

Journal of the Hellenic Veterinary Medical Society

Vol 77, No 1 (2026)



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doi: [10.12681/jhvms.37793](https://doi.org/10.12681/jhvms.37793)

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To cite this article:

Bahadoran, S., Khoozan-Chermahini, M., Hassanpour, H., & Farhadi, N. (2026). Melatonin modulates pulmonary hypertension syndrome through increase of nitric oxide and its enzyme in chickens. *Journal of the Hellenic Veterinary Medical Society*, 77(1), 10083–10090. <https://doi.org/10.12681/jhvms.37793>

Melatonin modulates pulmonary hypertension syndrome through increase of nitric oxide and its enzyme in chickens

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ABSTRACT: This study aimed to evaluate the effect of dietary melatonin in pulmonary hypertensive response by measuring nitric oxide and relative gene expression of endothelial and inducible nitric oxide synthases (eNOS and iNOS). A total of 144 one-day-old fast-growing chickens (Ross 308) were divided into three groups, with 48 birds per group. Each group was housed in one room and randomly split into three replicates, with 16 chickens per pen. The chicks were raised for 6 weeks under programmed cold stress. In experimental groups, cold stress was induced to study pulmonary hypertension syndrome (PHS), with melatonin supplemented at 0, 0.2, and 0.4 % diets. Post-experiment, serum total nitric oxide metabolites were measured and also heart (right ventricle) and lung tissues were analyzed for eNOS and iNOS gene expression by quantitative real-time PCR. Mortality rates were highest in the control group at 33.3%, followed by a decrement to 22.2% in the melatonin-0.2% treatment group, and further reduction to 13.9% in the melatonin-0.4% treatment group of chickens. The ratio of the right to total ventricular weight of heart as an indication of PHS was decreased in the melatonin-0.4% group of chickens at 42 days of age. A significant increase was revealed in the relative abundance of eNOS and iNOS genes within both the melatonin-0.2% and melatonin-0.4% groups of chickens compared to the control group ($P < 0.05$). However, no significant disparities in the expression levels of these genes were noted between the melatonin-0.2% and melatonin-0.4% groups ($P > 0.05$). There were no significant alterations in the relative quantities of these genes in both the melatonin-0.2% and melatonin-0.4% groups of chickens when compared to the control group ($P > 0.05$). It is concluded that melatonin may improve the cardiovascular system and decrease pulmonary hypertensive response through eNOS upregulation and the production of nitric oxide as a vasodilator.

Keyword: Ascites; Pulmonary hypertension; Melatonin; Nitric oxide

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Date of submission: 18-5-2024

Date of acceptance: 2-12-2025

INTRODUCTION

Pulmonary hypertension syndrome (PHS, ascites) in broiler chickens is a significant concern in the poultry industry, particularly in fast-growing commercial breeds. PHS, also known as ascites syndrome is a multifactorial disorder characterized by elevated pulmonary arterial pressure, and heart hypertrophy/failure. PHS in broiler chickens is a significant concern in the poultry industry that has detrimental effects on welfare, growth performance, and economic viability. Several factors including rapid growth, genetics, environmental factors (e.g., temperature, humidity, and air quality), and nutritional factors (e.g., high-energy diets) contribute to the development of PHS. The pathophysiology of pulmonary hypertension involves complex interactions between vascular, cardiac, and pulmonary factors. Chronic pulmonary vasoconstriction and vascular remodeling lead to increased pulmonary vascular resistance, right ventricular pressure overload, and subsequent hypertrophy and dilation of the right ventricle. Ultimately, right heart failure ensues, characterized by fluid accumulation in the abdominal cavity (ascites) and reduced cardiac output.

Vasoconstrictors (e.g., angiotensin II, endothelin, adrenaline, serotonin, and thromboxane A₂) (Hassanpour et al. 2016; Hassanpour et al. 2019; Hassanpour et al. 2011; Hassanzadeh et al. 2014; Chapman and Wideman Jr 2006) and vasodilators (e.g., nitric oxide, and bradykinin) play crucial roles in regulating the tone and diameter of blood vessels. Imbalances between them contribute significantly to the development and progression of the PHS (Hassanpour et al. 2015; Hassanpour et al. 2009; Teshfam et al. 2006; Hao et al. 2014). Nitric oxide (NO) is a crucial signaling molecule with diverse physiological functions, including vasodilation, neurotransmission, and immune response regulation. It is synthesized from the amino acid L-arginine through a family of enzymes called nitric oxide synthases (NOS). There are three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO is a potent vasodilator synthesized by endothelial cells (Andrabi et al. 2023). In normal conditions, NO diffuses into adjacent smooth muscle cells in blood vessel walls, activating soluble guanylate cyclase (sGC), leading to cyclic guanosine monophosphate (cGMP) production. This cGMP causes relaxation of smooth muscle cells, resulting in vasodilation and decreased vascular resistance. In PHS, there's impaired NO production or reduced bioavailability due to endothelial dysfunction, oxidative stress, or other

factors. This leads to inadequate vasodilation and increased pulmonary vascular resistance, contributing to the development of pulmonary hypertension (Tan et al. 2007). NO inhibits the proliferation and migration of vascular smooth muscle cells, which are key processes involved in vascular remodeling. NO has anti-inflammatory properties and inhibits platelet aggregation, which helps maintain vascular health. The reduced NO levels may contribute to an inflammatory state within the pulmonary vasculature, exacerbating vascular damage (Chirkov et al. 2022).

Melatonin is primarily known as the hormone that regulates the sleep-wake cycle and circadian rhythms. It is synthesized and released by the pineal gland in response to darkness. Beyond its role in circadian rhythm regulation, melatonin has antioxidant, anti-inflammatory, immunomodulatory, and neuroprotective properties (Ferlazzo et al. 2020). Melatonin has been shown to influence the activity of NOS enzymes, which catalyze the production of NO from L-arginine (Khalaf et al. 2020). This influence could be different on three NOSs and occurs through various mechanisms, including direct interaction with NOS isoforms and intracellular signaling pathways such as activation of protein kinase C (PKC), protein kinase B (Akt), regulation of the expression of transcription factors like nuclear factor-kappa B (NF- κ B) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Maity et al. 2023).

This study aimed to evaluate the effect of dietary melatonin in pulmonary hypertensive response through measurement of nitric oxide production and gene expression of nitric oxide synthases (eNOS and iNOS) in the heart and lungs of broiler chickens experimentally exposed to cold stress.

MATERIALS AND METHODS

Animals

The procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Shahrekord (approval code: IR.SKU.REC.1402.038). A total of 144 one-day-old fast-growing chickens (Ross 308) were divided into three groups, with 48 birds per group. Each group was housed in one room and randomly split into three replicates, with 16 chickens per pen. The chicks were raised for 6 weeks under a lighting schedule of 23 hours of light and 1 hour of darkness at an altitude of 2100 meters above sea level. They were kept in floor pens with wood shaving litter and had

ad libitum access to feed and water. The diet consisted of a mash form of normal basal diet formulated for the starting (1–10 days), growing (11–25 days), and finishing (26–42 days) growth stages, primarily composed of corn and soybean meal according to Faraji et al. (2020). Cold stress was induced in all groups of chickens using a temperature program outlined by Hassanpour et al. (2023) to induce PHS. In two groups of treatment, melatonin powder with purity $\geq 98\%$ (purity confirmed by Thin-layer chromatography) (RazakPharma Co. Tehran, Iran) was supplemented to diets in concentrations of 0, 20, and 40 mg/kg (melatonin-0.2% and melatonin-0.4% groups) according to previous studies that suggested these doses useful in the birds (Mohit et al. 2014; Patil et al. 2013). Throughout the study, daily mortality was recorded, and broilers that died during the experimental period were examined for heart failure and ascites lesions.

Dissection and sample preparation

After the rearing period (42 days), the blood samples were collected from the brachial vein of all chickens. Then, the chickens were weighed, euthanized, and their lungs and hearts dissected. The ventricles were isolated from the vessels and atria up to the atrial–ventricular valve plane. Ventricular weights were measured to calculate the right to total ventricular weight ratio (RV:TV ratio), an essential indicator of pulmonary hypertension. According to Hassanpour et al. (2015), an RV:TV ratio exceeding 0.29 indicates the presence of PHS. The segments of the lung and right ventricle of heart were immediately frozen in liquid nitrogen and stored at -70°C for subsequent experiments. The serum of each blood sample was separated and then stored in the same condition. Morbidity related to PHS was assessed based on this ratio or upon observation of ascitic fluid accumulation/hydropericardium. Chickens that

died after day 8 during the experiment underwent necropsy to identify all PHS-related morbidity.

RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA from lung and right ventricle heart tissues was isolated using RNX-Plus solution (Sinaclon Bioscience, Karaj, Iran) and the acid guanidinium thiocyanate-phenol-chloroform single-step method following the protocol outlined by Bahadoran et al. (2021). The resultant RNA pellet was dissolved in 40 μl of DEPC-treated water. The quality and integrity of RNA samples were assessed using spectrophotometry, with only samples exhibiting an A260/A280 ratio within the range of 1.8–2.2 deemed suitable for subsequent cDNA synthesis. Based on the manufacturer's guidelines, cDNA synthesis was performed using the Easy cDNA Synthesis Kit (Parstous Co.). The resulting cDNA was then stored at -20°C until further analysis by RT-qPCR (Hassanpour et al., 2015a).

To assess potential changes in the transcriptional levels of eNOS and iNOS genes across all experimental groups, relative RT-qPCR analysis was conducted utilizing the PCR kit specified, with YWHAZ serving as a stable control gene for normalization of cDNA input and relative quantification of target gene expression. Specific primers for the genes employed are listed in Table 1. Each sample underwent PCR in triplicate, with 10 ng cDNA and 400 nM of each specific primer in a total volume of 10 μl . PCR amplification was programmed as follows: initial denaturation at 94°C for 2 minutes, followed by 30–40 cycles of denaturation at 94°C for 15 seconds, annealing at $58\text{--}62^{\circ}\text{C}$ for 10–30 seconds, and extension at 72°C for 10–20 seconds. No-template and no-reverse transcriptase controls were included in each PCR reaction. Threshold cycle numbers and mean efficiency values were recorded and calculated using LinRegPCR software. Relative gene expres-

Table 1. Primers used for quantitative real time PCR analysis of chicken mRNAs

Target	Primer sequence (5'-3')	PCR Product	Accession No.
eNOS	GGCTGACTGGGTCTGGAT GTAACGGAAGGTGGGGCA	100 bp	JQ434756.1
iNOS	CAAGCAAACGGCCAAGATCC TTCCAGACCTCCACCTCAA	162 bp	NM_204961.2
YWHAZ	AGGAGCCGAGCTGTCCAATG TCCAAGATGACCTACGGGCTC	83 bp	NM_001031343.1

eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

sion (target/YWHAZ) was determined using the formula previously described (Hassanpour et al. 2015).

Measurement of nitric oxide metabolites

Serum samples were utilized to quantify nitric oxide metabolites (nitrate + nitrite) following the protocol outlined by Behrooj et al. (2012). This method is based on the reduction of nitrate to nitrite by cadmium. Initially, serum samples were deproteinized by the addition of ZnSO₄ (75 mmol/l) and NaOH (55 mmol/l) solutions. After centrifugation, the resulting supernatants were collected and diluted with glycine buffer (45 g/l; pH 9.7). Cadmium granules (2-25 g) were rinsed three times with deionized water and then immersed in a CuSO₄ solution (5 mmol/l) in glycine-NaOH buffer (15 g/l; pH 9.7) for 5 minutes for activation. The freshly activated cadmium granules were subsequently added to the serum samples. Following continuous stirring for 10 minutes, the samples were transferred to labeled tubes for nitrite determination using the Griess reaction. Griess reagent 1 (1% sulphanilamide in 5% phosphoric acid) was added to the sample tubes and kept for 10 minutes at room temperature. Griess reagent 2 (N-naphthylethylenediamine dihydrochloride in water) was then added to all samples, and absorbance was measured at 540 nm within 10 minutes using a spectrophotometer (Corning 480, USA).

Statistical analysis

The data were analyzed using the SPSS software (version 16). The One-way ANOVA was used to determine the statistical differences among the main effects of melatonin on RV:TV ratio, nitric oxide metabolites as well as eNOS and iNOS gene expression followed by Tukey's test. The data were expressed as mean \pm SD and differences between

the means at a level of $p < 0.05$ were considered as statistically significant.

RESULTS

Index of PHS and mortality rate

At 42 days of age, the RV:TV ratio, serving as an index for PHS severity, exhibited a significant decrease in the melatonin-0.4% group of birds compared to the control group ($P = 0.004$). Conversely, the reduction of this index was not statistically significant in the melatonin-0.2% group ($P = 0.056$) (Figure 1). Notably, mortality rates were highest in the control group at 33.3%, followed by a decrement to 22.2% in the melatonin-0.2% treatment group, and further reduction to 13.9% in the melatonin-0.4% treatment group of birds.

Assessment of nitric oxide

Results of overall nitrite and nitrate measurement (as an indicator of NO level) in the serum of all experimental groups are presented in Figure 2. The serum NO levels were significantly higher in the melatonin-0.4% groups of chickens than in the control and melatonin-0.2% groups ($P < 0.001$), whereas this parameter did not change in the melatonin-0.2% group of chickens compared to the control group ($P > 0.05$).

Relative expression of the eNOS gene in the lung and right ventricle of the heart

Figures 3 depicts the relative expression levels of the eNOS gene in lung and right ventricular tissues. The findings reveal a significant increase in the relative abundance of these genes within both the melatonin-0.2% and melatonin-0.4% groups of chickens compared to the control group ($P < 0.05$). However, no significant disparities in the expression levels of

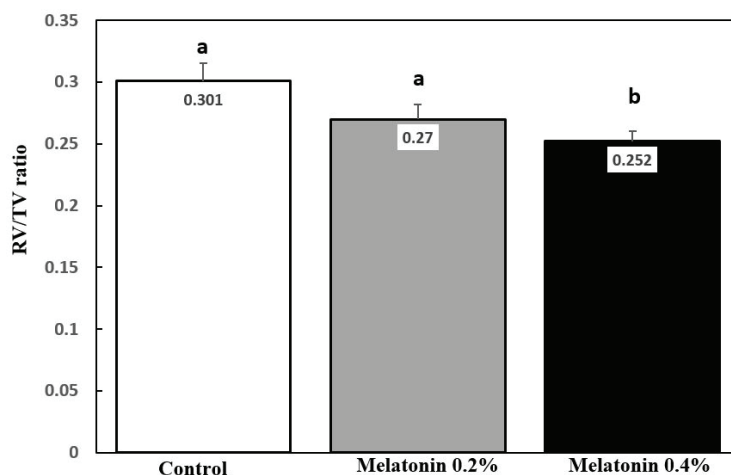
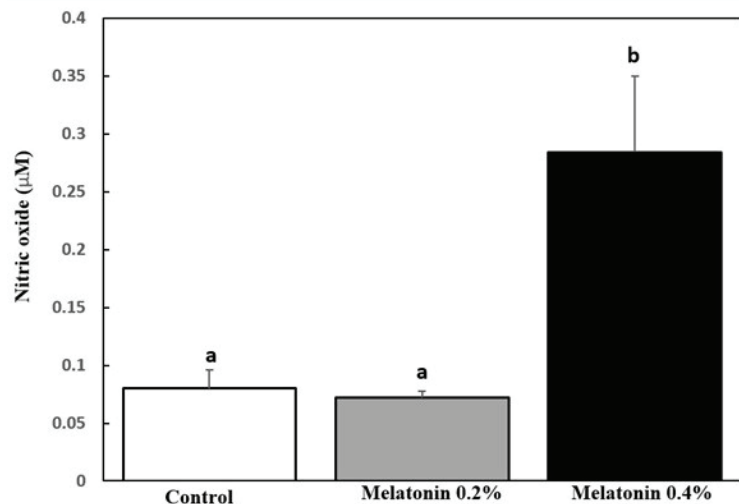


Figure 1. Right ventricle to total ventricles (RV:TV) ratio, as an index for pulmonary hypertension syndrome severity in the experimental groups of broiler chickens. Different letter shows significant difference at $p < 0.05$ level.

Figure 2. Serum nitric oxide levels in the experimental groups of broiler chickens. Different letter shows significant difference at $p < 0.05$ level.



these genes were noted between the melatonin-0.2% and melatonin-0.4% groups ($P > 0.05$).

Relative expression of the iNOS gene in the lung and right ventricle of the heart

Figures 4 displays the relative expression levels of the iNOS gene in lung and right ventricular tissues. The findings indicate that there were no significant alterations in the relative quantities of these genes in both the melatonin-0.2% and melatonin-0.4% groups of chickens when compared to the control group ($P > 0.05$).

DISCUSSION

This research was designed to investigate serum nitric oxide production and heart/lung NOS gene expression in chickens with cold-induced PHS fol-

lowing dietary supplementation of melatonin. As mentioned, the induction of pulmonary hypertension syndrome (PHS) through cold stress initiates a cascade of physiopathologic events, marked by heightened metabolism leading to right ventricular hypertrophy and eventual heart failure. As delineated by Bahadoran et al. (2021), the RV:TV ratio emerges as a dependable index, indicative of right ventricular hypertrophy, developmental PHS, and the incidence of ascites in broiler chickens. Our study, predicated on the RV:TV ratio, unveils the potential of melatonin to modulate clinical PHS and mitigate mortality rates.

The beneficial impact of melatonin supplementation on cardiovascular health is underscored by various studies (Baker and Kimpinski 2018; Zhou et

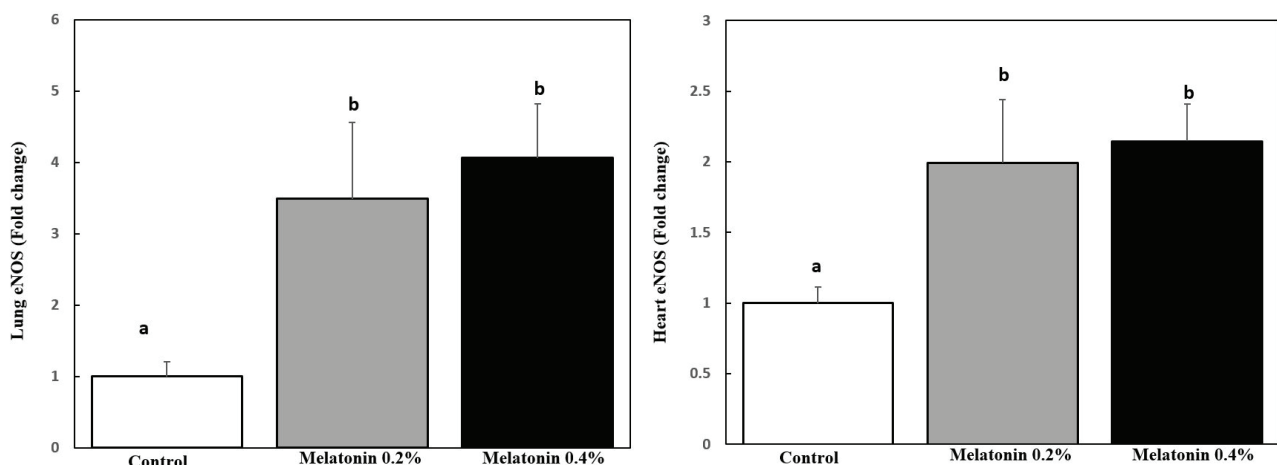


Figure 3. Relative expression of lung/heart endothelial nitric oxide synthase (eNOS/YWHAZ) in the experimental groups of broiler chickens. Different letter shows significant difference at $p < 0.05$ level.

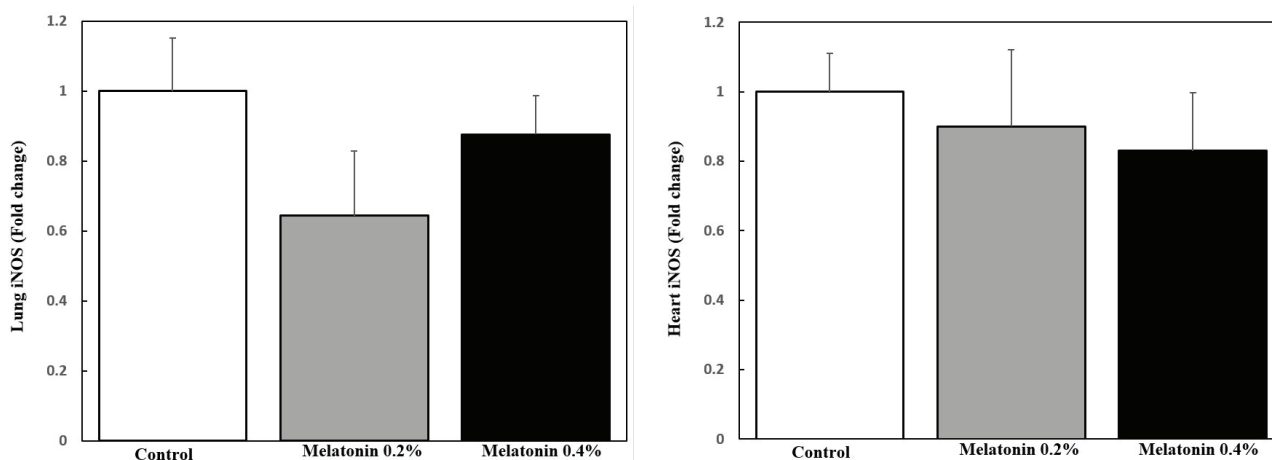


Figure 4. Relative expression of lung/heart inducible nitric oxide synthase (iNOS/YWHAZ) in the experimental groups of broiler chickens.

al. 2018; Nduhirabandi and Maarman 2018). Studies have demonstrated a decrease in plasma melatonin levels in acute and chronic human heart failure, with circulating melatonin emerging as a promising biomarker for assessing the severity of ventricular remodeling (Dzida et al. 2013; Dominguez-Rodriguez et al. 2014). Notably, melatonin administration has been associated with the normalization of blood pressure circadian rhythm, enhancement of ventricular function, and attenuation of hypertension in congestive heart failure patients (Nduhirabandi and Maarman 2018). These favorable effects are attributed, at least in part, to the release of nitric oxide as a vasodilator, inhibition of α 1-adrenergic activity, and augmentation of cholinergic tone (Eghbal et al. 2016). Such findings are in accordance with our results, accentuating the beneficial influence of melatonin on PHS, including a reduction in ascites-related mortality.

One of the key pathological mechanisms underlying PHS is the dysregulation of vascular tone and endothelial function, leading to increased pulmonary vascular resistance and elevated pulmonary arterial pressure (Hassanzadeh et al. 2014). NO plays a pivotal role in the regulation of vascular tone by promoting vasodilation and maintaining vascular homeostasis. Studies have shown that NO production is dysregulated in chickens with PHS. It has been confirmed that as the disease progresses and right ventricular hypertrophy worsens, NO bioavailability may be reduced due to endothelial dysfunction, oxidative stress, impaired NOS activity, and NOS gene dysregulation (Hassanpour et al. 2015; Hassanpour

et al. 2009; Tan et al. 2007). NO also modulates inflammatory and immune responses (Ricciardolo et al. 2004), which may further impact the pathogenesis of PHS. It exhibits anti-inflammatory properties by inhibiting the expression of pro-inflammatory cytokines and adhesion molecules, thereby attenuating inflammatory responses and tissue damage. However, dysregulated NO production and impaired NO signaling pathways may contribute to immune dysfunction and exacerbate inflammatory processes in PHS and heart failure (Tumurkhuu et al. 2019).

Melatonin has been shown to enhance NO production by upregulating the expression and activity of eNOS. By promoting eNOS activity, melatonin enhances NO-mediated vasodilation, improves endothelial function, and restores vascular homeostasis in heart failure (Tobeiha et al. 2022; Reiter et al. 2024). However, these studies are consistent with our data that showed elevations in serum NO and expression of eNOS gene in the heart and lung especially following the use of 40 mg/kg diet melatonin.

The effects of melatonin on iNOS have been the subject of many studies, yielding both supportive and contradictory findings. Numerous studies have demonstrated that melatonin can significantly inhibit iNOS expression and activity, contributing to its anti-inflammatory, antioxidant, and neuroprotective properties. Melatonin can reduce oxidative stress by scavenging free radicals and upregulating antioxidant enzymes, decreasing secondary induction of iNOS in response to cellular damage and inflammation (Esposito and Cuzzocrea 2010; Carrascal et al. 2018; Akyuz et al. 2021; Oktem et al. 2006).

melatonin's inhibition of iNOS helps protect neurons from nitrosative stress, highlighting its potential therapeutic benefits in neurological disorders (Akyuz et al. 2021). Despite this supporting evidence, a few studies have reported no significant impact of melatonin's inhibitory effects on iNOS (Yi et al. 2014; Xu et al. 2022). This controversy might be due to the timing, dosage, and duration of melatonin administration. Also, the effect of melatonin on iNOS may vary depending on the species and tissue type. However, the referenced studies might explain our findings regarding the non-significant effect of melatonin on iNOS expression in the lungs and heart of PHS-broiler chickens.

CONCLUSION

The present study indicated that melatonin supplementation (40 mg/kg diet) positively alleviates

PHS in broiler chickens, as evidenced by improved RV:TV ratios and reduced mortality rates. Our research supported melatonin's cardiovascular benefits, specifically its role in enhancing nitric oxide (NO) production. This enhancement is likely due to melatonin's promotion of eNOS gene expression, leading to increased NO-mediated vasodilation and improved cardio-respiratory function. Our study found no significant effect on iNOS expression in the lungs and heart of PHS-broiler chickens.

CONFLICT OF INTEREST

The authors have no conflict of interest.

ACKNOWLEDGMENTS

Current research conducted as part of Khoozan M.R.' DVM thesis, Shahrekord University, Shahrekord, Iran.

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