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#### ■ Keywords

Broiler, taurine, heat stress, heat shock protein, gene and protein expression.



## Taurine Reduces Heat Stress by Regulating the Expression of Heat Shock Proteins in Broilers Exposed to Chronic Heat

### ABSTRACT

The aim of the current study was to investigate the effects of taurine on heat stress by evaluating their RNA and protein expressions of HSP90, 70 and 60 in Ross broilers. Birds were distributed in a control group (CO) reared at 24°C, a heat-stress group (HS) maintained at 34°C (weeks 3-5) and not supplemented with taurine, and a heat-stress group (HST) maintained at 34°C (weeks 3-5) and supplemented with 0.1% taurine from 2 weeks of age. The final body weight was significantly higher in the HST group than in the HS group ( $p < 0.05$ ). The mRNA expression of all three genes in the liver and of HSP90 in the muscle were significantly lower in the HST than in the HS group ( $p < 0.05$ ). In the liver, the expression of HSP70 and HSP60 proteins was significantly higher in the HS group compared with the CO and HST groups ( $p < 0.05$ ), while HSP90 expression was not different ( $p > 0.05$ ). In the muscle, HSP70 expression was significantly lower in the HST group than in the CO and HS groups and HSP60 expression was dramatically decreased in HS group, whereas no differences in HSP90 expression were detected among groups. In conclusion, the broilers exposed to heat stress and supplemented with taurine showed lower expressions of heat shock genes and proteins both in the liver and muscle tissues, indicating that taurine supplementation improved broiler thermo-tolerance.

### INTRODUCTION

Due to global warming, there is an increasing interest in the research of heat stress and its impact on livestock production. Thermal conditioning has been shown to improve the heat tolerance of broilers submitted to heat stress (Vinoth *et al.*, 2015). Temperature is one of the main factors that negatively affect the production performance and has stronger effects on poultry compared with other livestock species. Chickens have no sweat glands and, due to their fast metabolic rates and high body temperature, are very sensitive to high ambient temperatures. Birds can only tolerate a very narrow temperature range, of 18-24°C (Rajkumar *et al.*, 2011). Heat stress causes several physiological and metabolic changes in chickens, which have negative effects on body weight gain, feed efficiency, and serum triglyceride and uric acid levels (Deyhim *et al.*, 1995), eggshell quality and livability (Quinteiro-Filho *et al.*, 2010; Mashaly *et al.*, 2004), in addition of increasing production costs (Rajkumar *et al.*, 2011).

Some dietary supplements may enhance physiological mechanisms that allow chickens to better resist heat stress. Yahav *et al.* (1997) suggest that the mechanisms enabling better survival of broiler involves those that reduced hyperthermia and protected the body tissues from heat stress. Some researchers found that the dietary supplementation



of taurine, betaine, vitamin C, and vitamin E may improve the growth performance and livability of birds under heat-stress situations (Ipek *et al.*, 2007; Shim *et al.*, 2006; Zulkifli *et al.*, 2004). Zulkifli *et al.* (2007) also reported that a palm oil diet reduced the body temperature and mortality rate of chickens during heat exposure.

Heat shock proteins (HSPs) are highly conserved protein families synthesized in response to physical or biological stressors, including heat exposure (Staib *et al.*, 2007). It is well documented that one of the most important HSP functions is the protection of the body from the toxic effects of heating (Arrigo, 2000). HSPs may play important roles in protein folding or unfolding and translocation (Zügel & Kaufmann, 1999), in protein (dis)assembly (Bukau *et al.*, 2000), as well as in damaged protein refolding (Maloyan *et al.*, 1999). HSPs are broadly classified into six distinct families according to their molecular weights, from 10 to 150 kDa (Benjamin & Mc Millan, 1998) and their sizes: small HSPs, HSP40, HSP60, HSP70, HSP90, and HSP110 (Habich & Burkart, 2007). The degree of stimulated heat tolerance is related to HSP expressions (Krebs and Bettencourt, 1999); 70 kDa appears to be closely associated with HSP heat tolerance (King *et al.*, 2002; Wang & Edens, 1998). HSP70 is the earliest responsive gene (Morimoto, 1998).

Taurine, a  $\beta$ -aminoethanesulfonic acid, is derived from free amino acids and found in large quantities in almost all animal tissues, and presents colorless and odorless properties (Shim *et al.*, 2006). It is a chemically-simple compound but has profound effects on cells and is an essential nutrient for many mammals (Schaffer *et al.*, 2010). Taurine has been shown to play a role in bile-acid binding (Ito *et al.*, 2004), intracellular osmotic pressure control (Schaffer *et al.*, 2010), antioxidant protection (Kocak-Toker *et al.*, 2005), and in alleviating heat stress (Shim *et al.*, 2006). However, to the best of our knowledge, there are no studies on the effects of dietary taurine supplementation on heat-shock proteins and gene expressions in the liver and muscles of broilers submitted to heat stress. Therefore, this study aimed at investigating the effect of dietary taurine supplementation on heat-shock proteins and gene expressions of broiler under heat stress.

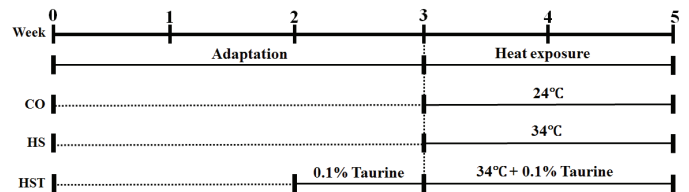
## MATERIALS AND METHODS

### Birds and experimental procedures

All experiments were undertaken in accordance with the guidelines of the Care and Use of Experimental

Animals, Chonbuk National University, Republic of Korea. The study protocol was approved by the Animal Care and Use Committee of Chonbuk National University. Maximum care was taken to minimize suffering of the birds.

One-hundred twenty one-day-old Ross broilers were obtained from a commercial hatchery (Dongwoo, Inc., Kimhae, Republic of Korea). The treatments consisted of a control group (CO), which birds were reared at 24°C and not supplemented with taurine; a heat-stressed group (HS), which birds were maintained at 34°C from 3 to 5 weeks of age and not supplemented with taurine; and a heat-stressed group (HST), which birds were reared at 34°C from 3 to 5 weeks of age and were supplemented with 0.1% taurine in the drinking water from 2 weeks of age. The experimental scheme is shown in Figure 1.



**Figure 1** – Experimental schedule, including the heat exposure and taurine supplementation periods.

The experiment was conducted at the experimental farm of Chonbuk National University, Jeonju, Republic of Korea. Broilers were reared in stainless steel cages equipped with nipple drinkers and hanging feeders at 10 birds per cage. Control group (CO) and heat-stressed groups (HS and HST) were reared in separate room to apply the temperatures of 24 °C and 34 °C respectively.

The environmental temperature on the day of placement was 34°C and was gradually decreased to 24°C until day 21, when birds were allocated to the three experimental groups, with four replicates of 10 birds each. The HST group received 0.1% taurine in drinking water from the second week on for adaptation to taurine. Heat exposure started on the third week, when the environmental temperature was maintained of 34°C for the HS and HST groups. Control birds were kept at 24°C during the experimental period.

Broilers were fed starter (1 to 21 days of age as mash) and grower (22 to 35 days of age as crumble) commercial feeds purchased from Purina (Cargill Agri Purina Inc., Seongam City, Republic of Korea). The chicks received a commercial basal diet containing 19% CP and 3,000 kcal ME/kg. Feed and water were supplied *ad libitum* throughout the experimental period.



During the whole experimental period, the lights were kept on all day and the humidity was maintained on an average of 60% respectively. At the end of experimental period (day 35), the birds were weighed after 12-h fasting, sacrificed and liver and muscle samples were collected for analyses.

### Sample collection

At the end of the experimental period, 10 birds per treatment were randomly selected and sacrificed by decapitation. Carcasses were manually eviscerated and liver and breast muscles were dissected, collected, and aseptically placed into 2 mL tubes. The tubes were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until analyses.

### RNA isolation and cDNA synthesis

Total RNA was extracted from liver and muscle tissues by using TRIzol Reagents (Invitrogen, NY, USA), according to the manufacturer's instructions. Total RNA was quantified by nano drop (ThermoFisher Scientific, USA) at 260 nm/280 nm absorbance. The quality of total RNA was assessed by using Experion™ Automated Electrophoresis System (BIO-RAD, Hercules, CA, USA) with RNA chip kits (Experion™ RNA StdSens Reagents, #700-7259, BIO-RAD) and good-quality RNA samples (1  $\mu\text{g}$ ) were selected for further reverse transcription (Figure 1) into cDNA, using iScript™ cDNA Synthesis kit (BIO-RAD). The reverse transcription was processed at

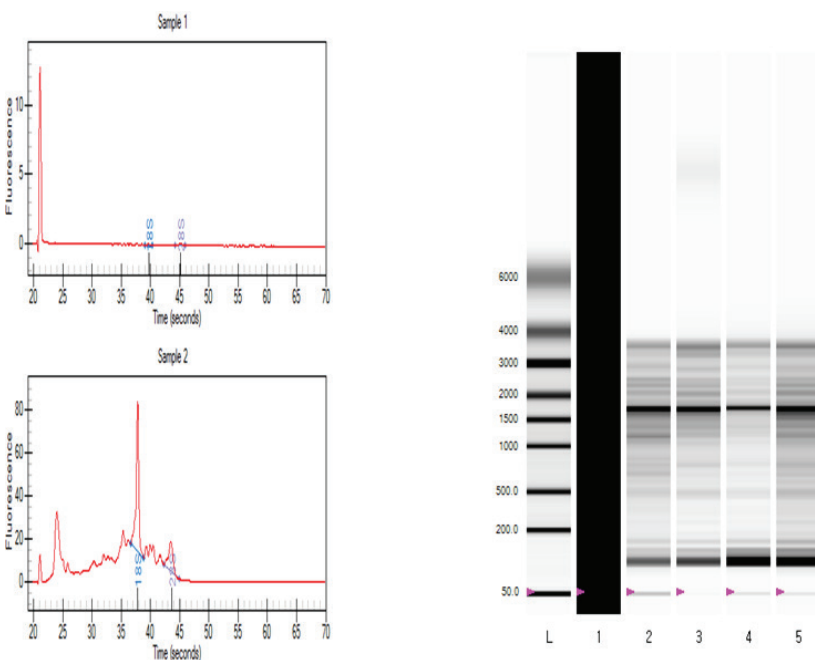
$25^{\circ}\text{C}$  for 18 s, followed by 11 cycles at  $48^{\circ}\text{C}$  for 4min, and the enzyme reaction was inhibited at  $55^{\circ}\text{C}$  for 18 s.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Real-time PCR was performed using SsoFast™ EvaGreen® Supermix (BIO-RAD, USA) and CFX96™ Real-Time PCR detection System (BIO-RAD). The cDNA of each gene was amplified and the reaction was carried out according to the manufacturer's instructions (BIO-RAD). Briefly, PCR was conducted in a 20  $\mu\text{L}$  total reaction volume containing 100 ng cDNA, 10  $\mu\text{L}$  SsoFast™ EvaGreen® Supermix, and 1  $\mu\text{L}$  of 10 pM primers. The first-strand cDNAs were used as a template to amplify gene-specific primers for HSP90, HSP70, HSP60, and the housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). The thermal cycling parameters were as follows:  $95^{\circ}\text{C}$  for 2min, followed by 40 cycles at  $95^{\circ}\text{C}$  for 5 s,  $59^{\circ}\text{C}$  for 5 s, and  $65^{\circ}\text{C}$  for 5 s. The expressions of the target genes were normalized with GAPDH. The primer sequences of the gene fragments are presented in Table 1.

### Western blot analysis

Chicken livers and muscles were homogenized in 1 mL RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxy cholic acid, 1% triton X-100. pH 7.5) and centrifuged for 20 min at 12,000 rpm. The supernatant was stored at  $-20^{\circ}\text{C}$ . Before western blotting, sample proteins were quantified using DC Protein assay kit (BIO-RAD, USA) following the manual's instructions, according to which BSA functions as the standard, and samples were measured at 750nm. Equal amounts of protein samples were loaded on 12 % SDS-PAGE gels to separate proteins according to molecular weight and then transferred to PVDF membranes (Immuno-Blot PVDF membrane Roll, BIO-RAD, USA) with a Semi-Dry Electrophoretic Transfer Cell (BIO-RAD Laboratories, USA). Membranes were blocked for 3 h at room temperature in 5% skimmed milk in TTBS (20 mM Tris, 137 mM NaCl, 5 mM KCl, 0.05% Tween-20 (v/v), pH 7.4). After blocking, membranes were washed three times in TTBS for 5 min at room temperature and incubated with HSP90, HSP70, HSP60, and  $\beta$ -actin in 3% skimmed milk as primary antibodies overnight at  $4^{\circ}\text{C}$ . The membranes were again washed three times for 10 min with



**Figure 2** – RNA integrity check. The 18S and 28S peaks are clearly visible in sample 2, but not in sample 1. In gel electrophoresis, Lane 1 shows totally degraded RNA, while in Lanes 2-5, RNA is intact, with clear 18S and 28S rRNA bands.



**Table 1** – Primer sequences used for gene fragments.

Primer	Accession number	Sequence (5'– 3')	Product size (bp)
HSP90	NM_206959.1	F- GCATTCTCAGTTCATTGGCTACC R- CTGTCTTCTCCTCCTTCTCCTCT	122
HSP70	J02579	F- ATGAGCACAAAGCAGAAAGAG R- TCCCTGGTACAGTTTTGTGA	95
HSP60	NM_001012916.1	F- AGAAGAAGGACAGAGTTACC R- GCGTCTAATGCTGGAATG	116
GAPDH	NM_204305.1	F- AGAACATCATCCCAGCGTCC R- CGGCAGGTCAGGTCAACAAC	130

HSP90: Heat shock protein 90, HSP70: Heat shock protein 70, HSP60: Heat shock protein 60, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

TTBS and incubated with rabbit anti-rat IgG-HRPgo at anti-rabbit IgG-HRP, as well as goat anti-mouse IgG-HRP as secondary antibodies for 1.5 h at room temperature. After incubation, membranes were washed TTBS three times for 10 min for a third time at room temperature, incubated in 6.30 mL of a HRP colorimetric reagent (BIO-RAD, USA) for 1 h at room temperature, and washed with DW. The membranes were scanned using a Bio-5000 plus Microtek (BIO-RAD, USA) and the band intensities were analyzed by the Quantity One Software (BIO-RAD Laboratories, USA). The protein band intensity value was normalized by the intensity of the  $\beta$ -actin band.

### Statistical analysis

The study data were analyzed by analysis of variance using the software SAS (SAS 9.1, USA). Duncan's multiple range test was applied to compare the means. The values were expressed as the mean  $\pm$  SE (standard error). Values were considered significantly different at  $p < 0.05$ .

## RESULTS

### Effect of heat stress on body and liver weights

Body and liver weight results are shown in Table 2. At the end of the study, final body weight was significantly higher in the HST group than in the HS group ( $p < 0.05$ ). Liver weight was higher in CO than the other groups and the liver/body weight ratios were significantly lower in the HST group compared with the CO and HS groups ( $p < 0.05$ ).

**Table 2** – Effect of taurine on final body weight, liver weight, and liver/body weight ratios of broilers.

Items	CO	HS	HST
Final body weight (g)	2336.50 $\pm$ 31.22 <sup>a</sup>	1881.70 $\pm$ 50.08 <sup>c</sup>	2067.90 $\pm$ 37.56 <sup>b</sup>
Liver weight (g)	63.83 $\pm$ 9.67 <sup>a</sup>	50.98 $\pm$ 7.81 <sup>b</sup>	49.18 $\pm$ 5.05 <sup>b</sup>
Liver weight/body weight (%)	2.73 $\pm$ 0.10 <sup>a</sup>	2.71 $\pm$ 0.10 <sup>a</sup>	2.38 $\pm$ 0.06 <sup>b</sup>

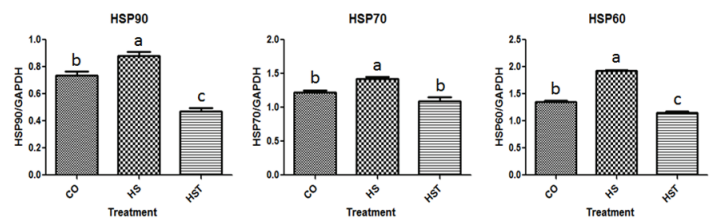
Mean values are presented as means  $\pm$  SE. Values in same row with different superscript letters are significantly different ( $p < 0.05$ ).

CO: Control group reared at 24°C and not supplemented with taurine; HS: heat-stressed group maintained at 34°C and not supplemented with taurine; HST: heat-stressed group maintained at 34°C and supplemented with 0.1% taurine.

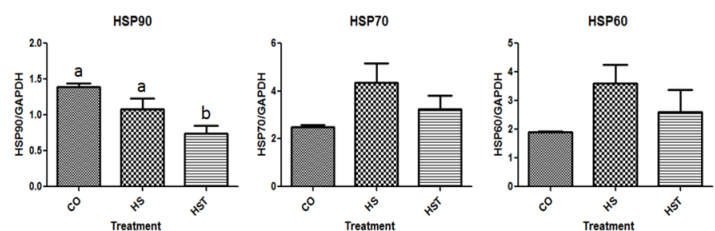
### Effect of heat stress on gene expression

The liver mRNA expression of all the three genes in the liver were significantly higher in the HS group and lower in the HST than in the CO group ( $p < 0.05$ ). However, muscle HSP70 and 60 mRNA expressions were not significantly different among treatments. The muscle mRNA expression of HSP90 was significantly lower in the HST group compared with the HS and CO groups (Figure 3A and 3B).

**A**



**B**



**Figure 3** – Heat shock protein (HSP) gene expression. A: liver tissue, B: muscle tissue. Values are expressed as mean  $\pm$  SE from individual tissue samples ( $n = 6$ ). Means with different superscripts indicate statistically significant differences ( $p < 0.05$ ).

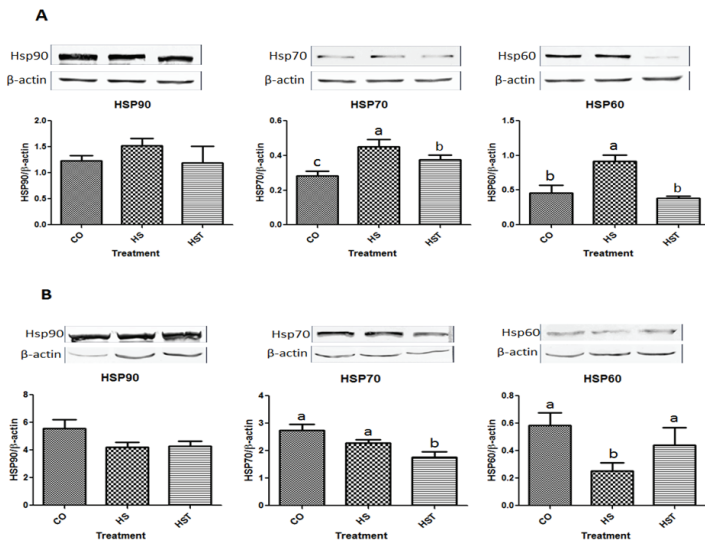
### Effect of heat stress on protein expression

In the liver, the protein expression of HSP70 and HSP60 was significantly higher in the HS group compared with the CO and HST groups ( $p < 0.05$ ) and





as lower in the HST group than the HS group, while HSP90 was similar among groups ( $p>0.05$ ) (Figure 4A). In the muscle, HSP70 expression was significantly lower in the HST group compared with the CO and HS groups, whereas HSP90 expression was not different among the groups (Figure 4B). However, HSP60 muscle expression was dramatically lower in the HS group with the HST group ( $p<0.05$ ).



**Figure 4** – Western blot of heat shock protein (HSP) expression. A: liver tissue, B: muscle tissue. Values are expressed as mean $\pm$ SE from individual tissue samples ( $n = 6$ ). Means with different superscripts differ significantly ( $p<0.05$ ).

## DISCUSSION

Final body weight was significantly lower in the HS group than in the CO group ( $p<0.05$ ), as previously found by Sohail *et al.* (2012) and Deeb & Cahaner (2002), who reported 10% body weight loss in broiler chickens exposed to chronic heat stress. The body weight of the taurine-supplemented group (HST) submitted to heat stress was higher (9.0%) than that of the HS group ( $p<0.05$ ), in agreement with Shim *et al.* (2006), who determined markedly higher body weight gain of broilers exposed to chronic heat stress of 34°C and fed dietary taurine compared with those not fed taurine. Similar to final body weight, significantly liver weight reduction was observed in broilers submitted to heat stress. These findings are consistent with those of Felver-Gant *et al.* (2012), who reported low liver weight in laying hens exposed to chronic heat stress. Higher liver weights were determined in the CO group than HS and HST groups; however, Shim *et al.* (2006) demonstrated that 0.8% taurine added to chickens under chronic heat stress had a high liver weight. Significantly lower liver/body weight ratios were determined in the HST group during the entire period

of heat stress exposure than in the other groups. Plavnik & Yahav (1998) previously observed that heat stress reduced relative liver weight due to decreased metabolic needs.

To our knowledge, this is the first study on the HSP gene and protein expressions in the liver and muscle tissues of broilers receiving taurine when exposed to heat stress. In general, all cells respond to increased temperature by rapid gene transcription and subsequent mRNA translation, yielding highly-conserved HSPs for their protection from damage caused by heat stress (Locke and Noble, 1995). However, previous reports on cellular mechanisms during long-term thermal conditions are contradictory and require further clarification.

In the present study, liver, heat stress increased the liver mRNA expressions of HSP60, HSP70, and HSP90 genes. Earlier studies reported higher expressions of HSP70 and HSP90 genes after heat stress. In broilers, acute heat stress enhanced HSP70 activation in the liver, the heart, the kidneys, and the lungs (Sun *et al.*, 2007; Yu, 2009) and HSP90 in the liver and the heart (Lei *et al.*, 2009; Yu *et al.*, 2008). In a study with ducks, HSP90 gene expression was increased in the liver by acute but limited heat stress after chronic heat stress (Wang *et al.*, 2013). However, lower HSP gene expressions were determined in the liver and muscle tissues of the taurine-supplemented group submitted to heat stress. Xie *et al.* (2014) reported that acute and chronic types of heat stress increased protein oxidation, but HSP gene expressions remained unaltered in muscles. Chen *et al.* (2014) demonstrated that HSP70 gene expression is tissue-dependent, with the highest expression determined in the muscles, followed by the liver and the heart during cold stress. In heat-stress chickens, HSP expression is also tissue-dependent (Gan *et al.*, 2013; Leandro *et al.*, 2004; Rajkumar *et al.*, 2017; Zhen *et al.*, 2006), as found in the present study, where liver tissues are more responsive than muscle tissues. Blake *et al.* (1990) stated that different tissues respond differently to heat stress, which corroborates our own findings. Nakai & Morimoto (1993) argue that tissue-dependent responses may be related to differences in the heat-shock-factor levels and that HSFs are responsible for the activation of heat shock elements under stress conditions (Mizuno *et al.*, 1997).

Taurine osmoregulatory activity appears to be an important determinant of cell survival. During heat stress, the birds' body cells are subjected to osmotic stress. During hyperthermia, such as exposure to



high ambient temperatures (heat stress), taurine and GABA may down regulate body temperature. Cells do not tolerate extreme variations in their sizes, which is why they possess volume-regulatory mechanisms to counteract the consequences of osmotic stress and to normalize their volumes. In most cell types, changes in taurine uptake and release contribute to the standardization of cell volumes. In the heart and the muscles, the taurine turnover is usually very slow. Yet, myocyte taurine content can be dramatically altered by changes in osmotic stress. However, there are still several unresolved issues regarding the role of taurine osmoregulatory actions in the muscles and the heart. Nonetheless, our present study and previous investigations demonstrate that the liver is the most and muscles are the least responsive to high-temperature challenges in HSP gene activation and protein expression, while taurine supplementation lowers both.

In conclusion, under heat stress, the synthesis of most proteins is delayed, but a group of highly-conserved proteins (heat shock proteins) is rapidly synthesized in broilers. The mRNA expressions of HSP90, HSP70, and HSP60 in liver and muscle tissues of broilers increased during heat exposure, as well as the expression of their proteins in the liver. The exposure of broilers to high temperature had negative effects but taurine supplementation improved their heat tolerance, as evidenced by the higher final body weights and reduced expressions of HSP genes and their protein expressions. The effect of taurine supplementation on the expression of HSPs was not evaluated in broilers maintained at thermal neutral conditions in the present experiment. Additionally, mRNA expressions of HSPs were tissue-dependent. The mRNA and protein expression levels of the evaluated HSPs were different in the liver and muscle tissues, which indicate that different organs respond differently to heat stress. This experiment suggests that taurine supplementation may be used in poultry industry to reduce heat stress. However, the mechanisms and the extension of taurine protection of HSPs in broilers are not fully known and require further research.

## ACKNOWLEDGEMENTS

This work was supported by the Basic Science Research Program, through the National Research Foundation of Korea (NRF) funded by Ministry of Education (Project No. NRF2017R1D1A3B03028490).

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