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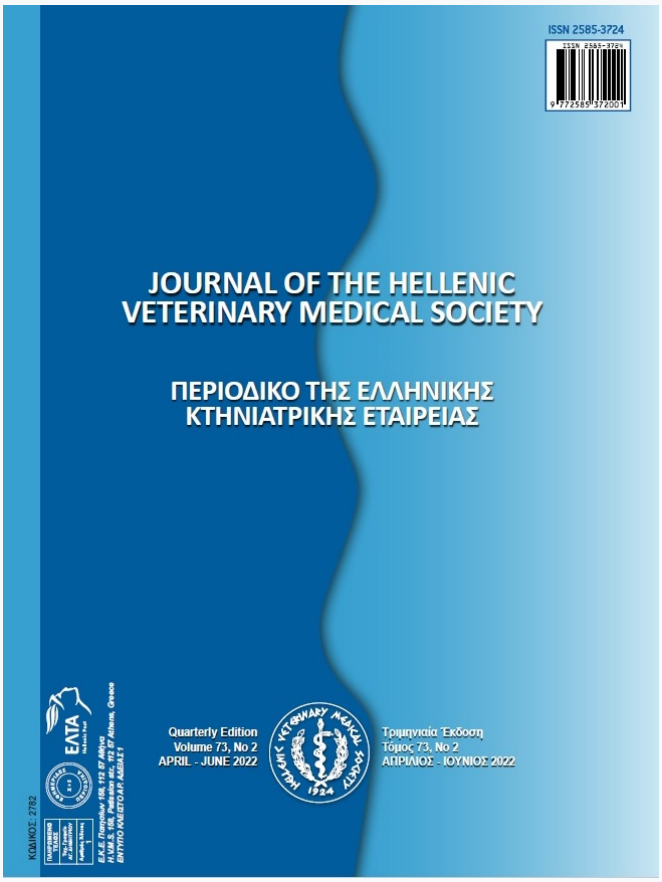

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Hamed Zarei, Edris Sadr Moghani

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Research article
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Natural antioxidants improve pulmonary hypertension syndrome and change blood profile in broiler chicken

H. Zarei[1*], E. Sadr Moghani[2]

[1]*Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran*

[2]*Department of Physiology, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar, Iran*

ABSTRACT: The objective of the present study was to investigate the effect of vitamins C, vitamins E and garlic powder on heterophil/lymphocyte and right ventricle (RV)/total ventricle (TV) ratio as well as lipid peroxidation levels in blood count (BC) with pulmonary hypertension syndrome (PHS) induced by T3 administration. A total of 225 chickens (*Ross 308,* 1 day old) were randomly divided into five equal groups (n=45) including the control (received standard basal diet (RSBD)+1.5 mg/kg of T3), sham (RSBD over period study), and the vitamin C (RSBD+1200 PPM of vitamin C+1.5 mg/kg of T3), the vitamin E (RSBD+400 mg/kg of vitamin E+1.5 mg/kg of T3), the garlic (RSBD +1% garlic+1.5mg/kg of T3) groups. T3 was administrated at days 7 to 49 of age. At days 21,35 and 49 post- reared, 15 chicks from each group were randomly selected, scarified and blood parameters including lymphocytes, heterophil, heterophil/lymphocyte and RV/TV ratio as well as lipid peroxidation were assayed. The decreased the RV/TV ratio in the all treatment groups compared to control group indicate that PHS-induced by administration of T_3improves by adding of vitamins E, vitamin C and garlic to basal diet.The highest and lowest mean percentage of heterophil was belonged to the control and sham groups on 35 and 49 days of age, respectively. The highest mean percentage of heterophil/lymphocyte ratio belonged to the control groups on days of 35, and 49 of age. The adding of vitamin C and garlic to basal diet resulted in a significant increase in the mean percentage of lymphocyte compared to the control and the vitamin E groups on days 35 and 49 of age. In conclusion, the natural antioxidants such as garlic, vitamins E and C can improve the increased RV/TV and heterophil/lymphocyte ratios in the blood profile of broiler chickens with PHS due to reduce lipid peroxidation.

***Keywords*:** Antioxidant, Blood profile, Broiler chickens, Lipid peroxidation, Pulmonary Hypertension Syndrome

Corresponding Author:
Hamed Zarei, Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran
E-mail address: h.zarei@iautmu.ac.ir

Department of Biology, Central Tehran Branch, Islamic Azad University, Asharafi Esfahani St., Poonak, Tehran, Iran

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INTRODUCTION

The broiler chickens with early life fast growth rate are accompanied by incidence a number of metabolic disorders such as to ascites or PHS because of the imbalance between the lungs asan oxygen-supplying organ and heart as an oxygen-demanding muscle (Abidin and Khatoon, 2013; Ahmadipour et al., 2015). Ascites is a multifactorial metabolic disorder that mainly caused by endogenous and/or exogenous factors and is characterized by PHS, hypoxaemia, and fluid accumulation in the abdominal cavity (Antolovich et al., 2002).Oxidative stress is an imbalance between oxidation and antioxidation. In addition, the extreme effects of humidity have been shown to increase mortality in chickens (Azis, 2012; Kang et al., 2020). The incidence of this syndrome in broilers has been also affected by feeding regime changes (Arab et al., 2006). It has been shown that some management practices such as feed restriction can reduce the PHS incidence (Arun and Gita, 2014). However, the feed restriction can act as a stressor on the broiler.The increased reactive oxygen species (ROS) production by oxidative stress has been widely accepted to contribute in the pathogenesis of the PHS in broilers chickens (Chang et al., 2003).

The presence of oxidative stress damages lipids, proteins and DNA, while the elevation of antioxidant enzymes activity (superoxide dismutase (SOD) and catalase) and also antioxidants content (glutathione peroxidase, reduced glutathione, ascorbate (vitamin C), tocopherol (vitamin E), and thiordoxin) could defeat these damages (Arslan et al., 2019; Bayr, 2005). Therefore, adding of antioxidant such as vitamin E, C and garlic to chicken diet can control the ROS production and reduce mortality rate from ascites syndrome (Chen et al., 2014; Faraji et al., 2019).

The antihypertensive and antioxidant properties of garlic as well as its positive effect on several cardiovascular risk factors have been widely studied in human and animal studies (Fathi et al., 2011).Vitamins Eand C are two biological antioxidant that reduce lipid peroxidation and neutralize free radicals and improved performance parameters and ascites related traits as well as decrees lipid peroxidation. In addition, vitamin C has been reported to have positive effects on limiting pathogenicity bacteria colonizing the intestine and caecum during the heat stress (Gurbanov and Shiliang, 2006; Gao et al., 2010; Gautam et al., 2010; Fazlolahzadeh et al., 2011; Saracila et al., 2020). Malondialdehyde (MDA) is an important product of lipid peroxidation, in which ROS degrade cell membrane unsaturated fats. Also, MDA is a significant marker of lipid peroxidation and leads to oxidative DNA damage (Aziz et al., 2015). It is also shown that antioxidants restore lipid peroxidation by increase of reduced glutathione, SOD, and catalase activity (Acaroz, et al., 2018). SOD and catalase are antioxidant enzymes that play a key role in preventing oxidative stress and lipid peroxidation (Acaroz, et al., 2019). The adding of garlic to chicken broiler reduced the cardiovascular risk factor, right ventricle/total ventricle ratio, ascites-related mortality rate (Faraji et al., 2019).The lipid peroxidation and the heterophil and lymphocyte ratio are increased in the stress oxidative condition. Therefore, the lipid peroxidation level as well as heterophil and lymphocyte ratio are two most parameters to assay the stress oxidative levels (Ahmadipour et al., 2015; Habibian et al., 2017). Based on our knowledge, up to now, the effects of garlic, vitamin E and C on serum lipid peroxidation level as well as heterophil/lymphocyte ratioas hematological parameters in broiler chickens with PHS induced by administration of T3 has yet investigated. Therefore, the objective of the present study was to investigate the effect of vitamins C and E and garlic powder on heterophil/lymphocyte ratios and lipid peroxidation levels in broiler chicken with PHS syndrome induced by T3 administration.

MATERIALS AND METHODS

All procedures involving live birds were approved by the Islamic Azad University Institutional Animal Care and Use Committee (Code: IR.IAU.VARAMIN.REC.1400.003).

A total of 225 chickens (*Ross 308,* 1 day old) were randomly divided into five equal groups (n=45) including the control (RSBD+ 1.5 mg/kg of T3 at day 7 to 49 of age), sham (RSBD), and the vitamin C (RSBD+1200 PPM of vitamin C+1.5 mg/kg of T3 at day 7 to 49 of age), the vitamin E (RSBD+400 mg/kg of vitamin E+1.5 mg/kg of T3 at day 7 to 49 of age), the garlic (RSBD+ 1% garlic+1.5 mg/kg of T3 at day 7 to 49 of age) groups each of 45 chickens per pen with 3 replicate pens per each group (n=15). The basal diet of all the groups was equally formulated based on corn and soybean. The basal diets were in mash form and formulated for starter (1 to 11 days), grower (12 to 25 days), and finisher (26 to 49 days) growth periods. The metabolizable energy (ME/kg of diet) and crude protein of the diet in each period were respectively as follows: starter, 13 MJ and 230 g/kg; grower, 13 MJ and 200 g/kg; finisher, 13 MJ and 180 g/kg (National Research Council, 1994). The chicks

were reared under same and standard condition such as humidity, ventilation, temperature, light cycle, and vaccination for 49 days with free access to water and diet. Over the course of the study the birds were fed with starter, grower, and finisher diets at days 1 to 11, 12 to 28, and 29 to 49 days of age, respectively. In the control and all three treatment groups 1.5 mg/kg of T3 (Sigma Aldrich, USA) was added to basal diet from 7 day of age to the end of the study to induce ascites (Hasani et al., 2018).

Lipid peroxidation assay

The blood was randomly sampled from jugular vein from 9 chicks of each group on days 21, 35, and 49 days of age and the sera were then isolated for lipid peroxidation assay using TBARS assay (Draper and Hadley 1990). Briefly, 200 µL of sera were incubated with 1.0 ml of 0.15M PBS (pH 7.4) for 40 minutes at 37 ºC and 2 ml of 0.8% thiobarbituric acid and 2 ml of 20% acetic acid (pH2.5) were added and then placed in boiling water for 10 minutes. Tubeswere allowed to cool at room temperature, and absorbance was recorded at 535 nm wavelengths using a spectrophotometer (Corning Inc., New York, USA).The TBARS concentration was calculated from standard curve that has been previously prepared and was expressed as µM mg^{-1} of the total proteins of the serum. As the amount of measured MDA is presented relative to the protein content of samples, 10 µl of each sample was removed before adding the thiobarbituric acid, for determination of protein concentration using the colorimetric method of Bradford (Bradford, 1976).

Smear preparation

Multiple cytology smears were made on slides and were air dried and fixed using 70% alcohol and stained by Giemsa stain to determine heterophil to lymphocyte ratio.

Assessment of cardiac parameter

At days 21, 35 and 49 of age the chicks were sacrificed and RVH was then determined as previously described by Hassanpour et al., (2014). The hearts were resected and total ventricles were weighted. The right ventricle/total ventricle ratio was calculated and recorded. When the right ventricle/total ventricle ration was greater than 0.28 the PHS had been induced.

Statistical analysis

The data were analyzed using the SPSS software (version 22). The One way ANOVA was used to determine the statistical differences among the main effects of the vitamin E, C and garlic on heterophil/lymphocyte ratio as well as lipid peroxidation capacity and followed by Tukey's test. The data were expressed as mean ± SD and differences between the means at a level of $p < 0.05$ were considered as statistically significant.

RESULTS

RV/TV ratio

The RV/TV ratio in the various groups at three interval times of rearing including 21, 35, and 49 days of age are presented in Table 1. No significant difference between any the five groups in term of the RV/TV ratio was seen on day 21 of age. A significant increase was detected in the vitamin E, vitamin C, garlic and control groups compared to the sham group respected to the RV/TV ratio on day 35 day of age (P<0.05) (Table 1). Finally, the control group showed significantly increasing of RV/TV indicating right ventricle hypertrophy compared to all treatment groups as well as the sham group. The vitamin E and garlic groups showed lower RV/TV ratio than that of the vitamin C group (P<0.05) on day 49 of age (Table 1).

CBC count

The mean percentages of heterophil, lymphocyte and heterophil/lymphocyte ratio were counted at three interval times of rearing including 21, 35, and 49 days of age (Table 2). In the control and garlic groups the mean percentage of heterophil were significantly increased compared to other treatment groups as well as the sham group on day 21 of age (P<0.05).

The garlic group showed higher mean percentage of lymphocyte than those of all treatment and the sham groups on day 21 of age (P<0.05). There was no significant difference amongst all three treatment groups in term of the mean percentage of heterophil/lymphocyte ratio. Whereas the lower and higher mean

Table 1. The RV/TV ratio in the various groups at two interval times

Age	Vitamin E	Vitamin C	Garlic	Control	Sham
21 day-old	0.21±0.00[a]	0.21±0.00[a]	0.21±0.00[a]	0.21±0.06[a]	0.20±0.01[a]
35 day-old	0.25±0.03[a]	0.25±0.02[a]	0.23±0.02[a]	0.27±0.07[a]	0.20±0.01[b]
49 day-old	0.25±0.00[b]	0.27±0.00[b]	0.24±0.01[b]	0.31±0.01[c]	0.22±0.01[d]

Different letter in each line shows significant difference at p<0.05 level. Each line that has one common letter showed no significant difference.

Table 2. The heterophil, lymphocyte and heterophil/lymphocyte ratio in various groups at three interval times

Variable/Group	Sham	Control	Vitamin E	Vitamin C	Garlic
Day 21 of age					
Heterophil	39.11± 1.07[a]	48.53± 0.09[b]	44.13± 1.12[c]	44.13± 1.03[c]	48.58± 0.28[b]
Lymphocyte	42.72± 1.82[a]	44.48± 1.08[ab]	42.85± 0.80[a]	42.15± 0.60[a]	45.95± 1.04[b]
H/L	0.91± 0.02[a]	1.09± 0.02[b]	1.02± .01[c]	1.04± 0.02[c]	1.05± 0.02[c]
Day 35 of age					
Heterophil	39.25± 1.55[a]	49.52± 0.24[b]	41.41± 0.81[c]	42.13± 0.81[c]	43.13± 1.04[c]
Lymphocyte	42.14± 1.58[a]	43.04± 1.04[a]	44.28± 0.78[a]	47.22± 0.57[b]	47.78± 0.70[b]
H/L	0.93± 0.04[a]	1.15± 0.02[b]	0.93±0.01[a]	0.68± 0.01[c]	0.90± 0.01[a]
Day 49 of age					
Heterophil	37.54± 1.07[a]	47.47± 1.04[b]	44.05± 1.52[c]	44.88± 1.23[c]	38.15± 1.88[ad]
Lymphocyte	55.08± 0.41[a]	42.21± 0.75[b]	54.41± 0.55[a]	58.21± 0.85[c]	57.19± 0.14[c]
H/L	0.68± 0.02[a]	1.07± 0.03[b]	0.80± 0.02[c]	0.77± 0.02[c]	0.66± 0.02[ad]

Different letter in each line shows significant difference at $p<0.05$ level. Each line that has one common letter showed no significant difference.

Table 3. Lipid peroxidation in the various groups at three interval times

Variable/Group	Sham	Control	Vitamin E	Vitamin C	Garlic
Day 21 of age					
Lipid peroxidation mol/L	0.89± 0.12[a]	1.41± 0.13[b]	1.01± 0.05[c]	1.07± 0.02[c]	1.19 ± 0.09[c]
Day 35 of age					
Lipid peroxidation mol/L	0.79± 0.23[a]	1.17± 0.09[b]	0.75± 0.04[a]	0.81± 0.07[a]	0.88± 0.01[a]
Day 49 of age					
Lipid peroxidation mol/L	0.77± 0.06[a]	1.70± 0.07[b]	0.87± 0.02[c]	0.91± 0.04[c]	0.88± 0.03[c]

Different letter in each line shows significant difference at $p<0.05$ level. Each line that has one common letter showed no significant difference.

percentage of heterophil/lymphocyte ratio was detected in the sham and the control groups than those of other groups on day 21 of age, respectively. The difference of the mean percentage of these blood cells between treatment with the control and sham groups are presented in Table 2.

Lipid peroxidation assay

Lipid peroxidation as results of free radicals activity was measured in various groups at three interval times of age (Table 3). A significant increase in the lipid peroxiation was detected in the control group compared to all treatment and sham groups at all three interval times of age ($P<0.05$). The lower lipid peroxiation was detected in the sham group at all three interval times compared to other groups ($P<0.05$).

DISCUSSION

The aim of this study was to investigate the effect of three antioxidant agents including vitamins C and E and garlic powder on blood cell analysis, heterophil, lymphocyte, and heterophil/lymphocyte ratio as well as lipid peroxidation levels in broiler chicken with PHS syndrome induced by T3 administration at three interval times of age. The increased RV/TV ratio more than 0.229 in the control group confirmed induction of PHS (Hassanpour et al., 2014).

The decreased the RV/TV ratio in the all treatment groups compared to control group indicate that PHS-induced by administration of T_3 improve by adding of vitamin E, vitamin C and garlic to basal diet from 7 days of age. Ascites or PHS is a metabolic disorder in broiler chickens in which hypoxia occur due to increasing of metabolic demand as well as decreasing of oxygen availability (Lara and Rostagno, 2013). The imbalance oxygen demand of tissues with oxygen supply induced by T_3 administration is accompanied with increasing of blood pressure within the pulmonary arteries followed by the progressive development of PHS (Nemati et al., 2017). T_3 administration induces PHS by increasing of cardiac output, basic metabolic rate, and hypoxia (Özkan et al.,2007; Habibian et al., 2017). Leading to induction of oxidative stress and ROS production (Pan et al., 2005). The increased ROS and enhanced nitric oxide synthase (NOS) expression and NO production have been previously reported in spontaneously hypertensive (Peng et al., 2013). The impaired NO synthesis in the heart ventricles has been also reported to be involved in the pathophysiology of cardiac failure in broilers chicken

with PHS (Hasani et al., 2018). NO production in the myocardiocytes is normal under standard or normal conditions. It plays an important role in regulation of cardiac function due to its role in the control of hypertrophic remodeling, myocardial regeneration, and subsequently improvement of ventricular diastolic distensibility (Mosqueira et al., 2021). The decreasing of RV/TV ratio indicating right ventricular hypertrophy in the all chicken with PHS treated with antioxidants agents can attribute to increase antioxidant capacity of chicken. The increased antioxidant capacity leads to decrease the RV/TV ratio resulted from omitting of ROS and NO production that plays critical role in regulation of myocardial hypertrophic remodeling and ventricular diastolic distensibility. The decreased antioxidant enzymes activity leading to reduce the broilers' defense system against ROS has been reported in the visceral tissue of broiler with PHS (Sahin et al., 2013; Pompeu et al., 2018).

Serum TBARS concentration as a biomarker of lipid peroxidation has previously reported (Selim et al., 2013). The decreased lipid peroxidation or lower TBARS values indicating of the free radical or ROS production in the treatment groups compared to the control group on different days of age was the one of the main findings of the present study. However, the higher lipid peroxidation in the treatment groups on days of 21 and 49 of age in all three interval times than that of the sham group shows the capacity of the vitamin E, vitamin C, and garlic at the used doses were not enough to scavenge all produced free radicals or ROS over the course of PHS. On day 35 of age we have found no significant difference between the sham with all treatment groups in term of lipid peroxidation levels. In addition we also found that vitamin E, vitamin C and garlic at the used dosages show equal capacity to destroy ROS and lipid peroxidation level at all three interval times of age. Several studies have shown that the adding of natural antioxidants, such as vitamins E and C as well as garlic alleviate the negative effects of lipid peroxidation and decrease ascites incidence Faraji et al., 2019; Şenay et al., 2019;Shaito et al., 2020). However, in this study the effect of vitamin E, C and garlic on the lipid peroxidation, RV/TV, and blood cell analysis was compared for the first time.

The obtained data from blood analysis showed that the highest men percentage of heterophil and lymphocyte was belonged to the garlic and control groups on 21 day of age. While the highest and lowest mean percentage of heterophil was belonged to the control and sham groups on 35 and 49 days of age, respectively. The adding of vitamin C and garlic to basal diet resulted in a significant increase in the mean percentage of lymphocyte compared to the control and vitamin E groups on days 35 and 49 of age. We have found that the lowest and highest mean percentage of heterophil/lymphocyte ratio are in the sham and the control groups than those of other groups on day 21 of age, respectively. There was no significant difference amongst all three treatment groups in term of the mean percentage of heterophil/lymphocyte ratio on 21 day of age. The adding of vitamin C to basal diet led to significant decreasing of the mean percentage of heterophil/lymphocyte ratio compared all groups. The highest mean percentage of heterophil/lymphocyte ratio was belonged to the control groups on days of 35, and 49 of age. These findings are in agreement with previous studies in which significant increases of blood heterophil to lymphocyte ratio value has been reported s in the broilers chicken with PHS (Varmaghany et al., 2015; Şenay et al., 2019). It has been recently reported that the adding of antioxidants to broiler diets have positive effects on stress response (Jill et al., 2020). The higher and lower heterophil to lymphocyte ratio in the control and treatment groups firstly can attribute to reduce lymphocyte resulted from ROS production and ROS neutralization, respectively. PHS is associated with oxidative stress from the over-production of ROS. Oxidative damage in human lymphocytes resulted from lipid peroxidation has been reported. Lipid peroxidation has been implicated in a number of deleterious effects such as increased and decreased osmotic fragility and membrane rigidity, respectively (Ogbuagu et al., 2018). The lower heterophil to lymphocyte ration can also attribute to increasing of heterophil. It has been reported that natural antioxidants such as garlic increase the ploy nuclear cells (Wideman et al., 2013).

CONCLUSION

Taken together, our research revealed that natural antioxidants including garlic powder, vitamin C, and vitamin E could improve the increased RV/TV ratio, and also change heterophil/lymphocyte ratio in blood profile of broiler chickens with PHS due to reduce lipid peroxidation.

CONFILICT OF INTEREST

The authors have no conflict of interest.

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