

## Original Article

## Vitamin E Can Down-regulate Some of Apoptotic Genes Involved in Pulmonary Hypertension Syndrome in Broiler Chicken

Hamed Zarei<sup>1\*</sup> , Behdad Gilvari<sup>2</sup>

1. Department of Biology, Faculty of Basic Sciences, Central Tehran Branch, Islamic Azad University, Tehran, Iran.

2. Department of Animal Sciences, Faculty of Agriculture, Garmsar Branch, Islamic Azad University, Garmsar, Iran.



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## ABSTRACT

**Background:** Ascites or pulmonary hypertension syndrome (PHS) is one of the significant problems in the poultry industry. Therefore, various studies have been conducted on its contributing factors.

**Objectives:** This study aimed to investigate the role of vitamin E in reducing the mRNA levels of caspase-1 (*CASP1*), caspase-2 (*CASP2*), and caspase-3 (*CASP3*) genes involved in the apoptosis pathway.

**Methods:** Ninety fast-growing 1-day-old chickens (Ross 308) were randomly assigned to three equal groups, including sham (basal diet), control (basal diet+1.5 mg/kg of triiodothyronine [T3]), and treatment group (basal diet+400 mg/kg of vitamin E+1.5 mg/kg of T3). To induce ascites, 1.5 mg/kg of T3 was added to basal diet from the seventh day to the end of the experiment. On the 21<sup>st</sup> and 49<sup>th</sup> days after rearing, 15 chicks from each group were randomly selected. The right ventricle/total ventricle weight ratio (RV/TV) and the expression levels of *CASP1*, *CASP2*, and *CASP3* genes in the lung and right ventricle of all three groups of broiler chickens were measured and compared.

**Results:** Although there was no significant difference between the three groups in terms of the RV/TV ratio on day 21 post-reared ( $P \geq 0.05$ ), a significant decrease was detected in the vitamin E-receiving group compared to the control group with respect to the RV/TV ratio on day 49 post-reared ( $P < 0.05$ ). Also, vitamin E reduced the relative expression of *CASP1*, *CASP2*, and *CASP3* at 49 days of age in the lung and heart tissues of broiler chickens with ascites ( $P < 0.05$ ).

**Conclusion:** Based on the results of this study, it seems that vitamin E can reduce some apoptosis genes (*CASP1*, *CASP2*, and *CASP3*) associated with pulmonary hypertension in broilers.

**Keywords:** Ascites, Birds, Caspases, Pulmonary hypertension syndrome (PHS), Triiodothyronine (T3)

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### \* Corresponding Author:

Hamed Zarei, Associate Professor:

Address: Department of Biology, Faculty of Basic Sciences, Central Tehran Branch, Islamic Azad University, Tehran, Iran.

Phone: +98 (21) 36726951

E-mail: [h.zarei@iautmu.ac.ir](mailto:h.zarei@iautmu.ac.ir)

## Introduction

Ascites or pulmonary hypertension syndrome (PHS) is still a significant problem for the chicken industry worldwide (Hoseinian et al., 2021; Khajali et al., 2016). PHS causes annual economic losses of around \$1 billion worldwide (Maxwell & Robertson, 1997). Studies have shown the role of oxidative stress in the pathogenesis of PHS in broiler chickens due to cellular damage caused by increased reactive oxygen species (ROS) production (Babaahmadi Milani et al., 2020; Bottje, 2019). An increase in ROS concentration rises oxidation and damages cellular lipids and DNA and finally leads to apoptosis (Su et al., 2019; Juan et al., 2021). However, the PHS mortality in broiler chicken is decreased by adding antioxidants to broiler diets (Ghiasi et al., 2023; Nemati et al., 2017).

It has been reported that feeding broiler chicken with coenzyme Q10 (CoQ10) decreases the induced cardiac hypertrophy, the right ventricular to total ventricular weight ratio (RV/TV), and losses due to ascites (Faraji et al., 2019). Vitamin E is a biological antioxidant that improves physiological performance, growth, and immunity in broiler chickens because of reducing lipid peroxidation and neutralizing free radicals in skeletal muscle and plasma (Khalifa et al., 2021; Calik et al., 2022). Recently, dietary vitamin E supplementation has been reported to affect lipid peroxidation and reduce muscle malondialdehyde production (Pompeu et al., 2018). The relationship between PHS induced by heat stress and oxidative stress in poultry has been widely studied (Akbarian et al., 2016; Badakhshan et al., 2023).

It has been shown that exposure of broiler chickens to oxidative stress increases the apoptosis of splenocytes due to the up-regulation of the expression of caspase-3 (*CASP3*) and caspase-9 (*CASP9*) genes, as well as impaired immune function (Oloruntola et al., 2023; Chen et al., 2019). In addition, other apoptosis genes, such as caspase-1 (*CASP1*), caspase-2 (*CASP2*), and *CASP3*, were up-regulated in the hearts and lungs of chickens with PHS (Hassanpour et al., 2014).

Although the underlying mechanisms of this syndrome are poorly understood, evidence of increased mRNA levels of *CASP1*, *CASP2*, and *CASP3* in the pathophysiology of PHS in broiler chickens has been reported. Therefore, the present study aimed to investigate vitamin E's role in reducing the genes mentioned above involved in the apoptosis in broiler chickens with PHS syndrome induced by 3,5,3'-L-triiodothyronine (T3) administration.

## Materials and Methods

### Study animals

A total of 90 fast-growing 1-day-old chickens (Ross 308) were randomly assigned to three equal groups, including sham (received standard basal diet over study), control (received standard basal diet+1.5 mg/kg of T3), and treatment (basal diet+400 mg/kg of vitamin E+1.5 mg/kg of T3). Each group consisted of 30 chickens, with 10 chickens per pen and 3 replicate pens per group. The chicks were reared for 49 days under standard conditions with free access to water and a standard basal diet. The basal diet was formulated as follows: The starter (1-11 d), grower (12-25 d), and finisher (26-49 d). To induce ascites, 1.5 mg/kg of T3 (Sigma Aldrich, USA) was added to the basal diet from 7 days of age to the end of the experiment (Hassanpour et al., 2014; Arab et al., 2006). The metabolizable energy was equal in all three starter, grower, and finisher diets (13 MJ). While the crude proteins of the diets were 230 g/kg, 200 g/kg, and 180 g/kg for the starter, grower, and finisher diets, respectively. The animals were treated according to the guidelines outlined in the care and use of laboratory animals (National Research Council (US) Institute for Laboratory Animal Research, 1996).

### Right ventricular hypertrophy assessment

On days 21 and 49 after rearing, 15 chicks from each group were randomly selected and sacrificed, and their right ventricular hypertrophy was determined, as described by Hassanpour et al. (2014) and Cueva et al. (1974). Briefly, after the heart resection, the total ventricle was weighed. The right ventricle was dissected from the left ventricle and septum and weighed; the RV/TV was then calculated and recorded. PHS was induced when RV/TV was greater than 0.29 (Wideman, 2001). The lungs and right ventricle tissues were stored at  $-70^{\circ}\text{C}$  for subsequent gene expression analysis.

### RNA extraction and cDNA synthesis

Total RNA was extracted from the dissected tissues using TRIzol reagent (Invitrogen, Karlsruhe, Germany). About 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 the 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 (Hassanpour et al., 2014).

**Semi-quantitative RT-PCR**

*CASP1*, *CASP2*, and *CASP3* gene expressions were determined by semi-quantitative real-time PCR (sRT-PCR) using the SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen, Karlsruhe, Germany). Primers were designed based on their nucleotide sequences in GenBank and validated before being used for experiments, and their sequences are listed in Table 1. Beta-actin (*ACTB*) was used as the internal comparator in parallel with the control sample. The reverse transcription cycle consisted of 50°C for 30 minutes followed by 40 cycles of 94°C for 40 seconds, 60-64°C for 50 seconds, and a cycle of melt curve consisting of 72°C for 50 seconds. For extensions of DNA, the products were held at 72°C for 5 minutes. The PCR products of *CASP1*, *CASP2*, *CASP3*, and *ACTB* were loaded on 2% electrophoresis gel and then stained with ethidium bromide to visualize the bands. The density of bands was calculated using Photo-Capt Image Software, version 99 and relative densities were expressed as *CASP1*, *CASP2*, and *CASP3*/*ACTB* density (Hassanpour et al., 2011; Teshfam et al., 2006).

**Statistical analysis**

All data are represented as Mean±SEM. The statistical analysis was carried out using GraphPad Prism Software, version 6. Comparisons were made between groups using the one-way analysis of variance. Differences were considered significant when P<0.05.

**Results**

The RV/TV ratios in various groups at two intervals of rearing, 21 and 49 days old, are presented in Table 2. Although there was no significant difference between three groups in terms of the RV/TV ratio on day 21 post-reared (P≥0.05), a significant decrease was detected in the vitamin E receiving group compared to the control group regarding the RV/TV ratio on day 49 post-reared (P<0.05).

The density expression of apoptotic genes, including *CASP1*, *CASP2*, and *CASP3* to *ACTB*, were assayed by sRT-PCR in the right heart ventricles as well as lung tissue of broiler chickens in the control, sham, and treatment groups and were compared on the 21 and 49 days of age (Figures 1, 2, 3 and 4). There was no significant difference between three groups in the lung tissues regarding the relative expression levels of *CASP1* at 21 days of age (P≥0.05). In contrast, the relative expression levels of *CASP2* and *CASP3* were significantly down-regulated in the treatment and sham groups compared to the control group on day 21 of age (P<0.05). Levels

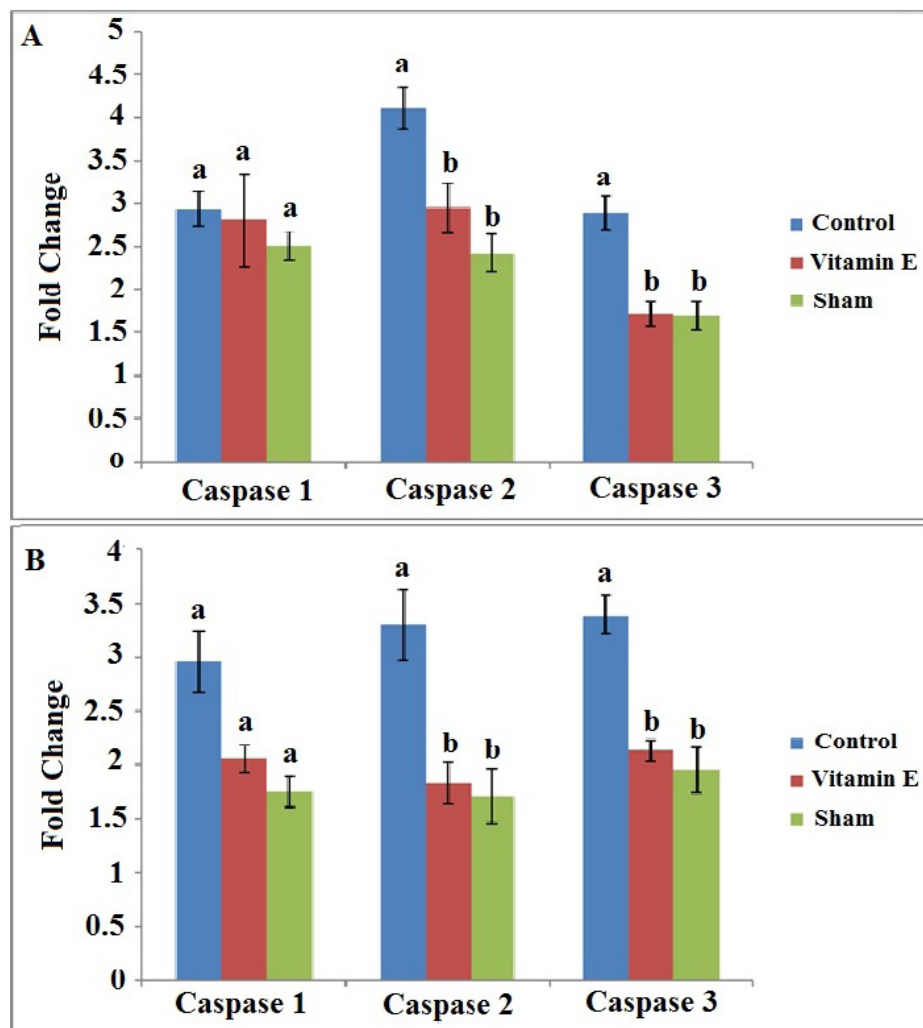
**Table 1.** Primer sequences used for evaluating genes expression

Gene	Sequence	Size
Caspase-1	5' CGGCCAGCGCCATCTTCATT 3' 3'AGGGAGCTGTACAGTGCCT5'	468 bp
Caspase-2	5'TGGCACTGATGGCAAACCTCC 3' 3'ATCGGAGCGGTAGGCAAAC5'	347 bp
Caspase-3	5'TTCAGGCACGGATGCAGATG 3' 3'TTCCTGGCGTGTTCCTTCAG 5'	238 bp
<i>β-actin</i>	5'ACTGGATTTCGAGCAGGAGAT 3' 3'TTAGAAGCATTGCGGTGGACAA 5'	426 bp

**Table 2.** The RV/TV ratio in various groups at two interval times

Age (d)	Mean±SEM		
	Vitamin E	Control	Sham
21	0.23±0.00	0.23±0.01	0.20±0.01
49	0.21±0.00 <sup>a</sup>	0.31±0.01 <sup>b</sup>	0.22±0.01 <sup>a</sup>

<sup>a, b</sup>Significant difference between treatments (P<0.05).



**Figure 1.** Comparing the relative density of *CASP1*/, *CASP2*/, and *CASP3/ACBT* PCR products in the lungs (A) and right ventricle (B) between control and PHS chickens at the age of 21 days

<sup>a, b</sup>Significant difference between treatments ( $P < 0.05$ ).

of apoptosis-related genes, i.e. *CASP1*, *CASP2*, and *CASP3*, showed a significant difference between the treatment and sham groups with the control group at 49 days of age ( $P < 0.05$ ).

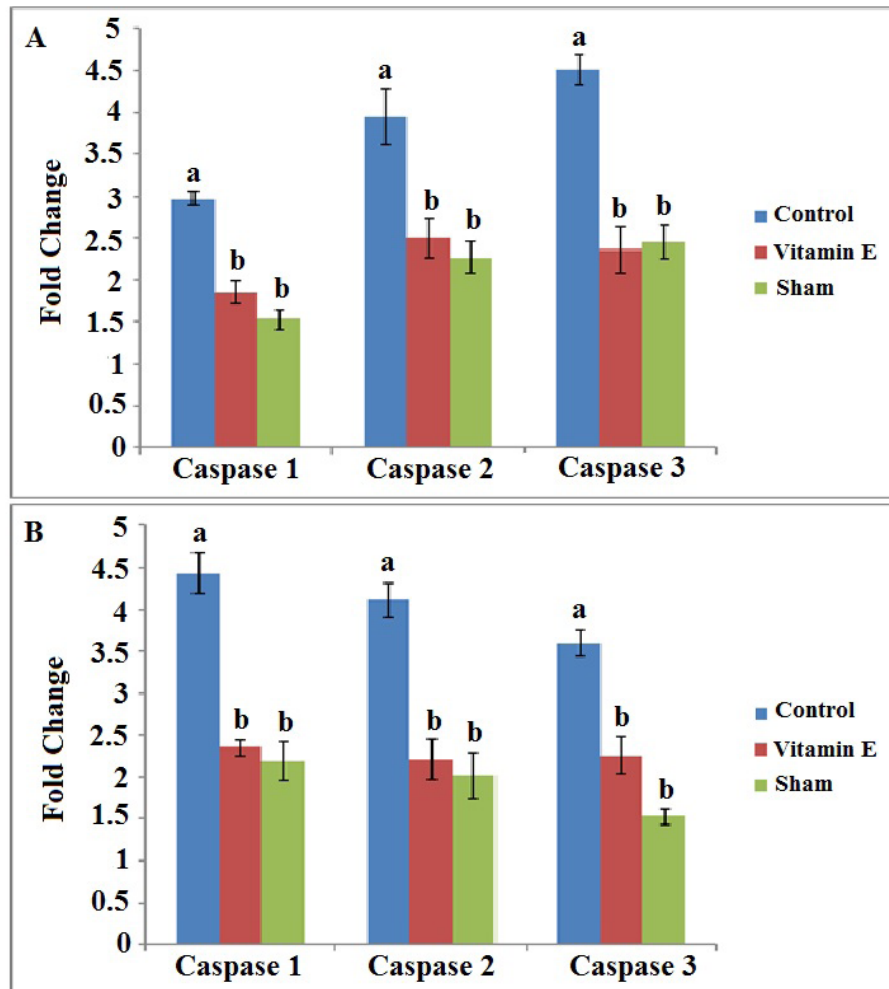
In the lung tissue, the decreasing amounts of the mean expression level of *CASP1*, *CASP2*, and *CASP3* genes in the treatment group were 72%, 62%, and 52% of the control group on day 49 of age, respectively. In addition, the treatment group's mean expression level of the *CASP3* gene was 63% of the control group at 21 days of age.

The right ventricle tissues of the treatment groups showed lower relative expression levels of *CASP1*, *CASP2*, and *CASP3* than that of the control group at

21 and 49 days of age ( $P < 0.05$ ). In the right ventricle tissues, the decreasing amounts of the mean expression level of *CASP1*, *CASP2*, and *CASP3* genes in the treatment group were 70%, 63%, and 53% of the control group on day 21 of age, respectively. In addition, the mean expression levels of *CASP1*, *CASP2*, and *CASP3* genes in the treatment group were 55%, 53%, and 62% of the control group at 49 days of age, respectively.

## Discussion

Research shows that the broiler chickens with PHS induce apoptosis pathway by up-regulation of some caspase genes such as *CASP1*, *CASP2*, and *CASP3* in the lung and right ventricles as well as *CASP3* and *CASP9* expression in the spleen (Chen et al., 2019; Hassanpour

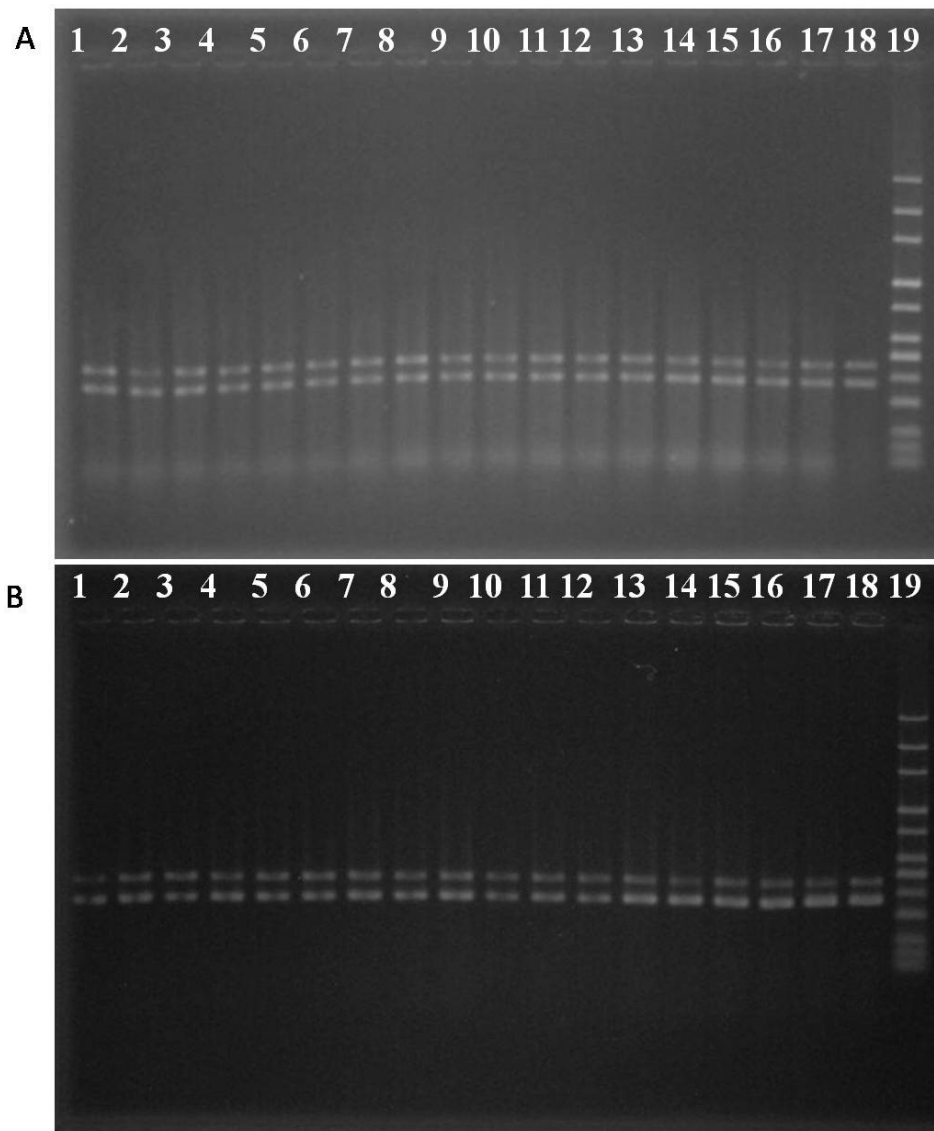


**Figure 2.** Comparing the relative density of *CASP1*/, *CASP2*/, and *CASP3/ACBT* PCR products in the lungs (A) and right ventricle (B) between the control and PHS chickens at the age of 49 days

<sup>a,b</sup>Significant difference between treatments ( $P < 0.05$ ).

et al., 2014). Therefore, this study investigated whether vitamin E can down-regulate the mRNA levels of *CASP1*, *CASP2*, and *CASP3* involved in the apoptosis pathway in broiler chickens' lung and right ventricles with PHS induced by T3 administration. The main finding of the present study was the effect of vitamin E on reducing the relative expression of *CASP1*, *CASP2*, and *CASP3* in the lung and heart tissues of broiler chickens with PHS as well as RV/TV ratio at 49 days of age ( $P < 0.05$ ). This finding can be attributed to the antioxidant activity of vitamin E. A decrease in the activity of antioxidant enzymes has been reported in the visceral tissue of broiler chickens, which may lead to a reduction of the defense system of broiler chickens against ROS or free radical attack, resulting in the activation of the caspase cascade and apoptosis (Panda & Cherian, 2014; Ozkan et al., 2007).

To confirm PHS induction, the RV/TV ratio was measured and considered as the onset of ascites when it was more than 0.229 (Walton et al., 2001). A significant decrease was detected in the vitamin E compared to the control group regarding the RV/TV ratio on day 49 post-reared ( $P < 0.05$ ). This finding indicates that PHS has been induced in the chicks, and vitamin E can reduce right ventricle hypertrophy clinically. Hypoxia has been well-known to be the primary cause of the development of metabolic disorders such as ascites in broilers (Wideman, 2000). Therefore, ascites in broilers increases with enhanced metabolic demand and reduced oxygen availability (Wideman, 2000). In this study, the dietary supplement T3 induced ascites by causing hypoxemic conditions through increased basal metabolism and high cardiac output. T3 administration to broilers has recently increased growth, feed conversion, and visceral organ mass (Chang et al., 2003). On the other hand, an imbal-



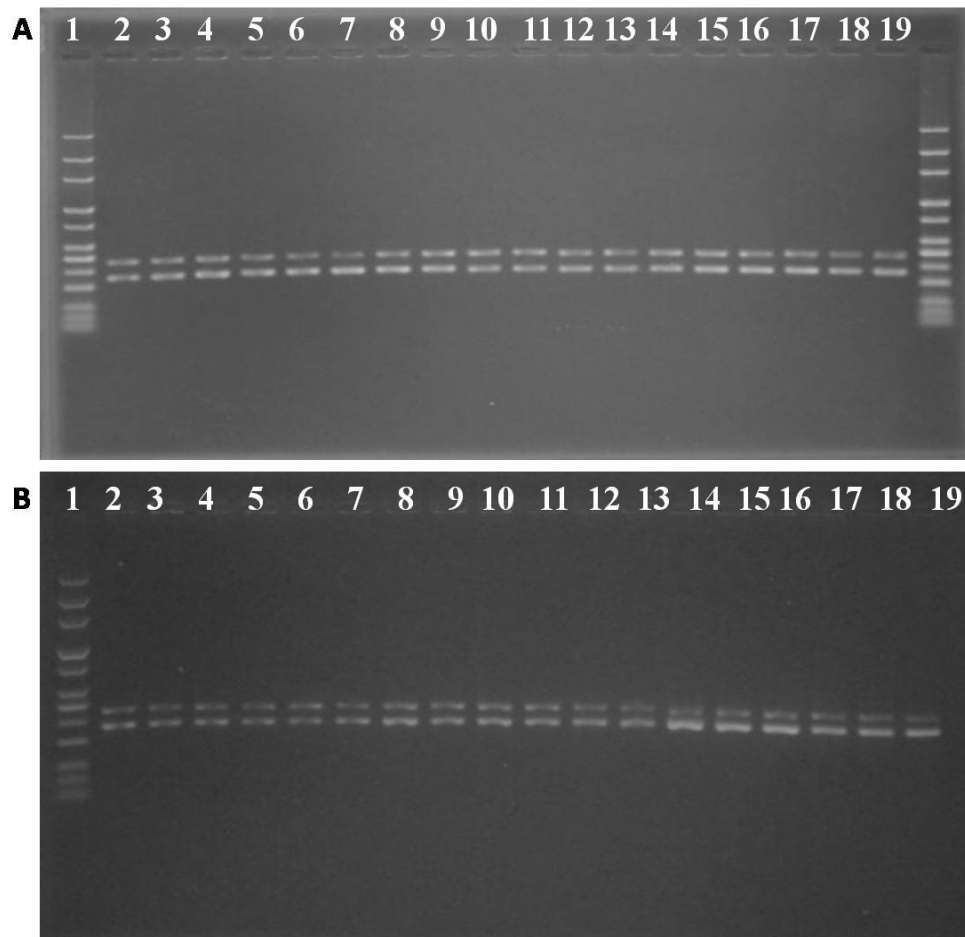
**Figure 3.** Results of the electrophoresis of semi-quantitative RT-PCR of *CASP1* extracted from the right ventricle (lanes 16-18) and lungs of the control groups (lanes 13-15), right ventricle (lanes 10-12) and lung of the vitamin E (lanes 7-9), right ventricle (lanes 4-6), and lungs of the sham group (lanes 1-3), and ladder (lane 19) on 21 (A) and 49 days of age (B)

Note: *ACBT* was the housekeeping gene.

ance between oxygen demand by tissues and oxygen supply increases blood pressure within the pulmonary arteries, leading to the progressive development of PHS (Habibian et al., 2017).

The obtained data from the expression of *CASP1*, *CASP2*, and *CASP3* in tissues showed that all genes mentioned above involved in the apoptosis and inflammatory pathway were up-regulated in both lung and heart tissues of broiler chicken with PHS at 49 days of age ( $P < 0.05$ ). These findings are consistent with results of Hassanpour et al. (2014) study. The higher expression of these genes in right ventricle tissues at 21 days of age than that of the treatment group shows that the apoptosis

pathway starts sooner in the heart than in the lung. Our data also showed that unlike caspase-3, caspase-1 and caspase-2 are up-regulated in the right ventricle before lung tissues in the broiler with PHS. However, compared to the control group, vitamin E could down-regulate any three caspase gene expression at 49 days of age in both heart and lung tissues ( $P < 0.05$ ). Apoptotic signaling is mainly mediated by caspase cascade and occurs under physiological and pathological conditions. It plays a critical role in homeostasis and development in multicellular organisms (Guo et al., 2016). The caspases are divided into three groups: i) Initiators (*CASP2*, *CASP8*, *CASP9*, *CASP10*, and *CASP12*), ii) Executive (*CASP3*,



**Figure 4.** Results of the electrophoresis of semi-quantitative RT-PCR of CASP2 extracted from the right ventricle (lanes 16-18) and lungs of the control groups (lanes 13-15), right ventricle (lanes 10-12) and lung of the vitamin E (lanes 7-9), right ventricle (lanes 4-6), and lungs of the sham group (lanes 1-3), and ladder (lane 19) on 21 (A) and 49 (B) days of age

Note: *ACBT* was the housekeeping gene.

*CASP6*, and *CASP7*), and iii) Inflammatory (*CASP1*, *CASP4*, and *CASP5*) (Julien & Wells, 2017). Although *CASP1* is an inflammatory caspase, it has been reported to be expressed during PHS in broiler chickens (Hassanpour et al., 2014) and heart failure (Merkle et al., 2007). The increased cardiac output, oxygen demand, and basic metabolic rate can produce ROS and induce apoptotic signaling (Pertwi et al., 2022; Majidi et al., 2023). It has been reported that apoptosis is involved in the pathophysiology and pathogenesis of mammal pulmonary hypertension, and free radicals are a critical factor in activating caspases and induction of apoptosis (Rafikova et al., 2019). The up-regulation of both *CASP2* and *CASP3* in the left ventricle of dogs with heart failure has also been reported by Heinke et al. (2001). The up-regulation of *CASP3* in the lungs of broiler chickens with PHS at 21 days of age may contribute to the activation of the mitochondrial apoptosis pathway. The initiation of apoptosis is associated with the release of cytochrome C from

mitochondria to the cytoplasm, leading to the processing of *CASP3* (Kang & Izumo, 2004).

## Conclusion

Vitamin E decreases the relative expression of *CASP1*, *CASP2*, and *CASP3* in the lung and heart tissues of broiler chickens with PHS and RV/TV ratio at 49 days of age. Our data also show that, unlike *CASP3*, the *CASP1* and *CASP2* are up-regulated in the right ventricle before lung tissues in the broiler with PHS. Finally, it seems that decreased mRNA levels of all three caspases in the hearts and lungs of broilers with PHS that received vitamin E are due to the down-regulation of the apoptosis pathway resulting from the antioxidant activity or neutralizing of ROS, which is involved in the pathophysiology of broiler chickens with PHS.

## Ethical Considerations

### Compliance with ethical guidelines

This study was approved by the local Ethics Committee for animal experiments of [Islamic Azad University, Garmsar Branch](#) (Code: IAUGB 2020.08.14).

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### Authors' contributions

All authors contributed equally to preparing this article

### Conflict of interest

The authors declared no conflict of interest.

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## مقاله پژوهشی

## ویتامین E می‌تواند بیان برخی از ژن‌های آپوپتوز دخیل در سندروم پرفشاری خون ریوی در جوجه‌های گوشتی را کاهش دهد

حامد زارعی<sup>۱</sup>، بهداد گیلوری<sup>۲</sup>

۱. گروه زیست‌شناسی، دانشکده علوم پایه، واحد تهران مرکزی، دانشگاه آزاد اسلامی، تهران، ایران.

۲. گروه علوم دامی، دانشکده کشاورزی، واحد گرمسار، دانشگاه آزاد اسلامی، گرمسار، ایران.

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## چکیده



زمینه مطالعه: آسیب یا سندرم پرفشاری خون ریوی (PHS) یکی از معضلات عمده صنعت طیور است و از این رو مطالعات مختلفی در خصوص عوامل مؤثر بر آن انجام شده است.

هدف: این مطالعه باهدف بررسی اثرگذاری ویتامین E در کاهش سطوح mRNA کاسپازهای ۱، ۲ و ۳ در مسیر آپوپتوز انجام شد. روش کار: ۹۰ قطعه جوجه یک روزه سریع رشد (۳۰۸ راس) به‌طور تصادفی در ۳ گروه شم (جیره پایه)، کنترل (جیره پایه+E ۱/۵ میلی‌گرم بر کیلوگرم تری‌یدوتیرونین (T3)) و گروه تیمار (رژیم غذایی پایه+E ۴۰۰ میلی‌گرم بر کیلوگرم ویتامین E ۱/۵ میلی‌گرم بر کیلوگرم T3) قرار گرفتند. به منظور القای آسیب ۱/۵ میلی‌گرم بر کیلوگرم T3 از روز هفتم تا پایان آزمایش به جیره پایه اضافه شد. در روزهای ۲۱ و ۴۹ پس از پرورش، ۱۵ جوجه از هر گروه به‌طور تصادفی انتخاب شدند و نسبت وزن بطن راست به کل بطن‌ها (RV/TV) و همچنین میزان بیان ژن‌های کاسپاز ۱، ۲ و ۳ در ریه و بطن راست هر سه گروه جوجه‌های گوشتی اندازه‌گیری و مقایسه شد. نتایج: اگرچه، تفاوت معنی‌داری بین هر سه گروه از نظر نسبت RV/TV در روز ۲۱ پس از پرورش وجود نداشت ( $P \geq 0.05$ )، کاهش معنی‌داری در گروه دریافت‌کننده ویتامین E در مقایسه با گروه کنترل باتوجه به نسبت RV/TV در روز ۴۹ پس از پرورش مشاهده شد ( $P < 0.05$ ). همچنین ویتامین E بیان نسبی CASP2، CASP1 و CASP3 را در ۴۹ روزگی در بافت ریه و قلب جوجه‌های گوشتی مبتلا به آسیب کاهش داد ( $P < 0.05$ ).

نتیجه‌گیری نهایی: براساس نتایج این مطالعه به نظر می‌رسد ویتامین E می‌تواند برخی از ژن‌های آپوپتوز مرتبط با فشارخون ریوی را در جوجه‌های گوشتی کاهش دهد.

کلیدواژه‌ها: آسیب، پرندگان، کاسپازها، T3، PHS

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## \* نویسنده مسئول:

دکتر حامد زارعی

نشانی: تهران، دانشگاه آزاد اسلامی، واحد تهران مرکزی، دانشکده علوم پایه، گروه زیست‌شناسی.

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رایانامه: [h.zarei@iautmu.ac.ir](mailto:h.zarei@iautmu.ac.ir)