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The key role of vitamin C in the treatment of pulmonary hypertension of meat-type chickens: role of caspase-3

Hamed Zarei[1*], Mohammad Reza Maleki[2]

[1] Department of Biology, Faculty of Basic Science, Central Tehran Branch, Islamic Azad University, Tehran, Iran
[2] Department of Animal Sciences, Faculty of Agriculture, Garmsar Branch, Islamic Azad University, Garmsar, Iran

*** Corresponding author email address**: h.zarei@iautmu.ac.ir

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A B S T R A C T

Ascites is a prominent example of the pathophysiological interaction between the heart and lungs in broilers. Previous experiments have shown that the amount of apoptosis increases in heart failure and pulmonary hypertension. In the current study, the effect of vitamin C on apoptosis was investigated by measuring the expression of the *CASP3* gene in the heart and lungs of chickens with ascites. For this purpose, 90 one-day-old meat-type chickens were divided into three groups (sham (basal diet), control (basal diet +1.5 mg/kg of triiodothyronine (T3)), and treatment group (basal diet + 1.5 mg/kg of T3 + 1200 ppm of vitamin C)) and bred for 49 days. On the 21st and 49th days after rearing, 15 chickens from each group were selected randomly, and the right ventricle/ total ventricle weight ratio (RV/TV), as well as the expression level of *CASP3* genes in the lung and right ventricle of all groups were measured and compared. The amount of mRNA related to *CASP3* gene at the age of 21 and 49 days demonstrated a meaningful decrease in the treatment group compared to the control group ($P<0.05$). This significant difference indicates the reduction of apoptosis in the group treated with vitamin C. Also, RV/TV as an index of the induction of this syndrome improved in the treatment group at the age of 21 and 49 days ($P<0.05$). Finally, according to the current study's findings, vitamin C has ameliorating effects in treating ascites in meat-type chickens.

Keywords: *Ascites, Apoptosis, Broiler; Caspase-3, Triiodothyronine, Vitamin C.*

1 Introduction

Ascites (pulmonary hypertension syndrome), a common disease in birds, can affect the respiratory and cardiovascular systems. This complication is a metabolic disorder and a multifactorial syndrome caused by the interaction between genetic, physiological, and environmental factors (1). The occurrence of ascites is associated with disorders such as hypoxemia, cardiopulmonary overload, blockage of blood vessels and heart, and right ventricular hypertrophy. It should be noted that heart failure is common in meat-type chickens with a

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high growth rate, and the respiratory system cannot provide efficient ventilation and gas exchange, ultimately leading to hypoxia (2). Increased blood flow, pulmonary vascular pressure, cardiac output, and right ventricular hypertrophy are compensatory outcomes of the cardiovascular system, which ultimately cause ascites (3, 4). Previous studies have shown that this syndrome produces large amounts of oxidants in damaged tissues. Also, in various studies, researchers investigated the most key environmental factors involved in the occurrence of ascites. The most important factors are altitude, lighting, cold stress, ventilation, air quality, diet with high nutrient density, and environmental conditions of hatching (5, 6).

On the other hand, the findings of several studies showed that the amount of apoptosis increases in heart diseases and pulmonary hypertension syndrome (7). Apoptosis usually occurs during aging and development as a homeostatic process to maintain the cell population in body tissues. Apoptosis can also be considered a defense mechanism, for example, in immune reactions or when cells are damaged by disease. Apoptosis is a highly conserved process with multiple pathways involved in several physiological and pathological processes. At the molecular level, major changes in this process include the formation of apoptotic bodies, cell shrinkage, activation of caspases, chromatin condensation, and DNA fragmentation. The main players in the process of apoptosis are caspases, specific cysteine proteases with proteolytic function. Caspases are enzymes that degrade other cytoplasmic proteins containing aspartic acid residues. Researchers have proven that the increase in caspase levels can be considered a parameter to detect the early stages of the apoptosis process (8, 9).

Antioxidants are chemical compounds that prevent oxidation at lower concentrations than the oxidizing agent. Biologically, antioxidants are compounds that protect the living system from harmful effects and prevent reactions that cause oxidation of molecular compounds with cellular structures (10). In the living system, when oxidation occurs, antioxidants combine with free radicals and weaken their activity (11). Ascorbic acid is one of the most important water-soluble natural antioxidants. This antioxidant stabilizes the free radical by moving the hydrogen atom in interaction with free radicals. For example, vitamin C reduces tocopherol, obtained from the combination of vitamin E with free radicals, to tocopherol and then to monoascorbate free radical. During enzymatic and non-enzymatic activities, this free radical is converted into ascorbate and hydroascorbate, neither free radicals. Also, vitamin C significantly reduces the effect of oxidants in destroying the endothelium of blood vessels, especially pulmonary vessels (12). Considering that the studies conducted on the pathophysiology of ascites syndrome have not addressed the cellular and molecular aspects affected by this syndrome in the heart and lungs of affected chickens, therefore we decided to measure the expression of *CASP3* gene as one of the indicators of apoptosis in the right ventricle and lungs of infected chickens and determine the effects of vitamin C on the amount of apoptosis caused in this syndrome.

2 Materials and Methods

2.1 Experimental groups

All experimental procedures were approved by the Animal Care Committee of the Islamic Azad University of Medical Sciences, and the animals were treated according to the guidelines outlined in the Care and Use of Laboratory Animals (NIH publication, revised 1996). A total of 90 fast-growing one-day-old Ross 308 chickens were randomly assigned to three equal groups, including sham (received standard basal diet over period study), control (received standard basal diet + 1.5 mg/kg of triiodothyronine (T3)), and treatment (basal diet + 1.5 mg/kg of T3 + 1200 ppm of vitamin C). Each group consisted of 30 chickens, with 10 chickens per pen and three replicate pens per group. The chicks were reared for 49 days under standard conditions with free access to basal diet and water. To induce ascites, 1.5 mg/kg T3 (Sigma-Aldrich, USA) was added to the diet from 7 days of age until the end of the study (8, 13).

2.2 Assessment of hypertrophy in the heart

On the 21st and 49th days after rearing, 15 chickens from each experimental group were selected randomly and sacrificed. Then, the right ventricle hypertrophy was determined according to the method used in previous studies (2, 8). The total ventricle was weighed Briefly after the resection of the heart. The right ventricle was dissected from the left ventricle and septum and weighed; then, the RV/TV was measured and recorded. Ascites was induced when RV/TV was greater than 0.29 (14). The lungs and heart tissues were stored at –70 °C for subsequent gene expression analysis.

2.3 *RNA extraction and cDNA synthesis*

Total RNA was extracted from the dissected tissues using TRIzol reagent (Invitrogen, Karlsruhe, Germany). 100 mg of heart and lung tissues were homogenized, digested in a digestion buffer, and mixed with chloroform. The mixture was centrifuged, and the settled total RNA in the upper aqueous phase was precipitated using isopropanol. The precipitated RNA pellet was rinsed in ethanol and re-suspended in DEPC-treated water. After removing residual DNA, the RNA was treated with DNase and qualified by spectrophotometry. RNA with an absorbance 260/280 ratio of ~ 1.9 was used for cDNA synthesis. Extracted RNA was electrophoresed on 2% agarose gel and stained with ethidium bromide to qualify RNA (8).

2.4 *Reverse transcription polymerase chain reaction (RT-PCR)*

All primers used in the current study are listed in Table 1. A cDNA synthesis kit (Invitrogen, Karlsruhe, Germany) synthesized cDNA using reverse transcriptase, Oligo (dt), and random hexamer. RT-PCR reaction was run, followed by 40 cycles. *Actb* gene expression was measured as an endogenous control. Finally, the density of bands was calculated using Photo-Capt V.99 Image Software, and relative densities were expressed as *CASP3*/*Actb* density (6, 15).

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

Gene	5'-primer	3'-primer	Cycles	Annealing temperature	Size of PCR product
Actb	ACTGGATTTCGAGCAGGAGAT	TTAGAAGCATTTGCGGTGGACCA	24	60 °C	448 bp
CASP3	TTCAGGCACGGATGCAGATG	TTCCTGGCGTGTTCCTTCAG	25	64 °C	426 bp

2.5 *Statistical analysis*

The findings were demonstrated as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 software. Comparisons were made between sham, control, and treatment groups using the One-way analysis of variance (ANOVA). $P<0.05$ was considered as a meaningful difference between groups.

3 Results

The RV/TV ratios in the different groups at two intervals of rearing, 21 and 49 days old, are presented in Table 2. At both 21 and 49 days of age, induction of ascites caused a meaningful increase in the RV/TV ratio in the control group, while administration of vitamin C decreased it to the normal level ($P<0.05$).

Table 2. The ratio of the weight of the right ventricle to the weight of both ventricles (RV/TV) in the studied groups at different ages

Age (days)	Vitamin C	Control	Sham
21	0.20 ± 0.01^{a}	0.23 ± 0.00^{b}	0.17 ± 0.00^{a}
49	0.22 ± 0.00^{a}	0.29 ± 0.00^{b}	0.21 ± 0.01^{a}

Different letters (a and b) in each line shows significant difference at $P<0.05$ level

The density ratio of CASP3/Actb of lung tissue in the sham, control, and treatment groups is compared in Table 3. According to the results, the amount of mRNA related to CASP3 in the lung tissue at both ages of 21 and 49 days in the treatment group has shown a significant reduction compared to the control group ($P<0.05$).

Table 3. Density ratio of CASP3/Actb resulting from PCR in lung tissue between study groups at different ages

Age (days)	Vitamin C	Control	Sham
21	1.15 ± 0.08^{a}	1.84 ± 0.18^{b}	0.86 ± 0.15^{a}
49	1.43 ± 0.15^{a}	2.18 ± 0.22^{b}	0.93 ± 0.15^{a}

Different letters (a and b) in each line shows significant difference at $P<0.05$ level

Table 4. Density ratio of CASP3/Actb resulting from PCR in right ventricle tissue between study groups at different ages

Age (days)	Vitamin C	Control	Sham
21	2.31±0.26[a]	3.41±0.25[b]	1.90±0.19[a]
49	2.68±0.36[a]	4.76±0.31[b]	2.23±0.17[a]

Different letters (a and b) in each line shows significant difference at P<0.05 level

In Table 4, the density ratio of CASP3/Actb in the right ventricular tissue in the sham, control, and treatment groups is compared with each other. According to the results, the amount of CASP3 mRNA in the right ventricular tissue at both 21 and 49 days of age decreased in the treatment group compared to the control group, which was remarkably significant (P<0.05).

4 Discussion

Recent studies have proven that under natural conditions, any factor that enhances metabolic activities in broilers increases the incidence of ascites. Increasing the growth rate, decreasing the temperature, feeding with high-energy rations, or consuming pelleted rations are among the factors that can increase oxygen consumption in poultry by increasing the metabolic function and eventually causing ascites, especially in broilers (16-18). Therefore, in the present study, in order to induce pulmonary blood pressure by increasing metabolism and, as a result, increasing oxygen consumption, T3 hormone was added to the diet of broiler chickens from seven days of age. In general, the increase in metabolic activities leads to an increase in the need for oxygen and the creation of tissue hypoxia, which increases cardiac output and pulmonary blood flow to compensate and satisfy tissue needs and ultimately leads to ascites.

Researchers have proven that hyperthyroidism enhances cardiovascular function by increasing cardiac output and decreasing systemic vascular resistance, leading to increased heart rate, systolic and diastolic function, and cardiac hypertrophy (19, 20). When the metabolic activity of different body tissues increases for any reason, the local blood flow in that tissue is strengthened, which leads to a reduction in the general blood pressure of the body. With this condition, the heart tries to compensate for the decrease in blood pressure by increasing its performance, which causes hypertrophy of the heart and its failure in the long run. RV/TV is one of the parameters to evaluate the incidence of cardiac hypertrophy. The RV/TV index shows the degree of right ventricular hypertrophy compared to the total ventricular mass. It determines what proportion of the weight of the ventricles is related to the right ventricle. As a result, an increase in this ratio compared to the normal state can indicate right ventricular hypertrophy. An increase in pulmonary artery pressure directly affects this index, and the RV/TV ratio can measure pressure on the right ventricle (21). Previous studies' findings have proven that broilers' lung growth is slower than body growth (4, 22). According to these results, the age-dependent increase in the RV/TV ratio in the control group can be attributed to this issue. In our research, this ratio was higher in the control group than in the sham and treatment groups in all experiment stages. In general, referring to Wideman's description in 2001 of pulmonary hypertension syndrome, which shows an increase in the ratio of RV/TV to more than 0.29 as a sign of the presence of this syndrome in birds (21) and according to the obtained data, This syndrome has completely affected the control group at of 21 and 49 days of age.

An increase in oxidative stress occurs in conditions where cells are unable to deactivate active free radicals. The high concentration of these factors damages the nucleic acid, lipid, and cell proteins by increasing lipid peroxidation and producing large amounts of malondialdehyde, which ultimately causes necrosis, apoptosis, and cell death (23, 24). According to the present study's findings, in a group of chickens that received vitamin C, this cardiac parameter was very close to its normal level, which can be caused by the antioxidant effects of vitamin C in this syndrome. So far, the predominant focus of studies has been on the pathophysiology of pulmonary hypertension syndrome, and mainly, factors such as increased pulmonary blood pressure, cardiac disorders, and free radicals have been proposed as the most important possible causes of ascites in poultry. Measuring the expression of *the CASP3* gene as an indicator in the right and left ventricles of the heart and lungs of affected broilers determines the amount of apoptosis caused by this syndrome (25, 26). Based on previous studies, it has been found that the activity of caspase-3 is significantly enhanced in hyperthyroid rats, and the increase in the level of thyroid hormones increases apoptosis (27). Also, several research studies showed that the rate of apoptosis in the heart of rats exposed to thyroid hormone increases, and the continued increase in the rate of apoptosis leads the heart to failure (28). It should be noted that several studies showed

that the amount of apoptosis increases in heart failure and pulmonary hypertension.

In the current research, the study conducted by RT-PCR on gene expression (*CASP3*) in broilers indicated that this level of expression in the group that received T3 hormone increased significantly compared to the sham and treatment groups, which is in line with the findings of previous experiments (29). According to the research of Hochhauser et al. in 2003, increased apoptosis plays a vital role in all types of ischemia, including myocardial ischemia that occurs due to incomplete blood supply and decreased oxygen levels (30). Also, Freude et al. suggested that damage caused by ischemia causes apoptosis, and if ischemia continues, it will eventually lead to necrosis (31, 32)

Various studies conducted on animal models such as mice, rats, rabbits, and dogs, as well as human models, have shown that cytosolic cytochrome C, which is released from mitochondria in response to apoptosis stimulation and apoptosis-activating proenzymes called Caspase is revealed in both animal and human models following heart failure. Also, the BCl-2 protein considered an apoptosis inhibitor, is up-regulated after acute coronary insufficiency, especially in the recovered myocardium (7). In a number of studies, it has been shown that the balance between BC1-2 as an inhibitor of apoptosis and Bax and Bad proteins as inducers of apoptosis along with caspase proenzyme is very important in increasing the rate of apoptosis in heart cells (33). Also, in experiments conducted on mouse and rat animal models, it has been determined that the apoptosis rate in heart cells after heart failure, such as ischemia and ventricular hypertrophy, reaches from 0.2% to 35%. In 2006, Gurbano et al., with a simultaneous study on humans and mice, found that following pulmonary hypertension, the amount of apoptosis in the cells of the blood vessels and lungs increases and programmed death of vascular smooth muscle cells is the main cause of changes in blood vessels (7).

As the results showed, in a group of chickens treated with vitamin C, the cardiac parameter of RV/TV improved to a large extent, which can be caused by the antioxidant effects of vitamin C in this syndrome (23). Usually, this vitamin is added to the diet of birds. However, birds have the ability to make this vitamin in their bodies, but in special conditions such as high growth rate, heat, cold, infection, the need for this vitamin increases and adding it to the diet seems necessary. It has been proven that vitamin C increases the degree of vasodilation. In particular, it has been proven that it can cause more release of nitrite oxide (vasodilator) from the vessel wall, a function of vitamin C that can effectively reduce pulmonary hypertension. The initial studies on ascorbic acid indicated that this vitamin can reduce mortality caused by environmental stress (12). Finally, the present study showed that vitamin C can have ameliorating effects on ascites in broilers, which is consistent with previous findings.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

Hamed Zarei: Conceptualization, Formal analysis, Writing - Original Draft

Mohammad Reza Maleki: Investigation

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

Ethical Considerations

This study was approved by the local ethics committee for animal experiments of Islamic Azad University, Garmsar Branch.

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