular growth within the ovary (Monsivais *et al.,* 2017).

The superoxide dismutase is active in eliminating superoxide dismutase anion radicals from extracellular stimuli (McCord & Fridovich, 1969). In addition to the human gene, the complete genomic sequences of the *SOD1* and *SOD2* genes have been isolated and identified for the other species of animals as well as for sheep (Mishra *et al.,* 2017). Oxidative stress indicates an imbalance between the production and emergence of oxygen free radicals and the ability of the biological system to detoxify or repair their destructive effects (Reuter *et al.,* 2010; Ďuračková, 2010). ROS include several oxygen-induced chemical reactive molecules used in balanced concentrations for better cell function. ROS causes DNA damage to the ovarian epithelium or cell apoptosis. However, it modulates cell oxidative status, follicular growth, corpus luteum formation, endometrial differentiation, and embryonic growth (Ghasemzadeh *et al.,* 2013).

At high concentrations, ROS can be an essential mediator of damage to the structure of lipids, proteins, and nucleic acids that eventually cause a condition called oxidative stress (Huang *et al.,* 2016). Free radicals have high energy due to free electrons and can damage tissues and cells (Kehrer & Klotz, 2015). To stop a reaction, the newly formed radical must react with another free radical to remove the unpaired electron or react with the free radical scavenger (Gülcin *et al.,* 2002).

However, no study has examined the relationship between fish oil as a source of omega-3 and antioxidants on genes involved in improving fertility in the ovary and ROS. This study was performed to evaluate the effect of vitamin E, L-carnitine, and omega-3 on fertility, ROS expression, and some ovarian genes in sheep. We assume that fish oil and antioxidants increase follicles and improve fertility in sheep.

MATERIALS AND METHODS

This experiment was approved by the Ethics Committee of Animal Care and Use of Islamic Azad University, Arak Branch, according to the guidelines of the National Research Council for the care and use of laboratory animals (P.O. BOX: 9655645613-ARAK-IRAN). This study was performed in winter. In total, 256 3-4 years old sheep were used in this study. We used a completely randomized design for our experiment. The experimental sheep were randomly divided into eight experimental groups, and four replicates in each experimental group, and eight ewes in each replication. The groups were divided according to parity, age, weight, and body condition score so that the mean of the groups was the same.

The experimental ewes were divided into eight groups, which include: 1) control (Con) (Base diet), 2) Base diet enriched with 800 international units/day of vitamin E (E) (Hill *et al.,* 2009), 3) Base diet enriched with L-carnitine (L) (500 mg/kg dry matter) (Foroozandeh *et al.,* 2014), 4) Base diet enriched with vitamin E + L-carnitine (EL), 5) Base diet enriched with Fish oil (F) (35 g/kg dry matter) (Ferreira *et al.,* 2014), 6) Base diet enriched with fish oil + Vitamin E (FE), 7) Base diet enriched with fish oil + L-carnitine (FL), and 8) Base diet enriched with fish oil + vitamin E + L-carnitine (FEL). According to NRC (2007), treatment diets were adjusted and the structures of diets are shown in Table 1.

In the current study, the experimental sheep were adapted for ten days. The sheep were fed with the treatment diet for 20 days. The diets were given to the experimental ewes in four meals with 6 hours intervals. Estrus synchronization was then performed. CIDRs (Source of progesterone) were used to synchronize the estrus cycles of experimental ewes. The CIDRs were placed in the vaginal area for 14 days, and after removing the CIDRs, PG-600 (400 IU PMSG + 200 IU hCG) were injected for all groups of experimental ewes and mating was done. After mating, the diet was continued for another 25 days. In this study, blood sampling and ultrasound were performed before the adjustment period for the health of sheep. Sonography was performed before mating, where the number of follicles above 3 mm was counted. Thirty-five days after the mating, an ultrasound was performed with a 7.5 MHz abdominal probe to diagnose pregnancy. Blood samples were taken from the jugular vein of the ewes before the acclimatization period and at the time of mating. Blood samples were collected in tubes containing anticoagulant EDTA. Plasma samples were stored in the freezer after centrifugation to measure the amount of FBS and estradiol using the ELISA kit. Data were analyzed using SAS software (version 9.4). Kolmogorov-Smirnoff normality test and the comparison of mean treatments were performed by Duncan test.

Procedure for Expression of Ovarian Genes and Intracellular ROS Levels

At the time of added rams, four sheep were selected from each treatment for slaughtering. One sheep was selected from each replication and a total of 32 sheep were sacrifized. After slaughtering, ovarian biopsy was performed to evaluate *SOD1*, *SOD2*, *PPAR-γ*, *TGF-βRI* genes and intracellular ROS levels. Samples were immediately stored in liquid nitrogen at -196 °C.

The frequency of transcription of *SOD1*, *SOD2*, *PPAR-γ*, *TGF-βRI* genes were measured using Reverse Transcriptase PCR (RT-PCR) followed by a real-time quantitative PCR (qPCR) for relative expression level. Gene-specific Forward and Reverse primers used in this study were designed from sequences in NCBI using Primer 3 plus software (Table 2).

RNA Extraction

Before the RNA isolation, samples were washed with 0.25% Trypsin-EDTA solution. The total RNA of the samples was isolated by Trizol method according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Briefly, the samples were mixed with 200 µL of Trizol and incubated for 10 minutes. They were then incubated for 10 minutes by adding 50 µL of chloroform again. The sample mixture was centrifuged at 4 °C for 15 minutes. The upper aqueous phase was collected and transferred to a new RNAse-free tube, then the sample was dried by incubation.

Evaluation of cDNA

The integrity of total RNA was checked on 1% agarose gel electrophoresis using 1x TAE buffer. Total RNA purity was assessed using a nanodrop with OD 260: OD 280 values, which was more than 1.8. Reverse Transcription (RT) using SuperScript III FirstStrand Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) was performed according to the manufacturer's instructions. The synthesized cDNA was stored at -20 °C until being used for RT-PCR and qPCR.

Procedure for Reverse Transcription PCR (RT-PCR)

RT-PCR for all genes were carried out with 1.25 U Taq DNA polymerase, 2.5 mM MgCl2, 0.4 mM dNTP mix, and 5 pM both forward and reverse primers.

The PCR reaction performed is as follows: A cycle of 95 °C for 15 minutes with 30-45 cycles of denaturation at 95 °C for 15 seconds, annealing at 59-64 °C for 30 seconds, extension at 72 °C for 15 seconds, and final extension of 72 °C for 5 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene in this study (Figure 1).

Procedure for Quantitative PCR (qPCR)

The relative expression level of genes in oocytes were quantified by qPCR using step one plus qPCR system (Applied Biosystem, Foster City, CA, USA). Relative quantification method was used to analyze the expression level of genes. The Maxima SYBR Green/ Rox qPCR (2X) combination was used for qPCR reac-

Table 1. Feed ingredients and chemical composition of treatment diets (% of diet dry matter)

Feedstuff	Treatments[1]							
	Con	E	L	EL	F	FE	FL	FEL
Alfalfa (%)	40.00	40.00	40.00	40.00	35.00	35.00	35.00	35.00
Wheat straw (%)	45.00	45.00	45.00	45.00	50.00	49.92	49.95	49.87
Milled barley (%)	3.00	3.00	3.00	3.00	2.50	2.50	2.50	2.50
Milled corn (%)	7.50	7.42	7.45	7.37	4.50	4.50	4.50	4.50
Soybean meal (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Molasses beet sugar (%)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Beet sugar pulp (%)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Mineral and vitamin supplements[2] (%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin E (%)	-	0.08	-	0.08	-	0.08	-	0.08
L-carnitine (%)	-	-	0.05	0.05	-	-	0.05	0.05
Fish oil (%)	-	-		-	3.50	3.50	3.50	3.50
Chemical composition of the feed:								
Dry matter intake (g/d)	1150	1150	1150	1150	1150	1150	1150	1150
Metabolizable energy (Mcal/kg)	2.09	2.09	2.08	2.08	2.12	2.12	2.12	2.11
Crude protein (%)	11.12	11.12	11.12	11.12	10.42	10.92	10.92	10.91
Neutral detergent fiber (%)	54.72	54.72	54.72	54.75	55.26	56.23	56.25	56.19
Acid detergent fiber (%)	39.74	39.74	39.74	39.75	40.65	40.98	41.00	40.95
Calcium (%)	9.41	9.41	9.41	9.41	8.76	8.76	8.76	8.76
Phosphorus (%)	2.55	2.55	2.55	2.57	2.37	2.37	2.37	2.37

Note=[1]Treatments: Con= control, E= vitamin E, L= L-carnitine, EL= Vitamin E + L-carnitine, F= Fish oil, FE= Fish oil + Vitamin E, FL= Fish oil + L-carnitine, FEL= Fish oil + Vitamin E + L-Carnitine. [2]Vitamin-mineral supplement= 6.19% calcium, 6.9% phosphorus, 1.7% sodium, 9.1% magnesium, 0.3% iron, 0.03% copper, 0.2% manganese, 100 ppm cobalt, 100 ppm iodine, 0.1 ppm selenium, 50×105 international units of vitamin A, 10×105 international units of vitamin D, no vitamin E.

Table 2. Sequences of primers used to amplify GAPDH genes as reference genes and *SOD1*, *SOD2*, *TGF-βRI*, and *PPAR-γ* in ewe ovaries

Gene	Primer	Product size	Annealing temperature (°C)
GAPDH	TGACCCCTTCATTGACCTTC	184 bp	62.6
	CACGTACTCAGCACCAGCAT		
SOD1	TTCGAGGCAAAGGGAGATAA	226 bp	59.7
	CAATGGCAACACCATTTTTG		
SOD2	GGAAGCCATCAAACGTGACT	186 bp	63.6
	AGCAGGGGGATAAGACCTGT		
TGF-βRI	GCAAAGGTCGTTTTGGAGAA	207 bp	62.0
	CTGACACCAACCAGAGCTGA		
PPAR-γ	GATAAAGCGTCAGGGTTCCA	187 bp	62.9
	TATGAGACATCCCCACAGCA		

Note: GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, *SOD1*: Superoxide Dismutase 1, *SOD2*: Superoxide Dismutase 2, *TGF-β*: Transforming Growth Factor–β, *PPAR-γ*: Peroxisome Proliferator-Activated Gamma Receptor.

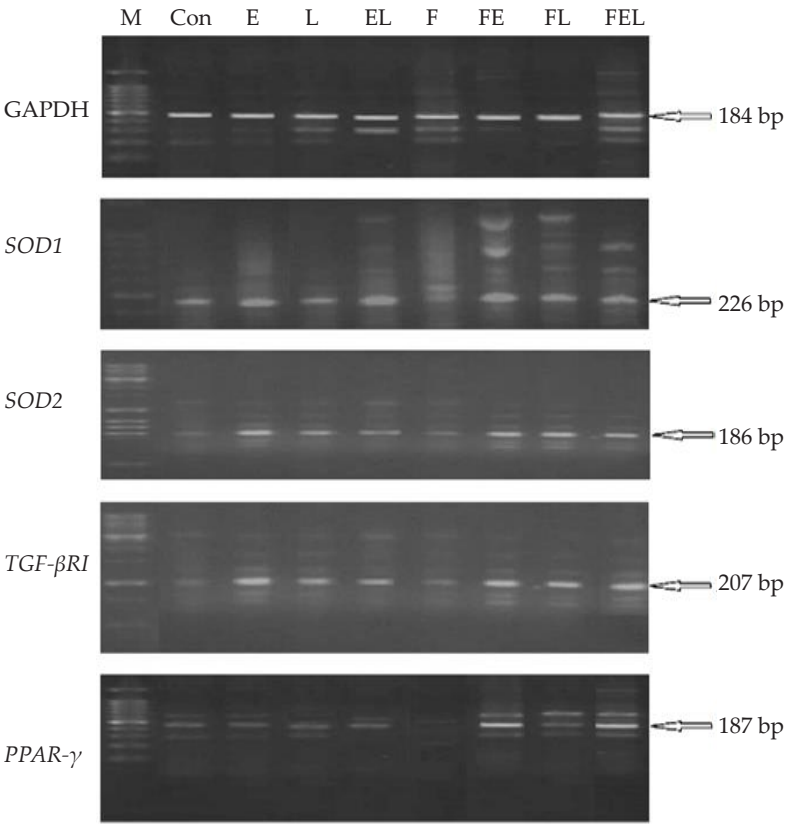

Figure 1. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) expression pattern as a reference gene and Superoxide Dismutase 1 (*SOD1*), Superoxide Dismutase 2 (*SOD2*), Transforming Growth Factor–β (*TGF-βRI*), and Peroxisome Proliferator-Activated Gamma (*PPAR-γ*) genes on several treatments in ewes ovaries. Note: control (Con), vitamin E (E), L-carnitine (L), vitamin E + L-carnitine (EL), Fish oil (F), Fish oil + Vitamin E (FE), Fish oil + L-carnitine (FL), Fish oil + Vitamin E + L-Carnitine (FEL).

tions (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). In each reaction, 5-µL qPCR master mix, 5 pM forward and reverse gene primers, and 2 µL cDNA samples were used. Temporal and temperature program used in PCR reaction includes: initial denaturation at 95 °C for 10 minutes with 40 cycles of denaturation at 95 °C for 15 seconds, and then annealing and expansion at 60 °C for 1 minute. The expression of each gene was measured relative to GAPDH.

Statistical Analysis

To determine the relative expression level of each mRNA from Ct (threshold cycle for target amplification) using the method $2^{-\Delta\Delta Ct}$ (normalized expression ratio) was used. The ΔCt = Ct (target gene) - Ct (housekeeping gene) and ΔΔCt = ΔCt (target gene sample) - ΔCt (calibrator). The RT-PCR and qPCR amplicons of genes were confirmed by ethidium bromide (0.5 µg/mL) stained 2% agarose gel electrophoresis. Gene expression levels in tissues were analyzed by ANOVA statistical analysis

using Graphpad Prism 5 (San Diego, CA, USA). $p<0.05$ was considered significant (Livak & Schmittgen, 2001). All experiments were performed in triplicates.

Expression of Reactive Oxidative Species (ROS)

Intracellular ROS levels was measured using fluorescent markers 2', 7'dichlorodihydrofluorescein diacetate was determined according to the manufacturer's instructions (Figure 2). The samples were incubated for 30 minutes in phosphate-buffered saline solution (PBS) with ten mM DCFH-DA solution at 37 °C in the dark. This marker has a high sensitivity to H_2O_2. After incubating and washing twice, ROS analysis was done using epifluorescence microscope with UV filters of 460 nm. DA-DCFH actively enters the cell, and by the action of cellular esterase, the diacetate group is cleaved, and the non-fluorescent compound DCFH is formed. In the presence of hydrogen peroxide, DCFH is oxidized to DCF, leading to an intense green fluorescence. This compound is not able to leave the cell and can be evaluated by fluorescence microscope. The fluorescence intensities of oocytes were analyzed by ImageJ software.

RESULTS

According to the results, the amount of FBS in the blood at the time of first blood sampling before adaptation (FBS1) and second blood sampling at the time of mating (FBS2) were not affected by the experimental treatments ($p>0.05$). In addition, the level of estradiol in the blood at the time of the first blood sampling (Est1) and the second blood sampling (Est2) did not show a significant change ($p>0.05$) (Table 3). The number of follicles in the first sonography (Sono1) was not affected by the experimental treatments. However, in the second sonography (Sono2), the number of follicles in the FEL, FE, FL groups showed a significant increase. In groups E and L, no change was observed compared to the control group, but group F had the lowest number of follicles. The highest pregnancy rate was observed in FE, FL, and FEL groups, and the lowest pregnancy rate was observed in group F ($p<0.01$) (Table 4). The presence of fish oil alone reduced pregnancy, which was improved with antioxidants. The highest level of ROS expression was observed in group F (23.01), and the lowest expression was observed in E, L, EL, FEL groups (13.80, 13.49, 13.51, 14.12) compared to the control group ($p<0.01$) (Figure 3). The use of fish oil alone has increased the amount of ROS in the tissue, which can be observed by adding antioxidants to the diet. Fish oil alone decreased the expression of genes in the ovaries by increasing the level of ROS in the tissue (Figure 2). Also, the highest level of *SOD1* expression was found in the groups fed with antioxidants and fish oil, and the lowest gene expression was found in the

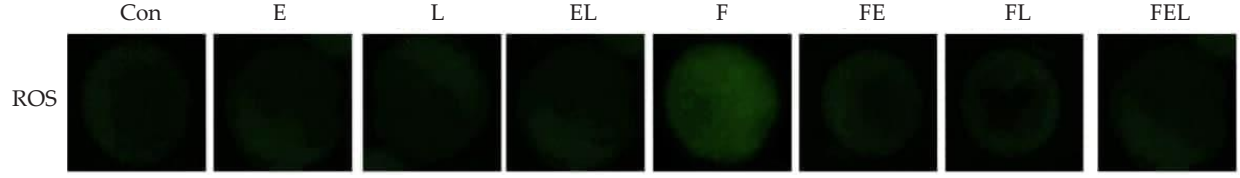

Figure 2. Detection of Reactive Oxygen Species (ROS) activity level on treatments control (Con), Vitamin E (E), L-carnitine (L), vitamin E + L-carnitine (EL), Fish oil (F), Fish oil + Vitamin E (FE), Fish oil + L-carnitine (FL), Fish oil + Vitamin E + L-Carnitine (FEL) in ewes ovaries.

Table 3. Fasting blood sugar and blood estradiol (mg/dL) in pre-adaptation (1) and pre-mating (2) periods in ewes treated with vitamin E, L-Carnitine, and fish oil

Item	Treatments[1]									
	Con	E	L	EL	F	FE	FL	FEL	SEM	p-Value
FBS (1)[2]	62.57	50.96	51.60	52.90	59.03	66.22	66.14	66.78	2.22	0.308
FBS (2)	57.28	54.74	55.26	55.08	55.16	56.61	58.08	56.87	0.59	0.852
Estradiol (l)[3]	94.96	92.95	93.37	93.56	90.94	87.68	87.75	83.73	2.16	0.935
Estradiol (2)	106.26	102.55	102.81	99.37	91.44	86.14	86.39	86.55	2.65	0.260

Note: [1]Treatments: Con= control, E= Vitamin E, L= L-carnitine, EL= Vitamin E + L-carnitine, F= Fish oil, FE= Fish oil + Vitamin E, FL= Fish oil + L-carnitine, FEL= Fish oil + Vitamin E + L-Carnitine. [2]FBS= Fasting blood sugar. [3]Estradiol levels.

Table 4. Number of follicles above 3 mm before adaptation (Sono1) and before mating (Sono2), and the percentage of pregnancy (%) in ewes during the flushing period treated with vitamin E, L-Carnitine, and fish oil

Item	Treatments[1]									
	Con	E	L	EL	F	FE	FL	FEL	SEM	P-Value
Sono1[2]	1.12	1.13	1.14	1.08	1.05	1.14	1.1	1.11	0.024	0.993
Sono2	1.03^{b}	1.14^{b}	1.15^{b}	1.22^{b}	0.81^{c}	1.50^{a}	1.46^{a}	1.67^{a}	0.058	0.000^{**}
Pregnancy	55.87^{b}	60.04^{b}	59.97^{b}	62.33^{b}	40.31^{c}	80.23^{a}	78.38^{a}	85.23^{a}	3.26	0.000^{**}

Note: ** and *, respectively= the probability level of 1% and 5% and the superscript of each number indicates a significant order. [1]Treatments: Con= control, E= Vitamin E, L= L-carnitine, EL= Vitamin E + L-carnitine, F= Fish oil, FE= Fish oil + Vitamin E, FL= Fish oil + L-carnitine, FEL= Fish oil + Vitamin E + L-Carnitine. [2]Sonograph.

groups fed with fish oil diet (p<0.01). The expression levels of the *SOD2* gene in FL, FE, and FEL groups (0.979, 0.991, and 0.993, respectively) showed the highest expression rate compared to control and F groups (0.676 and 0.662) (p<0.01). The expression level of the *TGF-β* gene in FL, FE, and FEL groups showed a significant increase compared to the other treatments. In addition, the highest amount of *PPAR-γ* was observed in FE and FEL groups (1.991 and 1.995), and the lowest amount of *PPAR-γ* expression was observed in F group (1.356) (p<0.05). In this study, the use of antioxidants did not affect the expression of genes; the reason can be related to the dose of antioxidants used. Still, the addition of fish oil as a source of omega-3s and antioxidants was able to increase the expression of genes and improve fertility and follicle count.

DISCUSSION

The use of fish oil in the basal diet showed a significant decrease in the percentage of pregnancy and the number of follicles. Fish oil is rich in omega-3 fatty acids (Olsen *et al.,* 1992). High levels of unsaturated fatty acids in fish oil expose it to peroxidation (Sherratt *et al.,* 2020), which results in oxidative stress. Oxidative stress leads to the development and spread of cellular dam-

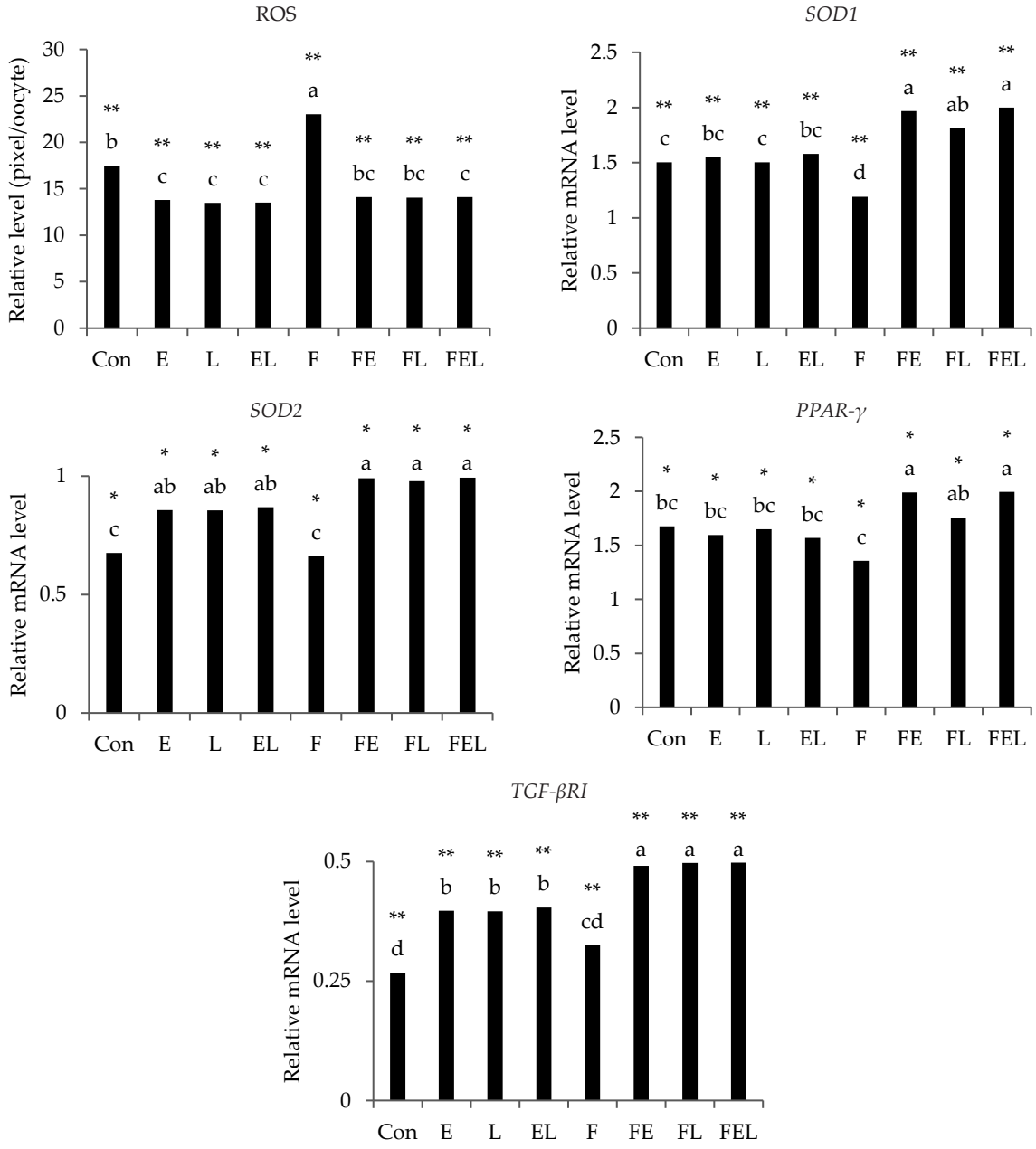

Figure 3. Reactive Oxygen Species (ROS) and expression of Superoxide Dismutase 1 (*SOD1*), Superoxide Dismutase 2 (*SOD2*), Transforming Growth Factor–β (*TGF-βRI*), and Peroxisome Proliferator-Activated Gamma (*PPAR-γ*) genes after RT-PCR in ewes ovaries treated with vitamin E, L-Carnitine, and fish oil. Note: control (Con), vitamin E (E), L-carnitine (L), vitamin E + L-carnitine (EL), Fish oil (F), Fish oil + Vitamin E (FE), Fish oil + L-carnitine (FL), Fish oil + Vitamin E + L-Carnitine (FEL). (** and * respectively the probability level of 1 and 5% and the superscript of each diagram specifies a significant order).

ages, including plasma membrane peroxidation (Hao *et al.*, 2006), amino acid oxidation (Heinecke, 2002), nucleic acid oxidation (Sung *et al.*, 2013), apoptosis (Lee *et al.*, 2014), and necrosis (Manickam *et al.*, 2017).

We also saw the highest expression level of ROS gene was found in the group of sheep fed with fish oil diet. ROS is involved in various physiological reproductive functions such as ovule maturation, ovarian steroidogenesis, ovulation, implantation, blastocyst formation, and corpus luteum function (Zhang *et al.*, 2005). ROS can regulate cell function by activating and controlling biological activity production and activating key cell-signaling pathways (Li *et al.*, 2017). Excessive ROS production has detrimental effects on cell function and may endanger reproduction and fertility (Adeoye *et al.*, 2018). It has been shown that ROS is produced in the follicle during the ovulation process. ROS can risk the ovulation process by rupturing the follicle (Lu *et al.*, 2018).

In the present study, the reduction of ROS production was observed by adding antioxidants to fish oil as a source of omega-3. Vitamin E consumption significantly increases the activity of antioxidant enzymes and reduces the level of fat peroxide (Jia *et al.*, 2017). The antioxidant effect of L-carnitine is related to the direct accumulation of free radicals. L-Carnitine can effectively reduce lipid peroxidation and free radicals (Shokrzadeh *et al.*, 2013). L-carnitine prevents lipid peroxidation by helping to maintain mitochondrial integrity and reduce the chance of ROS production (Kumaran *et al.*, 2005). The presence of *SOD* enzyme in the cells and tissues keeps the superoxide anion concentration at a very low level. The activity of *SOD* in the cells and extracellular environments is vital for the prevention of oxidative stress-related diseases and the prevention of various disorders (Matsuzawa & Ichijo, 2008; Wiemer, 2011). One study showed that ROS levels decreased with the activation of *SODs* (Sun *et al.*, 2012). *SODs* act as the first line of antioxidant defense to prevent oxidative stress (Ighodaro & Akinloye, 2018). *SODs* are also the first line of defense and among the most important defenses against ROS (Gill *et al.*, 2015).

This study, using vitamin E and L-carnitine as antioxidants, showed that the groups of experimental ewes which received antioxidants had lower ROS and higher *SOD* levels. In addition, the highest number of follicles and pregnancy was found in the groups of experimental ewes that had lower ROS levels, and the highest number of follicles and pregnancy was found in the groups of ewes fed with antioxidants and fish oil containing omega-3 diet, which is consistent with previous studies. Adding vitamin E to the diet was associated with reducing ROS (Cam *et al.*, 2004). Also, by adding L-carnitine to the diet, the production of ROS decreased, and the relative expression level of *SOD1* and *SOD2* genes in sheep ovarian tissue increased (Mishra *et al.*, 2016). The decreased ROS levels in female follicular fluid increased the number of follicles and increased fertility (Attaran *et al.*, 2000; Elizur *et al.*, 2014). The presence of vitamin E along with omega 3 in the diet can play a role in boosting follicle growth (Nateghi *et al.*, 2019). In the present study, it was shown that the use of vitamin E and L-carnitine increased the expression of *TGF-β* in the ovary, which led to the improved follicle function and pregnancy; this could be due to the improved inflammation in the tissue as well as the better function of the ovary. The ability of collagen to proliferate will promote the growth of follicles; *TGF-β* is involved in cell proliferation, differentiation, metabolism, fibroblasts, collagen synthesis, and extracellular matrix formation; collagen is essential for follicular growth. Various cytokines regulate it, *TGF-β* has been shown to have a regulatory effect on follicular collagen, and collagen plays an important role in follicular development (Zhou *et al.*, 2021). Activation of *TGF-β* is due to protein oxidation to ROS (Sun *et al.*, 2017). *TGF-β* proteins play an essential role in gonadal growth and follicular growth (Rosairo *et al.*, 2008). In mammals, *TGF-β* is known to regulate reproduction, including ovarian function (Raja-Khan *et al.*, 2014). *TGF-β* and its receptors are present in the ovarian follicles. In addition, a positive effect on oocyte growth has been shown *in vitro* (Rodrigues *et al.*, 2014).

One study showed that *PPAR-γ* may be used as an anti-inflammatory in various types of inflammation (Kim *et al.*, 2012). Therefore, in the present study, *PPAR-γ* can improve reproductive function by affecting inflammation and follicles (Hara *et al.*, 2013). *PPAR-γ* activation can regulate inflammatory responses (Kielian & Drew, 2003). Although omega-3s may be a small part of the fatty acids in the follicles, the relative frequency of omega-3s in the granulosa cells is physiologically higher than in the ovule. Therefore, it can be suggested that omega-3 fatty acids regulate ovule maturation and growth mainly by altering the metabolism and function of granulosa cells via *PPAR* (Zarezadeh *et al.*, 2019). Vitamin E can act as ligands for *PPAR-γ* activating receptors (De Pascale *et al.*, 2006). As a result, the presence of antioxidants (vitamin E or L-carnitine) and fish oil (omega-3s) improves the function of reproductive activity in the ovaries. We recommend the simultaneous use of fish oil and antioxidants to improve reproductive performance in sheep.

CONCLUSION

The use of fish oil along with vitamin E and L-carnitine improved follicle function and increased pregnancy rate by reducing ROS in ewes' ovaries as well as increasing the expression of *SOD1*, *SOD2*, *TGF-βRI*, and *PPAR-γ* genes. The use of fish oil along with antioxidants increases follicles and improves fertility in sheep.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

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