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Research Paper

Investigating the Role of Dietary Melatonin Supplementation in Ameliorating Pulmonary Hypertension Syndrome in Cold-stressed Broiler Chickens

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ABSTRACT

Background: Melatonin, an endogenously produced indoleamine, is a highly effective antioxidant and free radical scavenger. This study investigates the potential antioxidant effects of melatonin in birds exposed to conditions promoting pulmonary hypertension syndrome (PHS) under cold stress.

Materials and Methods: Two groups of birds were offered different concentrations of melatonin (0.2% and 0.4%), while a control group received no melatonin treatment. Serum and cardiac tissue samples were collected to evaluate glutathione peroxidase (GPX) and superoxide dismutase 1 (SOD1) activities and the relative expression of *GPX* and *SOD1* genes.

Results: The results showed a significant decrease in the right ventricular to total ventricular weight ratio in the melatonin 0.4% supplemented group compared to the control. Melatonin supplementation at 0.2% and 0.4% levels led to lower levels of malondialdehyde (MDA) in the serum and heart compared to the control group, indicating reduced lipid peroxidation. Both melatonin groups exhibited increased serum/cardiac (GPX) activities compared to the control group; however, the serum SOD1 activity was only increased in the melatonin 0.4% group of birds compared to the control group. Furthermore, the melatonin-0.2% and -0.4% groups displayed decreased relative gene expression of *GPX* and *SOD1* compared to the control group.

Conclusion: Melatonin, especially with a dose of 0.4%, when used as an antioxidant agent, can be beneficial in reducing the severity of PHS and heart right ventricular failure in birds.

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Introduction

Pulmonary hypertension syndrome (PHS) of birds, also known as ascites syndrome, is a current metabolic/nutritional disease in the poultry industry. The syndrome is typified by a rise in pulmonary artery pressure that eventually leads to right ventricular hypertrophy, right heart failure, and death [1]. Particular lesions found in PHS-affected birds include hydropericaditis, right heart enlargement, ascites, pulmonary congestion, and pulmonary edema. Pulmonary arterial hypertension is critical in developing PHS and ascites. Pulmonary artery vasoconstriction and pulmonary artery remodeling aggravate the PHS. This syndrome is usually influenced by different mechanisms, including hypoxia, endothelial dysfunction [2], vascular active material imbalance [3-6], oxidative stress [7], inflammation, thrombosis [8], and genetic properties [9]. The high production of reactive oxygen species (ROS) prone to PHS may cause this syndrome or exacerbate it. Proteins, DNA, and some molecules can be oxidized by ROS, resulting in a cascade of harmful reactions and initiation of tissue damage. Excessive production of ROS could cause damage to the pulmonary vascular endothelium and vascular remodeling [10-12].

For birds, the first line of defense against ROS is endogenous antioxidants, such as tocopherols, glutathione, and ascorbic acid. The levels of these antioxidants are reduced in liver and lung tissues and the mitochondria of ascitic birds in association with increased markers of ROS-mediated tissue injury, leading to oxidative stress during PHS [13]. As with the chemical antioxidants, cells are protected against oxidative stress by an interacting network of antioxidant enzymes, such as superoxide dismutases, catalase, and glutathione superoxide. The superoxide released by processes, such as oxidative phosphorylation is first converted to hydrogen peroxide (H_2O_2) and then further reduced to give water. This detoxification pathway results from mentioned enzymes, and various peroxidases removing hydrogen peroxide. Specifically, superoxide dismutase accelerates the dismutation reaction, which involves the conversion of superoxide radicals into H_2O_2 and oxygen. Glutathione superoxide reacts with H_2O_2 or organic hydroperoxides, reducing them to water. Catalase also catalyzes the breakdown of hydrogen peroxide into water and oxygen. In many studies, it has been attempted to improve the PHS by supporting the antioxidant status at the onset of the ascites-promoting condition [13-15].

Melatonin (N-acetyl-5-methoxytryptamine) is an indole amine derivative produced by the pineal gland and many extrapineal sources, such as skin, immune system cells, brain, and gastrointestinal tract. Progressive interest in this hormone as a potential therapeutic agent in several diseases is due to its pleiotropic effects [16, 17]. Melatonin plays a critical role in different physiological functions, including the regulation of circadian [18], immunity [19], oxidative stress [20], apoptosis [21], and mitochondria [22]. Most of these activities change during inflammation [16, 17]. Melatonin could easily enter the cellular organelles (e.g. nucleus and mitochondria), and act as a natural scavenger involving the neutralization and detoxification of several chemical agents such as H_2O_2, hydroxyl radicals, nitric oxide radical, and peroxynitrite [23]. This hormone contributes to oxidative stress protection via electron and hydrogen alterations [24].

This study investigates the serum and cardiac oxidative/antioxidative status and pulmonary hypertensive responsiveness following the use of melatonin in birds exposed to PHS-promoting conditions under cold stress.

Materials and Methods

Bird management and induction of PHS

A total of 108 one-day-old broiler chicks were purchased (Ross 308 strain) and randomly divided into three groups (one control and two treatment groups) with 36 birds in each group. In the two groups of treatment, melatonin powder with purity ≥98% (purity confirmed by thin-layer chromatography; RazakPharma Co. Tehran, Iran) was supplemented to diets in 0, 20, and 40 mg/kg concentrations (melatonin-0.2% and melatonin-0.4% groups) according to previous studies that suggested these doses useful in the birds [25, 26]. All groups occupied a single room. Birds were kept for 42 days and housing conditions and a standard ration were provided. Cold stress was induced in the rearing room by a gradual temperature decrease during 42 days of rearing, according to Hassanpour et al. (2023) [27] to provide PHS (Figure 1). The temperature was progressively decreased by about 17 ˚C over 21 days (from 32 ˚C to 15 ˚C) and subsequently remained at 15 ˚C until the last day of the experiment. The total mortality of birds was recorded, and dead birds were necropsied to evaluate the lesions.

Sample preparation and estimation of the PHS index

At 42 days, a total of 12 birds from each experimental group (4 birds/pen) were randomly selected for blood collection and serum separation. After collecting blood specimen (~3 mL) from the brachial vein, serum was

separated through centrifuging at 2500×g for 10 min. At the end of the experiment, all birds were sacrificed and their heart ventricles were harvested, according to Hassanpour et al. (2016) [6]. Then, the right ventricular to total ventricular weight ratio (RV:TV ratio) was calculated. This ratio was calculated as an index indicating the severity of PHS. Accordingly, the RV:TV ratio between 0.25 and 0.28 shows developmental pulmonary hypertension in birds, whereas the RV:TV ratio ≥0.29 is considered a clinical PHS in birds with clinical and necropsy signs of ascites [28, 29]. The right ventricular tissues were frozen in liquid nitrogen and stored at -70 ˚C until ribonucleic acid (RNA) extraction.

Ribonucleic acid extraction, DNase treatment, and cDNA synthesis of heart tissue

The right ventricular tissues of the heart were collected and homogenized. Total RNA extraction was performed using RNAxPLUS solution (Sinaclon Bioscience, Karaj, Iran), chloroform, isopropanol, and ethanol, following the methodology described by Ahmadipour et al. (2021) [30]. Briefly, the heart samples were digested using RNAxPLUS solution, followed by the addition of chloroform and subsequent centrifugation. Isopropanol was then added to the supernatant to precipitate the RNA, which was subsequently collected by centrifugation. The resulting RNA pellet was washed with 75% ethanol, centrifuged again, and dissolved in distilled water. The extracted RNA was purified by eliminating residual DNA with a DNAase kit (Sinaclon Bioscience). The quantity of RNA was determined by spectrophotometry (A260/280). The samples with an absorbance ratio within the range of 1.8-2.0 were considered suitable for cDNA synthesis [30]. For cDNA synthesis, the extracted RNA was immediately reverse-transcribed using the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan).

Relative quantitative real-time polymerase chain reaction analysis

The superoxide dismutase 1 (*SOD1*), glutathione peroxidase (*GPX*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*, as internal control) transcripts were amplified by real-time quantitative polymerase chain reaction (PCR) using SYBR® Premix Ex Taq™ II kit (TliRnase H Plus; Takara Bio Inc, Japan). *YWHAZ* has previously been confirmed as the best reference gene in the PHS of broiler chickens [31]. Table 1 presents the specific primers used for PCR amplification, designed as per Hassanpour et al. (2015) and Ahmadipour et al. (2021) [30]. The heart cDNA samples were subjected to amplification in triplicate using a real-time PCR cycler (Rotor-Gene Q 6000, Qiagen, USA). The thermal profile was an initial 95 ˚C for 30 s and 40 cycles of 95 ˚C for 15 s, 59-61˚C for 15 s, and 72 ˚C for 15 s. The no-template and no-reverse transcriptase samples were used to check the contamination of PCR reagents. The determination of the threshold cycle number (Ct) and calculation of the PCR reaction efficiency was done using LinRegPCR software version 2012.0 (Amsterdam, Netherlands). The transcript levels (gene/*YWHAZ*) were relatively estimated using the efficiency-adjusted Paffl methodology [32].

Malondialdehyde (MDA) measurement and *GPX/SOD* activity of serum and heart tissue

The chemicals utilized in this experiment were prepared from Sigma Aldrich (St. Louis, MO, USA). Following homogenization and sonication of tissue samples, the cell lysate was centrifuged to separate the supernatant for further assays. The thiobarbituric acid assay was applied to measure MDA (an indicator of lipid peroxidation) in the serum and heart right ventricle of experimental groups. According to Hassanpour, Khalaji-Pirbalouty [33], the trichloroacetic acid reagent was added to the serum and tissue supernatant to precipitate proteins. The top layer was retrieved after undergoing centrifugation. Next, an equivalent amount of trichloroacetic acid was introduced to the serum/tissue supernatants and subjected to incubation in a vigorously heated water bath (95 ˚C) for 10 min. Following the samples' cooling, their absorbance was measured at a wavelength of 532 nm. Serum MDA and heart tissue MDA data were expressed as µmol/L and nmol/g wet tissue weight, respectively.

GPX activity was measured as described by Hassanpour et al. (2015) [33]. In this method, a reagent containing potassium phosphate buffer, ethylenediaminetetraacetic acid, sodium azide, nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase, reduced glutathione, and H_2O_2 was prepared. This reagent was added to a homogenized/sonicated tissue sample, and then the color change was monitored in absorbance at 340 nm. The GPX activity was quantified as the rate of µmol NADPH consumption per min/mg of total protein in the sample.

One unit of GPX activity was reported as µmol NADPH consumed per min per mg total protein of sample, using the appropriate molar absorptivity coefficient for NADPH (6,220 mole/L/cm).

SOD activity was determined using the nitro blue tetrazolium (NBT) dye reduction test [33]. In brief, a mix-

ture containing xanthine, NBT, IU xanthine oxidase, and phosphate buffer was prepared. The homogenized/sonicated tissue sample was added to this mixture. In the reaction mixture, the superoxide radical generated within the system causes the reduction of NBT, resulting in the formation of a blue-colored dye known as NBTH2. The rate of NBT reduction was measured at a wavelength of 560 nm using spectrophotometry.

Statistical analysis

All data points are presented as Mean±SE. The SPSS software, version 26.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. The normality test of Kolmogorov–Smirnov was done for all data. To determine the significance of differences between the means of the experimental groups, a one-way analysis of variance was conducted. Differences were considered significant at $P<0.05$.

Results

Index of PHS and mortality rate

At 42 days of age, the RV:TV ratio, which represents an index PHS severity, was significantly decreased in the melatonin-0.4% group of birds (0.252±0.008, P=0.004) compared to the control group (0.301±0.014), while the decrease of this index was not significant in the melatonin-0.2% group (0.270±0.012, P=0.056).

The ascites mortality rates in the control, melatonin-0.2%, and melatonin-0.4% groups of birds were 33.3%, 22.2%, and 13.9%, respectively.

As observed among different experimental groups, there was a slight reduction of activity in two melatonin groups.

Relative expression of *SOD1* and *GPX* genes in the right ventricle of the heart

The relative expression levels of *GPX* and *SOD1* genes in the right ventricular tissue of the heart are presented in Figure 2 and Figure 3. The results showed that the relative amounts of these genes were significantly reduced in both the melatonin-0.2% and melatonin-0.4% groups of birds compared to the control group ($P<0.05$); however, no significant differences in the expression levels of these genes were observed between the melatonin-0.2% and melatonin-0.4% groups ($P>0.05$).

Assessment of lipid peroxidation, GPX/SOD activities

The results of MDA levels (lipid peroxidation) and GPX/SOD activities in the serum and right ventricle of the heart are presented in Table 2. The MDA levels were significantly lower in the serum and heart of melatonin-0.2% and melatonin-0.4 % groups of birds than in the control group ($P<0.001$), whereas this parameter did not change between the two melatonin groups of birds ($P>0.05$).

The GPX activity was significantly higher in the serum and heart of the chicken treated with melatonin at 0.2% and 0.4% levels as compared to the control group ($P<0.05$). Also, in the heart, the melatonin-0.4% group exhibited a higher GPX activity than the melatonin-0.2% group ($P<0.05$). However, no significant difference was observed between the two melatonin groups in the serum ($P>0.05$).

Regarding the SOD activity, it was increased significantly in the heart of chicken treated with the melatonin-0.2% and -0.4% compared to the control group ($P<0.05$), while differences between the two groups of melatonin were not significant ($P>0.05$). The SOD activity of serum was higher in the melatonin-0.4% group of birds than in the control and melatonin-0.2% groups ($P<0.05$).

Discussion

This study was designed to investigate oxidant/antioxidant status in the heart and serum of birds with PHS induced by cold stress. Lipid peroxidation was assessed as an index of oxidation status and we evaluated the activity/gene expression of *GPX* and *SOD1* as indicators of antioxidant status. The use of cold stress for the PHS induction switches a critical physiopathologic cascade consisting the increased metabolism, oxygen demand in tissues, vasodilation, elevated cardiac output, increased lung vasculature pressure, overload pressure in the right ventricle of the heart, right ventricular hypertrophy, and ultimately heart failure. The RV:TV ratio may be a reliable index to exhibit right ventricular hypertrophy, pulmonary hypertension (developmental PHS), and incidence of ascites (clinical PHS) in birds, as previously reported [34]. The results, based on the RV:TV ratio, demonstrated that melatonin could modulate clinical PHS and reduce mortality rates. The benefits of supplementing with melatonin and its function in cardiovascular health have been proven by numerous studies [35-37]. Plasma melatonin is reduced in human acute and chronic heart failure. Even circulating melatonin has been suggested as a beneficial biomarker for the

Table 1. Primers used for quantitative real time polymerase chain reaction analysis of chicken micro ribonucleic acids

Target	Primer Sequence (5′-3′)	PCR Product	Accession No.
GPX	Forward: GCTGTTCGCCTTCCTGAGAG Revers: GTTCCAGGAGACGTCGTTGC	118 bp	NM_001277853.1
SOD1	Forward: CACTGCATCATTGGCCGTACCA Revers: GCTTGCACACGGAAGAGCAAGT	223 bp	NM_205064.1
YWHAZ	Forward: AGGAGCCGAGCTGTCCAATG Revers: TCCAAGATGACCTACGGGCTC	83 bp	NM_001031343.1

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Abbreviations: *GPX*: Glutathione peroxidase; SOD: Superoxide dismutase 1; *YWHAZ*: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; bp: Base pair.

severity of heart ventricular remodeling [38, 39]. Melatonin administration has been shown to normalize blood pressure circadian rhythm, improve ventricular function, and ameliorate hypertension in congestive heart failure patients [37]. These functions may partly be due to the release of nitric oxide as a vasodilator, inhibition of α1-adrenergic, and increase of cholinergic tone [40]. These findings are consistent with our results, indicating the beneficial effects of melatonin on the PHS of birds, including a decrease in ascites mortality.

As mentioned above, oxidants are the main factors involved in PHS pathogenesis. Wang et al. reported increased hepatic MDA levels and decreased SOD activity indicating liver damage due to lipid peroxidation in PHS [41]. Hassanpour et al. (2015) evaluated the oxidant/antioxidant status of the brain of pulmonary hypertensive birds and reported a considerable lipid peroxidation/protein oxidation and weak antioxidant capacity in the hindbrain segment. Furthermore, they found decreased SOD activity in the brain of those chickens, while the GPX activity remained unchanged [33].

Evaluation of gene expression revealed downregulation of *SOD* transcript and upregulation of *GPX* transcript in the brain of pulmonary hypertensive chickens [33]. These studies confirmed a disruption in the cellular antioxidant defense system and cellular damage due to the oxidant accumulation in the PHS.

Melatonin scavenges oxidants and enhances the antioxidant system [42]. In line with previous findings, melatonin was shown to significantly lower levels of MDA in the blood, liver, heart, and kidneys of heat-stressed quail, according to Sahin et al. (2004) [43]. Several studies evaluated the effects of melatonin in different heart injuries of animal models [44-49]. These studies consistently reported that melatonin reduces lipid peroxidation in the injured heart, which is in line with our findings in the serum and heart of birds with PHS. On the other hand, some studies have reported an increase in serum or cardiac SOD and GPX activities following melatonin administration [45-47, 49], while others observed no significant change [44]. Meanwhile, melatonin may interact with SOD and GPX, leading to con-

Table 2. Comparison of MDA level, GPX and SOD activities in serum and right ventricle of heart between experimental groups of chickens after 42 days

Variables	Mean±SE			P
	Control	Melatonin-0.2%	Melatonin-0.4%	
Serum MDA (µm/L)	6.20±0.478[a]	3.96±0.125[b]	3.19±0.187[b]	<0.001
Heart MDA (µm/L)	5.17±0.338[a]	3.21±0.135[b]	2.56±0.163[b]	<0.001
Serum GPX (mU/mg)	9.01±0.470[a]	13.25±0.967[b]	15.80±1.052[b]	<0.001
Heart GPX (mU/mg)	5.64±0.275[a]	7.05±0.342[b]	9.02±0.518[c]	<0.001
Serum SOD1 (% inhibition)	14.02±0.634[a]	17.74±1.368[a]	23.44±0.832[b]	<0.001
Heart SOD1 (% inhibition)	13.99±0.581[a]	17.62±0.648[b]	19.65±0.883[b]	<0.001
Number of chickens	12	12	12	-

[a,b,c]Significant difference within row experimental groups (P<0.05).

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Abbreviations: MDA: Malondialdehyde; GPX: Glutathione peroxidase; SOD1: Superoxide dismutase 1.

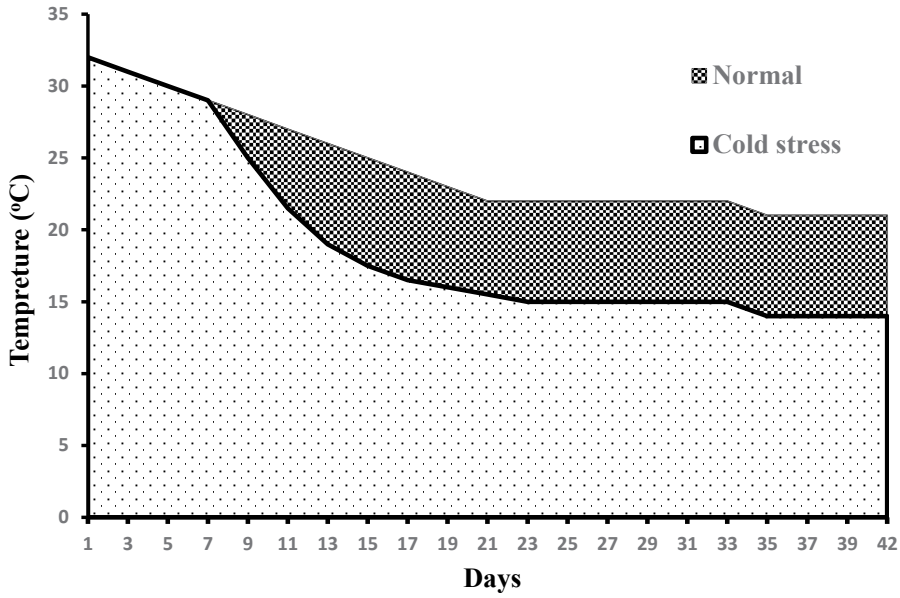

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Figure 1. A timeline diagram of temperature room changes in all experimental groups of chickens during rearing (compared to normal temperature) for the induction of PHS

formational changes that enhance its activity [50]. Also, melatonin can bind to metal ions (such as copper and zinc) that are cofactors for SOD and GPX, thereby promoting its enzymatic activity [51]. Although most mentioned studies agreed with our data (increased activities of serum/cardiac SOD and GPX), the observed conflict may be due to the type and severity of cardiac damage and the amount/duration of the melatonin administration. This conflict was also observed in the gene expression level of antioxidant enzymes. In this regard, Ghosh et al. (2007) indicated that in the presence of melatonin, the cardiac *SOD1* micro RNA increased in rat T3-induced heart failure while the expression of the cardiac *SOD2* gene remained unchanged [52]. Zare et al. (2021) also reported the same results for the gene expression of *SOD* and *GPX* [53]. In contrast, Yan et al. (2022) showed that melatonin may blunt the upregulation of oxidative stress-related genes (*SOD2*, *CAT*, and *GPX*)

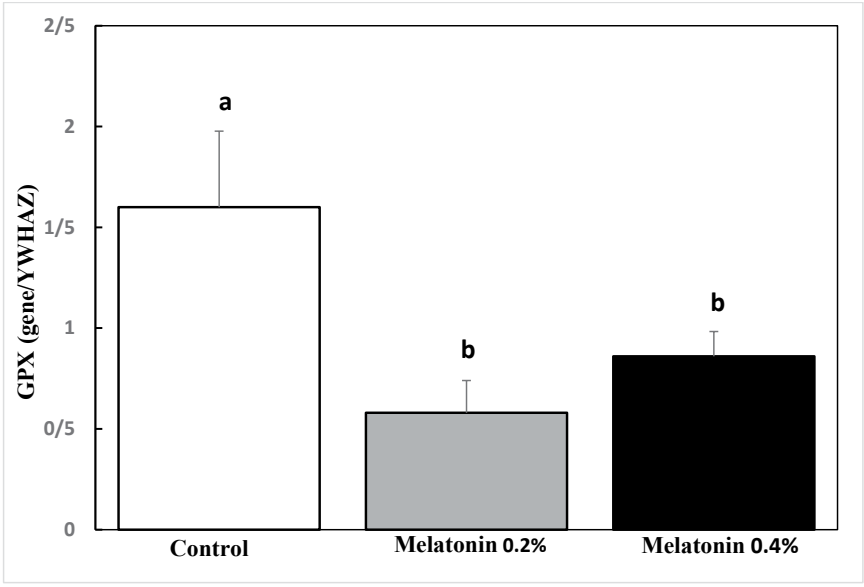

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Figure 2. Comparison of the relative expression of *GPX* gene among the different experimental groups at 42 days of rearing.
Notes: Values are represented as Mean±SE. [a,b]Significant differences among experimental groups (P<0.05).

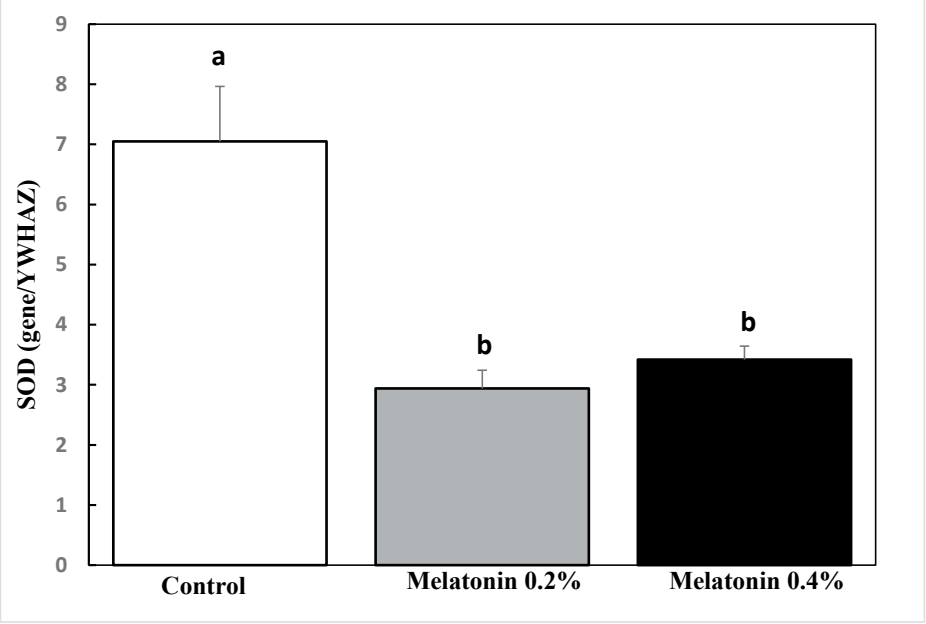

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Figure 3. Comparison of the relative expression of the SOD gene among the different experimental groups at 42 days of rearing
Notes: Values are represented as Mean±SE. [a,b]Significant difference between experimental groups (P<0.05).

in the heart of zebrafish [54]. Melatonin can activate various signaling pathways involved in cell survival and antioxidant responses, such as the PI3K/Akt and MAPK pathways. These pathways may, in turn, influence *SOD* and *GPX* genes and activities through downstream effectors [55]. However, several factors can influence cellular enzymatic antioxidants such as environmental conditions, genetic differences, and variations in feed composition [56, 57]. These factors may also change the impact of melatonin on antioxidant enzymes.

Our data shows a decrease in the gene expression of antioxidant enzymes (*SOD1* and *GPX*) in the heart of pulmonary hypertensive birds which contradicts the findings of many previous studies. However, it is important to consider that the protective action of melatonin can be influenced by the animal species and the stage of heart failure.

Conclusion

Melatonin especially with a dose of 0.4%, as an antioxidant agent, may be beneficial in reducing the severity of PHS and heart right ventricular failure in birds exposed to cold stress.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Shahrekord University (Code: IR.SKU.REC.1400.061).

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Authors' contributions

Study design: Shahab Bahadoran, and Hossein Hassanpour; Experiments, data analysis, and writing: Saeed Adinehvand, Hossein Hassanpour, and Abdonnaser Mohebbi.

Conflicts of interest

The authors declared no conflict of interest.

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