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Effect of thermal manipulation during embryogenesis on liver heat shock protein expression in chronic heat stressed colored broiler chickens

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ABSTRACT

Thermal manipulation during embryogenesis has been shown to improve thermo tolerance in broilers. Heat shock proteins are a family of proteins produced in response to variety of stress and protect cells from damage. The aim of this study was to evaluate the effect of thermal manipulation (TM) during embryogenesis on HSP gene and protein expression in the embryos and in chronic heat stressed 42nd day old chicks. On 15th day of incubation, fertile eggs from two breeds-Naked neck (NN) and Punjab Broiler-2 (PB-2) were randomly divided in to two groups, namely Control (C) eggs were incubated under standard incubation conditions and Thermal Conditioning (TC) eggs were exposed to higher incubation temperature (40.5 °C) for 3 h on 15th, 16th and 17th day of incubation. The chicks so obtained from each group were further subdivided and reared from 15th–42nd day as normal (N; 25 ± 1 °C, 70% RH) and heat exposed (HE; 35 ± 1 °C, 50% RH) resulting in four treatment groups (CN, CHE, TCN and TCHE). Embryos of two groups (C and TC) on 17th day and birds from four treatment groups on 42nd day were sacrificed. Liver was collected for analysis of gene expression by real-time PCR and protein expression by Western blot of Heat Shock Proteins (*HSP 90 alpha, HSP 90 beta, HSP 70, HSP 60, HSP 27 and ubiquitin*). The plasma collected on 42nd day was analyzed for biochemical parameters. Thermal challenging of embryos of both the breeds caused significant ($P \leq 0.05$) increase in all the HSPs gene and protein expression. The TCHE chicks had significantly ($P \leq 0.05$) lower HSPs gene and protein expressions and oxidative stress compared to CHE groups in both NN and PB-2. Based on these findings it can be concluded that TM during incubation provides adaptation to broiler chicks during chronic heat stress.

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1. Introduction

Adaptation by thermal conditioning during embryogenesis has been one of the options to mitigate the heat stress during the post-natal life of chicken to improve the heat tolerance, especially in broiler chicken. Temperature is one of the most important factors that exerts a negative influence on the performance of poultry and causes huge losses in terms of loss of productivity, reduced reproductive efficiency, increased stress, reduced immune competence and increased investment costs to mitigate the effects of climate change (Rajkumar et al., 2011), reduced growth rate, feed efficiency, intestinal injury, egg shell quality and survivability (Mashaly et al., 2004; Quinteiro-Filho et al., 2010). Poultry species are more vulnerable to heat stress due to increased environmental temperature as birds can tolerate a narrow zone of temperature range; 18–24 °C is the thermo-neutral zone for the birds. Increase in temperature beyond this range due to environment or other metabolic factors will lead to cascading effects on thermoregulation and could be lethal to the birds. High ambient temperature with high humidity affects thermoregulatory processes, heat dissipation, feed consumption and growth rate resulting in increased mortality

Thermal manipulation (TM) during embryogenesis (pre-natal) induces physiological memory due to epigenetic adaptation to high temperature eliciting improved thermo tolerance during the post-natal life (Yahav, 2009). Cyclical higher incubation temperature appears to improve the heat tolerance in chickens (Yahav, 2009), depending on the length and period of exposure. The central and peripheral nervous thermo regulatory mechanism and other body functions are well developed during the later stage of incubation (Tzschentke, 2007) which enables the embryos to adjust to the short term increase in temperature without any negative effects. Sudden changes in the temperatures are the earliest and most common phenomenon that cell has to cope with to

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preserve their structural and enzymatic integrity (Nadeau and Landry, 2006). The variation in environmental temperature will generally impose stress on the embryo, which may result in the evolution of adaptive genetic mechanisms to combat with extreme temperatures (Hoffmann and Parsons, 1991).

Heat shock proteins (*HSPs*) are a set of proteins synthesized in response to physical, chemical or biological stresses including heat exposure (Staib et al., 2007). The *HSPs* are broadly classified into 6 distinct families based on their molecular weights ranging from 10 to 150 kDA (Benjamin and Mcmillan, 1998). The stimulated thermal tolerance degree is related with the expression of *HSPs* (Krebs and Bettencourt, 1999); out of all the expressed *HSPs*, the one with a molecular weight of 70 kDA appears to be more closely associated with heat tolerance (King et al., 2002) followed by *HSP-27* (Samali et al., 2001)) and *ubiquitin* (Bond and Schlesinger, 1985). *HSP-70* gene is an early responsive gene and *HSP-27* is a late responsive gene. *HSP-27* facilitates the activation of the ubiquitin-proteasome pathway (Parcellier et al., 2003). The role of these proteins in the thermotolerance phenomenon in poultry is highly complex and has not been fully elucidated.

The aim of the present study was to investigate the possible effects of increased incubation temperature in the later stages of incubation on *HSP*-genes viz., *HSP -70, HSP -27, HSP -90 alpha, 90 beta, HSP -60* mRNA and *ubiquitin* expression and biochemical indicators in liver tissues in naked neck (NN) and Punjab Broiler-2 (PB-2) broiler chickens and also to elucidate the cumulative effect of pre embryonic exposure on the chicks.

2. Materials and methods

The experiment was conducted at ICAR-Directorate of Poultry Research, Hyderabad and Veterinary College and Research Institute, Namakkal, Tamilnadu. The experiment was approved by the Institutional Animal Ethics Committee (IAEC).

2.1. Experimental population

Chicks from NN and PB-2 were utilized for studying the effect of pre-thermal conditioning (15^{th}–17^{th} day) of embryos on the expression of HSP genes during post hatch life of chicks. PB-2, a synthetic colored broiler line was under long term selection for high 5 week body weight and 40 week part period egg production which is being utilized for the development of improved broiler chicken varieties. NN is an important ecotype of chicken distributed along the hot humid coastal regions of India and is known for its heat tolerance. The Na gene was introduced in to broiler population and the base population was developed after four successive generations of backcrossing and is maintained under mild selection pressure for six week body weight for the last nine generations.

A total of 904 eggs collected from PB-2 (384) and NN (520) lines were incubated under standard temperature and humidity till 14th day. On 15th day, the eggs were randomly divided in to two groups. One group (Thermal Conditioning: TC) of 192 (PB-2) and 260 (NN) eggs was exposed to higher temperature (40.5 °C), 3 °C above the standard incubation temperature (37.5 °C) for 3 h on 15th, 16th and 17th day of incubation. The relative humidity was maintained at 65% during the exposure. Other group (Control: C) with same number of eggs were incubated under standard incubation conditions.

2.2. Rearing and management

The chicks were reared under deep litter system till 14th day and on 15th day, they were randomly distributed at the rate of 6 birds per brooder pen placed in an automatically controlled environmental house. The chicks from each breed were further divided in to two groups; normal (N) and heat exposure (HE) in control and embryonic thermal conditioned groups making four groups in total (CN, CHE, TCN and TCHE) with 18 replicates. The HE groups were maintained at 35 ± 1 °C, 50% RH for 24 h throughout the experimental period (15th–42th day), while N groups were maintained at 25 ± 1 °C, 70% RH. The chicks were offered broiler ration (2900 K cal: ME, 22%: CP) *ad libitum* throughout the experimental period. The chicks were vaccinated against Marek's disease (1^{st} day), Newcastle disease (7th day), infectious bursal disease (14th and 24th day).

2.3. Collection of samples

Six embryos from C and TC birds in PB-2 and NN lines were sacrificed at the end of 17th day incubation and liver samples were collected aseptically for further processing.

On 42nd day of rearing, from 10 birds in each group blood samples were collected in EDTA containing tubes and centrifuged at $3000 \times g$ for 10 min to collect plasma for biochemical assay. Six birds from each group were sacrificed by decapitation, exsanguinated, manually eviscerated and liver samples were collected aseptically and stored at -70 °C until further analysis.

2.4. RNA Extraction and cDNA synthesis

Total RNA was extracted from Liver tissue using SV total RNA isolation system (Promega, USA) according to the standard protocol. The purity of the RNA was determined by measuring absorbance in Genova nano (Jenway, UK). cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied Biosystems, USA). First strand cDNA was synthesized from 1.5 μg of each total RNA samples using a random primer and reverse transcriptase enzyme according to the manufacturer's protocol.

2.5. Real-Time Quantitative PCR (RT-qPCR)

The expression of mRNA was quantified by SYBR green method using Mx-3000P spectro flourometric thermocycler (Stratagene, USA). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Hypoxanthine ribosyltransferase (HPRT) was used as an endogenous control. The first-strand cDNAs were used as template to amplify gene specific primers for *HSP 90 alpha, HSP 90 beta, HSP 70, HSP 60, HSP 27, Ubiquitin* and reference genes *GAPDH* and *HPRT*. The reactions were performed in a 25 μl volume of SYBR green master mix (Sigma, USA) with 10 pM of each primer (Table 1). The amplification protocol used was as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of cyclic denaturation at 94 °C for 15 s, annealing at 59 °C for 1 min and extension at 72 °C for 15 s. Primer amplification efficiency was assessed from the standard curve generated by using serial tenfold dilution of transcribed RNA. Regression of Ct values of standard curve was carried out to determine the amplification efficiency.

2.6. Immunoblot assay

Tissue homogenate was lysed in a 2X sample buffer (Laemmli, 1970) and boiled for 5 min at 95 °C, cooled in an ice bath and centrifuged at 12,000 g for 5 min. Protein samples were then quantified in Genova nano (Jenway, UK) and about 75 μg of samples was subjected to 12% SDS-PAGE. For immuno blotting analysis, protein bands in the slab gels were transferred to a PVDF membrane (Amersham, USA) by a semi-dry method using tris-glycine buffer. After transfer, the membrane was blocked with 5% bovine serum albumin and washed three times with TBST (tris

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