Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress[1]

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ABSTRACT Oxidative stress injury is one important factor in intestinal mucosal barrier damage. Expression of heat shock protein (*HSP*)70 is an endogenous mechanism by which living cells adapt to stress. This study was undertaken to investigate the protective effects of HSP70 on intestinal oxidative stress. Two hundred and forty broilers were injected intraperitoneally with HSP70 inducer L-(1)-glutamine or with the inhibitor quercetin. Twenty-four hours later, they were heat stressed for 0, 2, 3, 5, and 10 h, respectively, at 36 ± 1°C. The L-(1)-glutamine significantly increased HSP70 expression ($P < 0.001$). At 2 h or 3 h of heat stress, the HSP70 expression obviously elevated ($P < 0.001$). Levels of corticosterone and the heterophil:lymphocyte ratio significantly increased when HSP70 expression was inhibited ($P < 0.0001$). Serum corticosterone was negatively correlated with the HSP70 expression at 3 h of heat stress ($P = 0.0015$; R = −0.6537). Heat shock protein 70 significantly protected the integrity of the intestinal mucosa from heat stress, with significantly decreased lactic dehydrogenase when HSP70 expression was enhanced ($P < 0.001$). In addition, heat-stress time significantly affected the lactic dehydrogenase release ($P < 0.001$). Furthermore, HSP70 significantly elevated antioxidant enzyme activities (such as superoxide dismutase, glutathione peroxidase, and total antioxidant capacity) and inhibited lipid peroxidation to relieve intestinal mucosal oxidative injury ($P < 0.001$). These results suggest that HSP70 is capable of protecting the intestinal mucosa from heat-stress injury by improving antioxidant capacity of broilers and inhibiting the lipid peroxidation production.

Key words: broiler, heat stress, heat shock protein 70, intestine, oxidative stress

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INTRODUCTION

Heat stress is one of the most challenging environmental conditions affecting commercial poultry. Compared with other species of domestic animals, broiler chickens are more sensitive to high ambient temperatures (Geraert et al., 1993). As environmental temperature increases, feed consumption, growth rate, feed efficiency, and survival ability all decline (Mashaly et al., 2004; Yu et al., 2008).

Heat stress is one major source of oxidative stress. Heat shock could promote an increase in oxidative stress, thus creating a redox imbalance in favor of prooxidants. This could arise by increasing generation and reactivity of oxidants and by inactivating cellular antioxidant defenses. As a consequence, heat is likely to induce oxidative changes in cells (Altan et al., 2003; Mahmoud and Edens, 2003).

As living organisms, chickens have protective measures against environmental challenges. The heat shock proteins (**HSP**) are a set of proteins synthesized in response to physical, chemical, or biological stresses, including heat exposure (Ganter et al., 2006; Staib et al., 2007). Heat shock proteins are a group of evolutionarily conserved proteins that are, conventionally, classified according to molecular size, ranging from 10 to 150 kDa (Benjamin and McMillan, 1998). They play an important role in the protection and repair of cells and tissues. One of the most conserved and important protein families and has been studied extensively is HSP70 (Deane and Woo, 2005; Ming et al., 2010).

The gastrointestinal tract is particularly responsive to stressors, which can cause a variety of changes, including alteration of the normal, protective microbiota (Burkholder et al., 2008) and decreased integrity of the intestinal epithelium (Quinteiro-Filho et al., 2010). In variety of the in vitro model, oxidants such as hydrogen peroxide or superoxide (O_2^-) and others can increase the permeability of intestinal epithelial cells and

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disrupt the cytoskeleton through promoting excessive actin polymerization (Banan et al., 2001). Flanagan et al. (1998) used electron paramagnetic resonance spin trapping to demonstrate an increased flux of reactive oxygen species (**ROS**) in rat intestinal epithelial cell monolayers exposed to 45°C for 20 min.

Although there were many reports about the protection effects of HSP70 on the intestine under heat stress or other stress in the in vitro or in vivo models (Ehrenfried et al., 1995; Wischmeyer et al., 1997; Ren et al., 2001; Ohkawara et al., 2006), to our knowledge, studies on the effects of HSP70 following exposure to hot environments on the intestinal oxidative status of broilers are not available. L-(1)-Glutamine (Gln) is known to be protective to the intestinal epithelium and a potent enhancer of the synthesis of HSP both in vivo and in vitro (Wischmeyer et al., 1997; Kojima et al., 1998). Quercetin, namely 3,3',4',5,7-pentahydroxyflavone, is a naturally occurring flavonoid. Quercetin is a nonspecific chemical inhibitor of HSP, which inhibits not only HSP70 induction but also the cytoprotection provided by Gln supplementation (Wischmeyer et al., 1997). In a previous study, we established an HSP70 overexpression broiler model by L-(1)-glutamine (Gln) and quercetine. We assumed that the oxidative status of broiler intestines might be impaired by heat stress, and HSP70 might protect the intestine against oxidative damage under acute heat stress. To verify our hypothesis, we analyzed changes in the stress indicators and intestinal oxidative status.

MATERIALS AND METHODS

Experimental Birds

In total, 300 male broiler chickens (1-d-old; Arbor Acres Poultry Breeding Co., Beijing, China) were housed in wire-floored cages in an environmentally controlled room with continuous light. The room temperature was maintained at 35°C when the chickens were at the age of 1 to 3 d old, afterward the temperature was gradually reduced to 22°C. All birds were inoculated with inactivated infectious bursal disease vaccine on d 14 and 21 and with Newcastle disease vaccine on d 7 and 28. The birds had access to feed and water ad libitum. The diet was formulated to meet or slightly exceed the nutrient requirements suggested by NRC (1994). All procedures were approved by the Animal Care and Welfare Committee of the Chinese Academy of Agricultural Sciences.

Experimental Design

Two hundred and forty broilers with similar BW were put into 24 cages with 10 birds in each cage and randomly allocated to 4 treatments: control, enhancer, inhibitor, and enhancer + inhibitor group on 21 d of age. There were 6 cages per treatment. These birds were transported into temperature-controled metabolic chambers. On d 36 of age, the birds were injected intraperitoneally with 0.5 mL of saline, Gln (0.75 mg/kg of BW), quercetin (5 mg/kg of BW), and Gln (0.75 mg/kg of BW) + quercetin (5 mg/kg of BW), respectively. After injection, they were put back into the original cage. Twenty-four hours later, all of the chickens in the original cage were moved to the environmental chamber where they suffered from acute heat stress under 36 ± 1°C; stress times were respectively 0, 2, 3, 5, and 10 h.

Sample Collection

During the 0, 2, 3, 5, and 10 h in the heat-stress condition, chickens with approximately average weight in each cage were selected, taken out, and held by both shanks to minimize the distress. They were weighed and then blood samples (3 mL) were obtained via the wing vein for serum corticosterone concentration assay and heterophil and lymphocyte counts (EDTA, anticoagulant). Then, these chickens were immediately killed by cervical dislocation. The whole mucosa of the jejunum was collected using glass slides and stored at −70°C until analysis.

HSP70 Expression Analysis

Jejunal mucosa samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and centrifuged at 15,000 × *g* for 5 min at 4°C. Supernatants were assayed. Samples were heated at 95°C for 5 min and cooled on ice. Following the centrifugation at 15,000 × *g* for 5 min, 20-μL aliquots were resolved by electrophoresis (Bio-Rad, Richmond, CA) on 12% SDS-PAGE before being transferred electrophoretically to a PVDF membrane (Millipore, Billerica, MA). After blocking with TBST (0.05% Tween 20, 100 m*M* Tris-HCl, and 150 m*M* NaCl, pH 7.5) containing 5% skim milk for 30 min at room temperature, the membranes were incubated with primary antibodies [anti-mouse HSP70 (Stressgen, Gentaur Bvba, Belgium)] in a dilution of 1:4,000 at 4°C overnight. After washing in TBST 3 times, the membranes were incubated with a secondary antibody (horseradish peroxidase-labeled goat anti-mouse IgG, 1:10,000 dilution in blocking solution) for 40 min, and the antibody-specific protein bands were visualized with an electrochemiluminescence substract using a gel-imaging system (Tanon Science and Technology, Shanghai, China) with Image Analysis Software (National Institutes of Health, Bethesda, MD).

Measurement of Serum Corticosterone

The concentrations of serum corticosterone (**CORT**) were measured using a commercially available enzyme-linked immunosorbent assay kit (RapidBio Lab., Calabasas, CA) according to the manufacturer's instructions. Sensitivity of the assay is 0.7 nmol/L. The intra-assay and inter-assay variability were both 9.6%.

Table 1. Heat shock protein 70 expression in the jejunal mucosa of chickens under heat stress[1,2]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	0.70	0.75	0.68	0.71	0.71^{B}	0.054	8.79	<0.0001	<0.0001	<0.0001
2	0.73	0.95	0.88	0.93	0.87^{A}					
3	0.93	1.11	0.70	0.76	0.88^{A}					
5	0.59	0.81	0.68	0.85	0.73^{B}					
10	0.72	0.76	0.71	0.80	0.75^{B}					
Mean of time	0.73^{b}	0.88^{a}	0.73^{b}	0.81^{a}						

[a,b]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A,B]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 3 for each treatment group.

[2]Data are normalized to β-actin.

Measurement of Heterophil: Lymphocyte Ratio

Determination of the heterophil-to-lymphocyte (**H:L**) ratio was performed as described previously, with some slight modification (Campo et al., 2007). Two drops of blood were taken from a small puncture in the comb of each bird, 1 drop was smeared on each of 2 glass slides. The smears were stained using May-Grunwald and Giemsa stains, approximately 2 to 4 h after methyl alcohol fixation. One-hundred leukocytes, including granular (heterophils, eosinophils, and basophils) and nongranular (lymphocytes and monocytes), were counted on 1 slide of each bird (the other slide was supplementary) and the H:L ratio was calculated. Square-root transformation was used before analysis.

Assay of Lactic Dehydrogenase in Jejunal Mucosa

Assay kits for the lactic dehydrogenase (**LDH**) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Samples of the jejunal mucosa (100 mg) were homogenized in phosphate buffer (0.2 mol/L, pH = 7.4 and 4°C containing 50 mg of proteinase inhibitors/L and 0.1 mmol/L of phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 3,000 × *g* for 15 min and the supernatant was used for the assay. Then, the supernatants already prepared were subjected to measurement by a spectrophotometer (Leng Guang SFZ1606017568, Shanghai, China). All of the assays followed the instructions of the kits.

Assay of Antioxidant Indexes in Jejunal Mucosa

Assay kits for protein, total antioxidant capacity (**TAOC**), superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**) and malondialdehyde (**MDA**) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). For biochemical assays, samples of the jejunal mucosa (100 mg) were homogenized in phosphate buffer (0.2 mol/L, pH = 7.4 and 4°C containing 50 mg of proteinase inhibitors/L and 0.1 mmol/L of phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 3,000 × *g* for 15 min and the supernatant was used for the assay. Then, the supernatants already prepared were subjected to the measurement of TAOC, SOD, GSH-Px, and MDA levels by spectrophotometric methods using a spectrophotometer (Leng Guang SFZ1606017568, Shanghai, China). All of the assays followed the instructions of the kits. The TAOC was measured by the method of ferric reducing-antioxidant power assay (Benzie and Strain, 1996) and detected at 520 nm with the spectrophotometer. The SOD activity was measured by a

Table 2. Effects of treatments that affect heat shock protein 70 on the levels of corticosterone (nmol/L) in the sera of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	15.46	13.76	12.48	13.88	13.90^{A}	1.148	8.13	0.0007	<0.0001	<0.0001
2	12.18	10.93	16.59	14.67	13.59^{AB}					
3	11.00	10.61	15.04	11.48	12.03^{BC}					
5	9.37	12.13	11.91	9.22	10.66^{C}					
10	10.41	14.37	16.80	19.27	15.21^{A}					
Mean of time	11.68^{c}	12.36^{bc}	14.56^{a}	13.70^{ab}						

[a–c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A–C]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 4 for each treatment group.

Table 3. Effects of treatments that affect heat shock protein 70 on the heterophil:lymphocyte ratio in the sera of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	0.49	0.45	0.52	0.52	0.50	0.011	13.21	<0.0001	0.5159	<0.0001
2	0.45	0.47	0.53	0.50	0.49					
3	0.47	0.46	0.53	0.51	0.49					
5	0.49	0.48	0.52	0.51	0.50					
10	0.49	0.47	0.51	0.53	0.50					
Mean of time	0.48[b]	0.47[b]	0.52[a]	0.51[a]						

[a,b]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

xanthine oxidase method that monitors the inhibition of reduction of nitro blue tetrazolium in the sample (Winterbourn et al., 1975). The GSH-Px activity was detected with 5,5′-dithiobis-*p*-nitrobenzoic acid, and then we monitored the change of absorbance at 412 nm with the spectrophotometer (Hafeman, 1974). The MDA level was analyzed with 2-thiobarbituric acid, monitoring the change of absorbance at 532 nm with the spectrophotometer (Placer et al., 1966).

Statistical Analysis

All statistical analyses were performed using SAS statistical version 8.2 (SAS Institute Inc., Cary, NC). Statistical analyses were performed using a single 2-way ANOVA to estimate the effects of treatment and time and of the interactions between time and treatment. Differences among pairs of treatments and pairs of times were tested using Duncan's multiple-range tests. Correlation analysis was performed by linear regression test. Means were considered significantly different for values of $P < 0.05$.

RESULTS

HSP70 Expression

Heat shock protein 70 expression is shown in Table 1. Treatment, heat-stress time, and their interaction affected HSP70 expression ($P < 0.0001$). In terms of treatment, HSP70 expression in the enhancer group was significantly higher than those of the control and inhibitor groups. For heat-stress time, HSP70 expression at 2 h and 3 h of heat stress were significantly higher than all of the other times.

CORT

The level of CORT in the serum of heat-stressed broilers is displayed in Table 2. The CORT concentration was influenced by treatment ($P = 0.007$), heat-stress time ($P < 0.001$), and their interaction ($P < 0.001$). Compared among treatment, a marked elevation in the inhibitor group was noted compared with the control and enhancer groups. The CORT concentration decreased gradually from 2 to 5 h of heat exposure and increased dramatically at 10 h of heat exposure ($P < 0.05$).

H:L Ratio

Effects of treatment, heat-stress time, and their interaction on the blood H:L ratio in broilers are shown in Table 3. Treatment and the interaction of treatment and heat-stress time significantly affected the H:L ratio ($P < 0.001$). However, there was no statistical difference among different heat exposure times in H:L ratio ($P = 0.5159$). Besides, the level of H:L ratio increased

Table 4. Effects of treatments that affect heat shock protein 70 on the levels of lactic dehydrogenase (U/g of protein) in the jejunal mucosa of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	1,092.14	808.64	1,506.37	1,104.00	1,127.79[A]	74.01	22.68	<0.0001	<0.0001	<0.0001
2	1,528.37	807.49	1,272.84	1,013.79	1,155.62[A]					
3	1,138.42	723.59	1,170.44	880.28	978.18[B]					
5	757.35	637.39	990.94	1,044.81	857.62[C]					
10	1,048.26	678.61	1,051.79	1,026.93	951.40[BC]					
Mean of time	1,112.91[a]	731.14[c]	1,198.48[a]	1,013.96[b]						

[a–c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A–C]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

Table 5. Effects of treatments that affect heat shock protein 70 on the levels of glutathione peroxidase (GSH-Px; mg of GSH/g of protein) in the jejunal mucosa of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	41.10	63.35	31.86	48.04	46.09^{AB}	3.38	44.46	<0.0001	0.0404	<0.0001
2	44.97	82.40	29.93	45.89	50.80^{A}					
3	45.28	68.26	30.00	49.23	48.19^{AB}					
5	46.42	56.01	24.34	46.89	43.42^{B}					
10	50.09	60.14	29.35	49.88	47.37^{AB}					
Mean of time	45.57^{b}	66.03^{a}	29.10^{c}	47.99^{b}						

$^{a–c}$Means in the same row with no common superscripts differ significantly ($P < 0.05$).

^{A,B}Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

significantly in the inhibitor group ($P < 0.05$) compared with the control and enhancer groups.

LDH Release

Data on the levels of jejunal LDH are presented in Table 4. Treatment, heat-stress time, and their interaction significantly affected the LDH release ($P < 0.001$). No differences were found in LDH concentration between the control and inhibitor groups; however, there was a significantly lower LDH concentration in the enhancer group compared with those of the control and inhibitor groups. Jejunal LDH rapidly increased during the first 2 h of heat exposure. At that time, LDH level reached a peak, and from then until 5 h of heat exposure, the levels decreased rapidly ($P < 0.05$).

Lipid Peroxidation and Antioxidant Enzyme Activities

Tables 5, 6, 7, and 8 show the effects of HSP70 on the jejunal antioxidant indexes and lipid peroxidation, respectively. Treatment ($P < 0.001$), heat-stress time ($P = 0.0404$), and their interaction ($P < 0.001$) significantly influenced the concentrations of GSH-Px (Table 5). Higher concentrations of GSH-Px were detected in enhancer groups compared with that in all other groups. In terms of heat-stress time, GSH-Px concentrations at 2 h of heat exposure reached a peak, which was greater than 3 h and 10 h of heat exposure, to some extent ($P > 0.05$), and significantly higher as compared with 5 h of heat exposure.

Treatment ($P < 0.001$), heat-stress time ($P = 0.0001$), and their interaction ($P < 0.001$) also significantly influenced the concentrations of SOD (Table 6). Comparisons among all other groups revealed a significant elevated SOD concentration in the enhancer group. For heat-stress time, 2 h of heat exposure induced the highest level of SOD ($P < 0.05$).

Treatment and the interaction of treatment and heat-stress time significantly affected the concentration of TAOC ($P < 0.0001$); however, the heat-stress time did not affect levels of TAOC ($P = 0.0764$; Table 7). Significantly higher concentrations of TAOC were detected in the enhancer groups than in all of the other groups. With the extension of heat exposure time, there was an increased trend in TAOC levels that reached a peak at 10 h of heat stress.

With regard to MDA, there was a significant influence between treatment ($P < 0.001$), heat-stress time ($P = 0.006$), and their interaction ($P < 0.001$). Furthermore, MDA levels differed significantly among the 4 groups during the whole heat exposure, in which the level in the enhancer group was the lowest. Before 10 h of heat exposure, there was no significant difference among groups before and after heat exposure. At 10

Table 6. Effects of treatments that affect heat shock protein 70 on the levels of superoxide dismutase (U/mg of protein) in the jejunal mucosa of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	51.50	62.64	41.63	51.73	51.88^{BC}	1.99	40.85	<0.0001	0.0001	<0.0001
2	61.57	61.46	44.39	57.87	56.32^{A}					
3	47.57	60.27	39.96	49.74	49.39^{C}					
5	56.14	62.11	41.65	51.71	52.90^{B}					
10	53.74	63.33	41.52	52.21	52.70^{B}					
Mean of time	54.10^{b}	61.96^{a}	41.83^{c}	52.65^{b}						

$^{a–c}$Means in the same row with no common superscripts differ significantly ($P < 0.05$).

$^{A–C}$Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

Table 7. Effects of treatments that affect heat shock protein 70 on the levels of total antioxidant capacity (U/mg of protein) in the jejunal mucosa of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	0.80	1.33	0.51	0.65	0.82^{B}	0.058	46.60	<0.0001	0.0764	<0.0001
2	0.79	1.27	0.58	0.80	0.86^{AB}					
3	0.77	1.16	0.61	0.88	0.86^{AB}					
5	0.85	1.16	0.62	0.86	0.87^{AB}					
10	0.95	1.24	0.59	0.98	0.94^{A}					
Mean of time	0.83^{b}	1.23^{a}	0.58^{c}	0.83^{b}						

[a–c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A,B]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

h of heat exposure, however, the level of MDA was significantly elevated compared with those of the other times of heat exposure.

Correlation Analysis of HSP70 and CORT

To further explore the cytoprotective functions of HSP70 against environmental stresses, the correlation analysis between HSP70 and CORT were performed. The CORT was negatively correlated with HSP70 expression in the jejunal mucosa at 3 h of heat stress ($P = 0.0015$; R = −0.6537; Figure 1).

Correlation Analysis of HSP70 and Antioxidant Indexes

Figures 2, 3, and 4 demonstrate the correlation between HSP70 and the antioxidant enzyme activity at 3 h of heat stress. The amounts of GSH-Px (Figure 2), SOD (Figure 3), and TAOC (Figure 4) were positively correlated with HSP70 expression, and correlation coefficients were $P = 0.0002$, R = 0.7593; $P = 0.0028$, R = 0.6063; and $P = 0.016$, R = 0.4560, respectively. The correlation between HSP70 expression and MDA at 3 h of heat stress is shown in Figure 5. Malondialdehyde was negatively correlated with HSP70 expression ($P = 0.0073$; R = −0.5293).

DISCUSSION

Heat stress in birds is a major economic concern in poultry production due to its adverse effects on feed consumption, growth rate, hatchability, mortality, and health of birds (Bartlett and Smith, 2003; Ryder et al., 2004). Broiler chickens under current closed-production systems can be under heat stress during the summer season, unless the cooling system is fully operational. When living organisms are exposed to thermal stresses, the synthesis of most proteins is reduced, but a group of highly conserved proteins known as HSP are rapidly synthesized. It is well documented that as a major HSP, one of the most important functions of HSP70 is to protect organisms from the toxic effect of heating (Al-Aqil and Zulkifli, 2009; Zulkifli et al., 2009).

In the present study, the acute heat exposure model was employed and the data indicated that HSP70 expression played an important role in the oxidative stress response, which was caused by acute heat stress. Inhibition of HSP70 expression led to a more severe extent of stress, as there was a significant elevation of serum CORT and H:L ratio in acute-stressed chickens. On the other hand, HSP70 protected the intestinal mucosa from heat-stress injury by improving antioxidant capacity of broilers and inhibiting lipid peroxidation production.

Table 8. Effects of treatments that affect heat shock protein 70 on the levels of malondialdehyde (nmol/mg of protein) in the jejunal mucosa of chickens under heat stress[1]

	Treatment							*P*-value		
Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	Mean of treatment	Pooled SE	*F*-value	Treatment	Time	Treatment × time
0	19.15	14.37	25.43	19.67	19.66^{B}	1.31	28.94	<0.0001	0.006	<0.0001
2	25.09	11.86	25.89	20.47	20.83^{B}					
3	22.52	13.46	25.53	19.58	20.27^{B}					
5	19.92	14.39	25.80	20.26	20.09^{B}					
10	22.49	20.99	28.29	19.60	22.84^{A}					
Mean of time	21.83^{b}	15.01^{d}	26.19^{a}	19.92^{c}						

[a–d]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A,B]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

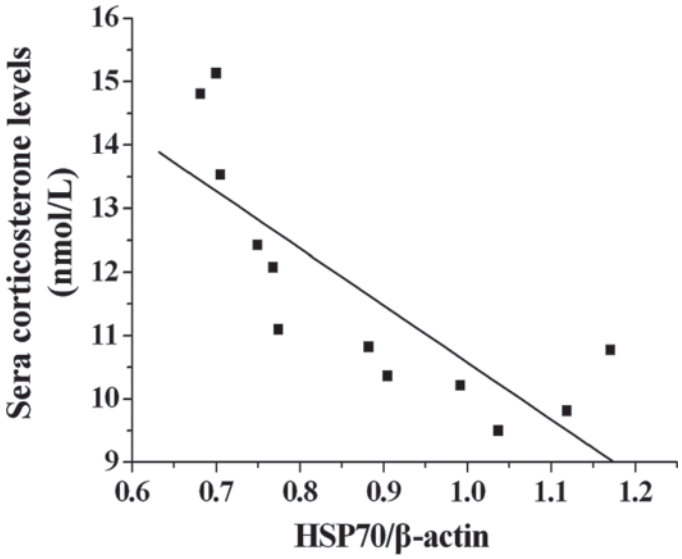

Figure 1. Correlations between heat shock protein (HSP)70 and the sera corticosterone levels at 3 h of heat stress. Regression equation was Y = −8.99922x + 19.57552; $P = 0.0015$; R = −0.6537. For all P-values and correlation coefficients, n = 12 observations.

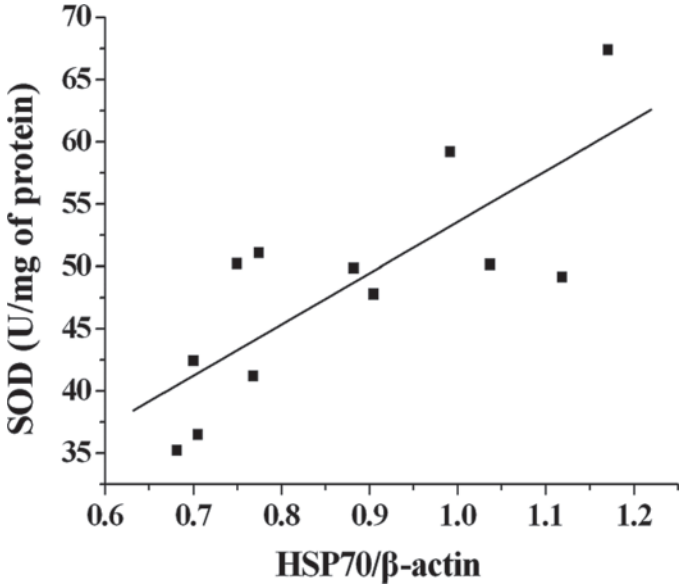

Figure 3. Correlations between heat shock protein (HSP)70 and superoxide dismutase (SOD) in the jejunal mucosa of chickens at 3 h of heat stress. Regression equation was Y = 41.08851x + 12.4599; $P = 0.0028$; R = 0.6063. For all P-values and correlation coefficients, n = 12 observations.

Serum CORT and H:L ratio are commonly used as effective biological indicators of acute stress response in poultry (McFarlane and Curtis, 1989; Zulkifli et al., 2009). The present findings confirmed that HSP70 expression may influence the levels of CORT and H:L ratio in heat-stress conditions, in which CORT and H:L ratio elevated when HSP70 expression was inhibited. Our correlation analysis between HSP70 and CORT also indicated that HSP70 might be required for thermal resistance. In addition, the results in this study showed that CORT concentration was significantly affected by heat-stress time, which gradually decreased from 2 to 5 h of heat exposure and increased dramatically at 10 h of heat exposure. This finding was not consistent with those of Zulkifli et al. (2003) and Mahmoud et al. (2004) that heat challenge elevated CORT in broiler chickens. One explanation of the discrepancies could be the timing of blood sampling after onset of exposure to heat (Zulkifli et al., 2009). It was presumed that there was an initial rapid increase in CORT followed

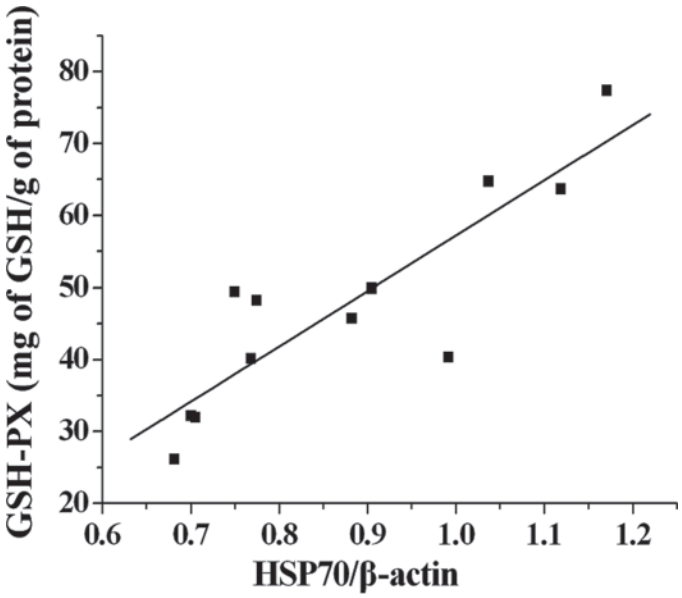

Figure 2. Correlations between heat shock protein (HSP)70 and glutathione peroxidase (GSH-Px) in the jejunal mucosa of chickens at 3 h of heat stress. Regression equation was Y = 76.81259x − 19.59994; $P = 0.0002$; R = 0.7593. For all P-values and correlation coefficients, n = 12 observations.

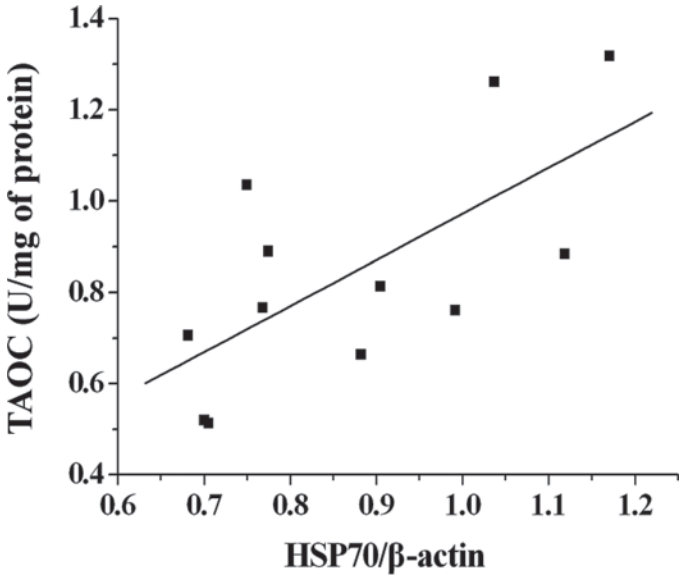

Figure 4. Correlations between heat shock protein (HSP)70 and total antioxidant capacity (TAOC) in the jejunal mucosa of chickens at 3 h of heat stress. Regression equation was Y = 1.00913x − 0.03718; $P = 0.016$; R = 0.4560. For all P-values and correlation coefficients, n = 12 observations.

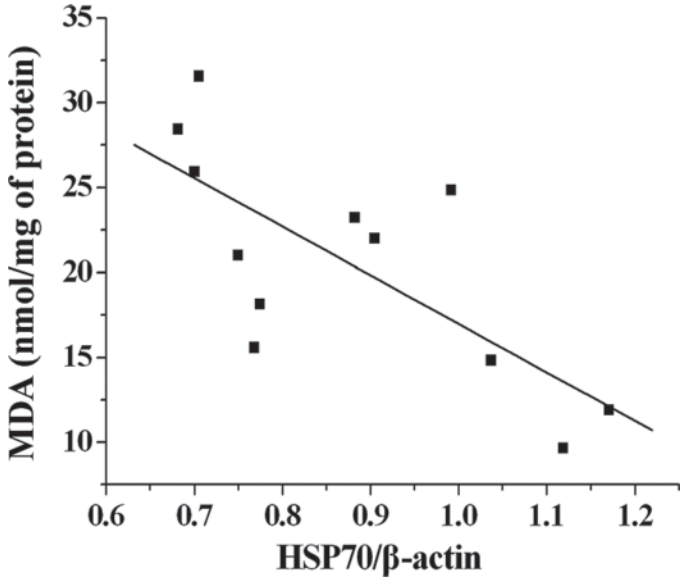

Figure 5. Correlations between heat shock protein (HSP)70 and malondialdehyde (MDA) in the jejunal mucosa of chickens at 3 h of heat stress. Regression equation was Y = −28.59012x + 45.57701; P = 0.0073; R = −0.5293.

by a quick normalization. There were some reports to support this hypothesis. Edens and Siegel (1975, 1976) reported that increases in CORT attributable to heat stress were maintained for only 70 min. The results of De Souza and Van Loon (1982) showed that the plasma CORT concentration peaked at approximately 15 to 30 min after the onset of restraint stress and returned to the control range by approximately 60 to 90 min. We speculated that broilers might experience heightened stress at the beginning of heat exposure and then became adapted to the perceived adverse environment later during heat stress. And the dramatic increased level of CORT at 10 h of heat exposure seemed to be positively related to survival time in such an environment (Edens and Siegel, 1976).

Lactic dehydrogenase is widely distributed in the cells of the body, and the cytoplasmic enzyme LDH is widely used as a marker of organ or tissue lesions in toxicology and clinical chemistry. The more seriously damaged, the more LDH released. Furthermore, LDH is generally associated with cellular metabolic activity, which is inhibited or elevated under oxidative stress (Das et al., 2004). The current research demonstrated that heat-stress time significantly influenced LDH activity, and increased expression of HSP70 significantly decreased the LDH levels in the mucosa. It indicated that HSP70 could antagonize intestinal mucosal injury caused by heat stress, reduce cell damage, and thus, inhibit LDH release into the blood.

Heat stress is one environmental stress that has been demonstrated to cause an increase in oxidative stress and an imbalance in antioxidant status (Gursu et al., 2004). It is known that superfluous ROS induced by heat stress can cause oxidative injury, such as lipid peroxidation and oxidative damage to proteins and DNA (Mujahid et al., 2007). Oxidative stress injury is one important factor in the intestinal mucosal barrier damage. Heat stress is shown to decrease nutrient digestibility possibly due to excessive ROS that oxidizes and destroys cellular biological molecules and finally causes a variety of impairments to intestinal tissues (Payne and Southern, 2005; Zhao and Shen, 2005). In the current study, an assay of the levels of GSH-Px, SOD, TAOC, and MDA in the jejunal mucosa were conducted. These measurements were used because they can sensibly reflect the oxidative status of the tissue. The content of MDA in the serum and tissue can indirectly reflect the extent of lipid peroxidation and overproduction of ROS in the body. Both GSH-Px and SOD are the main parameters to assess high oxidative stress and alteration of intestinal redox equilibrium to a more oxidized biological state (Assimakopoulos et al., 2006). Besides, we assayed the TAOC level, which can additionally reflect the nonenzymatic antioxidant defense system.

Heat shock proteins are the major stress proteins induced to protect cells from oxidative stress and other types of injuries (Hightower, 1991; Yenari et al., 1999). Ren et al. (2001) pointed out that short-chain fatty acids protected the intestinal epithelial cells from oxidative damage, this effect is in part through the induction of HSP generated. Some reports suggested that there was a strong relationship between oxidation and HSP70 synthesis. Aucoin et al. (1995) showed that oxidative stress caused a reduction in intracellular glutathione levels and induced *HSP70* mRNA. The study of Wong et al. (1998) showed that A549 cells could be resistant to oxidative stress damage by increased HSP70 expression. A similar result was also found in Mahmoud and Edens (2003) study, in which thiol oxidation contributed to HSP70 synthesis in stressed cells.

To investigate the relationship between jejunal oxidative injury and the induction of HSP70, we detected some indicators of intestinal oxidative status in different treatments that affect HSP70 during heat stress in the current study. We found that increased expression of HSP70 obviously improved the antioxidant defense system of broilers during heat stress, which was reflected by the observed elevation in GSH-Px, SOD, and TAOC and a decline in MDA. Furthermore, there was a strong positive correlation between HSP70 expression and GSH, SOD, and TAOC activity, whereas there was a strong negative correlation with MDA. That was to say, induction of HSP70 under heat stress promoted the production of GSH, SOD, and TAOC, but it inhibited the formation of MDA. In the enzyme antioxidant system, SOD and GSH-Px are the most important antioxidants. They work together to detoxify superoxide anions and hydrogen peroxide in cells. Superoxide first can be degraded into hydrogen peroxide by SOD and subsequently catalyzed to convert water by series of enzymes, including GSH-Px (Blokhina et al., 2003). Our results confirmed that elevated expression of HSP70 increased the antioxidant capacity of the intestine through both enzymatic and nonenzymatic

systems. This could be beneficial for the birds, as increased antioxidant activity ensures proper and rapid elimination of ROS, which could be formed during heat stress, and thereby protecting the body.

In summary, results in the current study indicated that, under heat stress, HSP70 expression may significantly reduce the damage of intestinal mucosa, which could effectively scavenge oxygen free radicals, improve body oxidation-reduction system imbalance, reduce oxygen free radical damage to cells in mucosal tissues, and improve the permeability of intestinal mucosa, thus effectively maintaining the structure and function of the intestinal barrier.

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