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Antioxidant Enzyme Activities Characterization in Pulmonary Hypertension Syndrome (PHS) in Broilers

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Abstract: Major objectives of this study were to assess antioxidant protection in plasma and whole liver in broilers with pulmonary hypertension syndrome. The experiment was conducted with 160, 1 day old male broilers (Ross 308). The chickens were divided in 2 groups of 4 replicates and 20 chicks for any replicate. One group of these chickens was raised in normal temperature (control without PHS) treatment and the other group was raised in cold temperature (with PHS) treatment for induce pulmonary hypertension syndrome. Mortality was inspected to determine cause of death and diagnose of PHS. For evaluation of antioxidant status, the parameters that were determined in plasma and liver include: Glutathione Peroxidase (GPX), Superoxide Dismutase (SOD), Total Antioxidant Status (TAS) and Malondialdehyde (MDA). Sampling of blood and liver tissue were determined at day 21 and 42. At 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 then ratio of Right Ventricle weight to Total Ventricle weight (RV/TV) calculated too. The results of the experiment indicated that in plasma (GPX) and (SOD) activity were elevated in broilers with PHS compared to controls. It was also, PHS group had low TAS compared to control. But in liver, just GPX (not for SOD) was elevated in PHS group than control. The levels malondialdehyde equivalents an indicator of lipid oxidation subsequent to generated oxidative stress at plasma and liver tissue was significantly higher in PHS group compared control. RV/TV ratio and mortality due to ascites, also were significantly affected by treatments as PHS group had greater RV/TV and mortality due to ascites mortality percentage compared to control.

Key words: Oxidative stress, antioxidant enzymes, ascites, mortality, broiler, GPX, MDA

INTRODUCTION

Modern strains of broilers are able to achieve market weight in <60% time than broilers from 40 years ago. Nevertheless, pulmonary and cardiac capacity of modern broilers is very similar compared with the old broiler strains which forces the cardiopulmonary system to work very close to its physiological limit. Consequently, pulmonary hypertension syndrome (PHS, ascites) is a common problem that causes considerable mortality (Julian, 1993). Oxidative stress is also involved in the pathophysiological progression leading to ascites (Maxwell *et al*., 1986).

Oxidative stress occurs when forces that favor oxidation outweigh antioxidant protection within cells (Yu, 1994). The involvement of oxidative stress in Pulmonary Hypertension Syndrome (PHS), in broilers has now been clearly demonstrated by findings that broilers with PHS exhibit increases in oxidized glutathione (GSSG), or the ratio of oxidized to reduced glutathione (GSSG/GSH) in tissue, increases in plasma lipid peroxides and decreases in major nonenzymatic antioxidants such as GSH, α-tocopherol and ascorbic acid in liver and lung (Enkvetchakul *et al*., 1993; Bottje and Wideman Jr., 1995; Bottje *et al*., 1995, 1997).

During the normal oxidative metabolic process, various Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are produced. During this normal metabolism, 1-2% of oxygen is converted to ROS (Sheeran and Pepe, 2006). ROS are known to be the cause of different disorders including thermal injury, inflammations, sepsis, mutagenesis, carcinoma, autoimmune diseases and ischemia reperfusion injury (Flohe *et al*., 1985; McCord, 1985; Halliwell, 1989). The role of ROS in the injury induced by ischemia reperfusion has been convincingly shown in different organs including 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 where the generated reactive

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species can alter the properties of lipids, proteins and nucleic acids, leading to cellular dysfunctions. Recent research findings from different laboratories suggest that ROS and RNS play a critical role in 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 subcellular organelles (mitochondria and sarco-endoplasmic reticulum) crucial for 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 the protein of contractile apparatus such as myosin and troponin T into the circulation (Maxwell *et al*., 1994). Oxidative stress changes activities of antioxidant enzymes. Recently, researchers have reported mitochondrial dysfunction in PHS characterized by increased radical production and oxidative stress (Iqbal *et al*., 2001a, b). Therefore, a major objective of this study was to extend these findings by determining antioxidant status (TAS), MDA levels and activities of antioxidant enzymes GSH-Px (GPX) and SOD in plasma and liver tissue obtained from broilers with or without PHS.

MATERIALS AND METHODS

Birds and diets: About 160, 1 day old male broiler chickens (Ross 308) were used in this experiment. Chickens allocated randomly in to 2 treatments groups with 4 replicates each and 20 chicks per replicate (per cage).

Two groups including broilers under normal (control) and cold environmental temperature (with PHS). All chicks were fed a basal corn-soybean meal diet including 22.04% CP and 3,200 kcal kg^{-1} of ME (1-21 days) or 20.26% CP and 3,200 kcal ME (22-42 days). Feed and water provided *ad libitum*.

Management and measurements: Broilers were divided in two groups (control and PHS). Broilers of control group were reared under normal temperature until end of experiment. For inducing ascites, the birds of the PHS group were raised under 32 and 30°C during week 1 and 2, respectively. The house temperature was decreased to 15°C during week 3 and maintained between 10 and 15 for the rest of the study (Iqbal *et al*., 2001a, b).

Mortality was recorded daily and all of the dead birds inspected for diagnosis of ascites. Diagnosis of ascites generally depends on observation of the following symptoms; right ventricle hypertrophy, cardiac muscle laxation, swollen and stiff liver and clear, yellowish, colloidal fluid in the abdominal activity (Geng *et al*., 2004).

Sampling: At day 21 and 42, one chick from each replicate was randomly chosen and after 3 h starvation, blood sampling from wing vein. After blood sampling, the bird were killed and thorax and abdomen were open sampling from liver tissue for antioxidant status evaluation and inspected for signs of heart failure and ascites. At the end of the experiment (week 6), 2 chickens from each replicate (pens) were randomly slaughtered. At the end of the experiment (week 6), 2 chickens from each replicate (pens) were randomly slaughtered.

The heart was removed and the right ventricle was dissected from the left ventricle and septum. The right and left ventricles were weighed separately and the ratio of Right Ventricular (RV) weight to Total Ventricular (TV) weight (RV/TV) was determined. Bird having RV/TV values >0.299% were considered to have ventricular hypertrophy (Julian, 1990). Blood samples were collected and centrifuged and plasma was collected and stored at -80°C until measurement of the other enzymatic and chemical analysis.

Blood and liver antioxidant indices: Blood and liver tissues were used for determination of antioxidant enzyme activities. Glutathione peroxidase, superoxide dismutase and total antioxidant capacity were detected spectrophotometerically using Rancel, Ranceland Randox kits, respectively. The activity of GSH-Px was determined according the method of Pagali and Valentine with some modifications. Glutathione Peroxidase (GPX) catalyses the oxidation of Glutathione (GSH) by cumene hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm for 5 min (25°C) is measured.

The liver homogenate (50 μL) was incubated with 25 mM potassium phosphate, 0.5 mM EDTA, pH 7.4, 0.5 mM $NaNO_3$, 0.3 mM NADPH, 0.64 U GSH-Rd and 1 mM GSH. The reaction was started with 0.1 mM cumene hydroperoxide. Values were corrected for nonenzymatic oxidation of GSH and NADPH by hydrogen peroxide. The result were expressed in unit of GSH-Px activity using a molar extinction coefficient of GPX U/L of sample = 8412 $\times\Delta$A 340 nm min^{-1} (ΔA = Deference blank with sample). The unit was defined as U mg^{-1} protein. For evaluation in blood whole blood (20 μL) was incubated with 0.5 mM potassium phosphate, 4.3 mM EDTA, pH 7.2, 4 mM GSH, 0.5 U GSH-Rd. The reaction was started with 0.18 mM cumene hydroperoxide. The unit was defined as U g^{-1} hemoglobin.

Plasma Superoxide Dismutase (SOD) activity was determined by Xanthine Oxidase (XOD) enzyme. This method employs xanthine and Xanthine Oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of (I.N.T) under the conditions of the assay. Where 0.5 mL of whole blood centrifuged for 10 min at 3000 rpm and then aspirated off the plasma. Then erythrocytes washed four times with 3 mL of 0.9% NaCl solution and centrifuged for 10 min at 3000 rpm after each wash. The washed centrifuged erythrocytes then increased to 2.0 mL with cold redistilled water, mixed and left to stand at +4°C for 15 min.

The lysate was diluted with 0.01 mol L^{-1} phosphate buffer pH 7.0 so that the % inhibition falls between 30 and 60%. Then, the lysate incubated for 5 min at 25°C with CAPS 40 mmol L^{-1}, pH 10.2, EDTA 0.94 mmol L^{-1}, Xanthine 0.05 mmol L^{-1}, I.N.T. 0.025 mmol L^{-1}, Xanthine Oxidase 80 U L^{-1}.The results were express as units of activity (U g^{-1} hemoglobin) (Randox ransod superoxide dismutase manual).

Liver Superoxide Dismutase (SOD) activity in liver was determined following the xanthine oxidase method described by McCord and Fridovich with modifications. Briefly, liver homogenate (50 μL) was incubated for 5 min at 25°C with 20 mM potassium phosphate; 1 mM EDTA, pH 7.8; 0.25 mM xanthine and 0.17 mM cytochrome c. The reaction was initiated by adding xanthine oxidase (0.16 U) and was assayed by following the reduction of cytochrome c at 550 nm for 5 min (25°C) in the presence or absence of xanthine oxidase and SOD. The results were expressed as units of activity (U mg^{-1} protein). One unit of the activity was defined as the amount of SOD that inhibited the rate of cytochrome c reduction by 50%.

For plasma Total Antioxidant (TAS) capacity, ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$. This has a relatively stable blue-green colour which was measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which was proportional to their concentration. For evaluation, serum (20 μL) was incubated at 37°C with 1 mL metmyoglobin chromogen (6.1 mM L^{-1}), ABTS 610 μmol L^{-1} then detected absorbance in 600 nm. Then this component incorporate with 250 μmol L hydrogen peroxide (25% μmol L^{-1}) and after 3 sec detected aberrance in 600 nm. Reduce amount in cationе $ABTS^+$ result in antioxidant would compared with standard. The results were expressed as units of activity (mmol L^{-1}).

For MDA determination, the blood was centrifuged at 1500×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. 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^{-1} of malondialdehyde dim ethyl acetyl.

Statistical analysis: The data were analyzed based on a completely randomized design using the GLM procedure of SAS. Duncan's multiple range were used to separate the means when treatments 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±SEM.

RESULTS AND DISCCUSION

Incidence of PHS: Overall during the course of this study, the mortality due to PHS between groups was significant different as PHS group had higher ($p<0.05$) mortality than control group (38% vs. 7.5%). Also, an indicator for incidence PHS, RV/TV ratio was higher ($p<0.05$) in PHS group than control (Table 1).

MDA equivalents levels in plasma and liver tissue: The MDA equivalents levels in plasma and liver tissue of broiler in two treatment groups are shown in Table 2. As shown the MDA equivalents levels showed that lipid

Table 1: RV/TV ration and mortality percentage of broilers with (PHS) and without PHS (control)

Treatments	RV/TV ratio	Total mortality due to ascites (%)
Control	0.22±0.02^b	7.5±1^b
PHS	0.31±0.01^a	38.0±4^a

Means within columns with different superscript letters are significantly different ($p<0.05$)

Table 2: MDA equivalents levels in plasma and liver tissue of broilers with (PHS) and without PHS (control)

Days	Treatments	MDA in plasma (nm m L^{-1})	MDA in liver (nm m L^{-1})
21	Control	1.30±0.31^b	0.85±0.03^b
	PHS	2.50±0.33^a	1.32±0.23^a
42	Control	1.60±0.20^b	1.10±0.04^b
	PHS	6.27±0.43^a	2.60±0.25^a

Means within columns with different superscript letters are significantly different ($p<0.05$)

Table 3: TAS equivalents and GPX and SOD activities in plasma of broilers with (PHS) and without PHS (control)

Days	Treatments	TAS (m mol L^{-1})	GPX (U g^{-1} Hb)	SOD (U g^{-1} Hb)
21	Control	0.71±0.10^{b}	33.00±1.92	735.0±102.0
	PHS	2.05±0.13^{a}	39.32±2.80	1371.0±265.0
42	Control	1.36±0.11^{b}	22.25±1.10^{b}	712.0±96.00^{b}
	PHS	2.97±0.25^{a}	30.72±0.82^{a}	913.0±112.0^{a}

Means within columns with different superscript letters are significantly different ($p<0.05$)

Table 4: GPX and SOD activities in liver of broilers with (PHS) and without PHS (control)

Days	Treatments	GPX (U g^{-1} Hb)	SOD (U g^{-1} Hb)
21	Control	0.23±0.02	7.40±0.22
	PHS	0.25±0.01	8.60±0.48
42	Control	0.19±0.01^{b}	8.75±0.41
	PHS	0.25±0.02^{a}	8.25±0.75

Means within columns with different superscript letters are significantly different ($p<0.05$)

peroxidation in both plasma and liver at day 21 and 42 were higher ($p<0.05$) in PHS group than control group.

GPX, SOD activity and TAS in plasma: As shown in Table 3, there is a significant different between groups in antioxidant activities in 42 days as SOD and GPX activities were higher ($p<0.05$) in PHS group compared to control group. Too, there was control group had higher TAS in both days 21 and 42 compared PHS group.

GPX and SOD activity in liver tissue: Finding from SOD and GPX activities in control and PHS groups are shown in Table 4. As shown the PHS group had higher ($p<0.05$) in GPX activities in 42 days and SOD activity was not significantly affected by treatments.

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 ascites (Buys *et al*., 1999). Cold temperature, despite increasing demand for oxygen consumption, leads to decreased ventilation and decreased oxygen availability in broiler houses (Buys *et al*., 1999). Hypoxemia initiates a cascade of events that results in ascites and death (Julian, 1993).

The antioxidant status of a cell or tissue is dependent upon a variety of factors that include the presence of a myriad of nonenzymatic and enzymatic antioxidants as well as forces that favor oxidation (Yu, 1994). Several studies indicate that oxidative stress is involved in the path physiology of PHS (Enkvetchakul *et al*., 1993; Bottje and Wideman Jr., 1995; Bottje *et al*., 1995, 1997). As the results indicate, higher production MDA in PHS group can implicate increased production of ROS theses chickens. These agents may cause lipid peroxidation in the membrane of the cells resulting in tissue injury in organs including lung and heart and liver (Arab *et al*., 2006).

Morphological changes observed in myocardial mitochondria the present study 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 measure the effects of free radicals is by measuring the oxidative damage (i.e., lipid peroxidation) to cellular membranes (Lykkesfeldt and Svendsen, 2007).

Increase in mortality and RV/TV ratio (Huchzemeyer and Deruyck, 1986) is often accompanied by ascites therefore, greater RV/TV (0.31 vs. 0.22) and mortality percentage (38 vs. 7.5%) of PHS birds than control birds in the study indicated the ascites development. Higher GPX and SOD activity in PHS birds in plasma and liver tissue could be due to up regulation of the expression of this enzyme and an important adaptive response to greater hydrogen peroxide production as a result of electron leakage from the respiratory (Iqbal *et al*., 2001a). Although, enzyme expression was not investigated in the present study, several studies have reported up regulation of GPX and SOD in response to oxidative stressing vascular endothelial, tracheobronchial epithelial and leukemia cells (Shull *et al*., 1991; Lu *et al*., 1993; Lee and Um, 1999).

Greater hydrogen peroxide production was observed in PHS birds than in healthy birds in plasma and liver tissue as a consequence of greater electron leak from the respiratory chain in liver and other tissues (Iqbal *et al*., 2001a).

These findings, therefore, indicate that PHS birds' liver experience greater degree of oxidative stress than do healthy bird's mitochondria that would potentate hydrogen peroxide formation and subsequently reduced in TAS. Lung mitochondria from PHS birds exhibited higher GPX that would help in catabolizing the greater hydrogen peroxide load (Iqbal *et al*., 2001a, b). Further studies are planned to examine expression of these antioxidant enzymes in PHS.

CONCLUSION

The results in the study, suggested that heart failure and mortality in broilers with hypoxia and subsequent PHS can associated with ROS production during oxidative stress. So, oxidative stress due hypoxia is the most initiate the problem with PHS. As ROS can cause cell injury and increase release enzyme in plasma including MDA in

plasma and liver tissue. ROS also can affect activities in antioxidant enzymes to neutralization theses free radicals and reducing oxidative stress.

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