Journal of Animal and Veterinary Advances 11 (21): 3914-3920, 2012
ISSN: 1680-5593

Effects of Cold Stress on Expression of *Hsp70* Gene in Blood Lymphocyte of Piglets

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Abstract: To explore effects of intensity and time of cold stress on expression of *Hsp70* gene in blood Lymphocyte of piglets, Western-blotting and real-time PCR were performed to detect expression level of *HSP70* gene, respectively. The results showed as followed: at −10 to −4°C, HSP70 expression increased significantly for 0.5 h and subsequently decreased slightly before increasing markedly again at both 6 and 24 h; HSP70 mRNA expression level increased sharply after piglets were exposed for 0.5 h. The condition maintained for 6 h then declined at 12 h it reached a level apparently higher than the level prior to cold exposure at 24 h the transcription level recovered to normal. At −3 to 3°C the expression level increased at 6 h but then returned to pre-exposure levels; mRNA increased significantly at 12 h. At 4-10°C, no marked difference in expression level was detected. In conclusion, HSP70 mRNA expression showed the faster regular pattern therefore, it can be used as an objective parameter to evaluate animal cold stress conditions.

Key words: Piglet, cold stress, HSP70, HSP70 mRNA, transcription, animal

INTRODUCTION

Cold stress occurs frequently to animals particularly young animals which results in tremendous loss to animal industry. It has become a major research area to explore parameters that can be used to evaluate cold stress level. Molecular biological indicator can provide information on biological reactions to the environment which has unique advantages to accurately and sensitively evaluate the early reaction of animal to environmental cold stress. Combination of technique for determining biological cold stress indicator and research on cold stress mechanism provides us with a new research direction in animal cold stress.

HSP70 of the HSP families is present in low levels in normal cells and its sequence is highly conservative; the most conservative region being that at 44 ku at the N tip. HSP70 levels rise markedly under some stressful conditions and consequently, it can serve as a biomarker of stress reactivity and the severity of danger experienced. As a result, HSP70 has become the focal HSP of interest. It has been confirmed that HSP is produced in the cells of various tissues at different levels during exposure to stressors such as hyperthermia, hypothermia, oxidizing agents, heavy metals, tumors and even psychological stress. High HSP70 expression levels are also present in eggs hatched under conditions of heat or cold stress, whereby the quantity varies according to the duration of exposure and the tissue sampled. Stress causes denaturation of nucleoproteins and the exposure and interaction of hydrophobic regions resulting in aggregation. At this time, HSP70 moves swiftly from the cytoplasm to the nucleus to combine with hydrophobic regions so as to restrict the extent of this aggregation and unfold those proteins which have already aggregated. Through this process, HSP70 allows a return to a normal protein conformation and exerts a protective role. Collectively, HSPs are considered to be one of the most primitive cellular mechanisms that allow the effects of deleterious stimuli to be mitigated.

It has proven that many stress factors including cold temperature can induce significant increase in HSP70 synthesis. Change in quantity of HSP70 is related to *HPS70* gene expression level under stress condition (Shyu *et al*., 2000) and it is also related to steady increase of HSP70 mRNA (Burdon, 1993).

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The mRNA measurement is a basic technology used to study physiological and pathological reactions at gene level. Real-time Fluorescence Quantitative Polymerase Chain Reaction (RFQ-PCR) is a mRNA quantification technology established on the basis of PCR technology and it has many advantages such as simple and easy for laboratory operation, direct result, high sensitivity, specificity and repeatability. It has become an important technique in gene determination and quantification (Yu and Jiang, 2003).

A series of collaborated research were conducted on stress molecular biological indicator with researchers at University of Alberta (Yang and Christopherson, 2004). Stresses of cold temperature, transportation and feed were applied to beef cattle, the quantity of HSP90, HSP70 and related mRNA expression in skeleton muscle, lymphocyte and rumen mucosa buds were determined with RT-PCR technique. Results showed that HSP70 in skeleton muscle increased significantly under cold stress; HSP70 mRNA, HSP90, HSP70 and HSC70 in lymphocyte increased significantly under transportation stress; the expression of HSP90, HSP70 and HSC70 in peripheral blood lymphocyte also increased under stress by concentrate feed.

In order to further reveal the general pattern of HSP70 mRNA expression in mammal animals under cold stress. The study was performed in 2005 to investigate the expression of lymphocyte HSP70 and its mRNA in Wistar rat under cold stress. Results demonstrated that under normal temperature, blood lymphocyte HSP70 and its mRNA showed basal quantity expression and transcription, however cold stress induced increase in the quantity of HSP70 expression and HSP70 mRNA transcription (Li *et al*., 2006a, b).

Researchers carried the research out with Junmu No.1 weanling pigs, quantitatively measured lymphocyte HSP70 mRNA expression under different cold stress intensity and different time in cold stress and analyzed the dynamic expression pattern of HSP70 mRNA with Real-time Fluorescence Quantitative PCR technology (RFQ-PCR). This study generated laboratory data for further study on reaction mechanism of animal to cold stress and on evaluation of HSP70 mRNA as molecular indicator for cold stress reaction.

Although, many experiments have demonstrated the existence of cold stress induced intracellular HSP70 expression, the effect of the severity of cold stress has not been reported. In the present study, HSP70 and HSP70 mRNA from Junmul piglets exposed to different levels of cold stress for various time periods were quantified by Western blot analysis to examine the dynamics of the HSP70 response. This allowed an exploration of the reaction mechanism and an assessment of the value of HSP70 or HSP70 mRNA as a molecular biomarker of the response to cold stress.

MATERIALS AND METHODS

Animals and procedures: About 30 Junmu No. 1 weanling pigs of 55 days old were equally allocated into 3 groups; each group was used as self control group. They were exposed to −10, −4, −3 −3, 4 and −10°C temperatures, respectively and fed normally during the test period. Blood samples were taken at 0, 0.5, 3, 6, 12 and 24 h of cold exposure. Cold exposure stopped at 24 h then animals were reared under normal feeding condition at 15 to −18°C temperature. Blood samples were taken again at 0.5, 3, 6, 12 and 24 h after cold exposure was terminated. Blood samples were taken from cephalic vein, 10 mL per sample per pig mixed with heparin, separated for lymphocyte, half of each sample separately and half of each sample added with 1 mL Trizol were kept in -84°C freezer for further analysis.

HSP70 procedures: Lymphocytes were separated by density gradient centrifugation and crushed by ultrasonic waves whilst bathed in ice. The protein concentration was assessed by the Braford Method, a standard curve was drawn and the sample solutions were calculated (total protein 200 μg). The separating gel concentration of SDS-PAGE was 10% with a spacer gel of 5%. The prepared samples and diluted marker solution were boiled for 5 min at 100°C to denature the proteins. The samples were then centrifuged (10000 RPM) and placed on ice before electrophoresis.

Nitrocellulose (NC) and WATMAN 3 mm filters were inserted into the buffer for 30 min before electrophoresis was completed. The gel and both filters were then placed into the electrophoresis tank to allow the transfer of protein from the samples to the NC filter (150 mA, 3 h at room temperature). Subsequently, the filter was removed and dried for 2-3 min using ponceau S to reveal the protein band before being washed with deionized water at room temperature placed in a Petri dish containing PBS and laid on the shaker to rinse (15 min, 3 repetitions) until the protein band disappeared. Following this process, the filter was put in 10% nonfat milk powder solution prepared with TBST, shaken for 3 h to seal it and rinsed out by TBST (15 min, 3 repetitions). The filter was then placed into a monoclonal antibody solution diluted by 2% nonfat milk powder solution, shaken for 3 h and again rinsed by TBST. Lastly, it was incubated with IG marked by horseradish peroxidase (1:5000) for 1 h, rinsed by TBST and developed by DAB.

HSP70 mRNA procedures: Promoters were designed according to conserved region gene sequence of HSP70 in rat, cattle and Sus scrofa. Up-primer sequence: gCCAAgCtggAC AAggC down-primer sequence: ttgAAgAAgtCCtgCAg. Total lymphocyte RNA preparation containing the transcripts of interest was prepared with Trizolo test kit, reverse transcribed with AMV reverse tanscriptase and followed by PCR reaction. The PCR reaction conditions included: pre-denaturation at 94°C for 5 min, 94°C for 30 sec, 51°C for 30 sec, 72°C for 30 sec, 40 cycles and elongation at 72°C for 2 min. Recovered DNA was linked with PMD18-T carrier, transferred to DH5α reception cell, DNA strand was extracted with Green DNA strand kit, PCR identification and enzymatic cleavage showed resulting sequence as follows: GCCAAGCTGGACAAGGCCCAGATCCACG ACCTGGTGCTGGTGGGGGGCTCGACGCGCATCCC CAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAA.

Fluorescence probe was designed according to the sequence of elongated transcript, the probe sequence: AgAtCCACgACCtggtgCtg (provided by Invitrogen). Fluorescence labeled 5' end fragment is FAM and Fluorescence labeled 3' end restriction fragment is TAMAR.

About 10 μL of identified bacterial sample was taken from the -84°C freezer and added to 5 mL LB culture media incubated at 37°C 200 RPM overnight. DNA strand was extracted with Green DNA plasmid kit. Molecular mass was converted into copy numbers based on molecular weight. After step dilution it was transferred to control quantification plate. About 1010 copies of DNA strand was diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 μL^{-1} used as standard control plate for quantitative florescence PCR optimization, standard curve establishment and unknown sample determination.

Statistical analysis: Both the empty band and protein band in the filter were differentially scanned at 580 nm with a 5.0×0.4 mm beam area. The intensity and significance of the peak area were assessed by the statistical analytical system to determine the relative HSP70 level in each band. Analyses were performed using t-tests.

The data obtained from quantitative fluorescence PCR analysis of the regulation of different cold stress intensity on blood lymphocyte HSP70 mRNA expression and the correlation were subjected to Analysis of Variance (ANOVA) of the SAS Software. Differences between control (without treatment) and cold stress treated groups were tested by Scheffe test. Differences were considered significant at $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

The standard curve of protein quantity: The standard curve was shown in Fig. 1 where the x-axis is protein concentration (μg/mL) and the y-axis is absorbance. The regression equation is $Y = 0.0733X + 0.0221$ with R^2 value being more than 0.99.

Results of HSP70 transcription: Western Blotting results were showed in Fig. 2. The interest protein is 70 kD. Different expression was shown in the Fig. 2 and the level of HSP70 expression in detail was shown in Table 1.

Results of HSP70 expression: The effect of different temperature and the duration of exposure on HSP70

Table 1: HSP70 expression in lymphocytes during cold stress (peak area, ±SD)

Time (h)	−10 to −4°C	−3-3°C	4-10°C
Cold exposure			
0	1,451.4±789.70	2,215.7±1,667.0	3,230.8±1,771.8
0.5	4,423.4±2,261.3*	2,519.2±1,045.4	2,721.0±1,407.6
3	2,765.0±1,747.4	3,407.4±576.600	4,188.4±2,277.3
6	3,366.0±2,560.6	4,342.9±1,432.7*	4,566.0±2,403.2
12	3,137.0±826.900*	2,794.2±1,199.7	3,926.5±770.700
24	2,983.0±386.700**	3,321.6±504.300	3,192.9±1,236.1
After exposure			
0.5	3,025.7±2,077.7	2,831.3±848.500	4,157.7±1,275.2
3	2,453.9±1,326.0	2,087.1±787.200	2,352.9±383.100
6	4,704.3±2,428.7*	3,673.8±1,572.0	2,594.0±1,526.0
12	6,045.6±803.200**	5,117.1±1,520.0*	-
24	9,319.7±5,703.7**	2,418.7±168.924	-

Comparison with 0 h cold exposure. *p<0.05,**p<0.01

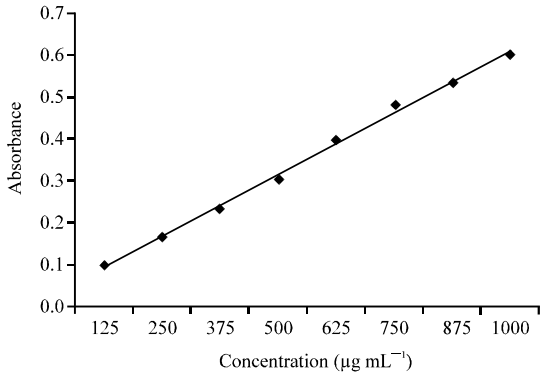

Fig. 1: The standard curve of BSA

Fig. 2: Results of Western blotting used to detect HSP70 expression levels in piglets

expression in piglet lymphocytes were shown in Table 1. At temperatures of −10 to −4°C, HSP70 expression rapidly increased to be three times greater at 0.5 h after the onset of cold stress ($p<0.05$) than the basal level. Subsequently, it decreased a little but remained high for the whole 24 h of exposure ($p<0.01$).

Especially, the expression level was significantly greater at 12 h ($p<0.05$) and 24 h ($p<0.01$) during the exposure and again higher at 6 h ($p<0.05$), 12 h ($p<0.01$) and 24 h ($p<0.01$) respectively after the cold exposure interruption than pre-stress levels. At −3 to 3°C, the expression level just began to rise significantly at 6 h after the beginning of exposure ($p<0.05$) and was followed by another rise at 12 h of the recovery period ($p< 0.05$). In contrast, the expression level remained largely unchanged at 4-10°C. From these results, it can be inferred that in a swifter and more prolonged intracellular HSP70 exposure to very cold temperatures (−10 to −4°C) results expression response. Piglets were moved into a warmer environment of 15-18°C after the exposure which may have an additional stress on top of the former one and induced a further increase in expression. The second elevation in expression, however occurred slower after the temperature change. Nevertheless, the expression began earlier and maintained for a longer duration when returned to a normal temperature from −10 to −4°C than from −3 to 3°C.

HSP homology comparison: Sequence icon showed that gene of recombined DNA strands validated the sequence of the designed fragments (Fig. 3). Multi-sequence comparison with DNA star showed the homology of *HSP70* gene sequence of piglets and wild pigs, cattle and rat are 86.3, 94.7 and 97.9%, respectively in Fig. 4.

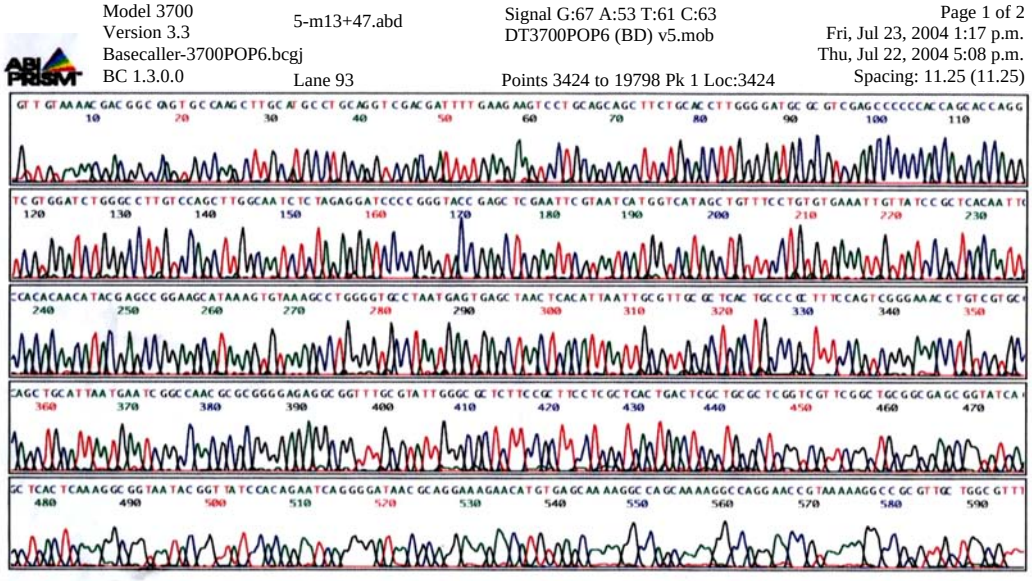

Fig. 3: *HSP70* gene sequence

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G C C A A G C T G G A C A A G G C C C A G A T C C A C G A C C T G G T G C T G G T G G G G G G C  piglet.SEQ
. . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . C . . . . . C . . . . .  Bovine.SEQ
. . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . C . . .  rat.SEQ
. . . . . . . . . . . . . . . . . . T . . . . . . . . T . . T A . T . . C . . A . . . . . T . . .  Sus scrofa.SEQ

T C G A C G C G C A T C C C C A A G G T G C A G A A G C T G C T G C A G G A C T T C T T C A A  piglet.SEQ
. . C . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  Bovine.SEQ
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  rat.SEQ
. . T . . C . . . . . . . . . . . . A . A . . . . . . . . T . . . . . . . . . . . . . . . . .  Sus scrofa.SEQ

Decoration 'Decoration #1': Hide (as '.') residues that match piglet.SEQ  exactly.
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Fig. 4: The analysis of homology in *HSP70* gene of pig, rat, bovine and *sus scrofa*

Standard curve and RFQ-PCR plot: Standard curve is shown in Fig. 5. Results of HSP70 mRNA amplification plot determined by quantitative florescence PCR are shown in Fig. 6. The standard curve page displayed the standard least-squares fit straight line. Standards are plotted as square symbols. The x-axis is the Log of the Starting Copy Number (Log CO) and the y-axis is the Threshold Cycle (CT). The slope, y-intercept and least squares fit correlation coefficient were displayed ($R^2 = 0.99$). The amplification plot displayed a visual graph of the sample amplification for all samples selected in the plate matrix (Fig. 6) where the x-axis is the cycle number and the y-axis is the copy number.

Results of HSP70 mRNA transcription: The transcribed copy number of lymphocyte HSP70 mRNA at different cold exposure time and intensity were shown in Table 2. The results showed that lymphocyte HSP70 mRNA transcription level increased sharply after piglets were exposed to −10 to −4°C temperature for 0.5 h ($p<0.05$). The increased level maintained for 6 h ($p<0.05$) then started to decline. Transcription level increased gradually after cold exposure was terminated then it reached a level apparently higher than the level pre-cold exposure at 12 h ($p<0.05$) and the transcription level recovered to normal at 24 h. When animals were exposed to −3 to 3°C, there was no significant change in lymphocyte HSP70 mRNA transcription in the first 6 h however, it increased significantly at 12 h ($p<0.01$) reached to 15.6 times the level prior to cold exposure and then continued to maintain in a high level to 24 h of the exposure ($p<0.05$). The level again increased sharply ($p<0.01$) reached a level 27.8 times higher at 0.5 h of the recovery period compared with the level prior to cold exposure and maintained the level to 3 h of the recovery period ($p<0.01$) before declining. When animals were exposed at 4-10°C, there was no significant change in lymphocyte HSP70 mRNA transcription in cold exposure and recovery period.

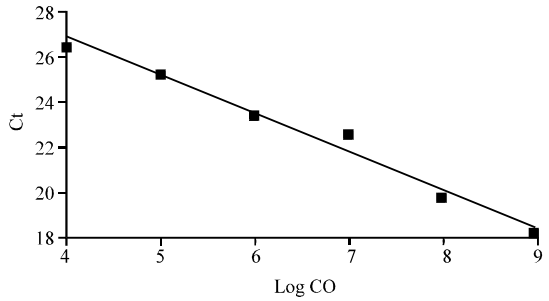

Fig. 5: The standard curve of RFQ-PCR

The correlation of HSP70 expression with HSP70 mRNA transcription in lymphocyte: At temperatures of −10 to −4°C, HSP70 expression with HSP70 mRNA transcription appeared similar trend (Fig. 7) and the point of HSP70

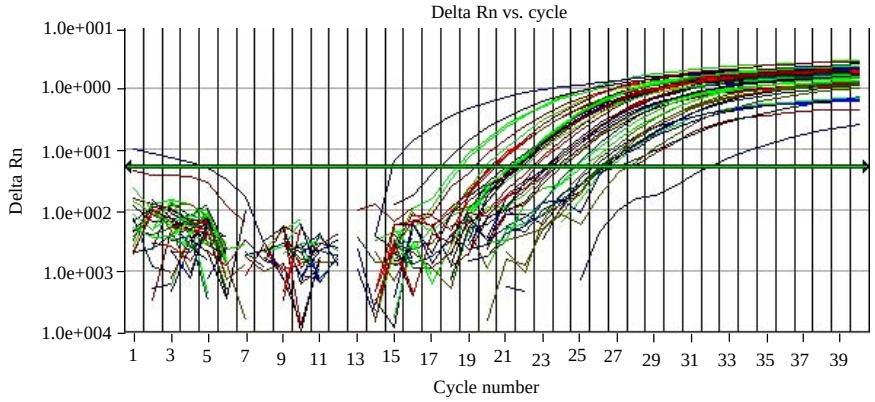

Fig. 6: HSP70 mRNA amplification plot

Table 2: Transcribed copy number of lymphocyte HSP70 mRNA at different cold exposure time and intensity ($\bar{X} \pm SD$)

Time (h)	−10 to -4°C	−3-3°C	4-10°C
Time of cold exposure 0	803.104±466.902	201.313±140.893	3,783.872±3,498.342
0.5	2,134.772±1,504.665*	214.092±87.504	3,092.439±2,836.341
3	3,063.287±2,652.354*	264.823±221.412	9,292.500±6,270.041
6	3,388.756±2,912.775*	127.869±10.289	3,680.000±1,808.010
12	2,700.202±3,151.377	3,146.666±209.605**	8,926.666±7,116.103
24	589.635±451.931	1,177.881±554.539*	6,038.279±8,289.724
Time after cold exposure 0.5	218.406±94.167	5,573.333±2,886.820**	5,136.666±2,597.813
3	936.565±359.054	1,476.961±702.887**	3,010.000±2,771.858
6	1,521.417±1,491.395	77.861±51.994	2,545.000±1,491.995
12	533.333±4,907.640*	307.430±128.828	-
24	869.885±367.857	-	3,783.872±3 ,498.342

Compared with 0 h cold exposure. *$p<0.05$,**$p<0.01$

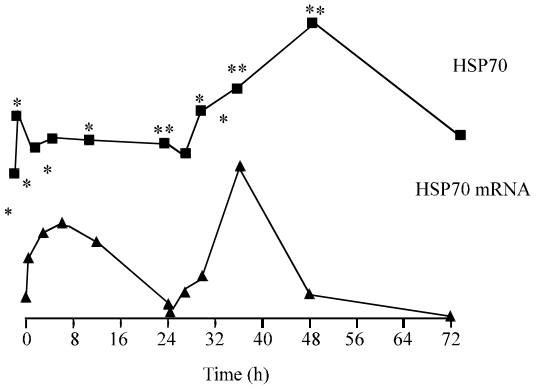

Fig. 7: The compared HSP70 expression with HSP70 mRNA transcription in lymphocyte

mRNA transcription was earlier than HSP70 expression. The correlation was analyzed at every time in the whole process. The correlation at 0 and 0.5 h of cold exposure and 24 h of the recovery period were positive strong (R^2 = 0.99735, 0.70961 and 0.94775). The correlation at 3 h of the recovery period was middle-degree positive strong (R^2 = 0.68822) but at 3, 6, 12 and 24 h of cold exposure and at 0.5, 6 and 12 h and after the recovery period were weak (R^2 = 0.3805, -0.25909, -0.35517, 0.13853, -0.36461, 0.21458 and -0.2354). In addition, the significance of the correlation were analyzed. The results showed that the correlation at 0 and 0.5 of cold exposure and 24 h of the recovery period have obvious significance ($p<0.05$) but other 8 correlation of were no obvious significance.

In this study, 50 days old Junmul pigs with identical genetic traits were selected to explore the level of *HSP70* gene expression. Previous HSP70 researches usually sampled using a single time point before and after stress and the stress origin fixed. HSP70 was not high-level expressed constantly after the stress. If the detection was conducted at a single time, it was easy to omit a meaningful time point or the test results could not reflect the true state of HSP70 expression. This study designed three levels of cold stress as well as a number of time points to detect the expression of HSP70 under cold exposure and during the recovery process. HSP70 expression could be determined dynamically from two way of different cold environments temperature and time, better reflecting the relationship of time-effect and revealing the regularity of changes in *HSP70* gene expression under cold stress.

The HSP70 results showed that more severely low temperatures cause greater damage to cellular structure and function and require a longer period of recovery. The results from the present study suggested that it took at least 48 h for expression levels to return to a normal level. During this process, HSP70 expression increased in the lymphocyte itself. The increase in HSP70 synthesis can encourage lymphocytes to enter into the cycle of cell division from the G0 stage thus facilitating their activation accelerating the adaptation to cold stimuli and protecting the organism from stress injury. The results showed that at 12 and 24 h during the exposure both the absolute value and percentage of lymphocytes in the blood were elevated above those of the pre-stress stage which was concurrent with an over-expression of HSP70 in the cells. During the study, the HSP70 level did not remain consistently elevating. During some stages of the response to stress, fluctuations in the level might reflect the activity of various cytokines produced by the lymphocytes in addition to simply being the result of individual differences or systematic errors.

It was apparent that the lower the cold exposure temperature, the higher the speed of HSP70 mRNA transcription. The fact that there was no significant difference in HSP70 mRNA transcription level between at 12 and 24 h when exposed to −10 −4°C could be due to accelerated dissolving of mRNA or damage to body cell functions by cold stress which resulted in malfunction of HSP70 mRNA expression or adaptation of animals to cold environment. When animals returned to room temperature (15-18°C) after cold exposure ceasing, it was equal to application of new heat stimulation to piglets that have primarily adapted to cold condition, therefore, the HSP70 mRNA transcription could be once again rebound. However, due to cell function damage at exposure to −10−4°C, HSP70 mRNA transcription failed to respond quickly.

It was found that there were similar performances but also significant differences of which the increase of mRNA transcription occurred slightly earlier than the expression of HSP70 from the comparison of the amount of lymphocyte HSP70 mRNA transcription dynamic curve and the expression levels of HSP70 during −10 to −4°C cold stress. In this study, the correlation was analyzed at every time in the process. The result showed that correlation of HSP70 and HSP70 mRNA was no obvious regularity. In addition, The majority of correlation have no obvious significance except that at 0 and 0.5 h of cold exposure and 24 h of the recovery period which may suggested that the level of protein expression could not be predicted from the expression level of mRNA, since there was no significant correlation between the abundance of HSP70 and HSP70 mRNA, however the higher correlation and significance in the early days of stress suggested that certain relation exists in the two index which were not explained distinctly and needed to studying and researching deeply. The amounts of protein

expression differed in dozens of times despite the same gene mRNA abundance and vice versa. Body protein syntheses are subjected to dynamic modification and processing and hence are not usually decided by the gene. It is impossible to observe a variety of cell function regulations in mRNA levels owing to many regulations occurring in protein domains; furthermore many proteins can display the functions only in combination with other molecules (Lu *et al*., 2003). HSP70 served as the ultimately functional protein in response to cold stress. *HSP70* gene regulatory mechanism and its effect on expression can be intensely explored from the gene levels starting with mRNA. Moreover, the detection of mRNA can reflect the expression of HSP70 to some extent. In the study, pig lymphocytes were recovered conveniently and real-time fluorescence quantitative PCR was sensitive to mRNA quantification and accurate. Therefore, lymphocyte HSP70 mRNA may be considered as a molecular indicator for the assessment of cold stress in animals.

CONCLUSION

Lymphocyte HSP70 mRNA responds fast to cold stress and this response shows the faster regular changes of stress intensity and duration. It demonstrated that transcription level of lymphocyte HSP70 mRNA can be used as an indicator of cold stress to animals. In practice, it can be used to monitor animal stress condition by a standard curve and HSP mRNA transcription level measured by RFQ-PCR.

ACKNOWLEDGEMENT

This research has been supported by the Natural Science Foundation of HeiLongjiang Province of China (ZD201116) and Educational Commission of HeiLongjiang Province of China (12521371). Thanks for Prof. Runqing Yang (Shanghai Jiaotong University, Shanghai) for the statistical analysis and Prof. S.Y. Li for the critical reading and editorial assistance of the manuscript.

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