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Effect of curcumin supplementation on TLR4 mediated non-specific immune responses in liver of laying hens under high-temperature conditions

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ABSTRACT

The liver performs a significant role in innate and adaptive immunity. Heat stress causes oxidative stress in liver tissues and reduces the immune responses of laying hens which can cause several diseases affecting poultry-production performance. Hepatic inflammation is a common trigger of liver disease, which is reflected by hepatic tissue damage leading to fibrogenesis and hepatocellular carcinoma. Dietary manipulation of curcumin has been proposed to ameliorate the immune status of chickens under heat stress. Thus, this study aimed to investigate the effect of curcumin supplementation on TLR4 mediated non-specific immune response in liver of laying hens under high-temperature conditions. Experimental groups contained two controls groups (high temperature and thermo-neutral control (HC and NC) fed basal diet) and three high-temperature curcumin treatments groups (HT100, HT200 and HT300). Laying hens in HC and HT groups exposed 6 h/day heat stress (32 ± 1 °C). The results of present study showed that heat stress curcumin treatment group had reduced inflammatory responses (IL-6, IL-1β, TNF-α) as compared to HC and NC group. Pathological lesions and DNA damage of immune tissues were decreased in heat stress curcumin supplementation as compared to HC and NC group. Furthermore, PCNA, TLR4 and its downstream gene expression as well as protein expression (TLR4, NF-κB and PCNA) were significantly down regulated in heat stress curcumin supplemented group as compared to HC and NC group. Therefore, it is concluded that heat stressed hens supplemented with dietary curcumin enhance the immunity of laying hens and combat stressful environmental conditions.

1. Introduction

The liver is a key, frontline immune tissue which plays an important function in both innate and adaptive immunity (Kubes and Jenne, 2018). It harbors various types of resident immune cells and produces immune mediators as well as regulatory molecules (Robinson et al., 2016). It is responsible for the synthesis of cytokines, chemokines, complement components and acute phase proteins that involve in the innate immunity (Robinson et al., 2016). The major role of the inflammatory response is to combat tissue injury and infection. Liver is more sensitive to environmental stress, toxins, food antigens and bacterial components (Gao, 2016). Innate immune cells identify danger substance or damage cells through surface expressed pattern recognition receptors (PRRs). Subsequently, activated PRRs initiate TLR4 signaling pathway that trigger the release of inflammatory response (Bieghs and Trautwein, 2013). The liver is located at a specific place in the body which acts as an important barrier between gut and systemic circulation. A study has reported that almost 70–80% of the hepatic blood supply is delivered from the gut through the portal vein. This blood supply is rich with environmental antigens and harmless dietary toxin as well as molecules from the microbiota of the gut. The liver must tolerate this immunogenic load while still providing immunosurveillance for pathogenic infections (Robinson et al., 2016; Kubes and Jenne, 2018).

High environmental temperature negatively affects the immune responses in the poultry birds. Thermal stress induces oxidative damage in liver tissues which, in turn, cause liver injury and tissue damage (Shini and Kaiser, 2009; Shini et al., 2010). A study has revealed that broiler constantly exposure to high environmental temperatures had reduced the non-specific immunity Fathi et al. (2017). Heat stress disturbs the microecological balance of the intestinal microbiota and causes bacterial translocation which induces the production of intestinal endotoxins (LPS) (Gu et al., 2012; Yu et al., 2012). These endotoxins reach the liver through circulation and activate the TLR4

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mediated response. TLR4 is highly expressed in both parenchymal and non-parenchymal liver cells (Guo and Friedman, 2010). Various authors have reported that most of liver diseases are associated with TLR signaling pathway (Takeuchi and Akira, 2010; Qian et al., 2014; Guven-Maiorov et al., 2015). Activation of TLR4 by LPS induces the initiation of NF-kB and MAPK pathways to promote the overproduction of pro-inflammatory cytokines (Kagan and Medzhitov, 2006). Therefore, it is necessary to reduce the inflammatory status by restraining the activation of TLR4-mediated NF- kB pathway.

From long time, antibiotics have been considered the first choice for liver inflammation. However, several antibiotics have banned due to their liver-carcinogenicity (Rahmani et al., 2017). Therefore, there is a need to find out new therapeutic approaches to treat liver inflammation. Recently, dietary curcumin has gained interest as an alternative feed additive to treat inflammatory diseases including acute liver and lung injury, endometritis and mastitis (Lv et al., 2015; Fathi et al., 2017). Curcumin has potential anti-inflammatory and anti-oxidative activity (Cleary and McFeeters, 2006; Singh et al., 2010; Zhang et al., 2014; Wang et al., 2015; Pulido-Moran et al., 2016; Amalraj et al., 2017; Hadisoewignyo et al., 2018). Therefore, the aim of this study was to explore the effects of dietary curcumin on TLR4 mediated non-specific immune responses in liver of laying hens under high temperature conditions for 9 weeks.

2. Material and methods

2.1. Reagents and antibodies

Curcumin was purchased from the Agricultural Vegetable Limited Company in Xi'an, China. Curcumin was composed of 77% curcumin, 18% demethoxycurcumin, and 5% bisdemethoxycurcumin (Rahmani et al., 2017). The purity of curcumin used in this study was 95%. Curcumin was first added to a small amount of basal diet and then thoroughly mixed with 100 kg feed. ELISA kits of IL-1β, TNF-α, and IL-6 was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-TLR4 (toll like receptor) polyclonal antibody (1:400) was purchased from Boster Biological Technology, Pleasanton CA, USA, and Catalog # PA1484. Mouse anti-PCNA (Proliferating cell nuclear antigen) monoclonal antibody (1:200) (Catalog # ab29) and rabbit anti-NF-κB (nuclear factor kappa B cells) polyclonal antibody (Catalog # ab16502) was obtained from abcam.com, China. Mouse anti-β-actin monoclonal antibody (1:1000) (Catalog # AF0003) was purchased from beyotime Biotechnology (Shanghai, China). Secondary antibodies known as horseradish peroxidase (HRP) goat anti-rabbit IgG (specific to NF-κB) and HRP-conjugated goat anti-mouse IgG (specific to TLR4, PCNA and β-actin) was obtained from beyotime Biotechnology (Shanghai, China). Green PCR Master Mix was purchased from Toyobo Life Science Co., Ltd (Shanghai, China). In addition, cDNA reverse transcription kit was purchased from Sangon Biotech Co., Ltd (Shanghai, China).

2.2. Chicken housing and management

Three hundred, 25 week old Roman egg laying hens were purchased from the poultry industry in Guangzhou, China. The chickens were distributed randomly in to 5 experimental groups, six replicates with 10 birds/replicate. Experimental group contained two control groups (normal temperature control (NC) and high temperature control (HC) groups fed basal diet (Table 1) and three high temperature curcumin supplemented groups (HT100, HT200 and HT300 mg/kg). NC was maintained at comfort temperature of (22–25 °C) with 55–65% relative humidity. Heat stress control (HC) group with basal diet and heat stress group with curcumin supplementation were maintained at (32 ± 1 °C) with 55–65% relative humidity for 9 week 6 h/day from 10.00 a.m. to 16.00 p.m. (Table 2). Chicks were reared on soft bedding cages and housed in well ventilated room previously disinfected with potassium permanganate and formalin. Artificial light was provided for 18 h/day with the intensity of 10–15 Lx until the end of experiment. Heaters were settled in experimental chickens room to regulate the environmental temperature according to the body requirement of chicks. Feed and water were offered ad-libitum.

2.3. Data collection

The blood collection was performed from the brachial vein, and the samples were stored in microtubes and then centrifuged under refrigeration (1,500 × *g*, 5 min, 8 °C) to obtain the serum, which was stored at −80 °C until analysis. The serum inflammatory cytokines concentration was determined using an ELISA kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The liver samples were collected at 3rd, 6th and 9th weeks of experiments for inflammatory cytokines analysis, liver tissues histology, q-RT-PCR genes analysis and western blotting.

2.4. Serum inflammatory cytokines analysis

Blood extracted from the brachial vein was incubated at 37 °C for 30 mints then centrifuged to obtain the serum. Inflammatory cytokines (IL-1β, TNF-α, and IL-6) were determined using chicken ELISA kits, according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All the samples and standards along with a blank control were run in duplicate and the readings were taken at 450 nm using a microplate reader (Bio-Rad Co., Ltd. Shanghai, China).

2.5. Histological examination

Liver samples were immediately collected from the birds for morphological and molecular studies. Liver tissues were excised and fixed in 4% paraformaldehyde solution in PBS, dehydrated and then embedded in paraffin for morphological analysis. After that, 4-μm tissue sections were cut using a Leica microtome (Nussloch Gmbh, Germany) and mounted on polylysine-coated slides (Boster Corporation, China). The rest of fresh liver samples were frozen in liquid nitrogen and then stored at −80 °C for q-RT-PCR and western blotting. Hematoxylin and eosin (HE) staining was performed by routinely used protocol for liver histopathological examination (Kim et al., 2016). Stained tissue sections were examined by light microscopy (Olympus BX51, Tokyo, Japan) with a digital camera (DP72; Olympus).

2.6. RNA extraction and cDNA synthesis

The total RNA was isolated from liver tissues according to the manufacturer's instructions (Fig. 1). Then total RNA was treated with RNase-free DNase I (TransStart; transgen.com.cn) to remove contaminating genomic DNA. The first strand cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (TransStart; transgen.com.cn). Briefly, 4 μg (0.4 μl) of total RNA from each sample was added to a mixture of 1 μl reverse transcriptase random primers (0.2 μg/μl), 4 μl of 5 × Reaction Buffer, 1 μl of RNase Inhibitor (20U/μl), 2 μl of dNTP Mix (10 mmol/L), 1 μl of RT(200U/μl), and 10.6 μl of RNase free ddH2O. The final reaction mixture was kept at 25 °C for 10min, and then heated to 42 °C for 50sec, followed by 85 °C for 15min, and finally cooled to 4 °C.

2.7. Quantification PCR analysis

Quantitative analysis of specific gene mRNA expression was performed via qPCR by subjecting the cDNA obtained from the above preparation to the Light Cycle instrument (Roche). The reaction mixture (20 μl) contained 0.4 μL of each forward and reverse primer, 10 μl of SYBR Green PCR master mix, 1 μl of cDNA sample, and 8.2 nuclease-

Table 1
Diets given to laying hens in this study.

Treatments	Abbreviations	Diet
Normal temperature control	NC	Basal diet
Heat stress control	HC	Basal diet
Heat stress treatment 1	HT100	Basal diet + curcumin 100mg/kg diet
Heat stress treatment 2	HT200	Basal diet + curcumin 200mg/kg diet
Heat stress treatment 3	HT300	Basal diet + curcumin 300mg/kg diet

Table 2
Ingredients and chemical composition of the experimental basal diet.

Ingredients	Percent (%)	Nutrients (Analyzed composition, %)[a]	Content
Corn CP 8%	62.8	ME/(MJ/kg) [a]	11.42
Soybean meal CP 44%	20.0	CP, %	18.17
Wheat bran 12%	2.0	Ca, %	3.7
Fish meal CP 62%	4.5	TP, %	0.58
Limestone %	9.0	Met, %	0.41
$CaHPO_{4\%}$	1.0	Cys, %	0.29
NaCl %	0.2	Lys, %	0.94
Premix[a]	0.5		

Notes: [1]The premix provided, per kg of diet: Vitamin A 9000 IU; Vitamin D 2500 IU; Vitamin E 20 IU; Vitamin B 1212 μg; Vitamin K 2.4 mg; Mn100 mg; Zn 60 mg; Fe 25 mg, Cu 5 mg; Co 0.1 mg (Mn, Zn, Fe, Cu, Co were provided in the form of sulfates); Se ($N_2SeO_{3\cdot5}H_2O$) 0.2 mg; I(KI) 0.5 mg.
[a] Calculated values were according to NRC (1994) values for feedstuffs.

Table 3
Primers used for Real-time PCR.

Gene	Primer sequences (5′–3′)	Accession no.	Product size (bP)
TLR4	f-TGAAAGAGCTGGTGGAACCC r-CCAGGACCGAGCAATGTCAA	NM_001030693.1	206
MyD88	f-AGGATGGTGGTCGTCATTTC r-TTGGTGCAAGGATTGGTGTA	NM_001030962.2	194
NF-κB	f-CTACTGATTGCTGCTGGAGTTG r-CTGCTATGTGAAGAGGCGTTGT	M86930.1	175
IL6	f-AAA TCC CTC CTC GCC AAT CT r-CCC TCA CGG TCT TCT CCA TAA A	NM-204628	106
IL-1β	f-ACCTACAAGCTAAGTGGGCG r-ATACCTCCACCCCGACAAGG	NM_204524.1	137
TNF-α	f-CAGATGGGAAGGGAATGAAC r-CACACGACAGCCAAGTCAAC	AY765397.1	204
PCNA	f- TCTGAGGGCTTCGACACCTA r- AACCTTTTCCTGATTTGGTGCTT	NM_204170.2	174
β- actin	f-TTGTTGACAATGGCTCCGGT r-TCTGGGCTTCATCACCAACG	NM_205518.1	153

free water. PCR product were analyzed on 1% agarose gel to confirm the gene sequencing (Table 3) and their specific temperature. Fig. 2 indicates that all mentioned primer sequencing were in their proper arrangement. The qRT-PCR reactions were performed on a Bio-Rad CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The qRT-PCR amplification conditions were as follows: pre-denaturation at 94 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 5 s, annealing at 55 °C for 15 s, and elongation at 72 °C for 10 s. The primers used in these assays are listed in Table 3. The mRNA expression levels were determined relative to the blank control after normalization to the β-actin level through the 2(-Delta Delta C (T)) method (Schmittgen and Livak, 2008). Analysis was carried out in triplicates.

2.8. Western blot analysis

Liver tissues were dissociated by RIPA lysis buffer (P0013B Beyotime Biotechnology, Jiangsu, China) supplemented with protease inhibitor mixture (Roche Applied Science, Indianapolis, USA) and centrifuged at 12,000 g for 15 min at 4 °C. Nuclear extracts for TLR4, NF-κB and PCNA were prepared from hepatic homogenates according to method described by Cheng et al. (2017). The protein concentrations were determined by using the BCA protein assay kit (Beyotime, China). Subsequently, same amounts of total proteins (40 μg) were fractionated using 10% SDS-PAGE (40 min at 80 V and after that 2 h at 120 V). Then, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (1:30 min at 80 V) (Merck Millipore, USA). To block non-specific antibody binding, these PVDF membranes were blocked with 5% bovine serum albumin (BSA) at 37 °C for 1 h. Subsequently, the membranes were incubated at 4 °C against rabbit anti-TLR4 (1:400) (Boster Biological Technology, Pleasanton CA, USA), anti-PCNA (1:200) (abcam.com, China), anti-NF-κB (1:1000) (abcam.com, China) and anti-mouse β-actin (1:1000) (Beyotime, China) antibodies for 12 h. After washing in 1X TBST buffer three to five times (12 mint/time), samples were incubated with the corresponding HRP labeled secondary antibodies (1:3000 dilution) at 37 °C for 1:30 mint and then washed in 1X TBST buffer three to five times (12 mint/time). Finally, protein level was determined using the enhanced chemiluminescent (ECL) reagent (Beyotime, China) and the images were captured with Azure Bio-imaging systems (California, USA).

2.9. Statistical analysis

Data were analyzed by using One-Way ANOVA and Duncan's multiple comparison Post Hoc tests (Duncan, 1955). Statistical analysis was performed using the statistical software package SPSS (SPSS, 2004). Statistical significance between mean values was set at ($P < 0.05$). Data were described as means and standard error.

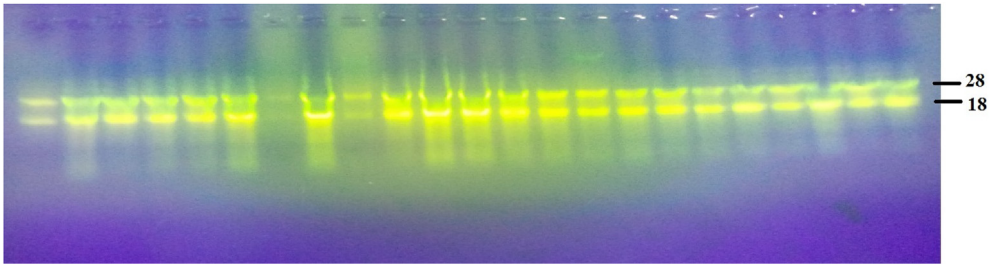

Fig. 1. RNA extraction.

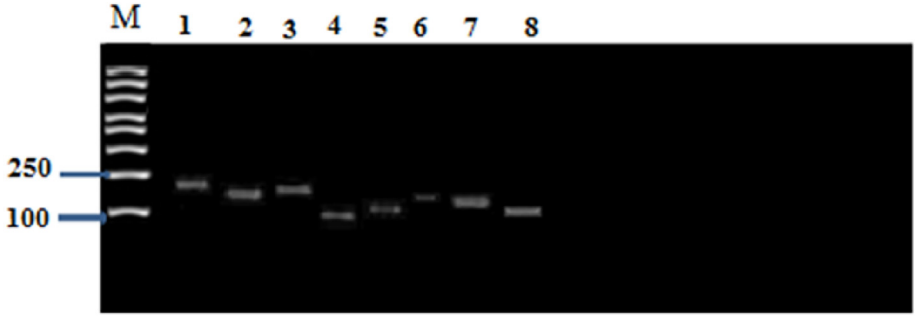

Fig. 2. PCR for gene sequencing confirmation. M; Marker, 1; TLR4, 2; MyD88, 3; NF-κB, 4; IL6, 5; IL-1β, 6; TNF-α, 7; PCNA, 8; β-actin.

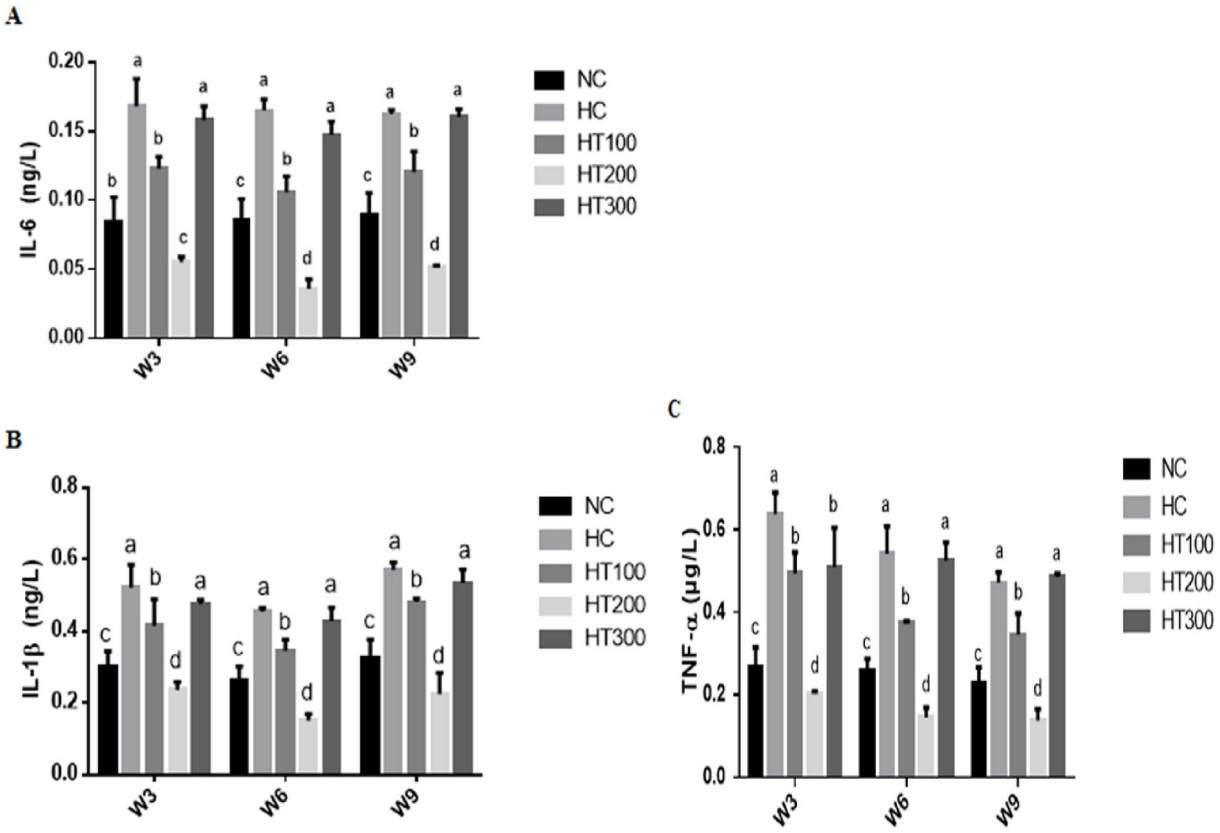

Fig. 3. Effect of curcumin supplementation on inflammatory cytokines IL6, IL-1β and TNF-α response during 9 week of heat stress experiment.(A) IL-6 response, (B) IL-1β and (C) TNF-α Note: Numbers with different lowercase letters are significantly different from each other ($P < 0.05$). Numbers not followed by different lowercase letters are not significantly different from each other ($P > 0.05$).NC; normal temperature control, HC; high temperature control, HT100; high temperature curcumin treatment 100, HT200; high temperature curcumin treatment 200, HT300; high temperature curcumin treatment 300.

3. Results

3.1. Effect of curcumin supplementation on serum pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) of heat stressed laying hens

The results demonstrated that serum inflammatory cytokine (IL-1β, IL-6 and TNF-α) response was increased in HC group during 9 week of experiment as compared to NC group. Whereas, heat stressed laying hens fed curcumin supplemented diets (HT100 and HT200) had significantly ($p < 0\ 0.05$) decreased proinflammatory cytokines (IL-6, IL-1β and TNF-α) response when compared with HC (without curcumin supplementation) group under week 3, 6 and 9 week of experiments. However, when compared HT group with NC, HT100 and HT200 group had significantly ($P < 0.05$) decreased inflammatory cytokines (IL-6, TNF-α and 1L-1β) response than NC group. Whereas, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to decrease inflammatory cytokines responses. But, there was no significant ($P > 0.05$) difference in inflammatory cytokines responses at HT300 dose as compared to HC group due to higher concentration of dietary curcumin which acts as pro-oxidant and increases the generation of ROS that damaged liver tissues and increased the inflammatory cytokines responses. Curcumin treatment significantly decreased the expression of IL-6, TNF-α and 1L-1β in a concentration dependent manner (HT100 and HT200). Thus, the results of current study indicated that dietary curcumin has excellent anti-inflammatory ability which inhibited the levels of pro-inflammatory cytokines induced by stressful conditions. Serum proinflammatory cytokines (IL-1β, TNF-α, and IL-6) activity are shown in Fig. 3.

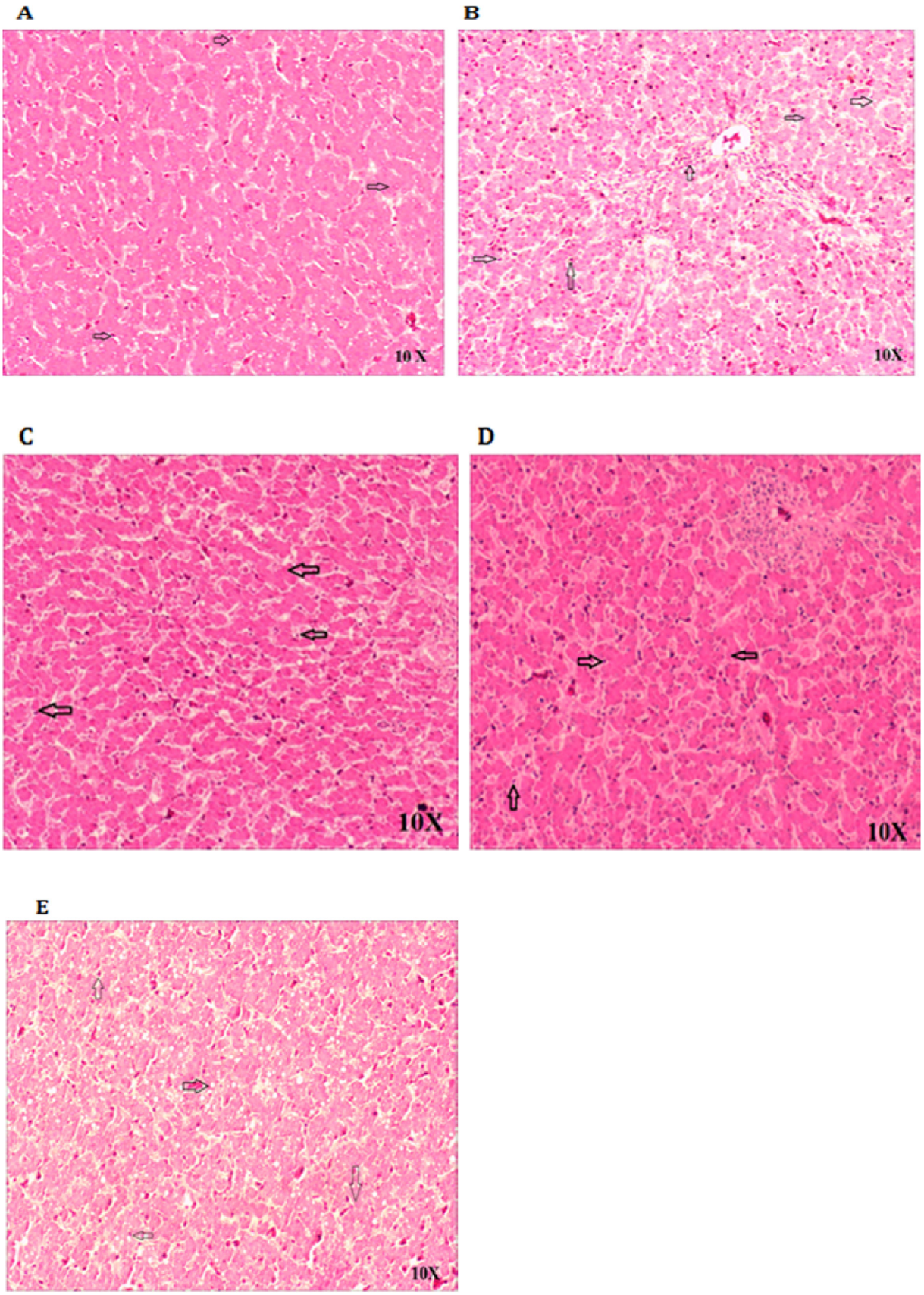

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Fig. 4. Effect of dietary curcumin on liver histology under week 3 of heat stress experiments. Histopathological changes in liver after heat stress exposure (10X). H and E staining was done on liver tissue sections. (A) Normal temperature control (NC) group, (B) high temperature control (HC) group, (C) high temperature curcumin treatment (HT100), (D) high temperature curcumin treatment (HT200) and (E) high temperature curcumin treatment (HT300 mg/kg) feed. Fig. 4A shows normal liver histology (hepatocyte, Kupffer cells, and sinusoidal cells). Fig. 4B shows fat vacuoles indicating fatty infiltration (right upper arrow), reduced size of a few hepatocytes (left lower arrow), dilated hepatic sinusoids (left arrow), and infiltration of inflammatory cells around the portal area (central arrow). Fig. 4C and D shows improved liver histology at 100 and 200 mg/kg curcumin supplementation. Fig. 4E shows almost similar liver histology with Fig. 4B. Fig. 4E shows fat vacuoles indicating fatty infiltration (central arrow), reduced size of liver hepatocytes (left upper arrow), dilated hepatic sinusoids (left arrow) and dilated Kupffer cells (right lower arrow).

3.2. Effect of curcumin supplementation on liver histology of heat stressed laying hens

The histological analysis was performed to examine the damaged liver tissues (Figs. 4, 5 and 6A-E). Heat stress control (HC) group had increased inflammatory cells infiltration around the central veins, dilation of sinusoidal capillaries, dilation of central veins, reduced size of few hepatocytes, and necrosis in the hepatic lobules when compared with NC group (Fig. 4B). But, the results of present study investigated that the inflammatory cells infiltration was significantly ($P < 0$ 0.05) reduced and the liver structure was comparatively improved in heat stress curcumin treatment groups (HT100 and HT200) as compared to untreated HC groups (Fig. 4C and D). However, when compared HT group with NC, HT100 and HT200 group had significantly ($P < 0$ 0.05) improved liver histology than NC group. While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to improve the liver histology. Whereas, there was no significant ($P > 0.05$) differences in liver tissue structure at HT300 dose as compared to HC group due to higher concentration of dietary curcumin which acts as pro-oxidant and increases the generation of ROS that damaged liver tissues. Hence, the results of present study showed that dietary curcumin (HT100 and HT200) has antioxidant ability that is reflected by improved liver histology.

3.3. Effect of curcumin supplementation on TLR4 and its downstream signaling molecules in liver of heat stressed laying hens

The effects of curcumin on TLR4 expression were determined by q-RT-PCR in liver tissues. Q-RT-PCR results suggested that HC group had significantly increased the mRNA level of PCNA, TLR4, MyD88, NF-κB, TNF-α, IL-6, 1L-1β as compared to NC group. But, the results of current study showed that heat stress curcumin treatment group at HT100 and HT200 significantly ($p < 0$ 0.05) reduced mRNA expression of PCNA, TLR4 and its down streaming molecules (MyD88, NF-κB, TNF-α, IL-6, 1L-1β) during 3rd, 6th and 9th week of experiment as compared to HC group. However, when compared HT group with NC, HT200 group had significantly ($p < 0$ 0.05) reduced mRNA expression of PCNA, TLR4 and its down streaming molecules (MyD88, NF-κB, TNF-α, IL-6, 1L-1β) than NC group. While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to reduce mRNA expression of PCNA, TLR4 and its down streaming molecules. Whereas, there was no significant ($P > 0.05$) differences in mRNA expression of PCNA, TLR4 and its down streaming molecules at HT300 dose as compared to HC group due to higher concentration of dietary curcumin which acts as pro-oxidant and increases the generation of ROS that damaged tissues and increased the mRNA expression of PCNA, TLR4 and its down streaming molecules. Thus, the result of present study showed that dietary curcumin (HT100 and HT200) has anti-inflammatory ability which is reflected by reduced mRNA expression of PCNA, TLR4 and its down streaming molecules which might helpful to develop heat resistant poultry breed in future (Fig. 7A–G).

3.4. Effect of curcumin supplementation on protein expression of TLR4, NF-κB and PCNA in liver of heat stressed laying hens

Western blotting indicated that PCNA, TLR4 and NF-κB protein expression was significantly increased in HC group as compared to NC group (Figs. 8–10). While, the results of our study indicated that heat stress curcumin treatment group had significantly ($P < 0$ 0.05) reduced the protein expression of TLR4, NF-κB and PCNA at concentration of HT100 and HT200 during week 3, 6 and 9 of experiment as compared to HC group fed only basal diet. However, when compared HT group with NC, HT200 group had significantly ($P < 0$ 0.05) reduced the protein expression of TLR4, NF-κB and PCNA than NC group. While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to reduce the protein expression of TLR4, NF-κB and PCNA. However, there was no difference in protein expression of PCNA, TLR4 and NF-κB at HT300 dose as compared to untreated HC group due to higher concentration of dietary curcumin which acts as pro-oxidant and increases the generation of ROS that damaged tissues and increased the proteins level of PCNA, TLR4 and NF-κB. Hence, the results indicated that the proteins level of PCNA, TLR4 and NF-κB was reduced dose-dependently in the heat stress curcumin treatment groups which showed its anti-inflammatory ability to protect the liver tissues against free radicles (ROS).

4. Discussion

In hot climatic areas of the world, high temperature causes oxidative stress in the poultry birds (Lin et al., 2006; Habibi et al., 2014). Oxidative stress is associated with diseases and tissue damage due to generation of reactive oxygen species (ROS) and cause various disorders in the liver. This imbalance between pro-oxidant and antioxidant metabolites can affect different signaling pathway including TLR4 which, in turn, suppress the specific and non-specific immunity of the chicken (Daneshyar, 2012; Ismail et al., 2013; Nawab et al., 2018). Recently, dietary curcumin, (a yellow pigment of turmeric), has been classified as a potential ingredient which is found in southern and southeastern Asia including Pakistan and China (Nouzarian et al., 2011; Wang et al., 2015; Ramos et al., 2017). It has various therapeutic and pharmacological characteristics including the property of anti-inflammatory, antioxidant, free radical scavenging, inhibition of lipid peroxidation, antiviral, antimicrobial, antiprotozoal and antitumor characteristics, and in addition, turmeric acts as an immune booster (Cleary and McFeeters, 2006; Singh et al., 2010; Zhang et al., 2014; Wang et al., 2015; Pulido-Moran et al., 2016; Amalraj et al., 2017).

The production of significant quantity of ROS can stimulate the release of inflammatory cytokines such as IL-6, IL-1β, TNF-α and they are regarded as critical inflammatory genes in the cytokines family (Laveti et al., 2013; Marcus et al., 2003; Korbecki et al., 2013). Serum inflammatory cytokines (IL-6, TNF-α and 1L-1β) response in laying hens was increased in HC group as compared to NC group fed only basal diet. But, the present study showed that heat stress curcumin treated laying hens had significantly ($p < 0.05$) reduced pro-inflammatory cytokines response such as IL-6, IL-1β, TNF-α at HT100 and HT200 dose during week 3, 6 and 9 of experiments as compared to the untreated HC group. However, when compared HT group with NC, HT100 and HT200

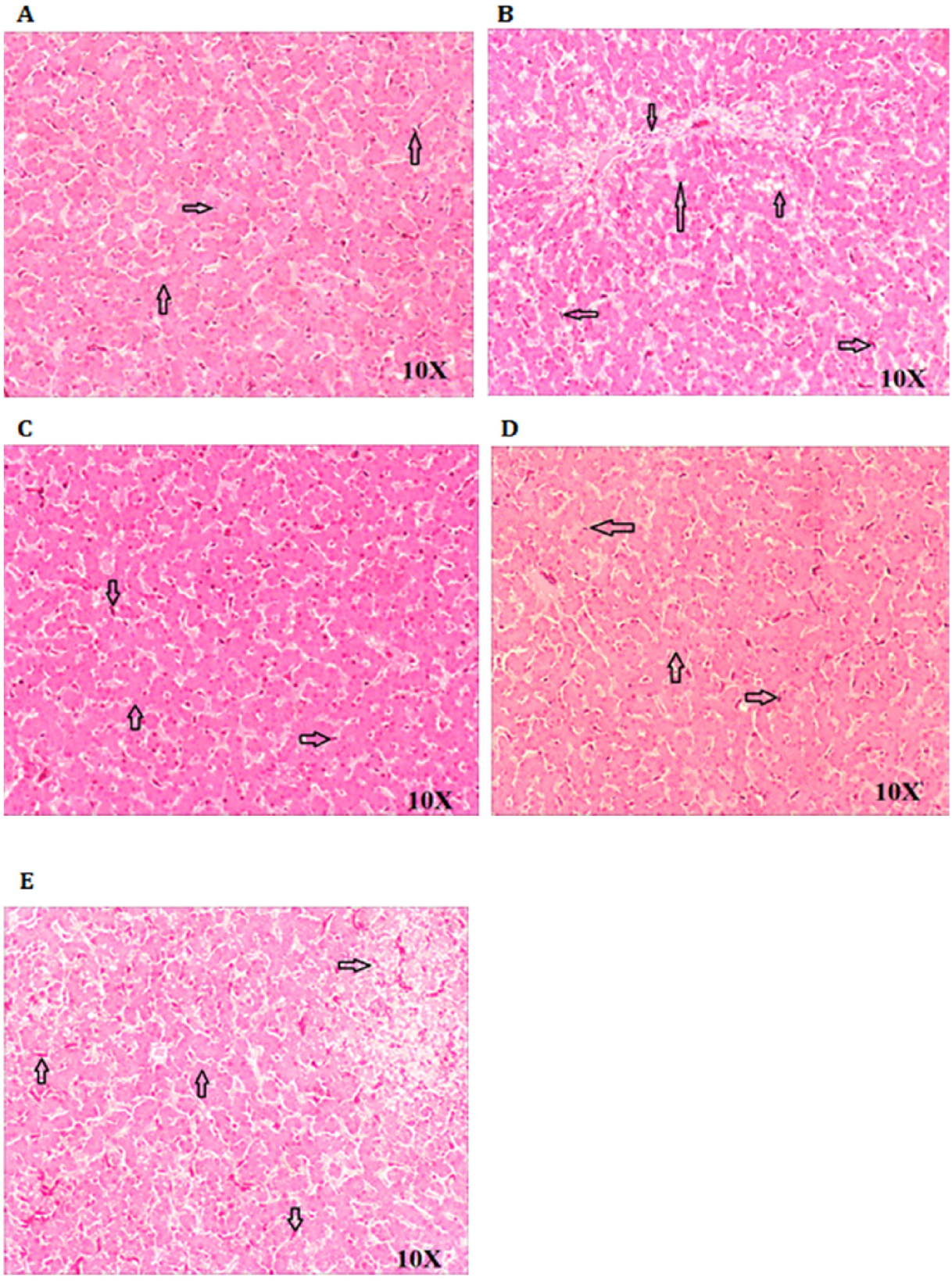

Fig. 5. Effect of dietary curcumin on liver histology under week 6 of heat stress experiments. Histopathological changes in liver after heat stress exposure (10X). H and E staining was done on liver tissue sections. (A) Normal temperature control (NC) group, (B) high temperature control (HC) group, (C) high temperature curcumin treatment (HT100), (D) high temperature curcumin treatment (HT200) and (E) high temperature curcumin treatment (HT300 mg/kg) feed. Fig. 5A shows normal liver histology (hepatocyte, Kupffer cells, and sinusoidal cells). Fig. 5B shows fat vacuoles indicating fatty infiltration (right upper arrow), reduced size of a few hepatocytes (left lower arrow), dilated hepatic sinusoids (left arrow), and infiltration of inflammatory cells around the portal area (central arrow). Fig. 5C and D shows improved liver histology at 100 and 200 mg/kg curcumin supplementation. Fig. 5E shows almost similar liver histology with Fig. 5B. Fig. 5E shows fat vacuoles indicating fatty infiltration (central arrow), reduced size of liver hepatocytes (left upper arrow), dilated hepatic sinusoids (left arrow) and dilated Kupffer cells (right lower arrow).

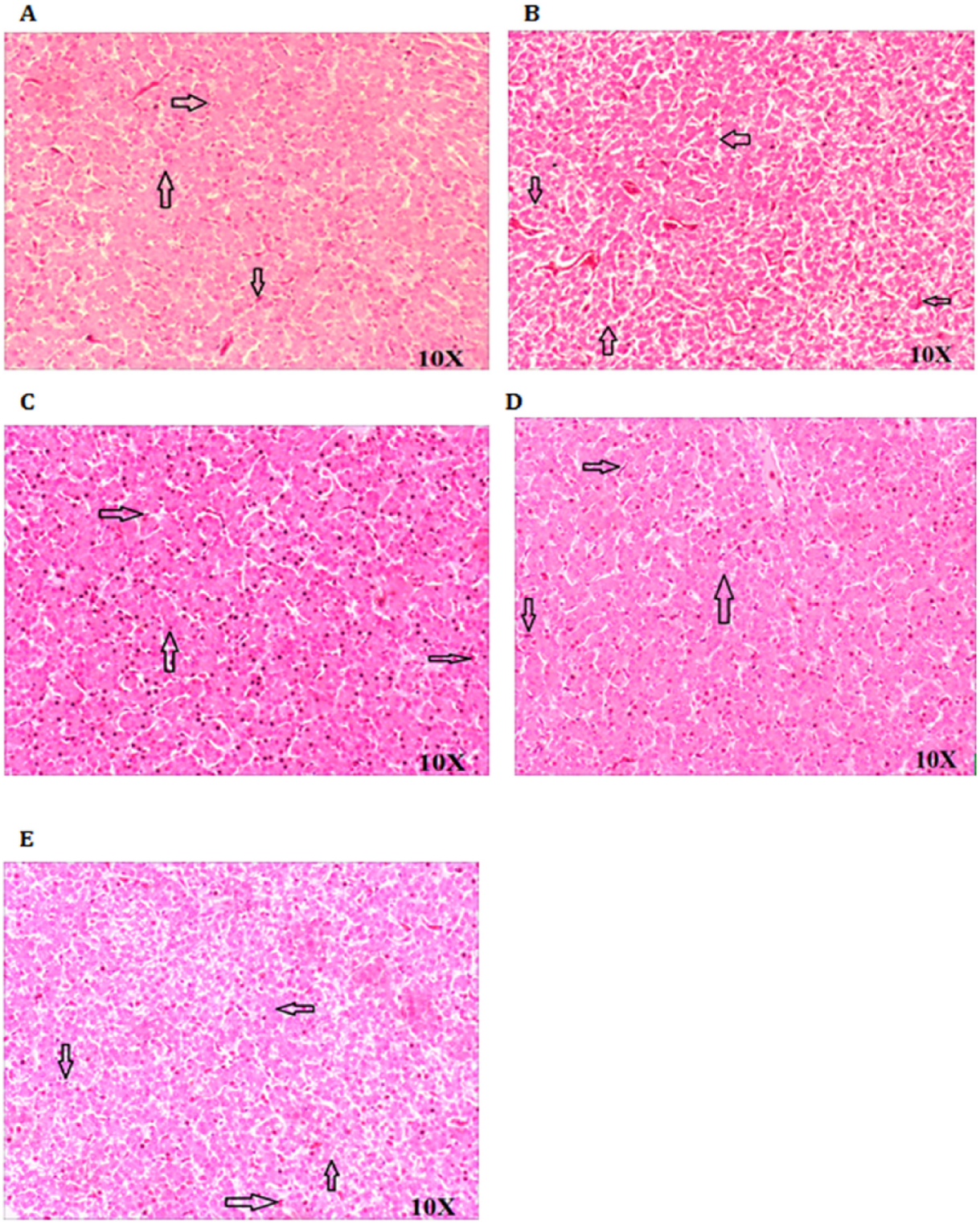

Fig. 6. Effect of dietary curcumin on liver histology under week 9 of heat stress experiments. Histopathological changes in liver after heat stress exposure (10X). H and E staining was done on liver tissue sections. (A) Normal temperature control (NC) group, (B) high temperature control (HC) group, (C) high temperature curcumin treatment (HT100), (D) high temperature curcumin treatment (HT200) and (E) high temperature curcumin treatment (HT300 mg/kg) feed. Fig. 6A shows normal liver histology (hepatocyte, Kupffer cells, and sinusoidal cells). Fig. 6B shows fat vacuoles indicating fatty infiltration (right upper arrow), reduced size of a few hepatocytes (left lower arrow), dilated hepatic sinusoids (left arrow), and infiltration of inflammatory cells around the portal area (central arrow). Fig. 6C and D shows improved liver histology at 100 and 200 mg/kg Curcumin supplementation. Fig. 6E shows almost similar liver histology with Fig. 6B. Fig. 6E shows fat vacuoles indicating fatty infiltration (central arrow), reduced size of liver hepatocytes (left upper arrow), dilated hepatic sinusoids (left arrow) and dilated Kupffer cells (right lower arrow).

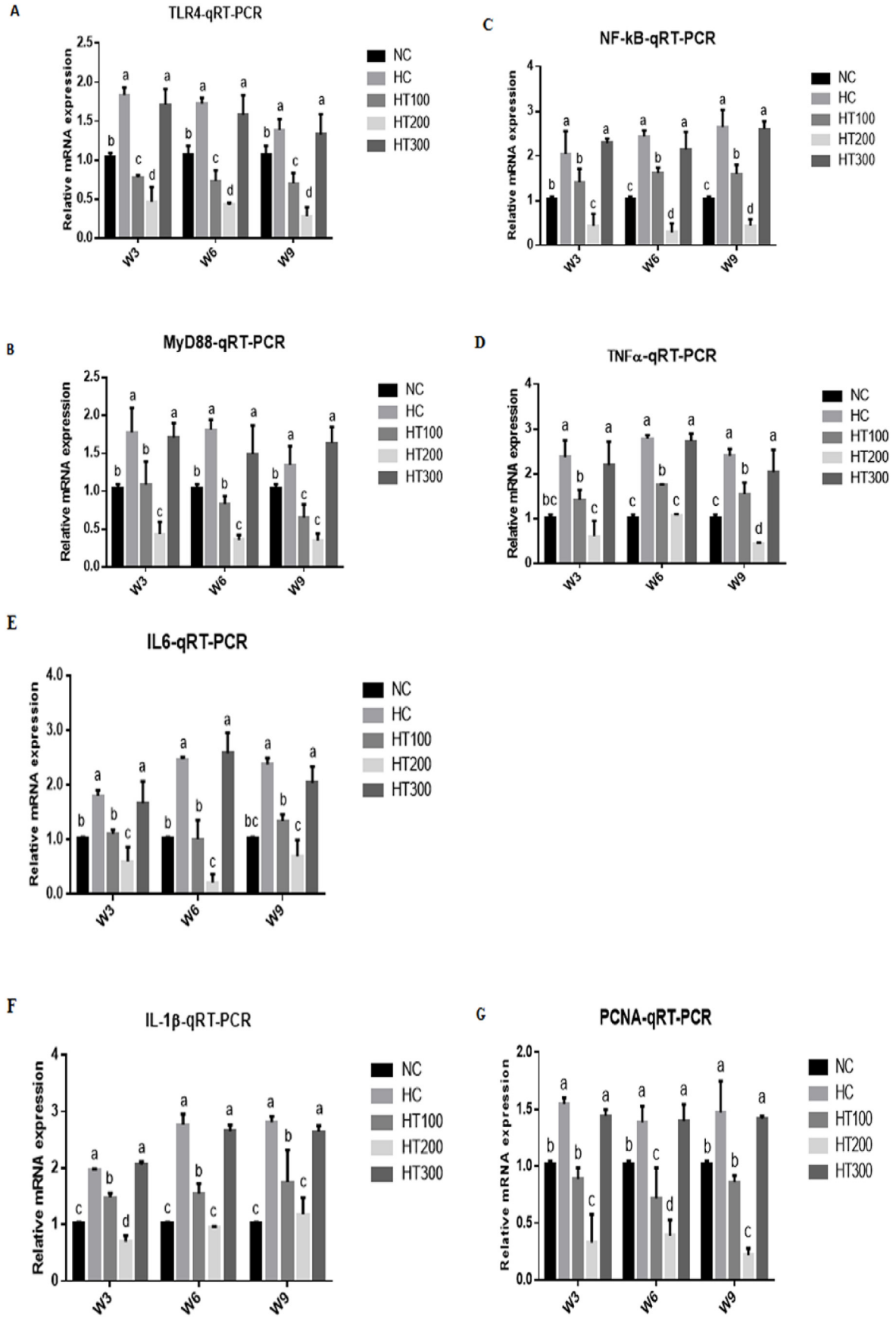

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Fig. 7. A-D. Effects of curcumin on TLR4, MyD88, NF-κB and TNF-α mRNA expression.(A) TLR4, (B) MyD88, (C) NF-κB and (D) TNF-α genes expression TLR4, MyD88, NF-κB and TNF-α genes expression were performed by q-RT-PCR and normalized according to the expression of β-actin.Note: Numbers with different lowercase letters are significantly different from each other ($P < 0.05$). Numbers not followed by different lowercase letters are not significantly different from each other ($P > 0.05$).NC; normal temperature control, HC; high temperature control, HT100; high temperature curcumin treatment 100, HT200; high temperature curcumin treatment 200, HT300; high temperature curcumin treatment 300. Fig. 7E–G Effects of curcumin on IL-6, IL-1β and PCNA mRNA expression.(E) IL-6, (F) IL-1β and (G) PCNA genes expression IL-6, IL-1β and PCNA genes expression were performed by q-RT-PCR and normalized according to the expression of β-actin. Note: Numbers with different lowercase letters are significantly different from each other ($P < 0.05$). Numbers not followed by different lowercase letters are not significantly different from each other ($P > 0.05$).NC; normal temperature control, HC; high temperature control, HT100; high temperature curcumin treatment 100, HT200; high temperature curcumin treatment 200, HT300; high temperature curcumin treatment 300.

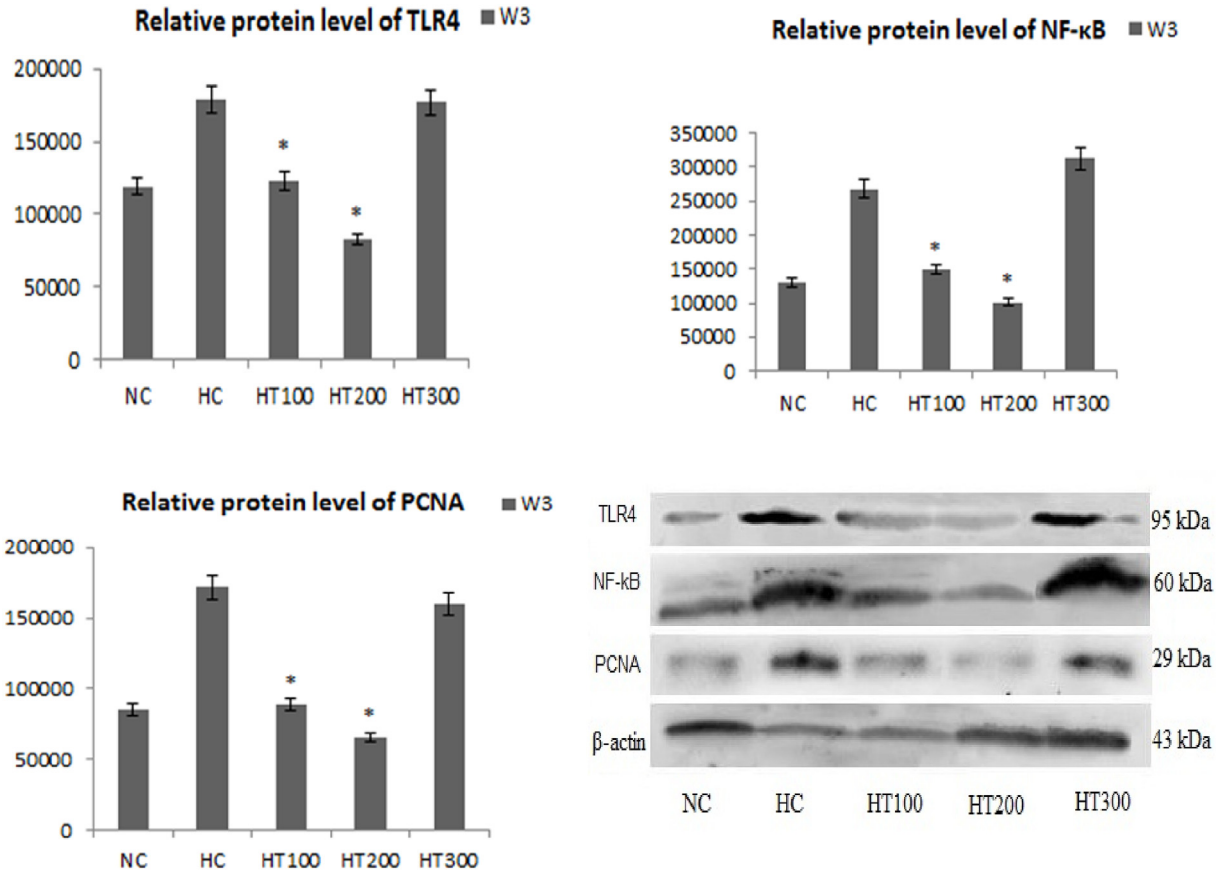

Fig. 8. Effects of curcumin on TLR4, NF-κB and PCNA protein expression under Week 3 of heat stress experiment. Protein expression of TLR4, NF-κB and PCNA was measured in liver tissues by western blotting using anti-TLR4, NF-κB and PCNA antibody. IOD (Integral optical density) shows increased TLR4, NF-κB and PCNA expression in untreated HC group and decreased in high temperature curcumin supplementation group. Immunoblots showed decreased TLR4, NF-κB and PCNA expression after curcumin treatment in chicken liver at HT100 and HT200 when compared with HC group without curcumin treatment. However, HT300 had no significant difference on TLR4, NF-κB and PCNA protein expression as compared to HC group. Data represent mean ± SEM.Note: Figures marked with sign * shows significant difference from each other ($P < 0.05$). Figures with no sign * are not significantly different from each other ($P > 0.05$).NC; normal temperature control, HC; high temperature control, HT100; high temperature curcumin treatment 100, HT200; high temperature curcumin treatment 200, HT300; high temperature curcumin treatment 300.

group had significantly ($P < 0.05$) decreased inflammatory cytokines (IL-6, TNF-α and 1L-1β) response than NC group. While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to decrease the inflammatory cytokines responses. Whereas, there was no significant ($P > 0.05$) differences in the inflammatory cytokines responses at HT300 dose as compared to HC group due to higher concentration of dietary curcumin (Nabavi et al., 2014), which acts as pro-oxidant and increases the generation of ROS that damaged tissues and increased the inflammatory cytokines level.

Regarding the above cytokines, TNF-α is secreted from activated macrophages and involves in exerting variety of biologic effects, which is considered to be an efficient pro-inflammatory factor (Wu et al., 2015). IL-6 is produced from various heat stress induced damaged/injured cells and IL-6 is necessary for tissue homeostasis (Cronin et al., 2016). IL-1β is mainly secreted from activated macrophages and is categorized as a key factor of mediating inflammation (Smirnova et al., 2002; Cheng et al., 2017). Hence, inflammatory immune diseases can be reduced by inhibiting the overproduction of inflammatory cytokines by application of dietary curcumin (Laveti et al., 2013). The results of present study were in accordance with (Yoshiaki et al., 1999; Zhou et al., 2011; Emadi and Kermanshahi, 2007; Kim et al., 2016) who proposed that curcumin decreases the production of inflammatory cytokines in human and chicken under stressful conditions due to decreasing the number of ROS. Consequently, the results of current study indicated that dietary curcumin has excellent anti-inflammatory ability which inhibited the levels of pro-inflammatory cytokines induced by stressful conditions.

Histological examination is good indicator of tissues damage which indicates the occurrence of inflammatory diseases (Kubota et al., 2012).

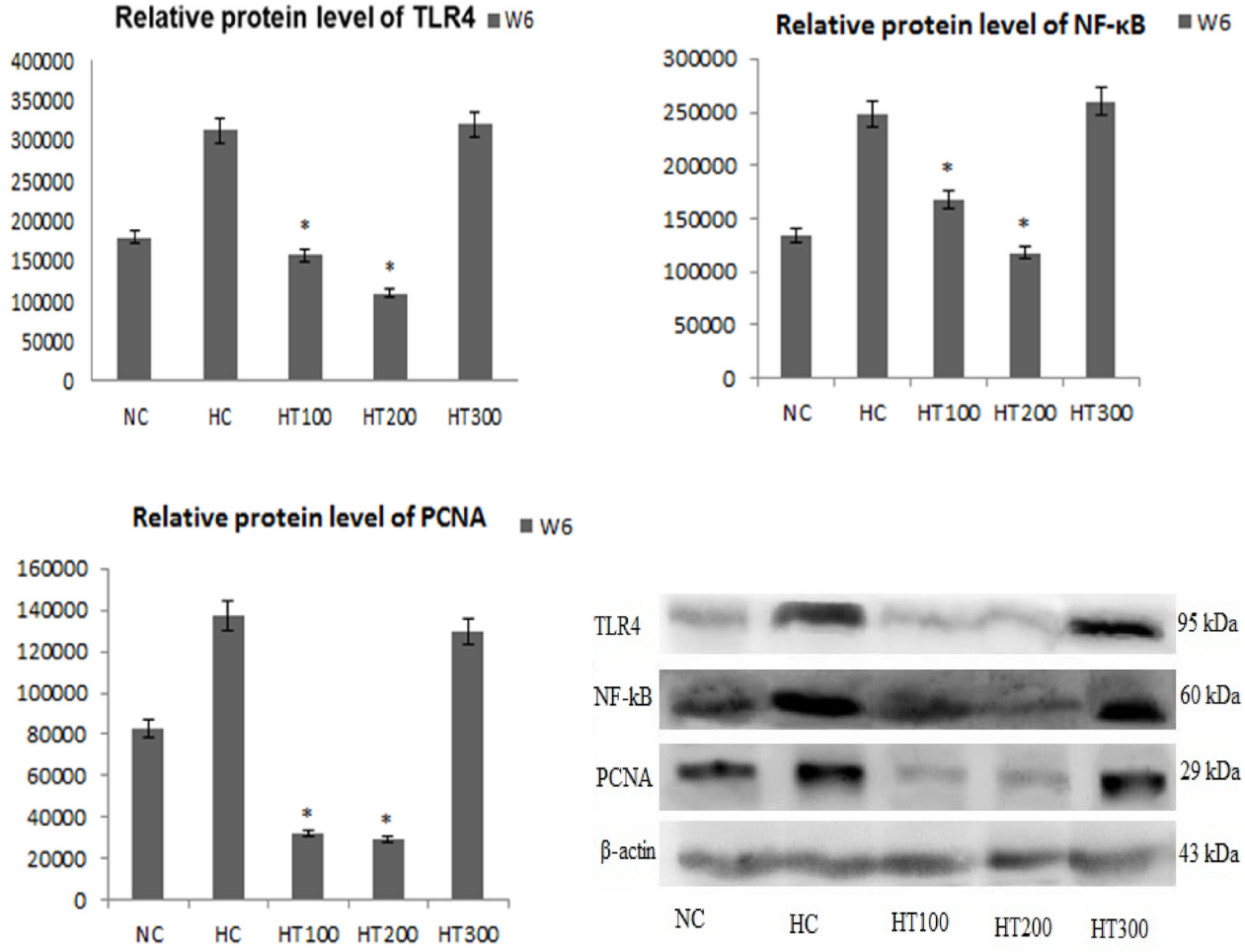

Fig. 9. Effects of curcumin on TLR4, NF-κB and PCNA protein expression under Week 6 of heat stress experiment. Protein expression of TLR4, NF-κB and PCNA was measured in liver tissues by western blotting using anti-TLR4, NF-κB and PCNA antibody. IOD (Integral optical density) shows increased TLR4, NF-κB and PCNA expression in untreated HC group and decreased in high temperature curcumin supplementation group. Immunoblots showed decreased TLR4, NF-κB and PCNA expression after curcumin treatment in chicken liver at HT100 and HT200 when compared with HC group without curcumin treatment. However, HT300 had no significant difference on TLR4, NF-κB and PCNA protein expression as compared to HC group. Data represent mean ± SEM.Note Figures marked with sign * shows significant difference from each other ($P < 0.05$). Figures with no sign * are not significantly different from each other ($P > 0.05$).NC; normal temperature control, HC; high temperature control, HT100; high temperature curcumin treatment 100, HT200; high temperature curcumin treatment 200, HT300; high temperature curcumin treatment 300.

Curcumin has potential anti-inflammatory activity which can be reflected by improved liver histopathological examination. The production of significant quantity of ROS stimulate the inflammatory cytokines such as IL-1β, IL-6 and TNF-α that are critical inflammatory genes in the family of cytokines (Laveti et al., 2013; Marcus et al., 2003; Korbecki et al., 2013). In this study, we explored the effects of dietary curcumin on heat induced damaged liver tissue. The results showed that HC group caused liver histopathological lesions like dilated sinusoidal capillaries, dilated central vein, reduced hepatocytes, dilated hepatic sinusoids, diffuse fatty infiltration, intracytoplasmic fat vacuole infiltration, RBC infiltration, and infiltration of inflammatory cells around portal area as compared to NC group (Huang et al., 2017). The present results showed that liver histopathological lesions were significantly ($P < 0.05$) reduced and liver tissues structure was improved in heat stress curcumin supplemented group (HT100 and HT200) under week 3, 6 and 9 of experiment as compared to untreated HC group. Hence, improved liver histology shows healthy liver immune response.

However, when compared HT group with NC, HT100 and HT200 group had significantly ($P < 0$ 0.05) improved liver histology than NC group. While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to improve the liver histology. Whereas, there was no significant ($P > 0.05$) differences in liver tissue structure at HT300 dose as compared to HC group due to higher concentration of dietary curcumin which acts as pro-oxidant and increases the generation of ROS that damaged liver tissues (Nabavi et al., 2014). On the other hand, our result was similar according to findings of Lee et al. (2017) and Lubbad et al. (2009) who stated that curcumin reduced the hepatic stress and colitis in rats which is reflected by improved colon histology of colitic rats after curcumin treatment (Lee et al., 2017; Lubbad et al., 2009). Another study was also in accordance with the present results which described that lung injury in mice exposed to LPS treatment was decreased by curcumin (Kim et al., 2016). Therefore, the result of present study showed that dietary curcumin (HT100 and HT200) has antioxidant ability that is reflected by improved liver histology.

Complex mechanisms involve in heat stress induced damaged liver tissue (Yao et al., 2016). In the present investigation, TLR4 mRNA expression and its down streaming molecules were examined in hepatocytes. TLR4 expression activates MyD88 dependent pathways and its downstream molecules which regulates the inflammatory cytokines and non-specific (innate) immune responses (Huang et al., 2017; Gorina et al., 2011; Mateu et al., 2015). Several convincing evidence have

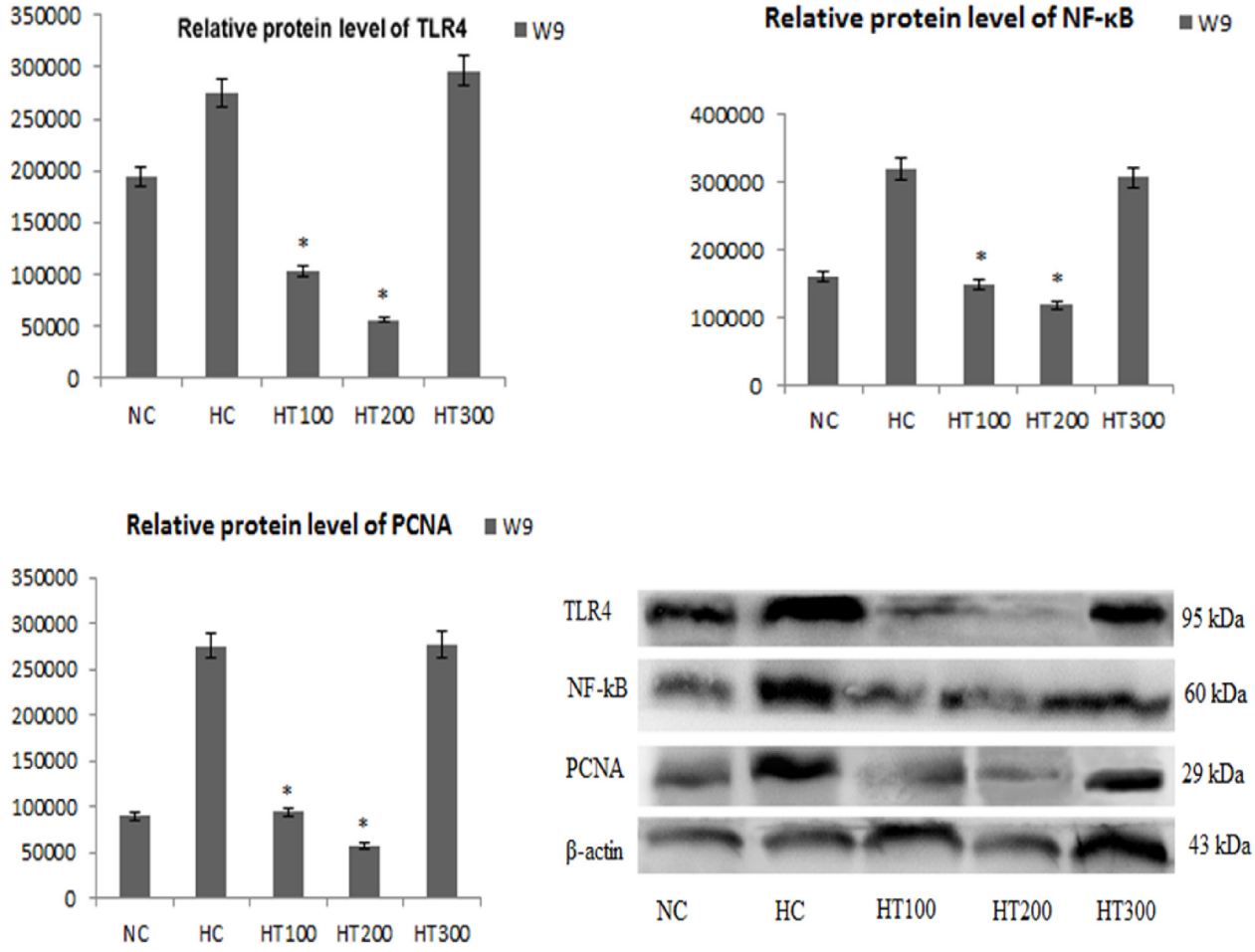

Fig. 10. Effects of curcumin on TLR4, NF-κB and PCNA protein expression under Week 9 of heat stress experiment. Protein expression of TLR4, NF-κB and PCNA was measured in liver tissues by western blotting using anti-TLR4, NF-κB and PCNA antibody. IOD (Integral optical density) shows increased TLR4, NF-κB and PCNA expression in untreated HC group and decreased in high temperature curcumin supplementation group. Immunoblots showed decreased TLR4, NF-κB and PCNA expression after curcumin treatment in chicken liver at HT100 and HT200 when compared with HC group without curcumin treatment. However, HT300 had no significant difference on TLR4, NF-κB and PCNA protein expression as compared to HC group. Data represent mean ± SEM.Note: Figures marked with sign * shows significant difference from each other ($P < 0.05$). Figures with no sign * are not significantly different from each other ($P > 0.05$).NC; normal temperature control, HC; high temperature control, HT100; high temperature curcumin treatment 100, HT200; high temperature curcumin treatment 200, HT300; high temperature curcumin treatment 300.

reported that NF-κB signaling pathway has been detected in various tissues including liver after exposure to stressful environmental conditions (Reuven et al., 2014; Kawasaki and Kawai, 2014; Huang et al., 2017), which is also significant for coordination in specific and non-specific immune responses. To explore the anti-inflammatory action of curcumin, we examined the effects of dietary curcumin on TLR4 mediated non-specific immune responses in damaged liver of laying hens under high temperature conditions. TLR4 expression in liver tissues by qPCR analysis suggested that HC group had significantly increased mRNA expression of TLR4, MyD88, NF-κB, TNF-α, IL-6, 1L-1β and PCNA as compared to NC group. But, the present results indicated that TLR4 and its down streaming molecules were significantly ($P < 0.05$) reduced in curcumin supplemented heat stress group (HT100 and HT200) under week 3, 6 and 9 of experiment as compared to untreated HC group.

However, when compared HT group with NC, HT200 group had significantly ($P < 0\ 0.05$) reduced mRNA expression of PCNA, TLR4 and its down streaming molecules (MyD88, NF-κB, TNF-α, IL-6, 1L-1β) than NC group. While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to reduce mRNA expression of PCNA, TLR4 and its down streaming molecules. Whereas, there was no significant ($P > 0.05$) differences in mRNA expression of PCNA, TLR4 and its down streaming molecules at HT300 dose as compared to HC group due to higher concentration of dietary curcumin (Nabavi et al., 2014), which acts as pro-oxidant and increases the generation of ROS that damaged liver tissues and up-regulated mRNA expression of PCNA, TLR4 and its down streaming molecules. However, the results presented in this study were similar with Lubbad et al. (2009) and Zhu et al. (2014) who investigated that curcumin significant reduced TLR4 and MyD88 mediated pathway in case of colitic rats and injured brain in mice (Lubbad et al., 2009; Zhu et al., 2014). Another study was also in accordance with the present results which stated that curcumin inhibits NF-κB pathway and inflammatory mediators associated with periodontal disease in rats (de Aquino et al., 2011). Thus, the results concluded that dietary curcumin (HT100 and HT200) has anti-inflammatory ability that is reflected by reduced mRNA expression of PCNA, TLR4, NF-κB and its down streaming molecules which might helpful to develop heat resistant poultry breed in future.

The protein expression of PCNA, TLR4 and NF-κB has been observed in injured liver tissues (Guo and Friedman, 2010; Huang et al., 2017). In the current study, the results of western blotting also indicated that PCNA, TLR4 and NF-κB protein expression was significantly increased in HC group as compared to NC group. During TLR4 signaling, MyD88

dependent pathway activate TLR4 and MD2 signaling complex under heat stress in chicken (Keestra and van Putten, 2008; Huang et al., 2017). Therefore, it has concluded that TLR4 and MyD88 dependent pathway and its down streaming molecules are involved in injured liver tissue of laying hens. While, the results exposed that heat stress curcumin treatment group had significantly ($P < 0$ 0.05) reduced the protein expression of TLR4, NF-κB and PCNA at concentration of HT100 and HT200 during week 3, 6 and 9 of experiment as compared to HC group fed only basal diet. However, when compared HT group with NC, HT100 and HT200 group had significantly ($P < 0$ 0.05) reduced the protein expression of TLR4, NF-κB and PCNA than NC group.

While, among all high temperature curcumin treatment groups (HT100, HT200 and HT300), HT100 and HT200 dose was suitable to reduce the protein expression of TLR4, NF-κB and PCNA. However, there was no significant effect on PCNA, TLR4 and NF-κB protein expression at HT300 as compared to HC group due to higher concentration of dietary curcumin which acts as pro-oxidant and increases the generation of ROS that damaged liver tissues and up-regulated the protein expression (Nabavi et al., 2014). But, the results of this study showed that curcumin (HT100 and HT200) obviously inhibited the protein expression of PCNA, TLR4 and NF-κB induced by heat stress which was similar with Lubbad et al. (2009) who stated that curcumin treatment significantly suppressed the increase protein level of TLR4, MyD88, and NFkB in rats damaged tissue which indicate that curcumin serves as an important beneficial anti-inflammatory feed additive in poultry industry to protect the several types of liver disease. Another similar study was also in accordance with our present results which identified that protein expression of NF-kB in the rats gingival tissues was significantly inhibited by the lower dose of curcumin (30 mg/kg body weight) (de Aquino et al., 2011). Therefore, the result of present study showed that dietary curcumin (HT100 and HT200) has anti-inflammatory ability to down regulate the protein expression of PCNA, TLR4 and NF-κB which might helpful to protect liver diseases and enhance disease resistance in the poultry.

5. Conclusions

The results of the present study observed that heat stressed laying hens fed curcumin supplemented diet had better heat tolerance and immune status than NC and HC groups fed only basal diet, which is reflected by reduced inflammatory cytokines response, down regulated TLR4 and its downstream gene expression and reduced TLR4, NF-κB and PCNA protein expression. The available findings also indicated that curcumin improves the immune status of birds and can be suitable feed additive as an alternative to synthetic immune booster in poultry diets.

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Availability of data and materials

Data sharing is not applicable to this article.

Authors' contributions

The author wrote the main text of the manuscript. The co-authors collected the data, contributed to the design and the drafting of the paper. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

According to rules and regulation of Animal Care Committee of Guangdong Ocean University (Guangzhou, People's Republic of China).

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