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PROTECTIVE EFFECTS OF γ -AMINOBUTYRIC ACID (GABA) ON THE SMALL INTESTINAL MUCOSA IN HEAT-STRESSED WENCHANG CHICKEN

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ABSTRACT

We explored the effects of heat stress (HS) and γ-aminobutyric acid (GABA) treatment on the structure and development of small intestinal mucosa in Wenchang chicken. One-day old male Wenchang chickens were randomly divided into control group (CK), heat stress group (HS) and GABA+HS group. The chickens from GABA+HS group were fed with 0.2 ml 0.5% GABA solution daily. In addition, the chicken from HS and GABA+HS groups were subjected to heat stress treatment at 40 ±0.5°C for 2h during 13:00-15:00 every day. Results showed that compared with CK group, HS group exhibited marked decline in villus length, mucosa thickness, intestinal wall thickness, and crypt depth in duodenum and ileum, and significantly fewer goblet cells ($P<0.05$). In contrast, compared with HS group, GABA+HS group exhibited enhanced villus length, mucosa thickness, intestinal wall thickness, and crypt depth in duodenum and ileum, as well as a much higher number of goblet cells ($P<0.05$). Therefore, heat stress caused significant structural damages to chicken small intestinal mucosa, and markedly reduced the number of goblet cells. GABA showed protective effects to alleviate HS-induced damages of the intestinal mucosa and increased the number of goblet cells.

Key words: GABA, heat stress, small intestinal mucosa, Wenchang chicken.

INTRODUCTION

Global warming is closely associated with the alarming trend of increasing frequency and intensity of heat waves (Meehl and Tebaldi, 2004); as a result, production and revenue of the livestock industry has been adversely affected (St-Pierre *et al*, 2003). In particular, hot weather is common in most areas of southern China. The high temperature environment exerts profound effects on the growth, development, production and reproduction of animals, which hampers the continuing increase of livestock production. Therefore, heat stress is a serious problem for animal production and reproduction.

Heat stress response refers to the overall non-specific physiological defense of animals in response to heat when the environmental temperature exceeds the upper limit of the comfort zone within the zone of thermoneutrality **(**Liu and Peng, 2001**)**. High temperature is the predominant source of heat stress. Animal metabolism undergoes dramatic changes as a function of the environmental temperature, and appropriate environmental temperatures allow optimal metabolic rates and heat production (Sahin *et al*, 2002; Fan *et al*, 2007; Ahmad *et al*, 2008). The range of such optimal temperatures is regarded as the zone of thermoneutrality or the thermal neutral zone. Raising livestock within the zone of thermoneutrality produces the best result, because lower temperatures may increase consumption of feeds, whereas higher temperature will diminish the production performance of animals (Han, 1998).Specifically, heat stress may significantly affect the growth of broilers, compromising their immune functions and increasing their disease incidence and mortality rate **(**Cooper and Washburn, 1998; Zulkifli *et al*, 2000; Gonzalez-Esquerra and Leeson, 2005**)**. It was also reported that body temperature of commercial broiler males was significantly higher in the 32°C environment after 7d of heat stress than at 21°C at all points through 21d of heat stress, with mean differences ranging from 0.5 to 1.0℃ (Chen and Wang, 2008; Ramnath *et al*, 2008).The body core temperature is the parameter that best reflects a bird's thermal status (Cooper and Washburn, 1998; Giloh *et al*, 2012).Heat stress can also cause increased intestinal permeability and significant damage to the epithelium, severely impairing the intestinal barrier function (Lambert *et al*, 2002).Therefore, extensive researches have been focused on the relief of heat stress in chickens, mainly through supplying various food additives to reduce deleterious effects of heat stress (Roussan *et al*, 2008; Silva *et al*, 2010; Haldar *et al*, 2011).

γ-Aminobutyric acid (GABA), an important neurotransmitter, is widely present in the nerve system of mammals. It can mediate the hyperpolarization of postsynaptic membrane, induce ion influx, and decrease the cell metabolism and oxygen consumption. GABA is involved in many functions including sedation, memory and sleep, anti-convulsion, reduction of blood pressure, regulation of respiration and appetite, and lowering of stress (Bongianni *et al*, 2006).GABA has been used in the food, pharmaceutical and cosmetic industry. It is reported

that it has been applied to animal production and has good effects on increasing the food intake, promoting the performance, the antioxidative capacity and anti-stress capacity of animals. Its application in forage industry has been increasing recently (Li, 2010).

The small intestine not only performs the digestion and absorption functions, but also plays a role as a barrier as part of the immune system. It is believed that properly functioning small intestinal mucosa is a critical physiological component involved in nutrient digestion, absorption and animal development (Han, 1991). However, the deleterious effects of heat stress on the digestive system have not been thoroughly documented. Meanwhile, previous studies in our laboratory showed that GABA can improve the outcome of heat-stressed broilers: increased food intake, body weight, average daily weight gain and decreased feed to gain ratio (Chen *et al*, 2002). However, it is unclear whether heat stress can damage the small intestinal mucosa and whether GABA can alleviate the damage. We therefore conducted this experiment to investigate the heat stress-induced damage to the small intestinal mucosa and the beneficial effects of GABA in Wenchang chicken.

MATERIALSAND METHODS

Animals: Ninety healthy 1-day old male Wenchang chickens were purchased from Yongji Wenchang Hatchery (Hainan, China), and weighted and numbered. They were randomly divided into 3 groups: the control group, the heat stress (HS) group and the GABA+HS group. Every morning the control and the HS group were fed with 0.2 ml physiological saline, and the GABA+HS group were fed with 0.2 ml of 0.5% GABA (Sigma, St Louis, MO, USA). All chickens were maintained by routine procedures, with unlimited access to distilled water and food (Zhanjiang Yilong Feed Factory, China). This experiment was conducted with approval from the Hainan Normal University Animal Experimentation Ethics Committee.

Heat stress treatment: Starting from day 1, the HS and GABA+HS groups were placed in a heat-box remodeled from a large-capacity incubator at a temperature of (40.5±0.5)°C and a humidity of (52.4±2.1)%for 2h (13:00-15:00) everyday. The temperature was controlled by HWMK-123 temperature control meter (Guangzhou Yier Incubation Equipment Co., Ltd., China). The control group was placed in a box at the same temperature and humidity as the raising condition. After 2h, chickens were returned to cages at the ambient temperature for maintenance (Chen *et al*, 2002; Ramnath *et al*, 2008; Yin *et al*, 2011).

Feed and weight gain: The chicks were provided with sufficient quantities of feed on the first day. Afterwards, the feed was added in the morning and afternoon every day. The weight (g) of the added feed was recorded with YP6001 (Shanghai Precision & Scientific Instrument Co., Ltd.).After heat stress on the 3, 6, 9, 12 and 15 days, the fasting body weight (g) of each chick and the remaining feed was weighed. The feed intake (g), the average weight gain (g) and feed to gain ratio were calculated.

Preparations of small intestinal mucosal tissue sections: Following heat stress for 3, 6, 9, 12 and 15 consecutive days, six birds were sacrificed by exsanguinations. The loop of duodenum, the mid-sections of jejunum and ileum were obtained and cleaned with physiological saline. The samples were then fixed in Carnoy's fixative for 8h, washed with70% ethanol and stored. The fixed tissues were subsequently subjected to routine paraffin sectioning and the thickness of sections was 5 micron (Zhao *et al*, 2004).

H.E. and PAS staining: Sections were dewaxed and oxidized with distilled water-periodate acid for 5 min. For H.E. staining, the samples were stained in hematoxylin for 1-2min, washed with distilled water, stained in eosin for 5-10s, and finally washed with distilled water again. Under the microscope, nucleus appeared blue and cytoplasm red. For PAS staining, the samples were stained in Schiff’s agent for 30-60 min at 37 , counterstained with hematoxylin to visualize nuclei, and destained with 0.5% HCl in ethanol. Under the microscope, glycogen granules appeared purple, glycoproteins pink, mucin and mucopolysaccharide red (Wang, 1994).

Data acquisition and analysis: Stained sections from various small intestinal segments were examined with an Olympus CX-21 microscope for morphological and structural changes in mucosa. MiPrd2.0 microscopic image analysis (Shangdong Yichuang Electronics Ltd., China) was conducted to measure the following parameters in each small intestinal segment: intestinal villus length, mucosa thickness, intestinal wall thickness, and crypt depth. Six sections were randomly chosen from each small intestinal segment, and for each section, 5 longest and well-aligned villi, 5 points of thickest mucosa and intestinal wall, and 5 points of deepest crypt were measured, and the average values were calculated. After selecting 5 parts of the section where there was a more uniform and dense distribution of the goblet cells, the number of goblet cells was counted among 100 small intestinal epithelial cells (IEC) from one chicken. Pictures were taken for all sections using YM310 digital camera (Shangdong Yichuang Electronics Ltd.).

Each parameter is defined as follows: villus length: the length from the top of the intestinal villus to the base of the intestinal villus; mucosa thickness: the thickness of mucous layer, which includes the annular plica, the intestinal villus, microvillus and intestinal gland; intestinal wall thickness: the distanceform the outer part

of the intestine to the joint of the muscular layer and the submucosa (ectoptygma thickness plus muscular layer thickness);crypt depth: the depth of the intestine gland.

All parametric data were analyzed using two-way ANOVA in SPSS software, version 16.0 (SPSS Inc, Chicago, IL, US) with time and treatment as factors. Differences among three groups were determined with Duncan's multiple range Test. Probability values less than 0.05 were considered statistically significant (P<0.05).

RESULTS

Effects of GABA on heat-stressed chicken: As shown in Table1, the body weight, feed intake and average weight gain were gradually increased with the increase of age, but the feed to gain ratio fluctuated between 1.41-1.93.Compared with the control group, the HS group showed a decline in body weight and average weight gain, which were statistically significant (*P<0.05*); feed intake decreased, but it was not significant; the feed to gain ratio increased, which was statistically significant (*P<0.05*) for several data points. Compared with the HS group, GABA+HS group showed significant and highly significant differences in body weight and the average weight gain(*P<0.05*); feed intake increased, but it was not significant; feed to gain ratio deceased, and it was statistically significant for several data points (*P<0.05*).

Effects of heat stress on the structure and development of small intestinal mucosa of Wenchang chicken: As shown in Fig. 1, the small intestinal mucosa in the control group showed intact structure with discrete layers. The striated border on the small intestinal mucosa surface was clearly defined; cell coat was thick, and the small intestinal villi and mucosal epithelial cells were clearly outlined and regularly aligned (Fig. 1; panels A, B, C). In comparison, the HS group showed relatively thin small intestinal wall. The small intestinal mucosal epithelial cells appeared to disperse outwards, indicative of compromised structural integrity. In many regions, mucosal epithelia were detached, accompanied by exposed laminapropria and ruptured small intestinal villi (Fig.1**;** panels D, E, F). As shown in Fig.2-4, the HS group showed marked decline in villus length, mucosa thickness, intestinal wall thickness, and crypt depth in duodenum, jejunum and ileum, which were statistically significant (*P<0.05*) comparing with the control group. And V/C of each group was changed, and some groups showed statistically significant changes (*P*<0.05; Fig.5).

Table 1.Effects ofGABAon the performance of heat-stressed chickens[1] (n=6)

Items	Groups	3d	6d	9d	12d	15d
Body weight（g）	Control	40.39±4.95^{a}	60.50±8.23^{a}	85.54±11.09^{a}	113.71±13.81^{a}	152.14±12.29^{a}
	HS	34.39±4.33^{b}	47.6±6.30^{b}	70.58±7.67^{b}	88.73±10.43^{c}	116.76±11.31^{c}
	GABA+HS	40.24±3.74^{a}	57.44±4.33^{a}	80.39±4.82^{a}	101.41±6.76^{b}	131.44±11.94^{b}
	Sign	NS	NS	NS	*	*
Average weight gain（g）	Control	10.86±3.90^{a}	13.77±3.05^{a}	17.81±1.60^{a}	26.40±5.15^{a}	36.90±4.87^{a}
	HS	5.50±3.99^{b}	10.84±2.67^{b}	12.83±1.65^{b}	19.28±3.82^{b}	27.70±4.18^{b}
	GABA+HS	10.14±4.36^{a}	12.94±3.22^{ab}	16.05±1.85^{a}	20.29±2.64^{ab}	31.45±5.29^{ab}
	Sign	NS	NS	NS	*	*
Food intake（g）	Control	13.81±2.22	20.55±2.03	26.97±1.02	49.54±2.82	53.59±2.95
	HS	12.08±2.83	18.89±0.97	26.63±0.78	44.08±6.61	44.68±11.80
	GABA+HS	12.94±2.06	20.52±0.56	26.54±0.38	45.43±5.21	45.81±11.82
	Sign	NS	NS	NS	NS	*
Feed to gain radio	Control	1.41±0.46^{b}	1.61±0.43	1.53±0.14^{b}	1.93±0.40	1.48±0.21
	HS	3.42±2.23^{a}	1.95±0.55	2.11±0.27^{a}	2.39±0.59	1.65±0.26
	GABA+HS	1.52±0.68^{b}	1.77±0.52	1.67±0.20^{b}	2.27±0.31	1.49±0.25
	Sign	*	NS	NS	NS	NS

[1] In the same rank, data with different superscript letters indicate statistically significant difference. * (p<0.05).

Effects of GABA on the structure and development of small intestinal mucosal tissue of Wenchang chicken under heat stress: After treatment of GABA, various small intestinal segments showed relatively intact mucosal tissue structure with discrete layers. The striated border on the small intestinal mucosa surface was clearly defined. In addition to thick cell coat, intestinal villi and mucosal epithelial cells showed uniform shape and compactly aligned with bright staining. The columnar cells showed abundant cytoplasm, with oval-shaped nuclei. These structural features were comparable with or superior to those of the control group (panels G, H, I). Result of statistical analysis showed an obviously increase in villus length, mucosa thickness, intestinal wall

thickness, and crypt depth on HS group when compared with GABA+HS groups were statistically significant (Fig.2-4).V/C of each group was restored or closed to normal after the treatment of GABA.

Effects of heat stress and GABA on the epithelial mucosal goblet cells: Goblet cells were largely scattered among columnar cells. On the other hand, they also aggregated and compactly aligned near the intestinal gland, while appeared more scattered near the tip of the villi. PAS staining showed characteristic purple color and goblet shape. As shown in Fig.6 and Table 2, the HS group showed diminished numbers of goblet cells in various small intestinal segments, and obviously lower than those of the control group. Remarkably, after treated with GABA, the GABA+HS group displayed much higher numbers of goblet cells than both the HS and control groups (Fig.6; Table 2).

Table 2. Comparison of the number of intestinal mucosal epithelial goblet cells among the control and experimental groups[1] (cells/100 IEC)

Items	Groups	3d	6d	9d	12d	15d
Duodenum	Control	39.33±3.11^{ab}	37.67±1.67^{a}	41.67±5.78^{a}	34.67±5.56	39.75±2.88^{a}
	HS	33.67±2.89^{b}	33.17±1.68^{b}	29.67±3.78^{b}	32.03±2.67	35.33±1.56^{b}
	GABA+HS	45.33±1.56^{a}	39.50±1.50^{a}	43.67±2.89^{a}	33.33±1.11	42.02±4.40^{a}
	Sign	NS	*	*	NS	*
Jejunum	Control	47.03±1.33^{a}	52.60±2.88^{a}	49.20±1.64^{a}	39.83±2.50^{a}	46.05±2.13^{a}
	HS	40.04±1.33^{b}	42.03±1.33^{b}	41.80±5.36^{b}	33.60±4.23^{b}	37.80±2.24^{b}
	GABA+HS	50.33±1.78^{a}	54.20±3.50^{a}	50.25±2.25^{a}	37.33±1.56^{a}	49.67±3.11^{a}
	Sign	*	*	*	*	*
Ileum	Control	57.50±3.25^{ab}	60.01±1.50^{a}	60.80±2.59	56.75±3.88	62.33±1.78^{a}
	HS	52.40±4.32^{b}	48.02±2.12^{b}	58.20±3.84	53.02±3.26	49.67±4.22^{b}
	GABA+HS	61.25±1.75^{a}	60.04±1.51^{a}	63.33±4.89	53.80±1.80	53.50±2.06^{ab}
	Sign	NS	*	NS	NS	NS

[1] In the same rank, data with different superscript letters indicate statistically significant difference. * ($p<0.05$)

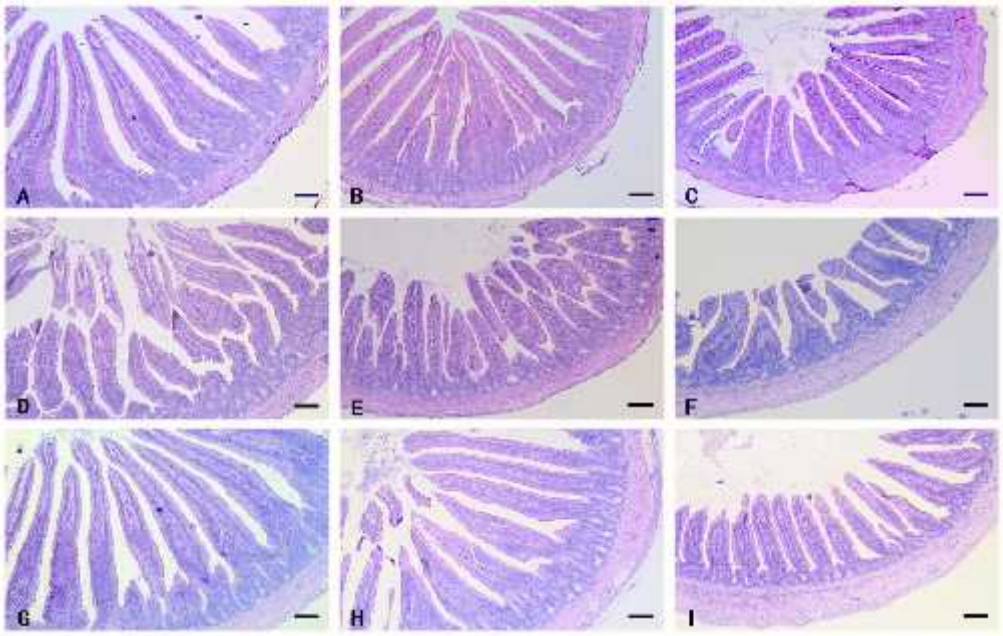

Figure 1. HE staining of the sections derived from chicken intestine tissues.
As shown in the picture, compared to the control group (A, duodenum; B, jejunum; C, ileum), the small intestinal mucosal parameters of the HS group (D, duodenum; E, jejunum; F, ileum) were declined; Meanwhile, the small intestinal mucosal parameters of the GABA+HS group (G, duodenum; H, jejunum; I, ileum) were restored to levels equivalent with or superior to the control group. Bar=100μm.

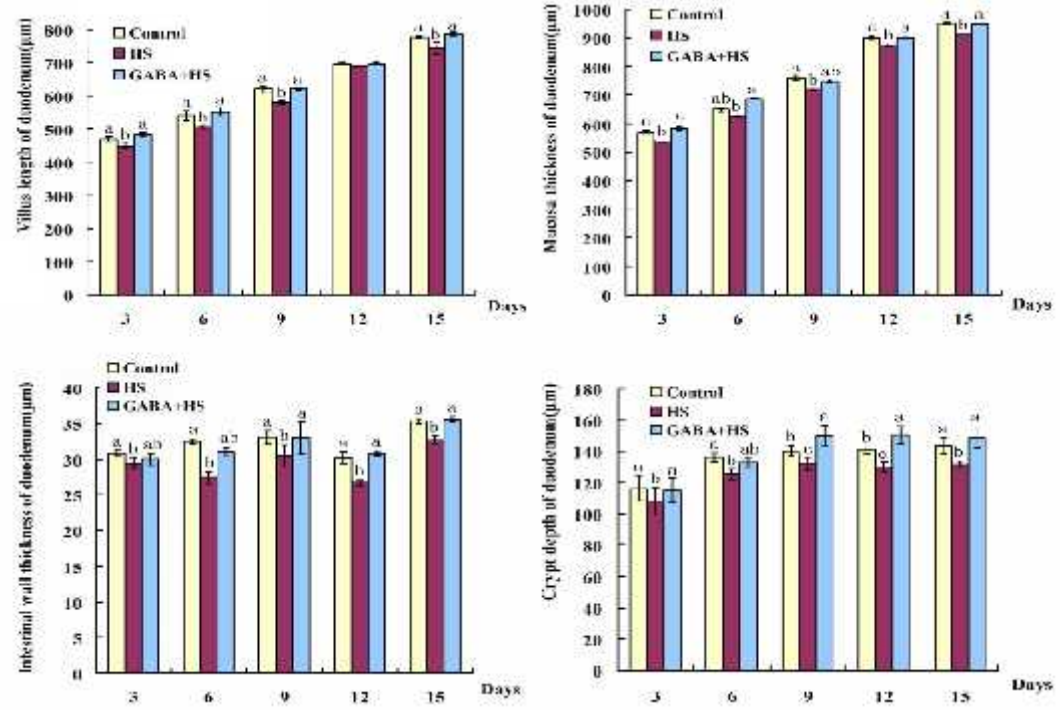

Figure 2. Effects of GABA on the structure and development of duodenum in heat stressed Wenchang chicken. The different letter means differ significantly (*P<0.05*), the same letter or no letter means no difference (*P>0.05*).n=6.

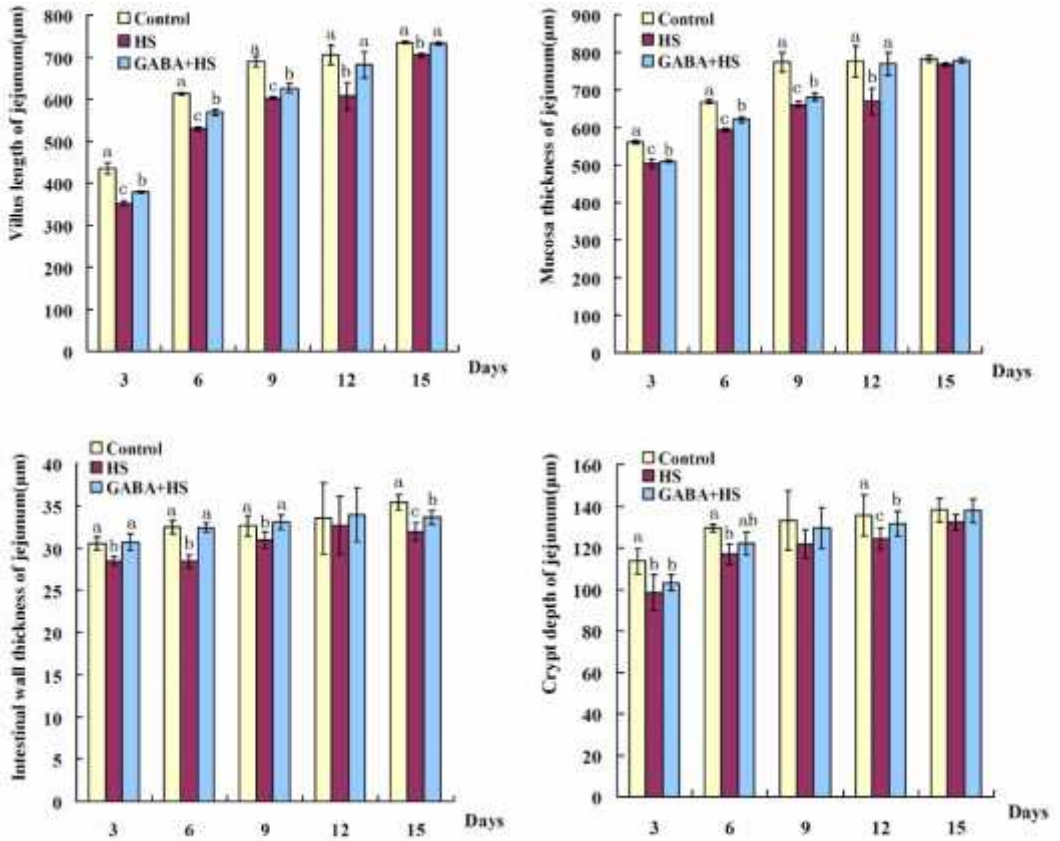

Figure 3. Effects of GABA on the structure and development of jejunum in heat stressed Wenchang chicken.The different letter means differ significantly (*P<0.05*), the same letter or no letter means no difference (*P>0.05*).n=6.

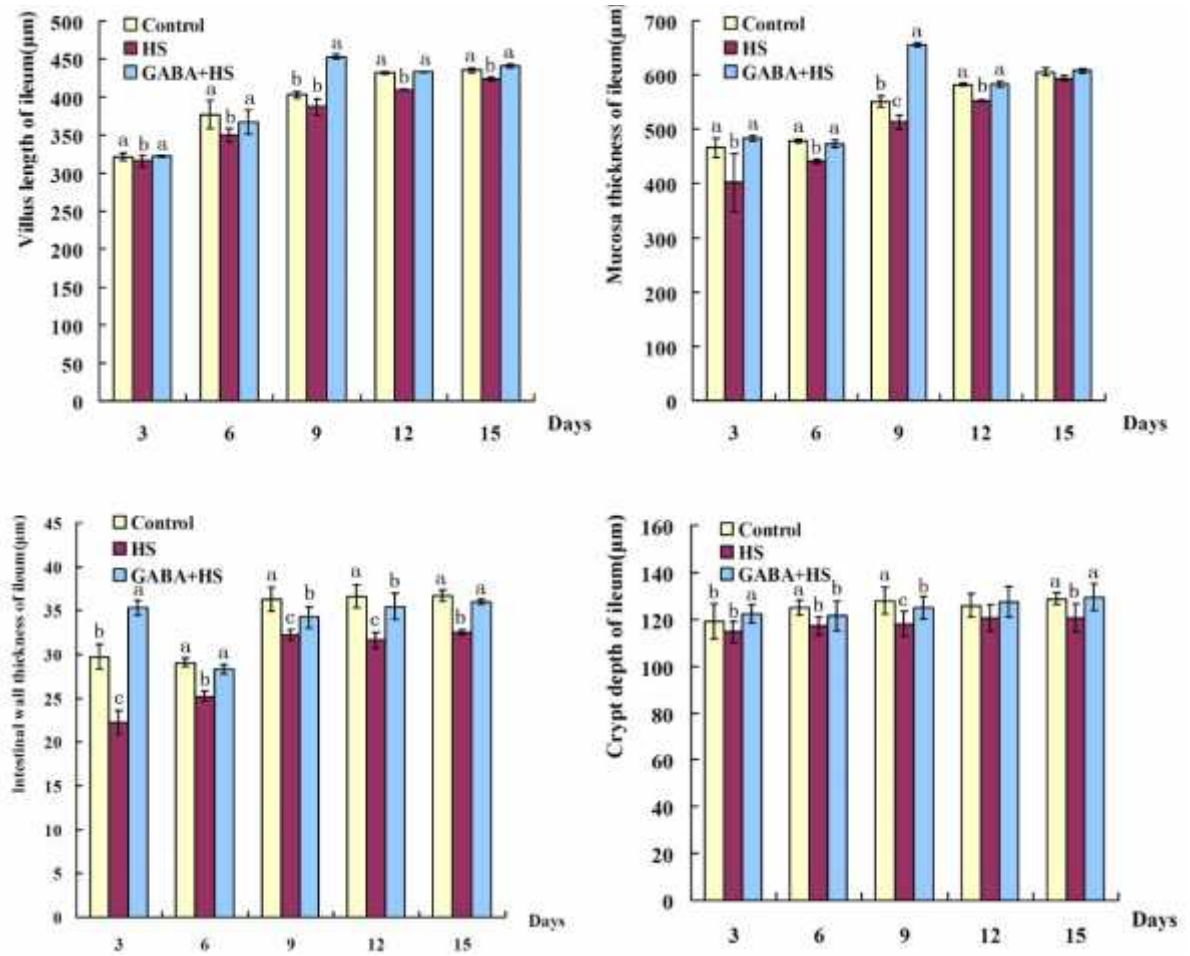

Figure 4. Effects of GABA on the structure and development of ileum in heat stressed Wenchang chicken. The different letter means differ significantly (*P<0.05*), the same letter or no letter means no difference (*P>0.05*).n=6.

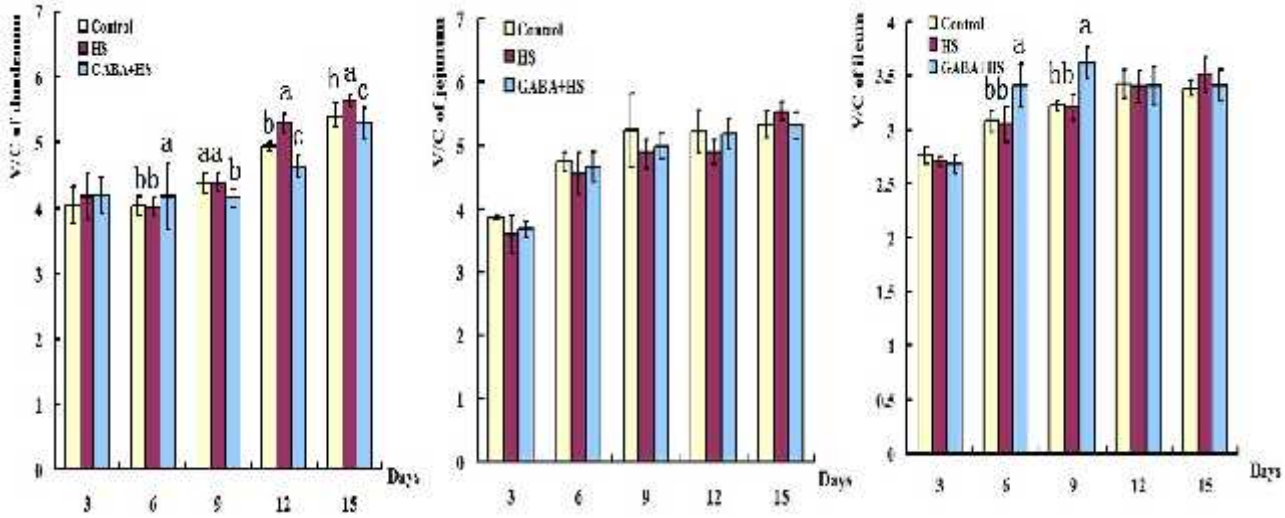

Figure 5. Effects of GABA on the V/C of thesmall intestine in heat stressed Wenchang chicken. The different letter means differ significantly (*P<0.05*), the same letter or no letter means no difference (*P>0.05*).n=6.

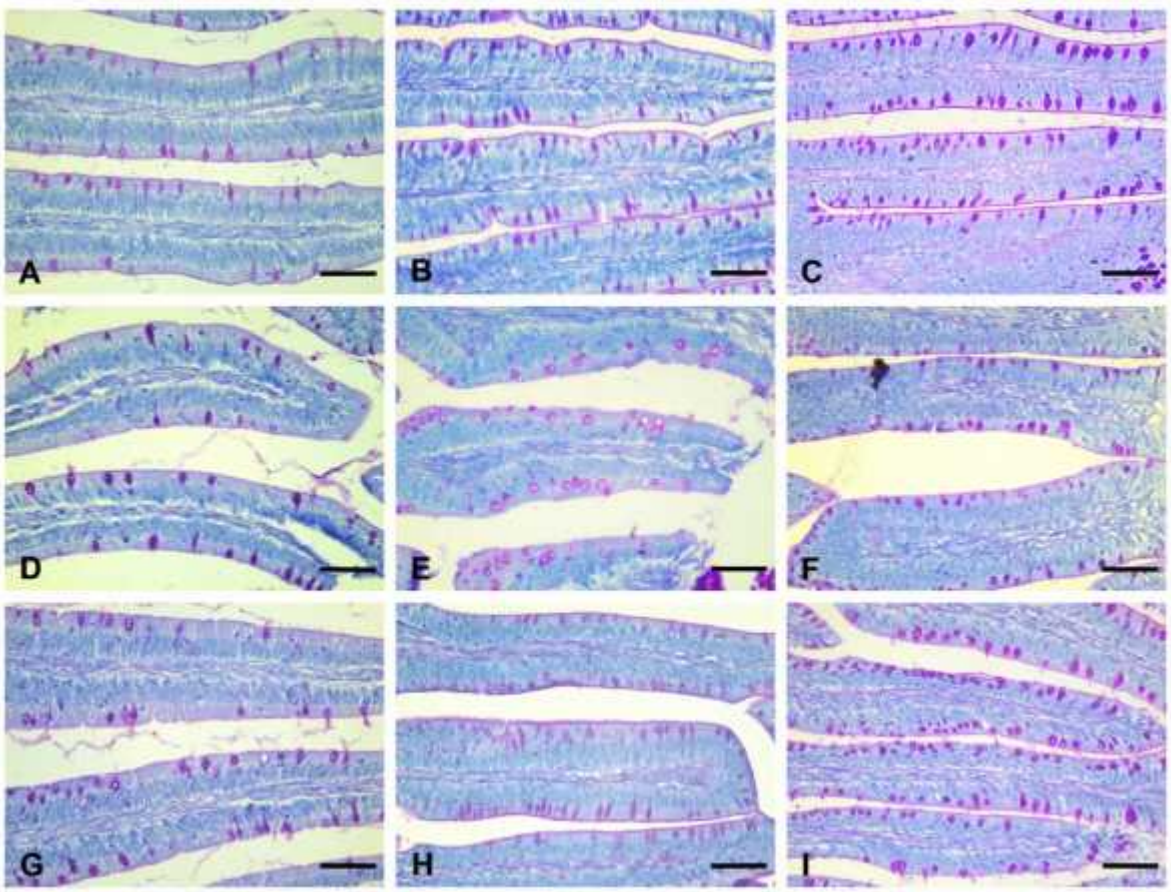

Figure 6. PAS staining of the sections derived from chicken intestine tissues. As shown in the picture, compared to the control group (A, duodenum; B, jejunum; C, ileum), The goblet cells numbers of the HS group (D, duodenum; E, jejunum; F, ileum) were declined; Meanwhile, the goblet cells numbers of the GABA+HS group (G, duodenum; H, jejunum; I, ileum) were much higher than both the HS and control groups. Bar=50μm.

DISCUSSION

In recent years, global warming has caused serious decline in the productivity of large-scale chicken farms, resulting in significant loss of revenue. Previous studies in our laboratory showed that the chicks under heat stress experienced less feed intake, more water consumption, increased respiration, high body temperature and listlessness, more rest instead of standing (Chen *et al*, 2002). In this study, they showed a decline in food intake, body weight, average weight gain and an increased feed to gain ratio. It has been reported that intestinal villus length, crypt depth, mucosa thickness and villus surface area represent key parameters for intestinal digestion and absorption functions (Han, 1991). Among them, intestinal villus length and width and epithelial cell (IEC) number are directly correlated with absorption area; for example, longer villi and greater width indicate greater absorption surface (Li *et al*, 2002). Importantly, the villi length/crypt depth ratio (V/C) is regarded as a general indicator of intestinal functional state. Reduction in the ratio indicates abnormal development or damages of mucosal epithelium, which in turn correlates with impaired digestion and absorption functions and growth. Nutritional physiology studies also supported the notion that increased V/C values are reflective of more robust intestinal functions (Han, 1991). Furthermore, Li and Zhu (2003) investigated the damage in the intestinal epithelial cell membrane as a result of heat stress in mice and reached the same conclusion. It is likely that under high temperature, the body accelerates peripheral blood circulation in order to expedite cooling; however, various tissues may suffer from insufficient supply of oxygen and iron, leading to many malfunctions.

Small intestinal mucosa, with highly specialized structure and physiological function, is most sensitive to oxygen and iron insufficiency. Consequently, stress induces ischemia and hypoxia, leading to the production of oxygen free radicals and inflammatory cytokines. Various downstream damages may follow, including

compromised structure integrity and detachment of intestinal mucosal epithelial cells, shrinkage of intestinal mucosa, villous necrosis, reduced surface area, and damages to intestinal immune-relevant cells such as intestinal intraepithelial lymphocytes, intestinal goblet cells and IgA^+ cells. These adverse events affect the digestion, absorption and immune functions of the intestine, ultimately resulting in reduced production of animals (Xu, 2006).In fact, the mucosa is also responsible for nutrient absorption and waste secretion, which require a selectively permeable barrier (Turner, 2009).Nutrients are in direct contact with the intestine so that they can be efficiently absorbed, and at the same time, intestine protects against the intrusion of harmful entities, such as toxins and bacteria, which may enter the digestive system with food (Furness *et al*, 1999). Goblet cells are typically scattered among columnar cells, and regulate local intestinal immune functions via specific or nonspecific immune mechanisms (Gaskins, 1996; Gu*et al*, 2002). For example, goblet cells secrete mucilage for the protection of the intestinal mucosa, and serve as structure components for the intestinal mechanical barrier. When under heat stress, the concentration of corticosterone in the blood increased, which might increase the number of apoptotic lymphocytes in mesenteric, increase intestinal permeability, and harm the intestinal mucosal immunity (Star *et al*, 2008; Sohail *et al*, 2010)

In the current study, rupturing of the tip of the villi was observed in duodenum, jejunum and ileum in the HS group. Furthermore, edema in the local region around the small intestinal villi was found; fracture and loss of villi were common, and in some areas villi were significantly truncated. In comparison, the small intestinal wall was relatively thick in the GABA+HS group. The small intestinal mucosal epithelial structure appeared intact, with thick and uniform cell coat. Both villus length and mucosa thickness were superior to those of the HS group. The control and GABA+HS groups showed greater numbers of goblet cells, and mucin secreted by the goblet cells also seemed to form an effective mechanical barrier. These results indicate the severe damage caused by heat stress and highlight the beneficial roles of GABA in heat stress response. As a nutritional regulatory factor, GABA could be converted into Gln indirectly in the enteric nervous system, which is a nitrogen source for cell proliferation and intestinal mucosa repair. Increased glutamine production can modulate the damage to the small intestine structure, reduce intestinal permeability, promote crypt cell proliferation and intraluminal secretion of cytosol and protect the physical barrier of small intestine (Sun *et al.*, 2004). V/C of the duodenum in the HS group was larger than that of the control group, while V/C of the jejunum and ileum was smaller than that of the control group. These results might be caused by the more serious crypt damage in the duodenum, and more serious villus damage in the jejunum and ileum. This result is consistent with their functions. The crypt for secretion in the duodenum is more developed, and the villus of jejunum and ileum, which is more important for digestion and absorption, is easy to be damaged. But the specific mechanisms still need to be clarified.

The observations of the current study suggest that heat stress caused damages to the small intestinal mucosa, which likely impaired the mucosal barrier and digestion and absorption functions. These effects eventually lead to abnormal growth and development, which has been reported by many researchers. Feeding chicken with GABA restored the normal structure and morphology of the small intestine, and increased the absorption area. Consequently, this appeared to promote absorption at the small intestine and proliferation of goblet cells, thus maintaining normal morphology and mechanical barrier functions of the small intestinal mucosa.

It has been previously reported that under high temperature conditions, daily diet supplemented with GABA enhanced the digestive enzyme activities, anti-oxidation activity and the immune activity (Zhang *et al*, 2012). During the entire experimental period, the average weight gain per day was increased by 13.08%, whereas the ratio of feed to weight gain was reduced by 7.81%. Glutathione peroxidase and superoxide dismutase activities were increased to varying degrees, and malondialdehyde (MDA) levels were reduced (Cao *et al*, 2008). Therefore, GABA supplement in daily diet enhanced the performance of pigs and alleviated heat stress. In addition, under high temperature conditions, GABA supplemented daily diet was also found to strongly promote growth in pigs, and enhance the secretion of growth hormones and melatonin as well as the levels of thyroid stimulating hormone (TSH) **(**Fan *et al*, 2007**)**.

Heat stress caused significant structural damages to chicken small intestinal mucosa, and markedly reduced the number of goblet cells, impaired the structural integrity of small intestinal tissues, which likely reduced the activity of mucosal digestive enzymes. As a result, the digestion and absorption functions, as well as the normal mechanical barrier functions of small intestinal mucosa may be adversely affected. On the other hand, GABA showed protective and reparative effects to alleviate HS-induced damages of the intestinal mucosa, increased the number of goblet cells, alleviated the heat stress by repairing the damages to the intestinal mucosa, thus promoting the absorption functions of mucosa and the clearance of excessive free radicals (Mujahid *et al*, 2005). These beneficial effects of GABA likely will lead to the optimal production of chickens, through enhancing small intestinal mucosal immune functions, promoting growth hormone secretion and inhibiting small intestinal somatostatin secretion. Therefore, our studies may

provide experimental support for the use of GABA as a food additive to improve broiler growth under heat stress conditions.

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