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# Chronic heat stress induces lung injury in broiler chickens by disrupting the pulmonary blood-air barrier and activating TLRs/NF-κB signaling pathway

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**ABSTRACT** As an important respiratory organ, the lung is susceptible to damage during heat stress due to the accelerated breathing frequency caused by an increase in environmental temperature. This can affect the growth performance of animals and endanger their health. This study aimed to explore the mechanism of lung tissue damage caused by heat stress. Broilers were randomly divided into a control group (**Control**) and a heat stress group (**HS**). The HS group was exposed to 35°C heat stress for 12 h per d from 21-days old, and samples were taken from selected broilers at 28, 35, and 42-days old. The results showed a significant increase in lactate dehydrogenase (**LDH**) activity in the serum and myeloperoxidase (**MPO**) activity in the lungs of broiler chickens across all 3 age groups after heat stress ($P < 0.01$), while the total antioxidant capacity (**T-AOC**) was significantly enhanced at 35-days old ($P < 0.01$). Heat stress also led to significant increases in various proinflammatory factors in serum and expression levels of HSP60 and HSP70 in lung tissue. Histopathological results showed congestion and bleeding in lung blood vessels, shedding of pulmonary epithelial cells, and a large amount of inflammatory infiltration in the lungs after heat stress.

The mRNA expression of TLRs/NF-κB-related genes showed an upward trend ($P < 0.05$) after heat stress, while the mRNA expression of MLCK, a gene related to pulmonary blood-air barrier, significantly increased after heat stress, and the expression levels of MLC, ZO-1, and occludin decreased in contrast. This change was also confirmed by Western blotting, indicating that the pulmonary blood-air barrier is damaged after heat stress.

Heat stress can cause damage to the lung tissue of broiler chickens by disrupting the integrity of the blood-air barrier and increasing permeability. This effect is further augmented by the activation of TLRs/NF-κB signaling pathways leading to an intensified inflammatory response. As heat stress duration progresses, broiler chickens develop thermotolerance, which gradually mitigates the damaging effects induced by heat stress.

**Key words:** heat stress, broiler, blood-air barrier, TLRs/NF-κB

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## INTRODUCTION

Broiler chicken, due to its high-quality protein and low-fat content, has become an indispensable food on human tables and has a huge breeding population worldwide. According to statistics from the Food and Agriculture Organization of the United Nations, the total number of broiler chickens raised globally in 2021 has reached 129.8 billion. Heat stress is one of the important environmental stressors in broiler chicken farming. High temperatures caused by the environment can affect efficient animal production and endanger animal welfare (Lara and Rostagno, 2013). Due to the high metabolism, fast growth rate, abundant feathers, and lack of sweat glands in poultry, they are more susceptible to heat stress during production and breeding (Nawab et al., 2018). High temperatures can cause severe damage to the health of poultry and affect their production performance, resulting in significant economic losses for commercial broilers (Baumgard and Rhoads, 2013; Hamidi et al., 2022). Studies have shown that heat stress causes damage to many organs in the body, including the heart, liver and lung (He et al., 2019; Zhang et al., 2020; Yang et al., 2021; Ma et al., 2022), and causes changes in the expression of many heat stress proteins (Hasan Siddiqui et al., 2020).

The lungs are an important gas exchange organ in the body, and various respiratory system diseases can cause different degrees of lung damage (Maldonado et al., 2020). According to research, various factors such as bacterial infection, toxin exposure, hypoxia, and stress are all related to lung injury (Chen, 2011; Dushianthan et al., 2011; Looney et al., 2014). Mammals mainly rely



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on sweat glands to dissipate heat and maintain body temperature balance (Yahav, 2015), but poultry lack sweat glands, so they primarily dissipate heat through respiration when the temperature is too high (Bell et al., 2001). High-frequency breathing leads to increased susceptibility of lung tissue damage in a heat stress environment. Research has shown that heat stress causes lung injury and results in the upregulation of various proinflammatory cytokines, including tumor necrosis factor TNF-a, IL-6, and IL-1$\beta$ (Wheeler et al., 2007; Wu et al., 2018). Furthermore, this process exacerbates inflammation through TLRs/NF-$\kappa$B signaling pathways (Wu et al., 2019).

Toll-like receptors (**TLRs**) are a class of receptors located on the surface of cell membranes that can recognize microbial molecules (such as bacteria, viruses, etc.) and activate signaling pathways (Kawai and Akira, 2011). The TLRs/NF-$\kappa$B signaling pathway is an important inflammatory response pathway that can promote the proliferation and differentiation of inflammatory cells, while also promoting the expression of inflammation-related genes. When TLRs receptors bind to their recognized ligands, they trigger a tyrosine phosphorylation cascade that activates downstream molecules such as MYD88, IRAK and TRAF6, ultimately leading to activation of the NF-$\kappa$B signaling pathway (Beutler, 2009; Kawai and Akira, 2010). The TLRs/NF-$\kappa$B signaling pathway plays a crucial role in combating infections and protecting the body against invasion by pathogens in the immune system (Hayden and Ghosh, 2012).

There is a close relationship between heat stress-induced lung injury and the TLRs/NF-$\kappa$B signaling pathway. Studies have shown that the activation of the TLRs/NF-$\kappa$B signaling pathway and subsequent inflammatory cytokine expression can be inhibited by reducing the expression of TNF receptor-associated factor 6 (**TRAF6**) and interleukin-1 receptor-associated kinase 1 (**IRAK1**). This inhibition can effectively attenuate the inflammatory response caused by lung injury (Perry et al., 2008; Ikari et al., 2014).

Changes in the permeability of the blood-air barrier are the main pathological changes in lung injury (Bhattacharya and Matthay, 2013; Herold and Gabrielli, 2013). The blood-air barrier is composed of alveolar epithelial cells, microvascular endothelial cells, and extracellular matrix between them (Leiby et al., 2020). Damage to the blood-air barrier can lead to increased lung permeability, impaired oxygen and carbon dioxide exchange function, and induce respiratory difficulties (Wang et al., 2020), further leading to various lung diseases such as tuberculosis and pulmonary inflammation (Planès et al., 1994). Tight junctions, adherens junctions, and gap junctions between lung epithelial cells and lung vascular endothelial cells collectively maintain the integrity of the alveolar epithelial barrier and endothelial barrier structure (Komarova et al., 2007). Changes in the expression of important tight junction proteins ZO-1 and occludin, as well as intercellular adhesion molecule-1 (**ICAM-1**), can cause changes in microvascular permeability in the lungs and damage to the blood-air barrier (Fang et al., 2018; Peng et al., 2020). MLCK is a calcium-dependent protein kinase that phosphorylates MLC, affecting the interaction force between actin and myosin, changing the cellular tension balance between tight junctions, adherens junctions, and cytoskeleton contraction, causing changes in the permeability of microvascular endothelial cells and affecting the blood-air barrier (Sun et al., 2015; Al-Sadi et al., 2017).

However, the roles of TLRs/NF-$\kappa$B signaling pathway and blood-air barrier in lung injury induced by heat stress are still unclear. Therefore, this study aimed to investigate the effects and mechanisms of heat stress on lung injury in broilers.

## MATERIALS AND METHODS

### Animals and Treatment Design

A total of 120 one-day-old male broilers were purchased from a commercial hatchery, and reared in a climate chamber at 25°C ± 1°C and 60% humidity from 1- to 21-days old, with standard vaccination procedures. At 21-days old, 100 broilers with similar body weights were randomly divided into 2 treatment groups, with 5 replicates per group and 10 chickens per replicate. The control group was maintained at a temperature of 23°C, while the heat stress (**HS**) group was maintained at a temperature of 35°C for 12 h per d, from 8:00 am to 8:00 pm. All broilers were provided with normal feed and water. All experimental animals in this study were approved by the Ethics Committee of Henan University of Science and Technology.

### Sample Collection

Samples were collected at 28-, 35-, and 42-days old. At each time point, 1 randomly selected chicken from each replicate was euthanized for blood collection and lung tissue sampling. Blood plasma was collected and stored at −25°C, while a portion of the lung tissue was fixed in 4% paraformaldehyde and another portion was transferred to −80°C for storage.

### Respiratory Rate Statistics, Serum pH Measurement

The abdominal feathers of broilers were observed for fluctuation, and the number of fluctuations per minute was counted as the respiratory rate. The pH value of serum was measured using a pH meter.

### Pathological Analysis

Lung tissues fixed in paraformaldehyde were dehydrated, cleared, and embedded, and then cut into 4 to 6 mm sections. Hematoxylin and eosin (**H&E**) staining was performed, and morphological characteristics of lung tissue were observed using an optical microscope (Olympus Corporation, Tokyo Japan).

### Measurement of Lactate Dehydrogenase, Total Antioxidant Capacity

Serum was diluted 50-fold and the lactate dehydrogenase (**LDH**) concentration was measured using a standard assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at OD450. The total antioxidant capacity (**T-AOC**) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to measure the enzymatic concentration at OD593, following the manufacturer's instructions.

### Myeloperoxidase Assay

Myeloperoxidase (**MPO**) levels are positively correlated with neutrophils in infected areas. Lung tissue was prepared as a 5% tissue homogenate in weight/volume ratio of 1:19 using homogenization medium. MPO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions, and the enzyme concentration was detected at OD460 using a microplate reader (Tecan Trading AG, Männedor Switzerland).

### Proinflammatory Cytokines Assay

According to the manufacturer's protocol, ELISA kits (Boster Biological Technology Co. Ltd., Wuhan, China) were used to measure the serum levels of cytokines TNF-$\alpha$ and IL-6.

### Quantitative Real-Time Polymerase Chain Reaction Assay

The expression levels of HSP60, HSP70, MLCK, MLC, NF-$\kappa$b, TLR4, TRAF6, MYD88, IRAK, and NLRP3 in lung tissue were detected using PCR. Total RNA was extracted from lung tissue using TRIzol reagent (Nanjing Vazyme Biotech Co. Ltd., Nanjing, China). The concentration and quality of the extracted RNA were measured using a spectrophotometer, and the RNA concentration was adjusted to 1,000 ng/$\mu$L. Reverse transcription was performed using the Hiscript III QRT Supermix kit (Nanjing Vazyme Biotech Co. Ltd.) according to the manufacturer's instructions. Quantitative PCR was performed using a sequence detection system (Thermo Fisher Scientific, Boston, MA) with a reaction volume of 20 $\mu$L. All primers used in this study are listed in Table 1, and $\beta$-actin was used as the internal control. The PCR program consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 95°C for 15 s, 60°C for 60 s, and a final extension at 95°C for 15 s.

**Table 1.** Information of sequences of the oligonucleotide primers.

| Gene | Sequences (5′−3′) |
|---|---|
| $\beta$-Actin-F | CCGCTCTATGAAGGCTACGC |
| $\beta$-Actin-R | CTCTCGGCTGTGGTGGTGAA |
| HSP60 F | GATGTGAAGTTCGGTGCGGA |
| HSP60 R | ATGGTGACAGCTACGGCATC |
| HSP70 F | TGTGGCCTTCACCGATACAG |
| HSP70 R | TGGGGTCATCATACTTGCGG |
| TLR4 F | CCAAACACCACCCTGGACTT |
| TLR4 R | CCATGGAAGGCTGCTAGACC |
| TRAF6 F | TTCCCTGACGGTAAAGTGCC |
| TRAF6 R | ACAAGAAACCTGCCTCCTGG |
| IRAK F | GGAGGTGCTCTGTGGAACTA |
| IRAK R | CACTGGCTGGTTGGGACTTC |
| NLRP3 F | TAGAGTACGCGGGTGAAGGA |
| NLRP3 R | CTGTGAAACTGCCCAACACG |
| MYD88 F | GAGGGATGATCCGTATGGGC |
| MYD88 R | ACACGTTCCTGGCAAGACAT |
| NF-Kb F | ACACCACTGGATATGGCAGC |
| NF-Kb R | TCTTGCTTGGATCAGGCGTT |
| MLCK F | CTCTGTCGGACCCGCTAC |
| MLCK R | CATCCCCCATGATGTGGACC |
| MLC F | CACATACGCGCAATGTGGAG |
| MLC R | CTTGTTTGGGTCTGCCAAGC |

### Western Blot

Approximately 20 mg of lung tissue was added to 200 $\mu$L of RIPA lysis buffer (Biyuntian Bio-Technology Co., Shanghai, China), followed by the addition of 2 $\mu$L of proteinase inhibitor. After homogenization, the supernatant was collected, and total protein was extracted. The protein concentration was determined using a BCA assay kit (Biyuntian Bio-Technology Co., Shanghai, China). Twenty micrograms of protein were separated on a 10% SDS-polyacrylamide gel and transferred onto a 0.45 $\mu$m PVDF membrane (Millipore, Darmstadt, Germany). The membrane was blocked with a 5% skim milk solution at room temperature for 1 h, followed by incubation with the primary antibody (Jackson ImmunoResearch Laboratories Inc., PA) diluted in 5% skim milk (1:5,000), and incubated overnight at 4°C. Subsequently, the membrane was incubated with a horseradish peroxidase (**HRP**)-conjugated secondary antibody (1:5,000) for 40 min. Chemiluminescent signals were detected using an enhanced chemiluminescence (**ECL**) kit (Nanjing Vazyme Biotech Co., Ltd.) and visualized using a chemiluminescent imaging system (Protein Simple USA, Inc., CA). The band intensity was calculated using Image J software.

### Statistical Analysis

Data were analyzed using IBM SPSS Statistics 26 software with 1-way analysis of variance (**ANOVA**) and independent sample $t$ test. Data were presented as mean ± standard deviation, and $P < 0.05$ was considered significant.

## RESULTS

### Respiratory Rate and Serum PH

The respiratory frequency of broilers was determined by observing the number of abdominal feather fluctuations. The results showed that heat stress significantly increased the respiratory frequency in the HS group of

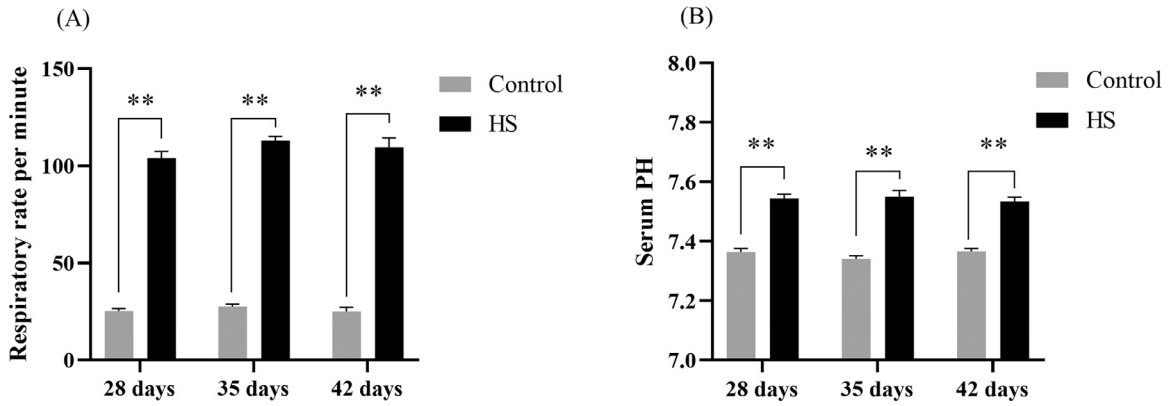


**Figure 1.** Effect of heat stress on respiratory rate (A) and serum PH (B) in broiler chickens. The data are presented as mean ± SE ($n = 5$) ($^{**}P < 0.01$).

broilers ($P < 0.01$). Furthermore, the serum pH value test results indicated that compared with the Control group, the serum pH of the HS group at all 3 ages was significantly elevated ($P < 0.01$). These findings suggest that heat stress not only increased the respiratory frequency of broilers but also caused respiratory alkalosis (Figure 1).

## Lung Histopathological Analysis

Histopathological changes in lung tissue were observed using HE staining, and the results are shown in Figure 2. Compared with the Control group, broilers in the HS group showed varying degrees of congestion and bleeding in lung tissue. At 28-days old, there was an increase in cell spacing and obvious inflammatory cell infiltration in the lung tissue of broilers in the HS group. At 35- and 42-days old, a large number of epithelial cells were shed from lung tissue of broilers in the HS group, and the endothelial structure of microvessels was damaged, with a significant increase in red blood cells.

## Serum Biochemical Parameters Analysis

Oxidative indicators and proinflammatory cytokines were measured in the serum. As shown in Figure 3, after heat stress, the serum LDH levels of the HS group chickens at all 3 ages were significantly higher than those of the Control group ($P < 0.01$). The HS group also exhibited significantly elevated T-AOC levels at both 35- and 42-days old ($P < 0.01$, $P < 0.05$), however this difference was not seen at 28-days old. Furthermore, compared with the Control group, the serum TNF-a levels were significantly higher in the 28-days old chickens after heat stress ($P < 0.05$), while there was no significant change at 35- and 42-days old ($P > 0.05$). Additionally

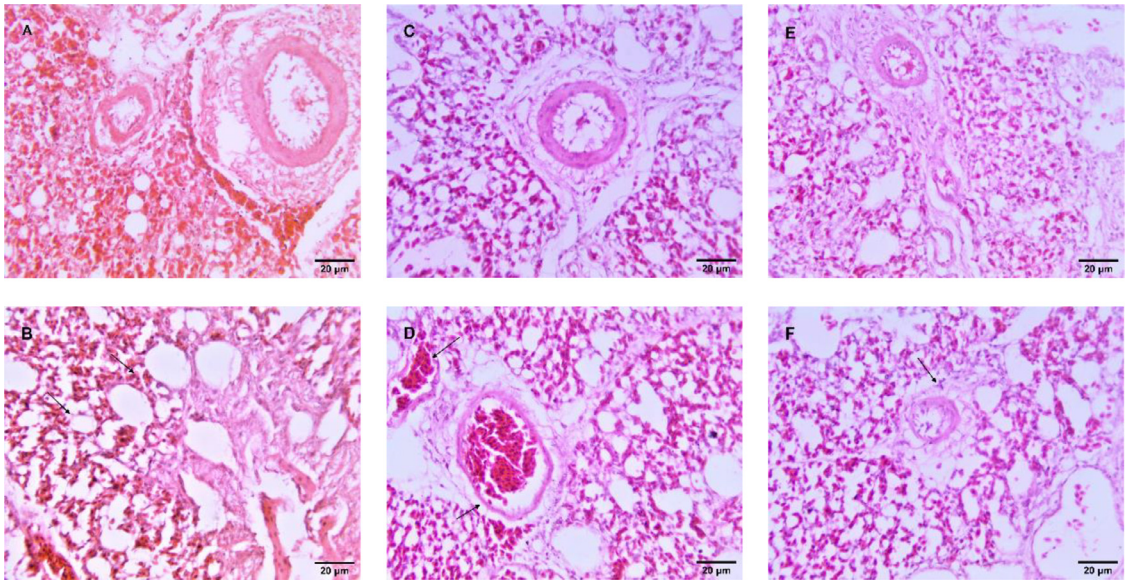


**Figure 2.** Effect of chronic heat stress on the histopathological changes in broiler lung (H&E stain; scale bar: 20 $\mu$m). Abbreviations: A, Control group at 28 d of age; B, Heat stress group at 28 d of age; C, Control group at 35 d of age; D, Heat stress group at 35 d of age; E, Control group at 42 d of age; F, Heat stress group at 42 d of age.

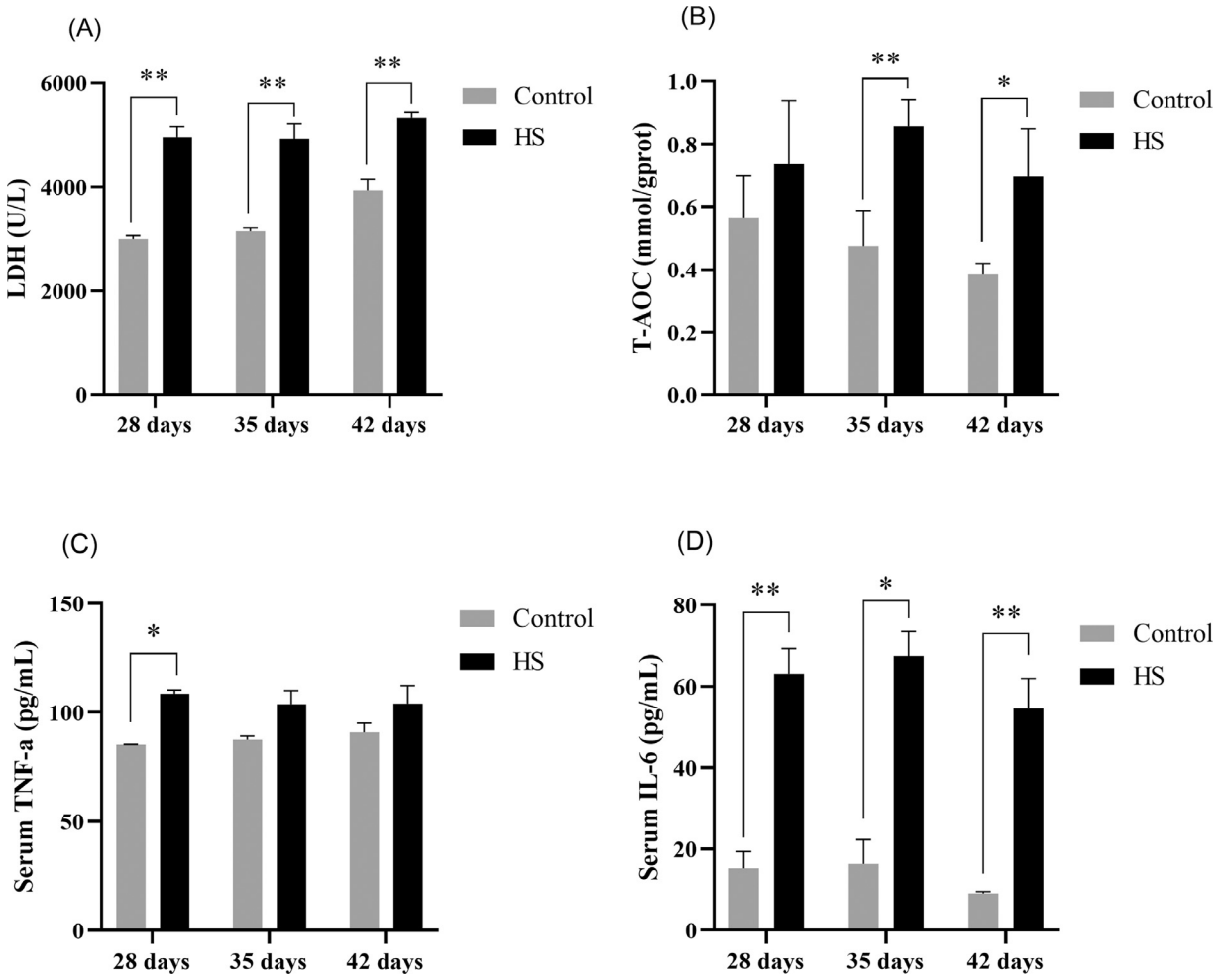


**Figure 3.** Effect of chronic heat stress on the expression of LDH (A), T-AOC (B), TNF-$\alpha$ (C), and IL-6 (D) in broiler serum. HS means heat stress. The data are presented as mean ± SE ($n = 5$) ($^{*}P < 0.05$, $^{**}P < 0.01$).

the serum IL-6 levels of the chickens at all 3 ages were significantly increased after heat stress ($P < 0.05$).

## Expression of Myeloperoxidase in the Lung

MPO activity was measured in lung tissue. As shown in Figure 4, after heat stress, the MPO activity in the lung tissue of chickens at all 3 ages was significantly higher than that of the Control group ($P < 0.01$).

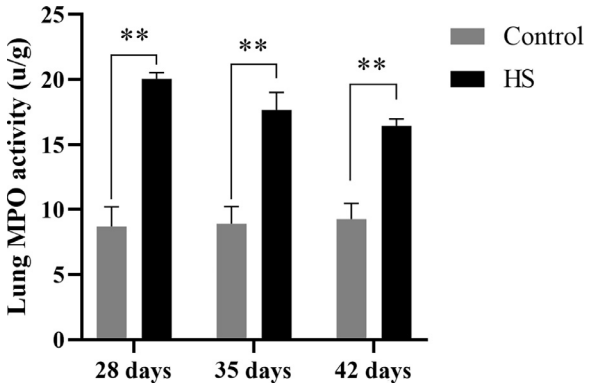


**Figure 4.** Effect of chronic heat stress on MPO activity in the lungs of broiler. HS means heat stress. The data are presented as mean ± SE ($n = 5$) ($^{**}P < 0.01$).

## Expression of Relevant Genes and Proteins in the Lung

The expression of heat shock proteins (**HSPs**) in lung tissue was detected by PCR. As shown in Figure 5, compared with the Control group, the expression of HSPs in the lungs of broilers at all 3 ages increased after heat stress. HSP60 exhibited a significant increase at 28- and 35-days old ($P < 0.01$), while HSP70 showed a significant increase at 35-days old ($P < 0.05$).

The expression changes of genes encoding proteins related to the blood-air barrier are shown in Figure 5. The mRNA expression of MLCK increased significantly after heat stress, particularly at 28-days old ($P < 0.01$). Conversely, the mRNA expression of MLC decreased significantly at 28- and 35-days old after heat stress ($P < 0.05$), but showed no significant change at 42-days old ($P > 0.05$).

The changes in TLRs/NF-$\kappa$B-related gene expression are shown in Figure 6. Compared with the Control group, the mRNA expression of MYD88 and TLR4 increased in the lung tissue of chickens at all 3 ages after heat stress, with significant changes observed at 28- and 35-days old ($P < 0.05$). Additionally, the mRNA expression of TRAF6, IRAK, and NF-$\kappa$B in the HS group at

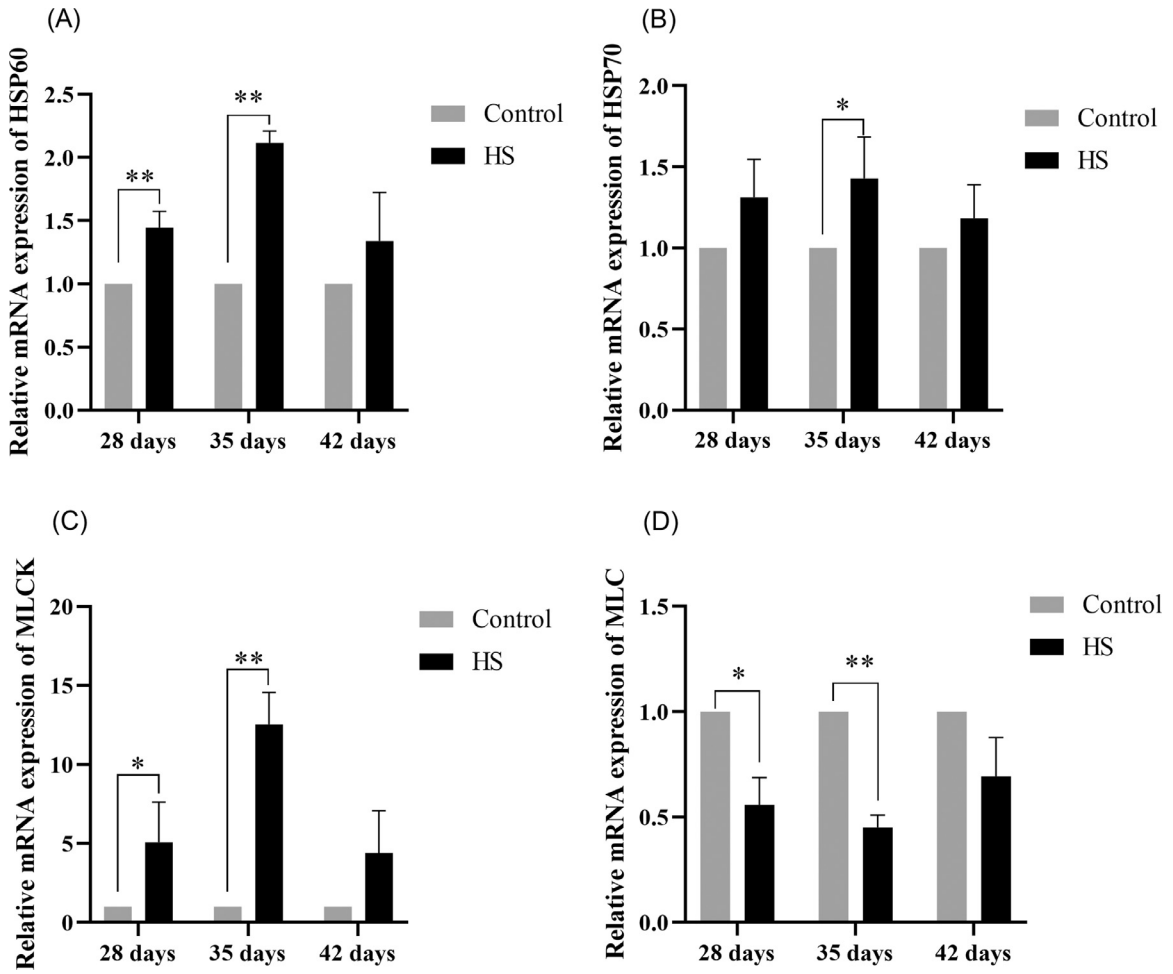


**Figure 5.** Effect of chronic heat stress on the mRNA expressions of HSP60 (A), HSP70 (B), occludin (C), MLCK (D), MLC (E) in broiler lung. The data are presented as mean ± SE ($n = 5$) ($^{*}P < 0.05$, $^{**}P < 0.01$).

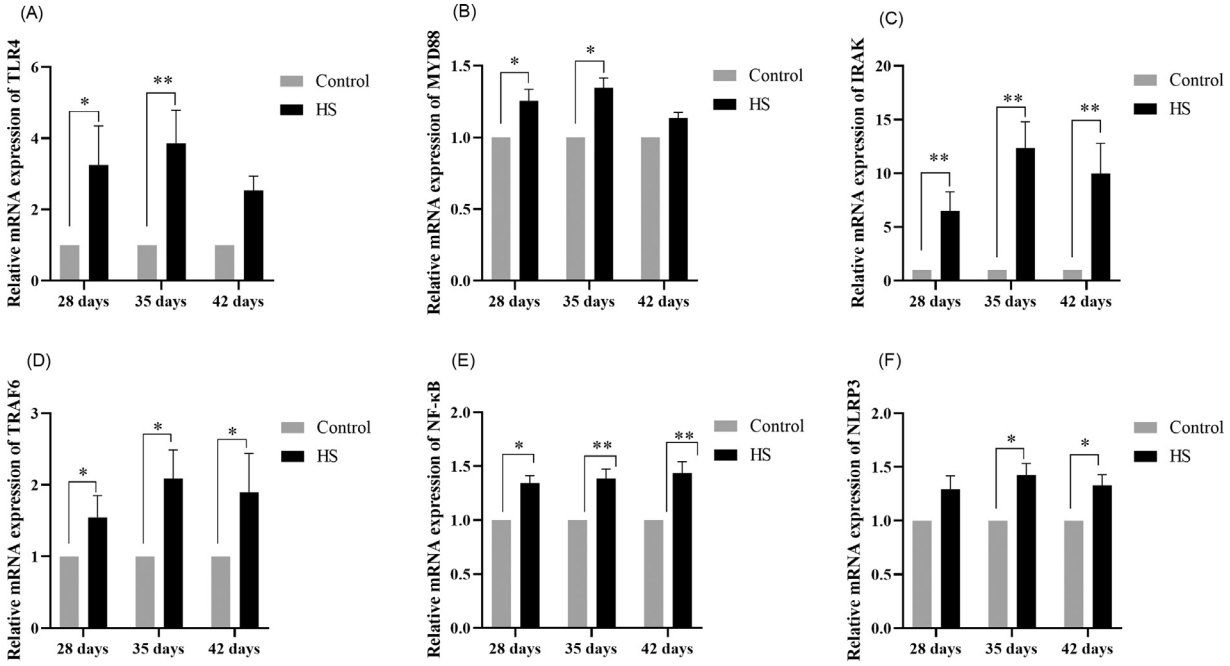


**Figure 6.** Effect of chronic heat stress on the mRNA expressions of TLR4 (A), MYD88 (B), IRAK (C), TRAF6 (D), NF-$\kappa$B (E), NLRP3 (F) in broiler lung. The data are presented as mean ± SE ($n = 5$) ($^{*}P < 0.05$, $^{**}P < 0.01$).

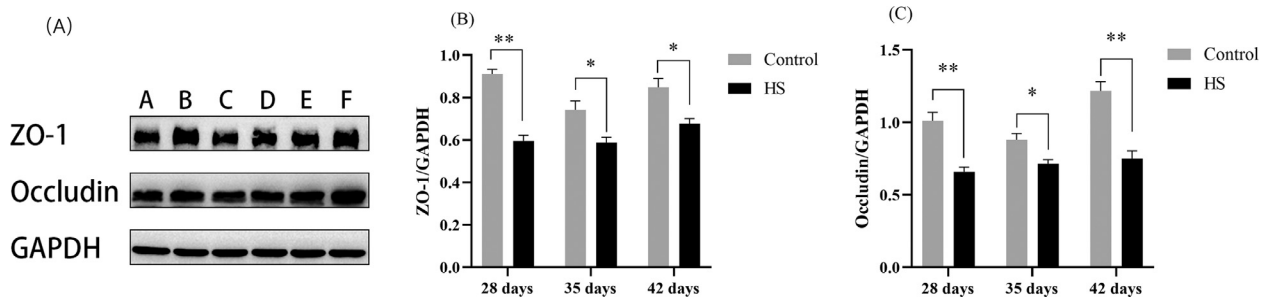


**Figure 7.** Effects of heat stress on proteins expressions of ZO-1 and occludin in lung. (A) Proteins levels of ZO-1 and occludin in lung of broilers. Relative expression of (B) ZO-1 and (C) occludin ($^{*}P < 0.05$, $^{**}P < 0.01$).

all 3 ages was significantly higher than that in the Control group ($P < 0.05$, $P < 0.01$, $P < 0.05$). Furthermore, the mRNA expression of NLRP3 in the lung tissue of the HS group showed a significant increase at 35- and 42-days old ($P < 0.05$), but no significant increase was observed at 28-days old ($P > 0.05$).

### *Western Blot*

The expression of tight junction proteins in lung tissue was detected by Western blot. As shown in Figure 7, compared with the Control group, the expression of the tight junction protein ZO-1 and occludin in the lung tissue of chickens at all 3 ages were significantly decreased after heat stress ($P < 0.05$). ZO-1 exhibited the most significant decrease at 28-days old ($P < 0.01$) and occludin showed the most significant decrease at 28- and 42-days old ($P < 0.01$).

## DISCUSSION

As an important stressor in large-scale poultry farming, heat stress severely affects the welfare and productivity of poultry. Numerous studies have shown that heat stress can cause damage to various organs such as the liver, heart, and intestines, consequently affecting their normal functions (Chen et al., 2020, 2021). However, there is currently limited research on the effects of heat stress on the lungs of broiler chickens. The lung, as an important respiratory organ in animals, plays a crucial role in adjusting the ratio of oxygen and carbon dioxide, as well as regulating the acidity and alkalinity of the blood. Additionally, the lung surface contains a plethora of immune cells and substances, such as mucus, that serve as barriers against viruses, bacteria, and other microorganisms, effectively preventing their entry into the body. Studies have shown that the breathing rate of animals increases in a heat stress environment, and the respiratory system becomes restricted. This restriction makes it difficult for the body to effectively eliminate carbon dioxide ($\mathbf{CO_2}$) produced by the alveoli, resulting in a decrease in $CO_2$ concentration and an increase in pH levels in the blood (Riesenfeld et al., 1996). The results of this experiment indicated that heat stress caused a significant increase in the respiratory rate of broiler chickens compared to the control group. Additionally, the blood pH value increased, suggesting that heat stress affected the respiratory function of the lungs in broiler chickens, resulting in respiratory alkalosis.

Histopathological examination revealed significant congestion and hemorrhage in the lung tissue of broiler chickens following heat stress. Furthermore, a substantial amount of shedding of epithelial cells and infiltration of neutrophils and lymphocytes were observed. When comparing different ages of broiler chickens exposed to heat stress, it was observed that the extent of lung injury escalated with longer durations of heat stress. However, there was no significant difference in the degree of lung injury between 42-day-old and 35-day-old broilers. This lack of distinction may be attributed to the heat adaptation mechanisms that develop during later stages of heat stress (Goto et al., 2022).

Poultry employ an active mechanism called evaporative heat loss to dissipate heat when subjected to heat stress. This process involves exchanging heat with the external environment through air sacs, which leads to an increased respiratory rate and panting (Wasti et al., 2020). During heat stress, the increased breathing rate of broiler chickens leads to excessive excretion of carbon dioxide ($CO_2$) from the body. This process contributes to an increase in blood pH, inducing oxidative stress in chickens. Researchers have found that after exposure to heat stress, there was a significant increase in the serum LDH and MPO activity of broiler chickens. Additionally, T-AOC was enhanced, which aligns with previous research on the effects of heat stress (Yang et al., 2021; Yin et al., 2021).

When cellular oxidative stress exceeds a certain threshold, it can impact the integrity and function of lung cell membranes, thereby affecting respiratory function and compromising the stability of the immune system. Furthermore, oxidative stress can induce pathological changes, including inflammation and cell apoptosis, which exacerbate the development of lung lesions (Kinnula and Crapo, 2003; Rahman et al., 2006; Reddy et al., 2009). Previous studies have found that heat stress can alter the expression of HSP proteins (Hasan Siddiqui et al., 2020). As a member of the HSP protein family, HSP70 can bind to TLR4 and trigger an inflammatory response, this activation subsequently promotes the secretion and synthesis of

proinflammatory cytokines such as tumor necrosis factor-alpha (**TNF-α**) and interleukin-6 (**IL-6**) in animals (Asea et al., 2000). TLR4 activates downstream molecules MYD88, IRAK, and TRAF6 to ultimately activate the NF-κB signaling pathway, further amplifying the inflammatory response (Kawai and Akira, 2010). The results of this experiment showed that the mRNA expression levels of HSP60 and HSP70 in lung tissue of broiler chickens in the HS group at 28- and 35-days old were significantly increased. Additionally, the expression of mRNA associated with the TLR4/NF-κB pathway in the lung tissue of the HS group was notably higher compared to the Control group. These findings suggest that heat stress-induced lung injury in broiler chickens through inflammation mediated by the TLR4/NF-κB pathway.

Lung injury often involves alterations in the permeability of the blood-air barrier. This barrier is maintained by important tight junction proteins, namely ZO-1 and occludin. These proteins play a crucial role in preserving the stability and integrity of the blood-air barrier. Some studies have found a close relationship between MLCK activity and the expression of ZO-1 in the barrier, where MLCK inhibits the stable expression of ZO-1 (Yu et al., 2010). MLCK promotes MLC phosphorylation and disrupts ZO-1 expression in epithelial cells, thereby altering the permeability of the blood-air barrier. Although there have been many studies on acute lung injury in mice and humans, research on changes in the lungs of broiler chickens after heat stress is limited. Therefore, we observed changes in the lung tissue of broiler chickens under different durations of heat stress. The results showed that the MLCK activity increased in the lung tissue of broiler chickens in the HS group at 28- and 35-days old, while MLC activity significantly decreased. Moreover, the expression levels of ZO-1 and occludin in the lung tissue of broiler chickens in the HS group were significantly lower than those in the Control group. This indicates that heat stress disrupts the intercellular tight junction structure of lung tissue, alters the permeability of the blood-air barrier, and consequently affects the normal function of the lungs.

## CONCLUSIONS

In this experiment, heat stress caused varying degrees of pathological damage to chicken lung tissue, and the expression of genes related to the TLR4/NF-κB pathway was affected by heat stress. Heat stress deepened the damage to lung tissue by activating the TLR4/NF-κB pathway and disrupting the blood-air barrier of the lung tissue. The difference in the degree of damage between different ages of chickens manifested as an increase in lung injury with increasing duration of heat stress, but there was no significant difference between 35-day-old and 42-day-old chickens. This may be due to the later development of thermal adaptation in chickens, which prevents further aggravation of heat stress injury, suggesting a significant period of heat stress-induced animal damage. Therefore, in large-scale broiler chicken farming, appropriate measures can be taken to prevent and mitigate losses caused by heat stress before the period of significant heat stress injury. Currently, the main methods to alleviate heat stress injury are ventilation and cooling. Based on the results of this experiment, it can be observed that heat stress primarily induces lung damage in broiler chickens through inflammatory responses. Therefore, apart from improving environmental conditions, exploring effective drugs to suppress inflammatory reactions can also be a potential approach to alleviate heat stress injury. These research results have important practical implications for discovering effective ways to alleviate chicken lung damage under high-temperature conditions and reduce the losses caused by heat stress to production.

## ACKNOWLEDGMENTS

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Ethics Declaration: All experimental animals in this study were approved by the Ethics Committee of Henan University of Science and Technology.

## DISCLOSURES

None of the authors has any conflicts of interest to declare.

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