Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2021 / v.23 / n.3 / 001-008

http://dx.doi.org/10.1590/1806-9061-2020-1430

Original Article

Effects of Heat Stress on Expression of Heat Shock Proteins in the Small Intestine of Wenchang Chicks

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■Keywords

Heat stress; heat shock proteins; small intestine; Wenchang chicks.

Submitted: 01/December/2020
Approved: 05/March/2021

ABSTRACT

In this study, immunohistochemistry and real-time fluorescent-based quantitative PCR were used to evaluate the expression of heat shock protein (HSP) 60, HSP70, and HSP90 in the small intestine of Wenchang chicks. Compared with the control group (CK), the positive expression of HSP60 and *HSP60* mRNA in the heat stress group (HS) were initially higher and subsequently lower in the duodenum ($p<0.01$). In the HS jejunum, the levels of HSP60 were higher ($p<0.01$) and the *HSP60* mRNA was lower ($p<0.05$). Whereas the levels of HSP60 in the HS ileum were higher ($p<0.05$) and then lower ($p<0.01$), and the *HSP60* mRNA was higher ($p<0.01$). The levels of HSP70 were higher ($p<0.01$) and the *HSP70* mRNA was lower in the duodenum ($p<0.05$), while the expression of both HSP70 and *HSP70* mRNA was higher in the jejunum ($p<0.01$) and the ileum ($p<0.05$) of the HS. In the HS duodenum the levels of HSP90 were lower and then higher ($p<0.01$), and the *HSP90* mRNA was higher ($p<0.01$). The expression of both HSP90 and *HSP90* mRNA was higher in the HS jejunum ($p<0.05$). The levels of HSP90 were lower while the *HSP90* mRNA was higher in the HS ileum ($p<0.01$). These results indicate that heat stress disturbed the expression of HSP60, HSP70, and HSP90 in the small intestine of chicks.

INTRODUCTION

The temperature-regulating system is not fully developed in chicks. The body surface of chicks is covered with feathers and contains no sweat glands. Excessive heat in the environment can therefore induce a heat stress reaction leading to adverse effects in chicks. Previous studies have shown that a high-temperature environment causes heat stress and hinders the normal development of chicks, manifesting in damage to the thymus (Liang, *et al.*, 2016), bursa of fabricius (Chen, *et al.*, 2016), and small intestine (Chen, *et al.*, 2015). Heat stress also disrupts the expression of the mitogen-activated protein kinases (MAPKs) signaling pathway (Li, *et al.*, 2020) and the GABAergic system (Liang, *et al.*, 2019) on the hypothalamic–pituitary–gonadal (HPG) axis in chicks. The intestine plays important roles in digestion, nutrient absorption, and substance transport in the body and is particularly sensitive to heat stress. A number of reports have shown that heat stress can cause damage to intestinal morphology and structure; impair intestinal digestion and absorption capacity, antioxidant capacity and intestinal mucosal barrier function (Yu, *et al.*, 2019). As a result, intestinal health is affected, thus hindering the normal growth and development of poultry. Heat shock proteins (HSPs) act as molecular chaperones in the body; heat stress stimulates the expression of HSPs in poultry to varying degrees (Yu, *et al.*, 2019). Studies have shown that acute heat stress causes a sharp decrease

in the total cell protein synthesis of chickens. When HSPs synthesis continues to increase, the total protein content of cells gradually increases, suggesting that HSPs have protective and reparative effects on cells in a high-temperature environment (Xie, *et al.*, 2018). The current study examined the effects of heat stress on the expression of HSPs in the small intestines of Wenchang chicks.

HSP60 is a typical mitochondrial protein in eukaryotes. Under stress conditions, HSP60 may bind to mitochondrial membrane proteins to protect the mitochondrial membrane potential and prevent apoptosis in cells (Tangw *et al.*, 2018).

HSP70 is the most common heat shock protein, which protects organisms from the toxic effects of heating. The expression of HSP70 is an endogenous mechanism of adaptive stress in living cells (Hao, *et al.*, 2012; Gu, *et al.*, 2012).

The N-terminal structure of HSP90 contains an ATP binding pocket, which enables HSP90 to have ATPase activity (Prodromou, *et al.*, 1997). As an ATP-dependent molecular chaperone, HSP90 relies on binding to ATP and the hydrolysis of ATP by the N-terminal to perform its function (Meyer, *et al.*, 2003).

MATERIALS AND METHODS

Experimental animals

One hundred one-day-old healthy male Wenchang chicks were purchased from Tanniu Chicken (Hainan Province, China) and were randomly divided into 2 groups: a control group (CK) and a heat stress group (HS). There was no significant difference in bodyweight between all chicks. All chicks had free access to feed and water, and the feed was formulated to meet the nutrient level suggested by the NRC (1994).

From 2 to 6 weeks of age, chicks in the HS were exposed to heat stress (40.00 ± 0.50°C) for 2 hours from 12:00 pm to 14:00 pm every day, beginning at 2 weeks of age; chicks in the CK were exposed to room temperature (28.69 ± 0.18 °C) during the same time frame. After heat stress, chicks were returned to their cages for routine feeding.

Sample collection

At 2 to 6 weeks of age, 6 chicks from each group were randomly selected after the completion of heat stress treatment and euthanized by asphyxiation, followed by immediate dissection of the duodenum, jejunum, and ileum tissues. Some of the tissues were fixed with Boiun's fixative for immunohistochemistry (IHC) (n=6). The remaining tissues were stored overnight at 4°C in 300 µl RNAstore sample preservation solution (Tiangen Biochemical Technology, Beijing, China) and then removed from the solution and stored at -20 °C for quantitative PCR (n=6). This experiment was approved by the Ethics Committee of Animal Protection Center of Hainan Normal University.

Expression of HSPs in the small intestine detected by immunohistochemistry

The small intestine tissues were paraffin-embedded and sectioned into 5 µm-thick slices and then baked at 50 °C for 2 hours prior to IHC staining using a immunohistochemistry kit for rabbit (mouse) primary antibody (Shanghai Yisheng Biotechnology, Shanghai, China), according to the manufacturer's instructions. The primary antibodies used for IHC were diluted in 1× PBS (anti-HSP60, 1:100, catalog number: orb67 315; anti-HSP70, 1:8,000, catalog number: orb67306; and anti-HSP90, 1:400, catalog number: orb86613; Biorbyt, Cambridge, United Kingdom). Tissue sections incubated with PBS instead of primary antibodies were used as negative control. Image Pro Plus 6.0 software were used to analyze the mean integrated optical density (mean IOD) of HSP60, HSP70 and HSP90 positive reactions.

Expression of HSPs in the small intestine determined by reverse transcription fluorescent-based quantitative PCR (qPCR)

An RNAprep pure kit, purchased from Tiangen Biochemical Technology was used for the extraction of total RNA from small intestine. The FastKing cDNA first-strand synthesis kit (Tiangen Biochemical Technology) was used to synthesize cDNA, and the SYBR Green SuperReal Fluorescence Quantitative Premix reagent (Tiangen Biochemical Technology) was used for qPCR. A 20 µl qPCR reaction system was prepared on ice for each sample, containing 10 µl 2× SuperReal PreMix Plus, 0.6 µL 10 µM forward primer, 0.6 µl 10 µM reverse primer, 1 µl cDNA template, and 7.8 µl RNase-free double-distilled water. The reaction conditions were 95°C for 15 min; 40 cycles of 95°C for 10 s and 60°C for 30 s; followed by 95°C for 1 min, 60°C for 30 s, and 95°C for 30 s. The 2-week-old chicks' data from the CK were used as the reference to calculate the relative expression of the target genes using the $2^{-\Delta\Delta Ct}$ method.

Table 1 shows the primer sequences used in the qPCR and synthesized by Sangon Biotech (Shanghai, China). β-actin was used as the internal reference.

Table 1 – Primer Information.

Target gene	Sequence number	Primer sequence (5′-3′)	Product size (bp)
HSP60 (Xie, et al., 2018)	NM_001012916	F: GGCTATGATGCGATGCTTGG R: ACTACTGCTTCTGCCGTTGA	134
HSP70 (Zhou, et al., 2016)	NM_001006685	F: CGGGCAAGTTTGACCTAA R: TTGGCTCCCACCCTATCTCT	250
HSP90 (Xie, et al., 2018)	NM_001109785	F: TCCTGTCCTCTGGCTTTA R: AGGTGGCATCTCCTCGGT	143
β-actin (Zhou, et al., 2016)	L08165	F: CACCACAGCCGAGAGAGAAAT R: TGACCATCAGGGAGTTCATAGC	135

Statistical analysis

GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was used to perform ANOVA analysis of the obtained data. The data are presented as mean ± SEM. $p<0.05$ was considered statistically significant, and $p<0.01$ was considered extremely significant.

RESULTS AND ANALYSIS

Expression of HSPs in the small intestine of chicks

The IHC staining results showed HSP60 positive reactions that were brown in color and present in the intestinal mucosal epithelium and crypts. Most of the HSP60 positive reactions were distributed in the crypts (Fig. 1A). As shown in Fig. 1B, compared with the CK: the expression of HSP60 in the HS duodenum was lower at 2 weeks of age ($p<0.01$), but higher at 3, 5, and 6 weeks of age ($p<0.01$); HSP60 expression in the HS jejunum was higher at 3 and 6 weeks of age ($p<0.01$); and HSP60 expression in the HS ileum was higher at 3 ($p<0.05$) and 4 ($p<0.01$) weeks of age and lower at 5 and 6 weeks of age ($p<0.05$).

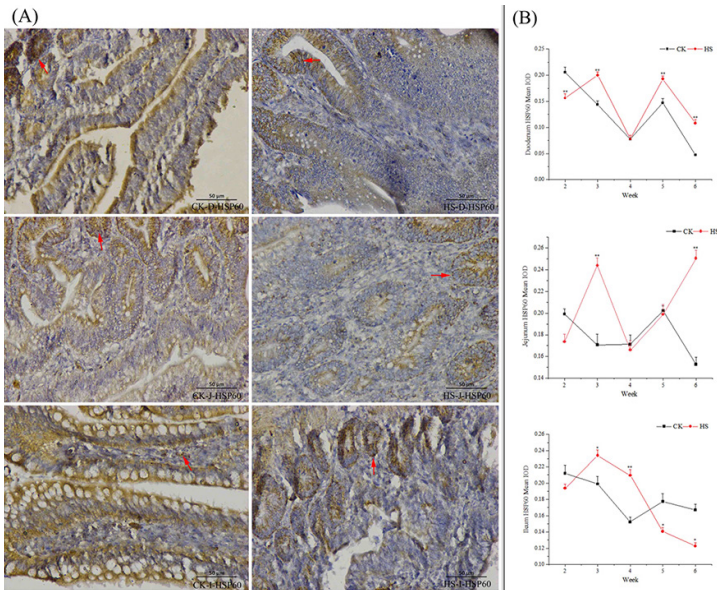

Figure 1 – Effects of heat stress on HSP60 expression in the intestine of chicks.

(A) Immunohistochemical results of HSP60 in the intestine of chicks (400×, Bar = 50 μm), (B) Effects of heat stress on mean IOD of HSP60 in the intestine of chicks.

The arrows point to positive reactions. CK = control group; HS = heat stress group; D = duodenum; J = jejunum; I = ileum. * indicates $p<0.05$ and ** indicates $p<0.01$ between the CK and the HS in the same weeks of age. The following is the same as here.

Immunohistochemistry showed HSP70 positive reactions that were brown in color and present in the intestinal mucosal epithelium and crypts (Fig. 2A). As shown in Fig. 2B, compared with the CK: the expression of positive reactions in the duodenum, jejunum, and ileum of the HS were increased at 4, 6, and 6 weeks of age, respectively ($p<0.01$).

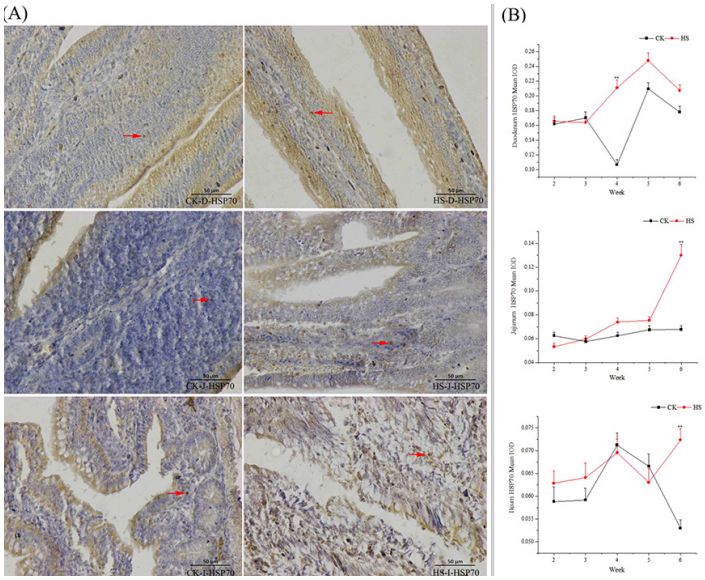

Figure 2 – Effects of heat stress on HSP70 expression in the intestine of chicks.

(A) Immunohistochemical results of HSP70 in the intestine of chicks (400×, Bar = 50 μm); (B) Effects of heat stress on mean IOD of HSP70 in the intestine of chicks.

Immunohistochemistry showed HSP90 positive reactions that were brown in color and present mostly in the intestinal mucosal epithelium and to a lesser extent in the crypts (Fig. 3A). As shown in Fig. 3B, compared with the CK: the HSP90 expression in the HS duodenum was lower at 2 weeks of age ($p<0.01$) and higher at 5 and 6 weeks of age ($p<0.01$); HSP90 expression in the HS jejunum was higher at 5 and 6 weeks of age ($p<0.01$); and lower at 3 and 5 weeks of age ($p<0.01$) in the HS ileum.

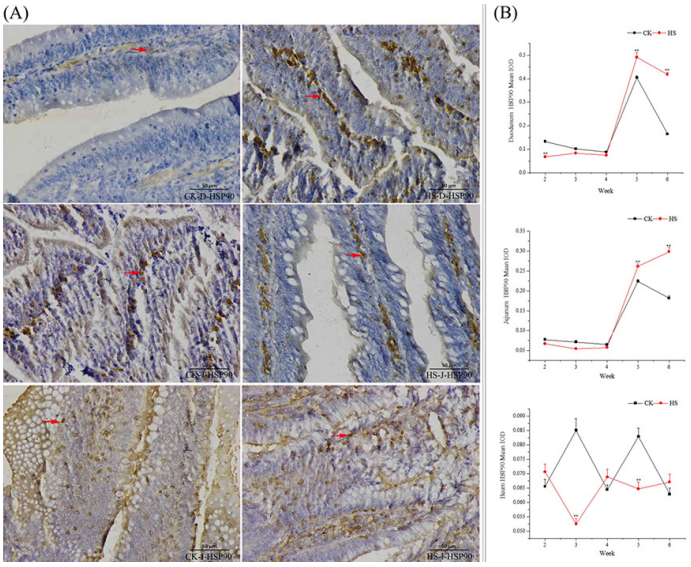

Figure 3 – Effects of heat stress on HSP90 expression in the intestine of chicks.

(A) Immunohistochemical results of HSP90 in the intestine of chicks (400×, Bar = 50 μm); (B) Effects of heat stress on mean IOD of HSP90 in the intestine of chicks.

Expression of *HSP* mRNAs in the small intestine of chicks

The qPCR results showed that compared with the CK (Fig. 4): *HSP60* mRNA expression in duodenum of the HS was lower at 2 weeks of age and higher at 5 weeks of age ($p<0.01$); *HSP60* mRNA expression in the HS jejunum was lower at 3 weeks of age ($p<0.01$), but higher at 5 weeks of age ($p<0.05$); and higher at 5 weeks of age in the HS ileum ($p<0.01$).

In Fig. 5, the qPCR results showed that compared with the CK: *HSP70* mRNA expression in the HS duodenum was lower at 3 weeks of age ($p<0.05$); but higher at the 4 weeks of age in the jejunum ($p<0.01$) and ileum ($p<0.05$) of HS.

As shown in the qPCR results in Fig. 6, compared with the CK: *HSP90* mRNA expression in the HS duodenum was higher from 4 to 6 weeks of age ($p<0.01$); in the HS jejunum was higher at 2 ($p<0.05$),

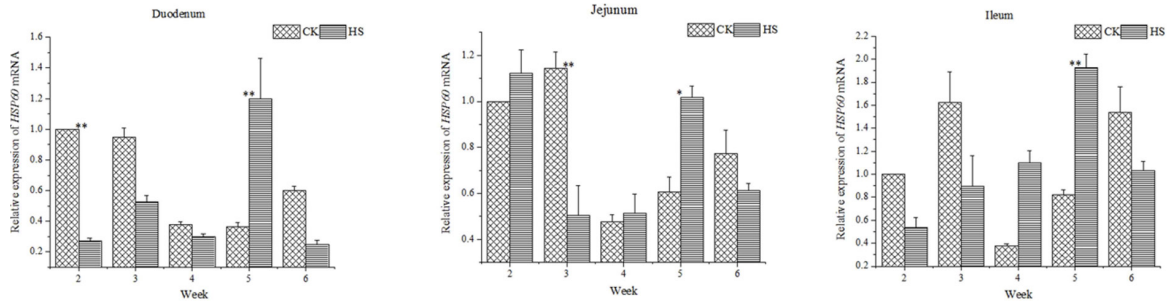

Figure 4 – Effects of heat stress on the expression of *HSP60* mRNA in the intestine of chicks.

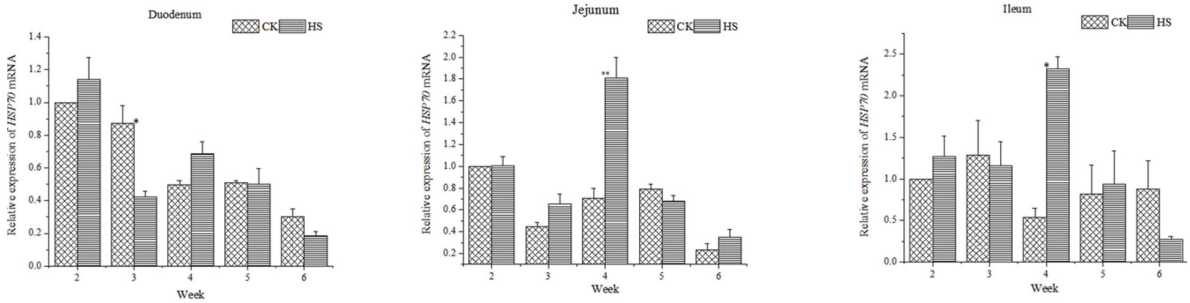

Figure 5 – Effects of heat stress on the expression of *HSP70* mRNA in the intestine of chicks.

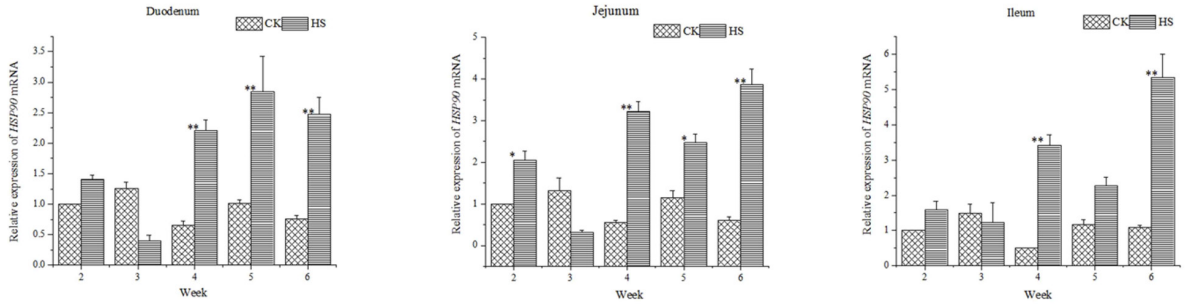

Figure 6 – Effects of heat stress on the expression of *HSP90* mRNA in the intestine of chicks.

4 (p<0.01), 5 (p<0.05), and 6 (p<0.01) weeks of age; and higher at 4 and 6 weeks of age (p<0.01) in the HS ileum.

DISCUSSIONS

Impacts of heat stress on the expression of HSP60 in the small intestine

HSP60 is expressed to various degrees in different tissues of broilers under high temperature. Heat stress causes myocardial damage, promotes the release of HSP60 from the mitochondria into the cytoplasm, and triggers the immune response of the body (Hoymans, *et al.*, 2008). A previous study showed that heat stress also significantly increases the HSP60 level in the heart, but decreases in the liver, suggesting that HSP60 expression is related to tissue damage under heat stress (Yan, *et al.*, 2009).

Results of the current study showed that the expression of HSP60 in the duodenum of the HS was significantly higher than in the CK. A study by Song *et al.* (2016) also indicated that acute heat stress may cause mitochondrial damage, which may induce a significant increase in the expression of HSP60 to maintain a steady mitochondrial membrane potential (Tang, *et al.*, 2018). In addition, HSP60 has ATP binding activity (Itoh, *et al.*, 1995). Heat stress significantly reduces the ATP content in the jejunum mucosa of broilers (Yang, *et al.*, 2018), reducing the binding between HSP60 and ATP, resulting in HSP60 overexpression. We had noticed that the protein expression of HSP60 was lower at 2 weeks of age, and then higher at 3, 5, and 6 weeks of age, but there was no significant at 4 weeks. The current study is difficult to explain this phenomenon, which will be the direction of our further research.

The HSP60 expression in the jejunum of the HS was higher than in the CK; while the *HSP60* mRNA expression of the HS was significantly less than that of the CK. A study by Werner *et al.* (2007) showed that the expression of HSP60 in the livers of heat-stressed green sturgeon larvae was lower than in the livers of normal larvae, and was similar to the expression of HSP60 in the livers of heat-stressed broilers in other studies (Tang, *et al.*, 2018; Xie, *et al.*, 2018; Yan, *et al.*, 2009). Continuous heat stress caused serious damage to the structure and integrity of the small intestine, which exceeded the ability of the small intestine to withstand the negative effect. By this time, the damage to the small intestine was irreversible. Moreover, the consumption of HSP60 in the body might be greater than the amount of HSP60 synthesized by heat stress. When the HSP60 level was lower than normal, the ability of the body to withstand the high-temperature environment was significantly reduced (Huckriede, *et al.*, 1995).

As the heat stress continued, the HSP60 expression in the HS ileum was first higher and then lower compared with the CK. Numerous studies have shown that excessively high ambient temperature often causes structural damage, such as deepening of the small intestinal crypts and exposure of the lamina propria (Li, 2013; Liu, *et al.*, 2016; Song, *et al.*, 2018), which may induce strong expression of HSP60 to protect cells. In the late stage of heat stress, the structural damage to small intestinal tissue was exacerbated and the HSP60 expression began to decline. The HSP60 mRNA expression was significantly higher in the HS compared with the CK. During heat stress, HSP60 is gradually consumed as a mitochondrial chaperone. When the consumption of HSP60 in the body was more than the synthesis due to heat stress stimulation, the protein level inevitably decreased, and negative feedback increased mRNA expression to synthesize more protein to meet the higher HSP60 demand.

Impacts of heat stress on the expression of HSP70 in the small intestine

Heat stress stimulates oxygen free radicals to bind with death receptors to activate mitochondrial signaling pathways and induce apoptosis. High expression of HSP70 significantly improves the heat tolerance of cells, thereby inhibiting the generation of oxygen free radicals, reducing the expression levels of death receptors as well as the upstream and downstream mitochondrial cascade signal, thereby inhibiting cell death induced by heat stress (Peng, *et al.*, 2019).

The experimental results showed that heat stress significantly increased the expression of HSP70 and reduced the expression of *HSP70* mRNA in the duodenum. Heat stress destroys the tissue structure of small intestinal villi and the duodenum is the most severely damaged (Wang, *et al.*, 2015), which stimulates the HSP70 expression in the duodenum and requires more *HSP70* mRNA to synthesize protein, thereby reducing the *HSP70* mRNA level.

Studies have shown that HSP70 alleviates the structural damage and oxidative damage of the intestinal mucosa induced by heat stress (Hao, *et al.*, 2012; Gu, *et al.*, 2012), thereby increasing the HSP70 consumption while reducing the protein content. The current study showed that *HSP70* mRNA expression in the jejunum of chicks is similar to the expression in the

jejunum mucosa of rats and pigs (Feng, *et al.*, 2014). Heat stress simultaneously enhances the expression of *HSP70* mRNA in the jejunum mucosa and activates the ERK signaling pathway in the intestine, suggesting that HSP70 may protect the intestine by activating the ERK signaling pathway under heat stress conditions (Feng, *et al.*, 2014).

Compared with the CK, the expression of *HSP70* mRNA in the ileum HS was higher during the early stage of heat stress. This may be a result of heat stress aggravating the metabolic burden of chicks, thus requiring more HSP70 to alleviate the heat stress damage, resulting in an increase in *HSP70* mRNA translation, leading to relatively higher levels of *HSP70*. The HSP70 expression increased at the later stage of heat stress. As the heat stress continued, the negative impact on the intestines intensified and induced high HSP70 expression, which significantly increased the activity of digestive enzymes (Hao, *et al.*, 2012), while inhibiting lipid peroxidation of the intestinal mucosa. These results indicate that HSP70 relieved the oxidative damage of heat stress to the intestinal mucosa of broilers by improving the antioxidant capacity of the body (Gu, *et al.*, 2012).

Impacts of heat stress on the expression of HSP90 in the small intestine

A high-temperature environment stimulates a change in the expression of HSP90 in broilers. *HSP90* mRNA in the hearts and livers of broilers was found to increase significantly after 2 hours of heat shock, but the *HSP90* mRNA in the heart decreased rapidly when the duration of heat stress was prolonged (Yu, *et al.*, 2009). In addition, expression of HSP90 in the hearts, livers, and kidneys of broilers increased after 2 hours of high-temperature exposure (Lei, *et al.*, 2009).

In this study, compared with the CK, the expression of HSP90 in the duodenum and jejunum of the HS was significantly higher. A previous study also showed that the expression of HSP90 rapidly increased in the intestinal mucosa of silky fowls during the early stage of heat stress, whereas during the late stage of heat stress, its expression decreased to a normal level and then rebounded (Liu, *et al.*, 2016). These results indicate that the heat tolerance of the small intestines of broilers is time-limited and that the body induces HSP90 expression to alleviate heat stress. The tolerance of normal cells to an excessively high-temperature environment is significantly stronger than that of cells with *HSP90* gene defects. High ambient temperature can induce the synthesis of *HSP90* mRNA and HSP90 protein to protect cells (Lei, *et al.*, 2009). In this study, the HSP90 expression in the ileum was different than in the duodenum and jejunum. The HSP90 expression in the ileum of the HS was significantly lower than in the CK, suggesting that the excessively high-temperature environment caused serious damage to the structure and function of the ileum and aggravated cellular metabolism. What's more, heat stress accelerates metabolism in the body, thus more ATP was likely required, which might have led to a continuation of HSP90 consumption. HSP90 protein expression is reduced when the consumption of HSP90 is greater than the synthesis of HSP90.

A previous study showed that the level of *HSP90* mRNA in both jejunum and ileum of chickens was significantly increased after heat stress exposure (38-39°C,8 h per day for 5 days) (Varasteh, *et al.*, 2015). In this study, the expression of *HSP90* mRNA in the small intestine of the HS was increased to different degrees compared with the CK. A study by Tamaki *et al.* (2011) showed that inhibition of HSP90 expression weakened the cytoprotective ability of IEC-6-90 cells, suggesting that HSP90 may play a role in protecting small intestinal epithelial cells from damage. Furthermore, heat-stress -induced high expression of HSP90 helped the small intestine resist high-temperature damage and accelerated the transcription of *HSP90* mRNA.

CONCLUSIONS

Heat stress disrupted the expression of HSP60, HSP70, and HSP90 in the small intestine of chicks in this study. The expression of HSPs in the duodenum, ileum, and jejunum was tissue-specific and time-specific, suggesting that HSPs may play different roles in physiological functions in the small intestines.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China under grants NSFC 31060312 and 31560680. We also express our high appreciation to Jiang-Wen Wu, Hainan Normal University, for the assistance with sample collection.

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