



## OVEREXPRESSION PATTERN OF HEAT SHOCK PROTEIN-70 IN JEJUNAL TISSUE OF BROILER CHICKEN UNDER ACUTE HEAT STRESS

<sup>a</sup>Sudhir Kumar Jaiswal\*, <sup>a</sup>Tyagi, J.S., <sup>a</sup>Meesam Raza<sup>1</sup>, <sup>b</sup>Ajay Chaturvedani, <sup>b</sup>Veerendra Kumar, <sup>c</sup>Leena Dilliwar, <sup>a</sup>Division of Poultry Science, <sup>b</sup>Division of Veterinary Extension, <sup>c</sup>Division of LES & IT, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P. 243122

\*Corresponding author email- sjsudhirjaiswal009@gmail.com

### ABSTRACT

The objective of this study was to investigate the over-expression pattern of heat shock protein 70 (HSP-70) under acute heat-stressed condition in broilers chicken. In total, 180 broilers chicken were injected intraperitoneally with glutamine (0.75 mg/kg of BW) or quercetin (5 mg/ kg of BW). Twenty-four hours later, they were heat stressed for 0, 2, 5, and 10 h respectively, under 40°C. The HSP-70 mRNA expression were obviously elevated at 2 h of heat stress, and glutamine induced the over expression of HSP70 in the jejunal mucosa at different heat-stress times ( $P < 0.05$ ). The present investigation established an HSP-70 enhanced expression in chicken model by glutamine which explicitly explained the HSP-70 expression under acute heat stress conditions.

**KEYWORDS:** Overexpression, Heat Shock Protein, Heat Stress, Jejunum, Broiler Chicken.

### INTRODUCTION

The present scenario of changing climate with increasing environmental temperature, heat stress is considered to be the major cause of loss of production and reduced profit in the poultry production worldwide (Sejian *et al.*, 2012), and highly suffering are the tropical and the subtropical zones. Being a tropical country, Indian poultry sector is also under pressure due to the slow and steady increase in ambient temperature. When living organisms experience thermal stress, the synthesis of most proteins is delayed, but a group of highly conserved proteins known as heat shock proteins or heat stress proteins (HSP) is rapidly synthesized (Al-Aqil and Zulkifli, 2009). These HSP play an important role in the survival of stressed cells and the stabilization of the internal environment (Gabai *et al.*, 1997). The first report of HSP appeared in 1962 after *Drosophila* salivary gland cells were exposed to 37 °C for 30 min and then returned to their normal temperature of 25 °C for recovery, a “puffing” of genes was found to have occurred in the chromosome in the recovering cells (Ritossa, 1962). According to the homology and molecular weights, HSP can be classified into 3 main families: HSP90 (~85–90 kDa), HSP70 (~68–73 kDa), and low molecular weight HSP (~16–47 kDa), (Basu *et al.*, 2002). Among the HSP, HSP 70 is one of the most conserved and important protein families and has been studied extensively (Deane and Woo, 2005; Ming *et al.*, 2010). Severe stress is destructive to the cell, causing cell cycle arrest and leading to apoptosis. Adaptation to stress condition caused by mild stress is usually beneficial to cell. Stress disturbs cell membrane fluidity that may result in activation of different signal transduction pathways, including the Ras single pathways and mitogen activated

protein kinases (MAPK) that among others lead to induction of heat shock responses (Park *et al.*, 2011). Heat stress induced increase in intestinal permeability was associated with massive generalized sloughing of small intestinal epithelial layer from the villus tips and lysis of intestinal epithelial cells, indicating that the increase in intestinal permeability was due to the extensive damage of the epithelial surface (Lambert, 2009). Heat shock protein 70 play important protective role in preventing the heat-induced disruption of intestinal barrier and suggested that HSP-mediated up-regulation of occluding protein expression may be an important mechanism involved in the maintenance of intestinal epithelial barrier function during heat stress (Doklandny *et al.*, 2006). Supplementation of glutamine, a non-essential amino acid has been reported to play an important role in alleviating the heat stress. Glutamine mainly targets small intestine and improves its integrity (Larson *et al.*, 2007 and Wu *et al.*, 1996), enterocyte architecture and population, activation of HSP-70 genes that ultimately ends with better nutrient availability and heat tolerance level to the bird during heat stress conditions. Generally chronic heat stress conditions even though cause mortality and suppress production, they improve the heat tolerance level at later stages. Studies of the protective effects of HSP-70 on the intestine mainly focus on rat, mice, or intestinal epithelial cells in vitro (Ehrenfried *et al.*, 1995; Wischmeyer *et al.*, 1997; Ren *et al.*, 2001; Ohkawara *et al.*, 2006). Although heat stress can induce intestinal injury in broiler chickens (Quinteiro-Filho *et al.*, 2010), yet there is limited information on HSP-70 activity. While acute heat stress levels may cause high mortality hence, it is hypothesized HSP-70 gene expression enhancers like

glutamine would have synergistic effect especially in acute heat stress conditions.

## MATERIALS & METHODS

### Experimental birds

One hundred eighty, day old CARIBRO Vishal broiler chicks were obtained from experimental hatchery section; ICAR-CARI, Izatnagar, India and the chicks were housed in multi-tier brooder cages. The temperature was maintained at 35°C when the chickens were at the age of 1 to 3 d old; afterward, the temperature was gradually reduced until 22°C. All birds were inoculated with an inactivated infectious bursal disease vaccine on d 14 and 21 and with a Newcastle disease vaccine on d 7 and 28. The birds had access to feed and water ad libitum. up to five weeks of age and reared under uniform husbandry conditions. The birds died during the study period were subjected to necropsy examination.

### Experimental design

One hundred eighty CARI-BRO Viashal broilers chicken with similar BW were put into 18 cages (10 birds /cage) and randomly allocated to 4 treatments, including control, enhancer and inhibitor at 21 d of age. There were 6 cages per treatment. These birds were transported into a temperature-controlled metabolic chamber. On 36 days of age, the birds were injected intraperitoneally with 0.5 ml of saline, Glutamine (0.75 mg/kg of BW) and quercetin (5 mg/kg of BW), respectively. After injection, they were put back into their original cages. Twenty four hours later, all of the chickens in their original cages were moved to the environmental chamber set controlled at 40°C. They suffered from acute heat stress fewer than 40 °C for stress times that were respectively 0, 2, 5, and 10 h.

### Sample collection

During the 0, 2, 5, and 10 h in the heat-stress condition, chickens that weighed similarly in each replicate per treatment average were selected and immediately killed by cervical dislocation. Mid segment jejunum samples were dissected out and collected aseptically, in tubes containing RNA *later* solution. The samples were stored at -20 °C till RNA extraction.

mRNA Expression Analysis: The HSP70 mRNA levels in mucosal samples of jejunum were analyzed by quantitative real-time reverse transcription PCR. The total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. One microgram of amount of RNA from each sample was taken as template and first strand cDNA was prepared by "Revert Aid" first strand cDNA synthesis kit (MBI, Fermentas) using random hexamer primers. Both HSP70 and GAPDH were amplified in the SYBR Green reaction mixture from the complementary DNA with specific primers. Housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as endogenous control. The following quantitative real-time reverse-transcription PCR primers were used: GAPDH (K01458) forward 5 -GTGTGCCAACCCCAA TG TCTCT-3, reverse 5 - CAGCAGCCTTCACTACCCT

CT-3; and HSP70 (HM587997) forward 5 -GGCACC ATCACTGGGCTT-3, reverse 5 -TCCAAGCCATAGGCA ATAGCA-3. Amplification was initiated by 2- and 5-min incubation periods at 50 and 95°C, respectively, followed by 40 cycles at 95°C for 30 sec, 55-59 °C for 45 sec and 72°C for 45 sec. Relative standard curves were obtained by plotting the cycle threshold (Ct) obtained after PCR amplification of serial dilutions of a steady quantity of a plasmid containing the corresponding complementary DNA. The PCR products of HSP70 were normalized to the GAPDH level.

### Statistical analysis

All statistical analyses were performed using SPSS software (version 20). Statistical analyses were performed using the one-way ANOVA to estimate the effects of treatment and time, respectively. Differences among means were tested using Duncan's multiple range tests. Means were considered significantly different for values of  $P < 0.05$ .

## RESULTS & DISCUSSION

The mRNA expressions of HSP 70 in jejunal mucosa are represented in Table 1. There was no significant difference in HSP70 mRNA expression before heat stress compared with that of the control; HSP70 mRNA expression was significantly upregulated in the jejunal mucosa of the enhancer group after 2 hour of heat exposure ( $P < 0.05$ ). The mRNA expression of HSP70 in the inhibitor group was only significantly lower than that of the control after 5 h of heat stress ( $P < 0.05$ ). The amounts of HSP70 mRNA in the enhancer were significantly higher than those in the inhibitor and control ( $P < 0.05$ ). A significant difference from heat-stress times on the expression of HSP70 was also detected ( $P < 0.05$ ). In the present study, the acute heat exposure (40°C) model was employed and glutamine (Gln) was used to establish an HSP-70 enhanced expression model in broiler chickens under acute heat stress for varied time periods. The results show significantly increased HSP-70 fold expression in the enhancer group during heat exposure and are in line with the findings of Hao *et al.*, 2012 which indicated Glu enhanced HSP-70 expression under heat stress with increased durations. Cia *et al.* (1991) also revealed that Glu induces the heat shock proteins expression. The gradual increase in HSP-70 expression from 0 hours to 10 hours duration reflects the combating strategies of bird to improve the digestive efficiency in response to acute heat stress exposure which is important for survival. This study also confirms that pretreatment with Glu induces a significant increase in HSP-70 mRNA expression in the jejunal mucosa under heat stress. The results of the present study concur with previous studies that Gln supplementation could stimulate an increase in HSP-70 protein or mRNA expression after heat stress (Ehrenfried *et al.*, 1995; Wischmeyer *et al.*, 2001). We established an HSP-70 overexpression chicken model by glutamine.

**TABLE 1:** Levels of heat shock protein 70 mRNA expression in the jejunal mucosa of broilers under heat stress

Time (hours)	Control	Enhancer	Inhibitor	P - value
0	1.361 <sup>A</sup> ±0.447	1.592 <sup>A</sup> ±0.414	1.001 <sup>A</sup> ±0.312	0.145
2	2.061 <sup>Aa</sup> ±0.319	6.365 <sup>Cc</sup> ±0.436	3.741 <sup>Db</sup> ±0.417	0.031
5	3.628 <sup>Cb</sup> ±0.618	4.012 <sup>Bc</sup> ±0.513	1.131 <sup>Ba</sup> ±0.412	0.027
10	2.853 <sup>Ba</sup> ±0.312	4.101 <sup>Bb</sup> ±0.404	2.252 <sup>Ca</sup> ±0.351	0.035
P - value	0.023	0.034	0.025	

<sup>A-C</sup>Means in the same column with no common superscripts differ significantly (P < 0.05).

<sup>ac</sup>Means in the same row with no common superscripts differ significantly (P < 0.05).

In recent years, studies have found that there is some correlation between high-temperature stress on the chicken pathological lesion and the expression of HSP-70 (Bao *et al.*, 2004; Sun *et al.*, 2007). The intestine is susceptible to heat stress, hypoxia, and other environmental factors, which result in mucosal damage. Studies found that a variety of stress factors in the intestinal tract, such as endotoxins, arsenite, ethanol, and ischemia may stimulate the production of HSP-70 (Beck *et al.*, 1995; Stojadinovic *et al.*, 1995; Tsuruma *et al.*, 1999; Tsukimi and Okabe, 2001). The results of the current study demonstrate the overexpression of HSP-70 may increase alkaline phosphatase activity which is thought to play an important role in the maintenance of normal intestinal barrier function (Bates *et al.*, 2006). Its change may reflect the change in intestinal digestion and absorption; however, the underlying mechanism is not clear. Glutamine (Gln) is a non-essential amino acid and considered to be essential nutrient during heat stress conditions. It is known to be protective of the intestinal epithelium and a potent enhancer of the synthesis of HSP both in vivo and in vitro (Wischmeyer *et al.*, 1997; Kojima *et al.*, 1998). The major site of dietary Gln absorption is small intestine (Souba, 1993; Tapiero *et al.*, 2002) and its absorption increases many folds during stress (Souba *et al.*, 1990). The down regulated pattern observed with HSP-70 expression in jejunum of birds treated with quercetin (HSP inhibitor) appraises that it inhibits not only HSP-70 induction but also the cytoprotection provided by Gln supplementation (Wischmeyer *et al.*, 1997). The proposed mechanism may probably be, that quercetin inhibits activation of one of the genes involved in dealing with resulting damage.

## CONCLUSION

The present investigation established an HSP-70 enhanced expression in chicken model by glutamine which explicitly explained the HSP-70 expression under acute heat stress conditions. However, future studies are required to realize the underlying physiological mechanisms between HSP-70 and protective under acute heat stress conditions.

## REFERENCES

Al-Aqil, A., and Zulkifli, I. (2009) Changes in heat shock protein 70 expression and blood characteristics in transported broiler chickens as affected by housing and early age feed restriction. *Poult Sci*, 88: 1358-1364.

Bao, E.D., Gong, Y.Y., Hartung, J., Fu, X.B., Wang, X.J., Zhang, H.T. and Wang, Z.L. (2004) Relation between pathologic damages and HSP70, changes in acute heatstressed broilers. *Sci Agric Sinica*, 37: 301–305.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M. and Iwama, G.K. (2002) Heat shock protein genes and their functional significance in fish. *Gene*, 295:173–183.

Bates, J.M., Mittge, E., Kuhlman, J., Baden, K.N., Chesman, S.E. and Guillemin, K. (2006) Distinct signals from the microbiota promote different aspects of zebra fish gut differentiation. *Dev Biol*, 297: 374–386.

Beck, S.C., Paidas, C.N., Mooney, M.L., Deitch, E.A. and DeMaio, A. (1995) Presence of the stress-inducible form of hsp-70 (hsp-72) in normal rat colon. *Shock*, 3: 398–402.

Cai, J., Hughes, C.S., Shen, J. and Subjeck, J.R. (1991) Induction of heat-shock proteins by glutamine “The feeding effect”. *FEBS*, 288 (1): 229-232.

Deane, E.E. and Woo, N.Y.S. (2005) Cloning and characterization of the hsp70 multigene family from silver sea bream: Modulated gene expression between warm and cold temperature acclimation. *Biochem. Biophys. Res Commun*, 330:776–783.

Dokladny, K., Moseley, P. and Ma, T.Y. (2006) Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *Ame J Physiol Gastrointestinal and liver physiol*, 290: 204-212.

Ehrenfried, J.A., Chen, J., Li, J. and Evers, B.M. (1995) Glutamine-mediated regulation of heat shock protein expression in intestinal cells. *Surgery*, 118:352–356.

Gabai, V.L., Meriin, A.B., Mosser, D.D., Caron, A.W., Rits, S., Shifrin, V.I. and Sherman, M.Y. (1997) HSP70 prevents activation of stress kinases. *J Biological Chemist*, 272: 18033-18037.

Hao, Y., Gu, X.H. and Wang, X.L. (2012) Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers Intestinal structure and digestive function. *Poult Sci*, 91: 781–789.

- Kojima, R., Tamaki, T., Kawamura, A., Konoeda, Y., Tanaka, M., Katori, M., Yokota, N., Hayashi, T., Takahashi, Y. and Kakita, A. (1998) Expression of heat shock proteins induced by L (1)-glutamine injection and survival of hypothermally stored heart grafts. *Transplant Proc*, **30**: 3746–3747.
- Lambert, G.P. (2009) Role of gastrointestinal permeability in exertional heatstroke. *Exerc Sport Sci Rev*, **32**(4):185-190.
- Larson, S. D., Li, J., Chung, D.H. and Evers, B.M. (2007) Molecular mechanisms contributing to glutamine-mediated intestinal cell survival. *Am J Physiol.-Gastr L*, **293**:1262-1271.
- Ming, J., Xie, J., Xu, P., Liu, W. B., Ge, X. P., Liu, B., He, Y. J., Cheng, Y. F., Zhou, Q. L. and Pan, L. K. (2010) Molecular cloning and expression of two HSP70 genes in the Wuchang bream (*Megalobrama amblycephala* Yih). *Fish Shellfish Immunol*, **28**: 407–418.
- Ohkawara, T., Nishihira, J., Takeda, H., Katsurada, T., Kato, K., Yoshiki, T., Sugiyama, T. and Asaka, M. (2006) Protective effect of geranylgeranylacetone on trinitrobenzene sulfonic acid induced colitis in mice. *Int J Mol Med*, **17**:229–234.
- Park, J. A., Kim, Y. E., Seok, H. J., Park, W. Y., Kwon, H. J. and Lee, Y. (2011) Differentiation and up regulation of heat shock 70 induced by a subset of histone deacetylase inhibitor in mouse and human embryonic stem cell. *Biochem Mol Biol Reports*, **10**:176- 181.
- Quinteiro-Filho, W.M., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M. L., Sakai, M., Sá, L.R.M., Ferreira, A. J. P. and Palermo Neto, J. (2010) Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poult Sci*, **89**:1905–1914.
- Ren, H., Musch, M.W., Kojima, K., Boone, D., Ma, A. and Chang, E.B. (2001) Short fatty acids induce intestinal epithelial heat shock protein 25 and IEC18 cells. *Gastroenterology*, **121**: 631–639.
- Ritossa, F. (1962) A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, **18**: 571-73.
- Sejian, V., Naqvi, S. M. K., Ezeji, T., Lakritz, J. and Lal, R. (2012) Environmental Stress and Amelioration in Livestock Production. (Eds.). 1–16. Springer-Verlag GmbH Publisher, Germany.
- Souba, W.W. (1993) Intestinal glutamine metabolism and nutrition. *J Nutri and Biochem*, **4**: 2-9.
- Souba, W.W., Herskowitz, K., Klimberg, V.S., Salloum, R. M., Plumley, D.A., Flynn, T. C. and Copeland, E.M. (1990) The effects of sepsis and Endotoxemia on gut glutamine metabolism. *Annals of surgery*, **211**: 543–549.
- Stojadinovic, A., Kiang, J., Smallridge, R., Galloway, R. and Shea-Donohue, T. (1995) Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology*, **109**: 505–515.
- Sun, P.M., Liu, Y.T., Zhao, Y.G., Bao, E.D. and Wang, Z. L. (2007) Relationship between heart damages and HSP mRNA in persistent heat-stressed broilers. *Agric Sci China*, **6**: 227–233.
- Tapiero, H., Mathe, G, Couvreur, P. and Tew, K.D. (2002) Free amino acids in human health and pathologies - II. Glutamine and glutamate. *Biomed and Pharmacother*, **56**: 446-457.
- Tsukimi, Y. and Okabe, S. (2001). Recent advances in gastrointestinal pathophysiology: Role of heat shock proteins in mucosal defense and ulcer healing. *Biol Pharm Bull*, **24**:1–9.
- Tsuruma, T., Yagihashi, A., Watanabe, N.T. Yajima, H., Kameshima, J., Araya and Hirata K (1999) Heat-shock protein-73 protects against small intestinal warm ischemia reperfusion injury in the rat. *Surgery* **125**: 385–395.
- Wischmeyer, P.E., Kahana, M.D., Wolfson, R., Ren, H., Musch, M.M. and Chang, E.B. (2001) Glutamine induces heat shock protein and protects against endotoxin shock in the rat. *J App Physiol*, **90**: 2403–2410.
- Wischmeyer, P.E., Musch, M.W., Madonna, M.B., Thisted, R. and Chang, E.R. (1997) Glutamine protects intestinal epithelial cells: Role of inducible HSP70. *Am J Physiol*, **272**: 879–884.
- Wu, G., Meier, S.A. and Knabe, D.A. (1996) Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. *J Nutr* **126**: 2578-2584.