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PROTECTIVE EFFECT OF γ-AMINOBUTYRIC ACID ON ANTIOXIDATION FUNCTION IN INTESTINAL MUCOSA OF WENCHANG CHICKEN INDUCED BY HEAT STRESS

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ABSTRACT

This study was conducted to explore the effects of heat stress (HS) and γ-aminobutyric acid (GABA) on antioxidation in intestinal mucosa of Wenchang chickens. One-day-old male chickens were randomly divided into GABA and control (CK) groups after 15 days feeding. The chicken from GABA group fed with 0.2 ml GABA solution and the chicken from CK group fed with 0.2 ml normal saline daily. At the age of 35 days, chickens in two groups were then divided into normal group (HS_B), heat stress group (HS_A) and heat stress recovery group (HS_R), respectively. HS_A and HS_R were conducted in an environment of 40 ± 0.5 °C for 3 h. Results indicated that the activities of antioxidase and T-AOC in HS_A group were lower than those in HS_B and HS_R groups; the content of MDA was higher than that in HS_B and HS_R groups. On the other hand, the activities of antioxidase and T-AOC in GABA group was higher than those in CK group and the content of MDA from GABA group was lower than that from CK group. In a word, heat stress caused a significant antioxidative damage to chicken small intestinal mucosa. In contrast, GABA can effectively alleviate the damage.

Key words: GABA; heat stress; intestinal mucosa; antioxidation function; chicken.

INTRODUCTION

In recent years, increasing global warming and heat waves with high frequency and high intensity result in a substantial reduction of livestock production and income (McMichael and Keith, 2010; Barnett *et al*, 2012). Heat stress can affect the growth, metabolism, immunity and reproduction of animals, and even can result in an immeasurable impact (Bloemhof *et al*., 2012; Nesamvuni *et al*., 2012). The latest statistics showed that animal damage caused by heat stress results in economic loss of nearly 728 million dollars in California and other states (Carroll *et al*., 2012). Due to the widespread application of feeding pattern in a high-density mode, the chickens during breeding process reveal gradually increased heat sensitivity. In addition, the lack of sweat glands in dense feathers leads to more vulnerable to acute and chronic heat stress in high temperature environment (Smith and Teeter, 1987; Copper and Washburn, 1998). Therefore, exploring the impact of heat stress on poultry organism and acquiring a better strategy to alleviate heat stress and ensure animal production in hot summer has practical significance.

Intestine is the major organ for the absorption of nutrients, and plays an important role in the maintenance of normal life, so that it is named as the central organ of stress responses (Wilmore *et al*., 1988). Previous studies have demonstrated that heat stress can lead to a significant increase in intestinal permeability and epithelial injury as well as intestinal barrier dysfunction (Oliver *et al*., 2012). Intestinal mucosa, as a major location of nutrient absorption and transportation, plays a key role in growth and development. The excellent status of intestinal mucosa is the physiological basis for nutrient uptake and normal growth of animals. Heat stress can lead to a series of pathological changes that are characteristics of epithelial cell shredding, laminal edema and intestinal villus fracture in duodenal, jejunal and ileal mucosa of chickens (Liu *et al*., 2011a). The vulnerability of intestinal mucosa to thermal stress is due to non-protein thiol groups at a high concentration level in intestinal mucosa. Under heat stress environment, the release of cortisol and catechol can produce a large number of active oxygen radicals that are capable of acting with thiol groups to result in protein denaturation and enzyme inactivation, thereby destroying enzymatic and non-enzymatic antioxidation systems in the body, even exacerbating oxidative stress (Yadav and Korde, 2011). In addition, oxygen free radicals can also bind to unsaturated fatty acids in membrane to induce lipid peroxidation (Li *et al*., 2011). Therefore, antioxidation function in intestinal mucosa under heat stress environment reveals its significance for ensuring normal physiological functions of intestinal tract. Currently, domestic and international researches on intestinal mucosa are mainly focused on the functions of digestion and absorption, barrier and immunity. However, antioxidation function of intestinal mucosa is less reported. Therefore, exploring the impact of heat stress on antioxidation function of intestinal mucosa in chickens

will play an important role in guiding practical production.

Gamma-aminobutyric acid (GABA), widely distributed in nervous system tissue, is regulated by $GABA_A$, $GABA_B$ and $GABA_C$ receptors on synaptic membrane, and involved in the regulation of a variety of behavioral and physiological responses in body (Sliwowska *et al*., 2006). Our previous studies have confirmed that GABA can alleviate heat stress to some extent in broilers (Chen *et al*., 2002). Similar result that GABA is a central nervous system sedative agent to resist thermal stress and promote growth and development is observed in animals (Jonaidi, 2012). A certain amount of GABA can result in a significant increase in the activities of Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), total antioxidation capacity (T-AOC) and an obvious reduction of malondialdehyde (MDA) in serum of pigs during the growth period in summer (Hu *et al*., 2008), suggesting that GABA can improve antioxidation capacity under high temperature conditions. However, whether GABA can alleviate heat stress-induced damage of antioxidation function in intestinal mucosa still needs to be further studied.

Currently, the impact of heat stress to antioxidation function has been explored to some extent, but most of them are concentrated on serum. The studies on intestinal mucosa are seldom. Whether heat stress can impact on antioxidation function in intestinal mucosa and GABA can mitigate heat stress-induced damage of antioxidation function in intestinal mucosa is rarely reported. Moreover, Wenchang chicken is one of poultry varieties with high quality in China. The strength of antioxidation function in intestinal mucosa can cause a direct impact on digestion and absorption of nutrients, and physiological development of Wengchang chicken as well as indirect impact of its economic benefits. In summary, understanding protective effect of GABA on the damage of antioxidation function in intestinal mucosa of Wengchang chicken induced by heat stress has an extreme significance for exploring heat stress-alleviating strategies and guiding practical production.

MATERIALS AND METHODS

Feeding and management of experimental animals: Two hundred and sixteen Wenchang male chickens with the age of 1 day were purchased from Hainan Yongji Wenchang Chicken Corporation. After feeding for 15 days, these chickens were randomly divided into GABA group and control (CK) group; each group consisted of six replicates with 18 chickens. The chickens from GABA group fed daily with 0.2 mL of 50 mg/kg GABA solution, while the chickens from CK group fed with same amount of saline until 35 days. The chickens from both groups were maintained at 26.5 ± 1.09 ℃ and provided free access to water and foods. Diets were formulated to meet the NRC (1994) nutrient requirements for all nutrients (Table 1).

Heat stress treatment and sampling: When the chickens were aged of 35 days, acute heat stress treatment was conducted. The chickens from CK group were randomly divided into three groups designated as normal temperature group (HS_B), heat stress group (HS_A) and heat stress recovery group (HS_R). Similarly, the chickens from GABA group were subjected to same treatment. The sampling in HS_B group was conducted before heat stress treatment. The chickens from rest 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 3 h (13:00-16:00) (Chen *et al*., 2002). After heat stress treatment, chickens in HS_A group were sampling immediately, and chickens in HS_R were subjected to 3 h recovery at 26.5 ± 1.09 °C and then sampled (Zhang, 2008). When completing corresponding treatment or recovery, the chickens were decapitated immediately and the duodenal, jejunal and ileal mucosa were harvested on ice bath, and then placed them in a glass homogenizer. The saline was added to homogenizer containing intestinal mucosa according to the mass ratio of 1:9 and 10% intestinal mucosa homogenate were prepared at 4 °C ice bath at 3000 r/min for10 min, respectively. The activities of GSH-Px, T-SOD and CAT, T-AOC and the content of MDA in supernatant were determined (Tan *et al*., 2011).

Index and methods of determination: Glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), catalase (CAT) activities, total antioxidation capacity (T-AOC) and malondialdehyde (MDA) content in intestinal mucosa were determined by corresponding kits according to manufacturer's instructions. All kits were purchased from Nanjing Jiancheng Bioengineering Institute, China.

Data processing and statistical analysis: The experimental data were subjected to the analysis of variance procedures that are appropriate for a completely randomized design using the General Linear Model (GLM) procedures of SPSS software, version 16.0 (SPSS Inc, Chicago, IL, US). Means were compared using Duncan multiple range test and statistical significance were set up at $P < 0.05$.

RESULTS

Effect of GABA on GSH-Px activity in intestinal mucosa of broilers induced by heat stress: As shown in Table 2, the activity of GSH-Px in HS_A showed a decrease trend compared with HS_B and had a significant difference in CK group of ileum. Under the condition of

HS_B, the activity of GSH-Px in GABA group did not exhibit an obvious difference when compared with CK group ($P > 0.05$). Under the condition of HS_A, the activity of GSH-Px in duodenal mucosa from GABA group was significantly higher than that from CK group ($P < 0.05$); similarly, the activity of GSH-Px in ileal mucosa from GABA group was significantly higher than that from CK group ($P < 0.05$), while the activity of GSH-Px in jejunal mucosa from GABA group did not reveal an obvious difference when compared with CK group ($P > 0.05$). Under the condition of HS_R, the activity of GSH-Px in ileal mucosa from GABA group was significantly higher than that from CK group ($P < 0.05$), but the activity of GSH-Px in duodenal and jejunal mucosa from GABA group revealed a decreasing trend, especially in duodenum.

Effect of GABA on T-SOD activity in intestinal mucosa of broilers induced by heat stress: As shown in Table 3, the activity of T-SOD in HS_A showed a decrease trend in general compared with HS_B and had a significant difference in CK group of jejunum. Under the condition of HS_B, the activity of T-SOD in duodenal, jejunal and ileal mucosa from GABA group showed an increase trend compared with that from CK group although no significant difference was observed ($P > 0.05$). Under the condition of HS_A, the activity of T-SOD in duodenal and jejunal mucosa from GABA group was significantly higher than that from CK group ($P < 0.05$), but the activity of T-SOD in ileal mucosa from GABA group showed a decrease trend compared with that from CK group but no significant difference was observed ($P > 0.05$). In contrast, under the condition of HS_R, the activity of T-SOD in intestinal mucosa from GABA group did not exhibit an obvious difference with that from CK group ($P > 0.05$).

Effect of GABA on CAT activity in intestinal mucosa of broilers induced by heat stress: As shown in Table 4, the activity of CAT in HS_A showed a decrease trend compared with HS_B and had a significant difference in both groups of duodenum, jejunum and in CK group of ileum. Under the condition of HS_B, CAT activity in intestinal mucosa from GABA group revealed no significant difference with that from CK group ($P > 0.05$). Under the condition of HS_A, CAT activity in jejunal mucosa from GABA group was significantly higher than that from CK group ($P < 0.05$); similarly, CAT activity in duodenal and ileal mucosa from GABA group was very significantly higher than that from CK group ($P < 0.05$). Under the condition of HS_R, CAT activity in duodenal mucosa was significantly higher than that from CK group ($P < 0.05$); although CAT activity in jejunum and ileum mucosa showed no significant difference compared with CK group ($P > 0.05$), but an increase trend was observed.

Effect of GABA on T-AOC in intestinal mucosa of broilers induced by heat stress: As shown in Table 5, T-AOC in HS_A showed a decrease trend compared with HS_B and had a significant difference in both groups of duodenum, jejunum and in GABA group of ileum. Under the condition of HS_B, T-AOC in intestinal mucosa from GABA group was higher than that from CK group and showed a significant difference in ileum mucosa ($P < 0.05$). Under the condition of HS_A, T-AOC in intestinal mucosa from GABA group was very significantly higher than that from CK group ($P < 0.05$). Under the condition of HS_R, although T-AOC in intestinal mucosa from GABA group showed an elevated trend, no significant difference was observed ($P > 0.05$).

Effect of GABA on MDA content in intestinal mucosa of broilers induced by heat stress: As shown in Table 6, the content of MDA in HS_A showed an increase trend in general compared with HS_B and had a significant difference in ileum. Under the condition of HS_B, MDA contents in duodenal, jejunal and ileal mucosa from GABA group revealed a decrease trend when compared with CK group, but no significant difference was observed ($P > 0.05$). Under the condition of HS_A, MDA content in jejunal and ileal mucosa from GABA group was significantly lower than that from CK group ($P < 0.05$), but showed an increase trend compared with that from CK group in duodenal mucosa ($P > 0.05$). Under the condition of HS_R, MDA content in ileal and duodenal mucosa from GABA group was significantly lower than that from CK group ($P < 0.05$); however, the content of MDA in jejunal mucosa from GABA group was significantly higher than that from CK group ($P < 0.05$).

DISCUSSION

Effect of GABA on CAT, T-SOD and GSH-Px activities in intestinal mucosa of broilers induced by heat stress: Antioxidation function in animal body requires the participation of antioxidation enzyme system, which includes GSH-Px, SOD and CAT. GSH-Px, an important antioxidation enzyme in body (Wei *et al*., 2011), specifically catalyzes reduced glutathione (GSH) to clear H_2O_2 and reduce the generation of lipid peroxides, thus protecting the structure and functions of cell membrane. SOD, an important component of radical-scavenging system in body, directly involves in antioxidation process. CAT, a class of enzymes conjugating iron porphyrin prosthetic groups, can eliminate hydroxyl free radicals and H_2O_2, and protect H_2O_2-induced damage in body (Ahmad *et al*., 2012).

In the present study, compared with CK group, the activities of GSH-Px, T-SOD and CAT in intestinal mucosa of broilers from GABA group did not exhibit a significant difference in normal temperature although total activity revealed an increasing trend, suggesting that

GABA can improve antioxidation enzyme activity in intestinal tract, which is consistent with the results from Huang's report (Huang, 2011). The possible reason is that increased GABA can promote the increase of glutamate level in the body. Glutamate, as the raw material for the synthesis of GSH in the antioxidation system, can promote the synthesis of GSH, and improve and maintain the reduced GSH level, thus enhancing the activity of GSH-Px in the antioxidation system. Under heat stress environment, the activities of GSH-Px, T-SOD and CAT in intestinal mucosa of broilers from GABA group were significantly higher than those from CK group, which indicated that GABA could effectively improve antioxidation enzyme activity in intestinal mucosa of broilers, alleviate the impact of heat stress on antioxidation enzyme activity. However, the activity of T-SOD in ileal mucosa of broilers from GABA group was lower than that from CK group, which is possibly due to that GABA completes its antioxidation capacity in ileal part mainly through improving GSH-Px and CAT activities, or antioxidation system in ileal mucosa is a dynamic balance status. The activities of SOD, GSH-Px and CAT in the antioxidation system can not reveal a simultaneous increase. The activation of one mechanism and corresponding inhibition of another mechanism may be the automatic protection mechanism in the body. However, this possible mechanism still needs to be further investigated and confirmed. During the recovery period after heat stress, the activities of GSH-Px, T-SOD and CAT in intestinal mucosa of broilers from both groups revealed an obvious increase, suggesting that the protection system of anti-lipid peroxidation is initiated, which is well agreement with previous study (Liu, 2011b). The antioxidation enzyme activity in intestinal mucosa of broilers from GABA group was obviously higher than that from CK group, which indicated that GABA has the promotion function to the recovery of antioxidation capacity after heat stress treatment. However, the activity of GSH-Px in duodenal mucosa of broilers from GABA group revealed an obvious reduction when compared with CK group, which may be due to the decreased pH in duodenal mucosa during the metabolism and conversion of GABA. The pH at the range of 6.5-7.5 in duodenal mucosa is not the optimal pH (8-9) for GSH-Px (Wang *et al*., 2011) so that the lower activity of GSH-Px is observed finally. In addition, the lower activity of T-SOD in jejunal and ileal mucosa of broilers from GABA group when compared with CK group may result from the elevated HSP70 protein under the heat stress environment. Previous study has demonstrated that HSP70 has antioxidation activity through inhibiting key enzymes for the production of oxygen free radicals and can improve SOD activity to reduce the generation of oxygen free radicals, thus realizing the protective function to the body (Aggarwal *et al*., 2012).

Effect of GABA on MDA and T-AOC in intestinal mucosa of broilers induced by heat stress: MDA is a final product of lipid peroxidation reaction chain. MDA content can reflect the level of lipid oxidation and the degree of cell membrane damage during the process of lipid peroxidation. T-AOC is a functional status indicator of antioxidation system in the body and can represent compensatory capacity of antioxidation enzymatic and non-enzymatic systems from external stimuli, as well as the metabolism status of free radicals. T-AOC, as the total antioxidation capacity, has much more advantages to evaluate oxidative damage than other individual antioxidation indicators. The significant reduction of T-AOC can reflect a large amount consumption of antioxidation substances in the body, which is the indirect indicator as the generation of a large number of free radicals (Song *et al*., 2008).

In our study, MDA content and T-AOC level in intestinal mucosa of broilers from GABA group is much higher than those from CK group at normal temperature, especially T-AOC in ileal mucosa, suggesting that GABA can significantly improve total antioxidation capacity and reduce the generation of free radicals in the body. Under heat stress stimulation, T-AOC level in intestinal mucosa of broilers from GABA group was significantly higher than that from CK group, but MDA content in jejunal and ileal mucosa was greatly reduced when compared with CK group, which indicated that total antioxidation capacity in intestinal mucosa from GABA group is significantly stronger than CK group, and jejunal and ileal mucosa has less damage in GABA group. All of these results are consistent with previous study (Zou, 2009). Followed by 3 h recovery after heat stress treatment, no significant difference in T-AOC level in intestinal mucosa between GABA and CK groups was observed, although T-AOC level in GABA group was still higher than that in CK group. Compared with CK group, MDA content in duodenal and ileal mucosa revealed a significant reduction in GABA group, which suggested that GABA can promote the recovery of antioxidation function after heat stress stimulation. The difference in MDA content in duodenal mucosa may be due to a large number of enzymes produced in duodenum that is close to pancreatic duct and common bile duct. These enzymes can easily eliminate oxygen free radicals produced by heat stress. The extremely higher MDA content in jejunal mucosa is possibly due to non-restricted feeding during the recovery period of heat stress. Jejunum is the major location of digestion and absorption of nutrients. Restricted energy intake can improve antioxidation enzyme activity and enhance radical-scavenging capacity as well as reduce radical-induced intestinal tissue damage (Yu, 1996).

In conclusion, GABA can significantly improve the activities of GSH-Px, T-SOD and CAT in intestinal mucosa of broilers under the stimulation of heat stress,

maintain higher radical-scavenging capacity in the body, and ensure redox chain stability and homeostasis. Meanwhile, GABA can reduce MDA content and improve T-AOC level to alleviate oxidative damage of tissues induced by free radicals and to ensure higher total antioxidation capacity in the body under the heat stress environment. All of these investigations clearly demonstrate that GABA plays an important role in alleviating the damage of antioxidation system in intestinal mucosa of broilers under the heat stress environment and preventing antioxidation function of intestinal mucosa. Its mechanisms need to be further explored.

Table 1. Composition and nutrition levels of the basal diet[1] (fed basis, %)

Ingredients	Content	Nutrient levels[2]	content
Corn	63.00	CP	15.50
Soybean meal	19.00	ME (MJ/kg)	11.24
Cottonseed meal	6.00	Ca	0.80
Rapeseed dregs	5.00	Lys	0.73
Fish meal	4.33	TP	0.60
Calcium bicarbonate	1.50	Met	0.35

[1] Formulated according to NRC (1994) to cover nutrients requirements.
[2] CP: Crude protein; ME: Metabolic energy, Values are deterministic values except ME.
Ca: Calcium; Lys: Lysine; TP: Total phosphorus; Met: Methionine.

Table 2. Influence of GABA on the activity of GSH-PX in intestinal mucosa of heat stress chickens (U/mL) [1]

Intestinal Segment	Group	HS_B[2]	HS_A[3]	HS_R[4]
Duodenum	CK	12.30^{b}	10.74^{b}	23.99^{a}
	GABA	12.54^{ab}	12.13^{b}	17.00^{a}
	S.E.M.	1.15	0.35	1.29
	Sign.	NS	*	*
Jejunum	CK	16.69^{b}	14.51^{b}	28.36^{a}
	GABA	16.48^{b}	15.33^{b}	27.98^{a}
	S.E.M.	0.79	1.04	1.16
	Sign.	NS	NS	NS
Ileum	CK	18.58^{a}	14.29^{b}	18.61^{a}
	GABA	18.52^{b}	17.19^{b}	23.09^{a}
	S.E.M.	0.75	0.52	0.95
	Sign.	NS	*	*

[1] Data with different superscript letters indicate statistically significantly difference.
* ($p<0.05$).
[2]HSB means normal temperature treatment, 3 HSA means heat stress treatment, 4 HSR means 3h recovery after heat stress. N=6.

Table 3. Influence of GABA on the activity of T-SOD in intestinal mucosa of heat stress chickens (U/mL) [1]

Intestinal Segment	Group	HS_B[2]	HS_A[3]	HS_R[4]
Duodenum	CK	58.02^{b}	56.31^{b}	66.97^{a}
	GABA	59.35^{c}	69.41^{b}	74.85^{a}
	S.E.M.	0.97	2.22	1.57
	Sign.	NS	*	NS
Jejunum	CK	57.29^{b}	43.98^{c}	71.85^{a}
	GABA	67.01	64.57	69.33
	S.E.M.	2.89	3.52	1.91
	Sign.	NS	*	NS
Ileum	CK	53.35	51.09	47.92
	GABA	56.52	48.82	47.36
	S.E.M.	3.23	2.22	0.71
	Sign.	NS	NS	NS

[1] Data with different superscript letters indicate statistically significantly difference.
* ($p<0.05$).
[2] HSB means normal temperature treatment, 3 HSA means heat stress treatment, 4 HSR means 3h recovery after heat stress. N=6.

Table 4. Influence of GABA on the activity of CAT in intestinal mucosa of heat stress chickens (U/mL) [1]

Intestinal Segment	Group	HS_B[2]	HS_A[3]	HS_R[4]
Duodenum	CK	11.92[a]	8.33[b]	8.25[b]
	GABA	11.88[a]	9.79[b]	10.01[b]
	S.E.M.	0.18	0.29	0.28
	Sign.	NS	*	*
Jejunum	CK	9.304[a]	7.588[b]	8.145[b]
	GABA	9.59[a]	8.45[b]	8.49[b]
	S.E.M.	0.25	0.18	0.13
	Sign.	NS	NS	NS
Ileum	CK	8.13[a]	6.78[c]	7.41[b]
	GABA	8.04[ab]	7.93[b]	8.34[a]
	S.E.M.	0.15	0.20	0.12
	Sign.	*	*	NS

[1] Data with different superscript letters indicate statistically significantly difference.
[*] ($p<0.05$).
[2] HSB means normal temperature treatment, 3 HSA means heat stress treatment, 4 HSR means 3h recovery after heat stress. N=6.

Table 5. Influence of GABA on the activity of T-AOC in intestinal mucosa of heat stress chickens (U/mL) [1]

Intestinal Segment	Group	HS_B[2]	HS_A[3]	HS_R[4]
Duodenum	CK	22.00[a]	13.31[b]	20.69[a]
	GABA	22.06[a]	18.29[b]	22.63[a]
	S.E.M.	0.38	1.03	0.51
	Sign.	NS	*	NS
Jejunum	CK	20.10[a]	10.25[c]	16.46[b]
	GABA	20.91[a]	15.03[b]	16.84[b]
	S.E.M.	0.71	0.91	0.46
	Sign.	NS	*	NS
Ileum	CK	10.91[ab]	9.19[b]	11.17[a]
	GABA	17.70[a]	10.61[b]	11.89[b]
	S.E.M.	1.15	0.29	0.32
	Sign.	*	*	NS

[1] Data with different superscript letters indicate statistically significantly difference.
[*] ($p<0.05$).
[2] HSB means normal temperature treatment, 3 HSA means heat stress treatment, 4 HSR means 3h recovery after heat stress. N=6.

Table 6. Influence of GABA on the activity of MDA in intestinal mucosa of heat stress chickens (nmol/mL) [1]

Intestinal Segment	Group	HS_B[2]	HS_A[3]	HS_R[4]
Duodenum	CK	2.81[b]	2.96[b]	12.11[a]
	GABA	2.79[b]	3.53[b]	6.30[a]
	S.E.M.	0.27	0.35	0.90
	Sign.	NS	NS	*
Jejunum	CK	4.73[b]	5.98[ab]	6.86[a]
	GABA	4.70[b]	4.51[b]	8.39[a]
	S.E.M.	0.46	0.30	0.28
	Sign.	NS	*	*
Ileum	CK	5.75[a]	4.59[b]	6.33[a]
	GABA	5.01[a]	1.16[b]	5.01[a]
	S.E.M.	0.25	0.53	0.30
	Sign.	NS	*	*

[1] Data with different superscript letters indicate statistically significantly difference.
[*] ($p<0.05$).
[2] HSB means normal temperature treatment, 3 HSA means heat stress treatment, 4 HSR means 3h recovery after heat stress. N=6.

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