Effects of Cyclic Heat Stress or Vitamin C Supplementation during Cyclic Heat Stress on HSP70, Inflammatory Cytokines, and the Antioxidant Defense System in Sprague Dawley Rats

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Abstract: A total of 21 male SD rats were divided into three groups to investigate the effects of consecutive cyclic heat stress or vitamin C under heat stress on heat shock protein (HSP) 70, inflammatory cytokines, and antioxidant systems. The heat stress (HS) and vitamin C supplementation during heat stress (HS+VC) groups were exposed to cyclic heat stress (23 to 38 to 23°C) for 2 h on each of seven consecutive days. The HS+VC group had free access to water containing 0.5% vitamin C throughout the experiment. Hepatic HSP70 mRNA in the HS group was significantly (P<0.05) higher than that in the control (CON) or HS+VC group. The mRNA levels of tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) in the HS group were greater (P<0.05) than those in the CON group. The HS+VC group showed significantly (P<0.05) lower mRNA levels of hepatic interleukin-6 and TNF-α than the HS group. However, thymic HSP70 and inflammatory cytokines were unaffected by treatments. In the hepatic antioxidant system, the mRNA and activity of glutathione peroxidase (GPX) were greater (P<0.05) in the HS than in the CON group, whereas the HS+VC group showed markedly (P<0.05) lower GPX mRNA and activity than the HS group. However, superoxide dismutase, glutathione S-transferase, and malondialdehyde were unaffected by treatments. In conclusion, cyclic heat stress activated hepatic HSP70, TNF-α, iNOS, and GPX genes, whereas vitamin C during heat stress ameliorated heat stress-induced cellular responses in rats.

Key words: antioxidant, cyclic heat stress, HSP70, inflammatory cytokines, vitamin C

Introduction

The lab animal’s environmental conditions, such as temperature, humidity, air ventilation, and noise, potentially affect not only basic physiological responses but also the results of studies in which researchers are used as models. Among various conditions, high ambient temperature beyond the range of the thermoneutral zone in rodents has been known as one of the most fatal stressors, which trigger various biological responses including poor performance and susceptibility to infectious disease [20, 41]. Moreover, the exposure of animals to the wide range of daily temperature fluctuations is big enough to induce large demands on their physiological and metabolic processes to compensate for changes in the thermal environment; this could affect the outcome of experiments [11]. In toxicological research, the intake and biological efficacy of many toxicants are exacerbated by exposure to heat stress [21]. Therefore, understanding the response to ambient heat stress is becoming increas-
ingly important in experiments with rodents.

At the cellular level, it is well documented that acute heat stress induces the expression of heat shock proteins (HSPs), which is closely associated with adapted thermotolerance against a sudden heat shock [18]. The expression of HSPs is known to be involved in an endogenous cellular defense mechanism that enables cells to cope with stressful conditions that induce inflammation and oxidative stress [7, 27]. In fact, induction of HSP caused by acute heat stress increased the production of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, which can eventually lead to hemorrhage and necrosis in multiple organs including the liver, heart, and brain [5, 16].

Acute heat stress-induced oxidative damage in animals has also been reported in numerous studies to date. There is substantial evidence that acute heat stress can profoundly disturb the balance between the generation of reactive oxygen species (ROS) and the antioxidant defense system [23, 37]. This oxidative stress induced by hyperthermal stress causes lipid peroxidation in membranes and oxidative damage to proteins and DNA [28, 41]. Excessive production of ROS has also been suggested as a crucial factor that induces HSP genes [1]. Thus, based upon previous studies, it is suggested that a mechanism by which oxidative stress caused by acute heat stress may be interactively associated with release of inflammatory cytokines via HSP genes. However, very limited research has been carried out to investigate whether consecutive cyclic heat stress in rodents could modulate inflammatory cytokines and the antioxidant defense system interactively.

The extent to which cellular damage causes oxidative stress relies on the balance between the production and removal of oxidants by the antioxidant system in animals. Therefore, excess oxidants are captured by antioxidant enzymes and antioxidants. Several studies [12, 29] have demonstrated that antioxidant enzymes and antioxidants prevented HSP induction, suggesting that the underlying mechanism by which hyperthermia causes HSP induction is via oxidative stress. Therefore, scavengers of free radicals such as vitamin C may play a pivotal role in preventing the cellular damage of organs and maintaining homeostasis during heat stress. Several studies with chickens have demonstrated that vitamin C supplementation appears to play a crucial role in the prevention of acute heat stress and subsequent improvement of poultry performance [24, 26]. However, there is a lack of evidence showing that dietary antioxidants can alleviate the adverse effects of consecutive cyclic heat stress on physiological parameters in rodents. Hence, it is hypothesized that vitamin C supplementation under cyclic heat stress may interactively modulate heat stress-induced physiological responses in rats.

The objectives of this study, thus, were 1) to investigate the effects of consecutive cyclic heat stress on mRNA expressions of heat shock protein, inflammatory cytokines, and the antioxidant defense system and 2) to examine the alleviating effects of vitamin C supplementation under cyclic heat stress on these physiological parameters in SD rats.

**Materials and Methods**

*Experimental animals and procedures*

The animal handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Gyeongnam National University of Science and Technology in Korea.

A total of 30 male Sprague Dawley (SD) rats (3 weeks), which originated from the Samtako Korea, were maintained under a barrier system with a regulated temperature (23 ± 2°C), humidity (50 ± 10%), and light/dark cycle (light on 07:00–19:00). Immediately after a two-week acclimation period, 21 rats showing a similar body weight were randomly allotted into three groups on the basis of body weight; the three treatment groups consisted of control (CON), heat stress (HS), and heat stress with vitamin C supplementation (HS+VC) groups. All rats were housed singly in polycarbonate cages according to the experimental design. A commercial diet containing 23% crude protein, 5% crude fat, 5% crude fiber, and 45% nitrogen-free extract and sterilized bottle water were fed *ab libitum*. To induce heat stress, the rats designated to the HS and HS+VC groups were subjected to cyclic heat stress (23 to 38 to 23°C) for 2 h on each of 7 consecutive days. A thermostat installed in the chamber box was used to maintain the desired temperature. The HS and HS+VC groups in the chamber box were maintained at 38°C for 1 h. It took 30 min to reach the desired temperature in the chamber box (38°C) and also about 30 min to return to the initial chamber temperature (23°C). We performed acute heat stress for 7 consecutive days, which was defined as cyclic heat stress. The relative humidity of the chamber box was not adjusted for cyclic heat stress. During the seven days of consecutive...
cyclic heat stress, all rats were awakened and had ad libitum access to feed and water. The HS+VC group received a bottle of water containing 0.5% vitamin C (DSM, Dalry Ayrshire, UK) throughout the experimental period. Body weights and daily water intake were measured during the experimental period.

Tissue harvesting

Twelve hours after the last day of the 7 consecutive days of cyclic heat stress, all rats (n=7 per group) were sacrificed with anesthetizing CO₂ gas. Immediately after taking blood from the heart for corticosterone assay, several organs, including the liver and thymus, were harvested and weighed. All organs were rapidly frozen in liquid nitrogen and stored at −70°C until further assay.

Analytical assays

Corticosterone assay: Corticosterone (CORT) was assayed by an enzyme immunoassay using a commercial kit (901-0907, Assay Designs, Inc., Ann Arbor, MI, USA) according to the instructions provided with the kit. Briefly, 100 μl of standard solutions and appropriately diluted plasma were loaded into an individual test well. Fifty microliters of conjugate and antibody were pipetted into each well and incubated for 2 h. After incubation, the contents of the wells were discarded and washed 3 times with a washing solution. A solution of p-nitrophenyl phosphate was added to each well and incubated for 1 h. Immediately after adding 50 μl of stop solution, the absorbance was determined using an ELISA reader (VMax, Molecular Devices, Sunnyvale, CA, USA) with its wavelength set at 405 nm.

Semi-quantification of mRNA expression of HSP70, cytokine, and antioxidant enzyme: To extract the total RNA from tissues, the method of RNAzol™ B (Tel-Test, Inc., Friendswood, TX, USA) was applied. Briefly, 100 mg of tissue was removed from each organ and added to 1 ml of RNAzol solution. The tissues were homogenized using a glass–glass homogenizer. The lysate was transferred to a microcentrifuge tube and added to 1/10 volume of chloroform to remove protein extract. The aqueous phase was separated by centrifugation for 15 min at 10,000 × g. Total RNA was precipitated with the same volume of isopropanol and centrifuged for 15 min at 10,000 × g. The precipitated total RNA was washed with 75% ethyl alcohol, dried, and diluted with diethyl-pyrocarbonate (DEPC)-treated water. The concentration of isolated total mRNA was determined by a spectrophotometer (GeneQuant pro, Amersham, Piscataway, NJ, USA) and confirmed on a 1.0% agarose gel stained with ethidium bromide (EtBr). A semi-quantification of mRNA using reverse transcriptase polymerase chain reaction (RT-PCR) was performed to quantify mRNA of biomarker genes including heat shock protein 70 (HSP70), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione S-transferase (GST). The cDNA primers

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<th>Product size (bp)</th>
<th>GenBank accession No.</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
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<td>TACTGAACCTCCGGGTTATGGTC</td>
<td>180</td>
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<td></td>
<td>Reverse</td>
<td>CGCCCTGTGCTCTTTGAGAAC</td>
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<td>SOD</td>
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<td>β-actin</td>
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</table>
used to amplify each gene are listed in Table 1. Briefly, for synthesis of the first strand cDNA, 1.0 µg of total RNA was incubated at 62°C for 10 min with 1.0 µg of oligo dT (Invitrogen Corporation, Carlsbad, CA, USA). Next, the resulting solution was incubated at 42°C for 50 min in a reaction mixture containing 2.5 mM dNTP and 200 units reverse transcriptase (Takara Bio Inc., Otsu, Japan). Following that, 3.2 units of RNase H were used to remove RNA hybridized with cDNA for 30 min at 37°C. Amplification of obtained RNA was performed for 32 cycles of denaturation at 94°C for 30 s, annealing at 50 min in a reaction mixture containing 2.5 mM dNTP, and 1 unit Taq polymerase (Takara Bio Inc.). We determined the number of cycles and kept the products within the exponential phase. The density of each product in agarose gel electrophoresis (1.5%) containing EtBr was measured using a gel densitometer (MiniBis Pro, DNR Bio-Imaging Systems, Jerusalem Israel). Levels of all mRNAs were expressed as the ratio of signal intensity for genes relative to that of β-actin.

Antioxidant enzyme activity and lipid peroxidation: To harvest cytosol and microsomes from the liver, the crude homogenized tissues were centrifuged at 10,000 × g, and the resulting supernatant was centrifuged at 105,000 × g in a Centrikon T-2080 ultracentrifuge. The final pellet (microsomes) was suspended in a phosphate buffer containing 150 mM KCl (pH 7.4) to produce a protein concentration of 20 mg/ml. The supernatant (cytosol) and a suspended pellet were frozen in liquid nitrogen and stored at −70°C until assay. The activity of Cu-Zn SOD was determined in the cytosol fractions using xanthine and a xanthine oxidase system for production of superoxide radical and subsequent measurement of cytochrome c as a scavenger of the radicals [9]. The SOD activity was expressed as units/mg of proteins, where one unit of activity was the amount of enzymes required to inhibit the rate of reduction of cytochrome c by 50%. GPX was measured at 37°C in the cytosol with cumene hydroperoxide as a substrate by monitoring the increase in absorbance at 340 nm [13]. One unit of activity was expressed as the amount of enzyme catalyzing the conjugated CDNB per min. The quantity of lipid peroxidation in the microsome was estimated by measuring amount of 2-thiobarbituric acid (TBA) reactive substances with a spectrophotometer at 532 nm [4]. TBA material is described in nanomoles of malondialdehyde (MDA) per milligram of protein. Protein was assayed by the BCA method (Tissue Assay Kit Pierce) and stored at −70°C until assay. The activity of malondialdehyde (MDA) per milligram of protein. Protein was assayed by the BCA method (Tissue Assay Kit Pierce) and stored at −70°C until assay. The activity of malondialdehyde (MDA) per milligram of protein. Protein was assayed by the BCA method (Tissue Assay Kit Pierce) and stored at −70°C until assay.

Statistical analysis

Data for the CON group versus the HS group or the HS+VC group were analyzed by Proc t-test at the P<0.05 level [35] to compare the effects of cyclic heat stress or vitamin C supplementation under heat stress. The level of probability indicating a statistical difference was determined as P<0.05. Data are presented as means ± SD.

Results

Apparent biological responses

The body and organ weights of the SD rats in the CON, HS, and HS+VC groups are presented in Table 2. During the 7 days of consecutive cyclic heat stress, there was no significant difference in body weight between treatments. The HS group was shown to have a significant (P<0.05) decrease in spleen weight (29% decrease) compared with the CON group without any effect on the weights of the liver and thymus. However, the weights of the liver, thymus, and spleen did not differ between the HS and HS-VC groups.

The effects of cyclic heat stress or heat stress with vitamin C supplementation on daily water intake are shown in Table 3. Based upon daily water intake, the HS+VC group consumed approximately 192 mg of vitamin C per day on average throughout the entire experimental period. On day 1, the HS group showed a significantly (P<0.05) higher water intake than the CON group. After that, there was no difference in daily water intake between the HS and Con groups. In addition, there was no effect on water intake in the heat-stressed rats with vitamin C supplementation compared with rats exposed to heat stress. These results indicate that cyclic heat stress or supplementation with vitamin C during cyclic heat stress did not affect daily water intake in rats.

The effect of cyclic heat stress or vitamin C supple-
Table 2. Effects of cyclic heat stress or vitamin C supplementation during cyclic heat stress on body and organ weights in SD rats

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^a)</th>
<th>(P)-value</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>HS</td>
<td>HS+VC</td>
</tr>
<tr>
<td>Initial BW, g</td>
<td>190.85 ± 13.24</td>
<td>188.57 ± 9.55</td>
<td>180.57 ± 10.69</td>
</tr>
<tr>
<td>Final BW, g</td>
<td>281.28 ± 11.74</td>
<td>280.00 ± 12.20</td>
<td>286.00 ± 13.30</td>
</tr>
<tr>
<td>Liver, g</td>
<td>12.47 ± 1.22</td>
<td>11.61 ± 1.66</td>
<td>12.06 ± 1.69</td>
</tr>
<tr>
<td>Thymus, g</td>
<td>0.69 ± 0.15</td>
<td>0.69 ± 0.07</td>
<td>0.73 ± 0.08</td>
</tr>
<tr>
<td>Spleen, g</td>
<td>0.75 ± 0.12</td>
<td>0.63 ± 0.07</td>
<td>0.65 ± 0.085</td>
</tr>
</tbody>
</table>

\(^a\)CON (thermoreutral), HS (7 days of consecutive cyclic heat stress), HS+VC (7 days of consecutive cyclic heat stress with vitamin C supplementation). \(^b\)CON group vs. HS group. \(^c\)HS group vs. HS+VC group.

Table 3. Effects of cyclic heat stress or vitamin C supplementation during cyclic heat stress on the daily water intake (ml) in SD rats

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^a)</th>
<th>(P)-value</th>
<th>(P)-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CON</td>
<td>HS</td>
<td>HS+VC</td>
</tr>
<tr>
<td>Day 1</td>
<td>44.35 ± 1.69(^d)</td>
<td>32.27 ± 8.09</td>
<td>41.13 ± 5.46</td>
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<tr>
<td>Day 2</td>
<td>32.77 ± 3.39</td>
<td>37.27 ± 6.73</td>
<td>39.21 ± 7.20</td>
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<tr>
<td>Day 3</td>
<td>37.27 ± 3.38</td>
<td>40.50 ± 4.50</td>
<td>36.00 ± 7.79</td>
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<tr>
<td>Day 4</td>
<td>35.35 ± 3.10</td>
<td>43.06 ± 7.97</td>
<td>33.42 ± 5.09</td>
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<tr>
<td>Day 5</td>
<td>36.63 ± 3.10</td>
<td>36.60 ± 5.46</td>
<td>41.13 ± 4.04</td>
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<tr>
<td>Day 6</td>
<td>34.06 ± 5.09</td>
<td>36.63 ± 4.01</td>
<td>39.21 ± 5.63</td>
</tr>
<tr>
<td>Day 7</td>
<td>35.35 ± 4.80</td>
<td>37.92 ± 7.73</td>
<td>34.71 ± 7.66</td>
</tr>
<tr>
<td>Average</td>
<td>36.54 ± 3.50</td>
<td>38.46 ± 6.35</td>
<td>37.83 ± 6.12</td>
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</table>

\(^a\)CON (thermoreutral), HS (7 days of consecutive cyclic heat stress), HS+VC (7 days of consecutive cyclic heat stress with vitamin C supplementation). \(^b\)CON group vs. HS group. \(^c\)HS group vs. HS+VC group. \(^d\)Contained 0.5% vitamin C.

The mRNA expression levels of HSP70 and inflammatory cytokines (IL-6, TNF-α, and iNOS) in the liver and thymus of SD rats exposed to cyclic heat stress or vitamin C supplementation during heat stress are shown in Fig. 2. In the liver, the mRNA expression level of HSP70 in the HS group was significantly \((P<0.05)\) higher than that in the CON group or HS+VC group (Fig. 2A). The mRNA expression level of TNF-α and iNOS in the HS group was significantly \((P<0.05)\) upregulated compared with that in the CON group. The HS+VC group showed significantly \((P<0.05)\) lower mRNA expression levels of hepatic IL-6 and TNF-α than the HS group and no affect on iNOS expression (Fig. 2A). Therefore, the activated mRNA expression levels of HSP70 and proinflammatory cytokines in response to cyclic heat stress were significantly \((P<0.05)\) attenuated by supplementation with vitamin C. In the thymus, however, there were no significant effects of cyclic heat stress or heat stress with vitamin C supplementation on the mRNA expression level of HSP70. Furthermore the proinflammatory cytokines, IL-6, TNF-α,
and iNOS, which are widely expressed in the thymus of birds, were not affected by cyclic heat stress or vitamin C supplementation under heat stress (Fig. 2B).

**mRNA expression and activity of antioxidant enzymes and lipid peroxidation in the liver**

The hepatic antioxidant defense system in rats exposed to cyclic heat stress or vitamin C supplementation under heat stress was examined, since a significant alteration of HSP70 and inflammatory cytokines was only observed in the liver. The mRNA expression level of GPX was significantly \((P<0.05)\) upregulated in the HS group compared with the CON group (Fig. 3). However, there was no significant effect of heat stress (the CON group vs. the HS group) or vitamin C (the HS group vs. the HS+VC group) on SOD and GST mRNA expression levels. Supplementation with vitamin C in rats subjected to cyclic heat stress markedly \((P<0.05)\) decreased GPX mRNA expression compared with the heat stressed rats without affecting SOD and GST expression.

The specific activity of hepatic GPX in the HS group was significantly \((P<0.05)\) higher than that in the CON group, whereas GPX activity in the HS group was markedly \((P<0.05)\) lower than that in the HS+VC group (Fig. 4B). However, the specific activities of SOD and GST and the level of MDA in the liver were not affected by exposure to cyclic heat or supplementation with vitamin C during the 7-day consecutive cyclic heat stress period, although the HS group tended to show an increase \((P=0.10)\) in the MDA level compared with the CON group (Fig. 4A, 4C, and 4D).

**Discussion**

In the present study, cyclic heat stress was applied for seven consecutive days to investigate the effects of cyclic
heat stress or heat stress with vitamin C feeding on cyclic heat stress-induced cellular responses in SD rats. We examined plasma CORT to clarify whether cyclic heat could induce a stress response in rats. CORT, widely recognized as a criterion of stress response, has been shown to increase when animals are exposed to acute stressors such as transportation and feed deprivation [33, 39]. However, cyclic heat stress or vitamin feeding under heat stress in our study did not affect the CORT level, which was similar to the result reported by Gibson et al. [10] who showed prolonged stress did not affect the CORT level.

This study demonstrated that consecutive cyclic heat stress significantly upregulated the mRNA expression levels of hepatic HSP70, TNF-α, iNOS, and GPX, whereas rats that received vitamin C during heat stress showed similar levels for parameters compared with the control rats. HSPs, a set of highly conserved proteins induced by heat stress, have been recognized as endogenous molecules that mediate the cellular defense system through the activation of nuclear factor (NF)-κB, which can in turn elicit inflammatory cytokine induction [2, 31]. In fact, HSPs are immunoregulatory agents involved in a physiological mechanism for regulation of proinflammatory disease [31]. According to our data, the rats subjected to cyclic heat stress showing upregulated HSP70 were found to have significantly higher TNF-α, and iNOS mRNA expression than the control rats, indicating that cyclic heat stress is also involved in inflammatory cytokine induction. In accordance with our observations, Asea et al. [2] reported that increased HSP70 markedly activated proinflammatory cytokines including IL-1β, IL-6, and TNF-α in human monocytes. High levels of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α in response to heat stress can eventually lead to hemorrhage and necrosis in multiple organs including the liver, heart, and brain [5, 16]. Several studies also demonstrated that an enhanced HSP70 level was associated with proinflammatory cytokine expression in certain pathophysiological conditions including pre-eclampsia, acute infection, arthritis, ageing, and acute heat stress [14, 27, 42]. Hagiwara et al. [14] reported that the production of nitric oxide (NO), which is associated with the inflammatory process, was significantly affected by heat stress, suggesting that the up-regulation of iNOS in this study might be associated with NO production after cyclic heat stress. At present, it has been reported that not only HSP70 induces the production of proinflammatory cytokines but also that these cytokines can activate HSP70 production [2, 32].

There also seemed to be a time-dependent onset of cellular responses induced by cyclic heat stress depending upon the types of signaling genes. In this study, the mRNA expressions of HSP70 and TNF-α were much

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Fig. 4. Specific activity of antioxidant enzymes (SOD, GPX, and GST) and lipid peroxidation (MDA) in the liver of SD rats exposed to cyclic heat stress or vitamin C supplementation during cyclic heat stress in SD rats (mean ± SD, n=7). An asterisk (*) indicates significant differences between two groups (CON vs. HS; HS vs. HS+VC) at P<0.05. NS represents nonsignificant.
more sensitively affected by cyclic heat stress compared with the mRNA expression of iNOS. Hagiwara et al. [14] reported that NO was much more slowly produced after heat stress compared with TNF-α in Wistar rats. Therefore, it is thought that induction and repression of each cellular signaling gene against stressors respond differently, depending upon the types of stressors, time-dependent manner of the gene, etc. [14, 36].

To investigate the alleviating effects of vitamin C on cyclic heat stress-induced cellular responses, the mRNA expression levels of HSP70 and inflammatory cytokines in the liver and thymus were examined. The proinflammatory cytokines IL-6, TNF-α, and iNOS, which are widely expressed in lymphoid (thymus) and nonlymphoid (liver) tissues, are the most powerful cognitive pathways in response to inflammatory process [17]. Supplementation of rats exposed to cyclic heat stress with vitamin C simultaneously decreased the mRNA levels of HSP70 and inflammatory cytokines (IL-6 and TNF-α) only in the liver compared with heat-stressed rats. In the thymus, a primary lymphoid organ, the mRNA expression levels of HSP70 and proinflammatory cytokines were not affected by cyclic heat stress or vitamin C feeding under heat stress.

From our observations, it can be interpreted that decreased HSP70 mRNA in the rat liver exposed to cyclic heat stress with vitamin C supplementation may be associated with down-regulated expressional levels of inflammatory cytokines. These results are supported by those of Mahmoud et al. [24], who reported that HSP70 expression was much lower in vitamin C-fed chickens than control chickens. In addition, Chang et al. [6] reported that a dietary antioxidant decreased a pro-inflammatory cytokine, IL-6, in humans. Several previous in vitro and in vivo studies also demonstrated that dietary antioxidants ameliorated proinflammatory cytokine production under acute heat stress [19, 33], suggesting that vitamin C, known to ameliorate heat stress, may function in the cellular defense system via scavengers of reactive oxygen metabolites.

It was reported that acute and prolonged heat stress could remarkably disturb the balance between the generation of ROS and the antioxidant defense system in birds [22, 23]. The activating effects of consecutive cyclic heat stress on mRNA expression of HSP70 and inflammatory cytokines in the liver can be attributed to oxidative stress, since enhanced metabolic reactions caused by high ambient temperature produce excessive ROS, which have profound effects on cellular antioxidant mechanisms. In the process of its antioxidant defense functions, the liver is the most active site for synthesizing the antioxidants and antioxidant enzymes. Therefore, we mainly focused on the hepatic antioxidant system in rats exposed to cyclic heat stress or vitamin C feeding under heat stress. In the present study, rats subjected to cyclic heat stress were shown to have a significant increase in hepatic HSP70 mRNA expression, which could be explained by the previous studies showing that hyperthermia caused HSP70 induction via increased ROS production as a result of activated metabolic rates [1, 29]. It was also observed that both the mRNA and specific activity of hepatic GPX were significantly increased by cyclic heat stress, which could produce excessive ROS in the rat liver. The extent of cellular damage relies on the balance between production of oxidants and removal by the enzymatic and nonenzymatic antioxidant system. Antioxidant enzymes including SOD and GPX are actively involved in the removal of excessive ROS induced by oxidative stress [8, 38]. Yang et al. [40] reported that heat stress resulted in a significant increase in production of ROS and antioxidant enzymes including SOD, catalase, and GPX, and in lipid peroxidation in chickens. In addition, Bhusari et al. [3] reported that chronic heat stress induced hepatic SOD and catalase, indicating that increased ROS in response to heat stress activated antioxidant enzymes. The current data from rats subjected to cyclic heat stress are partially in agreement with the previous studies in that GPX was significantly affected by cyclic heat stress or vitamin C feeding under heat stress.

In this study, the fact that there was no obvious change in SOD mRNA and activity in the liver of the rats subjected to cyclic heat stress was presumably due to the time point that was measured. In view of this consideration, it was reported that increased ROS under heat stress gradually returned to the control level within 12 h of removal of heat stress [40]. Hence, it is supposed that the hepatic SOD mRNA and activity of heat stressed rats might have return to a normal level at 12 h after cyclic heat stress in this study. In addition, we deprived the rats of feed for 12 h before sacrificing them. In particular, SOD might be significantly influenced by the withdrawal of feed, as demonstrated by the report of Marczuk-Krynicka et al. [25], which shows that starvation decreased the SOD activity in the rat liver.

The level of MDA in the liver of rats exposed to con-
secutive cyclic heat remained constant. It might be difficult to conclude that ROS was fully responsible for the increase of HSP70 mRNA, because the level of MDA in response to cyclic heat was not significant. However, several studies have demonstrated that ROS induced by oxidative stress have been involved in the HSP induction [23, 24]. We also observed that cyclic heat stress increased both HSP70 and GPX mRNA expression, which might be attributed to increased ROS production. Thus, it is assumed that if we measure the MDA level at an earlier time after cyclic heat stress, we might see a significant difference in MDA, since increased hepatic activity of GPX in response to cyclic heat stress may protect the lipid membrane of heat-stressed rats from ROS. Lin et al. [23] reported that the increased ROS during heat exposure had a significant impact on lipid peroxidation at earlier time points but not at other time points, although antioxidant enzymes were constantly upregulated. Although we did not observe a remarkable difference in MDA content and the mRNA and activities of SOD and GST, the mRNA level and activity of GPX were greatly affected by cyclic heat stress, leading to the conclusion that cyclic heat stress significantly disturbed the status of the antioxidant system in rats.

It is well known that vitamin C is an important water-soluble antioxidant that has a beneficial effect on reducing oxidative stress by the reduction of ROS [15]. Studies with chickens have well demonstrated that vitamin C can be applied to alleviating negative effects of high temperature [24, 34]. A study with rats reported that supplementation with vitamin C decreased the hepatic damage in a vitamin E-deficient status, although rats can synthesize vitamin C in the liver [8]. Furthermore, when vitamin C was removed from the diet in rats, tissue ascorbic acid and ascorbic acid-2-sulfate levels were decreased [30]. In the present study, hepatic GPX mRNA and activity were significantly increased with exposure to cyclic heat stress, whereas feeding vitamin C to heat stress-induced rats markedly decreased GPX. Therefore, it is postulated that vitamin C may partially function in the antioxidant defense system under cyclic heat stress to alleviate the level of ROS, which has been suggested as a key factor in inducing HSP family genes. Increased mRNA expressions of HSP70 and proinflammatory cytokines under cyclic heat stress are being counteracted by supplementation with vitamin C, suggesting that oxidative stress caused by cyclic heat stress is responsible for HSP70 and inflammatory cytokine induction in rats.

In conclusion, rats exposed to cyclic heat stressed showed a significant increase in HSP70, TNF-α, and iNOS mRNA expression and GPX activity and mRNA content in the liver compared with the control rats. This study suggest that there is a strong functional association between proinflammatory cytokines and the antioxidant defense system under cyclic heat stress in rats, which is in agreement with other studies of acute heat-stressed chickens. In addition, providing vitamin C to rats exposed to cyclic heat stress might have a potential protective function to attenuate heat stress-induced cellular responses in SD rats.

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