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Original Article

Effects of Glutamine on Lymphocyte Proliferation and Intestinal Mucosal Immune Response in Heat-Stressed Broilers

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■Keywords

Broilers, Glutamine, Heat stress, Intestinal mucosa immune function.

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ABSTRACT

To investigate the protective effect of glutamine (Gln) on lymphocyte proliferation and the intestinal mucosal immune response in heat-stressed broilers, 360 21-day-old Arbor Acres (AA) broilers were assigned to 4 groups in a completely randomized design, each of which included 6 replicates with 15 birds per replicate for 21 days. The chickens were fed a basal diet under no stress (NS group), a basal diet under heat stress (HT group), or a basal diet under heat stress with the addition of either 0.5 % or 1.0 % Gln. The results showed that the broilers in the HT group exhibited fewer proliferating peripheral lymphocytes, a lower growth performance, phagocytic rate and index of neutrophils, fewer goblet cells in whole intestine and intraepithelial lymphocyte (IEL) cells in the ileum, a lower sIgA content in the duodenum and the jejunum, a lower immunoglobulin content of serum and intestinal mucosa, than those of the NS group ($p<0.05$). Diets supplemented with Gln increased growth performance, the number of proliferating peripheral lymphocytes, the phagocytic rate and phagocytic index of neutrophils, the number of whole intestine goblet cells and ileum IEL cells, the sIgA contents of the duodenum and the jejunum, and the immunoglobulin contents of serum and intestinal mucosa ($p<0.05$) in broilers exposed to HT. In conclusion, Gln can enhance intestinal immune function in broiler chickens by stimulating T and B lymphocyte proliferation, increasing the number of goblet cells and IEL cells, as well as increasing the content of sIgA and immunoglobulin secretion.

INTRODUCTION

Heat stress is a stress response induced by high environmental temperature-relative humidity. Currently, heat stress is a major health problem worldwide for the poultry industry. There is accumulating evidence that high temperature decreases the growth of poultry and the quality of products and causes a series of nonspecific physiological reactions (Crespo & Shivaprasad, 2013; Wang *et al.*, 2010). These effects have been attributed to a decrease in the intestinal immune activity and to impairment of the normal intestinal tract immunologic barrier (Calefi *et al.*, 2014). The gut is one of the most important and longest digestive organs, and its permeability has been reported to be strongly correlated with heat stress (Song *et al.*, 2014; Nanto *et al.*, 2015). Many studies have evaluated the effects of biological barriers on intestinal permeability in heat stress animal models. These studies show that high temperature can affect nutrient transportation and absorption (Baumgard & Rhoads, 2013), cause a significant decline in the number of lymphocytes and goblet cells in the jejunum (Quinteiro-Filho *et al.*, 2015), and decrease the specific IgA (sIgA) antibody secretion contents of intestinal mucosa lymphatic tissue (Tsujita & Morimoto, 1999). Under these conditions, a functional imbalance of the immune system arises,

making poultry susceptible to pathogenic organism colonization and invasion and deleterious functional and structural changes in many organs (including the intestine) (Chen *et al.*, 2014), thus causing significant economic loss to poultry producers. Therefore, the intestinal mucosa immune barrier is an especially important target in the protection against heat-induced organ dysfunction and damage.

Glutamine (Gln) is traditionally considered a truly functional amino acid in nutrition. The compound is associated with beneficial effects for gut growth, development and health; is believed to provide ATP; and regulates cellular signaling pathways for fast-dividing enterocyte and lymphocyte cells (Wu, 1996). Gln can effectively stimulate protein synthesis in intestinal cells, protect intestinal morphology, modulate the intestinal immunity response, and enhance the physiological function of the intestinal mucosal barrier (Wu *et al.*, 2018). Although several beneficial effects of Gln on intestinal growth, development and health have been reported (Jiang *et al.*, 2009), detailed studies on the regulation of intestinal mucosal immune function by Gln, especially under stress conditions, have been reported only in recent years. These researchers have clearly indicated that the addition of Gln can reduce bacterial translocation, repair the damaged intestinal mucosa, and regulate intestinal expression and activation of immune response regulators (such as nuclear factor-κB (NF-κB), IKK, interleukin (IL) IgA, IgM and IgG antibody) (Mondello *et al.*, 2010), as well as improve gut barrier function under stress conditions, altogether suggesting a beneficial effect of Gln on gut immune function (Wang *et al.*, 2015).

Previous studies have discussed the changes in intestinal mucosal immunity during heat stress (Ashraf *et al.*, 2013), but the underlying immunity mechanisms in the gut under heat stress and what ultimately happens to gut immunity are not clear. Therefore, changes in the number of intestinal mucosal immune cells and proliferating peripheral lymphocytes, the phagocytic function of neutrophils, and the contents of sIgA and immunoglobulin in broilers under heat stress were tested to investigate whether Gln could protect intestinal mucosal immune barrier function by mediating proliferation of intestinal immune cells and T cells of peripheral blood in heat-stressed broilers.

MATERIALS AND METHODS

Birds, Experimental design, and Diet

The study was carried out at Henan University of Science and Technology, following the requirements of the experimental protocol of the Institutional Animal Care and Use Committee of Henan University of Science and Technology. A total of 360 Arbor Acres (mixed male and half) 21-day-old broiler chicks were selected, individually weighed chicks were allocated to 4 groups of 90 birds each in a completely randomized design, each of which included 6 replicates with 15 birds per replicate, and each replicate was placed into a cage (140 cm × 70 cm × 28 cm). The four experimental groups included a nonstressed control group (NS), which was fed a corn-soybean meal basal diet and kept in a thermoneutral room at 22 ± 1°C; a heat stress control group (HT), which was fed the same basal diet and kept at 33 ± 1 1°C for 10 h/d from 08:00 to 18:00 h and at 22 ± 1 1°C for the remaining time); and HT + Gln treatment groups, which were fed 0.5 % or 1.0 % Gln diet (*i.e.*, HT-G1 and HT-G2, respectively) and stressed by heat. Gln was obtained from Henan Honda Biological Medicine Co., Ltd., China (99 % purity, pharmaceutical grade) and was added on top of the basal diet.

Broilers were fed corn and soybean meal-based diets for 3 weeks. The formulations and nutrient composition of basal diets for finisher (21- 42 days) broiler growth periods are presented in Table 1, which were necessary to meet or slightly exceed the minimum nutrient requirements of broiler chickens, as recommended by the NRC (National Research Council, 1994). The room temperature was continuously regulated with electric heaters. From 22 to 41 days, the broilers were exposed to 33 ± 1 1°C and 55 ± 5 % relative humidity. All broilers were allowed free access to feed and water.

Growth performance

During the experimental period, body weight, feed intake of the chicks was recorded, and average daily gain (ADG), average daily feed intake (ADFI), and feed/gain ratio (F/G) were measured.

Blood sample collection

Six broilers (one bird per replicate) from each treatment group were randomly selected, and serum was sampled via the wing vein and collected in a heparinized centrifuge tube on day 21 of the trial. Half of each blood sample was centrifuged at 3500 × g for 15 min, and serum samples were obtained and frozen at -80°C for ELISA analysis. The remaining blood samples were used in the lymphocyte proliferation assay. Then, all of the broilers were euthanized immediately.

Table 1 – Ingredient and nutrient levels of the experimental diets (as-fed basis).

Ingredients (%)	22-42 d
Maize	43.5
Soybean meal	26.4
Wheat	20.5
Soybean oil	5.0
Limestone	0.90
Dicalcium phosphate	1.90
Salt	0.5
Premix[1]	1.0
Sodium chloride	0.3
Total	100.0
Calculated chemical composition[2]	
Apparent metabolism energy (MJ/kg)	12.85
Crude protein (%)	19.5
Calcium (%)	0.95
Available phosphorus (%)	0.41
Lysine (%)	0.95
Methionine (%)	0.43
Methionine + cysteine (%)	0.78

Note: [1]Vitamin mineral premix (per kg contained): chromium oxide, 0.5 g, L-lysine·HCl, 0.25 g; DL-methionine, 0.3 g; vitamin A (retinyl acetate), 5,000 IU; vitamin D3 (cholecalciferol), 2.50 IU; vitamin E (α-tocopherolacetate), 80 IU; menadione, 3 mg; thiamine 2.5 mg; riboflavin, 2.5 mg; nicotinamide, 25 mg; choline chloride, 800 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 0.3 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B1, 18 mg; vitamin B2, 6.6 mg; vitamin B6, 3 mg vitamin B12 (cobalamine), 0.02 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 8 mg; Mn (from manganese sulfate), 110 mg; Zn (Bacitracin Zn), 65 mg; iodine (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg; Na (sodium chloride), 1.3 g; Mg (magnesium oxide), 0.55 g; Co [cobalt-(II)-sulfate-heptahydrate], 0.30 mg.

[2]The nutrient levels are reported on an as-fed basis.

Collection and preparation of intestinal tissue and mucosa

After decapitation, small intestine tissue from the duodenum, jejunum, and ileum was dissected and carefully washed with normal saline and opened longitudinally. Approximately 3 cm of each intestinal segment was removed, fixed in 4 % paraformaldehyde, dehydrated and embedded in paraffin. Sections measuring 5 μm were cut, mounted, and stained with hematoxylin and eosin (H&E) stain.

The mucosa of the remaining intestinal segments was excised, homogenized, and centrifuged (4000 × g, 10 min, 4°C). The supernatant was then harvested frozen and kept for further analysis.

Lymphocyte proliferation assay

The peripheral blood lymphocyte assay was conducted against concavalin A (Con A) using the MTT method. Lymphocyte-rich mononuclear cells were isolated from blood collected with 7.2 mg dipotassium EDTA and separated on Ficoll-Histopaque density system (Sigma-Aldrich Co.) as described by Böyum (1968) and Nathanson (1982). Then, separated cells were washed (twice) with cold RPMI-1640 medium (2.5×10^5 cells/ml), resuspended in the media, and counted by the trypan blue dye exclusion technique. MTT solution was added to each well, and the plates were incubated at 37°C for 5 h. After this period, the plate was centrifuged at 1 000 × g for 5 min, and sodium dodecyl sulfate was then added to lyse the cells and solubilize the MTT crystals. Subsequently, the optical density was measured in a microspectrophotometer at 570 nm, and blood T and B lymphocyte proliferation was calculated by the corresponding equation and expressed as a mean stimulation index (SI).

Isolation and evaluation of phagocytic function of neutrophils

Broiler blood was layered onto 5 mL of Ficoll-Paque Plus and incubated at room temperature. Neutrophils were obtained using Histopaque 1077 (Sigma-Aldrich Co., Saint Louis, MO, USA) separating medium. Cells were centrifuged, withdrawn, detached, and resuspended in Hanks-Tris solution (5×10^5 neutrophils/mL), then were washed, suspended, smeared, and stained with acridine orange. All counts of phagocytosed neutrophils and dead bacteria were then performed. The phagocytic index (PI) was calculated as the average number of bacteria phagocytosed per 100 neutrophils. The phagocytosis percentage (PP) was calculated as the percentage of neutrophils taking part in phagocytosis.

Counting of intestinal intraepithelial lymphocytes and goblet cells

The numbers of intestinal intraepithelial lymphocytes (IEL) and goblet cells (GC) were counted on the H&E stained sections. The prepared glass slides carrying samples were observed under a Motic Med 6.0 CMIAS Image Analysis System (Motic China Group Co., Ltd., Guangzhou, China). A total of 3 fields per section and 5 sections per chicken were selected randomly. The mean values of IEL and GC among 100 enterocytes in different fields were then calculated for statistical analysis.

Determination of sIgA, IgA, IgG and IgM content by ELISA

Intestinal mucus concentrations of secretory IgA (sIgA) and immunoglobulins (IgA, IgG and IgM) were measured by enzyme-linked immunosorbent assay (ELISA, BlueGene, Shanghai, People's Republic of China) kits specific for chicks, following the instructions of the kit's manufacturer. The total protein concentration of

each intestinal mucosa was determined simultaneously by the Bradford brilliant blue method.

Statistical analysis

Statistical analysis of all data was carried out using SPSS version 20.0. Data for all of the treatment groups were compared using Tukey's range test. All data were expressed as means ± standard error of mean (SEM at levels of significance (p<0.05)).

RESULTS

Broiler production performance

During the feeding stages, the final weight, ADG, and ADFI were significantly lower in the HT group than in the NS group, and F/G were significantly higher in the HT group than in the NS group, (p<0.05) (Table 2). Supplementation with Gln significantly increased the final weight, ADG, and ADFI, and decreased the F/G compared to the number observed in the HT group (p<0.05).

Table 2 – Effects of glutamine on the growth performance in heat-stressed broilers.

Items	Diet treatments[1]			
	NS	HT	Gln1	Gln2
Initial weight (g)	955.60±20.07	955.07±20.13	955.42±20.10	955.51±20.15
Final weight (g)	2680.18±30.18[b]	2499.31±51.51[a]	2675.42±45.12[b]	2679.56±65.19[b]
Average daily gain (ADG) (g)	82.12±4.15[b]	73.54±3.9[a]	81.90±4.7[b]	82.10±5.0[b]
Average daily feed intake (ADFI) (g)	170.81±2.12[b]	159.58±8.25[a]	171.99±5.34[b]	169.94±2.58[b]
feed/gain ratio (F/G)	2.05±0.05[b]	2.17±0.09[a]	2.10±0.07[b]	2.07±0.04[b]

[1]NS = birds were kept at 22 ± 1°C and fed the basal diets; HT = birds were kept at 33 ± 1°C for 10 h (08:00 to 18:00 h) and 22 ± 1°C for the remaining time and fed the basal diet; G1 and G2 groups = birds were kept at 33 ± 1°C for 10 h (08:00 to 18:00 h) and 22 ± 1°C for the remaining time and fed the basal diet with 0.5 and 1.0% Gln, respectively.

[2]Standard error of the mean based on pooled estimate of variation.

[3a–b]Means within the same row that do not share a common superscript are significantly different (p<0.05); n = 8.

The number of proliferating peripheral blood T and B lymphocytes

The number of proliferating peripheral blood T and B lymphocytes was significantly lower in the HT group than in the NS group (p<0.05) (Fig. 1A and B). Supplementation with Gln significantly increased the number of proliferating peripheral blood T and B lymphocytes compared to the number observed in the HT group (p<0.05). The number of proliferating peripheral blood T and B lymphocytes approached nonheat stress levels (p>0.05). There was no significant difference between the HT-G1 group and the HT-G2 group (p>0.05).

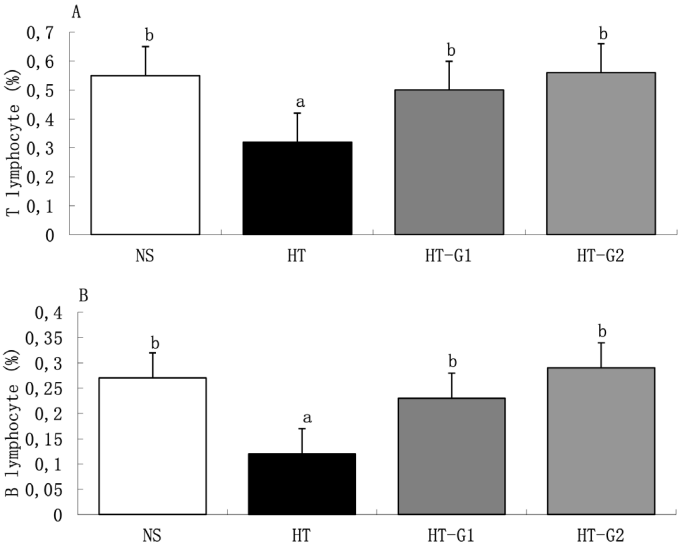

Figure 1 – Effects of Gln on the number of proliferating peripheral blood T and B lymphocytes in broilers under heat stress. Each column and the corresponding bar represent the mean and SEM, n=8, respectively. a-bMeans that possess different superscripts differ significantly (p<0.05). NS= birds were kept at 22 ± 1 °C and fed the basal diet; HT= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet; HT-G1 and G2 groups= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet with 0.5 % and 1.0 % Gln, respectively.

Phagocytic rate and phagocytic index of neutrophils

Compared to the NS group, heat stress induced a significant downregulation of the phagocytic rate and phagocytic index of neutrophils (p<0.05) (Fig. 2A and B). Supplementation with Gln significantly increased the phagocytic rate and phagocytic index of neutrophils compared to those of the HT group (p<0.05). The phagocytic rate and phagocytic index of neutrophils gradually approached nonheat stress levels (p>0.05). There was no significant difference between the HT-G1 group and the HT-G2 group (p>0.05).

The number of intestinal goblet cells

Exposure to heat stress (HS) significantly reduced the number of goblet cells in the duodenum, jejunum, and ileum compared to the numbers observed in the NS group (p<0.05) (Fig. 3A, B and C). Supplementation with Gln significantly increased the number of goblet cells in the duodenum, jejunum, and ileum compared to the HT group (p<0.05). The number of intestinal

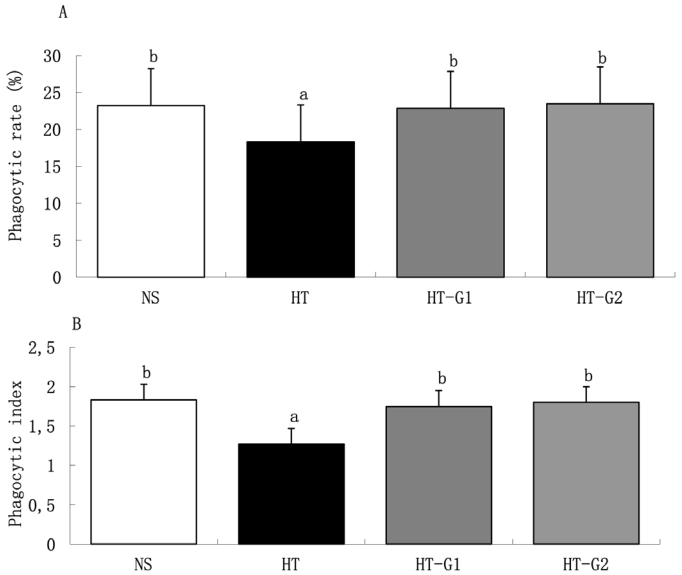

Figure 2 – Effects of Gln on phagocytic rate and phagocytic index of neutrophils in broilers under heat stress. Each column and the corresponding bar represent the mean and SEM, n=8, respectively. a-bMeans that possess different superscripts differ significantly (p<0.05). NS= birds were kept at 22 ± 1 °C and fed the basal diet; HT= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet; HT-G1 and G2 groups= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet with 0.5 % and 1.0 % Gln, respectively.

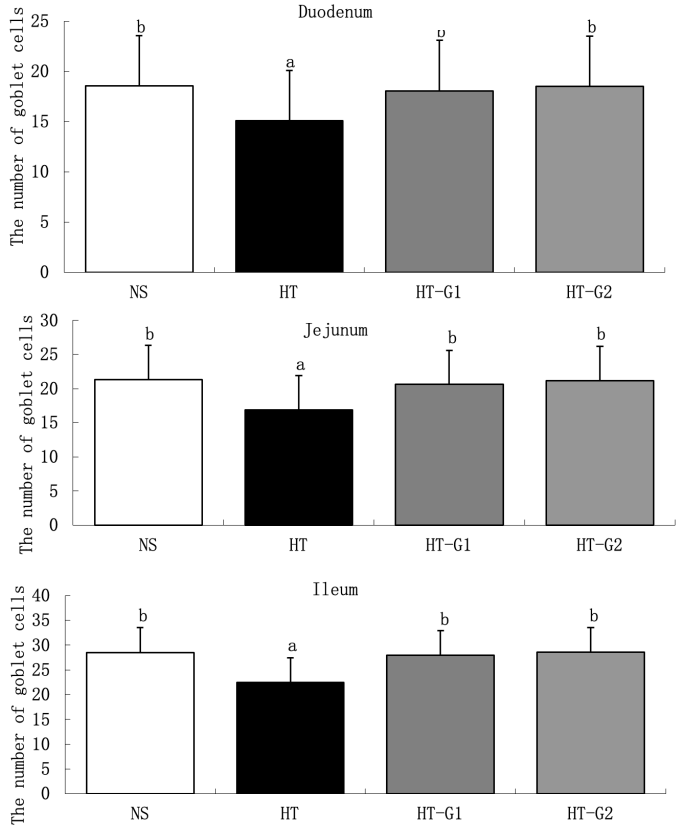

Figure 3 – Effects of Gln on the number of intestinal goblet cells in broilers under heat stress. Each column and the corresponding bar represent the mean and SEM, n=8, respectively. a-bMeans that possess different superscripts differ significantly (p<0.05). NS= birds were kept at 22 ± 1 °C and fed the basal diet; HT= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet; HT-G1 and G2 groups= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet with 0.5 % and 1.0 % Gln, respectively.

goblet cells gradually approached nonheat stress levels (p>0.05), and there was no significant difference between the HT-G1 group and the HT-G2 group (p>0.05).

The number of IEL-secreting cells

In the ileum, the number of IEL-secreting cells was significantly lower in the HT group than in the control group (p<0.05) (Fig. 4A, B and C). Supplementation with Gln significantly increased the number of IEL-secreting cells in the ileum compared to the number in the HT group (p<0.05). The number of IEL-secreting cells gradually approached nonheat stress levels (p>0.05). There was no significant difference between the HT-G1 group and the HT-G2 group (p>0.05). In the duodenum and jejunum, the HT group had fewer IEL-secreting cells than the NS group did, but there was no significant difference between the NS group and the Gln group (p>0.05).

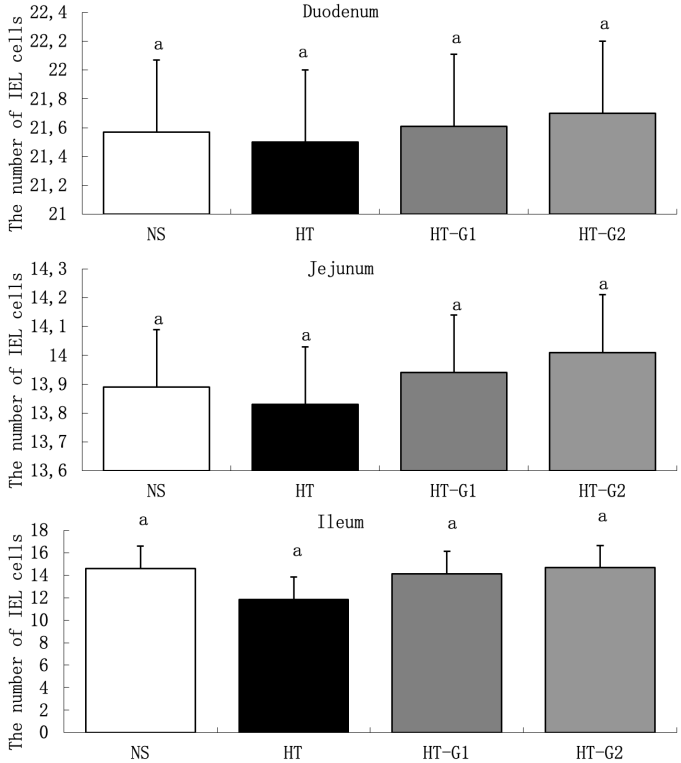

Figure 4 – Effects of Gln on IEL cells of the intestine in broilers under heat stress. Each column and the corresponding bar represent the mean and SEM, n=8, respectively. a-bMeans that possess different superscripts differ significantly (p<0.05). NS= birds were kept at 22 ± 1 °C and fed the basal diet; HT= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet; HT-G1 and G2 groups= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet with 0.5 % and 1.0 % Gln, respectively.

sIgA contents of the intestine mucosa

In the jejunum and ileum, the sIgA contents of the HT group was lower than that of the control group (p<0.05) (Fig. 5A, B and C). Supplementation

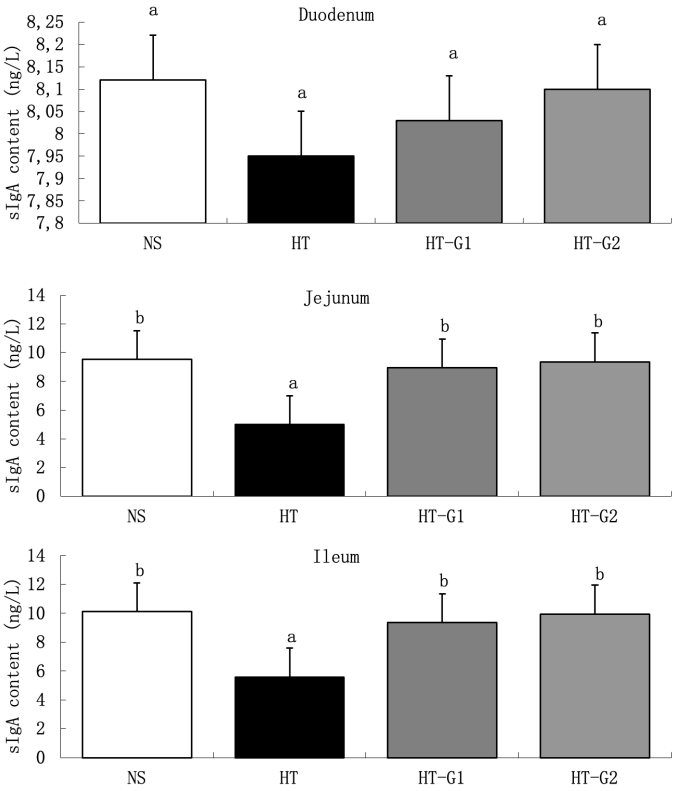

Figure 5 – Effects of Gln on sIgA contents of the intestine mucosa in broilers under heat stress. Each column and the corresponding bar represent the mean and SEM, n=8, respectively. a-bMeans that possess different superscripts differ significantly (p<0.05). NS= birds were kept at 22 ± 1 °C and fed the basal diet; HT= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet; HT-G1 and G2 groups= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet with 0.5 % and 1.0 % Gln, respectively.

with Gln significantly increased the sIgAs content in the jejunum and ileum compared to that of the HT group (p<0.05). The sIgA contents of the Gln group decreased, but there was no significant difference compared to the control group (p>0.05). In the duodenum, there was no significant difference among the groups (p>0.05).

The immunoglobulin contents of serum and intestinal mucosa

In the serum, duodenum, jejunum and ileum, the immunoglobulin contents of the HT group were lower than that of the control group (p<0.05) (Fig. 6A-D). Supplementation with Gln significantly increased the immunoglobulin contents in the serum, duodenum, jejunum and ileum compared to that of the HT group (p<0.05). The immunoglobulin contents of the Gln group decreased, but there were no significant differences compared to the control group (p>0.05). Moreover, there was no significant difference between the HT-G1 group and the HT-G2 group (p>0.05).

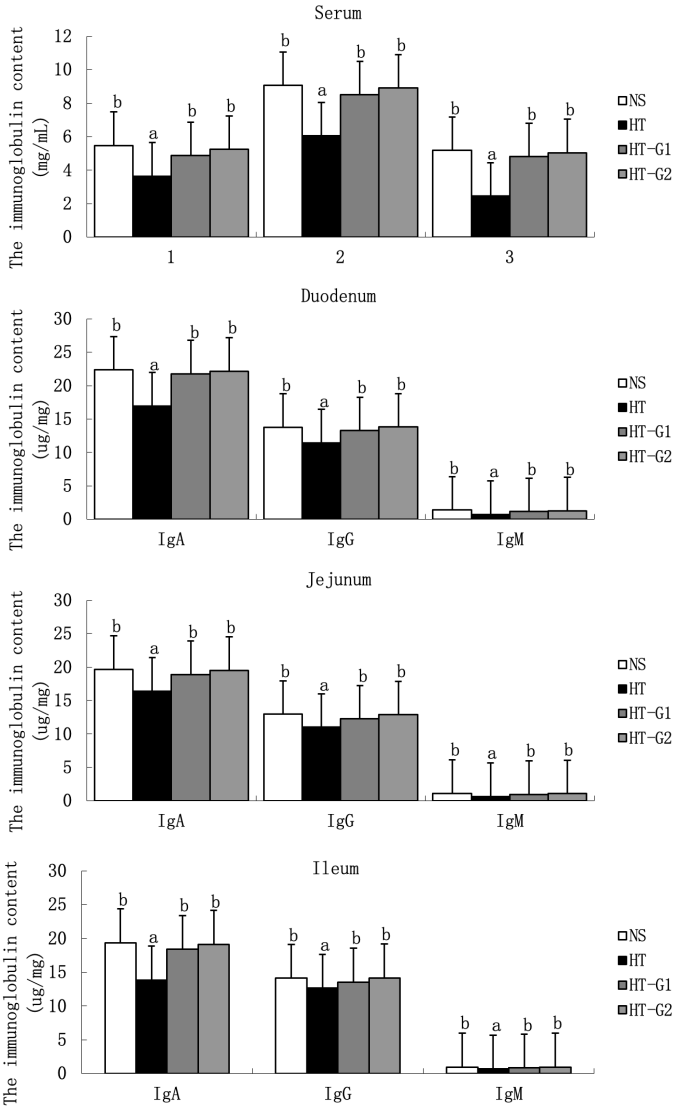

Figure 6 – Effects of Gln on the immunoglobulin contents of serum and intestinal mucosa in broilers under heat stress. Each column and the corresponding bar represent the mean and SEM, n=8, respectively. a-bMeans that possess different superscripts differ significantly (p<0.05). NS= birds were kept at 22 ± 1 °C and fed the basal diet; HT= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet; HT-G1 and G2 groups= birds were kept at 33 ± 1 °C for 10 h (08:00 to 18:00 h) and 22 ± 1 °C for the remaining time and fed the basal diet with 0.5 % and 1.0 % Gln, respectively.

DISCUSSION

As previously reported, the intestine is not only an organ for digestion and absorbing nutrients but it is also regarded as an important immunological organ (Steinert *et al.*, 2016). Fundamental immunological functions are provided by the intestinal mucosal immunity system, which is, in part, composed of gut-associated lymphoid tissue, IEL, GC, and abundant immunoglobulins. It has been shown that responsiveness of the intestinal mucosal immunity system to high temperature can reduce the number of goblet cells in the epithelia of the intestine and

damage immune function of the intestinal mucosa and mesenteric lymphoid tissues, thus decreasing the growth performance (Liu *et al.*, 2012; Ashraf *et al.*, 2013). The peripheral blood T and B lymphocytes were used to evaluate the influence of heat stress on cell-mediated immunity in our present study. There was a significant decrease in the number of proliferating peripheral blood T and B lymphocytes in the heat stress groups. Similarly, exposure to heat stress has been shown to decrease the growth performance and inhibit lymphocyte cell proliferation in cattle (Sunilkumar & Kataria, 2011). The decrease in the growth performance and lymphocyte proliferative response might be due to excessive production of reactive oxygen species due to heat stress, which initiates a cascade of lipid peroxidation of biological membranes, thereby reducing the growth performance and cell-mediated immune response (Goldstone & Hunt, 1997). In the present study, dietary supplementation with Gln significantly enhanced stress-stimulated lymphocyte proliferation and growth performance. These results might be observed because supplementation of Gln could enhance reactive oxygen species (such as GSH) production of lymphocyte cells and prevent lipid peroxidation by eliminating free active oxygen and lipid peroxides (Curi *et al.*, 1999), thereby increasing growth performance and preventing cell membrane disruption. Indeed, previous studies have demonstrated that glutamine is an important precursor of lipid synthesis under stress conditions (Curi *et al.*, 1999). However, these results may also be related to alterations in the biosynthetic processes of lymphocyte proliferation and cell cycle propagation. Glutamine is a primary fuel for lymphocytes and is used at high rates during lymphocyte proliferation (Chang *et al.*, 1999). Moreover, the influence of glutamine on lymphocyte proliferation can be explained by the requirement of glutamine for the production of IL-2 and IFN-γ (Rohde *et al.*, 1996).

It is generally recognized that neutrophils constitute the first line of defense against invading pathogens, are involved in the initiation of innate immune responses, and promote the protective inflammation response through the clearance of proinflammatory necrotic tissue and cell residues. The above findings are similar to the findings of Tsukamoto & Machida (2012); and Li *et al.* (2018) who found that the phagocytic capacity of neutrophils is significantly decreased under physiological stress. These results concluded that the general effect of physiological stress can cause neutrophil dysfunction, decrease the phagocytic ability of the broiler's neutrophils, and destroy microbial invaders (Tsukamoto & Machida, 2012). Supplementation with Gln enhanced the phagocytic rate and phagocytic index of neutrophils compared with those of the HT group. Sasaki *et al.* (2013) also showed that Gln supplementation enhanced neutrophil activity in male judoists after high-intensity exercise. Gln supplementation contributes to the maintenance of the body's immune functions (Wu *et al.*, 2019). This result is likely associated with the antioxidant effect of Gln; it has been shown that Gln can promote reactive oxygen species production and an oxidative burst of neutrophils in broilers (Sasaki *et al.*, 2013), thus explaining the good immune status of broilers in the present study. However, our study could not reveal the mechanism by which Gln affected the oxidative burst of neutrophils in detail; therefore, it is necessary to investigate the antioxidant capacity of glutamine during psychological stress.

Moreover, heat stress promoted a decrease in the number of goblet cells in broilers. A similar reduction of goblet cells has been observed in an animal model with prolonged heat stress (Shi *et al.*, 2015). These results indicate that heat stress can slow intestinal peristalsis and cause digesta retention and thereby promote the production of a large number of oxygen free radicals, ultimately resulting in the vulnerability of these segments and the intestinal mucosa barrier. Supplementation with Gln in the Gln group promoted an increase in the number of goblet cells in these broilers. Therefore, Gln supplementation caused beneficial protective effects by strengthening the intestinal wall of broilers. The observed increase in the number of goblet cells due to supplementation with Gln can be attributed to their antioxidant capacity and acceleration of differentiation and immunostimulatory effects (Martins *et al.*, 2016). Because Gln is the main metabolic fuel for migrating and proliferating cells of the intestinal mucosa, it can improve the immune response and intestinal barrier function by impairing reactive oxygen species production and, in turn, reducing bacterial translocation (Alves *et al.*, 2010).

The IEL compartment is mainly composed of epithelial cells and dispersed lymphocytes and is considered to be the first compartment of the immune system that plays an important role in mucosal immunity by gut-derived antigens (Shi *et al.*, 2015). Therefore, changes in the number of IELs can reflect the integrity status of the epithelial mucosa barrier in certain respects. The present study found that under heat stress conditions, the number of IELs in the ileum of broiler chickens was significantly decreased. Quinteiro-Filho *et al.* (2010)

reported similar effects under high-temperature conditions in birds, which reflect the potential of heat stress to impair the activity of intestinal mucosal immunity and initiate an inflammatory response. Our study indicates that the Gln-administered groups could increase the IEL number and ameliorate the inflammatory response triggered by heat stress. The increase in the IEL count in Gln-administered groups can be attributed to Gln-induced reduction in the level of proinflammatory cytokines (Isolauri *et al.*, 2001), thereby maintaining the defense capability of intestinal mucosa to some extent under heat stress, a phenomenon observed in our previous study (Wu *et al.*, 2018). The finding that Gln maintained epithelial barrier integrity and influenced the intestinal immune responses by mediating the proliferation of IELs (Horio *et al.*, 2008) supports the findings of the current study. We also found that Gln increased ileal IEL numbers. The reason for the discrepancy might be that the ileum hosts more microorganisms than do the other segments of the small intestine.

Some reports have indicated that the concentration of sIgA in mucosa is an indicator of specific immunity against psychological stress, decreasing under stress (Zuo *et al.*, 2014; Wu *et al.*, 2018). In this study, we found that intestinal sIgA levels were significantly reduced in the jejunal and ilial mucosa in the HT group. In some studies, secretory IgA has also been found to decrease significantly during stress (Matos-Gomes *et al.*, 2010). Moreover, a significant decrease in sIgA levels was found in the distal region of the small intestine; the underlying mechanism in the jejunum and ileum regions may be related to the regulatory effects of HT on redistribution and intestinal B cell homing via an adrenergic mechanism (Drago-Serrano *et al.*, 2012). Supplementation with Gln can increase the levels of sIgA. These findings indicate that Gln supplementation enhances the secretion of IgA. This result is similar to that reported by Lai *et al.* (2004), who showed that the addition of Gln to the rat diet can enhance sIgA secretion. Gln likely causes this effect by influencing the intestinal microbiota, the induction pathway (T-dependent and T-independent), IgA-secreting plasma cells, the expression of pIgR and even the transport of intestinal sIgA (Ren *et al.*, 2016).

In addition, IgA, IgG and IgM also participate in the intestinal mucosal immune response process. The low concentrations of serum and intestinal IgA, IgG and IgM in the HT group may indicate a decrease in the defensive capacity of the mucosal immune system. The results of this study agree with the data of Olfati *et al.* (2018), which demonstrated that the IgA, IgG and IgM concentrations were markedly decreased under heat stress in broilers. These physiological changes also increase the penetration of bacteria through the intestinal lining. However, heat stress produces negative effects on redox balance, exacerbating the production of reactive oxygen species (Olfati *et al.*, 2018) and contributing to the permeability of the gut lining to bacteria. This fact may be explained by the data reported by Quinteiro-Filho *et al.* (2015), which showed that heat stress increased serum corticosterone levels, the concentration of inflammation cytokines, and bacterial translocation in broiler chickens. This idea appears to be reinforced by the observed decreased percentage of T and B lymphocytes in chickens in the HS group, which also shows the suppressive effects of heat stress on humoral activity and its altering effects on the function of the intestine. Previous studies have reported modulatory actions of Gln on the humoral immune response, such as increased IgA, IgG and IgM production in humans and rats (Yeh *et al.*, 2005). In our study, dietary supplementation with Gln increased the serum and intestinal mucosa IgA, IgG and IgM levels of broilers under heat stress and greatly improved immune functions in heat-stressed broilers compared to those of the control group. These results suggest that Gln may mediate protection of mucosal surfaces in the intestinal mucosa. The beneficial effects of Gln on mucosal immunity might be explained by its antioxidant activity and bacterial translocation.

In conclusion, dietary supplementation with Gln could modulate intestinal immune function in broilers under heat stress by enhancing lymphocyte proliferation, the phagocytic rate and phagocytic index of neutrophils, the number of goblet cells and IELs, sIgA secretion, and blood serum and intestinal mucosa IgA, IgG and IgM levels. The results of this study indicate that Gln supplementation can regulate immune responses at mucosal surfaces and improve the immune response and function of the intestinal barrier. The beneficial effects of Gln supplementation are associated with its antioxidant activity and bacterial translocation.

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