Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

ROS Induce Cardiomyocyte Apoptosis in Ascitic Broiler Chickens

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ARTICLE HISTORY

Received: November 25, 2011
Revised: February 01, 2012
Accepted: February 09, 2012

Key words:
Apoptosis
Ascites
Lipid peroxidation
Reactive oxygen species (ROS)

ABSTRACT

It is believed that ascitic broilers die of right heart failure caused by pulmonary hypertension, but the underlying mechanisms of right heart failure are unknown. However, recent studies have shown that reactive oxygen species have the ability to damage heart cells. This study aimed to determine the changes of reactive oxygen species in serum and plasma, and the effect of this variation on myocardial cells during broiler ascites. We used hypoxia and a low-temperature method to induce broiler ascites in the fast-growing group. For controls, we treated a slow-growing group of broilers with 70% restricted feeding under the same circumstances as the fast-growing group. The results showed that hypoxia is a more effective and better way to induce broiler ascites than a low-temperature environment and high growth rate. In addition, reactive oxygen species levels were significantly increased in the fast-growing group compared with those in the slow-growing group. This significant increase in reactive oxygen species resulted in myocardial cell apoptosis in the fast-growing group. Our results suggest that cardiomyocyte apoptosis caused by increased reactive oxygen species levels of ascitic broilers is one of the most important reasons for causing heart failure.

To Cite This Article: Xi Z, S Yang, D Liu, L Wu, X Liu, J Zhao and D Guo, 2012. ROS induce cardiomyocyte apoptosis in ascitic broiler chickens. Pak Vet J, 32(4): 613-617.

INTRODUCTION

Broilers are more prone to the development of ascites compared with other classic strains of chickens because of their high growth rate. A recent epidemiological survey showed that the incidence of ascites has been maintained at a high level. It's estimated that there are approximately 40 billion broilers with ascites syndrome worldwide every year (Baghbanzadeh and Decuypere, 2008), which results in huge losses in the poultry breeding industry.

Many factors contribute to ascites syndrome, such as genetic, physiological, environmental factors and management (Singh *et al.*, 2011). Among these factors, genetic selection should be the most important. The growth rate of broilers can be increased by 4 to 5% every year due to the very successful genetic screening in the past 30 years. Compared with 40 years ago modern strains of broilers almost save 60% breeding time to achieve market weight (Baghbanzadeh and Decuypre, 2008).

Selection for rapid growth and efficient feed conversion has resulted in broilers with such a high rate of metabolism that their heart and lungs are barely capable of providing sufficient oxygen to sustain the body (Julian, 2005; Gupta, 2011). To meet the body's oxygen demand, broilers were forced to increase the cardiac output and heart rate. Those changes led to elevated vascular pressure in the lung and resulting pulmonary artery hypertension. Chronic pressure overload on the right ventricular muscle wall is followed by right ventricular hypertrophy, right atrioventricular valve insufficiency and right ventricular failure. This eventually leads to the occurrence of ascites.

Previous studies have mainly focused on the occurrence of ascites from physiological and pathological aspects. As well as metabolism disorders and development of ascites, broilers develop tissue hypoxia, skin cyanosis, right heart hypertrophy, and liver congestion. Recent studies have found that the heart muscle response to increased pulmonary or systemic blood pressure is hypertrophy of cardiac myocytes by adding sarcomeres (contractile protein units) in parallel with myocytes development and the ventricular wall becomes thinner (Hunter and Chien, 1999). At the same time, cardiac rhythm disturbance (Olkowski and Classen, 1998), hypercapnia and hypoxemia (Olkowskia *et al.*, 2005; Decuypere *et al.*, 2005), and elevated production of reactive oxygen species are common features in many ascitic

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broilers (Olkowski, 2007). Among these features, damage of reactive oxygen species to cardiomyocytes has been rarely reported.

Reactive oxygen species may damage cell membranes and compromise cellular function in a number of body systems (Currie, 1999). The current study aimed to investigate whether cardiomyocytes are damaged by elevated reactive oxygen species in ascitic broilers and how to mitigate this harm.

MATERIALS AND METHODS

Experimental design: Male chickens (Aviagen) were purchased from a commercial hatchery with a good reputation and raised in a temperature, humidity and air flow controlled henhouse with continuous fluorescent light. At 1 day of age, we selected 120 chickens from a total population of 200 according to body weight. Those with extreme weights were discarded. A total of 120 chicks were randomly divided into two groups: slow-growing (restricted feed) broilers (70% ad libitum) and fast-growing ad libitum fed broilers. The chickens were raised in a low temperature environment as previously described (Olkowski *et al.*, 1999). Briefly, the temperature was maintained at 34°C during the first 7 days, and gradually decreased to 21°C by the end of the third week, and finally down to 17°C by the end of the seventh week. Diets contained 3300kcal/kg energy and 24% protein to 3 weeks of age, and 20% protein from 3 to 7 weeks. Water was provided to both groups ad libitum.

All trials were conducted in Tibet's Nyingchi prefecture (altitude of 3700 m, Oxygen content is decreased by 40% compare to coastal areas). The lowered environmental temperature forced the birds to increase their metabolic rate, and lowered oxygen content, resulting in an increased burden on the cardiovascular system, making them more prone to the occurrence of ascites (Yahav *et al.*, 1997; Wideman *et al.*, 1998). At weekly intervals, body weight of 10 birds was measured and blood samples were taken on an individual basis. Whole blood samples were collected from the brachial vein, and an aliquot was stored at 4°C for serum precipitation. The remainder was centrifuged at 3000 rpm for 10 min to obtain plasma, and both were stored at -20°C for reactive oxygen species detection.

Ascites diagnosis: During the experiment, all dead birds were diagnosed for ascites according to abdominal fluid accumulation and the ratio of right ventricle to total ventricular weight (Rv/Tv). An Rv/Tv ratio above 0.29 was classed as right ventricular hypertrophy (Julian, 1987).

At the end of the experiments, all chickens were killed by cervical dislocation for detection of ascites. Hearts were also collected and the Rv/Tv ratio was calculated before placing in formal-saline for histological examination.

Hydrogen peroxide (H_2O_2) assay: Serum hydrogen peroxide content was determined by using a colorimetry kit (Jiancheng Co Ltd, Nanjing China) according to the manufacturer's instructions (Zhu *et al.*, 2009). The method is based on the principle that H_2O_2 reacts with molybdenic acid and forms a yellow complex. The total amount of complex can be calculated by spectrophotometer at 405 nm. H_2O_2 content can be counted by complex production measured.

Catalase (CAT) assay: CAT activity was determined by kit (Jiancheng Co., Ltd. Nanjing China) according to manufacturer's instructions. The reaction of CAT decomposition of hydrogen peroxide can be terminated quickly by adding ammonium molybdate. The remaining H_2O_2 reacts with ammonium molybdate to produce a light yellow complex. Through the amount of its production, the enzyme of CAT can be calculated.

Superoxide dismutase (SOD) assay: The xanthine and xanthine oxidase system produces superoxide anions, which oxidize hydroxylamine to form nitrite, resulting in a purple color. The absorbance was measured at 550 nm and SOD activity in serum was calculated from a standard curve.

Malondialdehyde (MDA) assay: MDA levels were determined by our previously described method (Zhu *et al.*, 2009). Degradation products of lipid peroxidation MDA can condensate with thiobarbituric acid to form a red product, which has a maximum absorption peak at 532 nm. MDA levels were derived from a formula with exact measured values.

Evaluation of myocardial cells apoptosis: The in suit DNA ligase method was used to detect myocardial cells apoptosis (Donath *et al.*, 2006). Briefly, biotin labeled hairpin oligonucleotides, which were labeled with fluorescent isothiocyanate (FITC)-avidin conjugate, can specifically identify apoptotic DNA strand breaks. To distinguish between cardiac myocytes and non muscle-derived cells, sections were labeled with anti-sarcomeric α-action antibody (1:200 dilution; Boster Co., Ltd. Wuhan China) and subsequently labeled with a tetraethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (1:500 dilution; Boster Co., Ltd. Wuhan China). Finally, nuclei were stained with DAPI (100ng/ml; Invitrogen Co., Ltd., New York USA) for 10 min. Sections were observed and photographed by an Olympus IX71 (Olympus Co., Ltd., Tokyo Japan) Cells were considered as apoptotic cardiomyocytes if they were labeled by FITC and TRITC together.

Statistical analysis: The results were analyzed with SPSS 17.0 statistical software. One-way ANOVAs were performed. A value of $P<0.05$ was considered as a significant difference.

RESULTS

We found that a hypoxic environment caused a higher incidence of ascites compared with a low-temperature environment and high growth rate.

Incidence of ascites syndrome: In the fast-growing group, 48 chickens had right ventriclular dilation (Rv/Tv > 0.29) among 60 broilers (data not shown) and 35 birds

had significant accumulation of ascites. Throughout the experiment, a total of 13 chickens died. From 3 to 7 weeks, the number of deaths of broilers was one, three, four, four, and one respectively. In contrast, only one of the slow-growing group chickens, which killed at 7 weeks of age, had ventricular dilation, but there did not appear to be fluid retention in the abdominal cavity.

Olkowski *et al.* (1999) and Nain *et al.* (2008) have reported that the incidence of ascites induced by a low-temperature environment and high growth rate was approximately 45%. Our results showed that a hypoxic environment, as an inducer of broiler ascites, increased the incidence of ascites by 25% compared with a low-temperature environment and high growth rate. The fast-growing group had an increase in body weight from 2 to 4 weeks. However, this difference was not significant at 5-7 weeks because of the occurrence of ascites. By the end of 7th week, the average weight of each group was 2300 g in the fast-growing group and 1930 g in the slow-growing group.

Gross lesions of ascites: We found that the pericardial and the intestinal peritoneal cavities (Fig. 1A) frequently contained fluid. Ascitic broilers were usually accompanied by right ventricular hypertrophy, pericardial effusion, an oozing jelly-like substance and liver congestion (Fig. 1B).

H_2O_2, CAT, MDA and SOD levels: We observed that H_2O_2 levels were significantly increased from the 4th week in the fast-growing group compared with those in the slow-growing group (Table 1). However, CAT, which can degrade H_2O_2 was decreased in the fast-growing group from the 3rd week compared with that in the slow-growing group. We observed a similar trend in MDA and SOD levels. Similar to H_2O_2, MDA levels were significantly reduced from the 4th week in the fast-growing group. SOD levels were significantly reduced in the fast-growing group from 3rd week compared with those in the slow-growing group. Plasma levels of H_2O_2, CAT, MDA and SOD showed similar results to those in serum (data not shown).

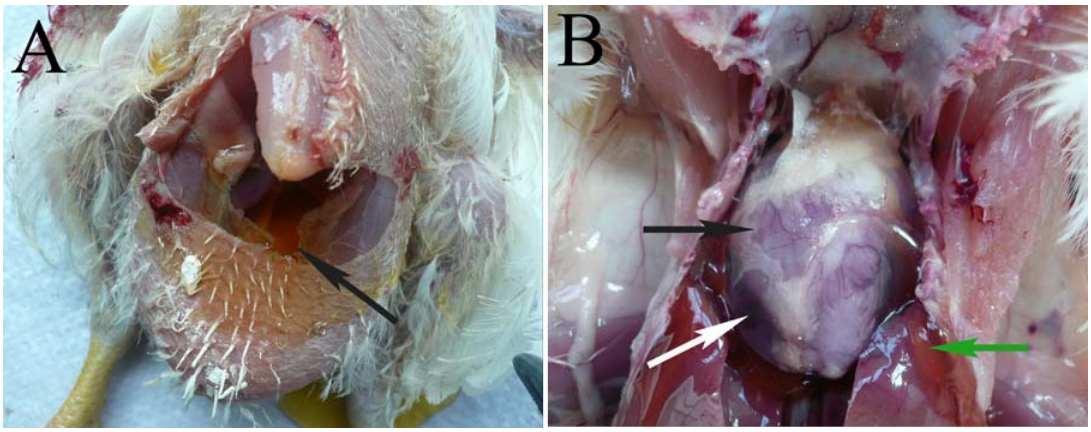

Fig. 1: Anatomy of ascitic chicken. (A) A large amount of yellow liquid (black arrow) is present in the abdominal cavity. (B) Broilers that died of ascites had right ventricular hypertrophy (black arrow), Pericardial effusion (white arrow), and an oozing jelly-like substance (green arrow).

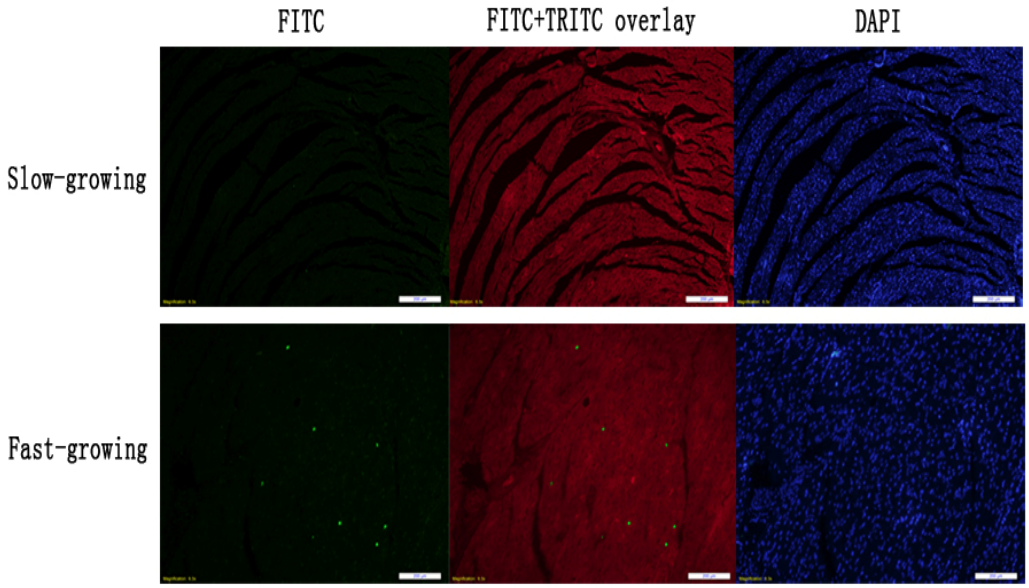

Fig. 2: Myocardial sections stained by the *in situ* DNA ligase method. Apoptotic nuclei are shown by bright green nuclear fluorescence (FITC). Sections were counterstained with anti-sarcomeric α-actinin antibody (TRITC) and DAPI to identify cardiac myocytes and nuclei.

Table 1: H_2O_2, CAT, MDA and SOD levels in serum of ascitic broilers

Parameters	SG/FG	2wk	3wk	4wk	5wk	6wk	7wk
H_2O_2 (mmol/L)	SG	85.93±2.78	86.44±2.68	86.99±2.32	90.07±2.46	96.17±1.71	100.37±2.80
	FG	86.68±2.36	88.81±2.99	91.37±2.91*	103.97±5.45**	120.76±6.93**	121.41±6.38**
CAT (U/ml)	SG	2.82±0.04	2.75±0.03	2.75±0.03	2.69±0.05	2.55±0.07	2.48±0.06
	FG	2.77±0.05	2.72±0.04*	2.64±0.05**	2.59±0.06**	2.32±0.06**	2.26±0.1**
MDA (nmol/ml)	SG	1.84±0.03	1.88±0.03	1.89±0.03	1.92±0.04	1.98±0.06	2.04±0.04
	FG	1.86±0.04	1.90±0.04	1.93±0.05*	2.00±0.07**	2.38±0.13**	2.55±0.04**
SOD (U/ml)	SG	254.40±3.13	252.10±2.56	248.00±2.94	242.50±3.27	236.00±3.16	234.00±2.02
	FG	251.20±3.73	245.60±3.67*	221.30±3.16**	210.30±3.06**	200.50±4.42**	188.40±3.17**

SG=Slow-growing; FG=Fast-growing; * P<0.05, **P<0.01 compared with the slow-growing group.

Cardiomyocyte apoptosis detection: We investigated the effect of the elevated reactive oxygen species content in individual myocardial cells. In the fast-growing group, we randomly selected eight cardiac tissues from broilers that had significant right ventricular hypertrophy and ascites or no obvious clinical features. At the same time, three cardiac tissues were randomly selected from the slow-growing group as controls. As shown in Fig. 2, we detected a significant amount of cardiac myocyte apoptosis in the fast-growing group. No apoptotic cells were observed in the slow-growing group. To ensure reliability of the results, three slices repeat were carried out in experiment for each tissue.

DISCUSSION

The intensive selection of broilers for maximal body mass has resulted in anatomical and physiological limitations of blood through the lung with consequent insufficient oxygenation of the tissues. Exposure to cold conditions and a hypoxic environment enhances this imbalance between oxygen supply and consumption (Gupta, 2011). This contradiction between metabolic oxygen requirement and tissue hypoxia leads to metabolic disorders, right ventricular hypertrophy and ascites syndrome.

Excessive abdominal fluid accumulation results in a reduction in the volume of air that is exchanged per respiration. In the current study, in chickens that developed ascites, fluid accumulated very rapidly once it was observed that chickens were always panting and had skin cyanosis (especially the comb and wattles). In the fast-growing group, chickens with ascites had many common characteristics. As well as right ventricular hypertrophy, we also discovered that clots of fibrin were usually present in pericardial cavities and on the surface of liver, sometimes accompanied by a congested liver. In addition, the pericardial and the intestinal peritoneal cavities frequently contained fluid.

During the normal oxidative metabolic process, approximately 1 to 2% of oxygen is converted to various reactive oxygen species (Sheeran and Pepe, 2006). Under hypoxic environment, there are reductions in the concentrations of the natural antioxidants SOD, ascorbic acid and glutathione peroxidase in both the liver and lung, which significantly reduce their ability against reactive oxygen production in these tissues. This fact is consistent with our results. The current study found that before H_2O_2 and MDA levels were increased from the 4th week in the fast-growing group, CAT and SOD levels were already decreased from the 3rd week. We speculate that the increase in H_2O_2 and MDA levels was caused by the decline in CAT and SOD levels. Since reactive oxygen species scavenge enzymes, the decrease in CAT and SOD levels may have been due to metabolic disorders caused by the excessively high growth rate, long-term hypoxia and the cardiovascular system, as well as liver damage of the broilers.

Several findings from the present study suggested that the deterioration of heart function in broilers with ascites syndrome was associated with lipid peroxidation, which was likely caused by reactive oxygen species (Nain *et al.*, 2008). Lipid peroxidation can alter the properties of cellular membrane and organelles crucial for maintenance of normal cardiomyocyte function. We detective the effect of the elevated reactive oxygen species content on individual myocardial cells. Surprisingly, we found cardiomyocyte apoptosis in myocardial tissue of the fast-growing group broilers. However, there was no apoptosis in the slow-growing group.

Cardiomyocyte apoptosis could be the result of lipid peroxidation caused by the increase in reactive oxygen species. In cardiac tissue from ascitic birds, many of the mitochondria have extensive ultrastructural damage (Maxwell *et al.*, 1996). Lipid peroxidation caused by reactive oxygen species within cell membranes has been identified as playing a significant role in the etiology of pulmonary hypertension (Bottje and Wideman, 1995). As a product of lipid peroxidation, MDA levels indirectly reflect the extent of damage to cells caused by reactive oxygen species. In our study, MDA content was remarkably increased from the 4th week compared with that in the slow-growing group. This could have been due to myocardial apoptosis in ascitic broilers, which was caused by lipid peroxidation from reactive oxygen species.

To reduce harm to myocardial cells caused by reactive oxygen species, researchers have attempted to alleviate the onset of ascites by increasing antioxidant substances. Roch *et al.* (2000) have confirmed that the combination of higher dietary levels of vitamin E and organic selenium (250 IU vitamin E and 0.3 ppm selenium yeast) significantly reduce the mortality caused by Pulmonary hypertension syndrome and improve body weight gain feed in cold-stressed broilers. Vitamin E may protect the birds by limiting the damage caused by reactive oxygen species to cellular DNA or act as a direct radical scavenger during acute oxidation of mitochondria. Furthermore, supplementation of vitamin C in broilers fed 400 mg/kg can effectively reduce lipid peroxidation in cardiac tissue (Nain *et al.*, 2008).

To date, most of the hypotheses regarding the etiology of ascites in broiler chickens have focused on lesions in right ventricular and right ventricular failure with the putative cause being primary pulmonary hypertension (Olkowskia *et al.*, 1999). However, our

experiments showed that myocardial cells were very sensitive to the insult of reactive oxygen species, and this insult resulted in apoptosis of individual cardiac myocytes. Myocardial cells are highly differentiated cells that typically do not replicate after birth, and loss of myocardial cells results in a loss of myocardial function (Wakeno *et al.*, 2006; Labovsky *et al.*, 2010), which contributes to the development of heart failure (Ripa *et al.*, 2006).

In conclusion, in the present study there were three important discoveries. First, hypoxia is a more effective and better way to induce ascites compared with a low-temperature environment and high growth rate method to induce ascites. Second, reactive oxygen species serum levels are remarkably increased in ascitic broilers, while antioxidant levels are significantly decreased. Third, high levels of reactive oxygen species lead to individual cardiac myocyte apoptosis and contribute to the development of heart failure. This could be one of the reasons for heart failure in ascitic broilers.

Acknowledgement: We thank Professor Jiakui Li for providing venues and facilities in Tibet's Nyingchi prefecture. This study is supported by the Research Fund for the Doctoral Program of Higher Education of China (20100146110001).

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