Dietary resveratrol supplementation inhibits heat stress-induced high-activated innate immunity and inflammatory response in spleen of yellow-feather broilers

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ABSTRACT The aim of this study was to investigate the effect of dietary resveratrol supplementation on innate immunity and inflammatory responses in the spleen of yellow-feather broilers under heat stress. A total of 288 yellow-feather broilers of 28-day-old were randomly assigned to 3 treatment groups with 6 replicates. A thermo-neutral group (TN) (24 ± 2°C) received a basal diet and another 2 heat-stressed groups (37 ± 2°C for 8 h/D and 24 ± 2°C for the remaining time) were fed the basal diet (HT) or basal diet with 500 mg/kg resveratrol (HT+Res) for 14 consecutive days. The results showed that heat stress decreased ($P < 0.05$) the growth index of thymus, spleen, and bursa of Fabricius, reduced ($P < 0.05$) the levels of complement C3 and C4 in serum. Heat stress also caused activation of inflammatory immune responses evidenced by increased ($P < 0.05$) the mRNA abundance of HSP (heat shock protein) 70, toll-like receptor (TLR)1, TLR4, TLR5, myeloid differentiation factor-88 (MyD88), nucleotide-binding oligomerization domain 1 (NOD1), Dectin-1, transforming growth factor-β-activated kinase 1 (TAK1), interleukin (IL)-1, IL-4, IL-6, and tumor necrosis factor (TNF)-α, but decreased the mRNA abundance of interferon (IFN)-γ, activated nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK), and phosphoinositide-3 kinases-protein kinase B (PI3K/AKT) signaling pathways. Dietary supplementation with resveratrol improved ($P < 0.05$) the growth index of thymus, spleen and bursa Fabricius, and increased ($P < 0.05$) the serum level of complement C3 under heat stress. In addition, resveratrol reduced ($P < 0.05$) the mRNA abundance of HSP70, TLR4, TLR5, NOD1, Dectin-1, and TAK1, and inhibited the NF-κB, MAPK and PI3K/AKT signaling pathway via down-regulated the phosphorylation of p65, extracellular signal-regulated kinases 1/2, c-Jun N-terminal protein kinase and AKT, as well as decreased the inflammatory cytokines expression, including IL-1, IL-4, IL-6, and TNF-α in the spleen under heat stress. Collectively, dietary resveratrol could have beneficial effects to regulate innate immunity and inflammatory response, via inhibiting the activation of NF-κB, MAPK, and PI3K/AKT signaling pathways induced by heat stress in the spleen.

Key words: broilers, heat stress, innate immune, inflammation, resveratrol

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INTRODUCTION

Heat stress has drawn greater public attention because of its detrimental impacts, especially for high-metabolic and productive poultry in tropical subtropical and arid regions. Moreover, the lack of sweat glands and the long-term breeding about genetic selection program of broilers for increased production performance has led to higher metabolic heat generation and therefore enhanced their susceptibility to heat stress (He et al., 2018). Exposure of birds to high ambient temperature has been shown to alter physiological homeostasis, such as systemic immune dysregulation, endocrine and electrolyte disorders which result in poor performance (Liu et al., 2016; Quinteiro-Filho et al., 2012). Higher levels of heat shock protein 70 (HSP70) mRNA expression were reported in broilers and laying hens under high environmental temperature conditions (Liu et al., 2016; Tang et al., 2018; Wang et al., 2018a). HSP70 proteins are a class of ATP-dependent molecular chaperone proteins involved in protein folding in response to cellular stress, it can be expressed in the nucleus, cytoplasm, endoplasmic reticulum, and mitochondria of eukaryotic cells, induced by various environmental threats (Muralidharan and Mandrekar, 2013), which can exert an inflammatory immune activating signal for host defencing against infection or an anti-inflammatory immunosuppressive signal to prevent excessive inflammation (Kaul and Thippeswamy, 2011).

It has been established that host's innate immune system detects the presence of microbial infection through germ line-encoded pattern recognition receptors (**PRRs**) (Kawai and Akira, 2009), which consist

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of the Toll-like receptors (**TLRs**), C-type lectin receptors (**CLRs**), nucleotide-binding domain, leucine-rich repeat-containing (or NOD-like) receptors (**NLRs**), RIG-I-like receptors (**RLRs**), and the AIM2-like receptors (Brubaker et al., 2015). After ligation of PRRs, nuclear factor kappa B (**NF-κB**) and mitogen-activated protein kinases (**MAPKs**) are activated, which leads to the production of pro-inflammatory cytokines and interferons (**IFN**). Under environmental stress conditions, due to the influences of the neuroendocrine and anti-oxidation systems, the expression of TLR is affected. Quinteiro-Filho et al. (2017) observed that the expression of TLR-2, but not TLR-4, was decreased in the spleen and caecal tonsils of heat stressed broiler chickens. However, the mRNA levels of TLR-4 were increased in the jejunum and ileum of heat stressed chickens (Varasteh et al., 2015) and peripheral blood mononuclear cell of Bama miniature pigs (Ju et al., 2014). The expression of TLR5 and nucleotide-binding oligomerization domain 1 (NOD1) gene was also induced in the heat and cold stressed fish, but mostly restricted in the blood (Basu et al., 2015). However, most PPRs and the related signal pathway of inflammation response are rarely reported in broilers under heat stress.

Resveratrol (*trans*-3,5,4′-trihydroxystilbene)is a nutraceutical that has attracted much attention due to its pharmacological potential, which is a phytoalexin polyphenolic compound found in many kinds of plants, including grapes, peanuts, and berries (Bitterman and Chung, 2015). It has been reported to exert many different biological effects, including anti-inflammatory, antioxidant, anti-aging, and anti-obesity (Aguirre et al., 2014; Francioso et al., 2014; Xia et al., 2017). Previous studies have also suggested that resveratrol regulates inflammation via various signaling pathways, such as NF-κB (Liu et al., 2016), MAPKs (Pirola and Froejdoe, 2008), and phosphoinositide-3 kinases (**PI3K**)-protein kinase B (AKT) (Pirola and Froejdoe, 2008). Liu et al (2016) also reported resveratrol as functional feed additive to influence performance and the expression levels of NF-κB, epidermal growth factor, and heat shock proteins of heat-stressed broilers. Based on these findings, we hypothesized that resveratrol could modulate the HSP70 and innate immunity in spleen of heat-stressed broilers. However, direct evidence for supporting this hypothesis is missing. Therefore, the major objective of this study is to investigate the effect of resveratrol on HSP70 expression, the activation of innate immunity, including the expressions of TLRs, NODs, RLRs, and CLRs, and pro-inflammatory cytokines in the spleen of heat-stressed yellow-feather broilers.

MATERIALS AND METHODS

Birds, Diets, and Experimental Design

All experimental trails including the usage of broilers were consulted to the Animal Care and Use Committee of Hunan Agricultural University, Changsha, PR China. All the male yellow-feather broilers were purchased from a commercial hatchery (Elite hatchery of yellow-feather broiler, Zhuzhou, China). From 1 to 21 D of age, the broilers were acquired commercial standard feeding and raising managements. In the following week adaptation period, the birds were supplied a basal diet and water ad libitum. Afterwards, a total of 288 birds with average weight of 717 g (individually weighed) were picked out from the flock and randomly allotted to 3 treatment groups, each of which included 6 cages of 16 birds per cage (with dimension of 160 × 90 cm). In thermos-neutral group (**TN**), birds were fed with the basal diet and housed in a temperature-controlled room at 24 ± 2°C for 24 h/D. In the remaining 2 groups, birds were subjected to cyclic heat stress in controlled room at 37 ± 2°C for 8 h/D (0930 to 1730 h) and followed by 24 ± 2°C for the remaining 16 h/D. Birds in these 2 groups were received the basal diet and basal diet supplementation with resveratrol (500 mg/kg), be set as HT group and HT+Res group, respectively. This treatment lasted for 14 D (from days 28 to 42). The basal diet was formulated to meet the nutrient requirements of broilers based on National Research Council (1994), and its nutrient profile is present in Table 1. Feed (crumbled) and water were provided ad libitum and checked thrice daily.

Table 1. Composition and nutrient levels of the basal diets (air-dry basis).

Item	Amount
Ingredient (%)	
Corn	58.50
Soyban meal	31.50
Rice bran	3.00
Soybean oil	3.24
Dicalcium phosphate	1.20
Limestone	1.13
Methionine	0.12
Salt	0.31
Vitamin-mineral-premix[a]	1.00
Total	100.00
Nutrent level[b]	
ME/(MJ/kg)	12.50
CP (%)	18.87
Ca (%)	0.90
Available P (%)	0.40
Lysine (%)	1.00
Methionine (%)	0.40

[a]The vitamin-mineral premix provides following for per kilogram diet: vitamin A 12,000 IU, vitamin D3 3,000 IU, vitamin E 25 IU, vitamin K3 2.2 mg, vitamin B1 3 mg, vitamin B2 8 mg, vitamin B6 4 mg, vitamin B12 0.02 mg, nicotinic acid 45 mg, pantothenic acid 12.5 mg, biotin 0.1 mg, folic acid 1 mg, copper 8 mg, iron 80 mg, zinc 60 mg, manganese 100 mg, selenium 0.15 mg, iodine 0.35 mg.

[b]Nutrient level: Lysine and Methionine were calculated values, whereas others were analyzed values.

Sample Collection and Preparation

On day 42, 1 bird (weighed) nearly the average weight were randomly taken out from each cage for blood samples collection from wing venous. Blood samples were centrifuged at 1,000 × *g* for 15 min at 4°C. Serum was

collected and frozen at −20°C until subsequence analysis. When birds were slaughtered, isolated, and weighed the spleen, thymus, and bursa of Fabricius. Afterwards, spleen was collected immediately, frozen by liquid nitrogen, and stored at −80°C until the extraction of total RNA and protein.

Chemicals

Resveratrol (≥98% purity) was extracted from Polygonum cuspidatum at the Hunan Engineering and Technology Center (Changsha, China). Antibodies against PI3K, iκBα, P65, P38, c-Jun N-terminal protein kinase (**JNK**) were purchased from Proteintech (Rosemont, IL). Antibodies against AKT, phosphorylated iκBα, phosphorylated P38 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against extracellular signal-regulated kinases 1/2 (ERK1/2), phosphorylated ERK1/2, phosphorylated JNK, phosphorylated P65 were purchased from Abcam (Cambridge, MA). Bicinchoninic acid (**BCA**) protein assay reagent was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Immune Organ Growth Index Determination

Immune organ index (mg/g) was calculated as the immune organ fresh weight (mg)/chicken weight (g) before slaughter.

Determination of Serum Complements Concentration

The concentrations of complement C3 and C4 in the serum were measured using the commercially ELISA kits according to manufacturer's instructions (Bogoo Biological Technology Co. Ltd., Shanghai, China), detailed by Liu et al. (2014).

Real-Time quantitative PCR

Total RNA was isolated from liquid nitrogen-frozen spleen using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the protocol provided by the manufacturer. We identified the purity of total RNA spectrophotometrically via usage of optical density (OD) 260 and OD280 measurements (Thermo Scientific, Waltham, MA, USA), and chose the samples with the 260/280 ratios of 1.8 to 2.0 for succeeding PCR reactions. Real-time PCR was performed as described previously (Liu et al., 2016). The primers of genes (Sangon Biotech, Shanghai, China) were shown in Supplemental Table 1. β-actin was used as a housekeeping gene to normalize target gene transcript levels. Relative expression was normalized and expressed as a ratio to the expression in TN group.

Table 2. Effect of resveratrol on growth index of immune organs in yellow-feather broilers under heat stress.

Items(mg/g)	TN	HT	HT+Res	SEM	*P*-value
Thymus	3.47[a]	1.20[c]	2.62[b]	0.168	0.001
Spleen	1.25[a]	0.92[b]	1.43[a]	0.062	0.002
Bursa Fabricius	1.84[a]	0.91[b]	1.79[a]	0.108	0.001

TN, thermoneutral treatment (24 ± 2°C); HT, high temperature treatment (37 ± 2°C).
[abc]Different superscript letters indicate significant differences ($P < 0.05$).

Western Blot

The protein concentration of the spleen was determined with the BCA protein assay kit at 562 nm wavelength according to the instructions of the manufacturer. Western blot analysis was conducted according to a previous study (Liu et al., 2016). Briefly, the equal amounts of proteins were separated by a reducing SDS-PAGE electrophoresis, subsequently, transferred to PVDF membrane. The membranes were blocked for 1 h at room temperature, in Trisbuffered saline containing 0.1% Tween-20 and 5% low-fat milk. The primary antibodies were incubated overnight at 4°C. After incubation with secondary antibody (HRP goat anti-rabbit IgG) for 1 h at room temperature, signals were detected using enhanced chemiluminescence kits (ECL-Plus, Thermo, Waltham, MA). Protein immunoreactive bands were photographed, and fluorescence was scanned for detection using the BioRad gel detection system. Each special banding gray value was digitized, and the gray value of the target protein was divided by β-actin and expressed as a relative level to group TN value.

Statistical Analysis

Values were presented as mean ± SEM. All data were analyzed by SPSS 21.0 (SPSS, Inc., Chicago, IL). Data among groups were analyzed using one-way ANOVA followed by Duncan's multiple range test, significance was considered as $P < 0.05$.

RESULTS

Growth Index of the Thymus, Spleen, and Bursa of Fabricius

The effects of heat stress and resveratrol supplementation on the growth index of the thymus, spleen, and bursa Fabricius are shown in Table 2. Compared with TN group, heat stress significantly decreased ($P < 0.05$) the index of the thymus, spleen, and bursa Fabricius. However, resveratrol supplementation alleviated ($P < 0.05$) the lower growth index of the thymus, spleen, and bursa Fabricius induced by heat stress. Additionally, broilers in HT+Res group exhibited the same level ($P > 0.05$) of the growth index of the spleen and

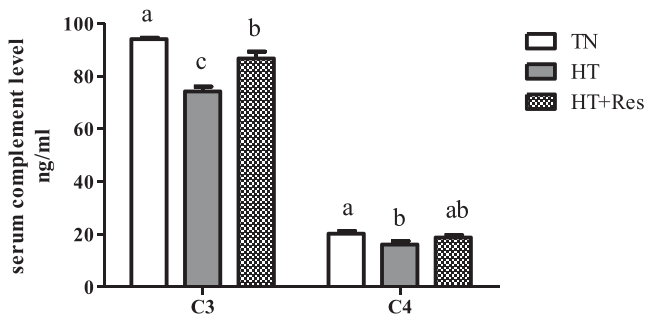

Figure 1. Effect of resveratrol on serum C3 and C4 levels in yellow-feather broilers under heat stress. Data are expressed as mean ± SEM (n = 6), [abc]Different superscript letters indicate significant differences ($P < 0.05$). TN, thermoneutral treatment (24 ± 2°C); HT, high temperature treatment (37 ± 2°C); HT+Res, HT+500 mg/kg resveratrol.

bursa Fabricius compared to broilers in thermoneutral conditions.

Serum Complement C3 and C4 levels

As shown in Figure 1, heat stress significantly decreased ($P < 0.05$) the levels of serum C3 and C4 compared with TN group. However, dietary resveratrol supplementation restored reduced serum C3 ($P < 0.05$) and C4 ($P > 0.05$) induced by heat stress. Interestingly, broilers kept under thermoneutral conditions showed ($P < 0.05$) the maximum levels of serum C3.

TLRs, CLRs, NLRs, and Immune Signal Pathway Related Gene mRNA Expression Levels

Based on our observation, compared with TN group, heat stress significantly increased ($P < 0.05$) TLR1, TLR4, and TLR5 mRNA expression, and decreased ($P < 0.05$) TLR2 mRNA expression in spleen. Moreover, dietary resveratrol supplementation significantly inhibited ($P < 0.05$) the higher expression of TLR4 and TLR5 in spleen of heat-stressed broilers. However, heat stress and dietary resveratrol supplementation had little effect on mRNA levels for TLR6 and TLR7 (Figure 2A). Likewise, heat stress also significantly increased ($P < 0.05$) expression levels of Dectin-1 and NOD1 mRNA, but dietary resveratrol supplementation significantly decreased ($P < 0.05$) Dectin-1 mRNA expression in spleen of heat-stressed broilers. Moreover, heat stress and dietary resveratrol supplementation did not alter mRNA levels for Dectin-2 and NLRP1L in this present study (Figure 2B).

As shown in Figure 2C, the expression levels of HSP70, myeloid differentiation factor-88 (MyD88), TAK1, NF-κB, and MAPK mRNA were greater ($P < 0.05$) but mTOR mRNA was lower ($P < 0.05$) in the spleen of broilers exposed to heat stress than the levels found in broilers in thermoneutral conditions. In contrast, HSP70, TAK1, NF-κB, and MAPK mRNA expression were inhibited ($P < 0.05$) in heat-stressed broilers fed with dietary resveratrol supplements, but improved ($P < 0.05$) mTOR mRNA expression compared with broilers in HT group.

NF-κB and PI3K/AKT Pathways

The expression of NF-κB pathway (iκBα, p- iκBα, p65, and p-p65) and PI3K and p-AKT proteins were shown in Figure 3 (wb). Heat stress markedly activated NF-κB evidenced by increased ($P < 0.05$) expression of p- iκBα (Figure 3B), p65 (Figure 3C), p-p65 (Figure 3D), and p-AKT (Figure 3F), and the down-regulation ($P < 0.05$) of iκBα (Figure 3A) expression when compared with TN group. Whereas, dietary supplementation with resveratrol significantly inhibited ($P < 0.05$) the expression of p65, p-p65, and p-AKT, and promoted ($P > 0.05$) the expression of iκBα and PI3K (Figure 3E) in spleen of yellow-feather broilers under heat stress.

MAPK Pathway

The effect of heat stress and resveratrol on MAPK pathway-related proteins expression in spleen is presented in Figure 4 (wb). Heat stress activated MAPKs based on the higher ($P < 0.05$) expression of p38 (Figure 4A), p-p38 (Figure 4B), p-ERK1/2 (Figure 4D), and p-JNK (Figure 4F) compared with these in TN group. However, resveratrol supplementation significantly inhibited ($P < 0.05$) the activation of p38 MAPK, and phosphorylation of ERK1/2 and JNK in spleen of broilers under heat stress. Moreover, the data shows that heat stress and resveratrol had no effect ($P > 0.05$) on the expression of ERK1/2 (Figure 4C) and JNK (Figure 4E).

Expression of Inflammatory Cytokines mRNA in the Spleen

As present in Figure 5, we investigated the effect of heat and resveratrol supplementation on the mRNA levels for inflammatory cytokines in the spleen, including IL-1β, IL-4, IL-6, TNF-α, and IFN-γ. Broilers in HT group exhibited higher ($P < 0.05$) mRNA levels for IL-1β, IL-4, IL-6, and TNF-α, but lower ($P < 0.05$) mRNA levels for IFN-γ compared to those in thermoneutral conditions. However, dietary resveratrol supplementation had significantly inhibited ($P < 0.05$) the mRNA over-expression of IL-1β, IL-4, IL-6, and TNF-α induced by heat stress. Moreover, dietary resveratrol supplementation increased ($P < 0.05$) the mRNA level of IFN-γ in the spleen of broilers exposed to heat stress.

DISCUSSION

Exposure of birds to high temperature environment can affect their growth performance and physiological homeostasis (Luo et al., 2018; He et al., 2019),

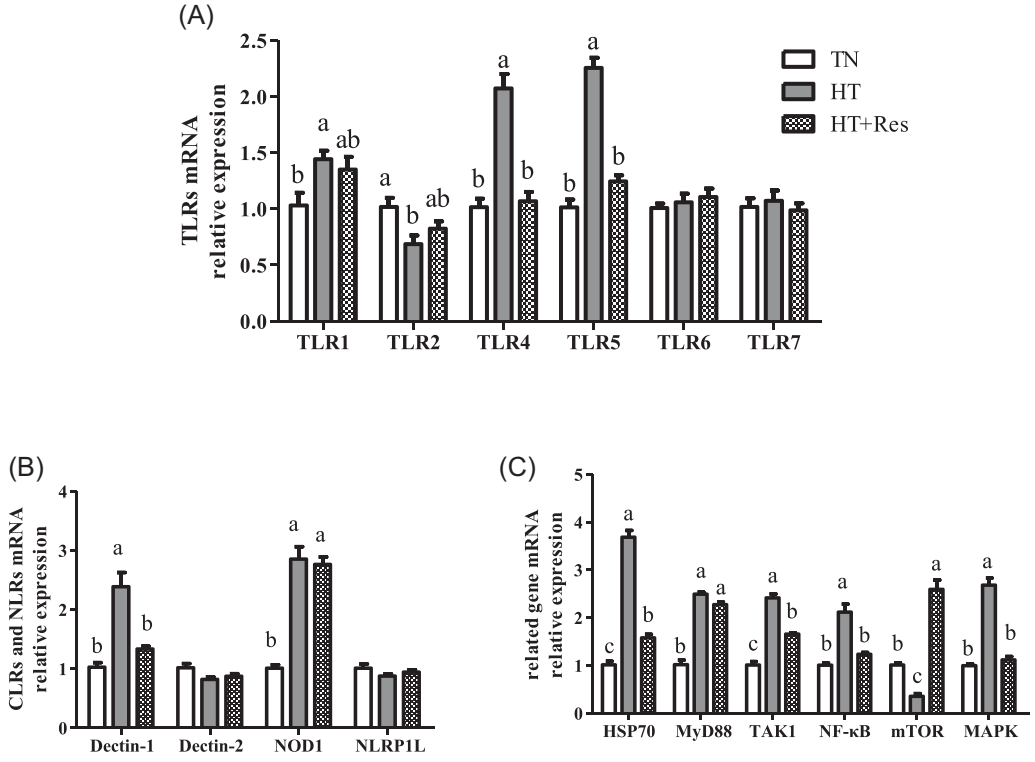

Figure 2. Effect of resveratrol on TLRs (A), CLRs, NLRs (B) and some related gene involved in immune signal pathway (C) mRNA expression in the spleen of yellow-feather broilers under heat stress. Data are expressed as mean ± SEM (n = 6), ^{abc}Different superscript letters indicate significant differences ($P < 0.05$). TN, thermoneutral treatment (24 ± 2°C); HT, high temperature treatment (37 ± 2°C); HT+Res, HT+500 mg/kg resveratrol. TLRs, Toll-like receptors; CLRs, C-type lectin receptors; Dectin, Dendritic cell-associated C-type lectin; NLRs, NOD-like receptors; NOD1, nucleotide-binding oligomerization domain; NLRP, NLR family PYD domain containing; HSP70, heat shock protein 70; MyD88, myeloid differentiation factor-88; TAK1, transforming growth factor-β-activated kinase 1; NF-κB, nuclear factor kappa B; mTOR, the kinase mammalian target of rapamycin; MAPK, mitogen-activated protein kinase.

but also have changes in the function of organs and tissues involved in neuroendocrine system and immune system (He et al., 2018). Classical studies on stress conducted by Hans Selye in 1936 demonstrated that stress as a syndrome characterized by adrenal gland hypertrophy, gastric ulcers and lymphoid organ atrophy (Selye, 1976). Among that, the spleen is the biggest peripheral immune organ, and bursa of Fabricius and thymus are central lymphoid organs in the immune system of poultry. Previously reported that exposed broilers to heat stress decreased the relative weight of thymus, spleen, and bursa (Calefi et al., 2016; Quinteiro-Filho et al., 2010), and also caused lower relative weight of spleen and bursa in Pekin ducks (Zhu et al., 2014). In this study, we also found that heat stress significantly decreased the growth index of the spleen, thymus and bursa of Fabricius by 26.4%, 65.4%, and 50.5%, respectively. This is an indication that heat stress caused damage to the immune organs. However, this present study shown that the growth index of the spleen, thymus and bursa of Fabricius of birds significantly increased upon dietary resveratrol supplementation during heat stress. Liu et al. (2014) also observed that diet supplemented resveratrol at 400 mg/kg can significantly increase the index of the bursa, thymus, and spleen in heat-stressed black-bone chickens. This result indicates that resveratrol may offer a potential nutritional strategy to inhibiting lymphoid organ atrophy and dysplasia caused by heat stress in yeller-feather broilers. A possible reason reported that resveratrol through the activation of the Nrf2 signaling pathway, thereby decreasing apoptosis in organs and tissue (Zhang et al., 2018).

Complement was discovered by Bordet in 1896 as a heat-labile component of serum, which recognized plays roles in the innate detection and elimination of pathogenic infections and in the modulation of adaptive immune responses (Song, 2010). In this present study, heat stress significantly decreased serum complement component C3 and C4. These results were in accordance with the data in reported by Zhang et al. (2012) and Hamidi et al. (2016). C3 was involved in the 3 complement pathway (classical, lectin, and alternative), and C4 also important for lectin pathway (Suresh et al., 2003). Therefore, heat stress can inhibit the immune function through decrease the content of serum C3 and C4 in broilers. However, dietary supplementation with resveratrol prevented the lower serum content of C3 and C4 induced by heat stress. The speculated reason is that resveratrol downregulates the complement-inhibitory protein cluster in result to improving the content of complement in serum under stress (Paul, 1993), but the mechanism still need further research.

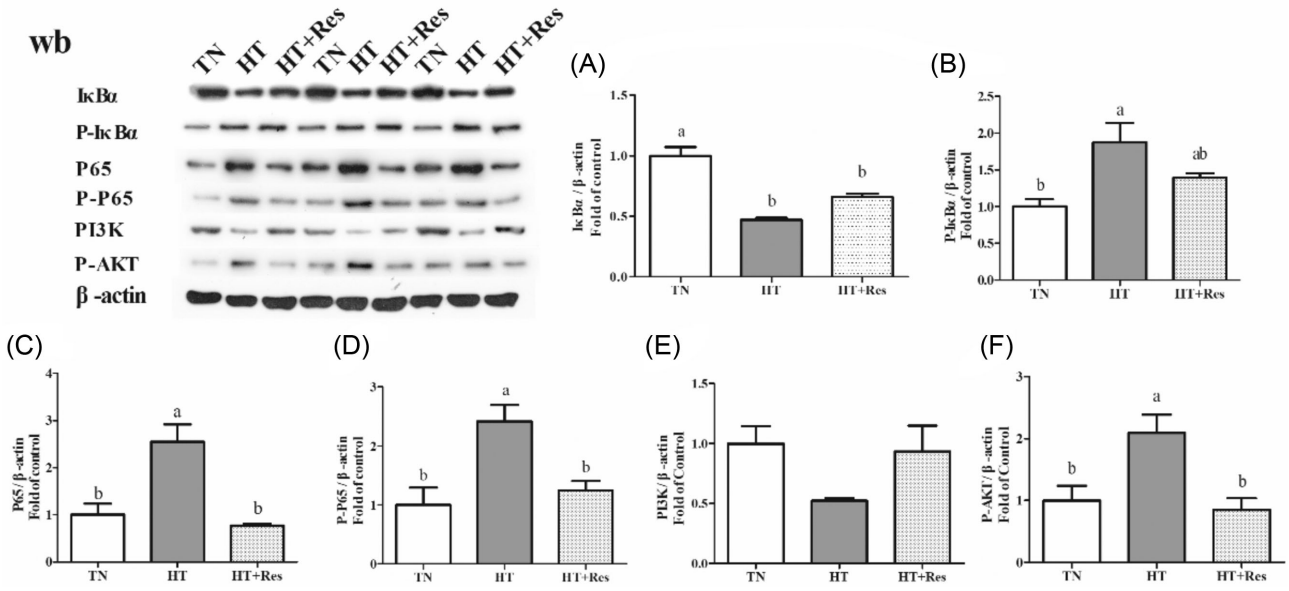

Figure 3. Effect of resveratrol on NF-κB and phosphoinositide-3 kinase–AKT pathway-related proteins expression in spleen of yellow-feather broilers under heat stress. Quantification of relative iκBα and phosphorylated iκBα abundance from data shown in A and B; p65 and phosphorylated p65 shown in C and D; PI3K and p-AKT shown in E and F. Data are expressed as mean ± SEM (n = 3), ^{abc}Different superscript letters indicate significant differences ($P < 0.05$). TN, thermoneutral treatment (24 ± 2°C); HT, high temperature treatment (37 ± 2°C); HT+Res, HT+500 mg/kg resveratrol. P-AKT, phosphorylated protein kinase B; PI3K, phosphoinositide-3 kinase.

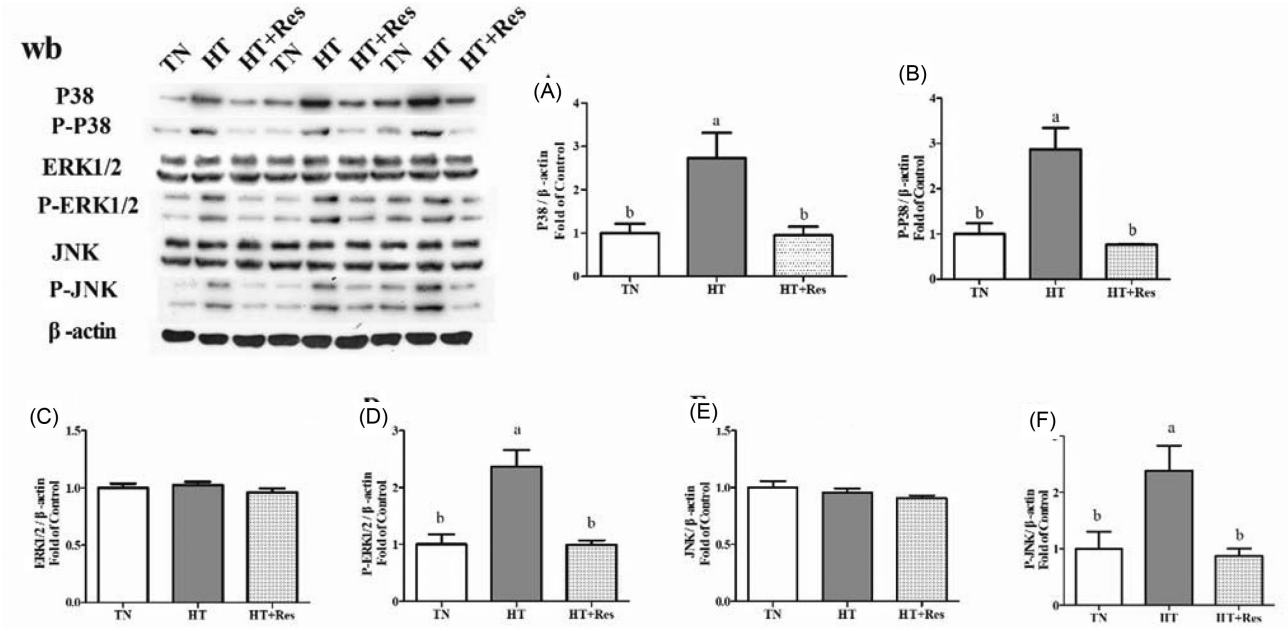

Figure 4. Effect of resveratrol on MAPK pathway-related proteins expression in spleen of yellow-feather broilers under heat stress. Quantification of relative p38 and phosphorylated p38 abundance from data shown in A and B; ERK1/2 and phosphorylated ERK1/2 shown in C and D; JNK and p-JNK shown in E and F. Data are expressed as mean ± SEM (n = 3), ^{abc}Different superscript letters indicate significant differences ($P < 0.05$). TN, thermoneutral treatment (24 ± 2°C); HT, high temperature treatment (37 ± 2°C); HT+Res, HT+500 mg/kg resveratrol. ERK1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun N-terminal protein kinase.

TLRs are PRRs that recognize pathogen-associated molecular patterns from microorganisms or danger-associated molecular patterns (**DAMPs**) from damaged tissue. It was reported that, TLR4 as the first TLR was discovered and be involved in lipopolysaccharide (**LPS**) recognition (Kawai and Akira, 2009). TLR2 was shown to sense bacterial lipopeptides and heterodimerizes with TLR1 to recognize triacylated lipopeptides, and heterodimerizes with TLR6 to recognize biacylated lipopeptides; TLR5 was reported to recognize bacterial flagellin, and TLR7 was shown to sense single-stranded viral RNA (O'Neill et al., 2013). Here, we established

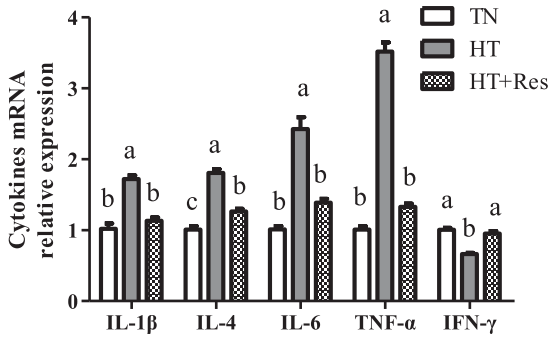

Figure 5. Effect of resveratrol on inflammatory cytokines mRNA expression in the spleen of yellow-feather broilers under heat stress. Data are expressed as mean ± SEM (n = 6), ^{abc} Different superscript letters indicate significant differences ($P < 0.05$). TN, thermoneutral treatment (24 ± 2°C); HT, high temperature treatment (37 ± 2°C); HT+Res, HT+500 mg/kg resveratrol. IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

an animal model of heat stress to yellow-feather broilers and determined the expression levels of PRRs and downstream signaling cascades. We found that TLR1, TLR4, and TLR5 were significantly up-regulated, while TLR2 was significantly down-regulated by heat stress. In contrast, previous studies reported that heat stress upregulates the expression of TLR2 and TLR4 in human monocytes (Zhou et al., 2005) and Bama miniature pigs (Ju et al., 2014), and upregulates TLR2, TLR4, and TLR5 in Indian carp (Basu et al., 2015). However, Quinteiro-Filho et al. (2017) found heat stress decreases expression of TLR2 in the spleen and cecal tonsils of broiler chickens. The reason of these inconsistence results possibly due to the form or duration of heat stress (Bharati et al., 2017). HSP70 is an endogenous molecules associated with cell damage, designated as DAMP can involve in inflammatory responses through binding to TLRs intracellularly and extracellularly (Feng et al., 2015). In the present study, we also found that heat stress significantly increased HSP70 expression in spleen, which is agreed with previous studies (Liu et al., 2014, 2016). Moreover, Dectin-1 and NOD1 were up-regulated by heat stress in this study. Previous studies reported that NOD1 gene was also induced in the heat and cold stressed fish (Basu et al., 2015, 2016). HSP70 can also bind to lipoxygenase-1, which is a member of CLR (Murshid et al., 2018), and small interfering RNA based knock down of acute heat shock may interfere in-vitro expression pattern of TLR1, TLR2 and NOD1, NOD2 (Sengar et al., 2018). Therefore, we speculate that heat stress may directly interact with TLRs (TLR1, TLR2, TLR4, and TLR5), Dectin-1 and NOD1, or indirectly promote the production of endogenous HSP70, as the DAMPs recognized by PRRs, consequently triggering the downstream inflammatory response signaling pathway. Interestingly, supplementation with resveratrol significantly down-regulated TLR4, TLR5, Dectin-1, and HSP70 in the spleen under heat stress. This observation of HSP70 is similar to the previous report that resveratrol inhibited the over-expression of HSP70 to alleviate circular heat stress-induced damage and inflammation in spleen and intestinal of black-bone chickens (Liu et al., 2014, 2016). Moreover, previous studies reported that resveratrol alleviate lysophosphatidylcholine-induced damage and inflammation, and against acute lung injury induced by lipopolysaccharide via inhibiting the myd88-dependent TLR4 signaling pathway, markedly decreased the expression of TLR4 (Zhang et al., 2014; Chen et al., 2018). However, it is rarely reported that resveratrol decreased the expression of TLR5 and Dectin-1 under heat stress. Certainly, resveratrol could directly regulated part PRRs and the ligands like HSP70 to regulate innate immune under heat stress in broilers.

When activated, TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR7) recruit adapter molecules (for example, MyD88) and subsequently initiate diverse downstream signaling cascades, resulting in the activation of transcription factors NF-κB and MAPK (Feng et al., 2015). Likewise, NOD1 recruits adapter molecule CARD-containing serine/threonine kinase RICK, finally activates NF-κB and MAPK (Hasegawa et al., 2008), and Dectin-1 recruits the spleen tyrosine kinase (Syk) activates downstream signaling through CARD9, finally activates NF-κB (Rogers et al., 2005). In the present study, we found that the expression of MyD88, NF-κB, MAPK, and TAK1 were significantly up-regulated in the spleen induced by heat stress. These finding suggested that in the heat stressed spleen, PRRs activation could further activate downstream signaling pathway molecules. TAK1, in a complex with TAK1-binding protein, subsequently activates 2 distinct pathway involving the iκB kinase complex or MAPK, and also, TAK1 is required for both MAPK and NF-κB activation during TLR and NLR signaling (Sato et al., 2005). Similarly, previous studies also reported that heat stress caused increased the mRNA and protein expression of NF-κB in jejunum mucosa of chickens (Liu et al., 2016), and thermal stress induced oxidative damage activated the MAPK pathway (Wang et al., 2018a). In present study, dietary resveratrol supplementation significantly inhibited the higher expression of TLR4 and TLR5 in spleen of heat-stressed broilers, and showed little effect on others like TLR1, TLR2, TLR6, and TLR7. Presumably, the action site of TLR4 and TLR5 is more sensitive to resveratrol under heat stress. The weakened TLR4 and TLR5 could further less activate downstream signaling pathway molecules, and suppress MAPK and NF-κB activation. Notably, the activated PRRs, like TLRs, Dectin-1, and NOD1, trigger immune responses through various downstream signal transductions, such as NF-κB, MAPK, and PI3K/p-AKT (Shang et al., 2018). In the present study, we found that the levels of p- iκBα, NF-κBp65, NF-κBp-p65, MAPKp38, MAPKp-p38, p-ERK1/2, and p-JNK were significantly increased, indicating that NF-κB and MAPK signaling pathway were significantly activated in spleen under heat stress. The NF-κB pathway plays an important role in activating host pro-inflammatory

responses to the stimulation of PRRs. In general, upon heat stress stimulation, iκB kinase phosphorylates iκB resulting in its degradation from the NF-κB complex, then the free NF-κB can enter the nucleus to function as transcription factors that initiate the expression of downstream specific genes, such as pro-inflammatory cytokines (Liu et al., 2016), in according with above, we also found that the level of iκBα was decreased by heat stress, resulted to decrease the binding of NF-κB in the cytoplasm. Moreover, previous research reported that ERK1/2 is activated by mitogens and growth factors, and not affect the inflammatory responses directly, while JNK and MAPKp38 cascades are usually triggered by stress and responses to inflammation (Kyriakis and Avruch, 2001). Besides NF-κB and MAPK, PI3K-AKT regulates the innate immunity (Wang et al., 2018b). We found that heat stress increased the activation of PI3K-AKT, significantly increased the level of p-AKT. The PI3K–AKT pathway also modulates innate immunity through regulating the phosphorylation of IkB kinases to activate the NF-kB pathway (Li et al., 2018). These above results suggested that several pathways contribute to the inflammation of spleen under heat stress. We also found that the mRNA expression level of mTOR was significantly decreased under heat stress. mTOR is a central regulator of cell growth and proliferation that integrates inputs from growth factor receptors, nutrient availability, intracellular ATP and a variety of stressors (Aramburu et al., 2014). Previous studies also reported that several well-characterized regulators and signaling circuits inhibit the activity of mTOR under stress (Sengupta et al., 2010; Yuan et al., 2015). Interestingly, we have identified that dietary resveratrol supplementation decreased the activation of muti-signaling pathways, including NF-κB, MAPK, and PI3K-AKT, by suppressing p65, p-p65, p-AKT, p38 p-p38, p-ERK1/2, and p-JNK levels in the spleen under heat stress. These were similar with early reports, that resveratrol also alleviated oxidative stress and LPS-induced inflammation responses by inhibiting iκB kinase phosphorylation and degradation of iκBα, reducing MAPKp38 phosphorylation, improving synthetic PI3K inhibitors and inhibiting PI3K/AKT pathway (Chen et al., 2018; Karuppagounder et al., 2014). Therefore, our results demonstrated that the inhibitory effect of resveratrol on NF-κB, MAPK and PI3K-AKT pathway could be an effective target for anti-inflammatory therapy of broilers under heat stress.

Recent studies have shown that innate immune PPRs-mediated signaling pathways (NF-κB and MAPK pathway) play a key role in inflammation responses in stress conditions (Bharati et al., 2017; Quinteiro-Filho et al., 2017). Another study suggested that heat stress induced higher levels of TNF-α and IL-4, but lower levels of IFN-γ and IL-2 in the spleen of broilers (Xu et al., 2014). Moreover, high environmental temperature prompts a considerable increase in the plasma levels of IL-1, IL-6, and TNF-α (He et al., 2018). We have also investigated the changes in the levels of IL-1, IL-4, IL-6, TNF-α, and IFN-γ, due to the activation of NF-κB and MAPK pathway and identified that heat stress had significantly increased the mRNA expression of IL-1, IL-4, IL-6, and TNF-α, whereas decreased the the mRNA expression of IFN-γ. It may be explained that IFN-γ synergy was independent activation of the transcription factors like NF-κB. Moreover, this result is accompanied by elevated levels of IL-4, which inhibits the activation of helper T cell, type 1 (Th1) cells that partially leads to a reduction of the secretion of IFN-γ (He et al., 2018). However, supplementation with resveratrol treated broilers showed significantly reduction in the levels of IL-1, IL-4, IL-6, and TNF-α, but increased the levels of IFN-γ under heat stress. Similarly, previous study also reported that resveratrol reduced the secretion of inflammatory cytokines via the TLRs/MyD88/NF-κB signal transduction pathway in lysophosphatidylcholine-induced inflammation (Chen et al., 2018). Therefore, our results demonstrate the inhibitory effect of resveratrol on the expression of major pro-inflammation cytokines. Moreover, IL-4 and IL-6 are associated with Th2 cell, while IFN-γ was related to Th1 cell (Heled et al., 2013), thus, we speculated that resveratrol also showed effect on the immunity shift from Th2 to Th1 cell in the spleen under heat stress.

In conclusion, we found that heat stress changes the innate immunity and inflammatory response, contributing to the activation of TLR, CLR, and NLR signaling in the spleen of yellow-feather broilers. Simultaneously, NF-κB, MAPK, and PI3K/AKT signaling pathways may be involved in these processes and induces expression of pro-inflammatory cytokines. In addition, we demonstrated that diets supplementation with resveratrol could inhibited inflammatory response via inhibited NF-κB, MAPK, and PI3K/AKT signaling pathways under heat stress condition. Thus, resveratrol may be act as a nutritional regulator to alleviate heat stress-induced high-activated innate immunity and inflammatory response in broilers. To our knowledge, this is the first study to investigate the impact of dietary resveratrol supplementation on innate immunity in heat-stressed animals.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Supplemental Table 1. Primer sequence used for the detection of mRNA levels.

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CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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