Effect of Low Ambient Temperature on the Concentration of Free Radicals Related to Ascites in Broiler Chickens*

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ABSTRACT : A flock of Arbor Acres chickens were reared in cages and provided with high energy pelleted feed. At 14 d of age, a total of 350 birds were separated into 3 groups randomly as follows: 100 birds were exposed to ambient temperature of 20°C as a control group, 150 birds were exposed to lower ambient temperature of 11°C to induce ascites (group I), and another group of 100 birds were exposed to lower ambient temperature of 11°C and fed diet containing 1% L-arginine for ascitic prophylactic treatment (group II). Blood and tissue samples (lung and liver) were collected from chickens at 3, 4, 5, 6 and 7 wk of age subsequently, to analyze the concentration and activities of free radicals, mononaldehyde (MDA), superoxide dismutase (SOD), Nitric Oxide (NO) and Nitric oxide synthase (NOS). The results showed that the prevalence of ascites in the control, group I and group II was 3%, 9.33% and 3% respectively ($p<0.01$). The concentration of free radicals in the lungs of 3 wks old preascitic broilers in group I was significantly higher than in the corresponding control group ($p<0.05$). The concentrations of free radicals in lung and liver in the 7 wk period, and that of NO and SOD in the plasma were significantly lower in group I than in the control group ($p<0.01$). However, the accumulated MDA contents in group I were higher than in the control group and group II ($p<0.05$), respectively. In the same way, the activity of NOS in group II was higher than both group I and control group ($p<0.01$) during the 7 wk period. There was no significant difference between SOD activities of group II and the control group ($p>0.05$), and also insignificant difference between NOS in group I and the control group ($p>0.05$). The results of this study indicate that there was a significant decrease in the concentration of MDA in group II. On the other hand, the concentration of free radicals decreased and MDA concentration increased in group I during the 7 wk period. The reduction in concentration of MDA in group II, following arginine supplementation may be associated with the scavenging activity of NO. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 8 : 1182-1187)***

Key Words : Ascites in Broilers, Free Radicals, MDA, SOD, NO, NOS, L-arginine

INTRODUCTION

Oxygen free radicals have been implicated as inducers of tissue injury in several pathologic conditions such as heart failure, right ventricular hypertrophy, ischemia-reperfusion, and stress. Oxygen-derived free radicals are generated by a number of cellullar reactions (Fridovich, 1978; Shah and MacCarthy, 2000). O_2^- is catalyzed to H_2O_2 by superoxide dismutase (SOD). H_2O_2 is then catalytically reduced in the cell to H_2O by catalase or glutathione peroxidase. During brief starvation, it is not only that the production of MDA in liver is increased (Dorota et al., 2003), but that there is also a loss of both SOD and CAT, which would result in an increase in the level of oxygen free radicals.

Ascites syndrome is a condition in which the body cavity is filled with serous fluid, leading to death or potential carcass condemnation. Several situations are known to influence the occurrence of ascites in broilers: the housing environment (temperature levels, respirable dust, carbon dioxide, and oxygen), rapid growth rates, high basal metabolic rate, and high energy rations (Julian, 1993; Julian, 2004). Physiologically, these conditions can create an oxygen deficit, which results in an increased cardiac output. The increase in blood flow results in increased pulmonary arterial pressure. The oxygen deficit causes increased hemoglobin concentration, packed cell volume, and erythrocyte number. These changes result in higher blood viscosity and may lead to pulmonary hypertension (Mirsalimi et al., 1993). Primary pulmonary hypertention triggers a pathophysiological progression leading sequentially to right-sided congestive heart failure, central venous congestion, pressure-induced cirrhosis of liver, and transudation of fluid into the abdominal cavity (ascites) (Julian, 1993; Wideman et al., 1995). Historically, the ratio of the right ventricle to the total ventricle mass, packed cell volume, and abdominal fluid have been used to access the index of ascites, but seldomly related to oxygen free radicals. Oxygen free radicals have been suggested to exert their cytotoxic effect by causing peroxidation of membrane phospholipids, which can result in an increase in membrane fluidity, increasing permeability, and loss of membrane integrity. The effect of oxygen free radicals on

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Table 1. Percentage diet composition with calculated nutrient content

Pelleted diet	ME kcal/kg	CP (%)	Ca (%)	Pi (%)	Crude fiber (%)	Crude ash (%)	Arginine (%)	Lysine (%)
Starter diet	3,990	21.0	1.0	0.4	6.0	8.0	1.22	1.27
Grower diet	3,885	19.0	1.0	0.4	6.0	8.0	1.08	1.24

cardiovascular. pulmonary functional ascites in broilers is not clear. A recent study (Bottji et al., 1995a, b) showed that, in the majority of chickens that developed ascites, the free radicals contribute, at least in part, to the pathogenesis of pulmonary hypertension, in agreement with this view. Iqbal et al. (2001, 2002) also reported that the ascites was associated with oxidative stress. Although considerable attention has focused on the ascites, little is known about how free radical activity may affect pulmonary function during the ascites. The objective of this trial deals with the effects of cold induce oxygen free radicals on plasma concentration of MDA, SOD, NO and NOS in broiler chickens.

MATERIALS AND METHODS

Animals

350 day-old broilers purchased from a commercial hatchery were housed in one cage of 30 birds each over two wk, they were brooded at 32 and 23°C during the first 2 wks, and were randomly divided into 3 groups from 3wk of age. Control group (n = 100), the ambient temperature of 20°C begun on day 15, and maintained until the experiment terminated on day 49. In group I (n = 150), and group II (n = 100), the ambient temperature of both of them was decreased from 23 to 11°C at 3 wk of age and was maintained till day 49. All birds received a standard pelleted broiler starter diet from 1 to 3 wk of age, a grower diet from 4 to 7 wk of age. Chickens in Group II were fed the same diet to which 1% arginine was added prior to pelleting from 3 to 7 wk of age. The composition of the pelleted rations is presented in Table 1. Feed and water were provided *ad libitum*.

Vaccination

All birds were vaccined using Newcastle (N_{79}) and infectious bursa disease (IBD) through eye and nasal route at 7 and 21 days of age.

Sample collection schedule and biochemical measurements

Blood was collected in heparin containing tubes from heart at the end of 3, 4, 5, 6 and 7 wks. After centrifugation at 3,000 rpm for 10 minutes, the supernatant was transferred to another tube, and placed at -20°C for later measurement of MDA, SOD, NO. Meanwhile, randomly selected preascites and ascites chicks of the control group and group I were killed respectively, and liver and lung collected for immediate oxygen free radicals measurement. Mononaldehyde (MDA) assay method was essentially the same as the one described by Yagi (1976). Superoxide dimutase (SOD) assay was determined using xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium to form a red formazan dye. The superoxide dismutase activity is then measured spectrophotometrically (505 nm and 30°C) as the degree of inhibition of this reaction (Nebot et al., 1993). Nitric Oxide (NO) was analyzed through the reduction of nitrate to nitrite (Green et al., 1987). Nitric oxide synthase (NOS) activity was measured from the conversion rate of L-arginine to L-citrulline (Wang et al., 1995).

Measurement protocol of oxygen free radicals

Liver and lung samples of 0.2 g each were weighed immediately, and 0.4 ml spectra of spin trapped by PBN (N-t-butyl-a-phenylnitrone, PBN, Sigma Chemical Co.) were added to organic grinder together for homogeneity, the mixture fluid transferred to centrifuged tube. After centrifugation at 2,000 rpm for 2 minutes, the 0.2 ml supernatant was transferred to quartz capillary tube (inner diameter 3 mm, outer diameter 4 mm, length 150 mm), the tube placed at -170°C liquid nitrogen for measurement of oxygen free radicals. The samples measured on the Bruker-ESP200D-SRC electron spin resonance (ESR) (Bruker Co., German). The parameters X wave, 20°C, Frequency 100 kHz, Field modulation intensity 1 Gauss, Gain 1-105, scan time 100 m, microwave frequency 9.48 Gauss/m, microwave power 20 mW, mid range 3376 Gauss, scan range 100 mT, aN = 27.5Gauss, aH = 1.65Gauss, g = 2.0074.

Statistical analysis

Statistical analysis of the results was performed with the use of SAS statistical software by analysis of variance (SAS Institute, 1994). A p value <0.05 was considered significant. All results are presented as mean±SD.

RESULTS

Status of occurrence

Group I with low ambient temperature resulted in a significant increase of mortality from ascites (9.33%). Group II with low ambient temperature and fed diet containing 1% L-arginine, however, showed a significant reduction in mortality from the syndrome (3%) compared with Group I. The maximum mortality rates from the

Table 2. The incidence of ascites in broiler chicken

Groups	Numbers	No. of sick chickens every week					Total sick No.	Incidence (%)
		3 wk	4 wk	5 wk	6 wk	7 wk		
Control group	100	0	0	0	2	1	3	3%
Group I	150	0	0	2	3	9	14	9.33%
Group II	100	0	0	0	2	1	3	3%

Figure 1. The same 6 wk age chicken (left: group II, body weight 1.662 kg, middle: group I, body weight 1.205 kg, right: control group, body weight 1.875 kg).

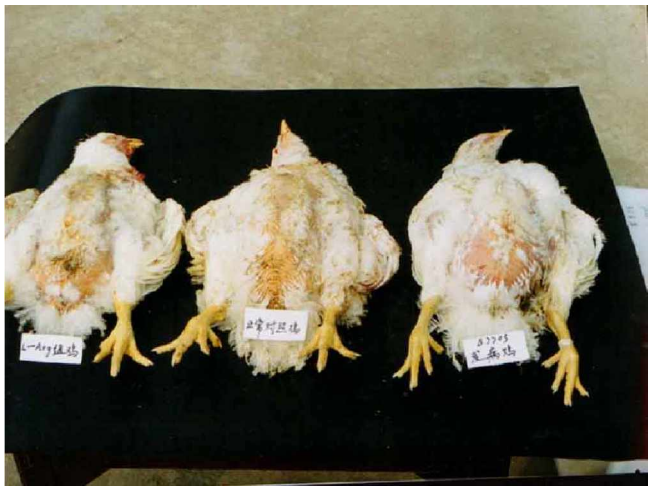

Figure 2. The necropsy finding of 6 wk age chicken (left: group II, middle: control group, right: group I).

syndrome were seen during the seventh week of age (Table 2), the control birds were without overt symptoms of ascites.

Clinical finding

The body weight of group I was significantly lower than any other groups at the same week (Figure 1). The syndrome was mainly characterized by accumulation of fluid in the abdominal cavity (Figures 2 and 3), ducklike locomotion, some of them exhibited open-mouth respiration, hematocrit elevation, and finally death. Prominent pathological changes were observed in the hearts from all birds that developed ascites, chicken showed dilation of the ventricle, especially right ventricular hypertrophy (Figure 4).

Figure 3. The ascites of broiler chicken in group I.

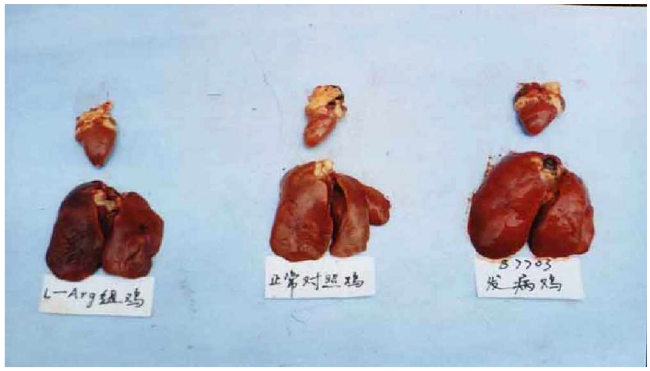

Figure 4. The heart and corresponding liver in the same age chicken (left: group II, middle: control group, right: group I).

swelling and atrophy of liver, other clinical signs were not noted.

ESR spectra of spin trapped by PBN (See Figures 5 and 6)

Free Radicals contents of lung and liver in broiler chicken

The results of free radicals were given in Table 3. The free radicals content of lung in group I preascites broilers increased significantly than the control group at 3 wk of age. However, the changes of free radicals in lung and liver of ascites in group I at 7 wk of age were significantly lower than the control group.

Plasma mononaldehyde (MDA) contents in broiler chicken

There was a tendency for increase in the plasma MDA

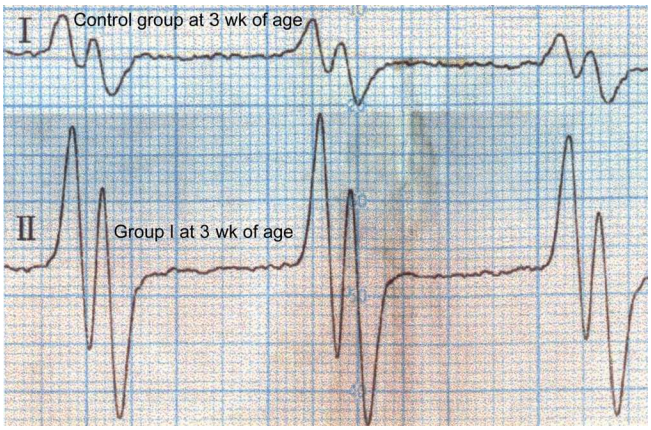

Figure 5. ESR spectra of spin trapped by PBN from lung at 3 wk of age: I control group; II group I.

Table 3. Free radicals contents of lung and liver in broiler chicken (mm/0.2 g)

Groups	Lung (3 wk)	Liver (7 wk)	Lung (7 wk)
Control	32.0 ± 12.5^{a}	365.0 ± 104.8^{A}	270.2 ± 121.1^{A}
Group I	43.6 ± 13.2^{b}	35.9 ± 18.3^{B}	17.1 ± 10.3^{B}

$^{A, B}$ Means within column indicates are significantly difference (p 0.01).
$^{a, b}$ Means within column with no common superscripts are significantly different (p 0.05), n – 7.

in group I and group II. also. the changes were significantly higher than the correspondent control group ($p<0.01$. $p<0.01$) (Table 4). Although MDA contents in group II at 3. 4 wk of age were not comparable to group I ($p>0.05$. $p>0.05$). it was significantly lower than the group I at the age of 5. 6 and 7 wks ($p<0.05$. $p<0.01$. $p<0.01$). The MDA of ascites in broilers plasma was significantly higher than the control group.

Plasma superoxide dismutase (SOD) activities in broiler chicken

As shown in Table 5. there was a decline in group I. particularly at the age of 6 and 7 wks. the SOD values were decreased significantly in group I comparing with control group ($p<0.05$). However. the SOD activity in group II coincides with control group ($p>0.05$).

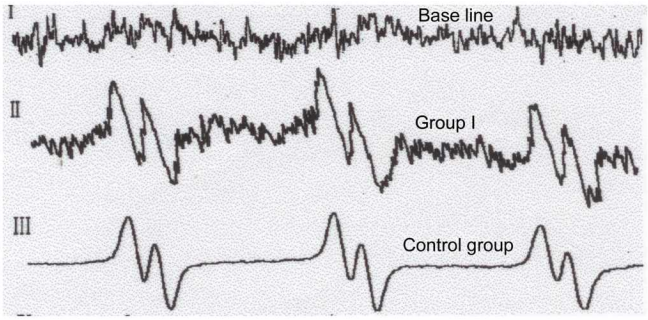

Figure 6. ESR spectra of spin trapped by PBN from lung and liver at 7 wk of age. I: base line; II: group I, the sensitivity of both them is 10 times for control group III: control group.

The changes of plasma NO contents in broiler chicken

After initial transient increased plasma NO level in group I at 3 wk of age. it began to decline at 4 wk of age. Although the remaining sustained higher. the finally NO value was rapidly decreased. The changes of plasma NO value in group II were higher than group I and similar to control group at the 4. 5. 6 wk of age (Table 6). but the values decreased in later 7 wk of age. The overall NO values in group I were less than the control group ($p<0.01$). The plasma NO of ascites in broilers was lower than the control group.

The changes of plasma NOS contents in broiler chicken

The NOS activities in group I were not significantly different with control group (Table 7). However. the NOS activities in group II were sustained higher than group I and control group during the 7 wks period ($p<0.01$. $p<0.01$. $p<0.01$).

Table 4. Plasma mononaldehyde (MDA) contents in broiler chicken (nmole/ml)

Groups	3 wk	4 wk	5 wk	6 wk	7 wk
Control	2.11 ± 0.26^{A}	2.76 ± 0.84^{A}	2.71 ± 0.95^{A}	2.86 ± 1.02^{A}	3.96 ± 1.20^{aA}
Group I	4.25 ± 1.42^{B}	3.86 ± 1.11^{B}	4.04 ± 0.81^{bB}	4.23 ± 1.33^{B}	4.69 ± 1.43^{B}
Group II	3.99 ± 0.57^{B}	3.55 ± 1.23^{B}	3.00 ± 1.22^{c}	3.24±1.40	3.50 ± 1.16^{c}
Ascites in broilers			13.53±0.30 (n = 2)	10.60±0.60 (n = 3)	6.63±2.08 (n = 9)

$^{A-C}$ Means within column with no common superscripts are significantly different (p 0.01).
$^{a-c}$ Means within column with no common superscripts are significantly different (p 0.05), n – 10.

Table 5. The changes of plasma SOD activities in broiler chicken (unit/ml)

Groups	3 wk	4 wk	5 wk	6 wk	7 wk
Control	51.01±26.12	65.04±13.67	60.67±22.34	91.93±14.56	78.82 ± 11.52^{a}
Group I	66.74±24.68	57.12±16.98	60.15±24.97	88.04±22.51	68.83 ± 18.81^{b}
Group II	69.08±22.55	72.98±21.16	63.75±29.41	97.77±23.19	85.49 ± 23.93^{a}
Ascites in broilers			63.77±18.13 (n = 2)	107.53±36.82 (n = 3)	77.58±26.53 (n = 9)

^{a,b} Means within column with no common superscripts are significantly different (p : 0.05), n – 10.

Table 6. The changes of plasma NO contents in broiler chicken (umol/L)

Groups	3 wk	4 wk	5 wk	6 wk	7 wk
Control	11.228±5.40^{A}	27.847±10.07^{A}	27.281±9.37^{a}	31.750±6.21^{A}	34.123±14.42^{A}
Group I	29.123±7.07^{B}	11.696±7.23^{B}	28.089±9.19^{a}	23.318±7.24^{B}	22.506±11.24^{bB}
Group II	24.211±12.14^{B}	20.175±9.63^{C}	31.813±14.90^{b}	24.912±9.62^{B}	18.105±6.51^{a}
Ascites in broilers				30.877±1.22 (n = 3)	21.895±8.68 (n = 9)

^{A,C} Means within column with no common superscripts are significantly different ($p<0.01$).
^{a,b} Means within column with no common superscripts are significantly different ($p<0.05$). n = 10.

Table 7. The changes of plasma NOS activities in broiler chicken (IU/ml)

Groups	3 wk	4 wk	5 wk	6 wk	7 wk
Control	0.790±0.38	1.922±1.69	1.785±1.25^{A}	1.527±1.01^{A}	1.976±1.34^{A}
Group I	0.624±0.35	0.911±0.67	1.420±0.81^{A}	1.683±1.06^{A}	2.081±1.41^{A}
Group II	0.931±0.24	0.995±0.64	2.586±0.28^{B}	2.832±1.57^{B}	4.795±2.22^{B}
Ascites in broilers				2.370±0.94 (n = 3)	1.860±1.20 (n = 9)

^{A,B} Means within column with no common superscripts are significantly different ($p<0.01$). n = 10.

DISCUSSION

Generally, the pathogenesis of ascites in broilers raised at low temperature is thought to accelerate ascites syndrome in broilers resulting from hypoxaemia (Han et al., 2004a). A similar changes were observed by Moreno and Hernandez (2003) in broilers that hypoxia induced ascites is associated with a decrease of endothelial-derived NO expression in pulmonary vessels, and by Cawthon et al. (2004) provide further characterization of the altered cellular oxygen utilization in broilers with ascites. In a simplified schematic outline the key features leading to the development of ascites are as following: rapid growth, increased oxygen requirement, hypoxia increased cardiac output, tissue hypoxia and hypoxia-triggered pulmonary vasoconstriction, or a chain of metabolic events resulting in pulmonary hypertension. The ensuing pathological changes were the causes of ascites. However, this is a hypothetical pathogenesis, which is based on the association of the pathological right ventricular hypertrophy with the most probable cause of ascites syndrome in broilers.

The term free radicals (called reactive oxygen species, ROS) is used to include not only O_2^- and OH^- but also non-radical derivatives of O_2, such as singlet oxygen ($^1O_2$), NO and hydrogen peroxide (H_2O_2). ROS have been shown directly to damage tissue, including pulmonary endothelium and supportive structures (Barnes, 1990) and increase pulmonary vascular pressures, both by enhancing the release of vasoconstrictive mediators (Barnes, 1990; Kehrer, 1993) and by disrupting the activity of NO (Seccombe et al., 1994). Pulmonary structural damage and hypertension induced by ROS may be two of factors that contribute to pathogenesis of ascites.

The g value is important for free radicals species determination, in the present study we obtained g value is 2.0074 within the ROS range of 2.0050-2.0074. The wave of ascites in broilers was 10 times less than control group. From the present data it might be suggested that the ROS results were also similar to Zhang et al. (2000) reports. MDA plays a very important role in ascites, because the MDA of induced ascitic broilers was apparently higher than the control group. This is significantly in response to a substantial increase in metabolic rate and oxygen consumption. It appears that ROS directly damage pulmonary structures, particularly the endothelium and degrade alveolar surfactant with subsequent collapse of the alveoli and a significant increase in pulmonary vascular resistance, they probably play a significant role in chicks pulmonary arterial hypertension. Also ROS depress the myocardial contractility and increase the tone in the peripheral vasculature, and leading to right ventricular hypertrophy. It would be erroneous to suggest that ROS are the primary mediators of the pulmonary disease response. Lipid peroxidation of biomembranes exacerbates the damage to pulmonary structures and releases lipid inflammatory mediators, which constrict pulmonary vasculature, release potent chemoattractant (Barnes, 1990). Pulmonary vascular tone is controlled by the vascular endothelium, the presence of ROS is known to affect pulmonary vascular tone. Iqbal et al. (2001) provided very elegant and convincing arguments for the possible involvement of ROS in the pathogenesis of ascites. It is noteworthy that ROS can be generated under hypoxemic conditions and can result in oxidative lesions in the tissue (Park and Kehre, 1991; Dawson et al., 1993; Han et al., 2004b). The probability of oxidative damage to the broilers' cardiomyocytes is further raised by a recent study of Maxwell et al. (1996) who showed that ultrastructural damage to the cardiomyocytes' mitochondria in ascitic birds may be associated with hydrogen peroxide activity.

NO is synthesized from L-arginine and O_2 catalysed by the activity of nitric oxide synthase (NOS). NO has a wide range of physiological and pathological activities, including

inhibition of platelet function, host defence, depression of the phagocytic cells, bronchodilation and neurotransmission. It regulates vascular tone in both systemic and pulmonary cumulation by activating soluble or cytosolic guanylate cyclase within vascular smooth muscle, initiating relaxation (Shah and MacCarthy, 2000). In our study, chicks exposed to cold remain decreased NO, the importance of NO to vascular flow becomes apparent when its synthesis or release is inhibited. A deficiency in the activity of endothelial NOS may be responsible for pulmonary hypertension. It has been suggested that ROS may initiate pulmonary hypertension during ascites as vascular endothelial cells are the primary targets of oxidant injury (Sala et al., 1993). ROS deactivates NO and inhibits the activity of NOS (Rengasamy and Johns, 1993). The present study also indicates that the diets supplemented 1% arginine can increase NO, antioxidant defences are thus essential to the activity of NO, as well as protecting the endothelial cell from oxidant injury.

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