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Variation in Expression of HSPs and Their Corresponding mRNA Transcripts in the Peripheral Blood Lymphocytes of Beef Cattle by Transportation Durations

[1, 2]Li Xin Deng, [1]Gao Feng Yang, [3]Chun Yan Zhang, [1]Zhi Ping Zhang, [1]Li Fan Xu and [1]Cong He
[1]Animal Husbandry and Veterinary Institute, Henan Agricultural University,
450002 Zhengzhou Henan, China
[2]China's Beef Industry Technology Research Center For Disease Control System,
[3]Ministry of Agriculture IPM, Key Laboraton of Crops in Southern North China,
Institute of Plant Protection, Henan Academy of Agricultural Sciences, 450002 Zhengzhou, China

Abstract: The expression of Heat Shock Proteins (HSPs) and corresponding mRNA transcription in Peripheral Blood Lymphocytes (PBL) of transported beef cattle are poorly documented despite potential health implications for consumers. Herein, concentrations of adrenal Cortisol (COR) and Adrenocorticotropic Hormone (ACTH), the expression of HSP70, HSP27, HSP90 and corresponding mRNA transcripts were examined in PBL of transported Xia Nan cattle, the first commercial breed used in China. Six cattle were transported and samples were collected prior to transportation at 1 and 0 h during transportation at 2, 3, 9, 10, 15 and 20 h and after transportation at 10, 20 and 30 h. As revealed by radioimmunoassay, COR and ACTH concentrations increased after 2 and 3 h of transportation, likely due to stress. Various HSPs in PBL had unique expression patterns. ELISA results showed that HSP70 expression was initiated at 2 h of transportation and the level was the highest at 2 h of transportation; however, HSP27 and HSP90 were primarily expressed just at 9 h of transportation. HSP70 mRNA transcripts was induced after 3 and 9 h of transportation. Additionally, a significant increase in HSP27 mRNA after 3 h of transportation was observed while no change in HSP90 expression was observed during transportation.

Key words: HSP mRNA, cattle serum, cattle blood plasma, peripheral blood lymphocytes, cattle stress, transportation stress

INTRODUCTION

The development and prosperity of the global livestock and poultry trades have made transportation a necessary link in the animal husbandry industry, particularly common in cattle. The process of transportation, however, induces stress that can not only reduce livestock immunity and production performance but also may cause severe injury and even animal death, resulting in a major point of economic loss during animal husbandry (Sehtte *et al*., 1994; Warriss and Brown, 1994). In beef cattle breeding, transportation has become a normal part of the commercial process. During long-distance transportation processes, lifestyle alterations cause changes in cattle physiological activity. These poorly documented changes may increase the risk of disease outbreaks such as the respiratory condition commonly known as Black Lung in Beef Cattle (BLBC) or Transit-Stress Syndrome in Beef Cattle (TSSBC) (Han *et al*., 2011).

Numerous earlier studies have shown that Hypothalamic-Pituitary-Adrenal axis (HPA) activity increases during periods of acute stress in many higher organisms (Yang and Li, 1999; Blake *et al*., 1991), promoting the release of Adrenocorticotropic Hormone (ACTH) from the anterior pituitary into peripheral circulation (Blake *et al*., 1991). ACTH promotes the transformation of cholesterol in the adrenal cortex to adrenal Cortisol (COR) and stimulates the adrenal cortex hormones involved in the stress response cycle. COR which is a Glucocorticoid (GC) produced by the adrenal cortex is not limited to stress response and also plays a role in regulation of carbohydrate, fat and protein metabolism as well as the maintenance of many normal tissue and organ functions. In addition, it possesses anti-inflammatory and immunosuppressive capabilities (Zhao *et al*., 2003). Up to now that stress-induced alterations in COR and ACTH patterns cause animal stress during transportation have not been studied yet.

Corresponding Author: Cong He, Animal Husbandry and Veterinary Institute, Henan Agricultural University, 450002 Zhengzhou Henan, China

At the cellular level, it is generally accepted that Heat Shock Proteins (HSPs) play a role in restoration of tissues subjected to various stresses thus protecting organisms from permanent stress-induced damage (Donnelly *et al*., 1992; Currie *et al*., 1993; Arrigo, 2000; Chiu *et al*., 2003; Gauthaman *et al*., 2005). Animals exposed to harmful stimuli usually exhibit rapid gene transcription and subsequent translation to produce a class of highly conserved HSPs (Lee *et al*., 2010; Arrigo, 2000). Based on the molecule mass of proteins, HSPs are classified into six major families: small HSPs, HSP27, HSP60, HSP70, HSP90 and HSP110 (Park *et al*., 2007; Yamagishi *et al*., 2001; Snoeckx *et al*., 2001). HSP70 is thought to be able to maintain cell homeostasis even when other molecular chaperones are involved in various forms of protein metabolism and function simultaneously (Locke and Noble, 1995). HSP70 is a priority-induced protein that occurs in specific regions of the brain and is involved in the regulation of the hypothalamic-pituitary-adrenal axis function. It is speculated that HSPs form complex interactions that make up the mammalian stress response (Blake *et al*., 1991). HSP90 proteins are classified as intracellular agents that participate in signal transduction. Along with HSP70s and other chaperones, HSP90s play an important intracellular regulatory function (Lee *et al*., 1996).

The aim of this study was to examine the expression kinetics of HSP70, HSP27, HSP90 and the corresponding mRNA transcripts of these compounds in the Peripheral Blood Lymphocytes (PBL) of beef cattle during different transportation processes. The relationship between HSPs, COR and ACTH were also examined to provide the link between stress during transportation processes and the regulation of various HSPs.

MATERIALS AND METHODS

Animals: Six female Xia Nan cattle which were part of the first breed introduced into China as approved by the Ministry of Agriculture were randomly selected. The weight of each cattle was 300±2 kg at 9 months of age. A clinical examination of health, feeding and management were conducted in a conventional manner. Cattle were transported by Futian single-layer trucks. Each truck was 9.8 m long, 2.4 m wide, 4.2 m high and possesses a standard canopy enclosure. The route chosen for transportation included a mix of roads, town traffic, state roads and highways. The cattle were transported at an average speed of 60-70 km h^{-1}. Temperature and humidity of the environment were recorded prior to transportation at 1 and 0 h during transportation at 2, 3, 9, 10, 15 and 20 h and after transportation at 10, 20 and 30 h.

The animal care and experimental procedures used in this study conformed to the regulations and guidelines of the regional Animal Ethics Committee and were approved by the Institutional Animal Care and Use Committee of the Henan Agricultural University.

Sample collection and isolation of peripheral blood lymphocytes: Anticoagulant blood (8 mL) and no-anticoagulant blood (1 mL) was collected from each cattle through jugular vein as mentioned above. Plasma and sera were stored at -20°C for ACTH and COR analysis, respectively.

Mononuclear cells were isolated from anticoagulant blood samples using the Ficoll-Hypaque Method. A volume of 3 mL of fresh anticoagulant blood was carefully mixed into Phosphate Buffered Saline (PBS) buffer (NaCl 4 g, KCl 0.1 g, Na_2HPO_4 0.72 g, KH_2PO_4 0.12 g, 500 mL ddH_2O, at pH 7.4) according to a 1:1 ratio. The mixture was transferred onto the surface of a 6 mL Ficoll-Hypaque tube and centrifuged at 3000 rpm for 15 min. An interface rich in mononuclear cells was recovered and washed three times with PBS. A volume of 1 mL of PBL was collected from the bottom of the centrifugal tube using a freezing pipe with 1 mL TRIzol® (Invitrogen, USA) and preserved in liquid nitrogen for HSPs and HSPs mRNA transcript analysis.

Determination of COR and ACTH concentrations: ACTH of plasma and COR of sera were determined by radioimmunoassay. About 100 μL plasma were mixed with ACTH-antisera in a polystyrene tube and then ^{125}I-ACTH was added. About 50 μL sera were tested for determination of COR. Radioactive intensity was determined by SN-695 intelligent RIA γ measurement (made in China). A standard curve was constructed. Concentrations of COR and ACTH was calculated by standard curve according to the combination rate.

Semi-quantitative detection of HSP70, HSP27 and HSP90: HSP70, HSP27 and HSP90 in PBL of transported cattle were detected by Enzyme-Linked Immunosorbent Assay (ELISA). The levels of HSP70, HSP27 and HSP90 were measured using commercially available ELISA kits (Calvin Biotechnology Ltd. China). Quantification of samples was performed using a standard curve. The β-actin (Calvin Biotechnology Ltd. China) was used to mediate bias caused by the protein extraction procedure. The assay was performed according to the protocol provided by the manufacturer and the sensitivity increased to 0.01 ng mL^{-1}. The quantity of HSP70, HSP27 and HSP90 in each sample was normalized using the following equation:

$$\text{Relative quantity of HSPs} = \frac{\text{Quantity of HSPs}}{\text{Quantity of } \beta\text{-actin}}$$

Real time PCR detection of HSP70, HSP27 and HSP90

Isolation of total RNA and RT-PCR: Total RNA was isolated from PBL using TRIzol® reagent (Invitrogen, USA) according to the instructions. The RNA was precipitated from the aqueous portion by mixing with isopropyl alcohol. After centrifugation, the pellet was washed with 75% ethanol and dried. Subsequently, the pellet was dissolved in ribonucleases-free water. Furthermore, the RNA was digested with ribonuclease-free deoxyribonuclease at 37°C for 15 min and the reaction was completed at 95°C for 5 min. The concentration of RNA was determined by spectrophotometry at 260 nm. RNA was serially diluted with ribonuclease-free water. cDNA was synthesized using the TRANScript MMLV kit (Qiagen, Germany) and then stored at -20°C until use.

Design of primers: Nucleotide sequences of HSP70, HSP27, HSP90 and β-actin were obtained from the National Center for Biotechnology Information (NCBI) database. The GenBank accession numbers of HSP70, HSP27, HSP90 and β-actin were AY662497.1, AB605262.1, AB072368.1 and BT030480.1, respectively. The primers HSP70, HSP27, HSP90 and β-actin were designed using primer premier 5.0 Software and summarized in Table 1. Primers were optimized for an annealing temperature of 60°C.

Fluorescence quantitative real time PCR: HSP70, HSP27 and HSP90 mRNA transcripts in PBL of transported cattle were detected by fluorescence quantitative real time PCR (FQ•RT-PCR). The 2 μL each cDNA sample diluted 25 times was suspended into 2×SYBR Premix Ex Taq™ (TaKaRa, Japan) with primer (0.2 μL) and double-distilled water was added to a total volume of 25 μL. Quantitative PCR was performed using an MX 3000 P real time PCR thermocycler (Stratagene). The thermal profile was 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 45 sec. A negative control tube without DNA was run together with the experimental samples. The amplification efficiencies of the target and reference were approximately equal. Therefore, HSPs mRNA of all lymphocyte samples could be normalized using the following equation:

Table 1: Specific primer pairs used in this study

Target gene	Primer name	Primer sequence (5'-3')	Expected product (bp)
HSP70	P1	5'-GACCTCAACAAGAGCATCAACC-3'	229
	P2	5'-TGTCCGAGTAGGTGGTGAAGAT-3	
HSP27	P3	5'-TACATTTCCCGTTGCTTCACT-3'	238
	P4	5'-GCTGGGCTAAGGGTCTTTACT-3'	
HSP90	P5	5'-GCGGAGAATAAAGGAGATTGTG-3'	179
	P6	5'-CTTCTATCTCGGGCTTGTCATC-3'	
β-actin	P7	5'-GATGTGGATCAGCAAGCA-3'	232
	P8	5'-CCTTCACCGTTCCAGTTT-3'	

$$\text{Relative quantity of HSPs mRNA} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = (Ct_{\text{HSPs mRNA}} - Ct_{\beta\text{-actin}})\ \text{control group} - (Ct_{\text{HSPs mRNA}} - Ct_{\beta\text{-actin}})\ \text{test group}$$

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

RESULTS

Environmental temperature, humidity and temperature humidity index: Temperature and humidity of the environment were recorded at different transportation times. Td and Tw were determined in a dry or wet bulb temperature readings (°C), respectively. The Temperature Humidity Index (THI) was calculated by a equation as follows:

$$THI = 0.72\ (Td+Tw)+40.6$$

According to the THI value judgment, heat stress states for test cattle were measured to be in ranges of the normal and safe state (THI<74), state of alert (THI 75-78), dangerous state (THI 79-83) and state of emergency (THI>84). Temperature and humidity showed in Table 2 indicted that the THI values were <74 at different transportation times of test cattle.

Concentration of serum COR and plasma ACTH: The results of COR and ACTH determination were shown in Table 3. COR levels are low prior to transportation and significantly increased ($p<0.01$) at 10, 15 and 20 h of transportation. The values were observed to return to levels almost identical to those observed before transportation directly after transportation. Plasma ACTH from experimental cattle was also low prior to transportation, though it significantly increased ($p<0.01$) at 3 h of transportation. This trend continued to 20 h ($p<0.01$) after transportation then virtually returned to normal values by 30 h after transportation.

Expression levels of HSP70, HSP27, HSP90 and corresponding mRNA transcripts: HSP70, HSP27 and HSP90 protein expression levels as well as their corresponding mRNA transcriptions, normalized to β-actin in PBL of experimental cattle transported for different lengths of time were shown in Fig. 1-3.

Table 2: The values of the average temperature, humidity and THI of the test cattle

	Before transportation				Transportation				After transportation		
Time	1 h	0 h	2 h	3 h	9 h	10 h	15 h	20 h	10 h	20 h	30 h
Index											
Td (°C)	19.04	18.96	18.90	18.02	18.42	18.89	20.95	19.11	19.03	19.06	19.21
Tw (°C)	21.35	22.64	25.25	23.28	23.64	24.14	23.55	22.47	23.99	23.15	23.68
THI	69.68	70.55	72.40	70.34	70.88	71.58	72.64	70.54	71.57	70.99	71.48

Table 3: The effect of transport stress treatment on serum COR and ACTH concentrations of Xia Nan Cattle (M±SD, n = 6)

	Before transportation				Transportation				After transportation		
Time	1 h	0 h	2 h	3 h	9 h	10 h	15 h	20 h	10 h	20 h	30 h
Index											
COR ($nmolL^{-1}$)	70.83±3.82	71.27±8.49	86.83±15.59	93.08±9.49	94.86±13.05	159.60±10.29**	127.18±18.80**	147.54±25.09**	98.90±4.27	84.20±13.38	80.00±5.31
ACTH (nmol L^{-1})	3.16±0.87	3.19±0.88	3.47±0.690	11.33±2.62**	3.16±0.370	8.97±1.870*	15.06±1.820**	10.38±1.160**	10.02±2.38**	9.45±2.120**	4.85±0.84

*p<0.05, **p<0.01, compared with 1 h before transportation

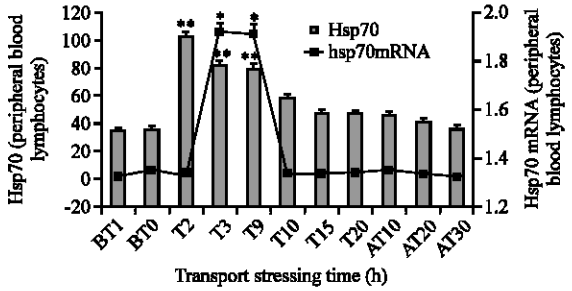

Fig. 1: Levels of HSP70 and HSP70 mRNA in PBL of transported cattle (n = 6). Values indicated are mean±SD. *p<0.05, **p<0.01 compared with BT 1 h; BT: Before Transportation, T: Transportation, AT: After Transportation

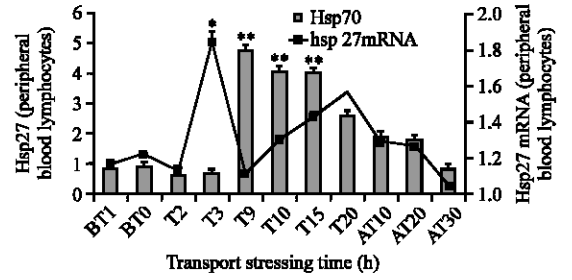

Fig. 2: Levels of sample1 HSP27 and HSP27 mRNA in PBL of transported cattle (n = 6). Values indicated are mean±SD. *p<0.05, **p<0.01 compared with BT 1 h; BT: Before Transportation, T: Transportation, AT: After Transportation

The level of HSP70 in PBL of the transported cattle significantly increased (p<0.01) at 2, 3 and 9 h transportation durations. Subsequent expression was restored to the levels observed prior to transportation. Transcription levels of HSP70 mRNA were similar to HSP70, showing a significant rise (p<0.05) at 3 and 9 h transportation durations (Fig. 1).

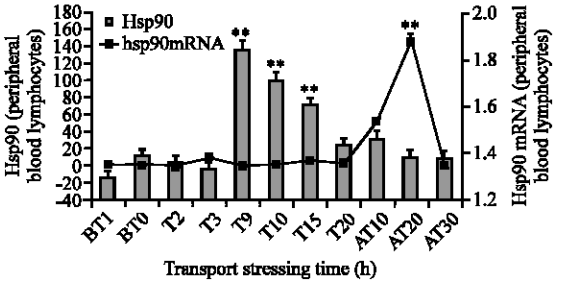

Fig. 3: Levels of sample1 HSP90 and HSP90 mRNA in PBL of transported cattle (n = 6). Values indicated are mean±SD. *p<0.05, **p<0.01 compared with BT 1 h; BT: Before Transportation, T: Transportation, AT: After Transportation

Expression level of HSP27 in PBL of the transported cattle displayed a similar tendency to that observed in HSP70. The expression of HSP27 significantly increased (p<0.01) at the 9 h transportation duration, thereafter demonstrating a gradual decline until restoration of levels equivalent to those observer prior to transportation. Transcription levels of HSP27 mRNA significantly increased (p<0.05) at the 3 h transportation duration. Transcription levels of HSP27 mRNA generally decreased as levels of HSP27 increased (Fig. 2).

The expression of HSP90 in PBL of transported cattle significantly increased (p<0.01) at the 9 h transportation duration. Transcription levels of HSP90 mRNA were elevated only at 10 h after transportation and reached a maximum value at 20 h after transportation before returning to normal pre-transportation levels (Fig. 3).

DISCUSSION

Transportation is thought to be among the most significant stress factors for cattle, leading to decreased immunity responsible for respiratory tract, gastrointestinal

tract and even systemic pathological reactions (Xin *et al*., 2008; Shi *et al*., 2008; Caswell and Archambault, 2007). The high morbidity and mortality of cattle in the transportation process has been commonly reported and this causes significant economic losses (Yang *et al*., 2011). In this study, the Xia Nan cattle breed during different transportation durations was used as a test model in order to correlate stress and levels of COR, ACTH, HSPs and HSPs mRNA transcription.

HSPs protect cells from injury, encourage repair and promote heat tolerance, serving as protective agents for the life activities of cells in stress conditions. Furthermore, HSPs enhance stress resistance and accelerate cell or organism recovery rates subsequent to various stress exposures (Xiao *et al*., 2009). The HSP70, HSP27 and HSP90 of cattle exhibit different expression patterns in the peripheral blood lymphocytes during transportation. Results in this study showed that HSP70 expression was induced after 3 and 9 h of transportation and a significant induction of HSP27 expression occured after 3 h of transportation.

Transcription levels of HSP27 and HSP90 mRNA were similar to those observed in HSP70, however but there was a decreasing trend of HSP70 as HSP27 and HSP90 increase. These results revealed that different HSPs may differ in their role in protection of cells in transported cattle responding to transportation-induced stress. HSP70 expression may affect HSP27 and HSP90 expression. In fact, these results in this study suggest that they be likely to coexist by forming complex interaction pathways thus providing the basis for the study of protein interactions in cattle during stress exposure. The experimental results showed that the HSP70 and HSP27 were superior stress indicators and might be preferentially selected over HSP90 in future studies.

Norepinephrine medulla animals can cope with stress without serious physiological consequences. The stress response pathway in these organisms has been previously determined to be associated with ACTH and glucocorticoid action (Wang, 1994). These results in this study showed that both ACTH and COR were significantly involved in the stress response of cattle, directly causing physiological changes in the blood and biochemical index. From the effect of hormones relative to the transportation before 1 h, at 10 h and at 15 h, COR concentrations were observed to increase by about 2 and 1.8 times, respectively. Furthermore, the concentration of ACTH increased by about 2.8 and 4.8 times, respectively. In the transportation recovery period at 20 h after stress, COR and ACTH concentrations were restored 1.2 and 3 times, respectively. At any given point, the effect of transport stress on the changes in ACTH level was more dramatic than that observed on COR levels. These findings indicate that ACTH is expected to become one of the primary indicators of cattle transportation applicable to commercial breeding in the future.

That the complex relationship among COR, ACTH and HSPs has not been completely clarified is needed for further investigation. That the expression of HSP70 and HPA axis functional connections restrained stress after 3-6 h in rat models was confirmed by Blake *et al*. (1991). The rat adrenal cortex and thoracic aorta demonstrated visible HSP70 expression. Resection of the pituitary gland of rats showed no *HSP70* gene expression after removal of the restraint stress. After administration of ACTH, however, HSP70 expression was induced, indicating its involvement with the stress reaction. Similarly, Vijayan *et al*. (1997) reported that COR levels increased without effect on hepatic HSP70 expression. These results in the current study showed that increases of COR and ACTH concentration are associated with stress during cattle transportation as found in other mammalian models. The levels of HSP70 in PBL of transported cattle were persistently increased during transportation at 3 h but the expression of HSP27 and HSP90 reached a maximum at much longer transportation durations.

CONCLUSION

The current findings suggest that the expression of HSP70 be positively correlated with COR and ACTH content. The level of HSP70 expression, however was between the expression level observed in HSP27 and HSP90. The mechanism of the relationship between COR and ACTH levels is an important topic to be addressed in future research studies. COR, ACTH and HSPs can be used as an objective index for reflecting the stress levels of cattle during transportation, potentially providing a powerful tool to reduce death, injury and pathogenesis of cattle thus reducing economic losses dramatically. This study provides a basis for the determination of stress conditions based on related indexes and the relationship between these indices.

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