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Araştırma Makalesi ***Research Article***

Response to Dietary Supplementation of Glutamine in Broiler Chickens Subjected to Transportation Stress

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Abstract

The main purpose of this study was to determine effects of glutamine supplementation on performance and blood parameters including Hsp70 and acute phase protein when chicken were subjected to transportation stress. A total of four hundred day-old-male cobb-500 chicks were obtained directly from a local hatchery. The chicks were allotted to two groups as: immediate placement (1 hour after hatching) with access to feed and water and placement after 24h transportation without access to feed and water. The experiment consisted of a factorial arrangement of 2 different diets and 2 different time of placement. Chicks from each placement group were fed either basal diet or basal diet + 1% glutamine from 1 to 21 days of age. The results indicated that dietary glutamine improved the body weight gain and feed conversion ratio significantly when chicks were subjected to delayed or immediate placement. In conclusion, supplementing chicken with glutamine in diet can reduce negative effects of delayed access to feed and water during transportation. Moreover, APP concentration and HSP70 level were positively affected when chicks supplemented with glutamine in the diet.

Introduction

The commercial broiler chickens are among the fastest growing farmed species which must be fed with adequate nutrients and raised in a suitable environment. However, generally the environment of broiler chickens is a composite of interacting stressors (Bartell and Batal, 2007). A bird's success in coping with environment stressor depends on the physiological ability of chickens to respond appropriately. Nutritional status of a bird is crucial in determining its ability to respond to environmental insults (Dai et al., 2009). Glutamine (Gln), traditionally recognized as a nonessential amino acid, plays a profound role in the response to injury, enterocyte metabolism, and maintenance of the intestinal epithelium. Hence, Gln supplementation could be beneficial in improving growth performance and well-being of chickens particularly under stressful conditions (Bartell and Batal, 2007; Soltan, 2009).

In commercial practice, the transportation of chicks and their delivery to the farms may take 24 to 48 hours. Such practice which involves deprivation of feed and water and may result in transportation stress and increase in mortality in chicks (Bergoug et al., 2013). Therefore, early access to feed and water is crucial to ensure optimum growth performance and well-being of broiler chickens. Corduk et al. (2013) and Daşkıran et al. (2012) showed that transportation stress and delay in access to feed and water can negatively affect body weight and intestinal development in chickens. Similarly, Bhanja et al. (2009) reported that 48h transportation and delayed placement reduced intestinal weight of 8-day-old chicks by 12%.

Moreover, Potturi et al. (2005) indicated that villi height decreased by 11% in 5 day-old turkey poults subjected to 48 h transportation. They also showed that transportation stress and delayed placement increased apoptosis in small intestine villi of poults which resulted in loss of enterocytes, limitation in nutrient uptake capacity and eventually poor growth rate. Therefore, a proper nutritional strategy has to be considered to enhance the gastrointestinal development of the chicks

to ensure optimum growth performance particularly after transportation stress.

Glutamine could be primarily synthesized from ammonia and glutamate in the skeletal muscles (Newsholme, 2001). However, Gln synthesis may not meet the required level under specific conditions such as stress, infection or injury (Newsholme, 2001; Obled, 2003). Dietary supplementation of Gln has been shown to improve growth performance, development of the gastrointestinal tract, humoral immune response and antioxidant level in poultry (Bartell and Batal, 2007; Dai et al., 2009; Yi et al., 2001). Under stressful condition, Dai et al. (2009) reported that Gln supplementation may reduce the detrimental effects of heat stress on growth performance, carcass characteristics and meat quality in broiler chickens. This could be related to Gln effect in reducing heat shock-induced cell death together with maintenance of cell growth that prevents intestinal mucosal atrophy (Gu et al., 2012).

Our earlier study (Shakeri et al., 2014) indicated that dietary Gln supplementation (0.5% w/w) have no beneficial effect on growth performance and intestinal morphology in broilers raised at different stocking densities. On the other hand, Soltan (2009) reported that supplementing dietary 1.5 and 2% Gln in broiler chickens depressed growth performances which suggest a possible toxic effect. In addition, previous studies in pigs and poultry (Soltan, 2009; Wu et al., 2007; Yi et al., 2001; Yi et al., 2005) suggested that the beneficial effects of Gln on development of digestive system were more obvious during early age period (21 days age).

Therefore, with considering the results of earlier studies, this experiment was conducted to evaluate the effects of 1% Gln supplementation for the first 21 days on growth performance, intestinal morphology, serum acute phase proteins (APPs) and hepatic oxidative stress biomarkers in chickens subjected to 24 hours transportation stress before placement.

Materials and Methods

Chickens, housing, management and experimental treatment

The present study was carried out at the department of agriculture at the University Putra of Malaysia. A total of 400 day-old-male Cobb-500 chicks were obtained directly from a local hatchery. The chicks randomly were divided in two equal groups consisting group 1: immediate placement (1 hour after hatching) with access to feed and water and group 2: placement after 24h transportation (chicken were put in boxes in a moving delivery vehicle) without access to feed and water. The chicks were wing-tagged, weighed and placed in the pens and exposed to brooding temperature (32 ^{o}C). Chicks from each placement group were fed either basal diet or basal diet + 1% Gln (Amresco, Solon, OH, USA) from 1 to 21 days of age. From the day 21 to 42, both treatments (Basal and Gln added diets) received the same feed (basal diet). The Gln was supplemented to the experimental diets just for the first 3 weeks of the study because development of villi in all parts of intestine happens in the first three weeks (Soltan, 2009). Composition of diets is shown in Table 1.

The experimental was a 2 × 2 factorial arrangement with 2 levels of diets and 2 levels of placement. Each treatment was housed in groups of 25 birds with 4 replicates in floor pens (1.8 m × 2.5 m) with wood shavings deep litter and cyclic temperatures (minimum, 24^{o}C; maximum, 35^{o}C). The relative humidity (RH) was between 75 and 90%. The temperature and humidity was determined by using digital Thermo hygrometer (Testo, Malaysia). The chicks received an intraocular live Newcastle disease (ND) vaccine (Sunvac ND Clone, Sunzen Biotech, Shah Alam, Selangor, Malaysia) on day 7 and 21. Feed and water were provided *ad libitum* and lighting was continuous. Body weight and feed intake were recorded weekly and FCR was calculated.

Sample collection

On day 21, five birds from each pen (20 birds per treatment) were randomly selected and killed by neck-cut. Blood samples (5 mL) were collected and after coagulation were centrifuged at 2,000 x g for 10 min and the serum was used to determine the acute phase proteins. The sample from duodenum in approximately 3 cm length was excised, tied at both ends, and filled and fixed in 10% formalin for villus height measurements. Samples from liver tissue (six samples per treatment) were collected with at least one bird representing each pen. The samples were snap-frozen in liquid nitrogen and stored at -80 ^{o} C to determine the expression patterns of Hsp70 protein and genes involved in antioxidant function and the immune system.

Intestinal morphology

The fixed duodenal samples were embedded vertically in paraffin wax (Baddeley et al., 1986). Slides were prepared using 5 µm sections, stained by haematoxylin and eosin and the villi length and crypt depth was measured using light microscope (Bancroft and Gamble, 2002). The distance from the tip of the villus to the villus crypt junction represents villus height, while crypt depth was defined as the depth of the invagination between adjacent villi. A total of 10 villi and 10 crypts per sample were measured.

Table 1. Nutrient composition of experimental diets (as fed basis).

Ingredients %	Starter (1 to 21 day)		Finisher (22 to 42 day)
	Basal	**%1 Gln**	**Basal**
Corn	49.00	49.00	54.54
Soybean Meal (39%)	40.00	40.00	34.94
Palm Oil	6.15	6.15	6.65
Dicalcium P	1.95	1.95	1.82
Limestone	1.21	1.21	1.05
Sodium chloride	0.44	0.44	0.30
Vitamin Premix [1]	0.30	0.30	0.30
Mineral Premix [2]	0.30	0.30	0.30
DL-Methionine	0.15	0.15	0.10
Gln[3]	0.00	1.0	0.00
Sea sand	1.0	0.00	0.00
Total	100	100	100
Calculated analysis			
ME (kcal/kg)	3050	3050	3150
CP, %	22	22	20
Calcium, %	1	1	0.9
P available, %	0.45	0.45	0.42

[1]Supplied per kilogram of diet: vitamin A: 1,500 IU; cholecalciferol: 200 IU; vitamin E: 10 IU; riboflavin: 3.5 mg; pantothenic acid: 10 mg; niacin: 30 mg; cobalamin: 10 μg; choline chloride: 1,000 mg; biotin: 0.15 mg; folic acid: 0.5 mg; thiamine: 1.5 mg; pyridoxine: 3.0 mg.
[2] Supplied per kilogram of diet: iron: 80 mg; zinc: 40 mg; manganese, 60 mg; iodine: 0.18 mg; copper: 8 mg; selenium: 0.15 mg; BHT: 100 mg.
[3] Gln: 1 % pure glutamine (Amresco, Solon, OH, USA)

Acute phase proteins measurement

The concentration of ceruloplasmin (CP) was determined by the rate of formation of a colored product from CP and the substrate, 1,4-phenylenediamine dihydrochloride according to Sunderman and Nomoto (1970). Briefly, 20.37 g of sodium acetate trihydrate was dissolved in 250 ml distilled water and adjusted to pH 6 using glacial acetic acid. 0.615 g of 1,4-phenylenediamine dihydrochloride (P1519, Sigma, St Louis, MO, USA) was added to the prepared buffer and kept in dark for a minimum of 45 minutes. One hundred µl of the above buffer and 100 µL of samples or standards were added to each microplate wells, shaken gently and kept in dark for 20 minutes. The absorbance was recorded spectrophotometrically using microplate reader at 450 nm. Standards were prepared with serial dilution of pig serum and saline buffer combination to achieve various concentration of 12.75, 6.37, 3.18, 1.59, 0.79, 0.39, 0.19 and 0.09 µg/mL ceruloplasmin.

The α-1 acid glycoprotein protein (AGP) content of serum was determined by radial immunodiffusion using a commercial ELISA kit (Life Diagnostics Institute, West Chester, UK) according to the manufacturer's instruction. The ovotransferin (OVT) concentration was determined using radial immunodiffusion as described by Mancini et al. (1965). Briefly, 1% agarose gel (A9539, Sigma, St. Louis, MO, USA) was prepared (0.13 g of agarose in 13 mL Tris-buffered saline (TBS)) in a water bath at 56˚C and 260 µl of rabbit anti-chicken transferring antibody (RabMAbs® Abcam, Cambridge, MA, USA) was added to the mixture and poured onto a gel membrane (Flow-Mesh™, Sigma Aldrich, St. Lois, MO, USA) in room temperature. Nine wells were punched in each gel and 10 µL of standard or serum samples were loaded in each well. The OVT standards were prepared at 0, 0.3125, 1.250 and 5 mg/mL (Albumin from chicken egg white Sigma Aldrich, St. Lois, MO, USA). Gels were incubated in dark and humid environment for 48 hours. Following incubation period, size of the ring around each well was measured and the concentration of OVT calculated using prepared standard.

Molecular biomarkers analysis

Heat shock protein expression

The Western blotting and SDS-PAGE were used to quantify the expression of Heat shock protein 70 (Hsp70) as described previously (Oskoueian et al., 2014; Soleimani et al., 2012). In brief, liver samples (0.3 g) were homogenized by Potter-Elvehjem tissue grinder (SigmaChemical Co., St. Louis, Missouri, USA), using 3 mL of chilled Tris buffer [20 mM Tris (pH 7.5), 0.75 M NaCl,

and 2 mM 2-mescaptoethanol] and 10 µl/mL of protease inhibitor cocktail (P8340, Sigma Chemical Co., St. Louis, Missouri, USA). The homogenate was centrifuged (23,000 × g) for 45 min at 4°C and supernatants was collected. The supernatant protein concentration was determined by bicinchoninic acid protein assay kit (BCA-1, B9643, Sigma-Aldrich, St. Louis, MO, USA) and BSA considered as the standard. The total protein (25 µg) was loaded and separated on 10% polyacrylamide gels (0.75 × 70 × 80 mm) containing SDS, using Mini-gel apparatus (Bio-Rad, Hercules, CA, USA). The gel was electrophoresed at 120 V until the tracking dye reached the base of the gel. The separated proteins were transferred to polyvinylidene difluoride membranes (MSI, Westborough, MA) using a Trans-Blot semidry electrophoretic transfer cell (Cleaver Scientific Ltd.). The 10 mL of cold blocking buffer (Kirkegaard and Perry Labs Inc., Gaithersburg, MD) used to block nonspecific biding sites after washing the membrane by distilled water for 60 min. 5 mL of blocking buffer plus antiserum (monoclonal mouse antibody; cat. no. ab6535, Abcam, Cambridge, MA) was prepared for incubation of membrane for 1 h in a 1:20,000 dilution against Hsp70. After Incubation, the membrane was washed by 10 mL of cold Tris-buffered saline Tween 20, 3 times (5 min each time). Following this process, blots were placed in a horseradish peroxidase conjugated rabbit antimouse secondary antibody 1:40,000 dilution (cat. no. ab6728, Abcam) and were incubated for 30 min. Blots were incubated in After rinsing with cold Tris-buffered saline Tween 20 (3 times, 5 min each), the blots were exposed to an enhanced chemiluminescent substrate (Chemi-Glow, Alpha Innotech, San Leandro, CA).

Visualization of bands was performed using a chemiluminescent imaging system (FluorChem 5500, Alpha Innotech) followed by quantification of the band summation density by Image-Pro Plus image processing and analysis software (Media Cybernetics, Silver Spring, MD). The sizes of the immunodetected proteins were confirmed by molecular weight markers (Precision Plus Protein, Bio-Rad, Hercules, CA). All solutions were made with Milli-Q water (Millipore, Bedford, MA).

Antioxidant and immune system associated genes expression

The liver samples (6 samples per treatment) were subjected to RNA extraction using RNeasy mini kit (Qiagen, Valencia, CA, USA) (Oskoueian et al., 2014). The total RNA quality control for concentration, integrity and size distribution was carried out. The Maxime RT PreMix kit (iNtRON Biotechnology, Sungnam, Korea) was used to convert the total RNA to cDNA. Two-step quantitative PCR using iQ SYBR Gree Supermix (Bio-Rad) and Real-time PCR thermocycler (Bio-Rad, CA, USA) was performed to determine the expression patterns of genes involved in antioxidant function [Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx)] and the immune system [interleukin 6 (IL6), interleukin 2 (IL2)] (Yarru et al., 2009). The optimum condition for the amplification of all genes were as follow: 95°C for 5 min (1X), then 95°C for 25 s, then 60°C for 20 s and 72°C for 30 s (35X). The expression of all genes were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin genes expression according to Vandesompele et al. (2002). The real time PCR results were analysed using CFX manager software version 2 (Bio-Rad Laboratories).

Statistical analysis

Data were subjected to ANOVA using the GLM procedure of SAS (2003). The study was designed as completely randomized design. All data were analyzed using feeding regimes, placement, and their interactions as main effects. When interactions between main effects were significant, comparisons were made within each experimental variable. When significant effects were found, comparisons among multiple means were modelled by Duncan's multiple range test. Mortality was calculated using the chi-square test.

Results

Growth performance

The body weight, feed intake and FCR values of broiler chickens receiving basal and Gln added diets are shown in the Table 2. The inclusion of Gln into diet did not affect the weight gain and FCR significantly ($P>0.05$) during the period of 1-22 days. However, these parameters were significantly ($P<0.05$) improved in the birds receiving Gln during the periods of 22-42 days and 1-42 days as compare to the birds fed with basal diet.

The addition of Gln to diet did not affect the feed intake significantly ($P>0.05$) throughout the experiment. The chickens exposed to transportation stress showed significantly ($P<0.05$) lower weight gain and feed intake during 1-21 days as compared to chickens with immediate placement. However, thereafter neither weight gain nor feed intake values showed significant ($P>0.05$) differences in both groups receiving basal diet and Gln added diets. Moreover, the transportation stress did not affect the FCR value significantly ($P>0.05$) throughout the period of study in birds fed by basal and Gln added diets.

The mortality rate in the birds receiving Gln added diet was significantly ($P<0.05$) lower than the group fed with basal diet. The interactions between diets and time for weight gain, feed intake and FCR parameters throughout the duration of study did not show any significant ($P>0.05$) differences (Table 2).

Table 2. Effects of diets and transportation stress on growth performance parameters.

	Body weight (g) Days			Feed intake (g) Days			FCR Days			Mortality (%)
	1-21	22-42	1-42	1-21	22-42	1-42	1-21	22-42	1-42	1-42
Diet										
Basal	775±8	1245±28	2020±31	1013±14	2865±55	3879±61	1.3±0.01	2.3±0.06	1.92±0.03	6.4
Gln[1]	796±9	1391±24	2183±26	1032±15	2922±44	3955±52	1.3±0.01	2.1±0.05	1.81±0.03	2.8
Time										
Immediate[2]	799±8	1309±35	2108±40	1052±11	2940±45	3992±51	1.31±0.01	2.26±0.07	1.89±0.04	4.8
Transported[3]	767±6	1328±36	2095±38	994±10	2847±51	3841±54	1.29±0.01	2.15±0.05	1.83±0.03	5.6
Analysis of variance				**Probabilities**						
Diet	NS	**	**	NS	NS	NS	NS	*	*	**
Time	*	NS	NS	**	NS	NS	NS	NS	NS	NS
Diet × Time	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1] Gln =1 % glutamine from day 1 to 21, [2] Immediate placement, [3] Placement after 24h transportation, *: (P<0.05), **: (P<0.01), NS: non-significant.

Villi length and crypt depth

The Table 3 shows the villi length and crypt depth of duodenum in broiler chickens receiving basal and Gln added diets. The averages of villi length and crypt depth in group fed by basal diet were 998±68 and 106.13±16 µm, respectively. These values improved significantly (P<0.05) upon incorporation of Gln to the diet. The results indicated that intestinal morphology was not affected by transportation stress at 21 days age as no significant (P>0.05) differences were observed between groups with immediate placement and placement after 24h transportation (Table 3). Further, the interactions between diets and time for villi length and crypt depth did not indicate any significant (P>0.05) differences.

Table 3. Effects of diets and transportation stress on villi length and crypt depth in broiler chickens.

Items	Villi length (µm)	Crypt (µm)
Diet		
Basal	998 ± 68	106.13 ± 16
Gln[1]	1167 ± 32	140.85 ± 26
Time		
Immediate[2]	1156 ± 56	127 ± 10
Transported[3]	1023 ± 45	121 ± 15
Analysis of variance	**Probability**	
Diet	*	**
Time	NS	NS
Diet × Time	NS	NS

[1]Gln =1 % glutamine from day 1 to 21, [2]Immediate placement, [3]Placement after 24h transportation, *: (P<0.05); **: (P<0.01); NS: non-significant.

Acute phase proteins

The concentrations of CP, OVT and AGP in serum of the broiler chickens receiving basal and Gln added diets are shown in the Table 4. The results of the present study showed significant (P<0.05) improvement in CP, OVT and AGP contents upon incorporation of Gln into diet. As indicated in Table 4, in the day 21, the CP, OVT and AGP serum contents in chickens exposed to transportation stress did not show any significantly (P>0.05) difference as compared to the chickens placed immediately. In addition, the interactions between diets and time for CP, OVT, AGP content of serum did not show any significant (P>0.05) differences.

Molecular biomarkers analysis

Table 5 shows the effects of Gln supplementation and transportation stress on the expression of hepatic Hsp70 and genes associated with antioxidant enzymes and immune system. The results indicated that supplementation of Gln to the birds for 21 days significantly (P<0.05) up-regulated the expressions of Hsp70, CAT, SOD, GPx, IL2 and IL6 genes (Table 6) as compared to the birds fed by basal diet. Moreover, the

birds with immediate placement or placement after 24h transportation after 21 days rearing, did not show any significant (P>0.05) differences in the expression of Hsp70, CAT, SOD, GPx, IL2 and IL6 genes. Similarly, no significant (P>0.05) differences were also observed in the interaction between diet and placement time.

Table 4. Effects of diets and transportation stress on CP (ceruloplasmin), AGP (α-1 acid glycoprotein protein) and OVT (ovotransferin) serum content in broiler chickens.

Item	CP (µg/ml)	AGP (ng/ml)	OVT (mg/ml)
Diet			
Basal	6.7 ± 0.61	744.9 ± 39.43	1.59 ± 0.33
Gln[1]	9.7 ± 1.32	1052.1 ± 81.45	2.19 ± 0.63
Time			
Immediate[2]	7.3 ± 1.22	865.8 ± 104.83	123.3 ± 2.21
Transported[3]	8.7 ± 1.14	955.4 ± 70.82	124.7 ± 2.64
Analysis of variance		**Probability**	
Diet	*	**	*
Time	NS	NS	NS
Diet × Time	NS	NS	NS

[1] Gln =1 % glutamine from day 1 to 21, [2] Immediate placement, [3] Placement after 24h transportation, *: (P<0.05); **: (P<0.01); NS: non-significant

Table 5. Effects of diets and transportation stress on expressions of hepatic heat shock protein 70, antioxidant and immune system associated genes.

Item	Hsp70	CAT	SOD	GPx	IL2	IL6
Diet						
Basal	38.4 ± 0.91	0.9±0.12	1.4±0.11	1.3±0.15	1.3±0.11	1.3±0.17
Gln[1]	41.8 ± 1.22	1.4±0.16	1.8±0.13	1.9±0.16	1.9±0.14	1.8±0.19
Time						
Immediate[2]	39.1 ± 1.13	0.8±0.16	1.5±0.18	1.2±0.18	1.4±0.27	1.6±0.21
Transported[3]	41.7 ± 1.24	0.9±0.23	1.3±0.26	1.1±0.17	1.5±0.14	1.5±0.19
Analysis of variance				**Probability**		
Diet	*	*	*	*	*	*
Time	NS	NS	NS	NS	NS	NS
Diet × Time	NS	NS	NS	NS	NS	NS

[1] Gln =1% glutamine from day 1 to 21, [2] Immediate placement, [3] Placement after 24h transportation, *: (P<0.05); NS: non-significant

Table 6. The primer characteristics used for the gene expression analysis.

Genes		Sequences (5' to 3')	References
Catalase (CAT)	F	ggggagctgtttactgcaag	(Soleimani et al., 2012)
	R	tttccattggctatggcatt	
Superoxide dismutase (SOD)	F	agggggtcatccacttcc	
	R	cccatttgtgttgtctccaa	
Glutathione peroxidase (GPx)	F	ttgtaaacatcaggggcaaa	
	R	tgggccaagatctttctgtaa	
Interleukin 2 (IL2)	F	tgcagtgttacctgggagaa	
	R	cttgcattcacttccggtgt	
Interleukin 6 (IL6)	F	gactcgtccggagaggttg	
	R	cgcacacggtgaacttctt	
GAPDH[4]	F	tgaaagtcggagtcaacggatt	(Yarru et al., 2009)
	R	ccacttggactttgccagaga	
β-Actin	F	caacacagtgctgtctggtgg	(Vandesompele et al., 2002)
	R	atcgtactcctgcttgctgat	

[4]Glyceraldehyde 3-phosphate dehydrogenase

Discussion

The results of this experiment showed that supplementing 1% Gln for 21 days did not improve the weight gain and FCR. This result is consistent with the result reported by Soltan (2009) who added 1% Gln o diet and observed no significant effect on body weight of broilers after 21 days. This result might be associated with the development of the digestive system in this period of time in chickens as Uni et al. (1999) and Nir and Levanon (1993) concluded that the gastrointestinal tract of chicks reached its maximum digestive development in 3 weeks post-hatch. Since the small intestine plays a profound role in the process of digestion and nutrient absorption, at least 21 days post-hatch needed for maximum digestive development. The present study clearly showed that 21 days supplementation of Gln through diet increased CP, AGP and OVT in chickens while no significant changes were observed for these values among chickens placed immediately or after 24 h transportation in the day 21. Earlier studies indicated that the level of APP increased within 3 days and will decrease after recovery from stressful condition (Eckersall et al., 2006; Niewold et al., 2003). Hence, there is a possibility that the chicks had recovered from the transportation stress before day 21. Besides, the analysis of stress molecular biomarkers indicated that Gln supplementation enhanced the expression of Hsp70 and genes associated with antioxidant (CAT, SOD and GPx) and immune system (IL2, IL6) in the liver (Table 5). The Hsp70 protein enhances the stress tolerance of the cells and thereby increase the cell survival (Oskoueian et al., 2014; Singleton and Wischmeyer, 2006). The CAT, SOD and GPx are also endogenous antioxidant enzymes which work in tandem to scavenge the free radicals. Zhu et al. (2011) reported a positive correlation with Gln content of the diet with the SOD and GPx activities where increase in the Gln concentration up to 1.5% in the diet of the fish increased the SOD and GPx enzyme activities. Moreover, the glutamine is an efficient precursor for glutathione production in the liver. Therefore, the up-regulation in the expression of hepatic antioxidant genes may be attributed to the effect of Gln in activation of antioxidant enzyme in the liver.

The results of this experiment were in agreement with earlier studies indicating improvement in 42d weight gain and FCR since 21d data is not significant in broiler chickens by addition of Gln into diet (Bartell and Batal, 2007; Soltan, 2009). Moreover, as shown in the Table 3 the birds supplemented with Gln possessed longer villi and deeper crypts than those fed control diet on day 21. These findings are in agreement with earlier work in chickens (Bartell and Batal, 2007) and turkeys (Yin et al., 2010) supplemented by 1% Gln in their diets. In fact, Gln stimulates gut mucosal proliferation, maintains mucosa structure (Khan et al., 1999) and reconstitutes the mucosa after damage (Newsholme, 2001). Increase in intestinal villi height may allow a more efficient utilization of nutrients and consequently improved growth performance. Hence, the improvement in the growth performance observed in this study attributed to the effect of Gln in increasing the villi length (Table 3). According to the previous studies, Gln significantly improved the villi length in duodenum and jejunum in the first three weeks after hatching (Murakami et al., 2007; Soltan, 2009; Wu et al., 2007). For this reason, in this research the Gln was supplemented for the first three weeks.

The alleviation of transportation stress symptoms in chicks and poults has been very important since under commercial practices, the delivery of chicks and poults to the farms might take about 24 hours or more. Several studies (Bartell and Batal, 2007; Bigot et al., 2003; Soltan, 2009; Uni et al., 1999; Yin et al., 2010) suggested that 24-48 hours of transportation was detrimental to growth performance, mortality rate, and development of the digestive system. Similarly, the results of this study indicated that transportation stress for 24 hours resulted in lower weigh gain and feed intake for 1-21 days.

Acute phase proteins are considered as non-specific innate immune components and shown by several studies as possible indicator of stress in farm animals (Eckersall et al., 2006; Murata et al., 2004). For example, shipment of pigs by road increased the levels of major APP (haptoglobin, serum amyloid A, and C-reactive protein) (Pineiro et al., 2007). Deprivation of feed and water as in delayed placement may elicit physiological stress response (Zulkifli, 1999). The present study clearly showed that 21 days supplementation of Gln through diet increased CP, AGP and OVT in chickens while no significant changes were observed for these values among chickens placed immediately or after 24 h transportation in the day 21. Earlier studies indicated that the level of APP increased within 3 days and will decrease after recovery from stressful condition (Eckersall et al., 2006; Niewold et al., 2003). Hence, there is a possibility that the chicks had recovered from the transportation stress before day 21. Shakeri et al. (2014) has also demonstrated that broiler chickens supplemented with 0.5% of Gln at 21 days of age had significantly higher CP, AGP and OVT than those fed control diet. The Gln-induced cell swelling may play a role in regulating the APP concentration in the serum (Lavoinne et al., 1998; Meisse et al., 1999). In addition, the research in medical science indicated that AGP had

anti-inflammatory effect by increasing the production of interleukin 1 (IL-1) receptor antagonist (Tilg et al., 1993) and limiting the migration of leukocytes through endothelium.

The IL2 and IL6 are considered as biomarkers of immune system where increases in the expressions of these cytokines indicate the activation of immune system function. In this study, the dietary supplementation of Gln resulted in up-regulation of IL2 and IL6 genes expression. In accordance with this result Wells et al. (1999) observed that dietary supplementation of 3.5% Gln in mice resulted in the increase in the macrophage production of IL2 and IL6. Similarly, several studies have also reported the elevation in the immune response upon inclusion of Gln in the poultry diet (Bartell and Batal, 2007; Dai et al., 2009; Yi et al., 2001). Indeed, supplementing the Gln through diet increases the plasma level of Gln and thymocytes, lymphocytes, neutrophils and macrophages uptake the Gln extensively. The Gln then enhances their proliferation, prevents the apoptosis and increases the antibody production in lymphocytes (Li et al., 2007).

Overall, the increase in the expression of Hsp70 and hepatic antioxidant and immune system associated genes may have been contributed to the lower mortality rate, enhanced body weight and FCR values in chickens fed diets supplemented with 1% Gln. The chickens placed immediately or after 24h transportation revealed no significant differences in the analyzed genes which could be due to the full recovery of the chickens exposed to transportation stress by day 21.

Conclusion

The present findings suggested that Gln supplementation at 1% for the first 21 days of age may improve 42d weight gain, FCR and gut morphology in broiler chickens subjected to 24 hours transportation stress. Moreover, supplementation of Gln may also aid chickens to cope with transportation stress through elevation in APPs, Hsp70 and hepatic associated antioxidant and immune system genes. Based on these results, dietary supplementation of 1% Gln in chickens challenged by transportation stress for the first 21 days is recommended.

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REFERENCES

Baddeley, A., Gundersen, H.J.G., Cruz-Orive, L.M., 1986. Estimation of surface area from vertical sections. Journal of Microscopy 142, 259-276.

Bancroft, J., Gamble, M., 2002. Theory and Practice of Histological Techniques, Churchill Livingstone, London, UK, pp. 217.

Bartell, S., Batal, A., 2007. The effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and humoral immune response of broilers. Poultry Science 86, 1940-1947.

Bergoug, H., Guinebretière, M., Tong, Q., Roulston, N., Romanini, C., Exadaktylos, V., Berckmans, D., Garain, P., Demmers, T., McGonnell, I., 2013. Effect of transportation duration of 1-day-old chicks on postplacement production performances and pododermatitis of broilers up to slaughter age. Poultry science 92, 3300-3309.

Bhanja, S., Devi, C.A., Panda, A., Sunder, G.S., 2009. Effect of post hatch feed deprivation on yolk-sac utilization and performance of young broiler chickens. Asian-Australasian Journal of Animal Sciences 22, 1174-1179.

Bigot, K., Mignon-Grasteau, S., Picard, M., Tesseraud, S., 2003. Effects of delayed feed intake on body, intestine, and muscle development in neonate broilers. Poultry Science 82, 781-788.

Corduk, M., Sarica, S., Yarim, G.F., 2013. Effects of oregano or red pepper essential oil supplementation to diets for broiler chicks with delayed feeding after hatching. 1. Performance and microbial population. The Journal of Applied Poultry Research 22, 738-749.

Dai, S., Wang, L., Wen, A., Wang, L., Jin, G., 2009. Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. British Poultry Science 50, 333-340.

Daşkıran, M., Önol, A.G., Cengiz, Ö., Ünsal, H., Türkyılmaz, S., Tatlı, O., Sevim, Ö., 2012. Influence of dietary probiotic inclusion on growth performance, blood parameters, and intestinal microflora of male broiler chickens exposed to posthatch holding time. The Journal of Applied Poultry Research 21, 612-622.

Eckersall, P., Young, F., Nolan, A., Knight, C.H., McComb, C., Waterston, M., Hogarth, C., Scott, E., Fitzpatrick, J., 2006. Acute Phase Proteins in Bovine Milk in an Experimental Model of Staphylococcus aureus Subclinical Mastitis. Journal of Dairy Science 89, 1488-1501.

Gu, X., Hao, Y., Wang, X., 2012. Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. Poultry Science 91, 790-799.

Khan, J., Iiboshi, Y., Cui, L., Wasa, M., Sando, K., Takagi, Y., Okada, A., 1999. Alanyl-glutamine-supplemented parenteral nutrition increases luminal mucus gel and decreases permeability in the rat small intestine. Journal of Parenteral and Enteral Nutrition 23, 24-31.

Lavoinne, A., Husson, A., Quillard, M., 1998. Glutamine et cellule hépatique: métabolisme, propriétés et concept de régulation du métabolisme par le gonflement cellulaire. In Annales de Biologie Clinique 5, 557-562.

Li, P., Yin, Y.L., Li, D., Woo Kim, S., Wu, G., 2007. Amino acids and immune function. British Journal of Nutrition 98, 237-252.

Mancini, G., Carbonara, A.T., Heremans, J., 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2, 235-236.

Meisse, D., Claeyssens, S., Husson, A., Lavoinne, A., 1999. Glutamine, a regulator of acute phase protein synthesis. Clinical Nutrition 18, 111-112.

Murakami, A., Sakamoto, M., Natali, M., Souza, L., Franco, J., 2007. Supplementation of glutamine and vitamin E on the morphometry of the intestinal mucosa in broiler chickens. Poultry Science 86, 488-495.

Murata, H., Shimada, N., Yoshioka, M., 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. The Veterinary Journal 168, 28-40.

Newsholme, P., 2001. Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? The Journal of Nutrition 131, 2515S-2522S.

Niewold, T., Toussaint, M., Gruys, E., 2003. Monitoring health by acute phase proteins. In: Animal Welfare and Acute Phase Proteins Proceedings of Fourth European Colloquium on Acute Phase Proteins Segovia, Spain. pp: 57-67.

Nir, I., Levanon, M., 1993. Research note: effect of posthatch holding time on performance and on residual yolk and liver composition. Poultry Science 72, 1994-1997.

Obled, C., 2003. Amino acid requirements in inflammatory states. Canadian Journal of Animal Science 83, 365-373.

Oskoueian, E., Abdullah, N., Idrus, Z., Ebrahimi, M., Goh, Y.M., Shakeri, M., Oskoueian A., 2014. Palm kernel cake extract exerts hepatoprotective activity in heat-induced oxidative stress in chicken hepatocytes. BMC Complementary and Alternative Medicine 14, 368-378.

Pineiro, M., Pineiro, C., Carpintero, R., Morales, J., Campbell, F.M., Eckersall, P.D., Toussaint, M.J., Lampreave, F., 2007. Characterisation of the pig acute phase protein response to road transport. The Veterinary Journal 173, 669-674.

Potturi, P., Patterson, J., Applegate, T., 2005. Effects of delayed placement on intestinal characteristics in turkey poults. Poultry Science 84, 816-824.

SAS., 2003. Statistical Analysis Institute (Version 9.1.3). In.: SAS Institute Inc., Cary, NC, USA.

Shakeri, M., Zulkifli, I., Soleimani, A., O'Reilly, E., Eckersall, P., Anna, A., Kumari, S., Abdullah, F., 2014. Response to dietary supplementation of L-glutamine and L-glutamate in broiler chickens reared at different stocking densities under hot, humid tropical conditions. Poultry Science 2700-2708.

Singleton, K.D., Wischmeyer, P.E., 2006. Effects of HSP70. 1/3 gene knockout on acute respiratory distress syndrome and the inflammatory response following sepsis. American Journal of Physiology-Lung Cellular and Molecular Physiology 290, 956-961.

Soleimani, A., Zulkifli, I., Hair-Bejo, M., Omar, A., Raha, A., 2012. The role of heat shock protein 70 in resistance to Salmonella enteritidis in broiler chickens subjected to neonatal feed restriction and thermal stress. Poultry Science 91, 340-345.

Soltan, M., 2009. Influence of dietary glutamine supplementation on growth performance, small intestinal morphology, immune response and some blood parameters of broiler chickens. International Journal of Poultry Science 8, 60-68.

Sunderman, F.W., Nomoto, S., 1970. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. Clinical Chemistry 16, 903-910.

Tilg, H., Vannier, E., Vachino, G., Dinarello, C., Mier, J., 1993. Antiinflammatory properties of hepatic acute phase proteins: preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1 beta synthesis by human peripheral blood mononuclear cells. The Journal of Experimental Medicine 178, 1629-1636.

Uni, Z., Noy, Y., Sklan, D., 1999. Posthatch development of small intestinal function in the poult. Poultry Science 78, 215-222.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, research0034.1 - research0034.11.

Wells, SM., Kew, S., Yaqoob, P., Wallace, FA., Calder, PC., 1999. Dietary glutamine enhances cytokine production by murine macrophages. Nutrition 15, 881-884.

Wu, G., Bazer, F.W., Davis, T.A., Jaeger, L.A., Johnson, G.A., Kim, S.W., Knabe, D.A., Meininger, C.J., Spencer, T.E., Yin, Y.L., 2007. Important roles for the arginine family of amino acids in swine nutrition and production. Livestock Science 112, 8-22.

Yarru, L., Settivari, R., Gowda, N., Antoniou, E., Ledoux, D., Rottinghaus, G., 2009. Effects of turmeric (Curcuma longa) on the expression of hepatic genes associated with biotransformation, antioxidant, and immune systems in broiler chicks fed aflatoxin. Poultry Science 88, 2620-2627.

Yi, G., Allee, G., Frank, J., Spencer, J., Touchette, K., 2001. Impact of glutamine, menhaden fish meal and spray-dried plasma on the growth and intestinal morphology of broilers. Poultry Science 80 (Suppl 1).

Yi, G., Carroll, J., Allee, G., Gaines, A., Kendall, D., Usry, J., Toride, Y., Izuru, S., 2005. Effect of glutamine and spray-dried plasma on growth performance, small intestinal morphology, and immune responses of Escherichia coli K88+-challenged weaned pigs. Journal of Animal Science 83, 634-643.

Yin, Y., Huang, R., Li, T., Ruan, Z., Xie, M., Deng, Z., Hou, Y., Wu, G., 2010. Amino acid metabolism in the portal-drained viscera of young pigs: effects of dietary supplementation with chitosan and pea hull. Amino Acids 39, 1581-1587.

Zhu, Q., Xu, Q.Y., Xu, H., Wang, C.A., Sun, D.J., 2011. Dietary glutamine supplementation improves tissue antioxidant status and serum non-specific immunity of juvenile Hybrid sturgeon (Acipenser schrenckii♀ × Huso dauricus♂). Journal of Applied Ichthyology 27, 715-720.

Zulkifli, I., 1999. Heterophil/lymphocyte response and performance of feed and water restricted broiler chickens under tropical conditions. Asian Australian Journal of Animal Sciences 12, 951-955.