

HEAT STRESS DAMAGES THE MORPHOLOGY OF DUODENAL ENDOTHELIUM VIA TLR4–MYD88–NF-κB SIGNALING IN BROILER CHICKEN

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ABSTRACT

Animal welfare and production are negatively affected by high ambient temperature. Although the gastrointestinal tract is extremely heat-sensitive, there is a dearth of information pertaining to how such heat stress affects different intestinal regions in chickens. This study therefore aimed to evaluate the morphological changes and gene expression of duodenal toll-like receptor 4 (TLR4) signaling in broilers that have been exposed to heat stress conditions on a controlled diet. At 2, 4, and 6 h of heat challenge, chickens were slaughtered, and duodenum samples were collected. Morphological changes were determined by hematoxylin and eosin staining. Five genes, TLR4, myeloid differentiation primary response gene 88 (MyD88), nuclear factor kappa B (NF-κB), toll-interleukin receptor-domain-containing adapter-inducing interferon beta (TRIF), and interferon regulatory factor 7 (IRF7), were selected to monitor the direction of the TLR4 signaling pathway. Results indicated that the duodenal intima was damaged by heat stress, TLR4 significantly responded to the heat stress, and increased heat challenge had no considerable impact on gene expression. Heat stress significantly increased MyD88 and NF-κB in MyD88-dependent signaling pathways. In conclusion, our results demonstrated that heat stress damages duodenal endothelium and upregulates duodenal TLR4–MYD88–NF-κB signaling genes in broilers.

Key words: Chicken; Duodenum; Heat stress; TLR4; TLR4 pathway

INTRODUCTION

Poultry, which have the limited capability of evaporation regulation, have been shown sensitive to heat stress (Wolfenson *et al.*, 2001). Heat stress could decrease the reproduction, immunity, and meat quality, finally inducing economic losses (Soleimani *et al.*, 2011; Kikusato and Toyomizu, 2013; Wang *et al.*, 2015). Several organs, including liver and hypothalamus (Figueiredo *et al.*, 2007), heart (Zhang *et al.*, 2017), and skeletal muscle (Kikusato and Toyomizu, 2013), can be affected by heat stress. The gastrointestinal tract can also be injured by heat-stress-associated changes in morphologies, functions, or gene expression (Garriga *et al.*, 2006; Quinteiro-Filho *et al.*, 2010, 2012; Chen *et al.*, 2014; Varasteh *et al.*, 2015).

TLR signaling pathways are a major class of type I transmembrane proteins of the innate immune system with outward-facing leucine-rich repeat domains and cytoplasmic conserved region toll-interleukin receptor (TIR) domains (Kumar *et al.*, 2009). TIR domains interact with different adapter proteins, including myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon beta (TRIF), which represents different MyD88-dependent and MyD88-independent signaling pathways (Yamamoto *et al.*, 2003; Piras and Selvarajoo, 2014). In the following cascades, MyD88 activates nuclear factor kappa B (NF-κB) (Diomedea *et al.*, 2017), while TRIF activates

interferon regulatory factor 7 (IRF7) (Ahmad *et al.*, 2011). In this study, AA broiler chicken was selected as experimental animal to assess how heat stress affects the histomorphological and TLR4-initiated pathway changes in the duodenum.

MATERIALS AND METHODS

Animals and sample isolation: 40 AA broiler chickens aged 35 d were randomized into 4 groups (one control group at 23 °C ±1 °C and three groups at 35 °C ±1 °C). The chickens were housed in two separate rooms, in which the environment was controlled, with one room being a control room and the other being the heat stress room. All broilers were fed one week for environment adaptation. After one week, the broilers were fed in the heat stress room for 2, 4, and 6 h. At the experimental endpoint, chickens were slaughtered, and intestine samples were collected. All samples were partly fixed in 4% paraformaldehyde for histological analysis, partly shock-frozen via liquid nitrogen, and transferred to a –80°C refrigerator prior to sample use to extracting total RNA. The experimental protocol was approved and was consistent with the “Guidelines for Experimental Animals” maintained by the Ministry of Science and Technology (Beijing, China).

Histomorphological determination: All duodenal samples were isolated from the same small intestine region and fixed with 4% paraformaldehyde. The sections

were hematoxylin and eosin stained (Quaedackers *et al.*, 2000). Morphological analyses of duodenum sections were performed using light microscopy. A total of 10 segments from each animal was assessed and imaged with an appropriate camera (Olympus), and image capture and analysis was conducted via computer-assisted digital image analysis system (Leica Microsystems, Germany).

Reverse-transcription polymerase chain reaction (RT-PCR): TRIzol (Invitrogen) was used to extract total

RNA, and the HiFiScript gDNA Removal cDNA Synthesis Kit from CW Biotech (Beijing, China) was used to generate cDNA based on provided protocols. Relative quantification of TLR4 was performed using UltraSYBR Mixture (CW Biotech, Beijing, China) as the standard protocol. GAPDH was a normalization control in these RT-PCR analyses. Primers for TLR4, MyD88, NF- κ B, TRIF, and IRF7 were designated by Primer Premier 5 (Table 1).

Table 1. Real-time PCR primers for TLR4 signaling pathway detection.

Primer	NCBI GenBank Number	Sequence (10pmol/ μ L)	Annealing Temp ($^{\circ}$ C)	Product Size (bp)
TLR4	NM_001030693.1	F:5'-AGAGCGGCTCCCACCGCGCTGA-3' R:5'-TCCTGTGCATCTGAAAAGCTGT-3'	60	129
MyD88	NM_001030962.1	F:5'-ATTCCGGTCAAGTGCAAGAC-3' R:5'-ATCACGCGCAGCAAGAGAGAT-3'	60	136
NF- κ B	NM_205134.1	F:5'-GCTGCTTTGACACAGATGGA-3' R:5'-CCTTTGCAAACTGGTTGGT-3'	60	139
TRIF	NM_001081506.1	F:5'-TCAGCCATTCTCCGTCCTCTTC-3' R:5'-GGTCAGCAGAAGGATAAGGAAAGC-3'	60	339
IRF7	NM_205372.1	F:5'-ACATGTTTCATGCTGCTGGAG-3' R:5'-GAGTGTTTTCCAAGGCCAAA-3'	60	174
GAPDH	NM_204305.1	F:5'-TGCCATCACAGCCACACAGAAG-3' R:5'-ACTTTCCCCACAGCCTTAGCAG-3'	60	123

Statistical analysis: All experiments for histomorphological analysis and gene expression detection were performed in triplicate. Data are means \pm s.d., SPSS v14.0 was used for all statistical testing. The comparative threshold cycle method which named $2^{-\Delta\Delta Ct}$ was used to assess relative gene expression (Livak and Schmittgen, 2001). Student's *t*-tests were used for comparing results between groups, and $P < 0.05$ was the significance threshold.

RESULTS

Heat stress triggers the damage of duodenal endothelium: We initially conducted a complete-morphometric analysis of duodenal samples collected from chickens that were either housed under control or

heat stress conditions. Villus height, crypt depth, villus height: crypt depth ratio, and muscle thickness were measured (Table 2). After heat challenge, all parameters were decreased. Except for the villus height ($P < 0.05$), the other three parameters reached extremely significant differences ($P < 0.01$).

After heat challenge, the width of the duodenal villi increased, the gap among the villi decreased, and the lobular protrusion was not evident (Figure 1A). The intrinsic villi layer exhibited obvious hyperemia (Figure 1B). The cavity in the intestinal tract was irregular and closed (Figure 1C). The endothelial layer of the duodenum was characterized by severe hyperemia and edema, accompanied by inflammatory cell infiltration; cytoplasm, and nucleus of epithelial cell were unstained; and the striatum was not evident (Figure 1D).

Table 2. Influence of heat stress on the morphological parameters of the duodenal mucosa.

Parameter	Control	Heatstress
Villus height (μ m)	2203.94 \pm 79.10 ^a	2099.19 \pm 140.86 ^b
Crypt depth (μ m)	335.14 \pm 34.62 ^A	280.74 \pm 40.81 ^B
Villus height: crypt depth ratio	6.32 \pm 0.67 ^A	7.41 \pm 0.94 ^B
Muscle thickness (μ m)	226.62 \pm 27.90 ^A	178.69 \pm 32.39 ^B

Data presented as mean \pm s.d. Different upper case superscript letters indicate extremely significant differences between control and heat-stressed groups, and lower case superscript letters indicate significant differences.

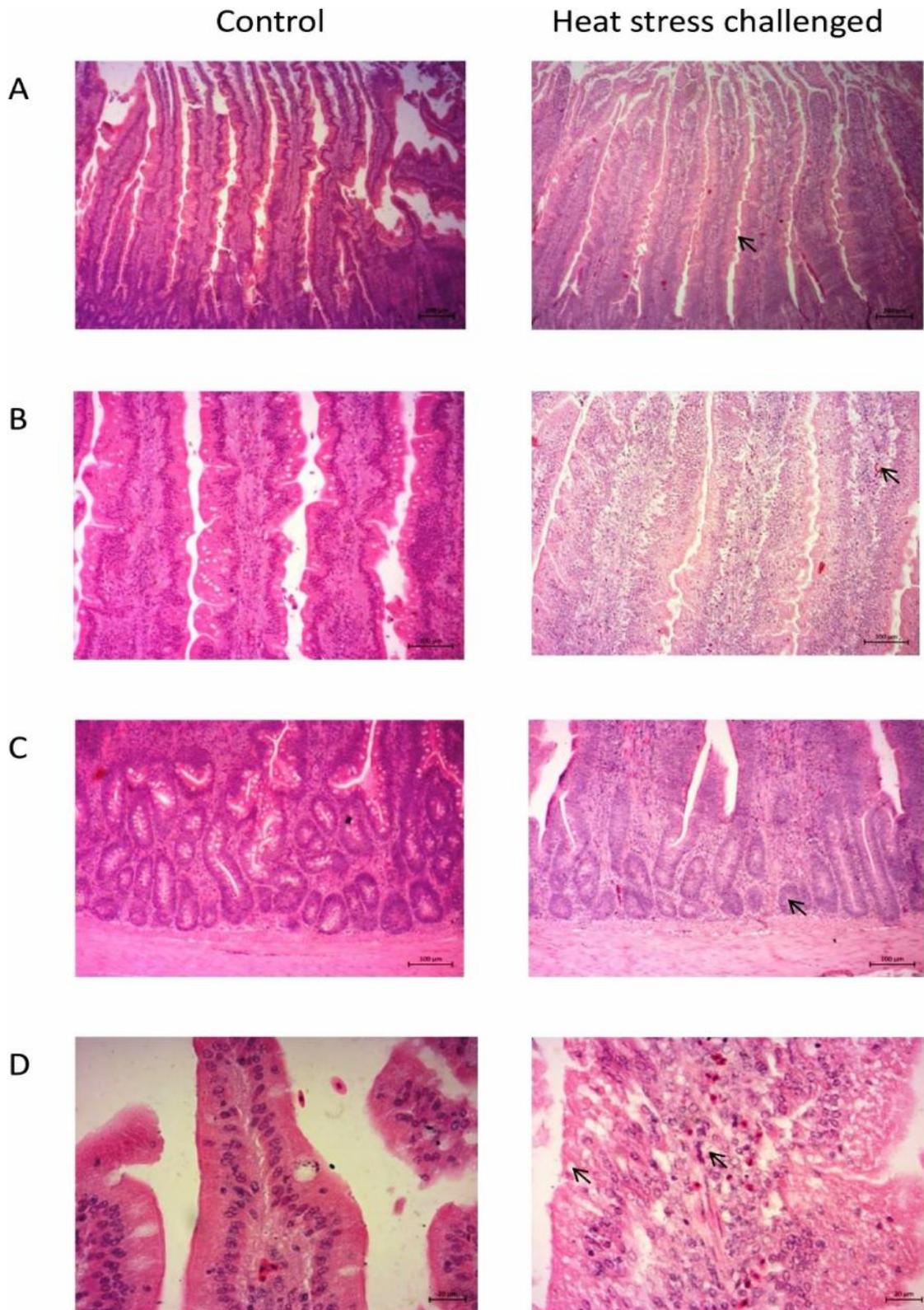


Figure 1. Illustrative images of hematoxylin -and-eosin-stained sections of normal small intestine (duodenum, jejunum, and ileum) from control and heat-stress-treated birds. A,B,C with 4× light microscope, and D with 40× light microscope. All the black arrows indicated the heat-induced phenotypic differences.

Expression analysis of duodenal TLR4 signaling genes: RT-PCR was employed to assess duodenal TLR4, MyD88, NF- κ B, TRIF, and IRF7 expression in control or heat-stressed broiler. 2h following initiation of heat stress, TLR4 was significantly upregulated in duodenum; after 4 or 6 h, the gene expression cannot be notably upregulated compared with that when the heat stress time was 2 h (Figure 2A). MyD88, NF- κ B, TRIF, and IRF7 expression

levels were analyzed in heat-stress and control groups to identify the heat-stressed TLR4 signaling pathway. MyD88, NF- κ B, and IRF7 were extremely upregulated in the heat-stressed group relative to controls, but the expression level of TRIF was not considerably changed in the two groups (Figure 2B-2E). These findings indicated that heat stress induced the upregulation of TLR4-MyD88-NF- κ B signaling genes in duodenum.

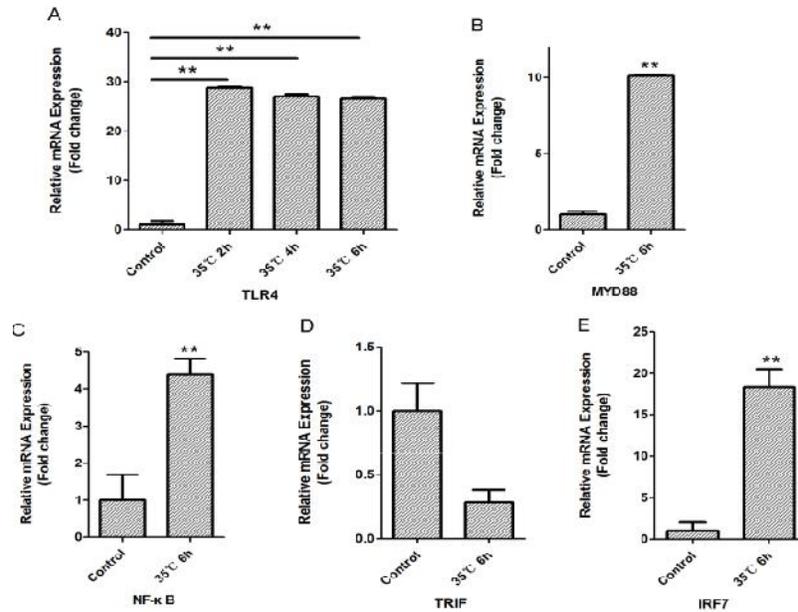


Figure 2. Expression of TLR4 pathway in heat-stress-challenged duodenum. The relative expression levels of TLR4 (A), MYD88 (B), NF- κ B (C), TRIF (D), and IRF7 (E) were measured in the heat-stress-induced duodenum in AA chicken using Real-time PCR. GAPDH was used as a loading control to normalize the expression levels. Data are expressed as mean \pm s.d. ($n=3$). * $P<0.05$, ** $P<0.01$.

DISCUSSION

Heat stress is a significant environmental stressor in the poultry industry, especially in broiler transportation. The gastrointestinal tract is a primary target of heat stress in chickens. This study showed that 6 h heat challenge can damage the duodenal endothelium. All four parameters were significantly changed ($P<0.01$). On the contrary, a report has indicated that no intestinal damage occurred during the initial 10 h of heat stress, and no differences were identified in villus height or intestinal crypt depth following 6 d of heat stress (Quinteiro-Filho *et al.*, 2012). The differences may originate from the used samples. Previously, chicks aged just 1 d were used, whereas we used 35 day-old chickens. The older chickens may therefore be more heat sensitive than young birds. The different breeds and environments may also affect results.

Previous studies had reported heat stress impacted signaling pathway, like the adenosine 5'-monophosphate-activated protein kinase signaling pathway in Small Intestinal Epithelium Cells (He *et al.*, 2018), insulin like

growth factor signaling pathway in muscle (Ma *et al.*, 2018), mitochondrial pathway in growth performance (Zhang *et al.*, 2015), to damage the growth performance, but little is known with TLR signaling pathway in chicken duodenal endothelium. In our study, the TLR4 pathway varied significantly ($P<0.01$) among duodenum in heat-stressed chicken. In the jejunum, TLR4 mRNA expression was increased after heat stress exposure (Varasteh *et al.*, 2015). All these reports indicated the TLR4 functions in the gastrointestinal tract of heat-stressed chicken. Genes involved in each pathway were assessed to further understand which pathway governs duodenum in heat-stressed chickens. In the TLR4 signaling pathway, MyD88 decides one route of signaling cascade, and the other is TRIF. Our result indicated that compared with the control, MyD88 was extremely upregulated in the heat-stressed group relative to controls, but not TRIF. Accordingly, the MyD88-dependent pathway follows TLR4 activation, which was confirmed by the expression of NF- κ B, which is an important downstream gene in the MyD88-dependent pathway. IRF7, a downstream gene of TRIF, was strongly

upregulated in the heat-stressed group relative to controls. Thus, another pathway for activating its expression requires further study.

In conclusion, heat stress upregulates duodenal TLR4–MYD88–NF- κ B signaling genes. This study provides insight into the molecular mechanisms of TLR4–MYD88–NF- κ B response pathway for heat stress regulation.

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