

Localization and expression of heat shock protein 70 with rat myocardial cell damage induced by heat stress *in vitro* and *in vivo*

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Abstract. The aim of the present study was to investigate the association between heat shock protein (Hsp) 70 expression kinetics and heat stress-induced damage to rat myocardial cells *in vitro* and *in vivo*. The results showed that the activity of heart injury-associated enzymes, including aspartate aminotransferase and creatine kinase, significantly increased and myocardial cells developed acute histopathological lesions; this therefore suggested that heat stress altered the integrity of myocardial cells *in vitro* and *in vivo*. Levels of Hsp70 *in vitro* decreased following the initiation of heat stress and then steadily increased until heat stress ceased at 100 min; however, *in vivo* studies demonstrated a gradual increase in Hsp70 levels in the heart cells of rats from the initiation of heat stress, followed by a sharp decline at 100 min. These results indicated that the cells sustained different degrees of injury *in vivo* compared with those sustained *in vitro*, this may be due to different regulatory mechanisms in the two environments. Intracytoplasmic Hsp70 signaling was significantly reduced at 60 min *in vitro*, compared with that of the *in vivo* study, indicating that Hsp70 consumption may have exceeded its production prior to 60 min of heat stress, and following 60 min the cells produced sufficient Hsp70 protein for their protection against heat stress. Hsp70-positive signals in the cytoplasm of heart cells *in vivo* were more prominent in the intact areas compared with those of the degenerated areas and the density of Hsp70-positive signals was significantly reduced following 60 min of heat stress. In conclusion, comprehensive comparisons of enzymes, cell morphology and Hsp70 levels indicated that decreased levels of Hsp70 were associated with the reduced protective effect on myocardial cells *in vitro* and *in vivo*.

Introduction

Heat stress is a severe environmental condition that affects animals. Animals respond to environmental stress with a series of reactions known as the 'fight-or-flight' response (1). Therefore, heat stress may result in economic losses in animal husbandry due to a decline in food consumption, growth rate, feeding efficiency and survival of animals as the environmental temperature increases (2-4). In addition, stressors, which can cause general adaptation syndrome, were reported to be responsible for eventual shock and sudden mortality of transported pigs and poultry that were exposed to high temperatures (5). Previous studies have demonstrated that chronic heat stress significantly altered physiological, metabolic, biochemical and cellular responses in murine and poultry models (6,7). However, animals have been shown to exhibit protective measures against environmental challenges; these include heat-shock proteins (Hsps), a set of proteins synthesized in response to physical as well as chemical or biological stresses, including heat exposure (8-10).

Hsps are ubiquitously expressed and highly conserved in prokaryotes and eukaryotes (11). Hsps are divided into six families of sequence-associated proteins on the basis of their molecular size, structure and function as follows: Small Hsps, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110 (12). Numerous Hsps were reported to be effective in cell survival and adaptation; however, certain Hsps exerted greater levels of cardiac protection than others (13), Hsp70 was suggested to have important roles in protection against stress-induced cardiac cell damage such as ischemia-reperfusion injury (14). In addition, Hsps were reported to minimize the size of cardiac infarctions and improve contractility of heart cells in humans and mice (15,16). Hsp70 has been extensively studied and was revealed to be expressed in the vertebrae, heart and brain, where it serves as a molecular chaperone and has a significant role in protecting cells against cellular stressors (17), including heat (18,19), hypoxia (20), ultraviolet irradiation (21) and oxidative stress (22).

Previous studies have indicated that significant increases of Hsp levels were the key step in the initiation of the heat-shock response (23-25). Hsp70 was also reported to be

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involved in the folding of nascent and misfolded proteins under non-stressful conditions (26). As a universal cytoprotective protein, Hsp70 was suggested to enhance the tolerance of cells to environmental changes or pathogenic conditions, increase the survival rate of stressed cells as well as have critical roles in cardiovascular disease, organism decay and cellular aging (27). Furthermore, one study demonstrated that Hsp70-overexpression accelerated ulcer healing via the inhibition of apoptosis as well as the promotion of proliferation and protein synthesis (28).

Stressful transports and increased environmental temperature were confirmed to be responsible for the shock and sudden mortality of pigs and broiler chickens, as a result of sudden heart failure preceded by cardiovascular damage (29). However, due to the numerous confounding environmental variables, investigating the mechanisms by which stress induces cell damage and alters cellular metabolism *in vivo* is challenging. The present study aimed to investigate the correlation between Hsp70 levels and cellular damage, using H9c2 cells subjected to heat stress *in vitro* as a model system. In addition, the present study aimed to evaluate the expression of Hsp70 in response to heat stress and examine its potential protective role against hyperthermia-induced cellular damage *in vitro* and *in vivo*.

Materials and methods

Heat-stress models of cell culture in vitro and experimental rats in vivo. H9c2 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; 11995-065; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; 16141079; Gibco-BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cellular viability of >95% was achieved and the cultured H9c2 cells were divided into six groups, which were exposed to heat stress in a water bath at 42°C for 20, 40, 60, 80 and 100 min. The control group was maintained at normal environmental conditions.

All experiments were performed in accordance with the guidelines of the Animal Ethics Committee of Jiangsu province (Jiangsu, China) and were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China). A total of 70 Sprague-Dawley (SD) rats, weighing 220±20 g, were purchased from Qinglongshan Farms (Nanjing, China). The rats were housed in cages with access to food and water *ad libitum*. Following three days of adaptation feeding at room temperature (RT), the animals were placed in controlled-climate chambers (RX8-500D, New Jiangnan Co., Ltd., Ningbo, Zhejiang, China) and then exposed to 42°C for 0 (control), 20, 40, 60, 80 and 100 min. Each group consisted of ten rats, with the exception of the 100 min group, which contained 20 rats. Mortality of the heat stressed rats *in vivo* was >40% following 100 min of heat stress. All the experimental rats were humanely sacrificed by decapitation at the end of the heat-stress period.

Enzyme detection in vitro and in vivo. Following exposure to heat stress, ~2 ml culture supernatants and ~1.5 ml serum

from the control and the heat-stressed rats, respectively, were collected from each group and the controls. The collected samples were then stored at -80°C until further use. Enzymatic activities of aspartate aminotransferase (AST; C010) and creatine kinase (CK; A032) were measured using commercial kits (Nanjing Jiancheng Biochemical Reagent Co., Ltd., Nanjing, China) according to the manufacturer's instructions and a microplate reader (Tecan Infinite M200PRO, Grödig, Austria). Each sample was analyzed five times, consecutively.

Cyto- and histopathological observations. H9c2 cells were cultured on glass coverslips (2-8x10⁴ cells in 35-cm² plates; Citotest Labware Manufacturing Co., Ltd., Haimen, Jiangsu, China) coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). Myocardial H9c2 cells were cultured at 37±1°C were heat stressed by incubating them in a water bath at 42±1°C for 0 (control), 20, 40, 60, 80 and 100 min. Following heat stress, cells were fixed in 4% paraformaldehyde for 30 min at RT and washed with phosphate-buffered saline (PBS) three times. Cells were then stained with hematoxylin and eosin (H&E) and examined using light microscopy (Axio Imager A2; Zeiss, Oberkochen, Germany).

Heart samples were obtained from rats of each group following heat stress and preserved in 10% formalin. Samples were embedded in paraffin and then cut into 5-µm serial sections. Sections were stained with H&E and the histopathological changes among the groups were examined. Images were captured using light microscopy.

Immunofluorescence staining. Following heat stress, myocardial H9c2 cells were washed with PBS and fixed with 95 and 100% ethanol, for 15 min each. Cells were washed with pre-cooled PBS for 15 min and then permeabilized with 0.5% Triton™ X-100 (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) in PBS for 15 min. Non-specific protein binding of permeabilized cells was blocked by incubation with 5% bovine serum albumin (BSA) for 30 min at RT. Cells were then incubated with Hsp70-specific mouse monoclonal antibodies (1:50; ADI-SPA-820-F; Enzo Life Sciences, Inc., Farmingdale, NY, USA) diluted with 1% BSA (Wuhan Boster Biological Technology, Ltd., Wuhan, Hubei, China) and subsequently incubated with fluorescein isothiocyanate (FITC)-conjugated antibody (BA1089; Wuhan Boster Biological Technology, Ltd.) in 1% BSA (1:50). Fluorescence images were obtained using light microscopy.

Serial sections of the heart tissue were immunostained using the standard avidin-biotin complex (ABC) immunoperoxidase detection system (AR1022; Wuhan Boster Biological Technology, Ltd.). Sections were then deparaffinized in xylene, hydrated with ethanol and rinsed with distilled water. Endogenous peroxidase was blocked using 3% H₂O₂ for 10 min at RT. The slides were blocked by incubation with 5% BSA for ~30 min at 37°C. Sections were then incubated with a 1:50 dilution of the Hsp70-specific primary antibodies for 2 h at RT. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; heavy and light chain) secondary antibodies for 1 h at RT. Following treatment with two drops of diaminobenzidine (DAB) substrate chromogen solution (AR1022; Wuhan Boster Biological Technology, Ltd.) for

Table I. Activity levels of AST and CK *in vitro* and *in vivo*.

Enzyme	Heat stress group/time (min)					
	0	20	40	60	80	100
<i>In vitro</i>						
AST (U/l)	9.17±0.98	9.67±1.37	11.17±0.75*	11.33±2.06*	12.83±2.13**	15.83±2.14**
CK (U/l)	8.67±1.19	10.17±2.12	10.33±1.79	11.67±2.51*	11.83±2.56*	12.00±2.45*
<i>In vivo</i>						
AST (U/l)	260.23±74.71	266.3±53.38	273.2±56.49	296.6±61.97	345.8±71.94*	406.1±97.12**
CK (U/l)	5596.2±1680.7	5864.5±2099.8	6057.8±1989.2	6496.5±1935.2	7064.5±1599.7	7924.8±1762.9*

Values are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. control. AST, aspartate aminotransferase; CK, creatine kinase.

10 min, the reaction was terminated by adding ~500 ml water. The sections were then counterstained with hematoxylin and fluorescence images were obtained using light microscopy. The corresponding negative controls were prepared in an identical manner omitting the antibody.

Western blot analysis. Heat-stressed H9c2 cells were washed three times with PBS and cell protein was extracted using an M-PER mammalian protein extraction reagent (78501; Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Heart tissue samples were homogenized in 1 ml protein extraction reagent (Thermo Scientific) on ice using an Ultra-Turrax homogenizer (623003; Fluko Equipment Shanghai Co., Ltd., Shanghai, China). Samples were then washed with ice-cold physiological saline and the resultant homogenates were centrifuged at 12,000 xg for 20 min at 4°C to remove cellular debris. The supernatant was collected and stored at -20°C until further use for protein quantification. The protein concentration was measured using a micro-bicinchoninic acid assay kit (23235; Thermo Scientific).

The H9c2 cell protein extracts (20 µg) and heart tissue protein extracts (80 µg) were separated using 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated overnight with Tris-buffered saline containing 0.05% Tween-20 (TBST) at 4°C. The membranes were then washed with TBST and incubated with antibodies against Hsp70 (ADI-SPA-820-F; Enzo Life Sciences, Inc.) and GAPDH (KC-5G4; Kangchen Bio-tech Inc., Shanghai, China) in blocking buffer for 2 h at 37°C. Following washing with TBST, the membranes were further incubated with HRP-conjugated goat anti-mouse IgG antibody (Wuhan Boster Biological Technology, Ltd.) at RT for 1 h. Antibody-antigen complexes were detected using western blotting luminal reagent (Bio-Rad Laboratories, Inc.). The bands were then quantified using Quantity One software version 4.6.2 (Bio-Rad Laboratories). GAPDH was used as a loading control.

Statistical analysis. Statistical analysis of the differences between the experimental groups and the control group were performed using the one-way analysis of variance followed by the least-significant-difference multiple comparison test using

Statistical Package for Social Sciences (SPSS) version 20.0 for Windows (International Business Machines, Armonk, NY, USA). Values are expressed as the mean ± standard deviation of at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference between values.

Results

Cell damage increases the levels of enzymes AST and CK in vitro and in vivo. Significant differences in AST and CK levels were observed in heat stressed rat myocardial cells compared with those of the control group (Table I). Following initiation of heat stress, enzymatic activity was significantly increased *in vitro* and *in vivo* at 100 min (P<0.05). The culture supernatant from *in vitro* myocardial cells showed a significant increase in AST levels from 40 min onwards and CK levels from 60 min onwards. However, *in vivo* levels of these enzymes were not significantly increased until 80 min for AST and 100 min for CK.

Cyto- and histopathological changes in vitro and in vivo. Figs. 1 and 2 demonstrate the effect of different durations of heat stress on cytological and histopathological changes of myocardial cells *in vitro* and *in vivo*, respectively.

No obvious pathological changes were observed in the control group (Fig. 1A). Following 20 min of heat stress, H9c2 cells exhibited acute degeneration with enlarged cell size and numerous granular cytoplasmic particles (Fig. 1B). Small granular particles with deeply stained nuclear condensation became more prominent in the cytoplasm of the enlarged myocardial cells following 40 and 60 min of heat stress (Fig. 1C and D). In the 80 min group, the primary indications of heat stress in myocardial cells were cytomorphosis (enlarged size) and karyopyknosis (Fig. 1E and F).

Following 20 min of heat stress *in vivo*, the gaps between the myocardial fibers were widened. The number of cytoplasmic granules in the intumesced myocardial cells of rats gradually increased with each group from the initiation of heat stress to its cessation at 100 min (Fig. 2B-F); of note, the number of granules was increased at 60 min (Fig. 2D).

Localization of Hsp70 in heat-stressed myocardial cells in vitro and in vivo. Figs. 3 and 4 reveal the distribution and

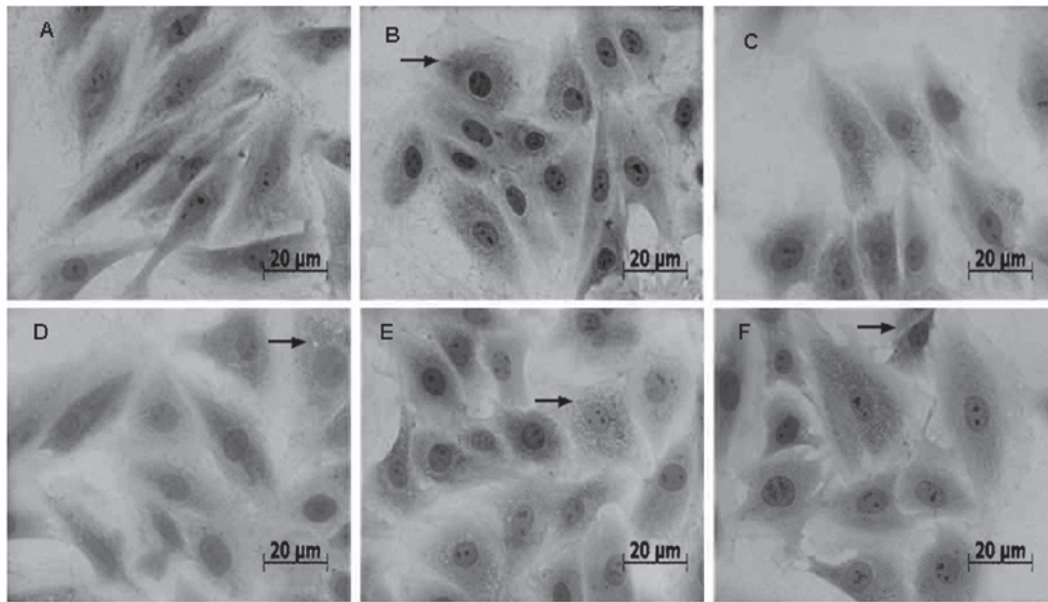


Figure 1. Representative images of *in vitro* heat-stressed myocardial cells in animals that succumbed prior to 100 min. Myocardial H9c2 cells were incubated at 37°C and subjected to heat stress at 42°C as follows: (A) Control, 0 min; (B) 20; (C) 40; (D) 60; (E) 80; and (F) 100 min. Cells were then stained with hematoxylin and eosin and examined using light microscopy. Arrow, cytoplasmic granular degradation.

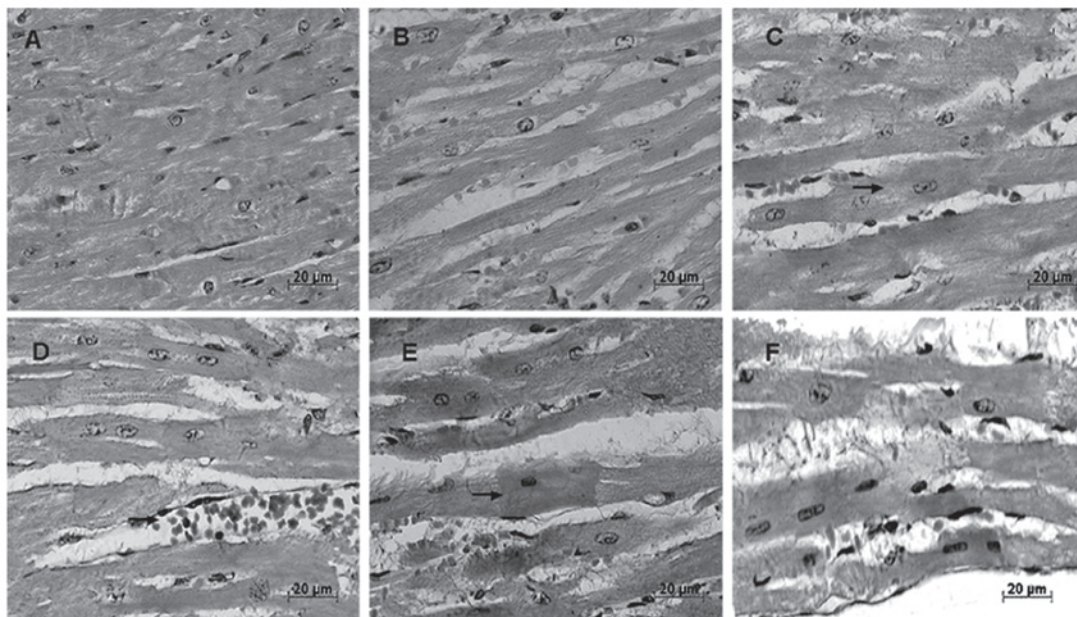


Figure 2. Representative images of the heat-stressed rat heart cells *in vivo*. Rats were subjected to heat-stress at 42°C as follows: (A) Control, 0 min; (B) 20; (C) 40; (D) 60; (E) 80; and (F) 100 min. Animals were sacrificed following 100 min heat stress and serial sections of the heart tissue were obtained from each group. Sections were then stained with hematoxylin and eosin and examined using light microscopy.

localization of Hsp70 in myocardial cells *in vitro* and *in vivo*, respectively. Hsp70 was located in the nucleus and the cytoplasm of myocardial cells following stress induction; however, stronger Hsp70 signals were detected in the cytoplasm and signals were identified in the heat-stressed groups as well as the control groups (Fig. 3A-F). Immunocytochemical staining revealed a decrease in cytoplasmic Hsp70-positive signaling following 40 min of heat stress (Fig. 3C) and the lowest density of Hsp70 signals was observed in the cytoplasm of the myocardial cells following 60 min of heat stress (Fig. 3D).

Immunohistochemical analysis revealed no observable differences in the distribution of Hsp70 in the myocardial cells of heat-stressed rats and normal control rats (Fig. 4). Hsp70-positive signals were primarily detected in the cytoplasm of the myocardial cells *in vivo*. Of note, following 60 min heat stress, Hsp70-positive signals in the cytoplasm of the myocardial cells were more prominent in intact areas than in degenerated areas (Fig. 3D-F). At 80 min of heat stress, the Hsp70-positive signals were unevenly distributed in favor of the cytoplasm of myocardial cells *in vitro* (Fig. 3E and F). Increasingly stronger cytoplasmic Hsp70-positive signals

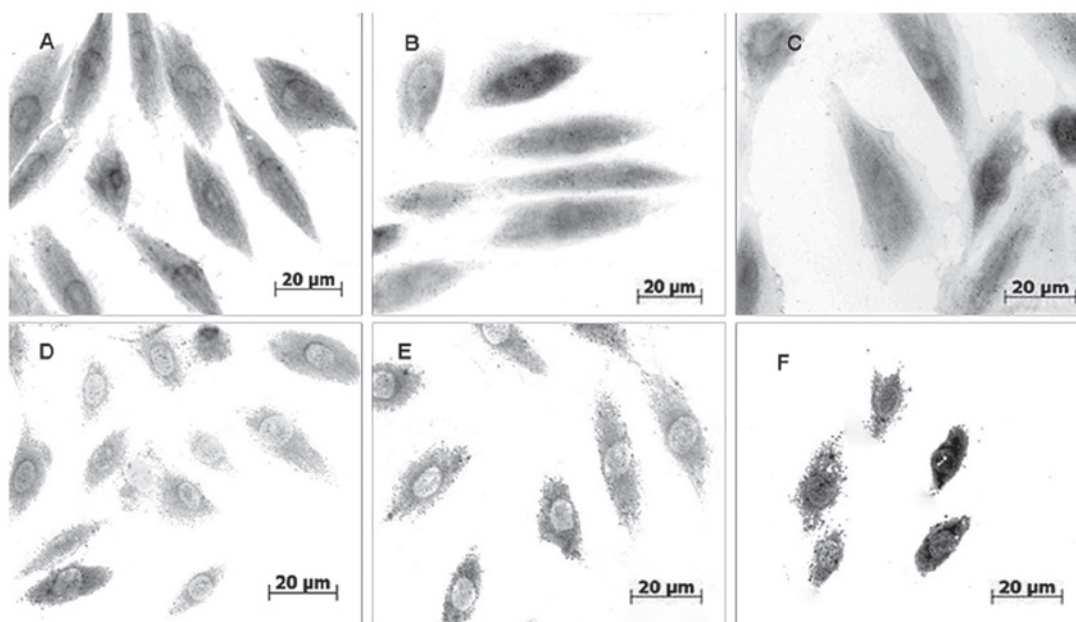


Figure 3. Distribution of heat shock protein 70 signals in the control and the heat-stressed H9c2 cells. Myocardial H9c2 cells were incubated at 37°C and subjected to heat stress at 42°C as follows: (A) Control, 0 min; (B) 20; (C) 40; (D) 60; (E) 80; and (F) 100 min. Cells were visualized using immunocytochemical staining and fluorescence microscopy.

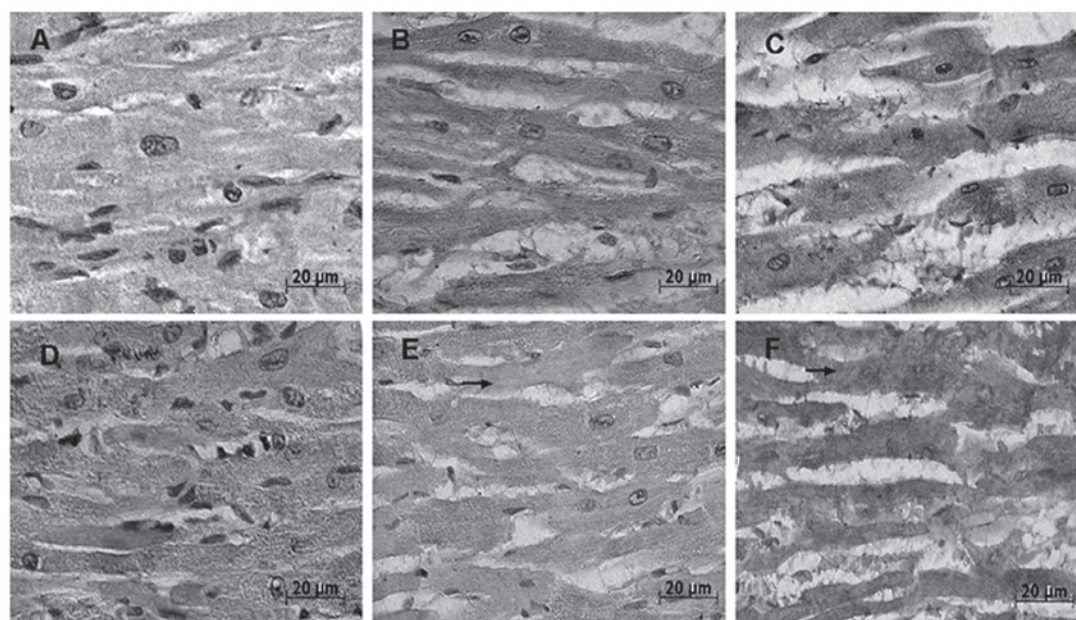


Figure 4. Distribution of heat shock protein 70 signals in the hearts of the control and heat-stressed rats. Rats were subjected to heat-stress at 42°C as follows: (A) Control, 0 min; (B) 20; (C) 40; (D) 60; (E) 80; and (F) 100 min. Animals were sacrificed following 100 min heat stress and serial sections of the heart tissue were obtained from each group. Sections underwent immunocytochemical staining followed by counter-staining with hematoxylin and eosin and visualized using fluorescence microscopy.

were observed in myocardial cells *in vivo* following 40 and 60 min of heat stress, respectively (Fig. 4C and D). In addition, following 80 min of heat stress, Hsp70 staining was observably lower in the cytoplasm of degenerated areas (Fig. 4E). As shown in Fig. 5, partial myocardial fiber collapse was observed in several rats (Fig. 5A), whereas no lesions were observed in the myocardium of normal rats. Hsp70-positive signals markedly decreased in the cytoplasm and were unevenly distributed in the myocardial cells of the rats (Fig. 5B).

Hsp70 expression levels in heat-stressed H9c2 cells in vitro and in heart tissues in vivo. Hsp70 expression in H9c2 cells *in vitro* decreased slightly, but not significantly, following exposure to heat stress; however, by 40 min, this decrease was significant and remained low until the cessation of heat stress ($P<0.05$) (Fig. 6).

Hsp70 expression levels in the myocardial cells of heat-stressed rats showed a slight, but not significant increase at 20 min following heat stress. Hsp70 expression in these cells

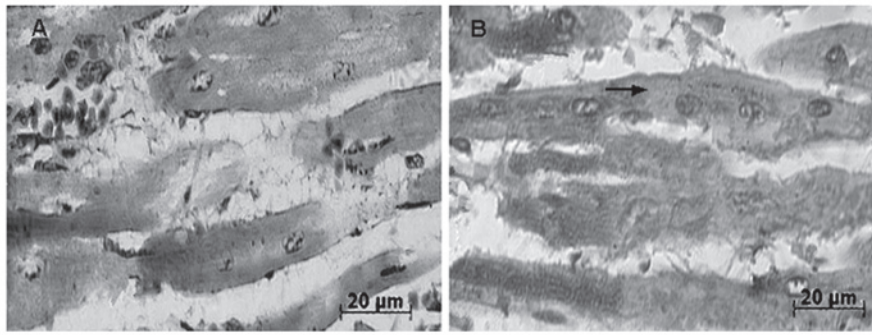


Figure 5. Post mortem histological images of myocardial cells from rats following heat stress. (A) Hematoxylin and eosin staining and (B) immunohistochemical staining of myocardial cells from rats which did not survive 100 min heat stress.

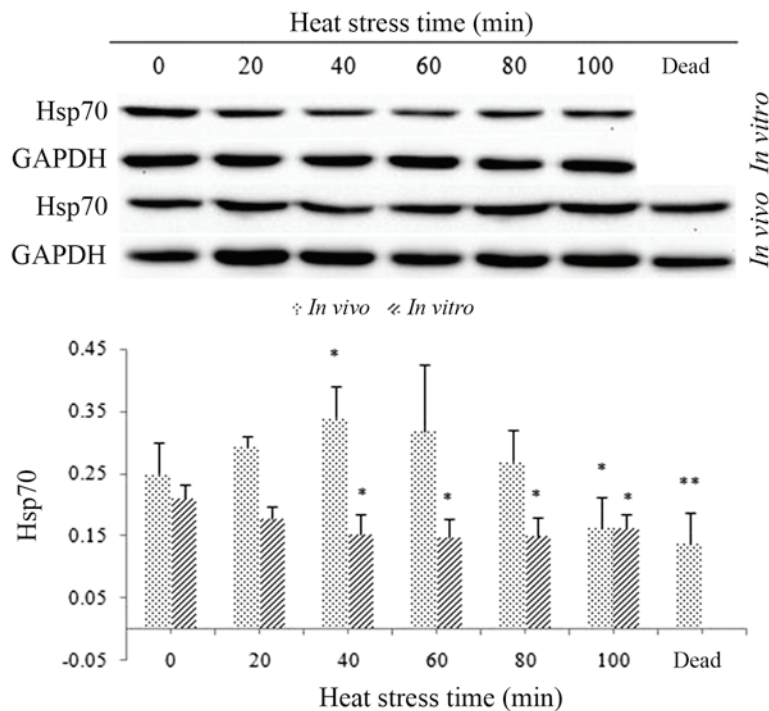


Figure 6. Western blot analysis and quantification of Hsp70 levels in heat-stressed H9c2 cells *in vitro* and in rat heart tissues *in vivo*. Values are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. control group (0 min). Hsp, heat shock protein.

peaked and was statistically significant at 40 min following heat stress; however, expression levels then decreased until 100 min at which point they were significantly decreased compared to those of the control group ($P < 0.05$). Of note, the protein levels of Hsp70 in the heart tissues of rats post-mortem were also significantly lower than those of the control rats ($P < 0.01$) (Fig. 6).

Discussion

Variations in CK and AST activities were reported to be associated with liver and heart disease (30,31). However, the present study revealed that activity levels of the two enzymes differed between the *in vivo* and *in vitro* experiments; in addition, activity levels of AST and CK in the supernatants of heat-stressed H9c2 cells as well as the sera of the heat-stressed rats exhibited significant variations. In H9c2 cells, the enzyme activities, markedly AST activity, increased immediately

following exposure to heat stress. Furthermore, acute degeneration was observed in the heat-stressed cells *in vitro* and *in vivo*, characterized by increased cell size, cytoplasmic granular degradation as well as nuclear condensation. Altered AST levels were found to correspond with the pathological changes that occurred following heat stress in the cytoplasm of rat myocardial cells *in vitro* and *in vivo*. This therefore indicated that H9c2 cells and myocardial cells were protected from damage, particularly at the beginning of heat stress. These results were comparable with the findings of previous studies (32,33). It has been reported that increased levels of AST and LDH were associated with heart disease (34), the proposed mechanism of this is thought to be due to external stresses that may damage myocardial cell membranes, leading to the release of CK into the supernatant *in vitro* (35). AST, which was used as a heat stress indicator in these experiments, may be beneficial for the further elucidation of the correlation between enzymatic activity induction and cardiac tissue damage (36).

Cytopathological results of the present study demonstrated acute degeneration, with increased cell size and numerous granular particles in the cytoplasm of H9c2 cells following heat stress for 20 min. Increased damage was observed with the duration of heat exposure. H9c2 cell cytoplasmic changes were consistent with the cytopathological changes in the myocardial cells *in vivo*. In addition, histopathological analysis revealed degeneration of the heart tissue and disordering of myocardial fibers following 20 min of heat stress as well as cell karyopyknosis *in vivo*; furthermore, following 60 min of heat stress exposure the damage observed had worsened. These results confirmed that heat stress at 42°C injured the myocardial cells *in vitro* as well as *in vivo*.

Hsps are important endogenous, protective proteins that have a significant role in the cellular response to stress (37). Hsps from different families exhibit different roles and functions and it was hypothesized that the localization of Hsps may be associated with the protection function of molecular chaperones (38). In the present study, immunocytochemical analysis revealed that Hsp70 was primarily distributed in the cytoplasm of myocardial cells, which was partially consistent with previous reports of Hsp family members being localized in the nucleus as well as the cytoplasm (39,40). However, in the present study, the cytoplasm of H9c2 cells demonstrated a lower density of Hsp70-positive signals *in vitro* following 60 min of exposure to heat stress. This therefore indicated that Hsp70 consumption may exceed its production prior to 60 min of heat stress, following which the cells produced sufficient Hsp60 in order to protect them against heat stress. In addition, immunohistochemical analyses revealed that Hsp70 was primarily expressed in the cytoplasm of muscle fibers *in vivo*; this was consistent with the results of previous studies where Hsp70 was detected in the muscle fibers of transported pigs. The present study reported that following 60 min of heat stress, Hsp70-positive signals in the cytoplasm of the heart cells were more prominent in intact areas than in degenerated areas; furthermore, the density of Hsp70-positive signals was reduced following 60 min of heat stress. These results were also consistent with those of previous studies (32,41,42). Of note, the present study demonstrated that Hsp70-positive signals with lower densities were unevenly distributed throughout the cytoplasm of myocardial cells of rats subjected to heat stress post mortem; however, these changes were not consistent with the *in vivo* and *in vitro* experiments. This inconsistency may be due to the different regulatory mechanisms *in vivo* and *in vitro*.

The expression of Hsp70 in the presence and absence of external stressors indicated the potential role of these proteins in physiological adaptation (43). The results of the present study showed that Hsp70 levels *in vitro* decreased following the initiation of heat stress and reached the lowest levels at 60 min, gradually increasing thereafter until the heat stress experiment was terminated at 100 min. These results were comparable with the results of the immunocytochemical analysis in the present study. By contrast, the Hsp70 levels in the heart cells of rats *in vivo* gradually increased from the initiation of heat stress and decreased sharply at 100 min of heat stress; this was not consistent with the results of the H9c2 cells *in vitro*, which may be due to different regulatory mechanisms *in vivo* and *in vitro*. It was reported that increased Hsp expression occurs

in the majority of cells in response to stress (44) or exposure to high temperatures (45). In addition, overexpression of Hsp70 was shown to confer substantial heat resistance and protect cells and proteins from thermal damage via the efficient recognition of denatured proteins (46). By contrast, decreased Hsp70 levels were reported to have a reduced protective effect on myocardial cells (47).

Hsps are synthesized in response to increased temperature in order to repair or degrade damaged proteins as a defense strategy to ensure cell survival (48-50). Hsp70 family members refold degenerated proteins by recognizing and binding to the cytoskeletal myosin heavy chain and actin in damaged gastric mucosa (51). In addition, Hsp70 has an important role in cytoprotection in numerous cell types by preventing denaturation as well as enhancing the proper assembly of cellular proteins and structure. Induction of Hsp70 was also associated with myocardial protection (52,53).

In the present study, increased expression of Hsp70 was observed in damaged heart tissue and myocardial cells, characterized by acute cell degeneration and release of specific enzymes. Hsp70 expression levels were compared with histopathological changes in myocardial cells and the results demonstrated that following heat stress, increased levels of specific enzymes and tissue damage were accompanied by the reduction of Hsp70 expression. Of note, within 100 min of heat stress there was a 45% mortality rate, accompanied by sustained severe heat-induced damage of myocardial cells in rats as well as a low uneven distribution of Hsp70 in the cytoplasm. This therefore indicated that heat stress injured myocardial cells *in vivo* and *in vitro*. However, the altered expression levels of Hsp70 in H9c2 cells *in vitro* did not correspond with those of rat myocardial cells *in vivo* following heat stress.

Hsp70 expression in myocardial cells was hypothesized to enable the organism to resist the adverse effects of stress. When exposed to heat stress, the heart rate is increased (54). Increased expression of Hsp70 in transgenic mouse hearts was reported to increase resistance to ischemia/reperfusion injury and significantly improve recovery of cardiac function (55). Furthermore, increased cellular levels of Hsp70 via gene transfer was demonstrated to significantly increase resistance in myocardial cells *in vitro* as well as in transgenic animals *in vivo* (55). A previous study reported that Hsp72 overexpression reduced the size of cardiac infarctions in *in vivo* transgenic mouse models of myocardial ischemia and reperfusion (56). These studies therefore indicated that enhanced Hsp70 expression may be a mechanism to improve cell survival in response to stressful environments, which may proceed via protecting proteins from degradation and facilitating their refolding (57,58). In the present study, Hsp70 levels decreased *in vivo* at the later stages of heat stress exposure, which may be due to the rats developing a tolerance to heat following several hours of exposure to heat stress, or due to material deficiencies following long-term stress. It has been previously reported that caloric restriction increased the induction of Hsp70 transcription and improved thermotolerance (59). It was also reported that inorganic phosphate deficiencies may affect the major cellular biochemical pathways controlling Hsp protein expression (60-63).

In conclusion, comprehensive comparisons of enzymes, cell morphology and Hsp70 levels indicated that decreased levels of Hsp70 were associated with the reduced protective effect on myocardial cells *in vitro* and *in vivo*. However, further studies are required in order to fully elucidate the mechanisms underlying the interaction between Hsp70 and tissue damage in heat-stressed cells *in vivo* and *in vitro*.

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References

- Banerjee Mustafi S, Chakraborty PK, Dey RS and Raha S: Heat stress upregulates chaperone heat shock protein 70 and antioxidant manganese superoxide dismutase through reactive oxygen species (ROS), p38MAPK, and Akt. *Cell Stress Chaperones* 14: 579-589, 2009.
- van der Hel W, Verstegen MW, Pijls L and van Kampen M: Effect of two-day temperature exposure of neonatal broiler chicks on growth performance and body composition during two weeks at normal conditions. *Poult Sci* 71: 2014-2021, 1992.
- Geraert PA, Padilha JC and Guillaumin S: Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. *Br J Nutr* 75: 195-204, 1996.
- Mashaly MM, Hendricks GL III, Kalama MA, Gehad AE, Abbas AO and Patterson PH: Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poult Sci* 83: 889-894, 2004.
- Lee WC, Lin KY, Chiu YT, *et al*: Substantial decrease of heat shock protein 90 in ventricular tissues of two sudden-death pigs with hypertrophic cardiomyopathy. *FASEB J* 10: 1198-1204, 1996.
- Hu Y, Jin H, Du X, *et al*: Effects of chronic heat stress on immune responses of the foot-and-mouth disease DNA vaccination. *DNA Cell Biol* 26: 619-626, 2007.
- Lu Q, Wen J and Zhang H: Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken. *Poult Sci* 86: 1059-1064, 2007.
- McCormick PH, Chen G, Tlerney S, Kelly CJ and Bouchier-Hayes DJ: Clinically relevant thermal preconditioning attenuates ischemia-reperfusion injury. *J Surg Res* 109: 24-30, 2003.
- Ganter MT, Ware LB, Howard M, *et al*: Extracellular heat shock protein 72 is a marker of the stress protein response in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 291: L354-L361, 2006.
- Staib JL, Quindry JC, French JP, Criswell DS and Powers SK: Increased temperature, not cardiac load, activates heat shock transcription factor 1 and heat shock protein 72 expression in the heart. *Am J Physiol Regul Integr Comp Physiol* 292: R432-R439, 2007.
- Li Z and Srivastava P: Heat-shock proteins. *Curr Protoc Immunol Appendix 1: Appendix 1A*, 2004.
- Lindquist S and Craig EA: The heat-shock proteins. *Annu Rev Genet* 22: 631-677, 1988.
- Liu L, Zhang X, Qian B, *et al*: Over-expression of heat shock protein 27 attenuates doxorubicin-induced cardiac dysfunction in mice. *Eur J Heart Fail* 9: 762-769, 2007.
- Lepore DA, Knight KR, Anderson RL and Morrison WA: Role of priming stresses and Hsp70 in protection from ischemia-reperfusion injury in cardiac and skeletal muscle. *Cell Stress Chaperones* 6: 93-96, 2001.
- Knowlton AA, Kapadia S, Torre-Amione G, *et al*: Differential expression of heat shock proteins in normal and failing human hearts. *J Mol Cell Cardiol* 30: 811-818, 1998.
- Gray CC, Amrani M and Yacoub MH: Heat stress proteins and myocardial protection: experimental model or potential clinical tool? *Int J Biochem Cell Biol* 31: 559-573, 1999.
- Oyake J, Otaka M, Matsushashi T, *et al*: Over-expression of 70-kDa heat shock protein confers protection against monochloramine-induced gastric mucosal cell injury. *Life Sci* 79: 300-305, 2006.
- Cvoro A, Dundjerski J, Trajković D and Matić G: Heat stress affects the glucocorticoid receptor interaction with heat shock protein Hsp70 in the rat liver. *Biochem Mol Biol Int* 46: 63-70, 1998.
- Evdonin AL, Guzhova IV, Margulis BA and Medvedeva ND: Extracellular heat shock protein 70 mediates heat stress-induced epidermal growth factor receptor transactivation in A431 carcinoma cells. *FEBS Lett* 580: 6674-6678, 2006.
- Dwyer BE, Nishimura RN and Brown IR: Synthesis of the major inducible heat shock protein in rat hippocampus after neonatal hypoxia-ischemia. *Exp Neurol* 104: 28-31, 1989.
- Li HM, Niki T, Taira T, Iguchi-Ariga SM and Ariga H: Association of DJ-1 with chaperones and enhanced association and colocalization with mitochondrial Hsp70 by oxidative stress. *Free Radic Res* 39: 1091-1099, 2005.
- Drummond IA and Steinhardt RA: The role of oxidative stress in the induction of *Drosophila* heat-shock proteins. *Exp Cell Res* 173: 439-449, 1987.
- Morimoto RI: Cells in stress: transcriptional activation of heat shock genes. *Science* 259: 1409-1410, 1993.
- Sorger PK: Heat shock factor and the heat shock response. *Cell* 65: 363-366, 1991.
- Wu C: Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* 11: 441-469, 1995.
- Beckmann RP, Mizzen LA and Welch WJ: Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* 248: 850-854, 1990.
- Njemini R, Bautmans I, Lambert M, Demanet C and Mets T: Heat shock proteins and chemokine/cytokine secretion profile in ageing and inflammation. *Mech Ageing Dev* 128: 450-454, 2007.
- Pierzchalski P, Krawiec A, Ptak-Belowska A, Barańska A, Konturek SJ and Pawlik WW: The mechanism of heat-shock protein 70 gene expression abolition in gastric epithelium caused by helicobacter pylori infection. *Helicobacter* 11: 96-104, 2006.
- Lee WC, Lin KY, Chiu YT, *et al*: Substantial decrease of heat shock protein 90 in ventricular tissues of two sudden-death pigs with hypertrophic cardiomyopathy. *FASEB J* 10: 1198-1204, 1996.
- Jabiry-Zieniewicz Z, Bobrowska K, Kaminski P, Wielgos M, Zieniewicz K and Krawczyk M: Low-dose hormonal contraception after liver transplantation. *Transplant Proc* 39: 1530-1532, 2007.
- Radosavljević T, Mladenović D, Vucević D, *et al*: Effect of acute lindane and alcohol intoxication on serum concentration of enzymes and fatty acids in rats. *Food Chem Toxicol* 46: 1739-1743, 2008.
- Yu J, Bao E, Yan J and Lei L: Expression and localization of Hsps in the heart and blood vessel of heat-stressed broilers. *Cell Stress Chaperones* 13: 327-335, 2008.
- Tang S, Lv Y, Chen H, *et al*: Comparative analysis of α B-crystallin expression in heat-stressed myocardial cells in vivo and in vitro. *PLoS One* 9: e86937, 2014.
- Mitchell MA and Sandercock DA: Creatine kinase isoenzyme profiles in the plasma of the domestic fowl (*Gallus domesticus*): effects of acute heat stress. *Res Vet Sci* 59: 30-34, 1995.
- Britton CV, Hernandez A and Roberts R: Plasma creatine kinase isoenzyme determinations in infants and children. Characterization in normal patients and after cardiac catheterization and surgery. *Chest* 77: 758-760, 1980.
- Zhang M, Xin L, Bao E, Hartung J and Yue Z: Variation in the expression of Hsp27, α B-crystallin mRNA and protein in heart and liver of pigs exposed to different transport times. *Res Vet Sci* 90: 432-438, 2011.
- Gullo CA and Teoh G: Heat shock proteins: to present or not, that is the question. *Immunol Lett* 94: 1-10, 2004.
- Georgopoulos C and Welch WJ: Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* 9: 601-634, 1993.
- Easton DP, Kaneko Y and Subjeck JR: The hsp110 and Grp1 70 stress proteins: newly recognized relatives of the Hsp70s. *Cell Stress Chaperones* 5: 276-290, 2000.

40. Geum D, Son GH and Kim K: Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. *J Biol Chem* 277: 19913-19921, 2002.
41. Kervinen H, Huittinen T, Vaarala O, *et al*: Antibodies to human heat shock protein 60, hypertension and dyslipidemia. A study of joint effects on coronary risk. *Atherosclerosis* 169: 339-344, 2003.
42. Bao E, Sultan K, Nowak B and Hartung J: Expression and distribution of heat shock proteins in the heart of transported pigs. *Cell Stress Chaperones* 13: 459-466, 2008.
43. Kilgore JL, Musch TI and Ross CR: Physical activity, muscle, and the HSP70 response. *Can J Appl Physiol* 23: 245-260, 1998.
44. Locke M and Noble EG: Stress proteins: the exercise response. *Can J Appl Physiol* 20: 155-167, 1995.
45. Lindquist S and Petersen R: Selective translation and degradation of heat-shock messenger RNAs in *Drosophila*. *Enzyme* 44: 147-166, 1990.
46. Simard JP, Reynolds DN, Kraguljac AP, Smith GS and Mosser DD: Overexpression of HSP70 inhibits cofilin phosphorylation and promotes lymphocyte migration in heat-stressed cells. *J Cell Sci* 124 (Pt 14): 2367-2374, 2011.
47. Matokanovic M, Barisic K, Filipovic-Grcic J and Maysinger D: Hsp70 silencing with siRNA in nanocarriers enhances cancer cell death induced by the inhibitor of Hsp90. *Eur J Pharm Sci* 50: 149-158, 2013.
48. Benjamin IJ and McMillan DR: Stress (heat shock) proteins molecular chaperones in cardiovascular biology and disease. *Circ Res* 83: 117-132, 1998.
49. Ciocca DR, Oesterreich S, Chamness GC, McGuire WL and Fuqua SA: Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J Natl Cancer Inst* 85: 1558-1570, 1993.
50. Harrington HM, Dash S, Dharmasiri N and Dharmasiri S: Heat-shock proteins: a search for functions. *Aust J Plant Physiol* 21: 843-855, 1994.
51. Otaka M, Odashima M, Tamaki K and Watanabe S: Expression and function of stress (heat shock) proteins in gastrointestinal tract. *Int J Hyperthermia* 25: 634-640, 2009.
52. Locke M and Tanguay RM: Diminished heat shock response in the aged myocardium. *Cell Stress Chaperones* 1: 251-260, 1996.
53. Plumier JC and Currie RW: Heat shock-induced myocardial protection against ischemic injury: a role for Hsp70? *Cell Stress Chaperones* 1: 13-17, 1996.
54. Knowlton AA: Heat-shock proteins, stress, and the heart. *Ann NY Acad Sci* 723: 128-137, 1994.
55. Plumier JC, Ross BM, Currie RW, *et al*: Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. *J Clin Invest* 95: 1854-1860, 1995.
56. Hutter JJ, Mestral R, Tam EK, Sievers RE, Dillmann WH and Wolfe CL: Overexpression of heat shock protein 72 in transgenic mice decreases infarct size in vivo. *Circulation* 94: 1408-1411, 1996.
57. Pratt W: The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J Biol Chem* 268: 21455-21458, 1993.
58. Hartl FU: Molecular chaperones in cellular protein folding. *Nature* 381: 571-579, 1996.
59. Heydari AR, Wu B, Takahashi R, Strong R and Richardson A: Expression of heat shock protein 70 is altered by age and diet at the level of transcription. *Mol Cell Biol* 13: 2909-2918, 1993.
60. Edens FW, Hill CH and Wang S: Heat shock protein response in phosphorus-deficient heat-stressed broiler chickens. *Comp Biochem Physiol B* 103: 827-831, 1992.
61. Belay T and Teeter RG: Effects of ambient temperature on broiler mineral balance partitioned into urinary and faecal loss. *Br Poult Sci* 37: 423-433, 1996.
62. Mahmoud KZ, Beck MM, Scheideler SE, Forman MF, Anderson KP and Kachman SD: Acute high environmental temperature and calcium-estrogen relationships in the hen. *Poult Sci* 75: 1555-1562, 1996.
63. Mahmoud KZ, Edens FW, Eisen EJ and Havenstein GB: The effect of dietary phosphorus on heat shock protein mRNAs during acute heat stress in male broiler chickens (*Gallus gallus*). *Comp Biochem Physiol C Toxicol Pharmacol* 137: 11-18, 2004.