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EXPRESSION ANALYSIS OF HEAT SHOCK PROTEINS IN DROMEDARY CAMEL (*Camelus dromedarius*)

M.T. Sadder[1,2], H.M. Migdadi[1], A.M. Zakri[1], K.A. Abdoun[3], E.M. Samara[3], A.B. Okab[3] and A.A. Al-Haidary[3]

[1]Biotechnology Lab, [3]Department of Animal Production, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia

[2]Biotechnology Lab, Faculty of Agriculture, University of Jordan, Amman, 11942, Jordan

ABSTRACT

Stress response in animals is a sequence of changes to certain challenges that animals can undergo. Arabian (Dromedary) camels were exposed to continuous heat stress under controlled environment. Blood samples were collected at 0, 3, 6 and 24 h of heat stress exposure. Total RNA was isolated and converted into cDNA. The differential expression of a group of heat stress responsive genes was assessed using real-time PCR. The genes HSP60, HSPA6, HSP105 and HSPA1L were over-expressed at 3 h point, followed by sharp drop at 6 h point, and then rebounded after 24 h of heat stress exposure. The expression of other heat stress responsive genes (HSP70, HSP90, HSPFB and CaHS) was marginally affected along the heat stress period. The integration of gene functions and physiological mechanisms will lead to better understanding of body homeostatic mechanisms in camels under heat stress.

Key words: Camel, differential expression, heat stress, HSPs

Camels possess the ability to apply remarkable adaptive thermoregulatory mechanisms (physical, biochemical and physiological) to survive under arid and semi-arid environments (Schmidt-Nielsen and Schmidt-Nielsen, 1952; Al-Haidary, 2006).

Several genes affecting animal evaporative heat loss have been identified except genes controlling sweat gland function and epidermal vascular supply (Olson *et al,* 2003; Mariasegaram *et al,* 2007). Selecting animals for their hair coat characteristics and homeorhetic responses to heat stress are considered as one of the methods to improve animal thermo-tolerance (Mariasegaram *et al,* 2007).

Cellular heat shock response involve systemic wide gene expression across the cellular and organs level and have been categorised into acute (thermo-tolerance "survival" ability at cellular level), acclimatory (ability to increase work at organ level) and adaptive (short and long-term genetic alterations) responses (Collier *et al,* 2008).

Cellular thermo-tolerance is maintained as long as pre-nonlethal heat stress expressed proteins family called heat shock proteins (HSPs) are elevated, which is lost by declining in HSPs gene expression under continuing increase of stress exposure which in turn activate apoptosis mechanisms causing cellular death (Sonna *et al,* 2002). Numerous stressors are known to induce HSPs transcription including cytokines (Jaattela and Wissing, 1993) and viral infection (Hall *et al,* 2000).

Identifying genes which are responsible for acquiring thermo-tolerance ability of camels is of great potential. The objective of present study was to estimate the potential role of cellular expression of HSPs in acquiring thermo-tolerance ability in dromedary camels.

Materials and Methods

Heat stress

Heat challenge in a controlled climatic chamber was conducted on 4 male dromedary camels of Almaghatir breed. Blood samples were collected by jugular venipuncture before exposure to heat stress (unstressed control, 29.5°C). Thereafter, camels were placed individually in a controlled climatic chamber (≃ 43.0°C) and blood samples were collected at 3, 6, and 24 h after heat exposure. Blood samples were collected in EDTA vacutainers, placed on ice and immediately delivered to the laboratory.

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Heat shock responsive genes

Sequences of HSP families and heat stress up regulated genes from previous studies on mammals were retrieved from public databases (NCBI, 2013). They were blasted on camel specific sequences generated from mixed EST libraries to find homologous sequences through pair-wise alignment (Al-Swailem *et al*, 2010). Forward and reverse primers for quantitative real-time PCR (qPCR) were designed using VectroNTI (Invitrogen, USA) (Table 1).

Gene expression

RNA was isolated from camel blood samples and each experiment was run in triplicates. Red blood cells were lysed using EL buffer (Qiagen, USA). The RNA isolation kit was used following the manufacturer instructions (Qiagen, USA). First strand DNA was generated using RT kit (Promega, USA). In order to quantify the relative expression profiles of specific genes, qPCR was conducted on generated cDNA. Expression was amplified with SYBR Green mix (Qiagen, USA). Real-time amplification data was collected with Applied Biosystems 7500 thermal cycler (ABI, USA). The fold change in gene expression relative to actin expression was determined from the CT values using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

The experimental animals were trained for a couple of weeks to enter and exit the unoperated climatic chamber to adapt to this procedure. This undertaking necessitated the involvement of a dedicated staff member to the training procedure.

Results

The climatic data {Ambient temperature (T_a) and temperature-humidity index (THI)} recorded during the experimental period are shown in fig 1. The ambient temperature and temperature-humidity index outside the chamber before exposing to heat stress (control, time zero) were 29.5°C and 25.7 units, respectively. Transferring animals to the preheated climatic chamber caused a drop in T_a. The recorded ambient temperature at 3 h point was 37.4°C, which continued to rise until it reached 43.4°C after 24 h. Likewise, the THI was 39.6 units after 3 h of heat stress and reached 45.0 units at the end of the experiment.

Designed primers were screened with conventional PCR using a pooled mix of generated first strand cDNA samples. All primer pairs could successfully amplify the expected fragments with correct sizes. Another screening was performed with qPCR using the same mix. All genes were successfully detected and their melting curves revealed their uniqueness, where amplicons of different genes with similar sizes had distinguishable melting curves. Moreover, clean one-peaked melting curves illustrated the amplification of single product.

Expressions of heat shock responsive genes were screened on individual samples of cDNA

Table 1. Designed real-time PCR primers for heat shock responsive genes in camel.

Description	Accession	Sequence (5'—3')	Tm	bp
Actin*	AB270711	F: TTACAATGAGCTGCGTGTGGCC	59.6	189
		R: ATCACGATGCCAGTGGTGCG	59	
HSPA6*	HQ214118	F: GCTTTGAGCTCAGTGGCATCCC	59.7	199
		R: TGCTCAGCCTCATGAACCATCC	58.6	
CaHS**	Camel3_plate_063_C03	F: CTGCAAATGCTTCTGTCGGTCC	58.4	165
		R: AGCTTCTCGTTCTTGGGCGG	58.1	
HSP105**	Camel2_plate_040_A14	F: CAATGCAGATGAAGCAGTGGCC	59.2	164
		R: TAAAGACCTCGTGGACGCCCTC	59.1	
HSP60**	Camel1_plate_006_E17	F: TTGAAGGCATGAAGTTTGATAGAGG	55.7	208
		R: GAGCTTCTCCATCCACATTTTCAGC	59.2	
HSP70**	CL3802Contig1	F: GAGATCATCGCCAACGATCAGG	58.1	161
		R: TTCCACGTCCGACCAATGAGC	59.4	
HSP90**	Camel3_plate_055_K13	F: TGGCAGCAAAGAAGCACCTGG	59.8	181
		R: ATCCTGTTGGCATGTGTCTGGG	58.3	
HSPFB**	CL716Contig1	F: TGTGCAGGATCTCACCTCTGTGG	58.7	172
		R: TTCCACTTCTTCCACCCCGG	58.3	
HSPAlL**	Camel1_plate_064_L14	F: TTCAATGACTCTCAGCGCCAGG	59	174
		R: CACATCAAATGTGCCTCCACCC	58.7	

* NCBI database, ** Al-Swailem *et al* (2010)

produced from all heat stress exposure periods (0, 3, 6 and 24 h). The qPCR showed prominent response for 4 major genes after 3 h (HSP60, HSPA6, HSP105, HSPA1L and HSP70). The relative gene expression was maximum for HSP60 with 10 fold increase compared to the control (unstressed), while increase in expression was around 7, 6, 4 and 1 fold for HSPA6, HSP105, HSPA1L and HSP70, respectively. On the other hand, the expression of HSPFB and CaHS genes were down-regulated after 3 h (Fig 2).

After 6 h, HSP60, HSPA6, HSP105 and HSPA1L showed sharp drop in expression compared to the prominent expression after 3 h, however, they still showed higher expression compared to the control with 2, 3, 2 and 2 fold increases, respectively (Fig 2).

The expression levels of HSP60, HSPA6, HSP105 and HSPA1L bounded up again after 24 h with 4, 10, 6 and 6 folds increase compared to the control, respectively (Fig 2). On the other hand, expression increases in HSP70 and HSP90 genes were relatively comparable along the stress period (3, 6 and 24 h), while HSPFB and CaHS expression dropped sharply below the control after 24 h compared to 3 and 6 h of stress periods (Fig 2).

Discussion

Inside the controlled climatic chamber, the ambient temperature and THI during the heat exposure period showed a range of 37.4 - 43.4°C and 39.6-45.0 units, respectively (Fig 1). This indicated that camels were exposed to severe heat stress according to LPHSI heat stress indices (1990). Animal acclimation is classified into short-term heat acclimation (STHA) that involves alteration of cellular signaling pathways, and long-term heat acclimation (LTHA) that involves expression of heat-acclimation phenotype. A group of the investigated HSPs were shown to be tightly associated with heat stress that could be related to the short-term heat acclimation. However, we found that the expression of some other genes was marginally affected by the applied heat stress. A number of influential factors can affect expression of HSPs. Age as a factor was found to be highly influential on HSPs and other heat stress genes in Fischer rats (Zhang *et al,* 2002). In this study, the expression level was found not only to be varied between young and old animals, but also can be reversed for certain genes from up-regulation to down-regulation or vice versa.

Expression of HSPs can also be up-regulated by other factors including IGF-I (Shen *et al,* 2007), glucocorticoids (Vijayan *et al,* 2003), insulin (Li *et al,* 2006) and melatonin (Bonior *et al,* 2005). Whereas, expression of HSPs are down-regulated by leptin (Figueiredo *et al,* 2007). In our case, HSPs expression was dropped after a heat stress exposure period of 6

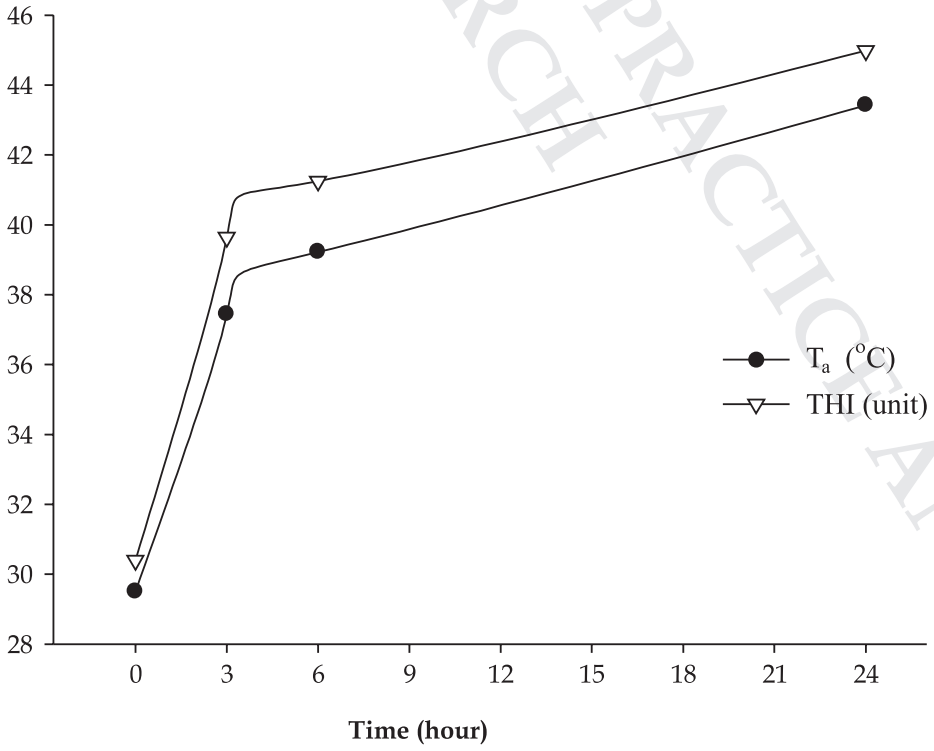

Fig 1. Ambient temperature (Ta) and temperature humidity index (THI) recorded during the experimental period.

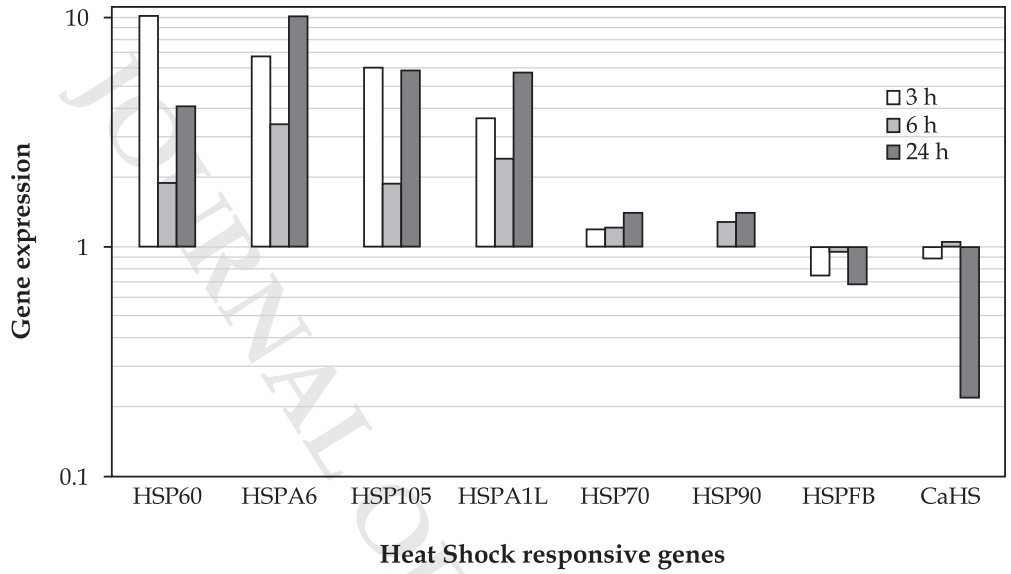

Fig 2. Relative expression of heat shock responsive gene in camel after 3, 6, and 9 h heat exercise compared to the control. Y axis is presented in a log10 scale.

h. These factors may control HSPs gene expression via activation of heat shock transcription factors (HSFs) (Collier *et al,* 2008). Therefore, improving our understanding of this process is the key to determine the fate of cells (survival and adaptation *vs* apoptosis and death) and linking it with animals' productivity will result in improving genetic selection of heat stress resistant genotypes. In *in vitro* experiments with mammalian cells, HSPs expression starts within minutes after the initiation of heat stress and peaks several hours later (Lindquist, 1986). However, for a live animal experiment and with a huge animal like camel, an incubation period is needed for the animal to acquire the surrounding heat, therefore, our first samples were taken at 3 h point. Nevertheless, 3 h is considered a short period for a living and huge animal. Expresison of HSP60, HSPA6, HSP105, HSPA1L and HSP70 showed significant increase in this relatively short period of heat stress (Fig 2). Most of HSP genes lack introns (NCBI, 2013) which facilitate their rapid expression, and explain how they can be expressed in the presence of stressors which can interfere with RNA splicing (Lindquist, 1986). Although, the intensity and duration of the heat stimulus needed to induce HSP expression vary from tissue to another, typical *in vitro* exposure involves heating mammalian cells at 42–45°C for 20–60 minutes and returning them to normothermic temperatures at 37°C (Sonna *et al,* 2002). However, to understand the downstream physiological mechanisms, living animals are needed for heat stress experiments (Zhang *et al,* 2002; Menéndez-Buxadera *et al,* 2012)

HSP60 has been considered as pro-apoptotic (Garrido *et al,* 2001). It showed the highest fold increase in expression after 3 h compared to other investigated HSPs in our study. On the other hand, HSPA6 gene, which codes for a heat shock 70 kDa protein, gave the 2nd most expressed genes after 3 h. HSP70s (HSP72, HSP73, HSP75, and HSP78) are ATP-binding proteins. They share common protein sequences and have 60–80% identity among eukaryotic cells (Craig, 1985). However, they are constantly produced under unstressed conditions (i.e. HSP73; also called HSP70), whereas, the HSP72 is induced in response to stress. Thermo-tolerance was illustrated by cellular manipulations of HSP70 level via plasmid transfection (Landry *et al,* 1989). Multiple cytoprotective functions have been reported for HSP70 even in unstressed cells, but the mechanisms for these functions are not entirely understood. These include chaperone activity (folding of new proteins; preventing aggregation of denatured proteins; refolding of denatured proteins), translocation of proteins across membranes and cellular compartments, stabilising the conformations of normal proteins, regulation of protein turnover and anti-apoptotic factors (Parsell and Lindquist, 1993). Moreover, they can be used as cellular injury biomarker in ischaemia- reperfusion injury to the heart (Yamashita *et al,* 1998).

Another important biochemical activity of HSP70 is the induction of immune system against viruses and tumours (Jaattela and Wissing, 1993; Hall *et al,* 2000). HSP70 mRNA expression has been demonstrated in a wide range of animal species. In

bovines, it was found in brain, heart, kidney, liver, lung, skeletal muscle, spleen and testis (Gutierrez and Guierriero, 1995) and cardiac endothelial cells (Rylander *et al*, 2005). In ovines, it was recorded in lung epithelial cells (Kramer *et al,* 2002) and cardiac muscle (Scharte *et al,* 2001). While in camels, HSP70 was expressed in lymphocytes (Ul'masov *et al,* 1990).

There are at least two regulatory elements in the 5' promoter region of HSP70 gene called heat shock elements (Amin *et al,* 1988) which are characterised by consensus penta-nucleotide inverted repeats sequence of 5'-nGAAn-3' motif. HSF interacts with HSP70 gene by DNA binding domain (Pirkkala *et al,* 2001) to activate its transcription. Three HSFs have been identified in mammalian species: HSF-1, HSF-2, and HSF-4, (Nakai, 1999). Under unstressed conditions, HSF-1 exists as a monomer molecule whereas, HSF-2 exists as a dimer molecule (Anckar and Sistonen, 2007; Sakurai and Takemori, 2007). HSPs preferentially bind to denatured proteins causing the release of HSF-1. HSFs then undergo activation by transition from monomer (HSF-1) or dimer (HSF-2) to trimer molecule. These activated HSF-1 and HSF-2 are translocated to the nucleus after these are tagged with SUMO-1 and co-localise to nuclear stress bodies. Finally, trimeric HSF-1 binds to the heat shock element (HSE) and activates HSP genes transcription.

Similar to our results for HPSA6, bovine HSP70 gene expression was dramatically stimulated between 1 and 2 h of heat stress and appeared to peak within 4 h of heat stress (Collier *et al,* 2006). This was followed by a down-regulation resulting in transcript levels close to baseline values after 8 h which is also in consistent with our 6 h sharp drop (Fig 2). However, they did not record another increase in expression after 24 h as we did. This might be related to the nature of the studied object. The presented expression in this study was reflected in a living animal where as, Collier *et al* (2006) used cultured primary BMEC, which were isolated from mammary gland tissue samples of a multiparous, pregnant, nonlactating Holstein dairy cow. On the other hand, it is important to note the 4 HSP70 genes were mapped and identified in cattle (Gallagher *et al,* 1993), while only three HSP70 genes were found in camel, which are clustered along one chromosome (Garbuz *et al,* 2011).

Conclusion

The genes HSP60, HSPA6, HSP105 and HSPA1L were over-expressed in dromedary camels within 3 hours of exposure to hot temperature. The presented data emphasise the importance of investigating living animals rather than cell cultures. Consequently, heat stress studies in controlled climatic chambers can mimic the natural heat stress situation and delivers more reasonable outcomes. It is recommended to investigate the herein over-expressed HSPs in different camel breeds and correlate them with productivity characters.

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