Expression Profile of Five Stress-Related Genes of Khorasan Native Chickens under Acute Heat Stress

Research Article

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ABSTRACT

High temperature is one of the main environmental factors causing economic losses to the poultry industry, as it reduces growth and production performance of chickens. The heat shock proteins (HSPs) play a key role in cellular defense mechanisms during exposure in a hot environment. The aim of this study was to evaluate the expression level of the candidate genes in the liver of Khorasan native chickens under acute heat stress. Sixteen 42 days old chickens were divided into two groups; the control (25 ˚C and 50% humidity) and heat-treated (42 ˚C and 50% humidity), and then the liver was sampled. The level of gene expression of *HSPB1*, *HSPB9*, *SERPINH1*, *HSPA2* and *HSP110* were evaluated using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method. The results of the analysis of variance revealed that the expression of *HSPA2* and *HSP110* was significantly increased. In the biological processes of gene ontology, three processes had *FDR* < 0.01. *HSPA2* and *HSPB1* involved in the processes that stimulated cells against increasing temperature. The results indicated that Khorasan native chickens have suitable resistance to acute heat stress. Furthermore, *HSPA2* has the ability to express under high ambient temperature in order to protect the structure of cellular proteins.

KEY WORDS gene expression, heat shock protein, heat stress, Khorasan native chicken.

INTRODUCTION

Global warming has negatively influenced animal production as well as poultry industry (Nardone *et al.* 2010). The high ambient temperature may slow growth performance, decrease immunity and reproduction ability of chickens (Rimoldi *et al.* 2015). Furthermore, heat stress affects meat quality in broilers (Hashizawa *et al.* 2013). Immediate physiological reactions of animals are crucial to ameliorate the effects of sudden changes in ambient temperature (Slawinska *et al.* 2016). Generally, chickens have no sweat glands or long and moist tongues to regulate body temperature. Thus, exposure to high environmental temperatures causes severe signs of heat stress (Li *et al.* 2015).

However, different breeds may show various behaviors against heat stress (Felver-Gant *et al.* 2012). Commercial breeds are more sensitive to stress because high selective programs deteriorate the immune function (Cahaner *et al.* 1995; Fouad *et al.* 2016). Indeed, continuous selection for fast growth has decreased resistance to heat stress in rapidly growing strains (Soleimani *et al.* 2011). High ambient temperature is also involved in oxidative stress as it causes a redox imbalance between pro- and antioxidants (Mujahid *et al.* 2007) and increases superoxide radicals (Rimoldi *et al.* 2015). One of the consequences of an increase of these harmful components is misfolding and degrading of intracellular proteins (Slawinska *et al.* 2016). At molecular level, heat stress induces the production of a group of pro-

teins with different weight called as heat shock proteins that their most important role is to protect cells from toxic effect of the heat, and also several of them function as intracellular chaperones for other proteins (Ganter *et al.* 2006; Staib *et al.* 2007; Rimoldi *et al.* 2015). These chaperones involved in binding and refolding the misfolded proteins and protein translocation (Whitley *et al.* 1999; Rimoldi *et al.* 2015).

Cell defense mechanism modulates gene transcription in the manner of down or upregulation to keep homeostasis against environmental changes (Lopez-Maury *et al.* 2008). Recently, high throughput technologies have been developed and assist to identify the expression level of many genes simultaneously (Wang *et al.* 2015; Lan *et al.* 2016; Jastrebski *et al.* 2017). However, qRT-PCR is still necessary to confirm the results of microarray or RNA sequencing (RNA-Seq) techniques. So far, Transcriptomic analysis has been performed to study gene expression changes in different organs of chickens under heat stress. The liver is an organ that plays a variety of vital roles such as energy metabolism, digestion, immune function, glycogenolysis, glycogen synthesis and detoxification. The results of studies revealed that different kinds of genes are regulated in the liver to protect the chickens against heat stress (Li *et al.* 2011; Coble *et al.* 2014; Luo *et al.* 2014; Xie et *al.* 2014; Lan *et al.* 2016). Among the genes, those that encode the heat shock proteins (HSPs) are commonly expressed in the chickens exposed to high ambient temperature. These conserved chaperone proteins help to maintain cell homeostasis through refolding the misfolded proteins, preventing aggregation of denatured proteins, participating in protein translocation and protein folding (Kregel, 2002). Previous studies indicated that the expression of HSPs increased in poultry exposed to heat manipulation (Figueiredo *et al.* 2007; Hwang *et al.* 2016; Cedraz *et al.* 2017). To our knowledge, the effect of heat stress on the expression of stress-related genes in the native chickens of Khorasan has not been studied so far. Therefore, the present study was performed to investigate the expression of *HSPB1* (*HSP20*), *HSPB9* (*HSP25*), *SERPINH1* (*HSP47*), *HSPA2* (*HSP70*) and *HSP110* (*HSPH1*) in the liver of Khorasan native chickens under acute heat stress.

MATERIALS AND METHODS

Experimental design

For this experiment, 80 one-day-old chicks were collected from the Khorasan native chicken breeding center. The chicks were kept in a controlled room. The temperature at first was 29 ˚C with a humidity of 50% and gradually decreased until the test day, which was maintained at 25 ˚C and 50% humidity.

At the age of 30 days, 30 chicks were transferred to separate rooms with the ability to control temperature and humidity and were accustomed to the new environment for five days (Lan *et al.* 2016). From day 36 to day 42, the ambient temperature for the thermal stress group gradually increased every day with an increase of 2 ˚C per day and finally was adjusted to 40 ˚C and 50% moisture. During the day before sampling, the temperature was maintained at 40 ˚C for the first 12 hours and 42 ˚C for the second 12 hours. The temperature was constant for the control group at 25 ˚C and 50% humidity. During the maintenance period, two hours of blackout was applied to each group during the day, and access to water and food was *ad libitum*. The energy and protein content of the diet were constant for both control and treatment groups. The starter diet contained 22.5% crude protein and 3000 kcal of metabolizable energy and the growth diet contained 21% crude protein and 2960 kcal of metabolizable energy. Then, eight chickens from each group were killed through cervical dislocation and their liver was sampled. The liver samples were frozen immediately in the liquid nitrogen and kept at -80°C until the extraction of RNA.

RNA extraction and the control of its quality and quantity

Trizol (SIGMA) was used to extract the total RNA based on the manufacturer protocols. Then, the concentration and quality of RNA were measured using a spectrophotometer (Microplate reader, Epoch, USA) by measuring optical absorption at 230, 260 and 280 nm. In addition, RNA integrity was examined by observing 28s and 18s ribosomal RNA bands on 1.5% agarose gel and staining with Gel-Red® (Figure 1).

RNA reverse transcription

A commercial kit (Thermo Fisher, USA) was used to produce cDNA library from the extracted RNA. Five micrograms of the total RNA were mixed with one microliter of Oligo (dT) primer and kept at 70 ˚C for five minutes. Then, 4 μL of 5X buffer, 3.2 μL of $MgCl_2$, 1 μL of dNTP, and 1 μL of reverse transcriptase were added to the mixture and the volume of the reaction reached to 20 μL with pure water. At first, the annealing was carried out at 25 ˚C for five minutes, then the extension was performed at 42 ˚C for 1 hour and eventually, the enzyme was deactivated at 70 ˚C for 15 minutes. The produced cDNA was stored for the next step at -20 ˚C.

RT-qPCR

SYBR® Green qPCR Master Mix (Thermo Fisher, USA) was used for the reaction of RT-qPCR through ABI 7300 Real Time PCR system.
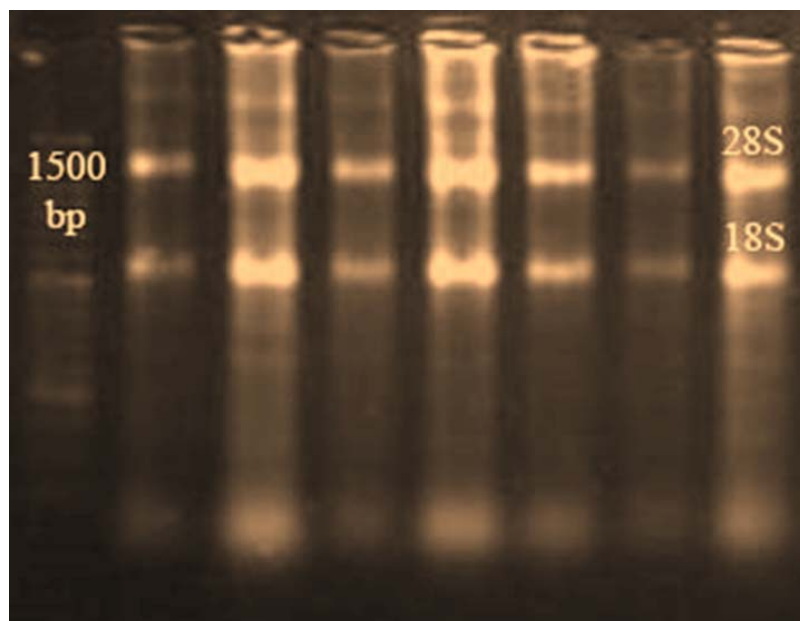
Figure 1 Quality and quantity of extracted total RNA bands on 1.5% agarose gel

The specific primers which were used are shown in Table 1. Each reaction was carried out in two replications. To determine the reaction efficiency, the standard curve was plotted according to the reaction at different concentrations. The amplification efficiency of each target gene was calculated according to the following formula: $E= 10(-1/slope)$ (Pfaffl, 2001). The negative control for the reactions was also considered. The reaction conditions were as follows: initial denaturation at 95 ˚C for 10 minutes and then 40 cycles with a temperature of 95 ˚C for 30 seconds, annealing at 62 ˚C for 30 seconds and extension at 72 ˚C for 30 seconds. Also, the drawing of the melting curve was performed in accordance with the protocol of the software used in ABI 7300 device. The reaction cycle threshold (Ct) value was corrected for statistical analysis using the following equation (Ciraci *et al.* 2010; Coble *et al.* 2014):

40- [(sample mean C_t) + (median GAPDH C_t–mean GAPDH C_t) × (sample gene slope/GAPDH slope)]

Statistical analysis
The results of RT-qPCR were analyzed using SAS® software version 9.1 (SAS, 2003). Mean adjusted Ct values of each duplicate of RT-qPCR were used in the analysis. Regarding that there was a treatment group and a control group, the Ct output was converted to 2^{-CT} (Livak and Schmittgen, 2001), and then the means of the control and heat-treated groups were compared by t-test. Also, the expression level of the target genes was compared with $2^{-\Delta\Delta CT}$ through one-way ANOVA.

Protein-protein interaction
The STRING database (www.string-db.org) was used to examine protein-protein interactions in silico. After entering the genes into the database, the data of protein interactions and the ontology of genes were processed. This database basically examines the functional and metabolic pathways of genes by connecting to other databases such as GO and KEGG. The paths with a value of P < 0.05 which were corrected by FDR multi-test method were considered as significant tests.

RESULTS AND DISCUSSION

Prior to analyzing the gene expression, the PCR reaction efficiency was evaluated. The optimum annealing temperature was determined for all primers at 62 ˚C. The slope of the standard curve was between -3.1 and -3.3 with a coefficient of determination (R^2) of 98% and the amplification efficiency between 1.99 and 2.
The lack of primer dimer and the formation of a specific product was confirmed by a dissociation curve. The expression of the genes in this study ranged from 16 to 30 cycles, according to Cedraz *et al.* (2017). The average expression of the genes versus the control group is shown in Table 2. *HSPB9*, *HSPA2* and *HSP110* upregulated between 5 to 7 fold in the heat treated group compared to the control group.
However, the results of the analysis of variance revealed that the expression of *HSPA2* and *HSP110* increased significantly (Figure 2).

Table 1 The primers sequences for RT-qPCR reaction

Gene name	Accession No.	Primer sequence	Fragment size (bp)
HSPB1	NM_205290.1	F: CGATGGGATGCTGACAGTG R: TACTTCTTGGCTGGTTCTTCC	114
HSPB9	NM_001010842.2	F: GTCTTCTGCTGAGAGGAGTG R: CGTTGTTCCGTCCCATCAC	117
SERPINH1	NM_205291.1	F: CTGATGGAGCCCTTATTGTCA R: TCACCATGAAGCCACGGTTA	98
HSPA2	NM_001006685.1	F: CAAGAACCAAGTGGCAATGAA R: CATACTTGCGGCCGATGAGA	72
HSP110	NM_001159698.1	F: GTAGTTTCGTTCGGCTCCAA R: CTGTGTTGTGGGCATGAGTAA	80
GAPDH	NM_204305.1	F: CATCACAGCCACACAGAAGA R: TGACTTTCCCCACAGCCTTA	124

Table 2 The average expression of the genes versus the control group (upper) and Network statistics of protein interactions (lower)

Average expression of the genes versus the control group	
Gene	**Fold change**
HSPB1	1.39
HSPB9	5.38
SERPINH1	1.95
HSPA2	6.05
HSP110	7.00
Network statistics of protein interactions	
Statistic	**Value**
Number of nodes	4
Number of edges	5
Average node degree	2.5
Protein-protein interaction (PPI) enrichment p-value	9.4e-08
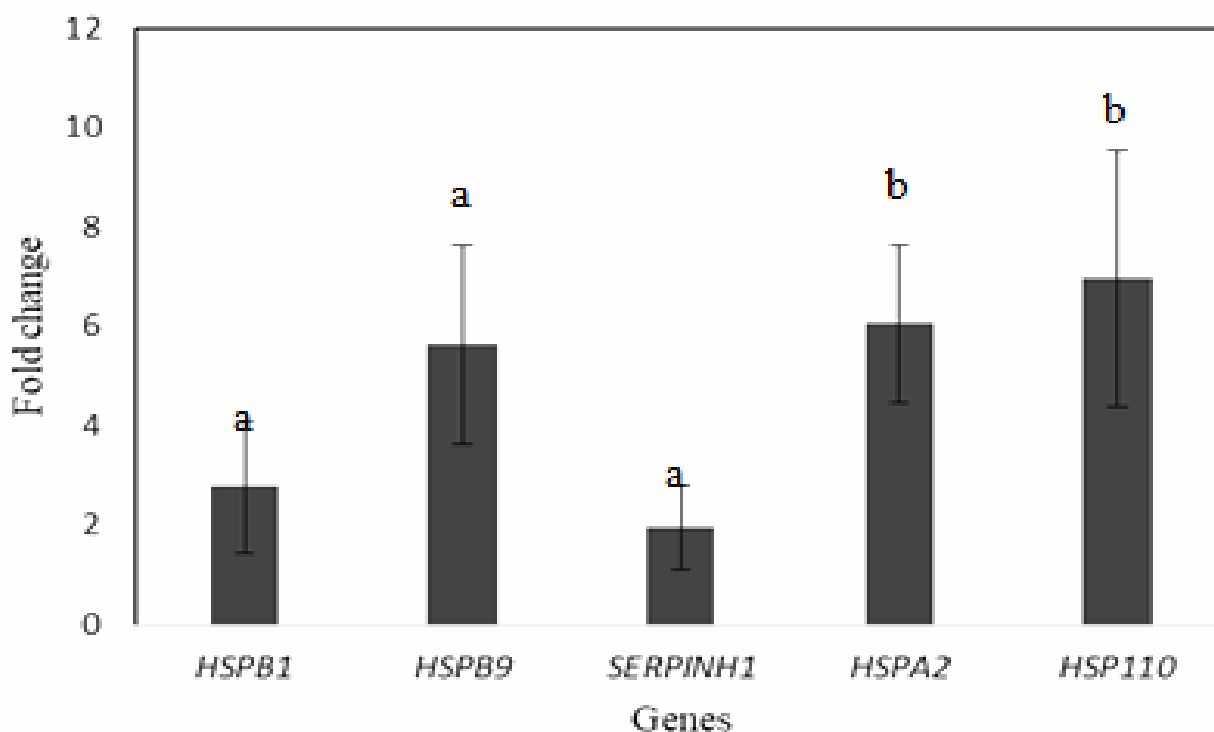
Figure 2 The comparison of gene expression level under heat stress
The means within the same column with at least one common letter, do not have a significant difference (P>0.05)

Comparing gene expressions between the control and the under thermal stress groups showed that *HSPA2* and *HSP110* had a significant upregulation in the chickens under heat stress (Figures 3). Furthermore, the expression of *HSPB1* and *SERPINH1* was not significant between the control and the treatment groups.

High temperatures can cause lots of damage to livestock, especially poultry. There are several physiological factors that can reduce the effect of heat on the animal. Increasing ambient temperature increases the expression of the genes associated with homeostases, such as heat shock factors and heat shock proteins, and reduces the expression of the genes which are less essential (De Nadal *et al.* 2011). Previously, the effect of heat stress on gene expression in laying chickens (Xie *et al.* 2014), broiler strains (Rimoldi *et al.* 2015), distinct chicken lines (Lan *et al.* 2016), and a different breed of broilers (Xu *et al.* 2018) was reported. However, it is the first time that in a native chicken of Iran the expression of five stress-related genes under hyperthermic stress is reported in this study. Heat shock proteins are produced in response to rising temperatures and other factors such as microbial infection, cancers and genetic damage (Ali and Banu, 1991). However, in this study, the expression of *HSPB9*, *HSPA2* and *HSP110* in the treatment group was 5 to 7 times more than the control group, but this increase was significant only for *HSPA2* and *HSP110* ($P<0.05$). The products of both genes are HSPs with high molecular weight. HSPA2 and HSP110 belong to the family of heat shock protein 70. The HSPA2 acts as a molecular chaperone in many cellular processes, including protecting proteins against stress, folding and transferring of newly synthesized polypeptides, activating the proteolysis of damaged proteins, and forming and decomposing protein compounds (Cedraz *et al.* 2017).

The expression of *HSP70* and *HSP90* in native chickens compared to the Cobb breed has indicated an increase under heat stress. The Cobb breed reveals more sensitivity to heat than native breeds. The commercial breeds have been selected for production traits and are not compatible with warm weather (Soleimani *et al.* 2011). Conversely, native breeds are more adapted to the surrounding environment and have the ability to withstand more heat (Cedraz *et al.* 2017). Increasing the expression of *HSPB9*, *HSPA2* and *HSP110* in Khorasan native chickens under heat stress confirms that this breed has the ability to tolerate heat. Meanwhile, there were no casualties due to heat. The expression of the *HSP70* increases under physiological stresses, diseases and environmental stresses (Eissa *et al.* 2017), and can be regulated by hormones (Wu *et al.* 2019). However, the regulation of the gene transcription is also related to the type of tissue.

Leptin injection reduced the expression of *HSP70* in the liver and the hypothalamus, but it did not affect the expression of the gene in the muscle of broiler chicks (Figueiredo *et al.* 2007). The expression of the heat shock factor family (HSF) genes is increased under ambient temperature which can increase the expression of *HSP* genes (Xie *et al.* 2014). However, the expression of *HSF* genes was not examined in the present study.

One reason for the increased expression of *HSP70* may be an increase in the level of reactive oxygen species (ROS) in the cell (Singh *et al.* 2014). Increased levels of ROS have been observed in chicken and cattle cells under heat stress (Singh *et al.* 2014; Cheng *et al.* 2018). In response to oxidative stress, HSF-1 is isolated from the HSP90 complex and transported to the nucleus for activation of transcription of HSP70, thereby preventing cell apoptosis (Rimoldi *et al.* 2015).

Heat shock proteins act as a cellular heat-sensing and may only respond to increased temperature and protein degradation (Figueiredo *et al.* 2007). The family of small heat shock proteins, such as HSP25, is expressed in terms of heat and chemical stress.

Even in the early stages of the embryo's blastoderm, the expression of *HSP25* and *HSP30CL* has been reported (Hwang *et al.* 2016). Also, the reduction of *HSP25* expression in blastoderm reduces the ability to differentiate cells and cell death increases (Hwang *et al.* 2016). However, the increase in expression of *HSPB1* and *SERPINH1* was not considered in the present study (Table 2). The expression of *HSP47* and *HSP60* increased from 2 to 4 hours of heat stress in broiler kidneys and then decreased to 24 hours (Tang *et al.* 2018). HSPB9 is the first cellular defense system against environmental stressors and is expressed before HSPB1 and HSPA2, although, its expression is low in early hours and then increases (Xu *et al.* 2019). The expression of *HSPB9* in the present study was 5 times higher in the treatment group (Table 2). Network statistics of protein interactions are shown in Table 2. On average, the proteins in the protein interaction network are associated with 2.5 proteins.

The P-value for the enrichment of protein-protein interaction (PPI) of the four proteins studied shows that they have a large intergroup interaction and are probably parts of a functional biological group (Franceschini *et al.* 2013). Totally, the proteins interacted in five biological pathways. HSPB9 was not included in the analysis, as STRING found no protein by this name for *Gallus gallus*. The interactions among proteins are shown in Figure 4. The analysis of biological pathways in STRING indicated that most of the pathways associated with cell and protein regulation are activated under thermal stress conditions (Table 3).
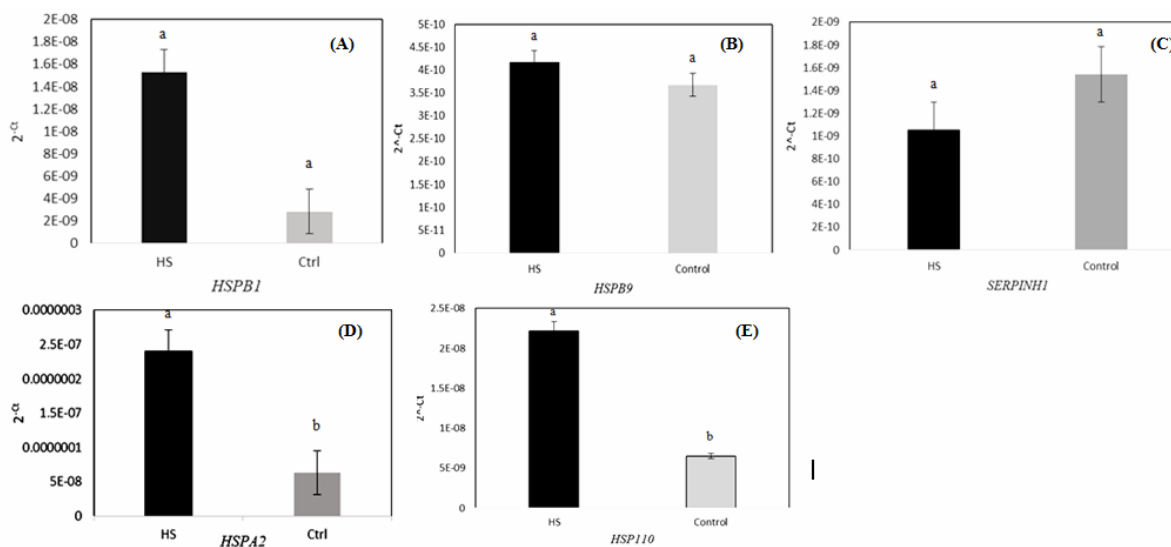
Figure 3 Comparison of candidate gene expression between control and under thermal stress groups: *HSPB1* (A), *HSPB9* (B), *SERPINH1* (C), *HSPA2* (D) and *HSP110* (E)
The means within the same column with at least one common letter, do not have a significant difference (P>0.05)

Two paths were important in KEGG biological pathways. The Protein Processing in Endoplasmic Reticulum pathway is involved in the regulation of the producing right proteins and the elimination of inappropriate proteins. The accumulation of damaged proteins in the endoplasmic network (ER) causes cellular tensions and, with the activation of the heat shock response system (HSR), the stress load decreases (Liu and Chang, 2008).

Heat shock protein 40, HSP70, and HSP90 play a role in the process of protein degradation. Low molecular weight HSPs, such as HSPB1, are not present in this biological pathway. The HSPB1 protein has an active role in MAPK signaling pathway, which controls many cellular activities, such as proliferation, differentiation and cellular transfer (Abhinand *et al.* 2016). The activation of MAPK signaling pathway and the presence of HSP27 are essential for the structural transformation of actin and cell migration (Golan *et al.* 2014).

In the biological processes of gene ontology, three processes had *FDR* < 0.01 (Table 3).

HSPA2 and HSPB1 involve in the processes that stimulate cells against increasing temperature, in terms of movement, secretion, enzyme production, gene expression, etc. as well as the reduction of enzymatic activity and down-regulation of cellular protein metabolic process (www.informatics.jax.org/vocab/gene_ontology/GO:0009408). Although few genes were analyzed in this study thus, the gene ontology may give only general aspects about the interaction between genes and their products.

In a study by Lan *et al.* (2016), heat shock proteins with a molecular weight greater than 70 influenced the expression of liver genes in broiler chickens under heat stress. While the difference in the expression of HSP genes was not found in the study by Coble *et al.* (2014). The reason for the variation in the expression of genes is related to the different ways of heat treatment in the experiments as well as the different breeds that have been used (Sun *et al.* 2015).

The molecular functions of the gene ontology observed in this study were similar to the report by Sun *et al.* (2015). HSPB1, HSPA2, and SERPINH1 play a role in intracellular functions (Table 3). The creation of a genetic network for high-expression genes in broilers exposed to severe heat stress showed the existence of the interaction between HSP genes and immune genes, while most HSP and immune genes showed upregulation (Lan *et al.* 2016). The elevated expression of *HSP90* in the early hours of stress may be due to its protective role in cells against environmental destructive effects and oxidative damage (Rimoldi *et al.* 2015).

In the present study, the increase in the expression of the *HSPB1* was not significant and the expression of *HSPA2* was significant at a confidence level of 0.05, which could indicate that the response of these genes is not severe under acute heat stress, but it may increase under chronic heat stress.

Meanwhile, indigenous chickens reveal greater resistance to temperature changes (Cedraz *et al.* 2017).
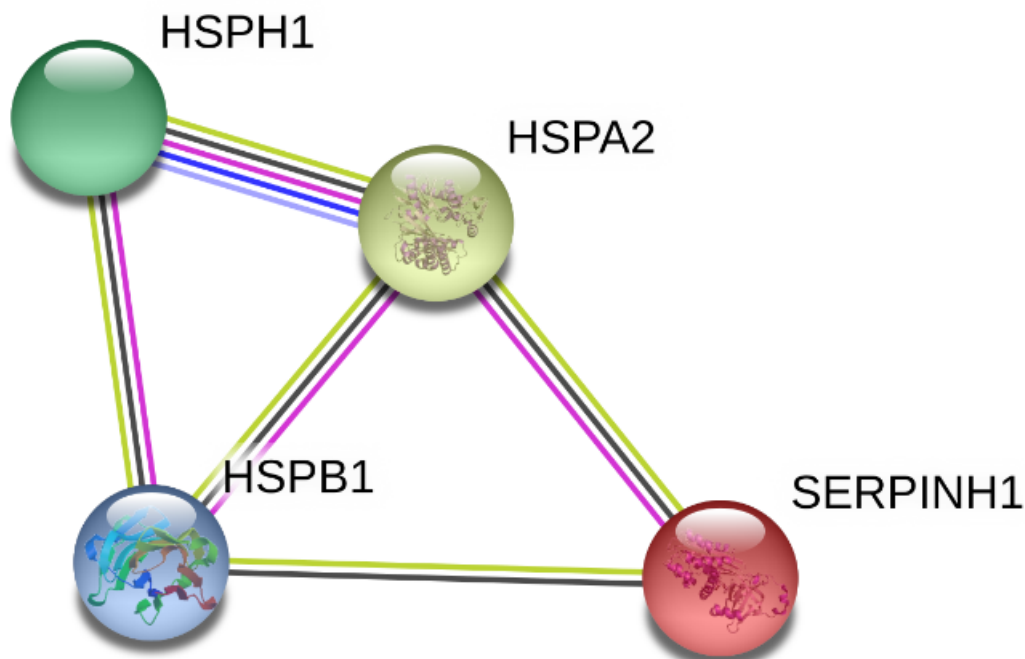
Figure 4 Protein-protein interaction in STRING

Table 3 Significant biological pathways in KEGG database (FDR<0.01) and Functional enrichments in gene ontology (GO) analysis

Term ID	Description	Protein	FDR
gga04141	Protein processing in endoplasmic reticulum	HSPA2,HSP110	0.0026
gga04010	MAPK signaling pathway	HSPA2,HSPB1	0.0039
Biological process			
GO-Term	**Description**	**Protein**	**FDR**
GO:0009408	Response to heat	HSPA2,HSPB1	0.0014
GO:0043086	Negative regulation of catalytic activity	HSPB1,SERPINH1	0.0082
GO:0032269	Negative regulation of cellular protein metabolic process	HSPB1,SERPINH1	0.0097
Molecular function			
GO:0004857	Enzyme inhibitor activity	HSPB1,SERPINH1	0.0029
GO:0005515	Protein binding	HSPA2,HSPB1,SERPINH1	0.0061
Cellular component			
GO:0044444	Cytoplasmic part	HSPA2,HSPB1,SERPINH1	0.0257
GO:0043231	Intracellular membrane-bounded organelle	HSPA2,HSPB1,SERPINH1	0.0373

The different cell lines and tissues give different responses to heat. The set of these responses depends on the combination of several factors including the genetic composition of the target species, genomic changes caused by past environmental stresses and the present state of the stressor (Sonna *et al.* 2002).

CONCLUSION

In this study, it was shown that the liver of Khorasan native chickens had an appropriate ability to express the *HSP* genes. Under heat stress, *HSPA2* and *HSP110* had higher expression than the control group, whereas *HSPB1*, *HSPB9* and *SERPINH1* did not show any difference compared to the control group. Considering the vital role of these proteins in maintaining the stability of cellular proteins, as well as the absence of casualties, it is concluded that Khorasan native chickens have the ability to tolerate acute heat conditions. This ability can be used to increase the capacity of the chicken temperature compatibility and to develop native resistant strains. Furthermore, *HSPA2* and *HSP110* can be considered as candidate genes in the marker-assisted selec-

tion program to improve heat tolerance in Khorasan native chickens. However, RNA-Seq analysis provides a lot of information on how different genes function, interact and involve in different biological systems, that based on this information new candidate genes can be introduced for further study.

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