

Effects of Glutamine Supplementation on Growth Performance and Antioxidant Status in Broilers with Pulmonary Hypertension Syndrome (PHS)

Research Article

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ABSTRACT

The present study examined the antioxidant defense of glutamine on plasma and liver antioxidant status in broilers under PHS with respect to changes in the levels of lipid peroxidation marker malondialdehyde (MDA), total antioxidant status (TAS) and the activities of anti peroxidative enzymes [superoxide dismutase (SOD) and glutathione dependent antioxidant enzymes glutathione peroxidase (GPX)] in the plasma and liver tissue. Sampling of blood and liver tissue were determined at d 21 and 42. At the end of the experiment, 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 the ratio of right ventricle weight to total ventricle weight (RV/TV) calculated too. Average BW gain and average feed intake were measured weekly from d 15 and average feed conversion ratio was calculated and reported weekly. The results showed that glutamine supplementation improve average feed conversion ratio ($P<0.05$). Moreover, in bird with PHS, supplementation of glutamine significantly ($P<0.05$) reduced the levels of MDA in plasma and liver tissue. A parallel increase glutathione peroxidase (GPX) activates in plasma and liver tissue. But SOD activity in plasma and liver tissue did not affected by glutamine supplementation. Also, glutamine significantly ($P<0.05$) reduced right ventricle weight to total ventricle weight (RV/TV) and mortality.

KEY WORDS ascites, antioxidant enzymes, glutamine, lipid peroxidation.

INTRODUCTION

Pulmonary hypertension syndrome (PHS) or Ascites is a metabolic disorder that mostly occurs in fast-growing broiler chickens. Broilers with PHS die due to right-sided congestive heart failure, which is associated with central venous congestion, pressure-induced liver cirrhosis, and transudation of ascetic fluid into the abdominal cavity (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 PHS in broilers has now been clearly demonstrated by findings that broilers with PHS exhibit increases in oxidized glutathione, or the ratio of oxidized to reduced glutathione in tissue, increases in plasma lipid peroxides, and decreases in major non enzymatic antioxidants such as GSH, α -tocopherol, and ascorbic acid in liver and lung (Enkvetchakul *et al.* 1993; Bottje and Wideman, 1995; Bottje *et al.* 1995, Bottje *et al.* 1997). One of the most important antioxidant molecules is glutathione. Since glutamine is a precursor of glutathione, its supplementation in the clinical diet can be used to maintain high levels of glutathione and to avoid oxidative stress

damage. Glutathione is the tripeptide g-glutamyl-cysteinyl-glycine. Since cysteine contains a sulfhydryl residue, and it is easily oxidizable, glutathione behaves as a very efficient sink of ROS. In a typical redox reaction, a ROS is reduced (and inactivated) through the generation of a disulfur bond between two glutathione molecules, yielding the oxidized glutathione pair. Once the ROS has been inactivated, two reduced glutathione molecules can be recovered through the enzyme reaction catalyzed by glutathione reductase (María Isabel Amores *et al.* 1999).

Glutamine, a multifaceted amino acid used as an energy substrate for most cells, is one of the principal free intracellular amino acids in mammalian heart cells (Rennie *et al.* 1994). It is important as a constituent of proteins and as a central metabolite for amino acid transamination via α -ketoglutarate and glutamate. It provides nitrogen for a number of biosynthetic pathways, serving as a precursor of the purine and pyrimidine rings of nucleic and nucleotides such as adenosine triphosphate (ATP) (Wilndmueller and Spaeth, 1974). Glutamine plays an important role in the nitrogen and carbon skeleton exchange among different tissues, where this amino acid fulfills many different physiological functions (Kovaccvic and McGivan, 1983). It has immunoregulative and cell-regulative capabilities, as recent investigations have shown (Roth *et al.* 2002). It also regulates endothelial nitric oxide metabolism in the heart tissue (Murphy and Newsholme, 1997). It is also involved in cell membrane stabilization, antioxidation, detoxification, and energy production (Rennie *et al.* 1994; Matilla *et al.* 2000; Fox *et al.* 1996). In the present study, we have investigated the role of glutamine the effects of glutamine on performance and plasma and liver antioxidant status in cold-induced PHS and heart failure in broilers.

MATERIALS AND METHODS

Birds and diets

One hundred sixty 1-d-old male broiler chickens (Ross 308) were used in this experiment. Chickens allocated randomly into 2 treatment groups with 4 replicates each and 20 chicks per replicate (per cage). All chicks were fed a basal corn-soybean meal diet, including 22.04% crude protein (CP) and 3200 kcal/kg of metabolizable energy (ME) (1 to 21 d), or 20.26% crude protein (CP) and 3200 kcal ME (22 to 42 d). Feed and water provided ad libitum. From d 1 to 14, all chicks were fed the basal diet (Table 1). From d 15, the diets were supplemented with diets were supplemented with 0 and 100, g/kg glutamine in the feed.

Average body weight gain (ABWG) and average feed intake (AFI) were measured weekly from d 15, and average feed conversion ratio (AFCR) was calculated and reported weekly.

Management and measurements

Broilers were divided into two groups (control and with glutamine). For inducing ascites, the all birds of both groups were raised under 32 °C and 30 °C during week 1 and 2, respectively.

Table 1 Composition of experimental diets

Item	Starter (0 to 21 d)	Grower (21 to 42 d)
Ingredients (%)		
Corn	54.47	59.25
Soybean meal (44% protein)	22.5	20.75
Corn gluten meal	7	8
Fish meal	6.16	3
Soybean oil	6	5.7
Dicalcium phosphate	1.72	1.22
Limestone	1.2	1.3
Vitamin and mineral premix ¹	0.5	0.5
Salt	0.25	0.25
DL-methionine	0.2	0
L-lysine	0	0.03
Total	100	100
Calculated analysis		
Metabolizable energy (kcal/kg)	3200	3200
Crude protein (%)	22.04	20.66
Calcium (%)	0.9	0.9
Available phosphorus (%)	0.4	0.35
Arginine (%)	1.3	1.3
Lysine (%)	1.14	1
Methionine (%)	0.53	0.4
Methionine + cystine (%)	0.9	0.75

¹ Supplied per kilogram of diet: vitamin A: 11000 IU; vitamin D₃: 5000 IU; vitamin E: 40 IU; vitamin K: 4 mg; vitamin B₆: 4 mg; vitamin B₁₂: 0.011 mg; Riboflavin: 5 mg; Niacin: 50 mg; Biotin: 0.01 mg; Thiamine: 3 mg; Zinc: 80 mg; Manganese oxide: 100 mg; Selenium: 10 mg and Iron sulfate: 80 mg.

The house temperature was decreased to 15 °C during week 3 and maintained between 10 and 15 for the rest of the study (Fathi *et al.* 2011; Fathi *et al.* 2012). 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: 1) right ventricle hypertrophy, cardiac muscle laxation; 2) swollen and stiff liver and 3) clear, yellowish, colloidal fluid in the abdominal activity (Fathi *et al.* 2011).

Sampling

At day 21 and 42, two chicks from each replicate was randomly chosen and after 3-h starvation, blood sampling from wing vein.

After blood sampling, the birds were killed and thorax and abdomen were open sampling of 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 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 RV/TV was determined. Bird is having RV/TV values more than 0.299% were considered to have ventricular hypertrophy (Julian, 1993). 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 spectrophotometrically using Rancel, Ranceland Radox kits respectively.

The activity GPX determination: 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 ethylenediaminetetraacetic acid (EDTA), pH 7.4, 0.5 mM NaNO₃, 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 results were expressed in unit of GSH-Px activity using a molar extinction coefficient of GPX U/L of sample = $8412 \times \Delta A_{340 \text{ nm/min}}$ (ΔA =deference Blank with sample). The unit was defined as U/mg protein. For evaluation in blood, whole blood (20 µL) was incubated with 0.5 mM potassium phosphate, 4.3 mM ethylenediaminetetraacetic acid (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 Hemoglobin.

Plasma 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. 0.5 mL of whole blood centrifuged for 10 minutes 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 minutes 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 minutes. The lysate was diluted with 0.01 mol/L 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, pH 10.2, EDTA 0.94 mmol/L, Xanthine 0.05 mmol/L, I.N.T. 0.025 mmol/L, Xanthine Oxidase 80 U/L. The results were expressed as units of activity (U/g Hemoglobin). (radox ransod superoxide dismutase manual).

Liver SOD activity in liver was determined following the xanthine oxidase method described by some studies 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 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 TAS capacity, ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxidase (metmyoglobin) and H₂O₂ to produce the radical cation ABTS⁺.

This has a relatively stable blue-green color, which was measured at 600 nm. Antioxidants in the added sample cause suppression of this colorful 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), ABTS 610 µmol/L then detected absorbance in 600 nm. Then this component incorporates with 250 µmol/L Hydrogen peroxide (25% µmol/L) and after 3 second detected aberrance in 600 nm. Reduce amount in question ABTS⁺ result in antioxidant would compared with standard. The results were expressed as units of activity (mmol/L).

For MDA determination, the blood was centrifuged at $1500 \times g$ 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 $3000 \times g$ 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 dim ethyl acetyl.

Statistical analysis

The data were analyzed based on a completely randomized design using the GLM procedure of SAS (SAS, 2002). Contrasts between treatments means were evaluated by Tukey's test at a significance level of 5%.

RESULTS AND DISCUSSION

Performance

Average Feed Intake (AFI), average body weight gain (ABWG) and average feed conversion ratio (AFCR) of control and glutamine treated birds are shown in Table 2. There were no significant differences for AFI and ABWG between 2 treatments at all weeks. But, Average feed conversion ratio was greater ($P<0.05$) just at whole period of study (3-6 week) in Control treated birds.

RV/TV ratio and mortality percentage

The RV/TV ratio (a better indicator of sustained hypertension) (31 vs. 28%) and total mortality percentage due to ascites during the whole period of study (38 vs. 25%) was significantly ($P<0.05$) greater in broilers of Control group as compared with broilers of Glutamine group (Table 3).

MDA assay in plasma and liver tissue

Findings from the level of MDA equivalents, an indicator of lipid peroxidation subsequent to generated oxidative stress, in plasma and liver tissue was higher ($P<0.05$) in broilers of Control group as compared with broilers of Glutamine group (Table 4).

Antioxidant enzyme activities in plasma

Table 5 depicts the levels of GPX and SOD and TAS in the plasma of Control and Glutamine broilers groups. There was a significant ($P<0.005$) increase in the activities of glutathione dependent antioxidant enzymes (GPX) in broilers with Glutamine was observed.

But, SOD activity and TAS levels in plasma was not affected by treatments.

Antioxidant enzyme activities in liver tissue

The levels of GPX and SOD in liver tissue of control and glutamine broilers groups are presented in Table 6. There was a significant increase in the activities of glutathione dependent antioxidant enzymes (GPX) in broilers with Glutamine was observed. But, SOD activity in liver tissue was not affected by treatments.

In the present study, it was clearly demonstrated that dietary Glutamine supplementation significantly reduced RV/TV index and ascites mortality in broilers (Table 3). Glutamine is utilized as a major energy source and drives mitochondrial ATP formation. The first step for glutamine catabolism in the mitochondria is its transport through the inner mitochondrial membrane by a proteincatalyzed process (Mates *et al.* 2002).

Sudden depletion of glutamine has been reported to result in a sharp decline in mitochondrial respiration (Darmaun, 2000). It is also, had reported that Ischemic cells are known to utilize glutamate for energy production and a fall in tissue glutamate does occur in the hearts of patients undergoing coronary artery bypass surgery (Subramaniam *et al.* 2007). It is possible therefore that in the present study, the conversion of glutamine to glutamate might have provided the much-needed substrate for the Krebs cycle for energy production to counteract isoprenaline-induced membrane disintegration in the myocardium. Reports by Dumaswala *et al.* (1994) indicated that glutamine could exert protective effects on membrane structure by preserving transmembrane ion gradient and lipid asymmetry. It is also, showed that, Higher IgA concentrations in the serum, bile, and intestines observed in the birds fed diets supplemented with glutamine support evidence reported by Burke *et al.* (1989) that rats fed diets supplemented with glutamine maintained higher serum IgA levels than the other treatment groups that were not fed diets with glutamine supplementation. Table 2 shows that, glutamine could improve feed efficiency.

Table 2 Growth performance of broilers treatments

Treatments	Week				
	3	4	5	6	1-6
Average feed intake (g)					
Control	295±40	441±54	702±51	882±107	2311±151
Glutamine*	324±37	650±66	637±53	916±113	2304±185
Average body weight gain (g)					
Control	141±21	295±27	363±31	420±41	1200±51
Glutamine*	170±19	439±31	428±28	345±43	1388±57
Average feed conversion ratio					
Control	2.25±0.22	1.47±0.10	1.92±0.11	2.19±0.14	1.95±0.10 ^a
Glutamine*	2.07±0.25	1.45±0.11	1.46±0.14	2.53±0.12	1.66±0.11 ^b

* 100 g glutamine per kilograms feed.

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

The birds fed diets supplemented with glutamine had significantly longer intestinal villi than the intestinal villi of birds fed the control.

Table 3 RV/TV ration and mortality percentage of broilers treatments

Treatment	RV/TV ratio	Total mortality percentage due to ascites (%)
Control	0.31±0.01 ^a	38±4 ^a
Glutamine*	0.28±0.03 ^b	25±3 ^b

* 100 g glutamine per kilograms feed.

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

RV/TV: right ventricle weight to total ventricle weight.

Table 4 MDA equivalents levels in plasma and liver tissue of broilers treatments

Day	Treatment	MDA in plasma (nm/mL)	MDA in liver (nm/mL)
21	Control	2.50±0.33	1.32±0.23
	glutamine*	2.57±0.67	1.27±0.93
42	Control	6.27±0.43 ^a	2.60±0.25 ^a
	glutamine*	4.57±0.48 ^b	1.95±0.03 ^b

* 100 g glutamine per kilograms feed.

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

MDA: malondialdehyde.

Table 5 TAS equivalents and GPX and SOD activities in plasma of broilers treatments

Day	Treatment	TAS (mmol/L)	GPX (U/g Hb)	SOD (U/g Hb)
21	Control	0.71±0.10	39.32±2.80	1371±265
	glutamine*	0.73±0.06	40.67±0.81	737±101
42	Control	1.36±0.11	30.72±0.82 ^b	913±112
	glutamine*	1.32±0.38	36.25±2.07 ^a	932±67

* 100 g glutamine per kilograms feed.

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

SOD: superoxide dismutase; GPX: glutathione peroxidase and TAS: total antioxidant status

Table 6 GPX and SOD activities in liver of broilers treatments

Day	Treatment	GPX (U/g protein)	SOD (U/g protein)
21	Control	0.25±0.01	8.60±0.48
	glutamine*	0.29±0.01	9.07±0.43
42	Control	0.25±0.02 ^b	8.25±0.75
	glutamine*	0.33±0.01 ^a	9.10±0.57

* 100 g glutamine per kilograms feed.

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

SOD: superoxide dismutase and GPX: glutathione peroxidase.

If the intestinal villi height can be increased early in the chick's life, then the chick may be able to utilize nutrients more efficiently earlier in life and thus have improved growth performance (Bartell and Batal, 2007). The increase in villi height that was reported by Bartell and Batal, (2007) might indicate that the birds fed diets supplemented with glutamine might have had greater nutrient absorption and utilization because increases in villi height result in more surface area for nutrient utilization. It could also suggest that in fact increased villi height does necessarily lead to

increased nutrient utilization and then increased performance Table 4 shows that Glutamine significantly reduced the MDA levels in plasma and liver tissue. A parallel increasing in the activities of glutathione-dependent antioxidant (GPX) in the plasma and liver tissue was also observed (Table 5 and 6). A growing body of evidence is emerging which suggests that reactive oxygen-derived free radicals play a crucial role in the pathogenesis of heart failure (Subramaniam *et al.* 2007; Nain *et al.* 2008; Lorenzoni *et al.* 2006). The effects of oxidative stress can be evidenced by cellular accumulation of lipid peroxides. Reports by Nirmala and Puvanakrishnan (1996) indicated that lack of antioxidant defense might have resulted in increased lipid peroxidation and subsequent deleterious effects on the cardiac and liver and other cells membrane.

The possible antioxidant effects of glutamine occur through the formation of nitric oxide. Earlier reports (Wink *et al.* 1993) indicated that nitric oxide derived from donor compounds showed marked protection against the cytotoxic effects of both hydrogen peroxide and alkyl hydroperoxide as well as hypoxanthine / xanthine oxidase-mediated cytotoxicity. NO released from glutamine either scavenges or prevents the formation of reactive oxygen species derived from hydrogen peroxide and superoxide (Maulik *et al.* 1996). It can rapidly react with superoxide anion ($O_2^{\cdot-}$), which is subsequently protonated to form OH^{\cdot} . Previous experimental evidence (1993), which supports the role of NO formed from glutamine as an antioxidant, indicates that the affinity of NO for $O_2^{\cdot-}$ is greater than that of SOD for $O_2^{\cdot-}$. In fact NO from glutamine even competes with SOD for $O_2^{\cdot-}$ supporting its role as an antioxidant. Direct action between NO and lipid peroxy radical forms a lipid nitroso adduct and this radical-radical interaction results in the termination of lipid peroxidation chain reaction and stops further initiation (Rubbo *et al.* 1994). Earlier Gonzales *et al.* (2005) reported the antioxidant property of glutamine against cobalt-induced lipid peroxidation in rat liver.

Earlier investigations by Mates *et al.* (2002) showed that glutamine was used to supply glutamate and cysteine, perhaps for glutathione biosynthesis. Reports by Prem *et al.* (1999) demonstrated that glutamine preserved total glutathione levels after injury / ischemia. The quenching of reactive oxygen species mediated reactions decreases oxidized protein levels and normalize index enzyme activities. Glutamine via glutamate and glutathione biosynthesis can prevent oxidation of redox of highly sensitive enzymes and thus protects the functions of the myocardium.

It has been shown previously that the administration of glutamine-supplemented nutrition protects the liver and improves the survival during acetaminophen-induced hepatic injury in rats, an effect probably due to the maintenance of liver glutathione (Hong *et al.* 1992). It is also pos-

sible that the glutamine itself may have provided the protective role. It has Reports by Karinch *et al.* (2001) has shown that during inflammatory process, concentrations of glutamine and glutathione are augmented within the endothelial cell. Our results lead to the conclusion that a relation may exist between those protective effects of glutamine and glutathione biosynthesis because glutathione is a tripeptide consisting of glutamate, cysteine and glycine.

CONCLUSION

The results of the present study indicate that beneficial effects of glutamine is probably related to a strengthening of the myocardial membrane by its membrane stabilizing action, or to a counteraction of free radicals by its antioxidant property, or to its ability to maintain near to normal status the activities of free radical scavenging enzymes and the level of GPX, which protect myocardial membrane from oxidative damage by decreasing lipid peroxidation. It is also, glutamine can improve the growth performance.

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