

Full Length Research Paper

The role of oxidative stress in the development of congestive heart failure (CHF) in broilers with pulmonary hypertension syndrome (PHS)

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The present study examined the possible role of reactive oxygen species in the pathogenesis of heart failure in broilers. Our experiment was conducted with 160 one-day-old male broilers (Ross 308) to investigate the mechanisms of cell injury in the pathogenesis of pulmonary hypertension syndrome. The chickens were divided into two groups of four replicates each, with 20 chicks per replicate. One group was raised in normal temperature (NT) while the other group was raised in cold temperature (CT) to induce the pulmonary hypertension syndrome. Mortality was inspected to determine cause of death and diagnose heart failure. Hematological, biochemical and pathological tests were used to determine the incidence of PHS: total red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), activity of alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH). Malondialdehyde (MDA) was used as an indicator of lipid oxidation, subsequent to generated oxidative stress. Samples of blood and liver tissue were taken at day 21 and 42 of age. At the end of the experiment (week 6), 2 chicks from each replicate were randomly selected and slaughtered. The heart was removed, the right ventricle was dissected away from the left ventricle and septum, and the ratio of right ventricle weight to total ventricle weight (RV/TV) was calculated. The results of our experiment indicated the significant difference between the two groups for RBC and HGB and for RBC, HGB and HCT at days 21 and 42 of age, respectively. However, there was no significant difference for activity of ALT, AST and LDH in plasma between the groups at day 21 of age. but CT birds had higher levels ($p < 0.05$) AST, ALT and LDH activities in plasma as compared with other birds. MDA content of plasma and liver was significantly higher ($p < 0.05$) in CT group at both ages. RV/TV ratio and mortality due to ascites were significantly higher in CT birds. In conclusion, heart failure and subsequent PHS can be associated with oxidative stress.

Key words: Oxidative stress, heart failure, ascites, hematological, broiler.

INTRODUCTION

Pulmonary hypertension syndrome (PHS) or ascites is a metabolic disorder that mostly occurs in fast-growing broiler chickens. High altitude, hypoxia, poor ventilation, low temperature and fast growth rate are known to be predisposing factors for the incidence of this syndrome (Huchzermeyer and DeRuyck, 1986; Maxwell et al., 1986; Wideman et al., 1995a, b; Hassanzadeh et al., 1997;

Balog, 2003). Pulmonary hypertension and cardiac dysfunction are the most important features of ascites. Pathological findings indicate that the creation of a cavity on the exterior surface of the right ventricular wall is the first sign of damage in pulmonary hypertension. As the injury progresses, it leads to dilation and hypertrophy of the right ventricle resulting in increased blood viscosity, reduced oxygen supply, congestive heart failure (CHF) and accumulation of fluids in the abdominal cavity (Julian, 1990, 1993; Odum, 1993; Owen et al., 1995).

Oxidative metabolism is a normal process in all tissues.

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Cardiomyocytes require a constant supply of oxygen for normal cardiac functions. However, oxygen associated metabolism in the myocardium sometimes can contribute to cardiac dysfunction, and may ultimately lead to heart failure (Giordano, 2005; Redout et al., 2007). During the normal oxidative metabolic process, various reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. During this normal metabolism, 1 to 2% of oxygen is converted to ROS (Sheeran and Pepe, 2006). ROS are implicated in different disorders, including thermal injury, inflammation, sepsis, mutagenesis, carcinoma, autoimmune diseases and ischemia reperfusion injury (Flohe et al., 1985; McCord, 1985; Halliwell, 1989; Farber et al., 1990). The role of ROS in the injury induced by ischemia reperfusion has been convincingly shown in different organs, including the brain, liver, skin, muscle, lung, intestine, kidneys and heart (Halliwell, 1989; Jaeschke, 1991; Diaz-Cruz et al., 1996).

However, under some circumstances, increased ROS/RNS production or decreased antioxidant defenses may lead to oxidative stress, in which case the generated reactive species can alter the properties of lipids, proteins and nucleic acids, leading to cellular dysfunction. Recent research findings from different laboratories suggest that ROS and RNS play a critical role in the development of human heart failure (Andreka et al., 2004; Sam et al., 2005; Nediani et al., 2007). Lipid peroxidation can alter the membrane properties of cellular and sub cellular organelles (mitochondria and sarco-endoplasmic reticulum) crucial for the maintenance of normal cardiomyocyte function. Broilers with congestive heart failure (CHF) show evidence of calcium overload in these sub-cellular components (Maxwell et al., 1993; Li et al., 2006) and evidence of breakdown and release of proteins of the contractile apparatus, such as myosin and troponin T, into the circulation (Maxwell et al., 1994).

The role of oxidative stress has long been debated in the pathogenesis of heart failure in human and animal models of cardiomyopathy. However, limited research has been carried out to investigate the possible involvement of oxidative stress in PHS and CHF in broilers. In order to further understand the physiological and biochemical disturbances leading to PHS and CHF in commercial broilers, we were interested in examining the possible role and molecular mechanisms of oxidative stress in the pathogenesis of these syndromes.

MATERIALS AND METHODS

Birds and diets

One hundred and sixty (160) 1-day-old male broiler chickens (Ross 308) were used in this experiment. Chickens were allocated randomly to 2 treatment groups, with 4 replicates each and 20 chicks per replicate (per cage). The two groups were broilers under normal temperature (NT) and broilers under cold environmental temperature (CT). All chicks were fed a basal corn-soybean meal

diet to meet requirement, including 22.04% CP and 3,200 kcal/kg of ME (1 to 21 days), or 20.26% CP and 3,200 kcal/kg ME (22 to 42 days). Feed and water provided as *ad libitum* consumption.

Management and measurements

Broilers of the NT group were reared at 32°C for the first week and then it was reduced 2°C per week up to week 5 which was kept at 22°C until the end of the experiment (Daneshyar et al., 2007; 2009). For inducing ascites, the birds of the CT group were raised under 32°C and 30°C during week 1 and 2, respectively. The room temperature was decreased to 15°C during week 3 and maintained between 10 and 15°C for the rest of the study (Igbal et al., 2001) Daneshyar et al., 2007; 2009). Mortality was recorded daily and all of the dead birds were inspected for diagnosis of ascites. Diagnosis of ascites generally depends on observation of the following symptoms:

- 1) Right ventricle hypertrophy, cardiac muscle laxation;
- 2) Swollen and stiff liver;
- 3) Clear, yellowish, colloidal fluid in the abdominal cavity (Geng, 2004).

Sampling

At days 21 and 42 of age, one chick from each replicate was randomly selected. then blood samples were taken from the wing vein after a 3-h starvation period. At the end of experiment, one bird per replicate was killed and its abdomen opened for signs of heart failure and ascites. About 5 grams of liver tissue were removed, homogenated and used for MDA determination. The heart was dissected and removed from the body to determine the ratio of right ventricular (RV) weight to total ventricular (TV) weight ratio. Birds having RV/TV values more than 0.299% were considered to have ventricular hypertrophy (Jolian, 1987). Blood samples were collected in tubes with EDTA anticoagulant tubes. Portions of each blood sample were immediately used for determining total red blood cell (RBC) count, hematocrit (HCT) and hemoglobin (HGB). The remaining was centrifuged and their plasma was collected and stored at -80°C for further enzymatic and chemical analyses.

Malondialdehyde (MDA): The blood was centrifuged at 1,500 × *g* for 5 min; plasma was collected in labeled tubes and stored at -80°C until analysis. After thawing, 500 µL of plasma was placed in a labeled glass tube and mixed with the reagents of a commercial kit for the measurement of thiobarbituric acid reactive substances (TBARS). Each tube was covered with a glass marble and incubated at 95°C for 45 min. The tubes were removed from incubation and allowed to cool in an ice bath for 10 min. Once cooled, the tubes were centrifuged at 3000 × *g* for 10 min and the supernatant carefully removed from the tubes for analysis. The absorbance of the supernatants was measured at 532 nm using a UV/VIS spectrophotometer (Gildford Instrument Laboratories, Inc., Oberlin, OH) and the results were compared against a standard curve made with 100, 50, 25, 12.5, and 0 nmol/mL of malondialdehyde dimethyl acetyl.

Statistical analysis

Data were analyzed based on a completely randomized design using the GLM procedure of SAS (SAS 9.1 institute2002). Duncan's multiple range tests were used to separate the means when treatment means were significant ($p \leq 0.05$); thus a probability level of $p \leq 0.05$ was considered statistically significant. Data were presented as means ± SD.

Table 1. RV/TV ratio and mortality percentage of broilers under normal (NT) and cold (CT) environmental temperatures.

Treatment	RV/TV ratio	Total mortality percentage due to ascites (%)
NT	0.02 ± 0.22 ^b	7.5 ± 1 ^b
CT	0.31 ± 0.01 ^a	38 ± 4 ^a

Data presented as the mean ± standard error. Means within columns with different superscript letters are significantly different ($p < 0.05$).

RESULTS

Incidence of heart failure

Total mortality due to PHS of CT birds during the the recent experiment was significantly higher ($p < 0.05$) than in the NT ones (38% versus 7.5%). Moreover, the RV/TV ratio of CT birds was greater ($p < 0.05$) than that of other birds (Table 1).

Hematology

The results of hematological values are summarized in Table 2, Blood RBC and HGB contents of CT birds was greater than that of NT ones at d 21 of age ($P < 0.05$). Moreover CT birds had the higher blood RBC, HGB and HCT contents at d 42 of age ($P < 0.05$).

Enzymes release

The activities of plasma ALT, AST and LDH are shown in Table 3, there was no significant difference between treatment groups for these enzymes at day 21 of age ($P < 0.05$), but at day 42, the CT birds had higher plasma ALT, AST and LDH activity than NT ones ($p < 0.05$).

Plasma and liver MDA contents

The plasma and liver MDA contents of broilers are shown in Table 4. CT birds had the higher MDA content in both the plasma and liver at both ages (day 21 and 42 of age) ($P < 0.05$).

DISCUSSION

Cold temperature is one of effective factors for inducing hypoxia and PHS. Hence this method was used for inducing heart failure and PHS in recent experiment. Cold temperature despite increasing demand for oxygen consumption, leads to reduced ventilation and decreased oxygen availability in broiler houses (Buys et al., 1999; Daneshyar et al., 2009). Hypoxia is thought to be the primary cause in the development of ascites; therefore, conditions that impose greater metabolic demand or decreased oxygen consumption increase incidence of

PHS (Buys et al., 1999). Cold temperature initiates a cascade of events that results in PHS and death (Julian, 1993; Wideman and Bottje, 1995). Greater RV/TV ratio of CT birds compared to NT ones (0.31 vs 0.22) is a consequence of higher oxygen demands. Higher mortality of CT birds compared to NT birds (38 vs 7.5 percent) in present study is a result of cold induced hypoxia. Moreover, this hypoxemia leads to some hematological changes such as hematocrit, hemoglobin, red blood cell, So the increased blood RBC, HCT and HGB of CT birds in our experiment shows these hematological changes due to PHS. Dilation and hypertrophy and increased PCV have been reported by some researchers (Julian, 1990, 1993; Owen et al., 1995). Despite the changes in hematological indices, hypoxia and be the major cause of ROS production. The low-flow circulation of blood that occurs in birds predisposed to ascites can induce anoxia in different tissues, including the heart. The reoxygenation induced via compensation efforts may happen continuously in the ischemic tissues, resulting in increased production of ROS (Dawson et al., 1993).

Higher plasma and liver MDA contents of CT birds in this experiment indicates the ROS production and lipid peroxidation. So it is suggested that the pathogenesis of ascites syndrome may be initiated by increased production of ROS. Hypoxia of cold temperature not only induces the ROS production, but even causes the injuries in some internal tissues. ROS may cause lipid peroxidation in the membrane of the cells and hence resulting in tissue injury in organs, including lung, heart and liver (Arab et al., 2006). In plasma and liver tissue started from day 21, rises in enzyme release (ALT, AST and LDH) (Table 3) and HCT (Table 2) were observed at day 42. It is suggested that the pathogenesis of ascites syndrome may be initiated by increased production of ROS. As the injury proceeds, it causes dilation and hypertrophy of the right ventricle, resulting in increased PCV, blood viscosity, and the accumulation of fluids in the abdominal cavity due to heart failure (Julian, 1990, 1993; Owen et al., 1995).

A transient hypoxia and then reoxygenation followed by frequent hypoxia can be the major cause of ROS production. The low-flow circulation of blood that occurs in birds predisposed to ascites can induce anoxia in different tissues, including the heart. The reoxygenation induced via compensation efforts may happen continuously in the ischemic tissues, resulting in increased

Table 2. RBC, HGB and HCT of broilers under normal (NT) and cold (CT) environmental temperature.

Day	treatment	RBC($10^6/\mu\text{l}$)	HGB(g/dl)	HCT (%)
21	NT	1.71 ± 0.12^b	6.12 ± 0.31^b	29.02 ± 0.80
	CT	2.42 ± 0.16^a	8.57 ± 0.49^a	34.27 ± 2.04
42	NT	2.0 ± 0.20^b	7.75 ± 0.35^b	32.0 ± 0.65^b
	CT	2.8 ± 0.17^a	11.20 ± 0.35^a	39.3 ± 2.27^a

Data presented as the mean \pm standard error. Means within columns with different superscript letters are significantly different ($p < 0.05$).

Table 3. ALT, AST and LDH levels in plasma of broilers under normal (NT) and cold (CT) environmental temperature.

Day	Treatment	ALT(U/L)	AST(U/L)	LDH(U/L)
21	NT	2.65 ± 0.57	211.75 ± 32	2975 ± 202
	CT	4.00 ± 0.41	223.50 ± 1	3100 ± 250
42	NT	3.75 ± 0.41^b	217.5 ± 1^b	3162 ± 320^b
	CT	7.37 ± 0.25^a	240.5 ± 7^a	4920 ± 674^a

Data presented as the mean \pm standard error. Means within columns with different superscript letters are significantly different ($p < 0.05$).

production of ROS (Dawson et al., 1993).

As the results indicate, increased production of ROS was shown at day 21 in the CT chickens. These agents may cause lipid peroxidation in the membrane of the cells resulting in tissue injury in organs, including lung, heart and liver (Arab et al., 2006) (Table 4). The increase in the amount of AST, ALT and LDH at day 42 is an indicator of a progressive liver cell injury accompanied by the increased production of ROS, resulting in the induction of a chain of oxidative reactions in the liver and other organs (Arab et al., 2006). As the results indicate, the amounts of ALT, AST and LDH have increased during days 21 to 42. There is evidence that serum values of ALT and AST are elevated before the clinical signs and symptoms of liver disease appear. As the injury proceeds, the gross damage (heart failure, fluid accumulation (ascites)) follows, resulting in death. This process can probably explain the pathophysiology of ascites in broilers.

Nain et al. (2008) reported that the morphological changes observed in myocardial mitochondria are consistent with oxidative damage. Notably, mitochondria are the major source of ROS, but because of their very high component of membranes, they are also a very sensitive target of ROS attack. The membrane lipids are very sensitive to oxidative damage due to the presence of polyunsaturated fatty acids, subsequently leading to lipid peroxidation (Halliwell and Gutteridge, 1985). Currently, one of the most common and well-recognized approaches to measuring the effects of free radicals is the estimation of oxidative damage (lipid peroxidation) to cellular membranes (Lykkesfeldt and Svendsen, 2007). So, the measurements from the lipid peroxidation in the present

study showed that in broilers with PHS, oxidative stress increases correlate with heart failure increases. The biochemical evidence of oxidative damage (elevated MDA) corresponds well with the observed morphological changes in the mitochondria, such as mitochondrial swelling, vacuolization, loss and disintegration of cristae (Nain et al., 2008).

The heart is one of the greatest energy-consuming organs in the body, which requires a constant supply of oxygen to maintain its metabolic functions (Giordano, 2005). In the cardiac tissue, mitochondria comprise 30% of the cardiomyocyte volume (Sheeran and Pepe, 2006). The major steps in ROS formation are complex I and complex III of the electron transport chain in the inner mitochondria membrane (Turrens and Boveris, 1980; Turrens et al., 1985). During normal metabolism, 1 to 2% of oxygen is converted to ROS. Hence, increased ROS or RNS production or decreased antioxidant defenses lead to oxidative stress. α -Ketoglutarate dehydrogenase (α -KGDH), one of the key rate-limiting enzymes of the tricarboxylic acid cycle, is involved in energy synthesis pathways. Studies in rats have demonstrated that α -KGDH is a sensitive target of hydrogen peroxide (H_2O_2).

In an anaerobic situation, LDH contributes to energy synthesis by anaerobic glycolysis. An increased production of ROS/RNS occurs during tissue hypoxia (Chen and Meyrick, 2004), which can negatively affect the activity of energy synthesis and transformation pathways. With hypoxia, activation of LDH enzyme by ROS may work as a force to counter the negative effect of other enzymes on energy synthesis and transformation pathways. Recently, higher LDH activity was observed in broilers developing CHF (Nain et al., 2008). Hence,

Table 4. MDA equivalents levels in plasma and liver tissue of broilers under normal (NT) and cold (CT) environmental temperature.

Day	Treatment	MDA in plasma (nm/m lit)	MDA in liver(nm/m lit)
21	NT	1.3 ± 0.31 ^b	0.85 ± 0.03 ^b
	CT	2.5 ± 0.33 ^a	1.32 ± 0.23 ^a
42	NT	1.6 ± 0.2 ^b	1.1 ± 0.04 ^b
	CT	6.27 ± 0.43 ^a	2.6 ± 0.25 ^a

Data presented as the mean ± standard error. Means within columns with different superscript letters are significantly different ($p < 0.05$).

increased activity of LDH in broilers developing CHF is most probably due to generated oxidative stress in the broilers. Insufficiency of creatine phosphate and ATP leads to deterioration in heart pump function in broilers (Nain et al., 2008; Olkowski et al., 2007). This suggests that the observed decline in energy phosphates with deterioration in heart functions might be associated with the decreased activity of these enzymes during oxidative stress.

In conclusion, the results of this study suggested that heart failure in broilers with hypoxia and subsequent PHS can be associated with ROS production during oxidative stress. So, oxidative stress due to hypoxia is the most initial problem with PHS and CHF. ROS can cause cell injury and increase the release of enzymes in plasma, including ALT, AST and LDH. ROS can also contribute to the deterioration of ATP synthesis in myocardium, subsequently leading to lowered energy reserve in themyocardium.

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