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Effect of Ascorbic Acid on mRNA Expression of HSP70 Gene in WLH Egg Type Growers During Heat Stress

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ABSTRACT

Meteorological factors such as high ambient temperature and high relative humidity exert adverse effects on poultry production. Heat stress results in poor growth performance, feed efficiency, egg production and higher mortality. The aim of the experiment was to explore and quantify the relative mRNA expression of HSP70 gene in relation to ascorbic acid supplementation in White Leghorn (WLH) egg type growers exposed to heat stress. A total of 96 WLH egg type growers of 10 weeks age, were randomly divided and maintained in controlled comfort (26±1.0°C) condition and heat stressed (40±5.0°C) conditions. Each group was divided into four subgroups with twelve birds each in two replicates for homogeneity of experimental design. G1 was designated to control group whereas, G2, G3 and G4 group was supplemented with 100, 200 and 300 mg ascorbic acid (AA) respectively. Relative expression analysis of HSP70 in liver tissue was done on day 42nd of the experiment using RT-PCR technique. The mRNA expression in egg type grower birds was significantly ($p<0.01$) down-regulated in all the treatment groups as compared to control group in both comfort and heat stressed condition. In comfort condition, maximum down-regulation (0.32 fold) was found in G3 group followed by G4 group (0.43 fold) as compared to control group. Similarly, in heat stressed condition, maximum down-regulation (0.31 fold) was found in G3 group, followed by G4 group (0.56 fold) in comparison to control group. The present investigation reveals that supplementation of ascorbic acid on the expression patterns of HSP70 gene provide an indication that AA may be useful in combating rigors of heat stress in chickens.

Keywords: mRNA expression, HSP70 gene, ascorbic acid, white leghorn, egg type growers

All living organisms possess surveillance and homeostatic mechanisms to adjust the demand of growth, differentiation and environmental stress. However, under certain circumstances, these mechanisms fail to adequately respond to imbalance and results in the accumulation of the mis-folded proteins inside the cell. To adapt to these environmental challenges and survive different types of injuries, cells have evolved networks of different responses which detect and control diverse form of stress. One of these responses, known as the heat shock responses (HSR) has attracted a great deal of attention as a universal fundamental mechanism necessary for cell survival under a variety of unfavorable conditions. The heat shock response is transient and lasts only a few hours (Lindquist, 1992). This phenomenon of HSR is a very well conserved regulatory network across all eukaryotes and is triggered by the synthesis of a group of proteins (Amrutkar *et al.,* 2014). In prokaryotic and eukaryotic cells, the synthesis of specific stress proteins increases under a wide variety of stress conditions. The most extensively investigated stressors is heat stress in which, a sudden increase in temperature induces the synthesis of heat shock proteins (HSPs). The role of HSPs in the protection of cells from heat stress is well established (Burdon, 1986). HSPs work as molecular Chaperones. Heat shock can induce the expression of specific stress-related genes, including heat shock protein genes that are translated into HSP to provide protection against the subsequent cellular injuries to cells and tissues (Hightower, 1991).

Substantial attention has been paid to the role of nutritional additives to minimize the effects of heat stress in poultry birds. The withholding of feed as well as the manipulation of dietary protein content, energy density, calcium, use of carbonated water and usage of vitamin C and E are believed to alleviate the effects of heat stress (Lee, 1992). The most significant increase in ascorbic acid demand take place during acute environmental stress such as excessive hot or cold weather. Stress increases the metabolic need for this vitamin or that decrease the innate capacity of biosynthesis. Under such conditions, supplementing the poultry diet with vitamin C may have a beneficial effect on performance (Konca *et al.,* 2009).

MATERIALS AND METHODS

The research was carried out in the Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur (M.P.). A total of 96 WLH egg type growers of 10 weeks age were randomly divided into eight groups in the experiment.

Four group of birds was maintained in natural summer conditions (May to June) maintained in heat stress (40±5.0°C) ambience, whereas other four group of birds was maintained in controlled conditions at 26±1.0°C (comfort temperature) using an air conditioner. Temperature and humidity of the experimental poultry unit was recorded using a digital temperature and humidity recorder. G1 group was taken as control, whereas, G2, G3 and G4 groups were supplemented with 100 mg, 200 mg and 300 mg AA in feed respectively in both comfort and heat stressed condition. Diets were formulated as per NRC specifications.

HSP70 expression analysis studies

Total RNA was isolated from the liver following standard TRIzol method (Oliveira *et al.,* 2012). The purity of RNA was checked before the preparation of first- strand cDNA. Prepared cDNA was stored at -20°C and later used for HSP70 gene expression studies. Expression of HSP70 gene was quantified using gene specific primer pairs using Real–Time PCR. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a reference gene.

RNA extraction

The birds were sacrificed following the appropriate standard procedure. Aseptically, liver tissue samples were collected from both heat stressed and comfort broiler and egg type grower birds for isolation of RNA. Total RNA was isolated from liver samples of birds using TRIzol reagent (Sigma-aldrich, USA).

First strand cDNA synthesis

The first strand cDNA was synthesized using RevertAidTM first strand cDNA synthesis kit (MBI Fermentas).

Primers

Primers for heat shock protein 70 gene (HSP70) and Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; used as housekeeping gene) were prepared as per the method described by Sun *et al.* (2007) and Jimian and Endong (2008) (Table 1).

Table 1: Sequence of gene specific primers for HSP70 and GAPDH

Sl. No	Gene	Primers	Annealing Temp.	Product Size (bp)
1	HSP70	F- AGCGTAACAC CACCA TTCC R- TGGCTCCCAC CCTAT CTC	58 ºC	372
2	GAPDH	F- TGAAAGTCGG AGTCA ACGGAT R- ACGCTCCTGG AAGAT AGTGAT	58 ºC	230

PCR reaction mixture

Readymix Taq PCR Reaction mix with $MgCl_2$ (Sigma Aldrich, U.S.A.) was used to prepare PCR reaction mixture of 20 µl (2 X ReadyMix Taq PCR Reagent Mix - 10 µl, forward primer (10 pM)- 0.1 µl, reverse primer (10 pM)- 0.1 µl and cDNA 1 µl) total volume was prepared and run in the thermal cycler (Bio-Rad laboratories Inc. USA). The PCR protocol (Table 2) designed for 35 cycles was kept same for both the primers used. The PCR products were tested for amplification of specific gene by agarose

gel electrophoresis using 2.0% agarose gel (Fig. 3 and 4) in 1x Tris Acetate EDTA Buffer (Sigma-Aldrich, U.S.A.).

Table 2: PCR standardization protocol

Sl. No.	Steps		HSP70	GAPDH
1	Initial Denaturation	Temperature	94°C	94°C
		Time	10 min	10 min
2	Denaturation	Temperature	94°C	94°C
		Time	1 min	1 min
3	Annealing	Temperature	58°C	58°C
		Time	45 sec	45 sec
4	Extension	Temperature	72°C	72°C
		Time	1 min	1 min
5	Final Extension	Temperature	72°C	72°C
		Time	10 min	10 min
6	Hold	Temperature	4°C	4°C
		Time	∞	∞

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR/Real Time PCR)

The relative expression of gene specific mRNA was quantified by qRT-PCR/Real-time PCR employing SYBR green chemistry (CFX Connect Real-time System, Bio-Rad laboratories Inc. USA).

Relative quantification

Comparative CT method (Livak and Schmittgen, 2001) was used for relative expression of target gene in the test sample (treatment G2, G3 and G4) relative to that of control sample (calibrator- G1). The relative expression of target genes was estimated in term of fold change in mRNA expression, using the following formula:

Fold change in expression of target gene = $2^{-\Delta\Delta CT}$

where,

ΔΔ CT = Δ CT test - ΔCT control/calibrator

Δ CT test = CT target gene - CT reference gene (In test / treatment group)

ΔCT control/calibrator = CT target gene – CT reference gene (In control/calibrator group)

where,

CT target gene = mean of the cycle threshold (CT) values of the gene being tested

CT reference gene = mean of the CT value of the housekeeping gene GAPDH

The recorded data was statistically analyzed using Completely Randomized Design. Various conditions and treatment groups were compared by using Duncan Multiple Range test (DMRT).

RESULTS AND DISCUSSION

The mRNA expression levels of HSP70 gene on day 42, was estimated in the liver samples of egg type grower birds supplemented with varying concentration of ascorbic acid (Table also depict calues recorded for birds under heat stress) Table 3 and Fig. 1 and 2.

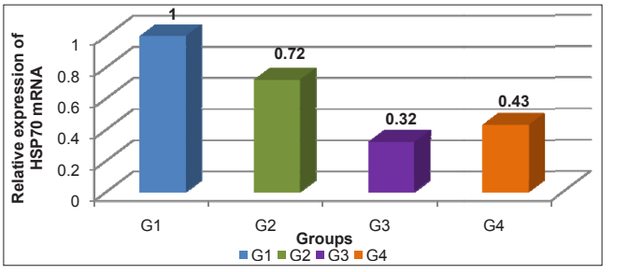

Fig. 1: Effect of ascorbic acid on mRNA expression of HSP70 gene in liver sample of egg type growers during comfort condition on day 42 of experiment

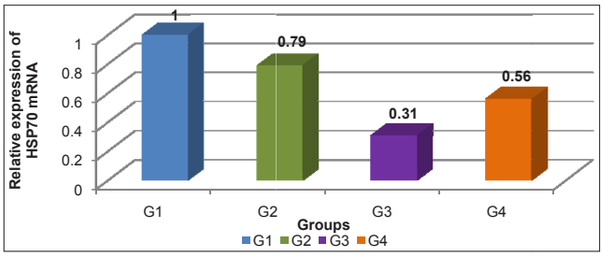

Fig. 2: Effect of ascorbic acid on mRNA expression of HSP70 gene in liver sample of egg type growers during heat stressed condition on day 42 of experiment

The mRNA expression level of HSP70 on day 42, was significantly ($p<0.01$) down-regulated in all the treatment groups as compared to control group in both comfort and

Table 3: mRNA expression of HSP70 gene in egg type grower birds

Birds	Condition	G1		G2		G3		G4	
		Δ Ct	$2^{-\Delta\Delta Ct}$	Δ Ct	$2^{-\Delta\Delta Ct}$	Δ Ct	$2^{-\Delta\Delta Ct}$	Δ Ct	$2^{-\Delta\Delta Ct}$
Egg type grower	Comfort	06.35^{D} ± 0.03	1	06.83^{C} ±0.04	0.72	08.00^{A} ±0.08	0.32	07.57^{B} ±0.04	0.43
	Heat	06.43^{D} ±0.02	1	06.77^{C} ±0.01	0.79	08.14^{A} ±0.02	0.31	07.28^{B} ±0.04	0.56

Means bearing different superscripts (^{ABCD}) within same row differ significantly ($p<0.01$).

Comfort (26±1º C), Heat (40±5º C)

G1 (Control), G2 (100 mg AA), G3 (200 mg AA), G4 (300 mg AA)

heat stressed condition. In comfort condition, maximum down-regulation (0.32 fold) was found in G3 group, supplemented with 200 mg AA, followed by G4 group (0.43 fold) supplemented with 300 mg AA. In heat stressed condition, expression level of HSP70 gene was significantly ($p<0.01$) lower in 200 mg AA comparison to group supplemented with 100 mg and 300 mg AA.

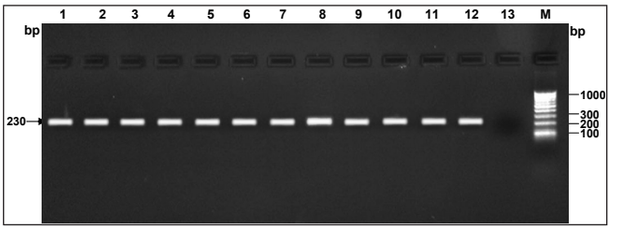

Fig. 3: Amplified PCR product (Lanes: 1-12) of GAPDH gene electrophoresed on 2% agarose gel, M: 100bp DNA ladder

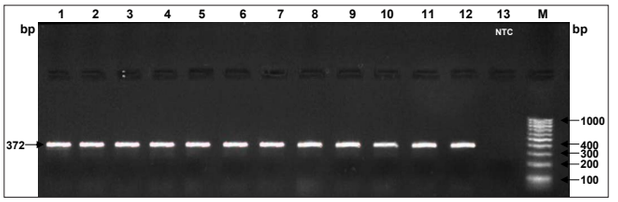

Fig. 4: Amplified PCR product (Lanes: 1-12) of HSP70 gene electrophoresed on 2% agarose gel, M: 100bp DNA ladder

Similarly, in heat stressed condition, maximum down-regulation (0.31 fold) was found in G3 group, supplemented with 200 mg AA, followed by G4 group (0.56 fold) supplemented with 300 mg AA. Moreover, between the treatment groups, the expression level of HSP70 gene was significantly ($p<0.01$) lower in 200 mg AA comparison to group supplemented with 100 mg and 300 mg.

The mRNA expression level of HSP70 on varying concentrations of AA supplementation at day 42, in the liver sample of egg type growers was significantly down-regulated in all the treatment groups as compared to control group in both comfort and heat stressed condition. Similar reports were enunciated by Mahmoud *et al.* (2003), who reported lower expression of HSP70, indicated less of a stress response in the AA-fed chickens. As per present findings, maximum down-regulation was found in group supplemented with 200 mg AA. It has been documented that HSP70 accumulation is associated with thermotolerance as measured by survival rate at a higher temperature. Heat stress preconditioning has been shown to be correlated with acquired thermotolerance. Wang and Edens (1993) reported that longer preconditioning time was associated with lower expression of HSP70 mRNA. This observation has been interpreted to mean that the development of tolerance to a stressor requires a greater stimulus to elicit a stress response in a greater magnitude (Wang and Edens, 1994). The results of the present study showed that AA-fed chickens have a lower HSP70 response to heat stress. Thus, AA-fed chickens appear to be better prepared for heat stress by having a higher threshold for HSP70 induction.

Felver-Gant *et al.* (2012) reported that HSP70 concentrations in the liver were greater in hens exposed to HS ($p<0.05$), which is in disagreement to our reports. Felver-Gant *et al.* (2014) reported that the concentrations of HSP70 increases in antioxidant treated WLH hens ($p<0.01$) exposed to HS. Similarly, HSP70 mRNA

expression tended to increase in treated hens, which is in contradiction to our findings. The possible mechanism for such reduction in mRNA expression might be due to AA, which is functionally involved in regulating steroid synthesis in the adrenals. Kitabchi (1967) hypothesized that high adrenal AA levels inhibit steroidogenesis. They postulated that adrenal AA functions down-regulate plasma corticosterone. Wang and Edens (1993) reported that corticosterone up-regulated HSP70 expression response of broiler cockerels. In the current study, corticosterone was measured and the HSP70 responses were compliant with aforementioned observations by Wang and Edens (1993) and Pardue *et al.* (1985). Dietary AA supplementation reduced the circulating levels of corticosterone in the heat-stressed chickens (Pardue *et al.,* 1985) and in accordance with Wang and Edens (1993) this could result in a decrease in the HSP70 expression. Wang and Edens (1993) reported that longer preconditioning time was associated with lower expression of HSP70 mRNA. This observation has been interpreted to mean that the development of tolerance to a stressor requires a greater stimulus to elicit a stress response in a greater magnitude (Wang and Edens, 1994). The results of the current study showed that AA-fed chickens have a lower HSP70 response to heat stress. Thus, AA-fed chickens appear to be better prepared for heat stress by having a higher threshold for HSP70 induction.

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