The swine industry in China has gradually adopted an intensive management system that has necessitated the transport of pigs at the age of 70 to 80 days. Transportation is inherently stressful for pigs. Currently, >20% of the total carcasses of pigs that are transported to the United States are composed of pale, soft, and exudative meat. Transportation can pose as a severe stressor for young pigs that manifests as weight loss caused by dehydration and feed withdrawal. Furthermore, stress can result in an increased incidence of diseases in young calves. The serum activity of creatine kinase and concentrations of cortisol, triiodothyronine, and thyroxine are reported to vary in response to stressors in pigs and calves, and these variations are often used to reflect the stress-coping characteristics and metabolic status of animals.

**Effect of transportation stress on heat shock protein 70 concentration and mRNA expression in heart and kidney tissues and serum enzyme activities and hormone concentrations of pigs**

Hong Yu, VMD; En-dong Bao, PhD; Ru-qian Zhao, PhD; Qiong-xia Lv, VMD

**Objective**—To determine the enzymatic and hormonal responses, heat shock protein 70 (Hsp70) production, and Hsp70 mRNA expression in heart and kidney tissues of transport-stressed pigs.

**Animals**—24 pigs (mean weight, 20 ± 1 kg).

**Procedures**—Pigs were randomly placed into groups of 12 each. One group was transported for 2 hours. The other group was kept under normal conditions and used as control pigs. Sera were used to detect triiodothyronine, thyroxine, and cortisol concentrations and alanine aminotransferase, aspartate aminotransferase, and creatine kinase activities. The heart and kidneys of anesthetized pigs were harvested and frozen in liquid nitrogen for quantification of Hsp70 and Hsp70 mRNA.

**Results**—No significant differences were detected in serum alanine aminotransferase activity and triiodothyronine and cortisol concentrations between groups; however, the serum creatine kinase and aspartate aminotransferase activities and thyroxine concentrations were higher in transported pigs. Densitometric readings of western blots revealed that the amount of Hsp70 in heart and kidney tissues was significantly higher in transported pigs, compared with control pigs. Results of fluorescence quantitative real-time PCR assay revealed that the Hsp70 mRNA transcription in heart tissue, but not kidney tissue, was significantly higher in transported pigs, compared with control pigs.

**Conclusions and Clinical Relevance**—Transportation imposed a severe stress on pigs that was manifested as increased serum activities of aspartate aminotransferase and creatine kinase and increased amounts of Hsp70 and Hsp70 mRNA expression in heart and kidney tissues. Changes in serum enzyme activities were related to the tissue damage of transport-stressed pigs. (Am J Vet Res 2007;68:1145–1150)

Heat shock proteins are widely distributed in nature, and they comprise a highly conserved group of proteins that are found in organisms. The stimulation of heat shock proteins following stress is extremely rapid and intense, as might be expected during an emergency response. Generally, the heat shocked or thermotolerant cells have a greater degree of resistance to environmental stress and to cell death. Additionally, evidence exists suggesting that heat shock proteins are involved in the stress caused by transportation in pigs. Stress represents the reaction of a body to stimuli that results in the disturbance of its normal physiologic equilibrium or homeostasis, and such disturbances are often associated with detrimental effects. Therefore, the purpose of the study reported here was to examine the relationship between Hsp70 production, Hsp70

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>FQ RT-PCR</td>
<td>Fluorescence quantitative real-time PCR</td>
</tr>
</tbody>
</table>

Received March 1, 2007. Accepted April 17, 2007.
From the Key Laboratory of Animal Physiology and Biochemistry, Department of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China.
Supported by grants (30430420, 30170682, and 30571400) from the National Science Fund of the People’s Republic of China.
Address correspondence to Dr. Bao.
Materials and Methods

The study protocol was reviewed and approved by an animal care and use committee, and the experiment was undertaken following the guidelines of a regional animal ethics committee.

Animals and experimental design—A total of 24 castrated Erhualian pigs were raised in individual pens (2.5 × 3.0 m²) at the China Boar Research Center (Wuxi City, China) according to the procedures necessary to meet the requirements of pigs. When the mean weight of pigs was approximately 20 ± 1 kg (mean ± SD), they were randomly placed into 2 (transported and control) groups of 12 pigs each. On the day of the transport trial, transport group pigs were individually housed in separate cages at 7:00 am, loaded onto a truck, and set off for a continuous 2-hour journey on a country road at the speed of 30 to 40 km/h. The control group was kept under normal conditions. Following the 2-hour period, all pigs were anesthetized with 3% pentobarbital sodium (10 mg/kg) by injection into the jugular vein while in the truck or animal house. Anesthetized pigs were brought to the dissection room where blood samples were collected by exsanguination and the sera obtained were frozen at −20°C and stored until further analysis. The heart and kidneys of all pigs were removed and frozen in liquid nitrogen to enable detection of Hsp70 and Hsp70 mRNA.

Determination of serum enzyme activities and hormone concentrations—Serum activities of aspartate aminotransferase, alanine aminotransferase, and creatine kinase were analyzed by use of commercial kits and a clinical autoanalyzer. Serum concentrations of cortisol, triiodothyronine, thyroxine, and cortisol were estimated on duplicate samples by use of commercially available radioimmunoassay double-antibody kits according to instructions of the manufacturer. Sensitivities of the assays were as follows: 2.0, 0.18, and 0.42 ng/mL for cortisol, triiodothyronine, thyroxine, and cortisol, respectively. Coefficients of variation within and between the assays were 4.8% and 6.4%, 8.9% and 5.6%, and 8.1% and 8.7%, respectively.

Western blot analysis—Tissue specimens were liquified of homogenization buffer (0.15M NaCl, 20mM Tris HCl [pH, 8.0], 1mM EDTA, 1mM phenylmethanesulfonyl fluoride, 0.1μM E-64, 0.08μM aprotinin, 0.1μM leupeptin, and 0.1% NP-40) by use of a homogenizer (tissue to buffer ratio, 1:10) on ice. Homogenates were then centrifuged at 12,000 × g for 20 minutes to remove the cellular debris. The supernatant was collected. Subsequently, the supernatant was mixed with an equal volume of Laemmli sample buffer, boiled for 5 minutes, and stored at −20°C for further analysis. Proteins were separated by use of 10% SDS-PAGE gels, and a total of 50 μg of proteins was separated. Following electrophoresis, proteins were transferred to a membrane. An equal amount of protein was loaded and confirmed with Ponceau S staining.

Following electrophoresis, membranes were immunoblotted with monoclonal antibodies (specific for human Hsp70 and reactive with swine Hsp70) against Hsp70 and β-actin (diluted 1:1,000 with Tween 20 Tris-buffered saline solution containing 0.5% bovine serum albumin). Subsequently, membranes were reacted with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature (approx 20°C). Blots were developed by use of a detection reagent and exposed to produce photographic images. Optical densities of the immunobands were quantified by use of a software program.

FQ RT-PCR assay—Total cellular RNA was isolated from tissues with Trizol reagent (2 mL/plate) containing phenol and guanidine isothiocyanate. The homogenized sample was treated with chloroform, and the aqueous portion containing the RNA was separated by centrifuging the samples at 12,000 × g for 15 minutes at 2°C to 8°C. The RNA was precipitated from the aqueous portion by mixing the latter with isopropyl alcohol. After centrifugation, the pellet was washed with 75% ethanol and dried. Subsequently, the pellet was dissolved in ribonuclease-free water. Furthermore, the RNA was digested with ribonuclease-free deoxyribonuclease I at 37°C for 15 minutes, and the reaction was completed at 95°C for 5 minutes. The concentration of RNA was determined by use of a spectrophotometer at 260 nm. Serial dilutions of RNA were prepared with ribonuclease-free water.

Primer sets were specifically designed to anneal to each target mRNA. The sequence of the Hsp70 mRNA (GenBank accession No. X68213) as well as that of the GAPDH mRNA housekeeping gene (GenBank accession No. CV874334) were obtained and analyzed by use of a software program, and the primer sequences were sent for synthesizing. The primers were used for the FQ RT-PCR assay as well as for the 1-step FQ RT-PCR assay. The primer sequences were as follows: Hsp70 sense primer 5′–GGCCCTGAATCCGCAAACT–3′, antisense primer 5′–TCGCCACGTAGGAAACG–3′; GAPDH sense primer 5′–GAAGGCTGGAGTGACGG–3′, and antisense primer 5′–CATGGGTAGAATGATCT–3′. Quantification of mRNA expression was performed by use of a PCR assay and a real-time detection system according to instructions of the manufacturer. In vitro reverse transcription of linearized plasmid DNA was used to prepare the standard curve. A 2-μL sample of RNA was added to 23 μL of the reaction mixture containing PCR buffer, ribonuclease inhibitor, SYBR Green I, 25 pmol of downstream and upstream primer, 1mM each of MgCl₂, avian myeloblastosis virus (reverse transcriptase), Taq polymerase, and ribonuclease-free water. The FQ RT-PCR assay conditions were as follows: 45 minutes at 42°C for reverse transcription; 1 minute at 95°C for avian myeloblastosis virus real-time inactivation and RNA-cDNA primer denaturation; 40 cycles for 30 seconds at 94°C, 20 seconds at 56°C, and 30 seconds at 72°C for the PCR; and 8 seconds at 80°C. To verify that only the specific product was amplified, a melting point analysis was performed after the final cycle by cooling the samples to 65°C and then increasing the temperature to 95°C.
at a rate of 0.5°C/s. A single product at a specific melting temperature was obtained for each target. Ethidium bromide–stained PCR products were separated on 1.5% agarose gels, viewed, and quantified by use of a software program.2 Sizes of the amplified products were 132 and 149 bp for Hsp70 and GAPDH, respectively. To compare the amplified products, quantification of the signals was performed with a standard curve. The use of GAPDH provided good control of the bias caused by possible degradation of the mRNA. The threshold cycle is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. We calculated the expression of the target gene relative to the housekeeping gene as the difference between the threshold values of the 2 genes.

Statistical analysis—All analyses were performed by use of a software program.7 Differences in serum protein concentrations, serum enzyme activities, tissue Hsp70 concentration, and tissue expression of Hsp70 mRNA were compared by use of a t test for independent samples between groups. Data were expressed as mean ± SD. Values of P < 0.05 were considered significant.

Results

Circulatory variables—Serum activities and concentrations of enzymes and hormones, respectively, were measured to evaluate stress in pigs during transport (Table 1). Obvious differences were found in serum activities of aspartate aminotransferase and creatine kinase and concentrations of thyroxine. Serum creatine kinase activity and thyroxine concentration in transported pigs were significantly (P = 0.001 and 0.008, respectively) higher than those in control pigs. Serum activity of aspartate aminotransferase in transported pigs significantly (P = 0.048) increased after the 2-hour transportation, compared with before transportation. Although serum activity of alanine aminotransferase and concentrations of triiodothyronine and cortisol in transported pigs were higher than those in control pigs, no significant difference in values was found between treatment groups.

Western blot analysis results—Immunoblotting results revealed that Hsp70, which reacted with the commercial Hsp70 monoclonal antibody (specific for human Hsp70 and reactive with swine Hsp70), was regularly detected in heart and kidney tissues of transported and control pigs. Protein bands of Hsp70 and β-actin were detected in heart and kidney tissues of transported and control pigs. Additionally, densitometric readings revealed that the concentration of Hsp70 in heart tissue was higher significantly higher in transported pigs than in control pigs. Activity of tissue Hsp70 concentration, and tissue expression of Hsp70 mRNA were compared by use of a one-way ANOVA test for independent samples between groups. Data were expressed as mean ± SD. Values of P < 0.05 were considered significant.

Table 1—Mean ± serum enzyme activities and hormone concentrations in pigs.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>CK (U/L)</th>
<th>T3 (ng/mL)</th>
<th>T4 (ng/mL)</th>
<th>Cortisol (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport</td>
<td>19.15 ± 9.94</td>
<td>83.66 ± 30.32</td>
<td>71.55 ± 21.12</td>
<td>0.32 ± 0.21</td>
<td>52.95 ± 10.02</td>
<td>269.08 ± 144.84</td>
</tr>
<tr>
<td>Control</td>
<td>14.39 ± 5.72</td>
<td>65.81 ± 18.22</td>
<td>41.40 ± 11.99</td>
<td>0.24 ± 0.20</td>
<td>43.47 ± 12.10</td>
<td>247.16 ± 116.39</td>
</tr>
</tbody>
</table>

*Within a column significant (P < 0.05) difference between groups. T Within a column significant (P < 0.01) differences between groups.

ALT = Alanine aminotransferase. AST = Aspartate aminotransferase. CK = Creatine kinase. T3 = Triiodothyronine. T4 = Thyroxine.

FQ RT-PCR assay—Amplification curves of Hsp70 RNA (with gradient dilutions of 1:10−2, 1:10−3, 1:10−4, and 1:10−5) were obtained from in vitro transcription of linearized recombination plasmids. Melting point curves (with gradient dilutions of 1:10−2, 1:10−3, 1:10−4, and 1:10−5) indicated that the production is specific; the melting point of the product was consistently 89°C, indicating that there was only 1 product. Amplified products were observed in 1.5% agarose gel electrophoresis.
Muscular activity or damage can result in increased activities of blood creatine kinase. Cytos into the bloodstream in response to physical stress. Serum cortisol concentration increases during loading and transportation in cattle and sheep have shown that cortisol concentrations have been used as reliable indicators of stress. Although substantial increases in serum concentrations of thyroxine are found in pigs in response to stress (indicating that metabolism is increased in response to stress), those of triiodothyronine vary according to the breed of pigs. Further, plasma cortisol concentrations have been used as reliable indicators of short-term physical stress in goats and pigs that are shipped. Studies regarding the effects of loading and transportation in cattle and sheep have shown that serum cortisol concentration increases during loading and initial transportation; however, cortisol concentrations decrease with time in cattle.

Creatine kinase leaks from the sarcoplasm of myocytes into the bloodstream in response to physical stress or exercise. In sheep, this leakage is attributed to increased permeability of muscle cell membrane. Additionally, physical injuries such as muscle fiber tears may result in increased activities of blood creatine kinase. Plasma creatine kinase activity is a good indicator of muscular activity or damage. In the present study, serum aspartate aminotransferase activities were obviously higher in transported pigs than in control pigs. However, no significant differences were found in the activity of alanine aminotransferase between groups. Because of the association between increased serum activities of aspartate aminotransferase and myocardial infarction and hepatitis, serum aspartate aminotransferase activity has been clinically useful in the diagnosis of heart and liver diseases. In Chinese experimental miniature pigs, stress results in an increased expression of aspartate aminotransferase, indicating that activities of certain enzymes in the serum can be used as indicators of stress. Aspartate aminotransferase is widely distributed in the myocardium, liver, and skeletal muscles; however, it is mostly concentrated in the myocardium. Niu et al. showed that stress could increase the activities of creatine kinase, alanine aminotransferase, and aspartate aminotransferase in serum, which indicates that stress results in some tissue damage to the heart and liver. In the present study, significant increases in the serum activities of creatine kinase and aspartate aminotransferase, but not alanine aminotransferase, were found, indicating that transportation resulted in some damage to the heart but not the liver, damage of which may depend on the type and duration of the stressors. Stress intensity caused by transportation was perhaps not severe enough to damage the liver and increase the release of alanine aminotransferase.

In rats, heat shock proteins are known to be expressed in brain cells in response to various stressors such as heat, trauma, and ischemia to protect against stress-induced tissue damage. Of the 4 or 5 genes in this family, one that has attracted the most attention is the gene for Hsp70. Generally, Hsp70 is found in normal and unstressed mammalian cells and can be detected in samples obtained from normal heart tissue of rats, rabbits, and pigs. Mechanisms regulating Hsp70 production have not been studied extensively in pigs. In the present study, vital organs (ie, heart and kidneys) were chosen to reflect the relationship between Hsp70 and tissue damage that may result in a medical emergency. Our immunoblotting results indicated that Hsp70 content in heart and kidney tissues was significantly higher in transported pigs than in control pigs. Khazzaka et al. showed that heat stress increased the concentration of Hsp70 in halothane gene-negative pigs, but not in the halothane gene-positive pigs. Bao et al. observed that Hsp70 content in the liver and skeletal muscles of pigs that were transported for 6 hours was not significantly different from those of the control pigs; however, they were significantly lower in kidney tissues. It remains to be investigated whether the increase in tissue concentrations of Hsp70 indicates damage and overuse and whether Hsp70 actually protects the muscles after transportation. Although most studies have revealed that Hsp70 accumulates to maximal tissue concentrations within 6 hours after heat shock, it is necessary to consider the fact that the cardioprotective effect of Hsp70 is usually detected > 24 hours after Hsp70 in-

(Figure 3). The Hsp70 mRNA and GAPDH mRNA of heart and kidney tissues were individually amplified in transported and control pigs. The initial copy number of each tissue sample was determined; results revealed that the amount of Hsp70 mRNA, as standardized by the amount of GAPDH mRNA, in heart tissues was significantly higher in transported pigs than in control pigs; however, no significant differences were found in values with respect to kidney tissues.

Discussion

When subjected to a stressful situation, metabolic and hormonal changes occur in animals to cope with stressful stimuli and acquire new ways to procure nutrients to ensure its survival. In this study, triiodothyronine and thyroxine were used to evaluate the metabolism of pigs, and aspartate aminotransferase, alanine aminotransferase, creatine kinase, and cortisol were used to assess tissue damage and transport-induced stress. Triiodothyronine and thyroxine are reported to respond in various manners to a variety of stressors in chicken. Although substantial increases in serum concentrations of thyroxine are found in pigs in response to stress (indicating that metabolism is increased in response to stress), those of triiodothyronine vary according to the breed of pigs. Further, plasma cortisol concentrations have been used as reliable indicators of short-term physical stress in goats and pigs that are shipped. Studies regarding the effects of loading and transportation in cattle and sheep have shown that serum cortisol concentration increases during loading and initial transportation; however, cortisol concentrations decrease with time in cattle.

Creatine kinase leaks from the sarcoplasm of myocytes into the bloodstream in response to physical stress or exercise. In sheep, this leakage is attributed to increased permeability of muscle cell membrane. Additionally, physical injuries such as muscle fiber tears may result in increased activities of blood creatine kinase. Plasma creatine kinase activity is a good indicator of muscular activity or damage. In the present study, serum aspartate aminotransferase activities were obviously higher in transported pigs than in control pigs. However, no significant differences were found in the activity of alanine aminotransferase between groups. Because of the association between increased serum activities of aspartate aminotransferase and myocardial infarction and hepatitis, serum aspartate aminotransferase activity has been clinically useful in the diagnosis of heart and liver diseases. In Chinese experimental miniature pigs, stress results in an increased expression of aspartate aminotransferase, indicating that activities of certain enzymes in the serum can be used as indicators of stress. Aspartate aminotransferase is widely distributed in the myocardium, liver, and skeletal muscles; however, it is mostly concentrated in the myocardium. Niu et al. showed that stress could increase the activities of creatine kinase, alanine aminotransferase, and aspartate aminotransferase in serum, which indicates that stress results in some tissue damage to the heart and liver. In the present study, significant increases in the serum activities of creatine kinase and aspartate aminotransferase, but not alanine aminotransferase, were found, indicating that transportation resulted in some damage to the heart but not the liver, damage of which may depend on the type and duration of the stressors. Stress intensity caused by transportation was perhaps not severe enough to damage the liver and increase the release of alanine aminotransferase.

In rats, heat shock proteins are known to be expressed in brain cells in response to various stressors such as heat, trauma, and ischemia to protect against stress-induced tissue damage. Of the 4 or 5 genes in this family, one that has attracted the most attention is the gene for Hsp70. Generally, Hsp70 is found in normal and unstressed mammalian cells and can be detected in samples obtained from normal heart tissue of rats, rabbits, and pigs. Mechanisms regulating Hsp70 production have not been studied extensively in pigs. In the present study, vital organs (ie, heart and kidneys) were chosen to reflect the relationship between Hsp70 and tissue damage that may result in a medical emergency. Our immunoblotting results indicated that Hsp70 content in heart and kidney tissues was significantly higher in transported pigs than in control pigs. Khazzaka et al. showed that heat stress increased the concentration of Hsp70 in halothane gene-negative pigs, but not in the halothane gene-positive pigs. Bao et al. observed that Hsp70 content in the liver and skeletal muscles of pigs that were transported for 6 hours was not significantly different from those of the control pigs; however, they were significantly lower in kidney tissues. It remains to be investigated whether the increase in tissue concentrations of Hsp70 indicates damage and overuse and whether Hsp70 actually protects the muscles after transportation. Although most studies have revealed that Hsp70 accumulates to maximal tissue concentrations within 6 hours after heat shock, it is necessary to consider the fact that the cardioprotective effect of Hsp70 is usually detected > 24 hours after Hsp70 in-

![Figure 3](https://example.com/f3.png) Gel electrophoresis of the FQ RT-PCR products with 1.5% agarose gels. Lane 1 contains the marker (from bottom to top, molecular weights are 100, 250, 500, 750, 1,000, and 2,000 bp). Lanes 2 to 5 contain Hsp70 amplification products. Lanes 6 to 9 contain GAPDH amplification products.
duction. Researchers have not been able to determine the precise mechanism underlying the cardioprotective effect of Hsp70. Concentrations of Hsp70 and Hsp70 mRNA were quantified in heart and kidney tissues of Erthualian pigs in our study, which may help elucidate the mechanism of Hsp70 induction. Although stabilization of the intracellular protein structure may be a possible mechanism, previous data indicate that increased Hsp70 concentrations are closely related to slower rates of depletion of ATP.40 However, the increased Hsp70 concentrations in heart and kidney tissues may also be related to a decrease in the available energy present in these tissues. It is reasonable to speculate that the duration of transportation can affect Hsp70 production.

With increased temperatures (3°C to 5°C above normal temperature), cells respond by rapid gene transcription and subsequent mRNA translation to yield a class of highly conserved proteins known as heat shock proteins.41 In most organisms, stress proteins constitute the predominant products of protein synthesis within the initial 15 minutes following exposure to high temperatures.42 In the present study, concentrations of Hsp70 mRNA in heart tissue, but not kidney tissue, of pigs were significantly higher in transported pigs than in control pigs, which indicated that stress-induced responses varied between different tissues. The induction of Hsp70 mRNA expression in heart tissue of transported pigs corresponds with the transcription of Hsp70 mRNA. It is reasonable to hypothesize that the myocardial cells respond to transport-induced stress by transcription of Hsp70 mRNA and subsequent mRNA translation. In rats, production of Hsp70 was increased shortly after the expression of the mRNA.43 Several studies44,45 have shown that prior induction of transfection or overexpression of Hsp70 proteins yields a beneficial response of protecting the myocardium from ischemia and reducing the infarction size in the heart of rats and rabbits. Heat shock protein production might be exploited from a therapeutic perspective as a means to protect the heart and kidneys from stress-induced insults. Although various methods may be used to induce Hsp70 production, careful attention must be given to how the induction methods affect the animal as a whole.

References