Original Research

Effect of Ascorbic Acid and Alpha-Tocopherol on Expression and Quantification of Heat Shock Protein 70 (HSP70) by Western Blotting during Heat Stress in Commercial Broilers

Amir Amin Sheikh[1*], Aditya Mishra[1], Rakshanda Bhagat[2], Kailash Kumar[2], Jaan Mohammad Wani[3] and Gowher Gull Sheikh[4]

[1]Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur-482001, Madhya Pradesh, INDIA
[2]Division of Veterinary Medicine, F.V.Sc & A.H., Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, R. S. Pura, Jammu-180009, Jammu and Kashmir, INDIA
[3]Division of Veterinary Gynaecology and Obstetrics, F.V.Sc &A.H., Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu , Jammu and Kashmir, INDIA
[4]Division of Animal Nutrition, F.V.Sc & A.H., Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shuhama, Alusteng, Srinagar-190006, Jammu and Kashmir, INDIA

***Corresponding author:** amirsheikh3468@gmail.com

Rec. Date:	Jan 06, 2018 14:01
Accept Date:	Sep 10, 2018 16:06
DOI	10.5455/ijlr.20180106020131

Abstract

The present study was carried to investigate the effect of ascorbic acid and alpha-tocopherol on expression and quantification of heat shock protein 70 (HSP70) during heat stress in commercial broilers by western blotting. For this study a total number of 168 day old broiler birds were randomly divided into fourteen groups and out of these seven groups were maintained at 37±5.0°C (heat stressed) and other seven groups were maintained at 26±1.0°C (comfort); each group consist of 12 birds. G1 (comfort) and G1 (heat) group was kept as control. Whereas G2, G3, G4 groups were supplemented with 100 mg, 200 mg and 300 mg ascorbic acid and G5, G6 and G7 were supplemented with 100 mg, 200 mg and 300 mg alpha-tocopherol respectively. On western blot expression analysis it was found that maximum HSP70 expression was observed in control i.e. G1 group and minimum HSP70 expression was observed in 200 mg ascorbic acid and 300 mg alpha-tocopherol supplemented groups. Therefore supplementation of 200 mg of ascorbic acid and 300 mg of alpha-tocopherol may be beneficial to alleviate the rigors of heat stress.

Key words: Alpha-Tocopherol, Ascorbic Acid, Broilers, Heat Stress, HSP70, Western Blotting

How to cite: Sheikh, A., Mishra, A., Bhagat, R., Kumar, K., Wani, J., & Sheikh, G. (2019). Effect of Ascorbic Acid and Alpha-Tocopherol on Expression and Quantification of Heat Shock Protein 70 (HSP70) by Western Blotting During Heat Stress in Commercial Broilers. International Journal of Livestock Research, 9(1), 343-349. doi: 10.5455/ijlr.20180106020131

Introduction

Meteorological factors such as high ambient temperature and high relative humidity exert adverse effects on poultry production. They also cause heat stress in poultry during the hot dry season (Ayo *et al.,* 2014). The high ambient temperature in sub-tropical climate leads to heat stress poultry, broilers in general and relatively lesser extent in layers. Heat stress results in poor performance in growth, feed efficiency and meat yield as well as higher mortality. HSPs work as molecular chaperones. The induction of heat shock protein (HSP70) has also been observed in variety of cells in response to heat stress, diseases and other cellular insults. 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. An important aspect of HSPs is that organisms which have reversed from previous mild stressful conditions expresses elevated level of stress proteins. Living organisms respond to changes in environmental temperature by activation of physiological mechanisms involved in heat loss or production, but the activation of these responses occur shortly and they are blocked to the non-stressing physiological state. If the animal or the cell is not able to overcome or adapt these environmental changes, homeostasis may be compromised and even death may occur. The withholding of feed, as well as the manipulation of dietary protein content, energy density, and calcium, use of carbonated water and usage of vitamin C and E are the practices believed to alleviate the effects of heat stress (Pardue and Taxton, 1986).

The extent of the deleterious effects of heat stress is determined not only by its magnitude but also by the status of cellular systems, particularly the cellular antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and water or lipid soluble antioxidants, such as ascorbic acid (AA) and alpha-tocopherol (vitamin E) (Pigeolet *et al.,* 1990). Hyperthermia is the most widely investigated phenomenon affecting the induction of heat shock protein. 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 cells and tissues (Hightower, 1991). Thus, this experiment focused on the influence of ascorbic acid and alpha-tocopherol supplementation during cyclical heating episodes on expression of HSP70 in broiler chickens.

Material and Methods

The proposed 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.), India.

Reagents, Chemicals and Equipments

The reagents and chemicals from standard manufacturers like Fermentas, Quiagen, Sigma Aldrich, Genxbio, Bio-Rad and Invitrogen were used. Standard protein marker (MBI- Fermentas), resolving gel and

DOI 10.5455/ijlr.20180106020131

staking gel kit (Sigma-Aldarich), Diaminobenzidine tablets (Sigma-Aldrich), protein extraction kit (Sigma-Aldarich), liquid nitrogen, Tween-20 (Sigma-Aldarich), skimmed milk powder (Premier milk foods, India), HSP70 primary and secondary antibodies (Santa Cruz, USA). Western blot apparatus (Bio-Rad, India), cooling micro-centrifuge (3500 Table-top micro refrigerated centrifuge, Cubota Corporation, Tokyo, Japan), tissue homogenizer (Polytron, Kinematica AG, Switzerland), micropipettes (Eppendorf AG, Germany), laminar air-flow apparatus (Tanco, India) were used in the study.

Birds and Husbandry

For this study a total number of 168 day old broiler birds were randomly divided into fourteen groups and out of these seven groups were maintained at 37±5.0°C (heat stressed) and other seven groups were maintained at 26±1.0°C (comfort); each group consist of 12 birds. G1 (heat) and G1 (comfort) group was kept as control. Whereas G2, G3, G4 groups were supplemented with 100 mg, 200 mg and 300 mg ascorbic acid and G5, G6 and G7 were supplemented with 100 mg, 200 mg and 300 mg alpha-tocopherol respectively. Diets were formulated as per NRC, 1994.

HSP70 Expression Analysis

Liver samples were collected on 45th day of the experiment and expression profile analysis of HSP70 in liver tissue was done on day 46th of the experiment. Cytosolic protein was extracted and quantified as described by Mahmoud *et al.* (2004). SDS-PAGE analysis was carried out in a vertical maxigel electrophoresis apparatus. Glass plates were cleaned and set in a gel moulding tray of electrophoresis apparatus with bottom and sides sealed with agarose (Towbin *et al.*, 1979). Gradient separating gel (7.4-15%) solution was poured between the two SDS-PAGE glass plates and 1 ml DDW was poured over the gel. After polymerization, DDW was removed by tilting the plates and 7 ml of 3% stacking gel was poured over the separating gel and later on suitable comb was inserted. The polymerization gel was mounted into the electrophoresis chamber and buffer reservoir was filled with 1X Tris glycine buffer. Before loading, 30µl sample buffer was added to each sample. The samples was boiled for 10 minutes and kept on ice. The samples were briefly centrifuged before loading. A standard protein marker (MBI- Fermentas) was included along with the samples. Electrophoresis was carried out at a constant current of 150 V and 150 mA, until the tracking dye reached the bottom of the gel. The gel was removed from the plates and stained with Coomassie brilliant blue for 1 hour and then destained with de-staining solution.

HSP70 protein was characterized by Western blot analysis in order to confirm specificity. The protein was run in 7.5-14% gradient SDS-PAGE gel along with pre-stained protein marker. After electrophoresis the gel was taken out from the plates and was kept in Western blot buffer. Four Whatman filter papers, PVDF membrane (Sigma, USA), SDS-PAGE gel, and four Whatman filter papers were stacked in respective order one by one on the anode plate of blotting apparatus (ATTO, Japan) after soaking with the Western blot

 DOI 10.5455/ijlr.20180106020131

buffer. Care was taken for avoiding air bubbles. The complete stack was saturated with ice cold transfer buffer before the cathode plate was placed in position over the stack and a current of 0.8mA/cm^2 was applied for 1hr. After the transfer, the gel was stained to check the efficiency of transfer of protein from gel to the membrane and the membrane was subjected to immunological detection. The membrane, after transfer, was incubated over night at 4˚C in 1% bovine serum albumin diluted with PBS. After blocking, the membrane was washed thrice with PBS-T (PBS+0.01% Tween 20) for 5 minutes each and incubated with HSP70 primary antibody 1:100 dilution (Santa Cruz, USA). After incubation, membrane was washed thrice with PBS-T (PBS+0.01% Tween 20) for 5 minutes each then added anti-rabbit goat IgG HRP (Horse Radish Peroxidase) conjugate for 1hr at 37^{0}C. After washing, the antigen antibody reaction was detected by incubating the membrane with substrate diaminobenzidene tetrahydrochloride (DAB).

The colour reaction was terminated by washing the membrane with distilled water to prevent background coloration. The band signals corresponding to the increasing concentrations of HSP70 protein was evaluated by densitometry and expressed as the ratio between the density of each band and the mean density of the reference standard, which was analysed in triplicate. HSP70 concentrations were then plotted against the density ratio of each concentration and a standard curve was constructed (Givisiez *et al.*, 1999). Density ratios between samples and the specific reference standard was calculated, and then the concentrations was determined according to the standard curve and expressed as ng HSP70/µg total protein.

Result and Discussion

Western blot results reveals that total protein expressed in ng/µg of total protein in liver samples was maximum in control group in both the conditions (heat stressed and comfort) shown in Table 1 and Fig. 1. However, minimum concentration of (2.682 ng/µg of total protein) HSP70 protein was observed in G3, which was supplemented with 200 mg ascorbic acid followed by G4 and G2 in both the conditions. In the vitamin E supplemented groups the total protein expressed in ng/µg of total protein in liver samples was maximum in control group in both the conditions. However, minimum concentration of (2.791 ng/µg of total protein) HSP70 protein was observed in G7, which was supplemented with 300 mg vitamin E followed by G6 and G5 in both the conditions.

Table 1: HSP70 expression analysis in liver tissue (ng/µg total protein) of experimental broilers

Birds	Condition	G1	G2	G3	G4	G5	G6	G7
Broilers	Comfort	4.524	3.261	2.682	3.091	3.293	3.123	2.791
	Heat	6.298	4.668	3.016	3.876	4.718	3.94	3.106

Comfort (26±1°C), Heat (37±5°C); G1 (Control), G2 (100 mg AA), G3 (200 mg AA), G4 (300 mg AA), G5 (100 mg vitamin E), G6 (200 mg vitamin E), G7 (300 mg vitamin E).

DOI 10.5455/ijlr.20180106020131

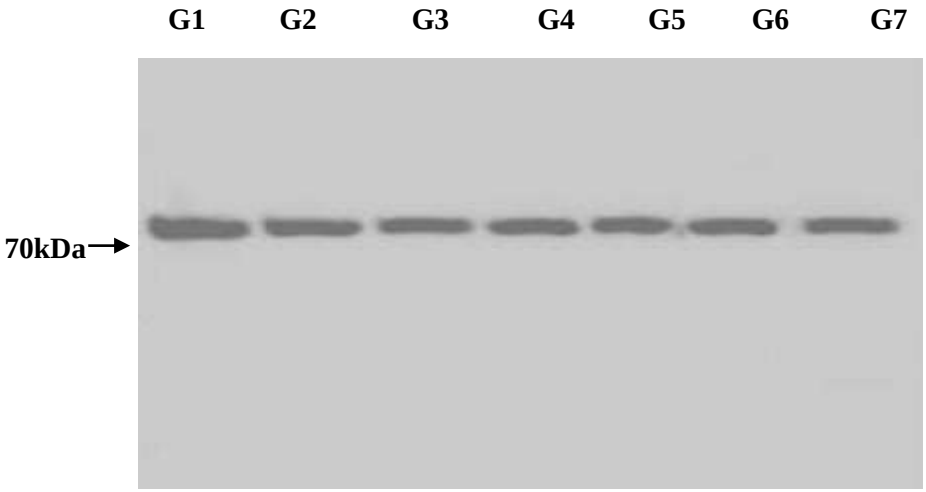

Fig 1: Detection of HSP70 in the liver samples of experimental broiler birds supplemented with varying concentrations of ascorbic acid and vitamin E by Western blotting. G1 to G7 broiler tissue sample.

In western blot analysis, the 70kd HSP70 protein was detected in all the treatment as well as in control group in AA supplemented groups. On density ratio calculations using standard curve the HSP70 concentrations was expressed as ng /µg total protein. In G3, the lowest concentration of HSP70 of 2.682 ng /µg total protein was observed in liver samples as compared to control group of comfort condition. Whereas, the lowest concentration of HSP70 of 3.016 ng /µg total protein was observed in liver samples of G3 during heat stress condition. Also, the 70kd HSP70 protein was detected in all the treatment as well as in control group in vitamin E supplemented groups. On density ratio calculations using standard curve the HSP70 concentrations was expressed as ng /µg total protein. In G7, the lowest concentration of HSP70 of 2.791 ng /µg total protein was observed in liver samples as compared to control group of comfort condition. Whereas, the lowest concentration of HSP70 of 3.106 ng /µg total protein was observed in liver samples of G7 during heat stress condition.

The expression of HSP70 was consistently higher in the N-AA group than in the AA-fed chickens throughout the cyclic heat stress episodes which are in accordance to present findings (Mahmoud *et al.*, 2004). This delayed response might be due to heat stress acclimation and could be indicative of acquired thermotolerance (Wang, 1992). Previous reports have shown that thermotolerance is associated with HSP70 induction and heat conditioning resulted in an increase in the expression of HSP70 when animals were further challenged with more stressful stimuli (Criag and Gross, 1991). Increased HSP70 induction may enable cells to recover from previous stressors and provide them with a transitory degree of protection (Li, 1995). It is well documented that AA supplementation alleviates the effects of heat stress (Pardue *et al.*, 1985) and previous studies have shown that the level of HSP70 induction is associated with improved

DOI 10.5455/ijlr.20180106020131

thermotolerance (Wang, 1992). Although these two well-established observations might appear to be in contradiction, the explanation stems from the physiological function of AA and factors that induce HSP70.

Conclusion

Thermal stress not only reduces the production and reproductive performances but also causes higher mortality in poultry resulting considerable economic losses. The most significant increase in vitamin C and vitamin E demand take place during acute environmental stress such as excessive hot or cold weather and stress conditions increases the metabolic need for this vitamin. Under such conditions, supplementation of diet with vitamin C and vitamin E may have a beneficial effect. On the basis of findings it was concluded that relative expression analysis of HSP70 in the liver samples of broilers enunciates that maximum decrease is observed by feeding of 200 mg ascorbic acid, followed by 300 mg ascorbic acid supplemented group. Similar results were found in the liver samples of broilers supplemented with 300 mg vitamin E followed by 200 mg vitamin E. This further indicates reduction in cellular stress by ascorbic acid as well as by vitamin E.

Acknowledgements

The authors would like to acknowledge, M. P. Biotechnology Council, Bhopal (M.P) for providing financial assistance for the research project.

References

1. Ayo JO, Egbuniwe I, Kawu MU and Sinkalu VO. 2014. Ameliorative effects of betaine and ascorbic acid administration to broiler chickens during hot-dry season in Zaria: A review. *African Journal of Biotechnology*.13: 2295-2306.
2. Craig EA and Gross CA. 1991. Is hsp70 the cellular thermometer. *Trends in Biochemical Sciences*.16: 135-140.
3. Givisiez PE, Ferro JA, Ferro MI, Kronka SN, Decuypere E and Macari M. (1999). Hepatic concentration of heat shock protein 70 kD (Hsp70) in broilers subjected to different thermal treatments. *British Poultry Science*, 40: 292-296
4. Hahn GM and Li GC. 1990. Thermotolerance, thermo-resistance and thermo-sensitization. In: Morimoto RI, Tissieres A and Georgopolous C. Stress Proteins in Biology and Medicine. 5th Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, pp 79–100.
5. Hightower LE.1991. Heat shock, stress proteins, chaperones and proteotoxicity. *Cell*,66: 191-197.
6. Li GC, Mivechi NF and Weitzel G. 1995. Heat shock proteins, thermotolerance and their relevance to clinical hyperthermia. *International Journal of Hyperthermia*.11: 459–488.
7. Mahmoud KZ, Edens FW, Eisen EJ and Havenstein GB. 2004. Ascorbic acid decreases heat shock protein 70 and plasma corticosterone response in broilers (*Gallus domesticus*) subjected to cyclic heat stress. *Comparative Biochemistry and Physiology*. Part B.137: 35-42.
8. NRC. 1994. Nutrient Requirement of Poultry 9th Rev Edn., National Research Council, National Academy Press, Washington, DC, 20418.
9. Pardue SL and Thaxton JP. 1986. Ascorbic acid in poultry: a review. *World Poultry Science Journal*. 42: 107-123. .

DOI 10.5455/ijlr.20180106020131

10. Pardue SL, Thaxton JP and Brake J. 1985. Role of ascorbic acid in chicks exposed to high environmental temperature. *Journal of Applied Physiology*. 58: 1511-1516.
11. PigeoletE, Carbisier P, Houbion A, Lambert D, Machiels C and Raes M. 1990. Glutathione peroxidase, superoxide dismutase and catalase inactivation by peroxides and oxygen derived free radicals. *Mechanism of Ageing and Development*.51: 283-297.
12. Towbin H, Staehelin T and Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *National Academy of Sciences of the United States of America*, 76(9): 4350–4354.
13. Wang S. 1992.Steroidal modulation of gene expression for heat-shock proteins in domestic chickens, Ph.D. Dissertation, The Graduate School, North Carolina State University, Raleigh, North Carolina.

DOI 10.5455/ijlr.20180106020131