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Omega-3 fatty acids mitigate histological changes and modulate the expression of ACACA, PFK1 and ET-1 genes in broiler chickens under environmental stress: a pulmonary artery, cardiomyocyte and liver study

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ABSTRACT The aim of this study was to investigate the effects of omega-3 fatty acids on blood biochemical parameters, histological changes in pulmonary artery, cardiomyocytes, and liver, as well as the expression of ACACA, PFK1, and ET-1 genes in broiler chickens under environmental stress (high stoking density). A total of 420 one-day-old male Ross broilers were used in a 2 × 2 factorial arrangements, with 2 levels of environmental stress (without and with stress; 9 and 17 birds/m^2, respectively) and 2 levels of omega-3 fatty acids (low and high; 0.057 and 0.5% of the diet, respectively) in a completely randomized design comprising 4 treatments and 5 replicates per each. The body weight decreased at d 40 because of environmental stress ($P \leq 0.05$). The ascites heart index (**AHI**) in broilers fed high omega-3 fatty acids diets was lower ($P = 0.062$) than broiler fed low omega-3 fatty acids diet (0.279 vs. 0.316). Stressed birds showed a higher neutrophil: lymphocyte ratio compared to non-stressed birds ($P \leq 0.05$). Broiler chickens receiving high omega-3 fatty acids diets exhibited elevated levels of hematocrit (**HCT**), hemoglobin (**HGB**), and lymphocytes ($P \leq 0.05$). The neutrophil: lymphocyte ratio, and serum concentration of alanine aminotransferase (**ALT**), and aspartate aminotransferase (**AST**) decreased in broilers fed high omega-3 fatty acids diets ($P \leq 0.05$). In stressed broilers on a high omega-3 diet, pulmonary artery wall thickness decreased (P ≤ 0.05). Additionally, under stress, myocardial cell diameter, hepatocyte and cell nucleus diameter significantly increased ($P \leq 0.05$). Stressed broilers showed an increased relative fold change in PFK1 enzyme activity but reduced ET-1 mRNA expression in the liver compared to stressed birds on a high omega-3 diet ($P \leq 0.05$). In conclusion, the results indicate that dietary omega-3 fatty acids have the potential to alleviate the adverse histological changes in the pulmonary artery, cardiomyocytes, and liver, while also modulating the expression of genes ACACA, PFK1, and ET-1 that are influenced by environmental stress in broiler chickens.

Key words: pulmonary artery, cardiomyocytes, liver, mRNA, stress, omega-3 fatty acid

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INTRODUCTION

Broiler breeders tend to produce more meat to increase income, which leads them to raise birds at high densities due to the short rearing period and the associated economic benefits. However, when broiler chickens are raised in high stocking density, they can be affected by various environmental factors such as air quality, competition for food, fear, diseases, and increased mortality, leading to stress (Delezie et al., 2007; Vallarino et al., 2006). Animals release glucocorticoid hormones through the hormonal cascade from the hypothalamus-pituitary-adrenal (**HPA**) axis when they encounter environmental stressors like high stocking density. The HPA axis plays an important role for maintaining the body's homeostasis in response to various stressors (Sapolsky et al., 2000; Romero et al., 2009). The increase in HPA activity due to stressful factors results in higher secretion of corticosterone. This leads to physiological and metabolic reactions such as increased blood glucose, decreased appetite, altered carbohydrate metabolism, reduced growth, heightened lipolysis enzyme activity, increased fat peroxidation reactions, heightened susceptibility to cardiovascular diseases, etc (Ali et al., 2008). Environmental stress in broiler chickens can lead to reduced growth rates and an

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imbalance between body weight and the relative weights of essential organs, such as the heart and liver, which are vital for overall growth. On the other hand, Broilers are more sensitive to stressors due to their genetic selection, which can cause disturbances in the growth and development of heart tissue. Various stressors, their interactions and the imbalance in weight between the body and the heart can cause pulmonary arterial hypertension (**PAH**) or ascites syndrome (**AS**) resulting in right ventricular hypertrophy, valvular inefficiency, and right ventricular failure. Breeding centers are currently researching the development of ascites-resistant strains; however, selecting against ascites is challenging due to the correlation between production traits and this disease.

Omega-3 fatty acids have been reported to play a role in protecting the heart. They participate in building cell membrane phospholipids, altering membrane properties and processes, and influencing the metabolism of eicosanoids (Nair et al., 1997). Several factors likely contribute to this, collectively resulting in increased antiarrhythmic changes, decreased development of antiatherogenic effects, and reduced reaction or accumulation of antithrombotic platelets (Mach et al., 2019). Research on the relationship between fish oil and cardiovascular disease in both animal and human models shows that this effect can be attributed to substrate competition between omega-3 fatty acids and arachidonic acid (**AA**, 20:4n-6) for cyclooxygenase enzymes that produce prostaglandins and thromboxanes (Dinicolantonio et al., 2014). Competition between omega-3 and AA fatty acids can have a beneficial impact on human and animal health. Additionally, omega-3 fatty acids have the potential to possess anti-arrhythmic properties for the heart (Hu et al., 2019). The mechanism of action involves the antiarrhythmic effect of Eicosapentaenoic acid (**EPA**) and Docosahexaenoic acid (**DHA**) by inhibiting ventricular fibrillation. This is achieved through the modulation of activity of specific ion channels in the myocardial sarcolemma (plasma membrane in muscle fibers; Mach et al., 2019).The liver in broilers is a sensitive tissue that can be affected by stress. During times of stress, glucocorticoids regulate fat metabolism in fat cells, the liver, and other tissues (Roldan and Herzig, 2015). The liver plays a crucial role in lipid metabolism in poultry, including synthesis, breakdown, and transport in poultry. Genome analysis of glucocorticoid-regulated gene networks shows that glucocorticoid receptors regulate various aspects of liver energy metabolism (Roldan and Herzig, 2015). More than 50 genes seem to be directly influenced by the regulatory actions of glucocorticoids. In some cases, glucocorticoid receptors also interact with other transcription factors to regulate liver gene networks, although only a few of which are currently known (Le et al., 2005). Additionally, glucocorticoids stimulate metabolic changes such as gluconeogenesis, alterations in lipid composition, and liver steatosis (Caro et al., 2007). Omega-3 fatty acids reduce triglyceride accumulation in the liver by increasing lipid oxidation and reducing the regulation of triglyceride biosynthesis due to their anti-stress effects (Clarke, 2001).

One of the negative effects of stress in broilers can be hypoxia. Hypoxia is considered the primary factor contributing to the development of PAH (Wang et al., 2008). Under these circumstances, glycolysis occurs preferentially to maintain energy in the bodies of broilers. One of the key enzymes involved in energy metabolic pathways is, glycolytic pathway: phosphofructokinase 1 (**PFK1**); fatty acid oxidation pathway: Acetyl- CoA carboxylase alpha (**ACACA**) in myocardial and liver tissues (Weljie and Jirik, 2011). In addition, Endothelia receptors: Endothelin-1 (**ET-1**) being a 21-amino acid peptide with vasoconstrictive activity. A substantial amount of evidence suggests that ET-1 plays a crucial role in endothelial dysfunction in a variety of cardiovascular diseases, including coronary artery disease, peripheral artery disease, stroke, and hypertension (Feldstein and Romero, 2007; Iglarz and Clozel, 2007; Abraham and Dashwood, 2008). Endothelial dysfunction appears to precede the clinical manifestations of many cardiovascular disorders (Schachinger et al., 2000; Abraham and Dashwood, 2008). Accumulating evidence also suggests that the endothelin (**ET**) system plays a significant role in the pathophysiology of congestive heart failure (**CHF**) and PAH (Shah, 2007).

It is believed that omega-3 fatty acids may be effective in alleviating the environmental stress faced by broilers. We hypothesized that supplementing broiler chickens' diets with omega-3 fatty acids may enhance cardiac energy metabolism and liver function under environmental stress. We investigated cardiac energy metabolism in clinical cases under stress, focusing on suspected AS, from the standpoint of energy metabolism and endothelin receptor. Accordingly, the aim of this study was to investigate the effect of dietary omega-3 fatty acids on cardiac and liver histology and mRNA expression of ACACA, PFK1 and ET-1 in the right ventricle and liver of broilers under environmental conditions to examine stress.

MATERIALS AND METHODS

Birds, diets and broiler chickens' management

Four hundred and twenty one-day-old male Ross 308 commercial broiler chickens were used in a 2 × 2 factorial arrangement, with 2 levels of environmental stress (without stress= low stocking density, 9 birds/ m^2; and with stress = high stocking density, 17 birds/ m^2) and 2 levels of omega-3 fatty acids (low and high; 0.057 and 0.5% of the diet, respectively) in a completely randomized design comprising 4 treatments, 5 replicates and 16 and 26 chicks per replicate (low and high stocking density, respectively). All birds in the normal and under environmental stress groups were placed in a room with a temperature of 20 to 25°C and provided ad libitum access to water and a standard diet [Starter: 2,870 apparent metabolizable energy (**AMEn**)/kg of diet, 220 g/kg crude protein (**CP**), Grower: 2,950 kcal/ kg of diet, 200.5 g/kg CP, Finisher: 3,025 kcal/kg of diet,

180 g/kg CP] formulated to meet the requirements for broilers (Table 2; Aviagen, 2019). Persia fat (Kimiya Danesh Alvand Co., Tehran, Iran) was used as the source of omega-3 fatty acids for the diet of broiler chickens. This product was made using fish oil. Persia fat had a crude fat content of 85% and contained approximately 19% total omega-3 fatty acids. Throughout the study, mortality rates were recorded. All experimental procedures used in the study were approved by the Animal Welfare Committee of the Department of Animal Science, University of Tehran, Tehran, Iran.

Blood and Serum Biochemical Parameters

Blood samples were taken from the wing vein of each replicate bird at 37 d of age. Routine blood tests were assessed for HCT, HGB, neutrophils, lymphocytes, and the neutrophil-to-lymphocyte ratio (**N/L**) using the Assay Kits (ZiestChem Diagnostics, Tehran, Iran). Serum biochemical indexes, including alanine aminotransferase (**ALT**) and aspartate aminotransferase (**AST**), were examined spectrophotometrically using the Assay Kits (Biorexfars BXC0215 and BXC0205, respectively, Shiraz, Iran) according to the method described by Richmond (1973).

Sample Collection and Tissue Preparation

At the end of the 40-day experimental period, male chicks from each group were randomly selected, weighed, and euthanized. The heart, pulmonary artery, and liver were removed. After removing the atrium and aorta, the atrioventricular valve plane was measured, followed by the weighing of the total ventricles (**TV**) (Wideman, 2001). To measure ascites heart index (**AHI**), the right ventricular (**RV**) wall was then dissected free of the left ventricle (**LV**) and septum. The RV was weighed, and the RV/TV ratio was calculated. Pulmonary arterial hypertension syndrome is defined as having a RV/TV ratio greater than 0.28 (Liu et al., 2017). The right ventricle and liver tissue were immediately frozen in liquid nitrogen and stored at -80°C for subsequent RNA analysis.

To evaluate the morphological changes in the broiler`s heart, pulmonary artery, and liver, samples were immediately stored in 10% formaldehyde and transferred to 4% paraformaldehyde for more than 24 h to ensure thorough fixation of the tissue structure. Then, tissue samples were placed in an ascending gradient of ethanol (70−99.5%) for dehydration. They were made transparent by dipping in xylene three times (4 min, 2 min, and 30 s). Then, they were placed into two beakers filled with paraffin for 1 hour. Finally, they were routinely sectioned and stained with hematoxylin-eosin (Luna, 1968). An ocular micrometer was used to measure the diameter of the cells and the hepatocyte nucleus diameter. The photomicrographs were captured using a micro camera attached to an Olympus BX-51 microscope, and the images were digitized using Zeiss 4.3 software, KS 400.3.

RNA Extraction of Right Ventricle and Liver Tissue and Real-Time PCR

To extract total RNA, 100 mg of right ventricle and liver tissues were homogenized in liquid nitrogen under aseptic conditions and then homogenized in 1 mL of TRIzol reagent (Kiazol, Iran). After adding chloroform, the mixture was centrifuged at 12,000 x g and 4°C for 15 min. The upper aqueous phase of the supernatant was separated, and the total RNA was precipitated with 100% isopropanol, then centrifuged at 12,000 x for 15 min). After discarding the upper liquid, the visible white pellet containing RNA was washed with 75% ethanol. Upon centrifugation at 7,500 x g for 5 min, the pellet was resuspended in diethylpyrocarbonate (**DEPC**)-treated water. To eliminate genomic DNA contamination, the extracted RNA was treated with RNase-free DNaseI and its buffer (Sinaclon Bioscience, Karaj, Iran). The Concentration and purity of RNA samples were assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). To determine the purity of the sample, its absorbance ratio (A260/280) was measured using spectrophotometry. Samples with a ratio between 1.8 and 2.2 were considered to have good quality RNA for cDNA synthesis (Hassanpour et al., 2015). Using a 1.5% agarose gel for electrophoresis, discrete 18S and 28S rRNA bands were considered as a qualification index for the extracted RNA. cDNA was synthesized by performing a cDNA synthesis kit (A101161, Parstous, Tehran, Iran), following the manufacturer's instructions.

Shortly after extraction, total RNA (2 μg) was reversely transcribed into cDNA (about 4 h) in a 20 μL reaction volume using the Parstous Kit, offered by the manufacturer (A10116, Parstous, Tehran, Iran). The thermal program for cDNA synthesis included the following 3 steps: 37°C for 15 min (reverse transcription), 85°C for 8 s (inactivation of the reverse transcriptase), and 4°C (cooling). The synthesized cDNA was then stored at -20°C (Ahmadipour et al., 2015).

Real-time PCR was used to determine the RNA transcription level of a candidate reference gene in the hearts and livers of chickens under stress with/ without omega-3 fatty acids. GAPDH was used as a reference gene. Gene expression was assessed using quantitative real-time polymerase chain reaction (**RT-qPCR**) with the Step-One RT-PCR system (AB Applied Biosystems, Foster City, CA) and primers designed for ACACA, PFK1, and ET-1 (the primers listed in Table 1). Quantification of real-time PCR was performed using the SYBR Premix Ex Taq II kit (TaKaRa Biotechnology, Dalian, Liaoning, P.R. China).

Specific primers for these genes were designed using Oligo 2 software (https://oligo-analyzer.software.informer.com/). The expected PCR products of the primers were tested in Nucleotide-Blast which

Table 1. Primers used for quantitative RT- PCR analysis of broiler mRNAs.

Target	Sequence (5′ → 3′)	Product length (bp)	Accession no
ACACA	F: CACCTTCGGCTGGGAAGCTTA	21	NM-205505.2
	R: AAGCATTGGCCTGCAAACACG	21	
PFK1	F: CCAGCCAGGTCTTTTATGGGCTT	23	NM-204223.2
	R: ATGCCCTAGAGACTCGACCCTT	22	
ET-1	F: ATACCAAAGGGCCATTCAGCAGA	23	XM-418943.6
	R: TCCAGTGCTGTTTTGGCGGTGT	22	
GAPDH	F: ATCACAGCCACACAGAAGACGG	22	NM-204305.2
	R: ACTTTCCCCACAGCCTTAGCAG	22	

revealed no similarity with other chicken (Gallus gallus)genes(www.blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Complementary DNA (2 μL) was added to 3.5 μL of SYBR Premix Ex Taq II Mix and 1 μL of each specific reverse and forward primer, resulting in a total volume of 10 μL. All reactions were performed in triplicate for each sample. The PCR program consisted of 40 cycles: 95°C for 10 min, 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Fluorescence was measured at the end of each phase for quantification. Possible contamination of PCR buffers and reagents was monitored using the no-template (water sample) and no-reverse-transcriptase controls.

The average cycle threshold (**Ct**) values were determined for each sample, and $2^{-\Delta\Delta CT}$ values were used to compare the target gene and reference gene (Livak and Schmittgen, 2001). The GAPDH gene was used as a housekeeping gene to normalize the data.

Data Analysis

The data analysis of carcass characteristics, mortality, blood biochemical parameters, and morphological changes were performed using the 2-way ANOVA procedure with the GLM procedure of SAS software version 9.4 (SAS Institute Inc, 2013). The least square means were estimated using the LSMEANS procedure and then separated using the PDIFF option. Tukey's multiple range comparison test was employed to assess significant differences among treatment means at a significance level of $\alpha = 5\%$.

Gene expression is presented as the relative fold change for each gene, calculated as the ratio between each dietary omega-3 fatty acids treatment under stress and the stressed group. The Fold change indicates whether a gene is up-regulated or down-regulated. Statistical analyses were conducted using an unpaired two-tailed Student's t-test, and graphical representations of the data were created using GraphPad Prism 9.1.0. (GraphPad Software, San Diego, CA). Data are presented as the mean ± SEM. Statements of statistical significance were based on $P \leq 0.05$.

Table 2. Ingredient composition and nutrient content of the basal diets.

	1−14 d		15−24 d		25−40 d	
	Low omeg-3	High omeg-3	Low omeg-3	High omeg-3	Low omeg-3	High omeg-3
Ingredient (% diet)						
Corn, Grain	59.15	57.22	60.94	61.66	68.35	69.09
Soybean meal- 46	34.84	35.23	31.90	31.76	25.06	24.91
Persia fat	0	2.30	0	2.34	0	2.36
Vegetable Oil	1.45	0	2.75	0.45	2.65	0.33
Dicalcium phosphate	1.96	2.92	2.11	2.11	1.60	1.60
Limestone	1.15	0.89	0.92	0.30	0.97	0.34
Common Salt	0.20	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.32	0.32	0.30	0.30	0.30	0.30
L-Lysine HCl	0.33	0.32	0.28	0.28	0.27	0.27
Vitamin premix[1]	0.30	0.30	0.30	0.30	0.30	0.30
Mineral premix[2]	0.30	0.30	0.30	0.30	0.30	0.30
Total	100	100	100	100	100	100
Calculated nutrients						
AMEn (kcal/kg)	2,870	2,870	2,950	2,950	3,025	3,025
Crude protein (%)	22.0	22.0	20.50	20.50	18.0	18.0
Crude fiber (%)	3.13	3.10	3.10	3.11	2.99	3.00
Calcium (%)	0.96	1.30	0.90	0.90	0.79	0.79
Available phosphorus (%)	0.48	0.65	0.50	0.50	0.39	0.39
Lysine (%)	1.18	1.18	1.10	1.10	0.93	0.93
Methionine (%)	0.63	0.63	0.58	0.58	0.55	0.55
Methionine + cysteine (%)	0.87	0.87	0.74	0.81	0.74	0.74
Measured nutrients						
Crude protein (%)	22.08	22.18	20.22	20.41	18.05	18.35
Crude fat (%)	2.49	4.39	2.48	4.49	2.60	4.63
Total omega-3 fatty acid (%)	0.057	0.500	0.061	0.500	0.057	0.500

[1]Provided the following per kilogram of diets: vitamin A, 9,000 IU (retinyl acetate); cholecalciferol, 2,000 IU; vitamin E, 36 IU (dl-α-tocopheryl acetate); vitamin B_{12}, 0.015 mg; menadione, 2 mg; riboflavin, 6.6 mg; thiamine, 1.8 mg; pantothenic calcium, 10 mg; niacin, 30 mg; folic acid, 1 mg; biotin, 0.1 mg; pyridoxine, 3 mg.

[2]Provided the following per kilogram of diets: manganese ($MnSO_4 \cdot H_2O$), 100 mg; zinc (ZnO), 85 mg; iron ($FeSO_4 \cdot 7H_2O$), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 10 mg; selenium (Na_2SeO_3), 0.2 mg; iodine (Iodized NaCl), 1 mg; choline, 250 mg.AMEn: apparent metabolisable energy corrected for nitrogen. Low and high omega-3 fatty acids diets contain 0.057 and 0.5% of the diet, omega-3 fatty acids respectively

Table 3. Effect of environmental stress and omega-3 fatty acids on live body weight, relative weight of liver and heart (% of LBW) and cardiac indices in broiler chickens on d 40.

Item	BW 40d (g)	Liver	Total heart	TV (g)	RV (g)	RV/TV
Environmental Stress						
Without	2197[a]	2.19	0.59	0.37	0.10	0.29
With	2032[b]	2.28	0.54	0.37	0.11	0.30
SEM	34.9	0.115	0.025	0.017	0.007	0.012
Omega-3 fatty acids (%)						
low	2140	2.10	0.52[b]	0.35	0.11	0.31
high	2089	2.37	0.61[a]	0.38	0.10	0.27
SEM	34.8	0.115	0.025	0.017	0.007	0.012
Environmental Stress× Omega-3						
Without low	2102	2.11	0.53	0.34	0.10	0.30
Without high	1961	2.27	0.65	0.40	0.11	0.27
With low	2177	2.09	0.51	0.37	0.12	0.33
With high	2216	2.47	0.57	0.37	0.10	0.28
SEM	49.3	0.162	0.036	0.024	0.011	0.017
Environmental stress	0.004	0.592	0.157	0.939	0.643	0.391
Omega-3 fatty acids	0.317	0.119	0.029	0.221	0.723	0.062
Stress× Omega-3	0.087	0.507	0.401	0.228	0.229	0.552

[a–b]Means with different superscripts within a column are different at $P < 0.05$.SEM = standard error of the mean.TV= total ventriculus weight.RV= right ventriculus weight.SEM = standard error of the mean.Low and high: low and high omega-3 fatty acids diets contain 0.057 and 0.5% of the diet, omega-3 fatty acids respectively.Environmental Stress: without stress= low stocking density, 9 birds/ m^2; and with stress = high low stocking density, 17 birds/ m^2.

RESULTS

Investigation of Associated Indexes of Autopsy and Mortality

Chickens experiencing environmental stress exhibited a decrease in body weight over a 40-day period ($P \leq 0.05$, Table 3). The total heart/body weight ratio in broilers fed high omega-3 fatty acids diets (0.5%) was significantly greater than in broilers fed low omega-3 fatty acids diets ($P \leq 0.05$). The AHI ratio was also lower ($P = 0.062$) in the broiler chickens fed high omega-3 fatty acids diets compared to those receiving low omega-3 fatty acid diets (0.279 vs. 0.316). This is an index for the determination of right ventricular hypertrophy and PAH (Wideman, 2001). Nevertheless, there were no statistically significant differences between the groups regarding relative weight of liver and TV, and RV/body weight ratio. The mortality of broiler chickens during the 40-day experimental period was positively influenced by the dietary omega-3 fatty acids ($P \leq 0.05$; Fig. 1).

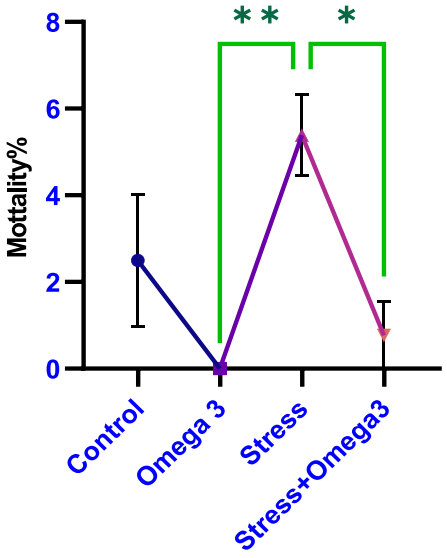

Fig. 1. Mortality of control, omega-3 (without environmental stress + high omega-3 fatty acid diets), stress (with environmental stress × low omega-3 fatty acid diets) and stress + omega-3 (with environmental stress + high omega-3 fatty acid diets) of broiler chickens on 1 to 40 d. The omega-3 and stress + high omega-3 fatty acid diets showed the lowest mortality rates, which expressed the positive impact of omega-3 on reducing broiler mortality. *$p \leq .05$; **$p < .01$.

Blood and Serum Parameters

The effects of environmental stress and dietary supplementation of omega-3 fatty acids on blood parameters and serum ALT and AST enzyme levels are presented in Table 4. There was no interaction effect of environmental stress × omega-3 fatty acids on blood traits and serum AST and ALT. Broiler chickens under environmental stress exhibited a significant increase ($P \leq 0.05$) in neutrophils and the neutrophil: lymphocyte ratio compared to the non-stressed birds. Additionally, stressed broilers displayed significantly lower lymphocyte values than their non-stressed counterparts ($P \leq 0.05$).

The HCT, HGB, and lymphocyte values were increased in the birds fed high omega-3 fatty acids diets ($P \leq 0.05$). Additionally, neutrophils, the neutrophil-to-lymphocyte ratio, as well as the levels of ALT and AST significantly decreased in the serum of broilers fed high omega-3 fatty acid diets ($P \leq 0.05$).

Histomorphology Changes in the Pulmonary Artery, Myocardial, and Hepatocyte

The effects of environmental stress and omega-3 fatty acids on the histomorphology of the pulmonary artery, myocardial and hepatocytes are presented in Table 5. The interaction between stocking density and omega-3

Table 4. Effect of environmental stress and omega-3 fatty acids on blood parameters and sera ALT and AST enzymes of broiler chickens on d 37.

Item	HCT (%)	HGB (g/dl)	Neutrophils (%)	Lymphocytes (%)	N/L	ALT (U/L)	AST (U/L)
Environmental stress							
Without	30.50	10.16	29.87	67.37[a]	0.44[b]	5.72	209.7
With	31.50	10.50	34.25[a]	62.87[b]	0.54[a]	6.05	202.6
SEM	0.540	0.179	0.667	0.739	0.014	0.510	3.638
Omega-3 fatty acids (%)							
Low	29.50[b]	9.83[b]	33.25[a]	63.25[b]	0.53[a]	6.94[a]	224.1[a]
High	32.50[a]	10.83[a]	30.87[b]	67.00[a]	0.46[b]	4.82[b]	188.2[b]
SEM	0.540	0.179	0.667	0.739	0.014	0.510	3.64
Environmental Stress × Omega-3							
Without low	29.00	9.66	30.50	66.00	0.46	6.49	227.7
Without high	32.00	10.66	29.25	68.75	0.42	4.95	191.7
With low	30.00	10.00	36.00	60.50	0.60	7.40	220.5
With high	33.00	11.00	32.50	65.25	0.49	4.70	184.7
SEM	0.763	0.254	0.943	1.045	0.020	0.721	5.15
				Probability			
Environmental stress	0.214	0.214	0.0006	0.001	0.0003	0.656	0.191
Omega-3 fatty ssacids	0.002	0.002	0.027	0.003	0.007	0.012	<.0001
Stress× Omega-3	1.000	0.996	0.256	0.357	0.121	0.438	0.981

[a-b]Means with different superscripts within a column are different at $P < 0.05$SEM = standard error of the means.Low and high: low and high omega-3 fatty acids diets contain 0.057 and 0.5% of the diet, omega-3 fatty acids respectively.Environmental Stress: without stress= low stocking density, 9 birds/ m^2; and with stress = high stocking density, 17 birds/ m^2. N/L: neutrophils to lymphocytes ratio.

fatty acids significantly affected the thickness of the pulmonary artery and diameter of hepatocytes, and hepatocyte nucleus ($P < 0.05$). Stressed broiler chickens fed high omega-3 fatty acid diets showed decreased thickness in the pulmonary artery ($P \leq 0.05$) compared to non-stressed birds fed low omega-3 fatty acid diets (Fig. 2D, pulmonary artery). The diameter of the hepatocyte and cell nucleus were highest in stressed broilers fed low omega-3 fatty acid diet ($P \leq 0.05$). These parameters decreased significantly when stressed broilers were fed high omega-3 fatty acid diets, leading to no significant difference compared to the unstressed birds ($P \leq 0.05$). During microscopic examination, an accumulation of inflammatory cells, bile duct hyperplasia, and generally mild liver damage were observed in the stressed broiler chickens receive low omega-3 fatty acid diets (Fig. 2C, liver).

The diameter of myocardial cells was notably increased in broilers under environmental stress ($P \leq 0.05$). Additionally, myocardial cells with granular degeneration, vacuole degeneration, fiber rupture, and increased capillary expansion were observed in broiler chickens subjected to environmental stress (Fig. 2C, cardiomyocyte).

Table 5. Effect of environmental stress and omega-3 fatty acids on histomorphology of pulmonary artery, heart and liver in broiler chickens.

			Liver	
Item	Pulmonary artery wall thickness (μm)	Heart Cell diameter (μm)	Hepatocyte diameter (μm)	Hepatocyte nucleus diameter (μm)
Environmental stress				
Without	649[b]	6.15[b]	7.95[b]	4.26[b]
With	703[a]	7.91[a]	12.43[a]	5.26[a]
SEM	12.8	0.459	0.735	0.222
Omega-3 fatty acids (%)				
Low	702[a]	6.69	8.58[b]	5.45[a]
High	650[b]	7.38	11.80[a]	4.07[b]
SEM	12.8	0.459	0.735	0.222
Environmental stress × Omega-3				
Without low	714[a]	7.24	8.22[b]	3.64[b]
Without high	693[a]	8.59	8.94[b]	4.51[b]
With low	691[a]	6.14	15.91[a]	6.89[a]
With high	607[b]	6.16	7.69[b]	4.02[b]
SEM	18.1	0.649	1.039	0.314
			Probability	
Environmental Stress	0.011	0.015	0.0005	0.0058
Omega-3 fatty acids	0.014	0.304	0.007	0.0005
Stress× Omega-3	0.007	0.321	0.002	<.0001

[a−b]Means with different superscripts within a column are different at $P < 0.05$.SEM = standard error of the means.Low and high: low and high omega-3 fatty acids diets contain 0.057 and 0.5% of the diet, omega-3 fatty acids respectively.Environmental Stress: without stress= low stocking density, 9 birds/ m^2; and with stress = high stocking density, 17 birds/ m^2.

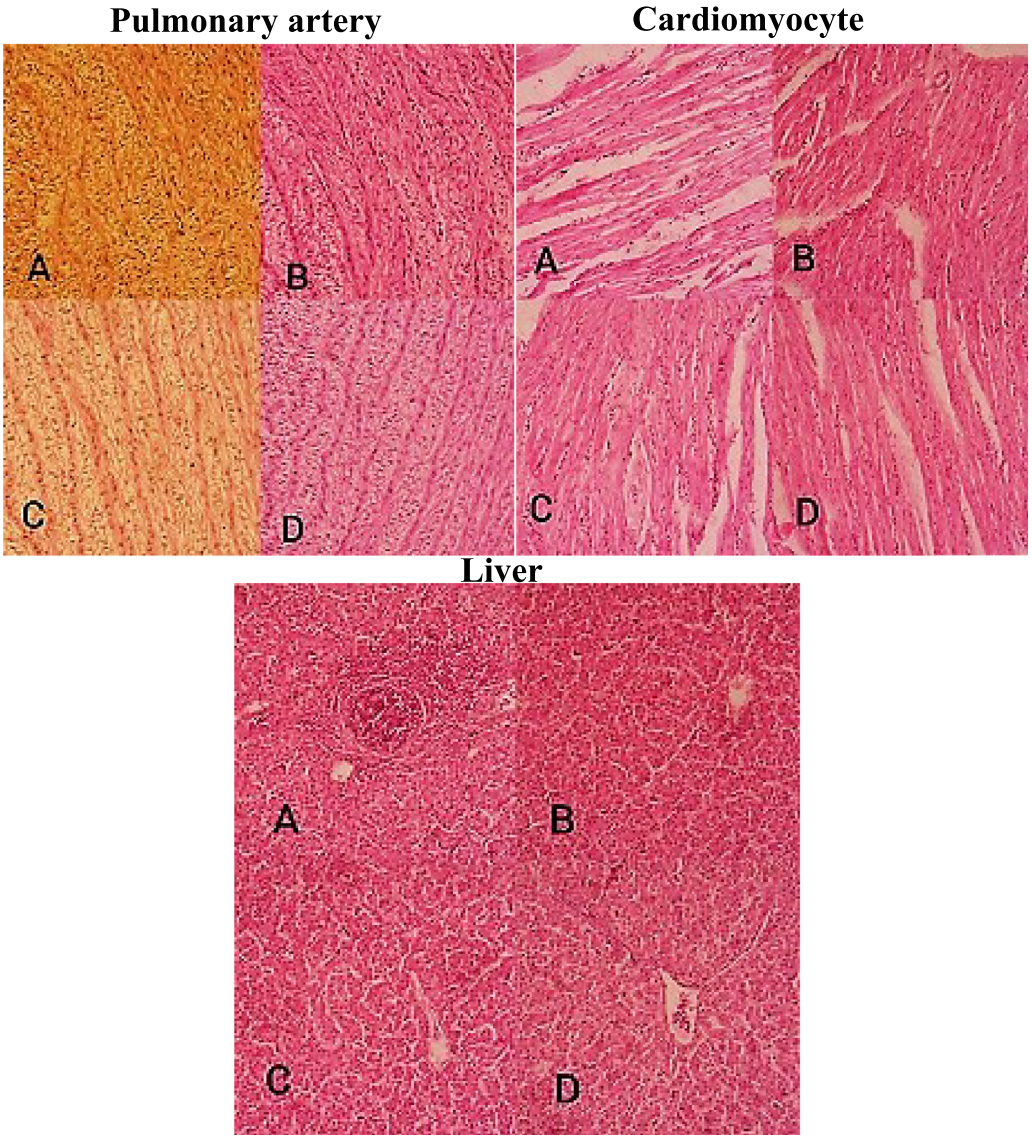

Fig. 2. Viewing of pulmonary artery, cardiomyocyte and liver sections of broilers (HXE, 100X): (A) Without environmental stress + low omega-3 fatty acid diets. (B) Without environmental stress + high omega-3 fatty acid diets. (C) With environmental stress + low omega-3 fatty acid diets. (D) with environmental stress + high omega-3 fatty acid diets. The wall thickness of (D), pulmonary artery is markedly decreased. In the (D) sample, the smooth muscle fiber got thinner. The cardiomyocyte of (C), the structure of myofibrils was disordered, granular degeneration and vacuole degeneration. In the liver of (C), accumulation of inflammatory cells and bile duct hyperplasia are observed.

Gene Expressions of ACACA, PFK1 and ET-1 in Broilers

Fig. 3 shows the expressions of the ACACA, PFK1, and ET-1 genes in the right ventricle and liver tissues of broilers exposed to environmental stress and received either a low or high omega-3 fatty acids diets.

The observed relative fold change indicated a numerical increase ($P = 0.084$) in the activity of the key enzyme PFK1 in the glycolytic pathway of the right ventricle of stressed broilers compared to those birds fed high omega-3 fatty acid diets (Fig. 3 PFK1, cardiomyocyte). This study found that stress in broiler chickens led to an increased energy supply in cardiomyocyte glycolysis. The expression levels of ACACA and ET-1 genes in right ventricle did not differ substantially between the stressed broilers compared to those birds fed high omega-3 fatty acid diets.

The ET-1 gene was expressed in the liver tissue (Fig. 3, liver) of both groups. The relative fold change in ET-1 mRNA expression was significantly lower in the broiler chickens subjected to stress compared to those fed high omega-3 fatty acids diets ($P \leq 0.05$). Birds fed high omega-3 fatty acids diets had significantly higher levels of ET-1 mRNA in their liver tissue ($P < 0.05$), with a 3.73-fold change increase compared to 1.27 in control tissue (Fig. 3 ET-1, liver). The relative expression of PFK1 mRNA, did not show a significant difference between broiler chickens fed high omega-3 fatty acids diets and those subjected to environmental stress. However, a significant reduction in PFK1 expression was observed ($P = 0.05$) in the liver tissue of broiler fed high omega-3 fatty acids diets (Fig. 3 PFK1, liver). There was no difference in the relative level of ACACA mRNA expression between birds subjected to

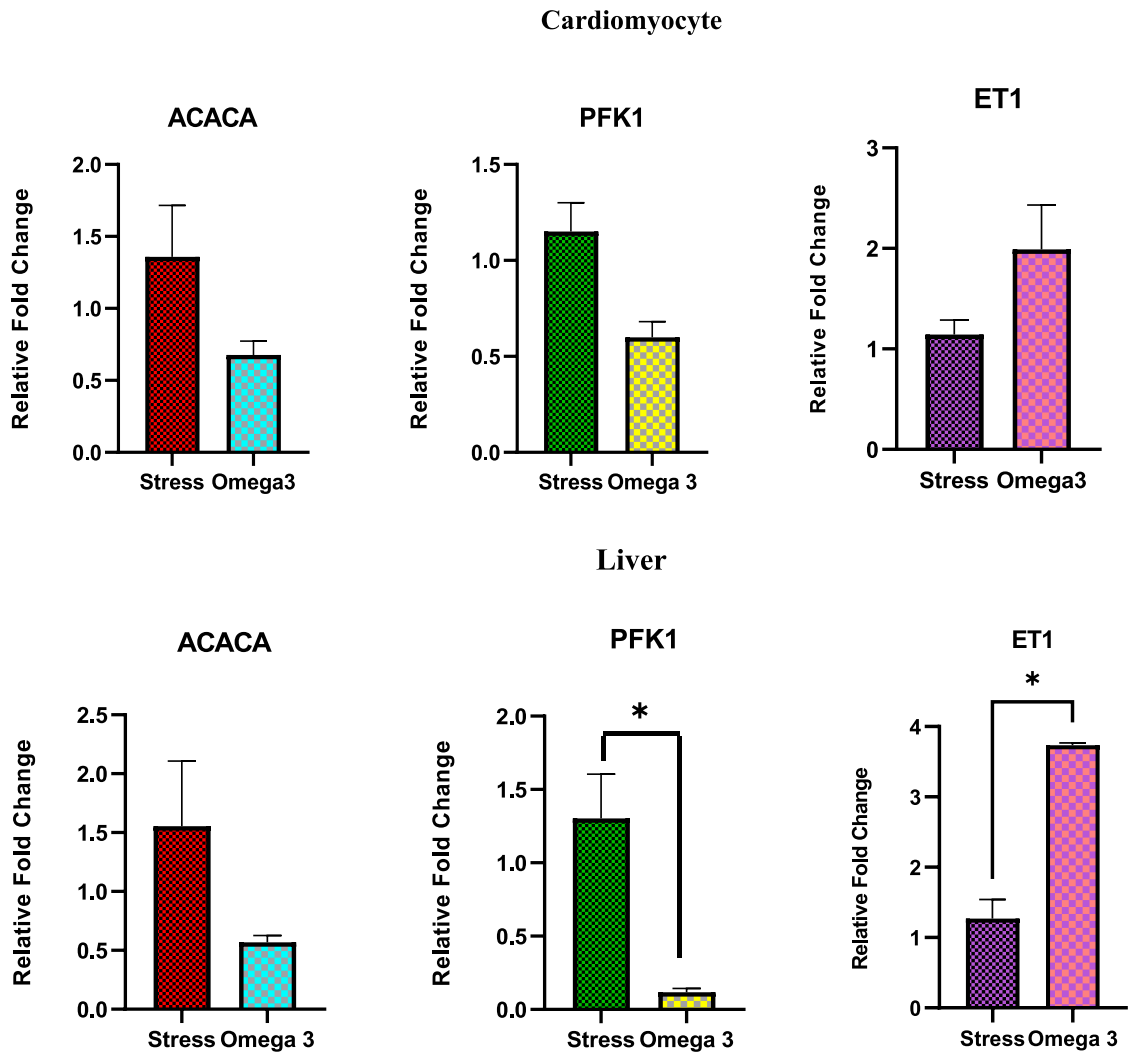

Fig. 3. Relative fold change ($2^{-\Delta\Delta CT}$) levels of ACACA, PFK1 and ET-1genes in the cardiomyocyte and liver tissue samples from environmental stressed group vs. environmental stressed group fed dietary high omega-3 fatty acids. Bars indicate the mean ± SEM of three independent experiments. *$P \leq 0.05$, as compared with the environmental stressed group (with unpaired two-tailed student's T test).

environmental stress and those fed high omega-3 fatty acids diets (Fig. 3 ACACA, liver).

DISCUSSION

The body weight of stress-treated broilers at 40 d of age was considerably lower than that of non-stressed broiler chickens in the current study. This study identifies stocking density of broilers as a source of environmental stress. Consequently, as the birds age and stocking density increases, their need for rearing space also rises. Overcrowding during rearing modifies the behavior of broiler chickens, adversely affecting their health and growth performance (Zuowei et al., 2011). When stocking density increases, broilers exhibit reduced movement and cover less distance, leading to decreased mobility (Estevez, 2007). Broiler performance declines due to difficulties in feed access, resulting from limited movement in high stocking densities (Sørensen et al., 2000). Consistent with these findings, previous studies have reported a negative impact of stocking density on body weight gain. Research has demonstrated that birds maintained at higher stocking densities exhibit a substantial decrease in their growth performance (Tomhave and Seeger, 1945; Heishman et al., 1952; Martrenchar et al., 2000).

In this study, Omega-3 fatty acids were used as a supplement to mitigate the negative effects of environmental stress in broiler chickens. Feeding high omega-3 fatty acids diets to stressed broiler chickens resulted in increases in total heart weight, and improvements in AHI (< 0.28), HCT (< 0.36%) and HGB levels, which indicate heart and vascular health, as well as the absence of ascites (Liu et al., 2017). It is worth noting that hypertrophy of the right ventricular wall is directly related to increased pulmonary arterial pressure. The AHI ratio serves as a measure of the elevated pressure load on the right ventricle and has been identified as the most crucial index for determining right ventricular hypertrophy and PAH (Wideman, 2001).

Archer et al. (1989) discovered that administering fish oil to rats reduces blood viscosity and right ventricular hypertrophy. Higher concentrations of unsaturated fatty acids are believed to increase the fluidity of the erythrocyte membrane and modify its function, improving erythrocyte deformability and potentially lowering the incidence of ascites. These changes, would reduce resistance to blood flow, enhance the movement of erythrocytes through capillaries, improve oxygen transport, and reduce the incidence of ascites (Walton et al., 1999).

In the current study, stress resulting from high stocking density increased the percentage of neutrophils and decreased the percentage of lymphocytes, leading to an elevated neutrophil to lymphocyte ratio. Dietary omega-3 fatty acids reduced the neutrophil/lymphocyte ratio, which serves as an indicator of oxidative stress. This reduction in lipid oxidation in the cell membranes of heart muscle contributes to their health. In line with the findings of this study, previous research has reported an increase in the proportion of lymphocytes in broiler chickens due to seaweed supplementation (Kotrbacek et al., 1994; Yan and Kim, 2013). Seaweed has also been shown to stimulate the immune system by activating macrophages and lymphocytes, strengthening defense mechanisms (Ghasemi et al., 2010). In this study, feeding broiler chickens diets high in omega-3 fatty acids increased HCT and HGB levels in the blood. This was accompanied by a decrease in the neutrophil/lymphocyte ratio, primarily attributed to a reduction in the percentage of neutrophils. These results are consistent with previous reports on the positive effects of omega-3 fatty acids (Yan and Kim, 2013). Furthermore, our study indicated that diets containing omega-3 fatty acids were associated with a significant decrease in the levels of ALT and AST in the serum, which may reflect improved liver function. It is important to note that alanine aminotransferase is typically found in various tissues. Elevated serum ALT activity serves as a reliable marker for liver parenchymal damage (Bergmeyer et al., 1986). This enzyme is mostly active in cardiomyocytes but less active in the kidneys, skeletal muscle, pancreas, spleen, and lungs.

Apartate aminotransferase is found in most human and animal tissues; however, it is particularly active in cardiomyocytes. Aspartate aminotransferase is an enzyme that is found in both the cytoplasm and the mitochondria of cells. In instances of mild tissue damage, the release of this enzyme predominantly originates from the cytoplasmic compartment rather than the mitochondrial source. Severe tissue damage significantly enhances the release of AST from mitochondrial sources. This elevation in AST levels is indicative of various pathological conditions, including cardiomyocyte disorders such as myocardial infarction, liver diseases, muscle dystrophy, and other organ injuries (Bergmeyer et al., 1986). According to the findings of the current study, dietary omega-3 fatty acids reduced levels of ALT and AST. This reduction indicates their potential to alleviate heart and liver tissue damage, ultimately leading to a significant decrease in bird mortality.

In the current study, morphological observations highlighted pulmonary artery remodeling, characterized by thickening of the artery wall, especially in the medial intima comprised of vascular smooth muscle cells, accompanied by a denser tunica adventitia (Table 5; Fig. 2, pulmonary artery). The morphological changes observed in the pulmonary artery reveal a significant increase in artery thickness in broiler chickens subjected to environmental stress. Furthermore, the smooth muscle layers at the margins were noted to be discontinuous and irregular. Moreover, the mesenchyme between the muscle layers exhibited looser associations in broilers under environmental stress compared to unstressed birds. Previous morphological studies have also reported similar characteristics of pulmonary artery remodeling in broilers with PAH. This remodeling was attributed to hypertrophy, proliferation, and restricted migration of various cell types, such as endothelial cells, smooth muscle cells, fibroblasts, inflammatory cells, and platelets (Deng et al., 2010; Qiao et al., 2012). Pulmonary vascular remodeling is a key morphological factor contributing to the development of susceptibility to AS. It leads to an increase in pulmonary vascular resistance and prolonged elevated arterial hypertension (Nain et al., 2009). Among vascular cells, the proliferation and hypertrophy of vascular smooth muscle cells are considered key contributors to pulmonary vascular remodeling. These processes play a significant role in the progression of AS (Wang et al., 2007; Yang et al., 2013).

In response to stress, cardiomyocyte hypertrophy serves as a compensatory mechanism that may evolve into a detrimental morphological characteristic over time, ultimately leading to structural and functional failure of the cardiomyocyte (Shi et al., 2014). Edema is the most prominent anatomical change in the heart of chickens with AS, while myocardiofibrillar disorders are the most common lesions (Tišljar et al., 2011). Furthermore, the heart tissues of stressed broilers showed loosely arranged myocardial fibers and blurred plasma membrane boundaries compared to non-stressed chickens. This finding is consistent with the consensus of several researchers (Naeije and Manes, 2014; Shi et al., 2014; Ahmadpanah et al., 2017; Ge et al., 2020). Studies on the thickness of the pulmonary artery revealed the impact of omega-3 fatty acids under stress conditions. Research indicates that omega-3 fatty acids benefit both animal and human health for various reasons, including their ability to inhibit the production of arachidonic acid by competing for the enzyme Delta-6 desaturase. This enzyme is crucial for the synthesis of long-chain unsaturated fatty acids. Long-chain omega-3 fatty acids compete with AA in the cell membrane, reducing their levels and thus allowing them to enter the sn-2 position of triacylglycerol phospholipids. For example, when the ratio of EPA to AA in cell membranes is altered, this results in a shift in eicosanoid production from prostaglandin i2 (13-PGI2) and thromboxane A2 (TXA2) to anti-accumulation eicosanoids such as thromboxane A3 (TXA3). in platelets and prostaglandin PGI3 in endothelial cells (Manson et al., 2019). These activities lead

to reduced platelet accumulation, resulting in antithrombotic (anticoagulant) and anti-inflammatory effects (Innes and Calder, 2020). On the other hand, omega-3 fatty acids may have the ability to prevent cardiac arrhythmias (Hu et al., 2019).This is due to the antiarrhythmic effect of EPA and DHA, which inhibits ventricular fibrillation. Omega-3 fatty acids can modulate the activity of specific ion channels in the myocardial sarcolemma (Mach et al., 2019).

The above-mentioned physiological changes increased the sensitivity of broilers to PAH, resulting in higher mortality rates in broilers under stress like what was observed in this experiment (Fig. 1). Mortality in broiler chickens represents a significant economic loss to producers, especially when the birds are approaching slaughter age, as birds gain weight before reaching sexual maturity due to SDS or PAH. The results of this study showed that omega-3 fatty acids can effectively reduce mortality rates in broilers and prevent economic losses.

Based on our findings, hepatocyte and nucleus diameters were decreased significantly when stressed broilers were fed diets high in omega-3 fatty acids, resulting in no significant difference compared to the non-stressed birds. This indicates a decrease in the functional activity of hepatocytes due to the reduction in nucleoplasm content in stressed broiler chickens. We also observed bile duct hyperplasia and mild hepatic steatosis in stressed broiler chickens. In chickens, such hyperplasia and fibroplasia of the bile ducts are classified as nonspecific lesions and are associated with systematic changes in liver metabolism after injury to the liver parenchyma (Hochleithner et al., 2005). Overall, environmental stress can lead to histological liver damage in broilers.

Several mechanisms may be involved in the improvement of liver morphological properties by omega-3 fatty acids. Omega-3 fatty acids act as regulatory messenger molecules in liver metabolism, increasing the antioxidant, anti-stress and anti-inflammatory effects in the liver via direct and indirect mechanisms (Song et al., 2003; Houstis et al., 2006; Calder, 2012).

Numerous studies have shown that mRNA expression is tissue and cell specific and can be disrupted in disease. In addition, the expression levels of many mRNA are associated with disease severity (Grant et al., 2013; Xu et al., 2016). Many mRNAs have been found to be associated with PAH or AS in broiler chickens. In rapidly growing broilers, there is increased stress on the cardiopulmonary system due to hypoxemia, resulting in right ventricular hypertrophy and a flaccid heart. This condition also leads to excessive cell proliferation, resistance to apoptosis, inflammation, fibrosis and vasoconstriction, ultimately leading to death (Riddell, 1991; Luger et al., 2003). Many researchers believe that AS is related to environmental stress (low temperature, high stocking density and oxygen deficiency), nutritional, management, and genetic factors (Julian, 2000; Olkowski et al., 2007; Baghbanzadeh and Decuypere, 2008). Pulmonary arterial hypertension or ascites syndrome in chickens can lead to relative hypoxia in the body and cause several pathophysiological disorders. These changes may include PAH, pulmonary vascular remodeling, cardiac hypertrophy and failure, and free radical production that could increase lipid peroxidation (Wideman et al., 2013).

In this study, we examined the activity of key enzymes involved in glycolysis, the fatty acid oxidation pathway, and endothelin-1 expression in the cardiomyocytes and liver tissues of stressed broilers fed diets high in omega-3 fatty acids. The data showed that the mRNA expression level of PFK1 was significantly reduced in the cardiomyocytes ($P = 0.08$) and liver of stressed chickens fed diets high in omega-3 fatty acids. The expression levels of a key enzyme in the fatty acid oxidation pathway, ACACA, were numerically increased in the cardiomyocyte and liver tissues of the stressed broilers. However, the expression of the ACACA gene was reduced by feeding feed high in omega-3 fatty acids to stressed broilers. This indicated that the energy supply in the cardiomyocyte and liver tissue of the stressed broilers was directed towards the oxidation of fatty acids. However, the occurrence of these phenomena could have occurred for the following reasons: Phosphorylation is a crucial post-translational modification in glycolysis (Tripodi et al., 2015). This process activates several metabolic enzymes that interact with carbohydrate substrates (Donthi et al., 2004), including protein kinases (**PK**) and the 2 subunits of phosphofructokinase (PFK1 and PFK2). These are key factors in glucose metabolism. Undoubtedly, glycolytic shift is involved in the pathogenesis of AS (Bonnet and Paulin. 2019). Many studies have shown that the bifunctional enzymes 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatases (PFKFB3) are upregulated in hypoxia (Minchenko et al., 2003) and that they can both synthesize and degrade PFK1. Furthermore, activated AMPK phosphorylates and inhibits ACC, ultimately leading to inhibition of malonyl-CoA synthesis. This in turn alleviated the inhibition of CPT-1 in the transport of fatty acyl-CoA to the mitochondrion and ultimately promoted fatty acid oxidation (Kudo et al., 1995; Dyck et al., 1999). Therefore, these results suggest that cardiac energy metabolism in ascitic broilers may promote fatty acid metabolism via the MIF-AMPK-ACC-CPT-1 pathway, which is consistent with the findings of Stanley et al. (1997).

Researchers suspected that the high concentration of acetyl-CoA in the myocardium of stressed broilers reduced the rate of glucose metabolism. This phenomenon is attributed to the fact that during the complete oxidative metabolism of glucose and fatty acids, acetyl-CoA, an intermediate of both pathways, finally enters the tricarboxylic acid (**TCA**) cycle for aerobic oxidation. Acetyl-CoA, an intermediate of energy metabolism in mitochondria, is produced primarily through glycolysis and fatty acid oxidation (Stacpoole, 2017). Once produced, acetyl-CoA is the main site for complete oxidation in the TCA cycle. The TCA cycle is an aerobic catabolic process. Because stressed broilers are in a relatively oxygen-deficient (anoxic) state, the rate of the TCA cycle is low, indicating inefficient use of acetyl-

CoA upon entering the TCA cycle. However, glycolysis and fatty acid oxidation, which occur before the production of acetyl-CoA, require relatively less oxygen to produce more acetyl-CoA. Although the source of acetyl-CoA remains relatively constant, its utilization in the TCA cycle is reduced, leading to accumulation of acetyl-CoA (Li et al., 2022). In addition, qRT-PCR analysis revealed a significant increase in ET-1 expression in liver tissue of broilers exposed to stress and fed diets high in omega-3 fatty acids ($P \leq 0.05$). Like other neurohumoral systems, the ET system is activated in broilers with CHF. ET appears to have different effects on normal and failing myocardium. It has a positive inotropic effect in the normal heart and a negative inotropic effect in the failing heart (Pieske et al., 1999). Plasma concentrations of ET-1 have been confirmed to be increased in broilers with heart failure regardless of the cause. There is a positive correlation between myocardial damage and ET-1 levels in progressive heart failure (Zolk and Bohm, 2000; Yazici et al., 2005). The results of measuring serum ET confirm the role of circulating ET in the pathophysiology of CHF in chickens with PAH. Zhou et al. (2008) also reported that exogenous ET accelerated the development of right ventricular hypertrophy, promoted pulmonary artery hypertension, and increased susceptibility to ascites-related mortality in stressed broilers (Hassanpour et al., 2011). In summary, the results of this study show that broiler chickens exposed to stress experience significant changes in blood biochemical parameters and histomorphological features, particularly in the pulmonary artery, cardiomyocytes and liver. Our results show that dietary supplementation with omega-3 fatty acids alleviates the negative effects of environmental stress on morphological traits in the pulmonary artery, cardiomyocytes and liver and reduces the expression of ACACA, PFK1 and ET-1 genes in broilers. This may ultimately reduce mortality due to sudden death syndrome and pulmonary arterial hypertension injuries.

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DISCLOSURES

The authors declare that they have no conflicts of interest to disclose.

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