

# Chronic heat stress promotes liver inflammation in broilers via enhancing NF- $\kappa$ B and NLRP3 signaling pathway

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## Research Article

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# Abstract

## Background

The study was to investigate the effects of chronic heat stress on liver inflammatory injury and its potential mechanisms in broilers. Chickens were randomly assigned to 1-week control group (Control 1), 1-week heat stress group (HS1), 2-week control group (Control 2), and a 2-week heat stress group (HS2) with 15 replicates per group. Broilers in heat stress groups exposed to heat stress ( $35 \pm 2^\circ\text{C}$ ) for 8 h/d with 7 or 14 consecutive days. Growth performance and liver inflammation injury were examined for the analysis of liver injury.

## Results

The results showed that heat stress decreased the growth performance, showed obvious blutpunkte, lowered liver weight and liver index, which resulted in significant liver damage of broilers. Both the gene and protein expressions of HSP70, TLR4 and NF- $\kappa$ B in the liver were significantly enhanced by heat stress. Furthermore, heat stress obviously enhanced IL-6, TNF- $\alpha$ , NF- $\kappa$ B P65, I $\kappa$ B and their phosphorylated proteins expressions in the liver of broilers. In addition, heat stress promoted the activation of NLRP3 with increased NLRP3, caspase-1 and IL-1 $\beta$ .

## Conclusions

These results suggested that heat stress can cause the liver inflammation via activation of TLR4-NF- $\kappa$ B and NLRP3 signaling pathway in broilers.

## Background

Heat stress jeopardizes humans and animals' health, and results in enormous economic loss in public health care and livestock production. Heat stress occurs when the net amount of energy flowing from a poultry's body to its surroundings is less than the heat it generates (1). For feedback heat stress, body temperature, respiratory rate, heart rate and rectal temperature will increase adaptively, which will affect feed intake and production efficiency of poultry, thus adversely affecting production economy (2). In addition, the long-term genetic selection of poultry for improved performance induced higher metabolic heat production and increased sensitivity to heat stress (3). Research reported that heat stress reduced annual economic losses averaged 128-240 million dollars for poultry industries in the United States, and the unfavorable influence will be progressively deteriorating as global temperatures rise (4). Alleviating heat stress has great significance to reduce economic loss of livestock industry.

Except for foreign substances such as food antigens, toxins, and bacterial components, the liver is also sensitive to ambient stress (5). Research found that heat stress can induce liver oxidative stress and reduce the immune responses of laying hens which resulted in decreased poultry production performance, such as reducing body weight and food consumption. Continuous exposure to high

temperature in broilers can lead to liver tissue damage and decreased non-specific immunity (6) and have other negative effects on immune response (7). At the same time, the intestinal stress caused by high temperature results in bacterial translocation and intestinal flora out of balance, and induces intestinal endotoxin entering the liver through the internal circulation (8).

The innate immune cells in the liver recognize dangerous substances or damaged cells through pattern recognition receptors (PRRs) thus activates TLR4 signaling to promote inflammatory responses (9). NF- $\kappa$ B, which acts downstream of TLR4 (10, 11) and other immune receptors (12), can increase the overproduction of proinflammatory interleukin 6 (IL-6), IL-1 $\beta$  and tumor necrosis factor-alpha (TNF- $\alpha$ ), which leads to the occurrence of inflammatory response (13). It's reported that NF- $\kappa$ B activated NLRP3 while inducing a variety of inflammatory chemokines (14), cytokines and cytokine precursors, including pre-IL-1 $\beta$ , and is therefore important for inflammasome initiation and assembly (15). However, whether heat stress mediated the inflammation in liver of broilers and it's relation with NF- $\kappa$ B-NLRP3 is still unclear. In this study, we designed a model of chronic heat stress in broilers to investigate whether heat stress activates NF- $\kappa$ B-NLRP3 to exacerbate liver inflammation.

## Results

### **Chronic heat stress inhibited the growth performance of broilers.**

Growth performance indexes of broilers are mainly reflected in body weight growth and feed conversion capacity. The daily body weight and feed intake of broilers were monitored in this study, and the results were shown in table 2. Compared with the control 1 group, the body weight gain of broilers in the HS1 group were significantly decreased ( $P < 0.01$ ), and the feed intake and gain feed ration also inhibited by heat stress but it is no significantly. With the enhanced of adaptability, these adverse reactions gradually ameliorate, and there is no significant difference between control 2 and HS 2 group, however, the performance of HS2 group still affected by heat stress.

### **Effects of heat stress on liver weight, index and pathological changes of broilers.**

The liver of broilers after necropsy was observed (Figure. 1A). It showed that the texture of the liver surface in the control 1 group (a) and the control 2 group (c) broilers were uniform, without obvious bleeding and necrosis points. HS1 group (b) and HS2 group (d) broilers showed obvious blutpunkte on the liver surface. The results showed that heat stress had a promotion on liver tissue damage. We also measured the body weight, liver weight and liver index, and the results showed that the above 3 indicators were significantly reduced after 2 weeks of heat stress, compared with control 2 group.

### **Chronic heat stress enhanced proteins expressions of TNF- $\alpha$ and IL-6 in liver of broilers.**

As shown in Figure.2, compared with the control 1 and 2 group, heat stress significantly increased the protein expression of IL-6 in liver after 1 and 2 weeks of heat stress ( $P < 0.01$ ,  $P < 0.01$ , respectively). The TNF- $\alpha$  protein levels in broilers with heat stress for 1 and 2 weeks were higher than that of in control 1 and 2 group broilers ( $P < 0.01$ ,  $P < 0.01$ , respectively).

### **Chronic heat stress activated the NLRP3 pathways in liver of broilers.**

As shown in Figure 3. compared with the control 1 and 2 group, the NLRP3 and caspase1 protein levels in HS1 and HS2 group both significantly increased ( $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.01$ , respectively). The level of cleaved-IL-1 $\beta$ /pro-IL-1 $\beta$  in liver of HS1 and HS2 group broilers were obviously higher than that of in control 1 and 2 group ( $P < 0.01$ ,  $P < 0.01$ ). The trend of cleaved-IL-1 $\beta$ /pro-IL-1 $\beta$  was consistent with proteins expressions of NLRP3 and caspase1.

### **Chronic heat stress strengthened NF- $\kappa$ B pathways in liver of broilers.**

We simultaneously detected the expression of NF- $\kappa$ B, I $\kappa$ B- $\alpha$  and their phosphorylated protein levels. The results were shown in Figure 4. Compared with control 1 and 2 group, 1 and 2 weeks of heat stress significantly increased the ratio of phosphorylation of NF- $\kappa$ B to NF- $\kappa$ B ( $P < 0.01$ ,  $P < 0.01$ , respectively), and also enhanced the ratio of phosphorylation to non-phosphorylation of I $\kappa$ B- $\alpha$  ( $P < 0.01$ ,  $P < 0.01$ , respectively).

### **Chronic heat stress promoted the proteins expressions of TLR4 and HSP70 in liver of broilers.**

As shown in the Figure 5, the proteins expression of TLR4 and HSP70 in the liver of broilers were measured. Heat stress for 1 and 2 weeks significantly up-regulated the protein expression of TLR4, compared to control 1 and 2 group ( $P < 0.01$ ,  $P < 0.01$ , respectively). HSP70 protein has a protective effect on cells, and its nonspecific expression can be increased when the body is subjected to stress. Our results found that, compared with the control 1 and 2 group, the protein expression of HSP70 in HS1 and HS2 group were significantly increased ( $P < 0.01$ ,  $P < 0.01$ , respectively).

### **Chronic heat stress up-regulated the genes expressions of TLR4, HSP70 and NF- $\kappa$ B in liver of broilers.**

As shown in the Figure 6, heat stress for 1 and 2 weeks enhanced TLR4 gene expression ( $P < 0.01$ ,  $P < 0.01$ , respectively), especially at 2 weeks of heat stress. An increase in HSP70 mRNA was observed in the liver after heat stress for 1 and 2 weeks, compared to control 1 and 2 group ( $P < 0.01$ ,  $P < 0.01$ ,

respectively). Consistently, NF- $\kappa$ B gene expression was also significantly upregulated during heat stress.

## Discussion

High temperature or hot climate has adverse impact on the growth performance of poultry due to more water intake and lower feed intake. At the same time, high temperature affects the digestive and absorption function of gastrointestinal tract, and results in significantly lower weight gain (WG) and feed conversion rate (FCR). High temperature also causes tissue damage, especially intestinal and liver, which affects the normal function of the poultry (16, 17). Heat stress for 1 week decreased weight gain and heat stress for 2 week reduced body weight of broilers. The relative organ weight reflects the growth and development of organs in some degree, and then affects their functions (18). Under the condition of continuous heat stress, the organ index of liver and other immunity organs and cellular immunity would decrease (17, 19), which was consistent with our results. In this study, heat stress for 2 weeks decreased the liver weight and index of broilers, indicating that heat stress impaired liver growth and development in broilers. Moreover, heat stressed broilers showed obvious blutpunkte on the liver. As the largest digestive organ in the body, liver injury may affect the digestive and absorption functions of the body, which may explain the growth performance of broilers decreased to a certain extent after heat stress in the experiment.

Liver is susceptible to the environmental stress. The overexpression of HSP70 as an indicator of various stress responses, including heat stress. In this study, increased HSP70 mRNA and protein levels were observed in the liver of the heat stress group broilers. Extracellular or exosomal-bound HSP70 binds to TLR2 or TLR4 to activate inflammatory response in animals (20, 21). TLR4 is thought to be the endotoxin receptor that receives the validation response (22). Activation of TLR4 stimulates the associated inflammatory signaling pathways. Research have shown that heat stress can significantly up-regulate the abundance expression of TLR4 mRNA in the liver of broilers (17). Activation of TLR4 can activate NF- $\kappa$ B, cause the synthesis and secretion of pro-inflammatory cytokines, further amplifying the inflammatory response. Research have shown that the expression of NF- $\kappa$ B protein was significantly increased by heat stress (23). Our results showed that heat stress for 1 and 2 week up-regulated the gene and protein expressions of TLR4 and NF- $\kappa$ B, and heat stress also increased the ratio of NF- $\kappa$ B and I $\kappa$ B- $\alpha$  protein phosphorylation to non-phosphorylation. We further detected the expression levels of inflammation-related proteins in the liver. Our results found that heat stress up-regulated TNF- $\alpha$  and IL-6 protein levels in the liver of broilers. So, heat stress activates NF- $\kappa$ B signaling pathway and promotes the secretion of inflammatory factors TNF- $\alpha$  and IL-6, leading to the occurrence of inflammation in liver of broilers.

Heat stress, as a kind of high temperature stimulation, can induce liver tissue damage (24). Yang's study found that the NF- $\kappa$ B/NLRP3 signaling pathway is inhibited in response to acute heat stress (25), and Greene's study also confirmed that heat stress can reduce NLRP3 inflammasome (26). However, most studies showed that NLRP3 protein level increased with heat stress temperature (27). Pei's study showed that protein level of NLRP3 increased with the extension of exposure time under heat stress (28). Studies

have shown that stress triggers activation of NLRP3 inflammasome (29), and excessive accumulation of inflammasome can trigger inflammation, which mediates liver injury (30). Except for NLRP3 activation, heat stress can also promote the activation of NF- $\kappa$ B signaling, P38, and ERK pathways (31, 32). Therefore, we observed the protein changes in the NLRP3 pathway of inflammasome. The results showed that the protein content of NLRP3 in the liver of broilers was significantly increased after heat stress, and the content of IL-1 $\beta$  precursor was significantly decreased, while the content of IL-1 $\beta$  in the mature and caspase-1 were significantly increased, indicating that heat stress activated NLRP3 inflammasome.

## Conclusion

In this study, chronic heat stress can inhibit broilers' growth performance, increased liver damage and contributed to promote the release of inflammatory factors. The expression of TLR4 in liver of broilers with heat stress is akin to the expression of inflammatory factors, NF- $\kappa$ B and NLRP3, indicating that chronic heat stress promoted the liver inflammation via activation of TLR4-NF- $\kappa$ B and NLRP3 signaling pathway in broilers (Figure 7).

## Methods

### Animals treatment

60 two-week-old broilers were purchased from a commercial hatchery (Nanhai Poultry Corporation, Foshan, China). One week of adaptive feeding, twenty-one-day-old male broilers were randomly divided into 1-week control group (Control 1), 1-week heat stress group (HS1), 2-week control group (Control 2) and 2-week heat stress group (HS2), with 15 replicates in each group. The control group was kept at  $23\pm 2^{\circ}\text{C}$ , while the heat stress group was kept at the  $35\pm 2^{\circ}\text{C}$ , and the humidity control as about 70%. The broilers of heat stress groups continuous treatment with high temperature for 8 h every day (8:00-16:00 every day) for 1 or 2 weeks. All broilers had freely drinking water and basal diet, which consists of corn, wheat, soybean meal, soybean oil and other conventional feed additives, amino acids, trace elements and vitamins. We recorded the body weight and the amount of feed consumed every day which were used to analyze the average weight gain (WG) and gain feed ratio (GFR). Broilers were sacrificed at the end of heat stress, all broilers were sacrificed quickly by CO<sub>2</sub> inhalation, and liver samples were collected for subsequent testing.

### Pathological changes and the organ index of liver

The liver of broilers in each group was photographed to observe the changes of pathological injury during the slaughter of broilers. Complete liver was taken, washed with autoclaving saline, dried with absorbent paper, weighed and recorded for calculating organ index (organ index = (liver weight / body weight)  $\times 100\%$ ).

### Western blot analysis

Liver samples were extracted with a lysate containing RIPA and phosphatase-inhibitors and phenylmethylsulfonyl fluoride (PMSF) on ice, supernatant protein quantification by BCA kit (Beyotime, Shanghai, China) before centrifuged at 13000 r/min at 4°C for 10 min. Proteins (20-40 µg from each sample) were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membrane. Then, the membrane was blocked with 5% skimmed milk (skim milk powder in Tris-buffered saline-Tween 20) for 1 hour and incubated with primary antibodies (IL-6, TNF-α, NLRP3, caspase 1, pro-IL-1β, cleaved-IL-1β, p-p65, p65, p-IκB-α, IκB-α, TLR4, HSP70 and GAPDH. Cell Signaling Technology, Danvers, MA, USA). Subsequently, the membrane was incubated with secondary antibodies which contain peroxidase-conjugated, followed by visualized with the Chemiluminescence System. The relative intensity of target protein was analyzed by the Image J software.

### Quantitative real-time PCR

Total RNA from the liver tissue using the Trizol reagent (Ambion, Austin, TX, USA), and reversely transcribed through 1st Strand cDNA Synthesis kit (Takara, Tokyo, Japan) according to the protocol. Then, quantitative real-time polymerase chain reaction using the TB Green™ Premix Ex Taq™ II (Takara, Tokyo, Japan) and a Roche LightCycler 480 system (Roche, Basel, Switzerland). Gene mRNA levels were normalized using the expression of housekeeping gene β-actin and the relative fold changes were calculated using the  $2^{-\Delta\Delta Ct}$  method. All primers used for qRT-PCR are listed in Table 1.

### Statistics

Data are expressed as means ± standard deviation. Statistical significance was performed using a two-tailed Student's test.  $P < 0.05$  were considered statistically significant, \* $P < 0.05$ , \*\* $P < 0.01$  vs. control 1; # $P < 0.05$ , ## $P < 0.01$  vs. control 2. SPSS 24.0 software (SPSS, Chicago, USA) was used for statistical analysis. The measured data were statistically plotted with GraphPad Prism 8.0 software.

## Tables

**Table 1** The primers used in qPCR are as **Table 1** follows

Gene	GenBank	Primer sequences(5'-3')	Produce size (bp)
<b>TLR4</b>	NM_001030693	F: GATGCTCTCTATGGGCTTCTCTGATG R: GAGGCTGCTTGGAACTACTGGATGG	132
<b>HSP70</b>	AY178442.1	F: CAGGGCAATGCTAGTGTGTACTIONCATC R: AGGGTCTTTCTTTGGTGTGTTCCATACG	120
<b>NF-κB</b>	NM_205134	F: CGAGTGCTTTGTCTACGAGATGGAG R: AGGTCAGCCGCTTCAATCTTCTTC	131
<b>β-actin</b>	NM_205518	F: ACGTCTCACTGGATTTCGAGCAGG R: TGCATCCTGTCAGCAATGCCAG	298

**Table 2** Effects of heat stress on growth performance of broilers.

<b>Growth Performance</b>			
<b>Treatment</b>	<b>Body Weight Gain (g)</b>	<b>Feed Intake (g)</b>	<b>Feed conversion rate (g:g)</b>
<b>Control 1</b>	147.49±9.86	401.13±5.30	0.37
<b>HS1</b>	91.81±23.06**	255.99±16.38	0.26
<b>Control 2</b>	338.22±84.16	847.89±15.62	0.40
<b>HS2</b>	248.09±47.76	758.74±11.95	0.33

\*\* $P < 0.01$  vs. control 1

## Abbreviations

HS	Heat stress
HSP70	Heat shock protein 70
WG	weight gain
FCR	Feed conversion rate
PRRs	Pattern recognition receptors
TLR4	Toll-like receptor 4
NF- $\kappa$ B	Nucleus factor kappa B
NLRP3	Nucleotide-binding oligomerization domain-like Receptor Family, Pyrin Domain Containing 3

## Declarations

### Ethics approval and consent to participate

All animals work was carried out as per the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398) and was approved by the Laboratory Animal Management Committee of Foshan University.

### Consent for publication

The author confirms that the work described has not been published before and its publication has been approved by all co-authors.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Competing interests

No potential conflict of interest was reported by the authors.

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## Authors' contributions

Conceptualization and funding acquisition, Lu-Ping Tang and Yong-Ming He; Investigation, experiment and operation, Yi-Lei Liu and Kang-Ning Ding; Methodology and data curation, Xing-Ling Shen and Yu-Qing Liu; Software, Yi-An Zhang and Han-Xiao Liu; Validation and writing original draft, Yi-Lei Liu.

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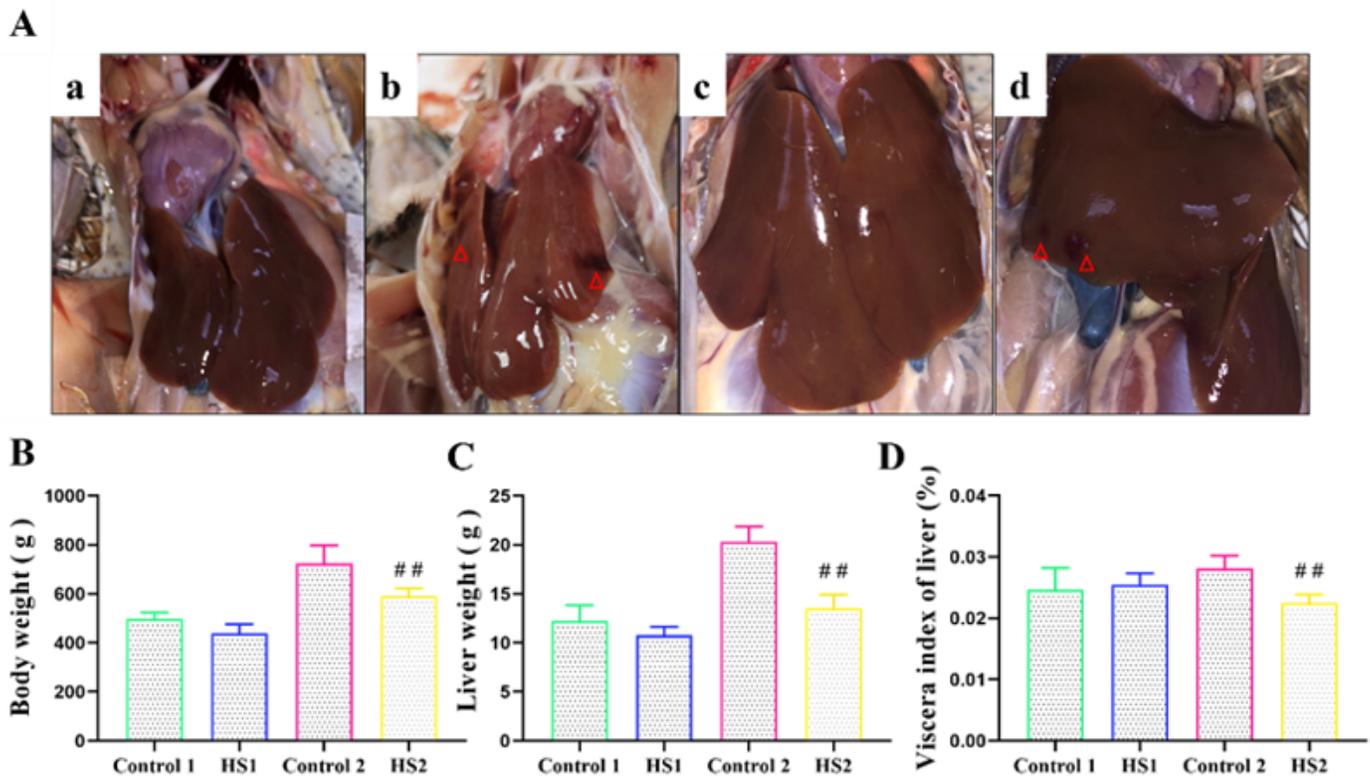
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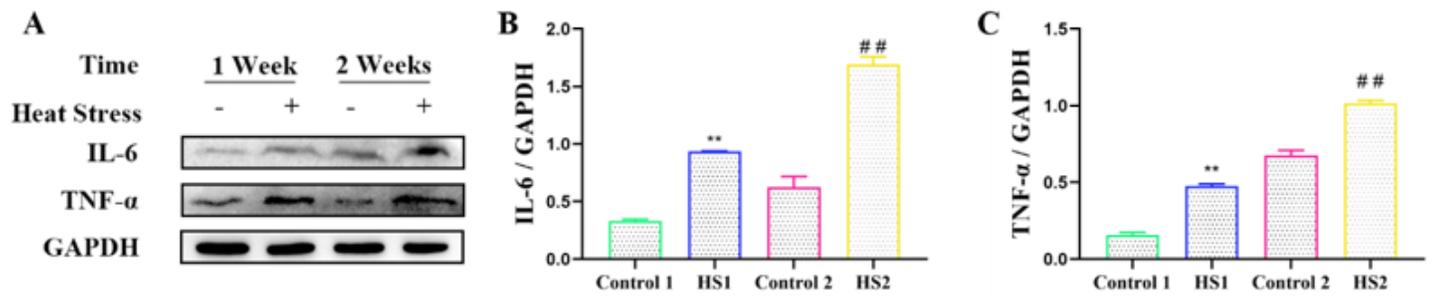
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## Figures



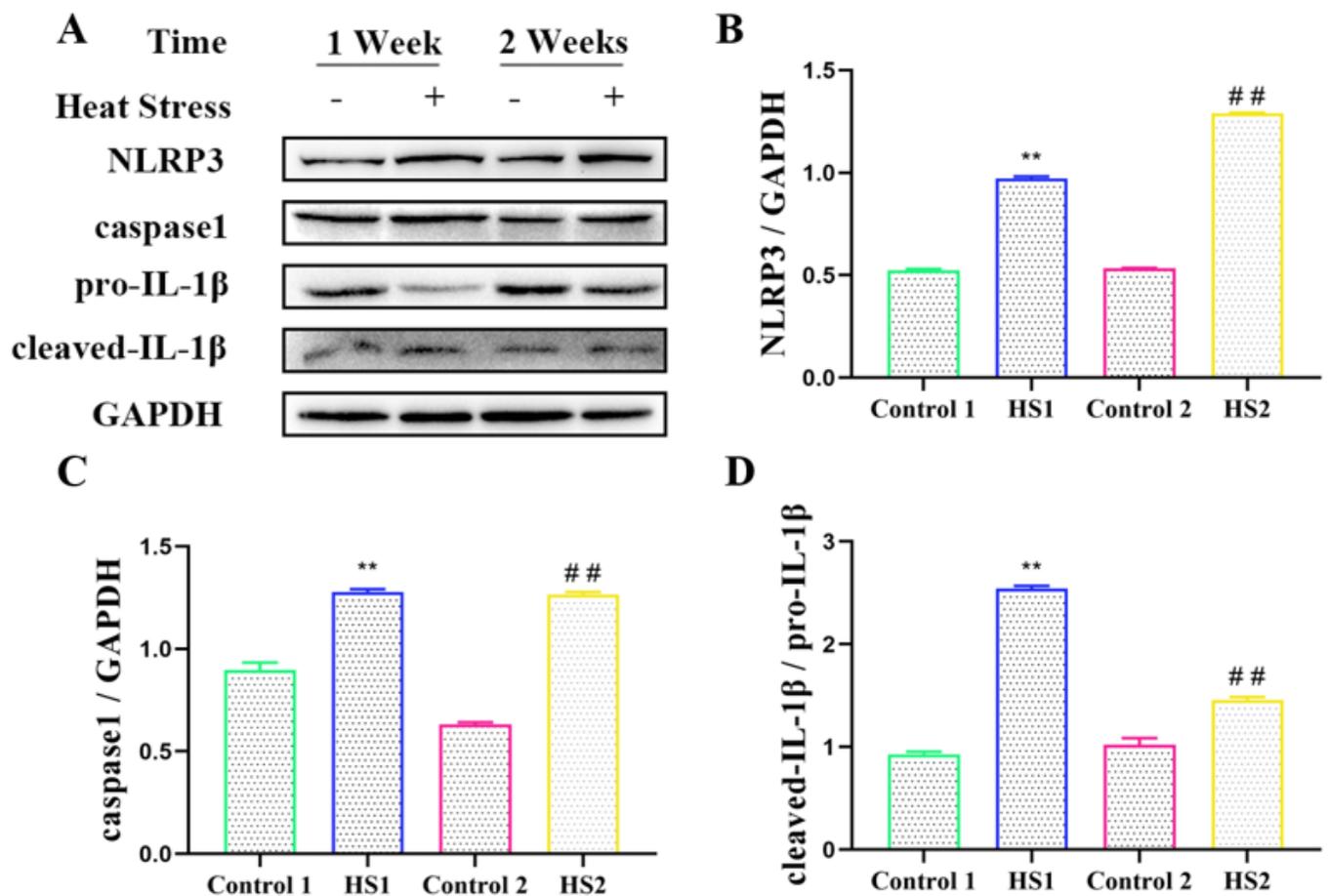
**Figure 1**

Effects of heat stress on liver changes of broilers. (A) Liver pathological changes. (a) control 1 group; (b) HS1 group; (c) control 2 group; (d) HS2 group. Red triangle represents necrosis points. (B) Body weight of broilers. (C) Liver weight of broilers. (D) Liver index of broilers. <sup>##</sup> $P < 0.01$  vs. control 2.



**Figure 2**

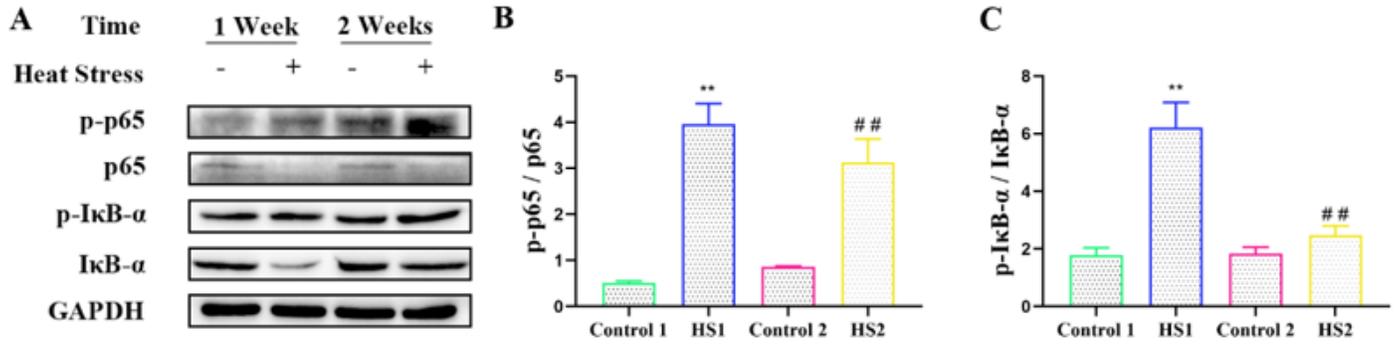
Effects of heat stress on liver proteins levels of TNF-α and IL-6 in broilers. (A) Protein expressions of liver TNF-α and IL-6 in broilers. Relative expressions of (B) IL-6 and (C) TNF-α. \*\*P < 0.01 vs. control 1; ##P < 0.01 vs. control 2.



**Figure 3**

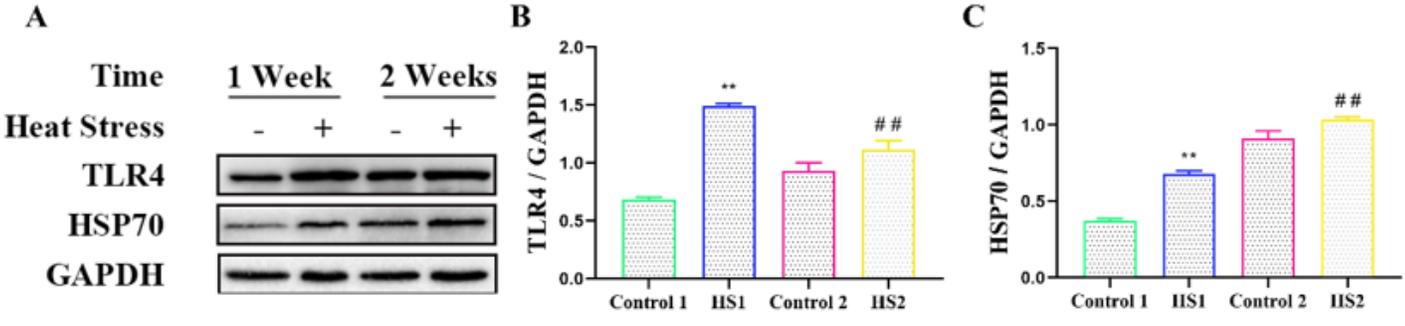
(A) Protein expressions of NLRP3 pathways in liver of broilers were measured by western blot. Relative expression of (B) NLRP3, (C) caspase 1 and (D) cleaved-IL-1β/pro-IL-1β. \*\*P < 0.01 vs. control 1; ##P <

0.01 vs. control 2.



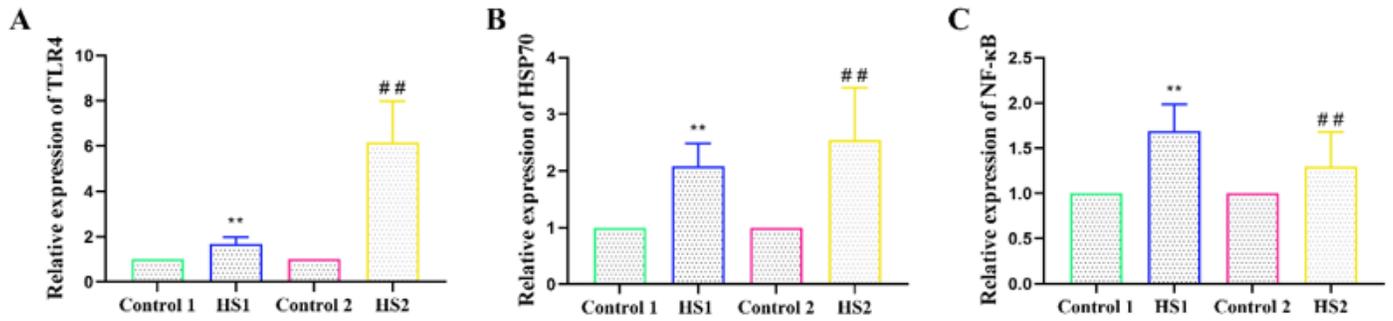
**Figure 4**

Effects of heat stress on NF-κB pathways in liver of broilers. (A) Protein expressions of NF-κB pathways in liver of broilers. Relative expression of (B) p-p65/p65 and (C) p-IκB-α/IκB-α. \*\*P < 0.01 vs. control 1; ##P < 0.01 vs. control 2.



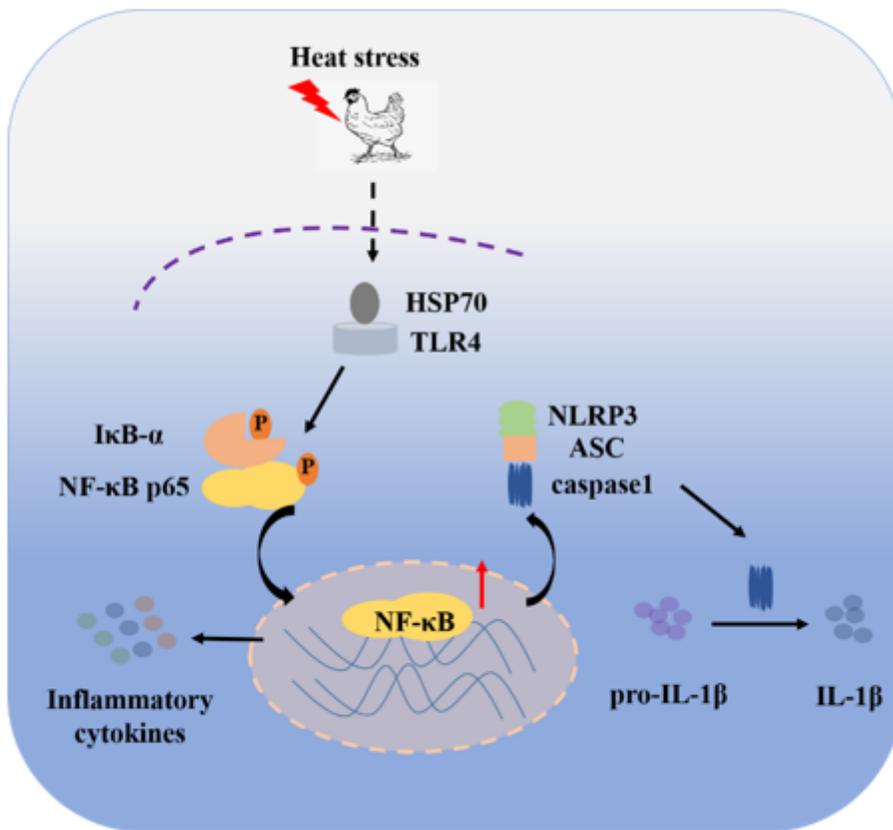
**Figure 5**

Effects of heat stress on proteins expressions of TLR4 and HSP70 in liver. (A) Proteins levels of TLR4 and HSP70 in liver of broilers. Relative expression of (B)TLR4 and (C) HSP70. \*\*P < 0.01 vs. control 1; ##P < 0.01 vs. control 2.



**Figure 6**

Effects of heat stress on genes expressions of TLR4, HSP70 and NF-κB in liver of broilers. (A) TLR4, (B) HSP70 and (C) NF-κB genes expressions in liver were measured by qRT-PCR. \*\*P < 0.01 vs. control 1; ##P < 0.01 vs. control 2.



**Figure 7**

Mechanism of heat stress induced liver inflammation injury of broilers. Heat stress activates TLR4-NF-κB and NLRP3 signaling pathway, which induced the secretion of pro-inflammatory factors.