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Effect of Acute Heat Stress on Heat Shock Protein 70 and Its Corresponding mRNA Expression in the Heart, Liver, and Kidney of Broilers*

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ABSTRACT : The objective of this study was to investigate the expression and localization of heat shock protein 70 (Hsp70) and its mRNA in the heart, liver, and kidney of acutely heat-stressed broilers at various stressing times. Male AA broilers (n = 100) were randomly divided into 5 groups of 20 birds per group. After 30 d of adaptive feeding at ambient temperature, 80 experimental broilers were suddenly heat stressed by increasing the environmental temperature from 22±1°C to 37±1°C. The 4 groups were heat stressed for 2, 3, 5, and 10 h, respectively. The localizations of Hsp70 protein and mRNA, determined by immunohistochemical staining and *in situ* hybridization, respectively, were demonstrated to be tissue dependent, implying that different tissues have differential sensibilities to heat stress. Intense Hsp70 staining was identified in the vascular endothelial cell of heart, liver and kidney, suggesting an association between expression of Hsp70 in vascular endothelial cell and functional recovery of blood vessels after heat shock treatment. Ante-mortem heat stress had a significant effect on the expression of Hsp70 protein and mRNA. The quantitation of Hsp70 protein and mRNA were both time and tissue dependent. During the exposure to heat stress, the heart, liver and kidney of broiler chickens exhibited increased amounts of Hsp70 protein and mRNA. The expression of *hsp70 mRNA* in the heart, liver and kidney of heat-stressed broilers increased significantly and attained the highest level after a 2-h exposure to elevated temperatures. However, significant elevations in Hsp70 protein occurred after 2, 5, and 3 h of heat stressing, respectively, indicating that the stress-induced responses vary among different tissues. (**Key Words :** Heat Stress, Heat Shock Protein 70, Localization, Quantitation, Broilers)

INTRODUCTION

Heat stress is one of the most challenging environmental conditions affecting commercial poultry. Compared to other species of domestic animals, broiler chickens are more sensitive to high ambient temperatures. They have no sweat glands, a rapid metabolism, and high body temperature. Furthermore, fast-growing lean broilers generate more heat than their free-living counterparts living in the wild (Geraert et al., 1993). These physiological characteristics, in combination with confined housing, make it difficult for broilers to regulate their heat balance. As environmental temperature rises, food consumption, growth rate, feeding efficiency, egg shell quality, and survivability all decline (van der Hel et al., 1992; Geraert et al., 1996; Mashaly et al., 2004). Pale, soft, exudative-like changes in meat quality have been observed in broilers exposed to acute or short-term heat stress immediately pre-slaughter (Northcutt et al., 1994; Sandercock et al., 2001).

As living organism in the world, chickens have their protective measures against environmental disadvantages. Having no sweat glands, birds dissipate heat via the respiratory system during heat stressing course (Marder and Arad, 1989). Marder and Arad (1983) also proved that modulation of tidal volume during thermal panting might play a major role in acid-base regulation. Except for panting, chickens can improve their thermal resistance and adaptability by enhancing chemical regulation, such as changing the levels of plasma hormones (Tankson et al., 2001). Wang et al. (2007) demonstrated that heat exposure could significantly increase the concentration of serum cortisol which is involved mainly in carbohydrate, lipid, and protein metabolism. Both T_4 and T_3 are mainly involved in increasing metabolism by decreasing the rate of glucose oxidation and increasing the amount of metabolic heat produced (Tao et al., 2006). According, changes of cortisol

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and T_3 after heat stress are helpful to chickens coping with the disadvantages from high temperature.

Chickens also have other physiological mechanisms to improve thermal resistance. Yahav et al. (1997) suggest that the mechanism enabling better survival of broiler chickens involves mechanisms that help to reduce hyperthermia thus protecting the body tissues from being exposed to the stress. Shabtay and Arad (2005) suggested that early age conditioning enable Bedouins to obtain heat shock resistance by the improved the effectiveness of the homeostatic mechanisms for body temperature regulation. Some researchers found that diet supplement such as taurine, betaine, vitamin E and vitamin C, can increase the growth performances, survivability and egg shell quality of birds under heat stress conditions (Zulkifli et al., 2004; Chee et al., 2005; Shim et al., 2006; Ipek et al., 2007). Zulkifli et al. (2007) reported that palm oil diet reduced mortality rate, body temperature and serum creatine kinase level of broiler chickens during heat exposure and suggested the uncertainty of how much dietary fat to put into diets for heat stressed broilers can be overcome by allowing them to select their own consumption.

When living organisms are exposed to thermal and nonthermal stressors, the synthesis of most proteins is retarded; however, a group of highly conserved proteins known as HSPs are rapidly synthesized (Baqchi et al., 2001; Ogura et al., 2007; Park et al., 2007). These proteins are essential for organisms living at the edge of their thermal range. It is well documented that one of the most important functions of HSPs is to protect organisms from the toxic effects of heating (Arrigo, 2000). HSPs may play important roles in protein assembly and disassembly (Bukau et al., 2000; Hartl and Hayer-Hartl, 2002), protein folding and unfolding (Hartl, 1996; Mayer and Bukau, 2004; Zietkiewicz et al., 2004), protein translocation (Ryan and Pfanner, 2001), and the refolding of damaged proteins (Hightower, 1991; Glover and Lindquist, 1998; Maloyan et al., 1999). Of the many expressed HSPs, those with a molecular weight of approximately 70 kDa appear to be most closely associated with heat tolerance (Craig and Gross, 1991; Wang and Edens, 1998; King et al., 2002).

Several investigations have demonstrated that heat shock response occurs in a variety of tissues (Craig, 1985; Guerriero and Raynes, 1990; Gutierrez and Guerriero, 1991; Dechesne et al., 1992; van Laack et al., 1993; Knowlton, 1995). Amongst all members of the 70 kDa family, the one that has attracted most attention is the Hsp70. However, the mechanisms regulating Hsp70 gene expression in broiler chickens have not been extensively studied. Being physiologically important tissues, the heart, liver, and kidney were selected in order to determine the localization of Hsp70 and the changes in the expression levels of Hsp70 during the process of heat stressing. This study aimed to demonstrate the relationship between the expression of Hsp70 protein and mRNA, and the localization of Hsp70 protein and mRNA in the heart, liver, and kidney of acutely heat-stressed broilers at various stressing times.

MATERIALS AND METHODS

Animals and experimental design

One-day-old male AA broiler chicks (n = 100) were obtained from the Nanjing Changjiaying Commercial Fowl Company. The birds were housed in large coops (20 birds per coop) and the coops were placed in a controlled-climate chamber. The birds were given a 4-week period to acclimate to their new surroundings and to recover from environmental stress. During this period, the broilers were reared under standard conditions. The relative humidity of the chamber is maintained at 60±10%. The room temperature (RT) was maintained at 34±1°C from day 1 to day 3. As the chickens grew, the RT was decreased and maintained at 31±1°C from day 4 to day 7. Afterwards, the RT dropped by 1°C every 2 days until 21±1°C and maintained at this temperature by controlled ventilation and heating until day 30. The total broiler population was vaccinated against Newcastle disease and infectious bursal disease on the 7th and 14th days, respectively. At 30 days of age, the RT was suddenly increased from 22±1°C to 37± 1°C and the 100 AA broilers were divided randomly into 5 groups defined by the duration of heating: 0 (control), 2, 3, 5, and 10 h of heat stress. In order to regulate the heat, the temperature was monitored in the center of each coop. The relative humidity of the chamber during heat stress course is maintained at 50±5%. The birds were given access to a commercial broiler feed and water *ad libitum* during heat stressing. On terminating the heat treatment, each of birds was sacrificed by decapitation. Following exsanguinations, the birds were manually eviscerated, and the heart, liver, and kidneys were quickly dissected and placed into 1.5 ml tubes. The tubes were frozen in liquid nitrogen, and then stored at -70°C until used. Further tissues samples (1.5 cm thick) were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, for histological analysis.

All experiments were undertaken in accordance with the guidelines of the regional Animal Ethics Committee and were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Immunohistochemical detection of Hsp70

Tissue preparation for histological analysis : After fixation, samples were dehydrated in graded alcohols and embedded in a paraffin block. Sections (4 μm) were cut,

floated in a water bath containing diethylpyrocarbonate (DEPC)-treated water, and mounted on polylysine-coated slides for immunohistochemistry (IHC) and *in situ* hybridization (ISH). The slides were dried at 60°C for 2 h, and stored at -20°C.

Procedures of IHC : The sections from different tissues were examined immunohistochemically using the streptavidin-biotin-peroxidase complex (sABC) procedure (ZYMED, USA). The sections were dewaxed and then rehydrated. In order to unmask the antigen, the sections were placed in 10 mM citric acid buffer (pH 6.0) and then heated twice in a microwave oven at 800 W for 3 min and at 400 W for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. Nonspecific binding sites were blocked by incubating for 20 min in blocking solution (ready-to-use). Sections were overlaid at 4°C overnight with Hsp70 monoclonal antibodies (SPA-820, cross-react with Hsp70 in chickens; StressGen, USA; diluted 1:800 with Tween 20/Tris-buffered saline containing 0.5% BSA). Although raised against human Hsp70, these antibodies are able to recognize chicken Hsp70. Samples were then treated with a biotinylated anti-mouse secondary antibody for 20 min at RT, followed by horseradish peroxidase (HRP)-streptavidin for 20 min at RT. The reaction was developed with 3-3′-diaminobenzidine (ZYMED, USA). The slides were counterstained with Mayer's hematoxylin for 30 s and then mounted. The specificity of the technique was controlled by substituting the primary specific antibody with normal mouse serum. This control gave a negative result.

Localization of *hsp70 mRNA* by ISH

Design of a probe for hsp70 mRNA detection : An hsp70 mRNA ISH System was purchased from HAO YANG Biological Manufacture Co., Ltd (Tianjin, China). The sequence of the biotinylated deoxyribonucleotide probe was: 5′-CTGGG AGTCG TTGAA GTAAG CGGGC AC-3′. The probe was used at a working concentration of 8 μg/ml.

Procedures of ISH : The procedures for *hsp70 mRNA* ISH were conducted according to the manufacturer's instructions with minor modifications. Briefly, after deparaffinization and rehydration, sections were rinsed in 3% hydrogen peroxide in methanol for 20 min in order to block the activity of endogenous peroxidase. The sections were then digested for 20 min at RT with a mixed digestion working solution (1 μg/ml proteinase K, 20 μg/ml pepsin, and 100 μg/ml EDTA Na_4, in 0.1 M citric acid buffer). Following 5 min washes in DEPC-PBS and DEPC-H_2O, the sections were overlaid with prehybridization solution (50% (v/v) formamide, 0.5% (w/v) SDS, 100 μg/ml denatured salmon sperm DNA, 5×SSC, and 5×Denhardt's solution), and incubated at 37°C for 1.5 h in a humid chamber. After three 5 min washes in 0.2×SSC at RT, each section was overlaid with hybridization solution, covered by a siliconized coverslip, and then hybridized overnight at 37°C in a humid chamber. Negative controls were overlaid with prehybridization solution alone. The following day, in order to remove the excess probe, sections were washed 3 times with 2×SSC for 10 min, once with 0.2×SSC for 10 min, and twice with 0.1 M TBS for 10 min, all at 37°C. The sections were then incubated at 37°C for 50 min with biotin-labeled antidigoxigenin Fab fragments. After removal of unbound Fab fragments using several 0.1 M PBS washes, the sections were overlaid with an HRP-labeled avidin-biotin-peroxidase complex at 37°C for 50 min. After removal of the unbound complex using several PBS washes, the staining reaction for HRP was performed in the dark in HRP substrate solutions. The slides were then counterstained with Mayer's hematoxylin for 30 s. After a further rinse in deionized water, the slides were dehydrated in graded ethanol and cleared in xylene. The sections were then mounted in glycerol gelatin and observed under an Olympus BH-2 microscope (Olympus, Japan).

Quantification of Hsp70 by enzyme-linked immunosorbent assay (ELISA) : After completely washed in ice-cold physiological saline, the tissue samples were homogenized in 10 volumes of homogenization buffer (0.15 M NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM PMSF, 0.1 μM E-46, 0.08 μM aprotinin, 0.1 μM leupeptin, and 0.1% NP-40) (Shaila et al., 2005) using an Ultra-turrax homogenizer on ice, and the homogenates were centrifuged at 12,000×*g* for 20 min at 4°C in order to remove the cellular debris. The supernatant was collected and stored at -20°C for protein quantification. The quantity of protein was detected using an enzyme immunoassay kit (QRCT-332031EIA\UTL, goat anti-chook Hsp70; Adlitteram Diagnostic Laboratories, USA). Quantification of samples was performed using a standard curve. β-actin was used to control the bias caused by the procedure of protein extraction. Immunological detection of the proteins was performed essentially according to the manufacturer's instructions. Briefly, 50 μl of standards and specimens (50 μl/well) were dispensed into individual goat anti-chicken antibody-coated wells. A biotin conjugate reagent was then added to each well, followed by an enzyme conjugate reagent. The samples were then gently mixed for 15 s and incubated at 36±2°C for 60 min. Following incubation, the reaction mixtures were discarded, and the empty microtiter wells were rinsed 5 times with washing buffer. Residual water droplets were removed from the wells by sharply tapping the plate on absorbent paper. Color A and color B reagents were then added to each well, followed by gentle mixing for 5 s, and then incubation at 36±2°C for 15 min. The reaction was stopped by adding stop solution to each

well. The samples were gently mixed for 30 s and the optical density at 450 nm read within 30 min using a microtiter plate reader. The quantity of Hsp70 in each sample was normalized using the following formula:

Relative quantity of Hsp70
= quantity of Hsp70/quantity of β-actin

Detection of *hsp70 mRNA* by real-time RT-PCR

Isolation of RNA : After washed in ice-cold physiological saline, approximately 0.5 g of each tissue stored at -70°C was ground in liquid nitrogen with a pestle and mortar. Total RNA was isolated from the ground tissue using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instructions. Each RNA pellet was resuspended in 50 μl of RNase-free water and stored below -70°C until used. Total RNA purity was determined by calculating the ratio of the absorbance readings at 260 nm and 280 nm.

Design of primers for hsp70 mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA detection : The primers used for detecting the hsp70 sequences were designed from the sequences of broiler hsp70 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) held in the GenBank database using Primer Premier Software Version 5.0. The *hsp70 mRNA* primers used were as follows: 5′-AGCGT AACAC CACCA TTCC-3′ (forward) and 5′-TGGCT CCCAC CCTAT CTC-3′ (reverse). *GAPDH mRNA* was detected using the frequently described oligonucleotides that are specific for broiler *GAPDH mRNA* sequences (Sun et al., 2007). The Primers used were as follows: 5′-TGAAA GTCGG AGTCA ACGGA T-3′ (forward), and 5′-ACGCT CCTGG AAGAT AGTGA T-3′ (reverse). The primers for *hsp70 mRNA* and *GAPDH mRNA* were synthesized and purified by TaKaRa Biotechnology Co., Ltd. (Dalian, China). According to the primers, the length of the PCR products of *hsp70 mRNA* and *GAPDH mRNA* were predicted to be 372 bp and 230 bp, respectively.

Preparation of hsp70 mRNA and GAPDH mRNA standards : In order to quantify the *hsp70 mRNA* and *GAPDH mRNA* expression, mRNA standards were prepared for the construction of standard curves. One-step reverse transcription-polymerase chain reaction (RT-PCR) was performed using the primers described above. The amplification was carried out in a 25 μl reaction volume. The RT-PCR products were purified using a TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Japan), according to the manufacturer's protocol. The purified products were inserted into pGEM-T Easy vectors (Promega, USA) according to the manufacturer's specification. Recombinant clones were transformed into *Escherichia coli* DH5α high-efficiency competent cells. White colonies were selected from X-Gal/IPTG ampicillin agar plates and grown in Lauria-Bertani/ampicillin liquid media. The plasmids were purified using a TaKaRa MiniBEST Plasmid Purification Kit Ver. 2.0 (TaKaRa, Japan). The cloned PCR fragments were sequenced by TaKaRa Biotechnology Co., Ltd. in order to assess the direction of insertion. The sequence-confirmed plasmids were linearized using the vector-specific restriction enzyme *Sca*I (TaKaRa, Japan) and subjected to *in vitro* transcription (IVT) using a Riboprobe *in vitro* Transcription System (Promega, USA) at 37°C for 2 h. The IVT products were then treated with DNase I and incubated at 37°C for 15 min in order to remove the remaining DNA. The excess nucleotides and pyrophosphate remaining inside the IVT products were then removed by phenol/chloroform extraction. The RNA pellets were resuspended in DEPC-treated water. The amount of the IVT-generated *hsp70 RNA* and *GAPDH RNA* fragments were determined spectrophotometrically and converted to the number of molecular copies using the following formula:

$$Y\ (\text{molecules}/\mu l) = (X\ (g/\mu l)/(\text{transcript length (bp)} \times 320)) \times 6.02 \times 10^{23}$$

A further 10-fold dilution series of the RNA transcript was used for the construction of standard curves by plotting the cycle threshold (Ct) values obtained against the known concentration of the serially diluted standard RNA.

Relative quantification of samples by SYBR Green I-based real-time RT-PCR : SYBR Green I-based one-step real-time quantitative RT-PCR amplification was performed using an iCycler iQ Real-Time PCR Detection System (Bio-Rad, USA). Test samples were assayed in 25 μl reaction mixtures containing 5.4 μl of reaction mix, 1 μl of SYBR Green I, 0.5 μl of each forward and reverse primer, 2 μl of RNA, 0.5 μl of reverse transcriptase, and 15.6 μl of nuclease-free water. Template control was also included in the tests. The thermal profile for SYBR Green I-based one-step real-time RT-PCR consisted of 50 min reverse transcription at 42°C, and one 3 min cycle of Taq DNA polymerase activation at 95°C, followed by 40 cycles of PCR at 94°C for 30 s (denaturation), 58°C for 30 s (annealing), and 72°C for 30 s (extension). Following amplification, a melting curve analysis was performed in order to verify the authenticity of the amplified product by its specific melting temperature (Tm). In each run, a dilution series of the *in vitro*-transcribed standard RNA was also included along with the clinical RNA samples. The Ct values of each samples were determined by one-step real-time RT-PCR, and the copy numbers of samples were obtained from the standard curves of *hsp70 mRNA* and *GAPDH mRNA*. The *hsp70 mRNA* of all samples was

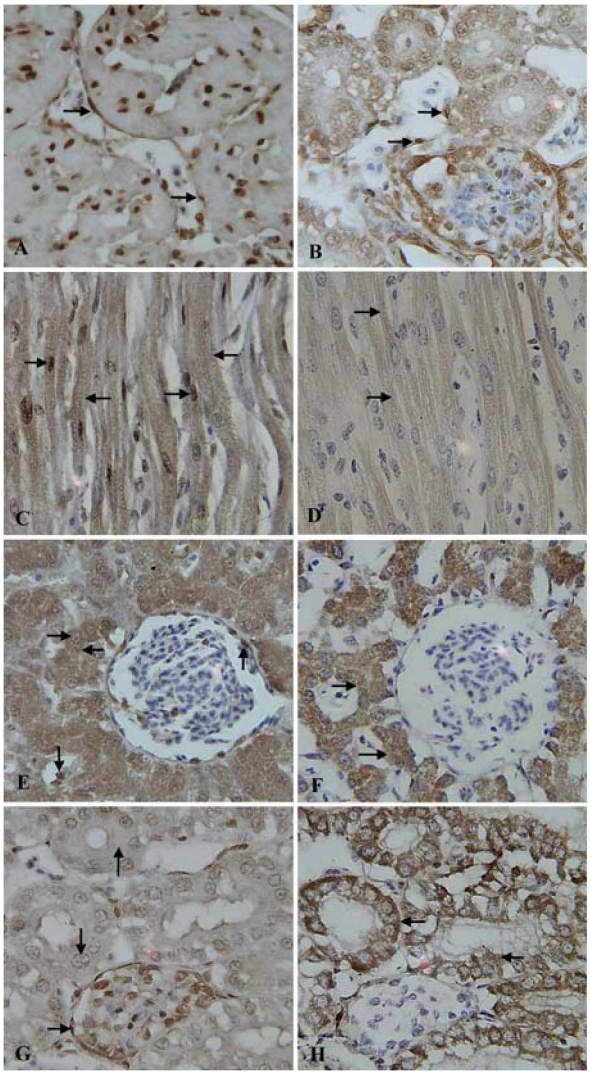

Figure 1. Detection of Hsp70 protein and mRNA in the heart, liver, and kidney of broilers (All images are at ×400 magnification). Positive staining of Hsp70 protein is localized mainly in the venule of heart (→ in Figure 1A) and kidney (→ in Figure 1B) of broiler chicks exposed to heat. Positive staining of Hsp70 protein revealed that it is localized mainly in the nucleus (→ in Figure 1C) and cytoplasm (← in Figure 1C) of cardiomyocytes. Positive staining of *hsp70 mRNA* is localized mainly in the cytoplasm of cardiomyocytes (→ in Figure 1D). Positive signals of Hsp70 protein indicate that it is localized mainly in the nucleus (→ in Figure 1E) and cytoplasm (← in Figure 1E) of hepatocytes, vascular endothelial cells in the sinusoid (↓ in Figure 1E), and central vein endothelial cells (↑ in Figure 1E) of the liver. Positive staining of *hsp70 mRNA* is localized mainly in the cytoplasm of hepatocytes (→ in Figure 1F). Positive Hsp70 protein signals are also detected in the nucleus (↓ in Figure 1G) and cytoplasm (↑ in Figure 1G) of renal tubular epithelial cells, renal glomerular endothelial cells (→ in Figure 1G) in kidney. Positive staining of *hsp70 mRNA* is localized mainly in the cytoplasm of renal tubular epithelial cells (← in Figure 1H).

normalized using the following formula:

Relative quantity of *hsp70 mRNA*
= copy number of *hsp70 Mrna*
/copy number of *GAPDH Mrna*

Statistical analysis

Statistical analysis of the differences between each group was carried out using a one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS version 11.5). Comparison of the mean value of the control group with that of each experimental group was performed using the Duncan test for multiple comparisons. Differences were regarded as significant at $p<0.05$.

RESULTS

Hsp70 localization by IHC

The immunohistochemical localization of Hsp70 is shown in Figure 1. Hsp70-positive signals were detected in the nucleus and cytoplasm of the myocardial cells, the hepatocytes, and the epithelial cells of the renal tubules of both heat-stressed and control broilers. The intensely Hsp70 staining was identified in the venule of heart (Figure 1A) and kidney (Figure 1B). The positive signals in the nucleus in all examined tissues were particularly intense in the perinuclear membrane and nucleolus. Hsp70 staining was identified both in the nucleus and the cytoplasm of myocardial cells (Figure 1C). Similarly, both the nucleus and the cytoplasm of hepatocytes exhibited positive staining for Hsp70 (Figure 1E). Positive signals were also detected in the central veins of the liver (Figure 1E). The positive Hsp70 signals were also detected both in renal glomerular endothelial cells and vascular endothelial cells in kidney (Figure 1G). No staining for anti-Hsp70 antibodies was detected in the negative control sections from the heart, liver, and kidney.

Localization of *hsp70 mRNA* by ISH

Hybridization signals for *hsp70 mRNA* were also detected in the heart, liver, and kidney of both heat-stressed and control broilers. *hsp70 mRNA* positive cells typically exhibited a brown reaction product. It can be concluded from Figure 1 that the nucleus of the myocardial cells, the hepatocytes, and the epithelial cells of renal tubules in broilers exhibited no *hsp70 mRNA* positive signals (Figure 1D, F, and H). Strong signals were, however, obtained from the cytoplasm of the parenchyma cells in all 3 examined tissues. However, no positive *hsp70 mRNA* signals were detected in the renal glomerulus of broiler kidney. Sections from negative control exhibited no hybridization signal for *hsp70 mRNA*.

Table 1. Changes in Hsp70 protein levels in the tissues of heat-stressed broilers

	Period of exposure to heat stress				
	0 h (control)	2 h	3 h	5 h	10 h
Heart	0.027 ± 0.007^{Aa}	0.041 ± 0.005^{Bb}	0.034 ± 0.006^{ABab}	0.035 ± 0.006^{ABab}	0.034 ± 0.009^{ABab}
Liver	0.058 ± 0.003^{Aa}	0.063 ± 0.003^{Aa}	0.067 ± 0.008^{ABa}	0.098 ± 0.003^{Cc}	0.080 ± 0.008^{Bb}
Kidney	0.037 ± 0.006^{Aa}	0.036 ± 0.005^{Aa}	0.052 ± 0.006^{Bb}	0.042 ± 0.005^{ABa}	0.046 ± 0.009^{ABab}

Different capital and small letters in the same line signify significance at the 0.01 and 0.05 levels, respectively.

Variations of Hsp70 concentration by ELISA

Standard curves for Hsp70 and β-actin proteins are shown in Figure 2. Table 1 presents the expression ratio (level of Hsp70/level of β-actin) data for each sample. The level of Hsp70 in the heart increased with increasing heat stress and reached a peak level ($p<0.01$) after 2 h of heat stress. There were no significant inductions, however, after 3, 5, and 10 h of heat stress compared to the control. The level of Hsp70 in the liver gradually increased shortly after the broilers were exposed to sudden heat stress. A significant induction of the Hsp70 occurred after 5 h of heat stress ($p<0.05$) and reached a peak level. After 10 h of heat stress, the level of Hsp70 had decreased significantly ($p<0.01$) compared with level after 5 h of heat stress, but was still higher ($p<0.01$) than the control. The level of Hsp70 in kidney decreased slightly ($p>0.05$) after 2 h of heat stress

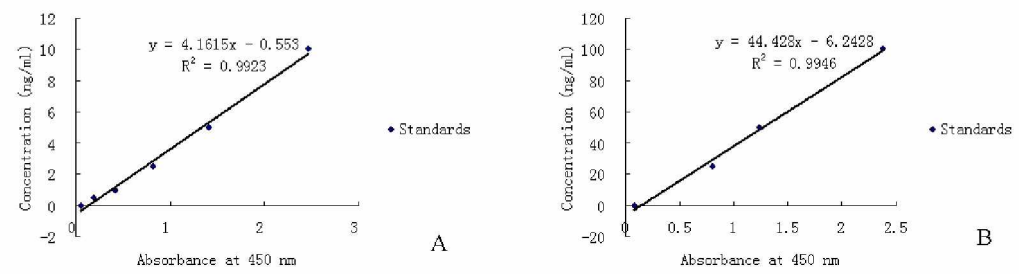

Figure 2. Hsp70 protein (A) and β-actin protein (B) standard curves.

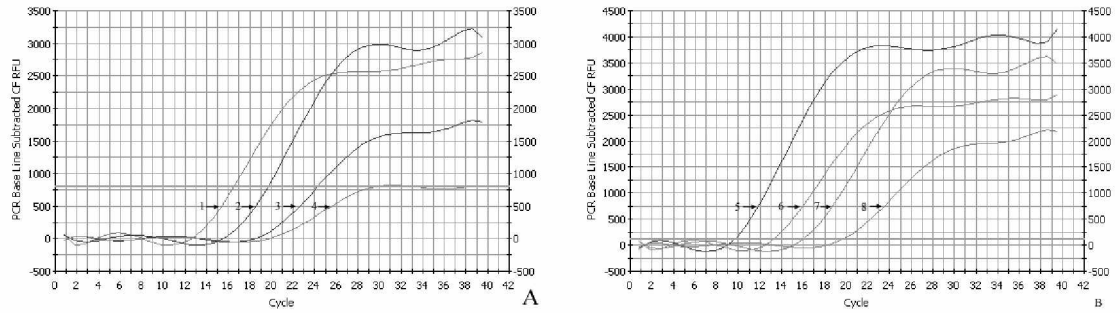

Figure 3. *hsp70 mRNA* (A) and *GAPDH mRNA* (B) PCR amp/cycle graphs. Lines 1 to 4 represent the amplification curves of *hsp70 mRNA* (A) obtained from *in vitro* transcription of linearized recombinant plasmid at dilutions of $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$, respectively. Lines 5 to 8 represent the amplification curves of *GAPDH mRNA* (B) obtained from *in vitro* transcription of linearized recombinant plasmid at dilutions of $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$, respectively.

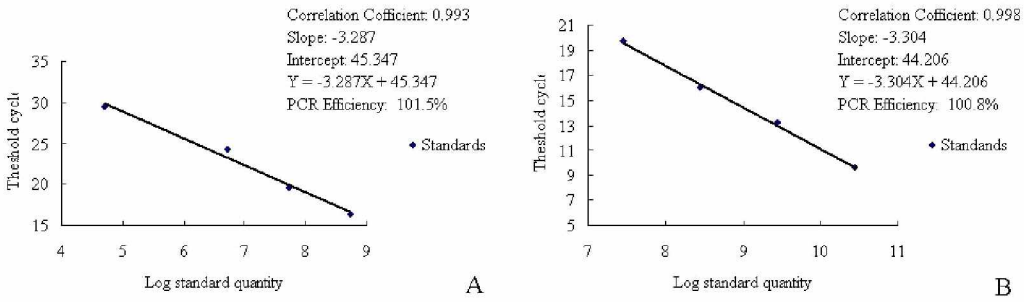

Figure 4. *hsp70 mRNA* (A) and *GAPDH mRNA* (B) standard curves.

Table 2. Changes in *Hsp70 mRNA* levels in the tissues of heat-stressed broilers

	Period of exposure to heat stress				
	0 h (control)	2 h	3 h	5 h	10 h
Heart	0.971 ± 0.007^{Aa}	1.168 ± 0.021^{Bb}	0.979 ± 0.019^{Aa}	0.945 ± 0.054^{Aa}	0.985 ± 0.029^{Aa}
Liver	0.882 ± 0.017^{Aa}	1.203 ± 0.067^{Bc}	0.973 ± 0.021^{Ab}	0.926 ± 0.040^{Aab}	0.987 ± 0.043^{Ab}
Kidney	0.979 ± 0.006^{Aa}	1.209 ± 0.020^{Cc}	1.004 ± 0.015^{Aa}	0.982 ± 0.007^{Aa}	1.051 ± 0.001^{Bb}

Different capital and small letters in the same line signify significance at the 0.01 and 0.05 levels, respectively.

but increased sharply ($p<0.01$) after 3 h compared with the control. However, there was no statistically significant induction after 5 and 10 h compared with the control.

Detection of *hsp70 mRNA* by real-time RT-PCR

The amplification curves for *hsp70 mRNA* and *GAPDH mRNA* obtained from *in vitro* transcription of linearized recombination plasmids at gradient dilutions of $1:10^2$ to $1:10^5$ are shown in Figure 3. Figure 4 shows the standard curves of *hsp70 mRNA* and *GAPDH mRNA* obtained from serially diluted standards. The correlation coefficients indicate that the standard equations are reliable and perfect. Table 2 shows the expression ratio (copy number of *hsp70 mRNA*/copy number of *GAPDH mRNA*) data for each sample. The results demonstrate that the levels of *hsp70 mRNA* normalized by *GAPDH mRNA* in the heart were significantly higher ($p<0.01$) in 2-h heat-stressed broilers than in control birds. However, compared to the control, there were no significant differences among birds heat stressed for 3, 5, and 10 h. The levels of *hsp70 mRNA* in liver increased with heat stress compared with the control. A significant induction was demonstrated after 2 h of heat stress ($p<0.01$), and after 3 h and 10 h of heat stress ($p<0.05$), but not after 5 h. The levels of *hsp70 mRNA* in kidney in the treatment groups clearly increased after 2 h of heat stress ($p<0.01$). After 3 and 5 h of heat stress, *hsp70 mRNA* levels in the kidney gradually decreased ($p<0.01$) compared with the level after 2 h, but were still higher ($p>0.05$) than those in the control. However, the levels of *hsp70 mRNA* after 10 h of heat stress had increased significantly ($p<0.01$) compared with the control.

DISCUSSION

The IHC signals provided scientific evidence for the presence of Hsp70 protein and its localization in specific tissues and particular cell types of broilers. In the present study, the expression of Hsp70 was detected in the nucleus and cytoplasm of myocardial cells, hepatocytes, and renal tubular epithelial cells. It was demonstrated that the Hsp70-positive signals in the nuclei of these 3 tissues were particularly intense in the perinuclear membrane and nucleolus; this localization of Hsp70 may be related to the function of molecular chaperones. As revealed by IHC, the heart Hsp70 was primarily expressed in the myocardial and vascular endothelial cells, consistent with previous findings in rats (Amrani et al., 1998). The expression of Hsp70 in the myocardial cells is probably very important for organisms, in that it may enable them to resist the adverse effects of stress. When exposed to heat stress, the work rate of the heart is increased (Knowlton, 1994; McCully et al., 1995). Wang et al. (1998) reported that actin microfilaments and intermediate filaments in 9 L rat brain tumour cells damaged after heat stress and reverted after 8 h of recovery. The recovery of cytoskeleton structures from heat treatment may be related with the induction of Hsp70. Therefore, in the present study the overexpression of Hsp70 in myocardial cells, hepatic cells and renal cells may be related with the protection of cytoskeleton. The function in protecting cytoskeleton also exist in the small HSPs. Miron et al. (1991) first report that as an HSP homologous to human Hsp27 (Hickey et al., 1986), Hsp25 in avian muscular tissues (heart, gizzard) is the predominant heat responsive HSP and presented a potential link between the induction of Hsp25 and the reorganization of actin. Afterwards, multiple publications indicate that the small heat shock proteins protect actin filaments from fragmentation induced by heat shock (Lavoie et al., 1995; Bryantsev et al., 2002; Pivovarova et al., 2005). The present finding highlights the importance of the blood vessels and endothelium in myocardial protection, and suggests an association between Hsp70 in blood vessels and functional recovery after heat shock treatment. White (1980) firstly found that one protein of 71,000 molecular weight accumulated in the microvascular fraction when the telencephalon slices were incubated in vinblastine. The inducible protein which was verified in the following research belongs to HSPs. Afterwards, a lot of researchers were devoted to the protection of HSPs in blood capillary. Wang et al. (1995) reported that induction of Hsp72 in human endothelial cells (ECs) by a thermal or nonthermal mechanism could prevent activated human polymorphonuclear neutrophil leukocytes mediated ECs necrosis, which may favor increased vascular permeability during systemic inflammatory response syndrome. McCormick et al. (2003a) reported that clinically applicable thermal preconditioning attenuated leukocyte-endothelial interactions associated with an increased expression of Hsp72. Zhang et al. (2000) reported that the expression of Hsp70 in the hepatocytes of mouse livers is localized around the central veins, and presumed that this distribution is related to the supply of blood. However, the cellular

distribution of Hsp70 in the present study was inconsistent. These observed differences in distribution may be related to differences in the manner of induction. The positive signals in the vascular endothelial cells of the central veins of liver imply that the expression of Hsp70 may be related to liver blood supply and the functional recovery from stress damage. The IHC signal of Hsp70 was detected in the glomerular capillary endothelial cells, vascular endothelial cells, and renal tubular epithelial cells. This observation suggests that the function of Hsp70 in the kidney may be related to its localization. After heat stressing, renal tubular epithelial cells exhibit an acute pathological change, leading to a disturbance in kidney homeostasis. This loss of homeostasis may cause the accumulation of metabolic products, or disturbances in the liquid, electrolyte, and acid-base balances in organisms. The consequences of such disturbance will influence normal physiological function and threaten the existence of the organism. The strong Hsp70 signals in the renal tubular cells and glomerular capillary endothelial cells may be related to the functional recovery of kidneys from stressing damage.

In the present study, we also demonstrated that hybridization signals for *hsp70 mRNA* were present in the cytoplasm of myocardial cells, hepatic cells, and renal tubular epithelial cells in all 3 examined tissues, suggesting the possible existence of inducible Hsp70 in these 3 cell types (Figure 1D, F, and H). The localization of *hsp70 mRNA* was coincident with the distribution of Hsp70 protein determined by IHC. Surprisingly, however, the expression of *hsp70 mRNA* could not be detected in cardiac vascular endothelial cells, hepatic vascular endothelial cells, renal vascular endothelial cells, and glomerular capillary endothelial cells that stained positively for Hsp70 protein. The reason for these seemingly contradictory results may be related to the experimental methods used or the precision of these experimental methods. On the other hand, the discordances of the localization of Hsp70 and *hsp70 mRNA* in the tissues may be give us novel evidence that Hsp70 shuttle from cytoplasm to nucleus after heat shock. Velazquez and Lindquist (1984) reported that drosophila Hsp70 concentrated strongly in nuclei while a small quantity remains cytoplasmic during heat shock and distributed throughout the cytoplasm during recovery. With a second heat shock it is rapidly transported back into the nucleus. Afterwards, many researches indicated that nuclear translocation of Hsp70 existed in different organisms exposed to different stressors (Martin et al., 1993; Lin et al., 1994; Mariéthoz et al., 1997; Jakubowicz-Gil et al., 2005).

The present study researched the kinetics of Hsp70 and its mRNA in broiler chickens after 10 h heat exposure. Many studies (Levy et al., 1997; Maloyan et al., 1999; Lepore et al., 2000) have shown that after heat preconditioning HSPs are induced during the recovery from the heat stressed period and can function as a repair system in the recovery phase (Mccormick et al., 2003b). Therefore, further research on the localization of Hsp70 protein and its mRNA in the recovery phase after heat exposure will be required in the future.

Enhanced Hsp70 expression may be a response to stressful environments, and may improve cell survival by protecting proteins from degradation and facilitating their refolding (Pratt, 1993; Hartl, 1996). Indeed, immunoreactivity against the anti-Hsp70 antibody was also noted in non-heat-stressed broiler tissues, although no enhancement of expression was found in these tissues using the IHC method. Thus, the expression of this protein in broiler heart, liver, and kidney tissues could not be used as a marker for heat stressing.

In the present study, it was demonstrated that the heart, liver, and kidney of broiler chickens exposed to high temperature exhibited increased amounts of Hsp70 protein and mRNA. The induction of Hsp70 protein and mRNA were time and tissue dependent, and occurred during the hyperthermic state. The data pertaining to protein synthesis and mRNA transcription are similar to the findings of previous studies (Wang and Edens, 1993; Gabriel et al., 1996). Under normal growth conditions, Hsp70 is synthesized constitutively; however, its expression increases following thermal challenge or stimulation from a variety of other environmental stressors. It was concluded from our primary results that significant elevations of Hsp70 in heart, liver, and kidney occurred after 2, 5, and 3 h of heating stressing, respectively. This indicated that the stress-induced response varies between different tissues. Many scientific researches have confirmed that the expression of HSPs is regulated mainly at the level of transcription by heat shock transcription factors (HSFs) (Morimoto, 1993; Wu, 1995; Morimoto, 1998). Michael et al. (1995) revealed that the levels of the two isoform of HSF1 mRNA were regulated in a tissue-dependent manner in mice. Shabtay and Arad (2006) concluded from their research that the activation of HSF1 and HSF3 is observed *in vivo* in the brain and in the blood tissues of chickens after heat shock, respectively. Accordingly, we can presume that the tissue-dependence of HSF activation may be a possible reason for the tissue-dependence of Hsp70 expression in the present study. The concentration of Hsp70 in heart reached a peak level after 2 h of heat stressing. This rapid heat shock response suggests that, among the organs examined, cell damage in heart after 2 h was the severest. The heart appears to be more susceptible to heat stress than either the liver or kidneys. This high susceptibility may be due to the fact that heat stress increases the work rate of the heart by increasing its rate of contraction (Knowlton 1994; McCully et al., 1995).

In the present study, the level of Hsp70 decreased at the

later stage of heat stressing course. The reason for this may be that the broilers developed tolerance to heat after several hours of heat stressing, or due to material deficiency after long-term stressing. Heydari et al. (1993) reported that caloric restriction increases the induction of Hsp70 transcription and improves thermotolerance. It has been reported that inorganic phosphate (Pi) deficiency may affect the major cellular biochemical pathways that control HSP protein expression (Edens et al., 1992; Belay and Teeter, 1996; Mahmoud et al., 1996; Mahmoud et al., 2004). Although Hsp70 expressions in the heart and kidney of broilers that were heat stressed for 10 h were not significantly different from those of the controls, we cannot ascribe it to the induction of feed consumption.

Table 2 shows that *hsp70 mRNA* expression in all organs increased significantly and reached its highest level when chickens were exposed to heat stress for 2 h. It has been documented that in chicken's *hsp70 mRNA* expression in all organs increases significantly after 1 h of heat stress compared with the control (Mahmoud et al., 2004). Gabriel et al. (1996) reported that the level of *hsp70 mRNA* in broiler livers increased after the first hour of heat stress and peaked at 3 h. The time required to reach the peak level in the present study, however, differed from the time reported by Gabriel et al. (1996). This discrepancy may be due to many different experimental variables such as the strain and age of the chickens used.

In conclusion, this study demonstrated that heat stress induces an increase in the levels of Hsp70 protein and mRNA in the heart, liver, and kidney of broiler chickens, and that the Hsp70 translation and transcription were time and tissue dependent. The localization of Hsp70 protein and mRNA expression was different in the 3 tissues examined.

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