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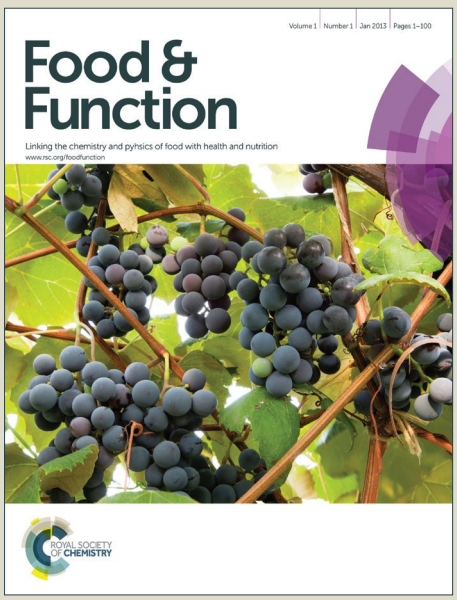

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Resveratrol modulates intestinal morphology and jejunal mucosa HSP70/90, NF-κB and EGF expression in black-boned chicken exposure to circular heat stress

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Abstract

The aim of this study was to investigate whether supplementation with resveratrol could alleviate intestinal injuries and to explore how resveratrol regulates heat shock protein (HSP)70, HSP90, nuclear factor kappa B (NF-κB) and epidermal growth factor (EGF) expression in the jejunal mucosa of black-boned chickens under circular heat stress. A total of 300 black-boned chicks of 42-d-old were randomly assigned to five treatment groups. The positive control chickens were kept in a normal-temperature (NT, 24 ± 2°C) chamber and fed with a basal diet. The other four groups were kept in a circular high-temperature (HT, 37 ± 2°C) chamber for 8 h and fed a basal diet supplemented with 0, 200, 400, or 600 mg/kg of resveratrol for 15 days. The results showed that the heat-stress responses damaged the villus structures of jejunum and ileum, resulting in shortened intestinal villi, deepened crypts, reduced villus height to crypt depth (V/C) ratio and decreased the numbers of goblet cells and lymphocytes. Heat stress also caused increased mRNA and protein expression of HSP70, HSP90 and NF-κB, and reduced EGF in the jejunal mucosa. Dietary supplementation with 400mg/kg of resveratrol improved the villus morphology, increased the numbers of goblet cells and lymphocytes, attenuated the mRNA overexpression of *HSP*70*, HSP*90 and *NF-κB* on the 6^{th}, 10^{th} and 15^{th} days of heat stress (P <0.05), activated the expression of *EGF* (P <0.05) in the jejunal mucosa. Resveratrol reduced protein expression of HSP70, HSP90 and NF-κB in the jejunal villi after 15-days heat stress, increased EGF expression from the lamina propria toward the epithelial cells of the villi. These results suggest that dietary resveratrol offers a potential nutritional strategy to improve intestinal morphology and alleviate jejunum mucosa injuries by modulating the mRNA and protein expression of HSPs, epithelial growth factor and transcription factor in black-boned chickens subjected to circular heat stress.

Keywords

Black-boned chicken; Heat stress; Jejunum mucosa; HSP70/90; NF-κB; EGF

Introduction

Intestinal mucosa acts as an interface that mediates communication between the interior of the body and the external environment. Its main functions not only include nutrient digestion and absorption but it also acts as an important barrier against the invasion of bacteria, viruses, parasites and some allergenic macromolecules.[1] The intestinal mucosa of poultry in the growing phase often exhibit immaturities in anatomy and function, and high-temperature environment can directly affect the intestinal development and cause the malabsorption of nutrients. Burkholder (2008) reported that heat stress can destroy normal microbial flora and villous structures in small intestine of chickens.[2] Quinteiro-Filho (2010) also proved that heat stress can induce intestinal injury and acute enteritis in poultry.[3] Damage to the intestinal mucosal barrier or the inhibition of immune function caused by heat stress can induce to the invasion of microbes or endotoxin in the intestinal cavity, resulting in a disturbed local intestinal or systemic balance between pro-inflammatory and anti-inflammatory cytokines.[4] Therefore, it was proposed that the effective antioxidant added to the dietary of chickens to alleviate intestinal injuries and improve immune function.

Heat shock protein 70 (HSP70), an important class of nonspecific cytoprotective proteins, plays an important role in the regulation of tolerance to various kinds of stress and resistance to stress.[5,6,7] HSP90 can interact with the main components of the cytoskeleton, such as actin and tubulin,[8] and it can also bind to denatured proteins to restore their normal structures.[9,10] Nuclear factor kappa B (NF-κB) is a key transcription regulatory factor and resides in the cytoplasm, involving in cell growth, inflammation, immunity, and death.[11] Upon extracellular stimulation, NF-κB becomes highly active, and freely dissociated NF-κB enters into the nucleus.[12] Epidermal growth factor (EGF) is an important intestinal epithelial growth and repair factor, which binds to its membrane receptor to controls cell metabolism, growth, and differentiation via intracellular signaling,[13] and to repair damaged intestinal mucosa.[14] Clearly, the investigation to the changes of pathological morphology and of heat shock proteins and growth factors in the intestinal mucosa is critical to further improve the intestinal absorption and barrier functions of chickens caused by heat stress.

Resveratrol has anti-oxidation,[15,16] anti-tumor,[17] anti-inflammatory,[18,19] neuroprotection and

cardiovascular protection effects.[20] Numerous studies have shown that resveratrol can effectively eliminate reactive oxygen species (ROS),[21] reduce DNA fragmentation, and enhance the regulatory effects on growth factors and anti-inflammatory cytokines.[22,23] Sahin (2011) reported that resveratrol can inhibit the high-levels of HSP70/90, NF-κB proteins in quail liver during heat stress.[24] Liu (2014) showed that resveratrol induce antioxidant capacity and attenuated the heat stress-induced overexpression of *Hsp*27/70/90 mRNA in the bursa of Fabricius and spleen of black-boned chickens.[25] However, the patterns by which resveratrol regulate the mRNA expression of *HSP*70/90*, NF-κB* and *EGF* in the intestinal mucosa of black-boned chickens after different periods of heat stress currently remain unclear. Further, the effects of resveratrol on the distribution of these proteins in the intestinal mucosa upon heat stress are also unknown. Therefore, this study aimed to conduct an in-depth investigation of the *HSP*70/90*, NF-κB* and *EGF* transcription levels under resveratrol mediated regulation of heat stress responses in black-boned chickens and of the protection effects by which the corresponding proteins are involved in anti-stress jejunum.

Materials and methods

Birds, diets, and experimental design

Birds and experimental design were same as the previous reported paper.[25] Three hundred female Xuefeng black-boned chickens, 28 d of age, were supplied a basal diet and water adlibitum. Resveratrol (≥98% purity; molecular weight, 228.25) was provided by Hunan Engineering and Technology Center for Natural Products (Changsha, Hunan, China). After a 2-wk adaptation period, the birds were individually weighed (the average weight was 293 g) and divided into 5 groups, each group of birds were further subdivided into 6 replicate groups (10 chickens/replicate), In group one, 60 chickens were fed a basal diet and maintained at 24 ± 2°C for 24 h/d (normal temperature, NT). The remaining 4 groups which included 240 black-boned chickens were housed in temperature-controlled rooms at 37 ± 2°C for 8 h/d (high temperature, HT; 0930–1730) followed by housing at 24 ± 2°C for the remaining16 h/d. This experimental treatment lasted for 15 d (from d 42 to 57). During the treatment period, the birds of NT group received a basal diet and the birds in the HT groups received 1 of 4 diets:

the basal diet or basal diet supplemented with 200, 400, or 600 mg/kg resveratrol. The basal diet was formulated based on NRC requirements (NRC, 1994) and the compositions and nutrient content were listed in Table 1. Feed was provided to the birds two times per day at 8:00 and 18:30, and any uneaten food was weighed in the morning of the next day. Six birds per treatment were sacrificed for sampling after feed deprivation for 10 h (treated day at 18:00) at 1, 3, 6, 10, 15 days of heat stress. All animal procedures were carried out in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Hunan Agricultural University.

<Table 1>

Sample

Black-boned chickens were slaughtered and the small intestine was excised. About 2 g of jejunal mucosa was collected immediately, frozen by liquid nitrogen, and stored at -80°C until the extraction of total RNA and protein. Segments of 1 cm length were cut respectively from the jejunum and ileum and fixed in 10% neutral formaldehyde for subsequent morphological and immunohistochemical analysis.

Jejunal morphology and histology analysis

Formalin-fixed samples were embedded in paraffin, and sectioned in transverse (5 μm thick) using AO820 microtome (WI, USA). After deparaffinization and dehydration, the sections of the jejunum were stained with hematoxylin and eosin (H&E). The structure of the mucosa was observed using a BX43 Olympus microscope (Olympus, Tokyo, Japan) and analyzed using Motic images advanced 3.2 software system (Motic, Xiamen, China). Villi height and crypt depth of the jejunum at least five well-oriented villi were measured and recorded. Intraepithelial lymphocytes (IELs) and goblet cells (GCs) were identified and enumerated per 100 villus epithelial cells for each crosssection in the coincident visual field.

Real-time quantitative PCR

Total RNAs were extracted from the jejunal mucosa from each of the six chickens at each time point (on day 1, 3, 6, 10 and 15 after treatment of heat stress) and purified using an

RNeasy Mini Kit (TaKaRa, Tokyo, Japan). The mRNA expression level of *HSP*70, *HSP*90, *NF-κB* p65 and *EGF* in the jejunal mucosa were detected by real-time quantitative PCR. The *β-actin* gene was used as the housekeeping gene. All primers from *G. gallus domesticus* were designed (Table 2), and synthesized and purified by Sangon Biotech Co. Ltd (Shanghai, China). The real-time PCR reactions were performed in a 20 μl total reaction volume, which included 10 μl of 2×SYBR® Premix EX Taq™ master mix (TaKaRa, Tokyo, Japan), 0.4 μl of ROX Reference Dye (50×), 0.8 μl each of the forward and reverse primers (10 μM), 1 μl of cDNA template, and 7.8 μl of sterilized water. The PCR were carried out on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA). The thermal cycler parameters were as follows: 30 s at 95 °C; 40 cycles for 5 s at 95 °C, 31 s at 59 °C, and an additional dissociation cycle of 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, and 15 s at 60 °C. The target gene expression was normalized to that of the selected reference gene, and the relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method.[26]

<Table 2>

Western blot

The protein concentration of jejunal mucosa from each group on day 15 of heat stress was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, America). The optical density (OD) was measured in the microplate reader at 562 nm wavelength. The protein concentration of each sample was calculated. A 50-μg protein was used for western blot analysis, after adding 5 × concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating the samples for 5 min at 95℃. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and transferred to polyvinylidine fluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature (RT), in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies HSP70 (ab5439; Abcam, England) at a final dilution of 1:5000, or EGF (BS-4568; RayBiotech, America) at a final dilution of 1:200 or β-actin (600008-1; Proteintech, America) at a final dilution of 1:4000 was performed overnight at 4 ℃. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at

RT with alkaline phosphatase-linked secondary antibodies HRP goat anti-rabbit IgG at a final dilution of 1:3000 (074-1506; Proteintech, America) in TBS-T with 1% low-fat milk. Protein immunoreactive bands were photographed, and fluorescence was detected on imaging analysis software (GIS1000). Each special banding gray value was digitized, and the gray value of target protein was divided by internal reference β-actin so as to correct errors.

Immunohistochemical staining

The fixed jejunal samples were cast in paraffin, sliced into 3-μm sections, then collected on silanized slides. After deparaffinization and hydration through xylenes, slides were subjected to microwave for antigen retrieval and then cooled down at RT. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 20 min at RT, followed by incubation with rabbit serum of the species from which the secondary antibody was produced for 30 min. Subsequently, sections were incubated overnight at 4 °C in a humidified chamber with a 1:100 dilution of the primary antibody, respectively HSP70 (HSP70-Lot11712; ZSGB-Bio, China), HSP90 (HSP90-LOT74610D222; Boster, China), NF-κB p65 (nfkBP65-LOTNO 78611P114; Boster, China), EGF (Y-07D06A-DA0250; Boster, China). The sIgA was then detected using a commercial immune-peroxidase staining kit (ZSGB-Bio, China). Briefly, the sections were incubated with a 1:100 dilution of biotinylated secondary rabbit-anti-goat antibody (Fas-L-BA0049; Boster, China) for about 10 h at 4 °C, followed by the avidin-biotin-peroxidase complex (ABC) reagent incubation for 30 min at RT. Bound antibody conjugates were visualized using 3, 3-diaminobenzidine (DAB) as a chromogen to develop a brown staining and sections were counterstained with hematoxylin and mounted with glycerol gelatin. Negative control sections were stained under identical conditions by substituting the primary antibody with 0.01 M PBS. Each slice in the light microscope (×400) randomly selected five fields of vision with a fixed window area. HSP70, HSP90, NF-κB, EGF protein expression was reflected by the size of the average integrated optical density values.[27]

Statistical analysis

The data was analyzed by analysis of variance (ANOVA) using the general linear model

(GLM) procedure of the statistical analysis system (SAS Institute, 2000). Paired *t*-test analysis was used to evaluate the environment effect between NT and HT treated without resveratrol, or the resveratrol effect between HT and HT treated with 400 mg/kg resveratrol. Duncan's multiple range test was applied for comparing the differences among treatments from different levels of resveratrol (0, 200, 400, or 600 mg/kg) under HT environment. The difference was considered to be significant at $P < 0.05$, and be very significant at $P < 0.01$.

Results

Changes in intestinal morphology

As presented in Fig. 1, the villus in the jejunum and ileum were neatly arranged and had clear regular shapes in the NT group. However, the connections from the jejunal epithelial membrane were broken, villus missing or shortened, the mucosal epithelium was degenerated with villus atrophy, the intestinal glands were prominent, and most of the lamina propria was exposed in the HT group. The mucosal villi of the ileum had edema and were enlarged, the epithelium was broken off from the tip of the intestinal villi, and the glands were swollen under the HT condition. The villus in the jejunum and ileum of black-boned chickens supplemented with 400 mg/kg of resveratrol under heat stress also had occasional shedding and breakage. Compared with the NT group, the jejunal villus height and the ratio of the villus height to the crypt depth (V/C) of the HT group were decreased ($P < 0.05$), the jejunal crypt depth of the HT group was increased ($P < 0.05$), and the ileal villus height and V/C ratio were reduced ($P < 0.01$). Dietary addition of 400 mg/kg of resveratrol significantly increased the jejunal villus height and the V/C ratio of black-boned chickens under heat stress, the jejunal crypt depth was decreased ($P < 0.05$); however, the ileal crypt depth and V/C ratio did not show significant differences ($P > 0.05$). The numbers of goblet cells and lymphocytes in the jejunum and ileum of the HT group (Table 3) were significantly lower than those of the NT group ($P < 0.05$). Dietary supplemented with of 400 mg/kg of resveratrol increased significantly the numbers of goblet cells and lymphocytes in the jejunum.

<Fig. 1>

<Table 3>

Jejunal mucosal *HSP*70/90, *NF-κB* and *EGF* mRNA expression

Fig. 2 showed that both the *HSP*70 and *NF-κB* mRNA expression levels in the jejunal mucosa of the HT group was significantly increased compared with the NT group during the entire period (P <0.05), while *HSP*70 expression was very significantly increased on the 3rd and 6th days, *HSP*90 was very significantly increased on the 3rd day, and *NF-κB* and *EGF* showed significant increases or decreases on the 6thand 10th days of heat stress (P <0.01). Dietary supplementation with 400 mg/kg of resveratrol significantly suppressed the expression of *HSP*70 and *HSP*90 on the 6th day of heat stress (P <0.05); 400 and 600 mg/kg of resveratrol significantly inhibited *NF-κB* expression on the 6th, 10th and 15th days of heat stress (P <0.05). With prolonged heat stress, 200, 400 and 600 mg/kg of resveratrol significantly stimulated *EGF* expression levels in jejunal mucosa on the 10th and 15th days of heat stress (P <0.05).

<Fig. 2>

Jejunal mucosal HSP70 and EGF protein expression

Western blot analysis was utilized to detect the protein expression of HSP70 and EGF in jejunal mucosa of black-boned chicken after 15-day heat stress, as shown in Fig. 3. As presented a highly significant increase in HSP70 level and lower in EGF level (P <0.01). Supplementation with 400 or 600 mg/kg resveratrol were significantly lower in HSP70 levels than without resveratrol during heat stress (P <0.05); the EGF level in the group provided 400 mg/kg of resveratrol was higher significantly than that in the 600 mg/kg group (P <0.05).

<Fig. 3>

Distribution of HSP70/90, NF-κB and EGF protein in the jejunal villus

The color signals of HSP70/90 and NF-κB protein expression in the HT group were lighter than those in the NT group, EGF protein expression became dim; however, the color signals of these protein expression in HT+Res400 group exhibited intermediate between the HT and the NT group (Fig. 4). The integral optical densities of HSP70/90 and NF-κB protein expression were higher, of EGF protein expression was lower in the HT group (P <0.05) than those in both the NT and HT+Res400 groups; the integral optical densities of HSP70/90 and

NF-κB protein expression in the HT+Res400 group was reduced compared with those of the HT group, and EGF protein was increased obviously expression from the lamina propria toward the epithelial cells of the villi (Table 4).

<Fig. 4>

<Table 4>

Discussion

Heat stress can cause pathological damage to the chicken intestine, and the serious lesions mainly involved in the shedding of mucous layer and mucosal epithelial cell, the edema of lamina propria, the fracture of intestinal villus,[28] which are consistent with the results of this study in the jejunum of black-boned chickens. Crypt depth reflects the cell generation rate, cells constantly migrate from the base of the crypt to the top of the villi, followed by the differentiation and formation of intestinal villus cells with absorption functions to compensate for villus epithelial shedding, and increases in the ratio of villus height to crypt depth (V/C) reflect increased digestion and absorption capacities.[29] Heat stress-induced morphological changes to the small intestine of poultry mainly consist of a shortened villus height, increased crypt depth and reduced V/C ratio,[2] which will inevitably lead to significantly reduced intestinal digestion and absorption area, sharply reduced function and efficiency in digestion and absorption, and significantly reduced average daily weight gain. This is consistent with our previous study on heat stress and the production performance of black-boned chickens.[25] The results of this study showed that resveratrol could effectively increase the V/C ratios of jejunum and ileum in black-boned chicken, to some extent, alleviate the damage to intestinal mucosa caused by heat stress. Studies also have reported that heat stress can cause hypoxia, ischemia, or even edema in intestinal epithelium of poultry as well as rupture of the connections between epithelial cell membranes and cells, cell necrosis, and shedding of the epithelium from the tip of intestinal villi or even from the entire villus layer.[30] These types of damage lead to increased permeability of the intestinal epithelium and bacterial translocation. Lymphocytes within the intestinal epithelium are the major source of cytokine secretion and are the pioneer protectors of the intestinal mucosal immune system.[31] The surface of intestinal epithelial cells is covered with a protective layer of mucus secreted by goblet cells, which can

protect intestinal epithelial cells, prevent the invasion of pathogens and play a key role in the repair process after injury to the intestinal mucosa.[32] Therefore, the presence of a certain number of lymphocytes and goblet cells is important for maintaining barrier function in the intestinal mucosa. Resveratrol had antioxidant and anti-inflammatory functions[33] and significantly increased the number of lymphocytes and goblet cells in the jejunal mucosa of black-boned chickens upon heat stress. It was also found that the intestinal villus height is directly proportional to the numbers of intestinal goblet cells and lymphocytes and inferred that resveratrol can improve the intestinal villus height by inhibiting damage to the intestinal mucosa caused by intestinal inflammatory factors and some oxides stress, thereby enhanced intestinal immunity response.

Previous researches in poultry have shown that heat stress is associated with induction of heat shock proteins and nuclear transcription factors resulting in an increase in the expression level of HSP70/90 and *NF-κB*p65 in quail livers,[24] HSP70/90 in chicken hearts and spleens.[5,25] Yu (2010) and Lu (2011) confirmed that *HSP*70 and *HSP*90 expression were increased in the small intestine of porcine and rat exposure to heat stress using Microarray analysis of gene expression profiles.[34,35] The results of present studies showed that on the early stage of heat stress treatment of black-boned chickens, the transcription levels of *HSP*70 and *HSP*90 in the jejunal mucosa rapidly increased to respond to cell damage caused by heat stress and that the abundance levels were relatively high on the 3rd and 6th days. The reason for this finding might be that the early phase of heat stress is the critical "sensitive period" and "confrontation" period, thus it is possible to dramatically mobilize *HSP*70*, HSP*90 and related genes for high abundance expression. In the late phase of heat stress, *HSP*70 expression was in a relatively stable high expression state at the 10th and 15th days. This finding may have occurred because after a certain period of resistance against heat stress, black-boned chickens gradually adapted to heat stress and developed a certain degree of tolerance in the late phase. The expression of *HSP*90 was close to that of the NT group on the 10th day and then showed a sudden increase on the 15th day, indicating that the heat stress tolerance of black-boned chickens has time limitations. This observation could also be caused by the resistance fatigue of the body due to long-term heat stress. Therefore, the defense system induced the high abundance expression of some genes, such as *HSP*90, to resist heat stress. After 15 days of

heat stress, the transcriptional level and the corresponding protein expression level of *HSP*70 in the resveratrol-treated group were not consistent with each other, which may be due to the multi-level regulation of transcription and protein translation in the body as a complex entity. Resveratrol can regulate antioxidant defense responses upon heat stress in quail liver[24] and black-boned chicken bursa and spleen[25] and can reduce the expression levels of *HSP*70 and *HSP*90 and the corresponding proteins, which share some similarities with the results of this study in the jejunal mucosa of black-boned chickens. These results suggest that black-boned chickens utilize the dual effects of the internal defense system and external resveratrol intervention to resist heat stress and protect the immune functions.

NF-κB interacts with inhibitor proteins and exists in an inactive form inside of cells. When cells are stimulated by certain cytokines, toxins and stress, NF-κB is activated immediately, subsequently translocates into the nucleus, binds with specific DNA binding sites to regulate the expression of various genes, and thereby regulates biological states, such as cell proliferation, apoptosis, and inflammation.[36,37] Sahin (2010; 2011) and Orhan (2012) reported that heat stress increases the NF-κB expression level in quail liver and induces the expression of multiple genes.[23,24,38] The current study also showed that *NF-κB* mRNA expression levels in jejunal mucosa of black-boned chicken were significantly increased throughout the period of heat stress and reached highly significant levels on the 6^{th} and 10^{th} days. This result also suggests that at this time point, the high abundance of *NF-κB* expression might be related to the degree of pathological morphology and that abundance of *NF-κB* mRNA expression possibly stimulates a series of genes related to jejunal mucosal injuries to stay in highly active conditions. Resveratrol-mediated inhibition of *NF-κB* mRNA expression in jejunal mucosa started to show a significant effect on the 6^{th} day, and we also found that 400 or 600 mg/kg of resveratrol significantly reduced the abundance of *NF-κB* expression. Combined with the current observation that resveratrol can down-regulate *HSP*70 and *HSP*90 mRNA expression levels in jejunal mucosa upon heat stress, further confirmed that NF-κB can act as a transcription factor that plays a positive regulatory role in the expression of *HSP*70 and *HSP*90.[39]

While screening for differentially expressed genes in pigs under heat stress, Liu (2009) found that genes such as epidermal growth factor receptor (EGFR) and EGF were

significantly down-regulated in jejunal tissue.[40] The results of this study found that heat stress down-regulated the expression of *EGF* mRNA in jejunum of black-boned chicken, which is consistent with the results reported by Yu (2010)[34] for small intestinal epithelium of porcine. Compared with normal temperature conditions, the *EGF* mRNA expression levels in black-boned chicken jejunal mucosa appeared to be significantly reduced on the 6th day of heat stress, indicating that in the early phase of heat stress, the expression of growth factors such as *EGF* decreased due to damage of the jejunal villus epithelial cells.[40] Intestinal villus inflammation and was aggravated during sustained high temperature environment, endogenous EGF may be exhausted, leading to more severe jejunal mucosal injuries. Daily dietary supplementation of 400 mg/kg of resveratrol significantly stimulated an increase in *EGF* mRNA expression in black-boned chicken upon heat stress, and showed a dramatic increase on the 15th day. This finding was similar to the trend of changes in EGF protein revealed by western blot, indicating the consistent expression of EGF protein and its mRNA. These observations further indicate that resveratrol can promote the secretion of EGF by jejunal mucosa, induce cell proliferation during the process of heat stress-induced jejunal villus injury in black-boned chickens, and resist heat damage caused by a high temperature environment.

The localization of HSPs is closely related to their chaperone function.[41,42] After heat stress, the HSP70-positive signals in the epithelial cells of the intestinal villi were much stronger than those in the non-heat stress conditions. This finding may have occurred because HSP70 expression in the nucleus can significantly inhibit nucleolar separation to protect the integrity of the cytoskeleton.[43] The HSP90-positive signals spread to the end of the entire villi of the intestinal mucosa, and the nuclei of the villus epithelial cells showed an uneven distribution of HSP90 in different injury sites. These proteins may differ in their functions as chaperones to participate in the self-protection of cells, stabilization of the cytoskeleton, promotion of the correct assembly of protein higher-order structures and removal of denatured proteins. Strong positive signals of NF-κB protein were also found in the entire jejunal mucosa after heat stress; in particular, the signal was significantly enhanced in the epithelial cells of the intestinal villi. As we have mentioned, resveratrol can down-regulate the transcription levels of *HSP*70, *HSP*90 and *NF-κB* mRNA in the jejunal mucosa to alleviate stress response, which

was further confirmed changes of HSP70 and HSP90 protein and revealed that resveratrol plays an important role in the repair of jejunal mucosa under heat stress.

Under normal conditions, the cell membrane surfaces of epithelial cells in the intestinal villi, lamina propria cells and crypt epithelial cells in the intestine are covered with large quantities of EGF protein, and endogenous EGF functions by specifically binding to autocrine and paracrine receptors.[44] EGF can improve hydrolase activity in the intestinal mucosal brush border, strengthen small intestinal secretion, accelerate cell differentiation and proliferation, regulate the secretion of digestive glands, stimulate mucosal proliferation and repair intestinal mucosal structures.[45] Heat stress caused a significant reduction in the distribution of EGF signals in the jejunal villus epithelial cells and glandular cells, whereas the lamina propria showed the expression of strong signals. Resveratrol could significantly increase the distribution of EGF-positive signals in epithelial cells and glandular cells of jejunal villus, which was distributed more sparsely in cells of the lamina propria. This finding suggests that resveratrol may induce the transduction of EGF-positive signals under heat stress conditions, that is, the transfer of EGF from the lamina propria of the jejuna mucosa to the nuclei and cytoplasm of villus epithelial cells, providing the EGF required for the rapid renewal of epithelial cells.

In summary, heat stress resulted in a negative impact on morphological alterations, with increased mobilization of heat shock proteins and transcription factors in the small intestine of black-boned chickens. The present results have demonstrated that dietary supplementation with resveratrol provided a protective strategy against circular heat stress and alleviated jejunal mucosa injuries in black-boned chickens through modulating the mRNA expression and protein location of heat shock genes and transcription factor, and through activating epithelial growth factor expression to further improve intestinal morphology and immunity.

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Figure Captions

Fig. 1. The representative figure of jejunal and ileal mucosa morphology in NT, HT, HT+Res400 groups (H & E Staining, ×100)**.** Black-boned chickens were fed a basal diet under normal temperature (NT) or high temperature (HT) condition, or fed a basal diet supplemented with 400 mg/kg resveratrol under HT condition (HT+Res400) for 15 days.

Fig. 2. The relative expression abundance of *HSP*70 (a), *HSP*90 (b), *NF-κB* (c) and *EGF* (d) mRNA in jejuna mucosa of black-boned chickens. Black-boned chickens were fed a basal diet under normal temperature (NT) or high temperature (HT) environment, or fed a basal diet supplemented with 0, 200, 400, 600 mg/kg resveratrol under HT environment (HT+Res200, 400, 600, n=6). * Means $P<0.05$, ** Means $P<0.01$. $^{a, b,c}$Means with different superscripts in the same day differ ($P<0.05$).

Fig. 3. HSP70 and EGF protein expression (a), the relative gray values of HSP70 protein / β-actin and EGF protein / β-actin (b) in jejuna mucosa. Black-boned chickens were fed a basal diet under normal temperature (NT) or high temperature (HT) environment, or fed a basal diet supplemented with 0, 200, 400, 600 mg/kg resveratrol under HT environment (HT+Res200, 400, 600, n=6) for 15 days. * Means $P<0.05$, ** Means $P<0.01$. $^{a, b,c}$ Means with different superscripts in the same protein differ ($P<0.05$).

Fig. 4. Distribution and expression of HSP70, HSP90, NF-κB, EGF protein in jejunal villus (Immunohistochemical staining, ×400). Black-boned chickens were fed a basal diet under normal temperature (NT) or high temperature (HT) condition, or fed a basal diet supplemented with 400 mg/kg resveratrol under HT condition (HT+Res400) for 15 days. The arrow points to the detective protein changes in the same location of jejunal villus.

Tables

Table 1. Composition and nutrient levels of the basal diet.

Ingredients	Air-dry basis, %	[b] Nutrient levels	
Corn	59.92	ME (MJ/kg)	12.41
Soybean meal	29.37	Crude protein (g/kg)	18.65
Wheat bran	3.24	Calcium (g/kg)	1.03
Limestone	1.18	Phosphorus (g/kg)	0.45
Dicalcium phosphate	1.63	Lysine (g/kg)	0.85
Salt	0.38	Methionine (g/kg)	0.36
Corn oil	3.28		
[a] Premix	1.00		
Totall	100.00		

[a] Provided with per kilogram diet: Vitamin A, 12,500 IU; Vitamin D3, 3,000 IU; Vitamin E, 25 IU; Vitamin B1, 3 mg; Vitamin B2, 6.5 mg; Vitamin B12, 0.2 mg; Vitamin K3, 3.25 mg; Biotin, 0.08 mg; Folic acid, 1.5 mg; D-pantothenic acid, 12.5 mg; Nicotinic acid, 45 mg; Chloride choline, 1 g; D, L-methionine, 0.8 g. Copper, 8 mg; Iron, 80 mg; Zinc, 40 mg; Manganese, 60 mg; Selenium, 0.15 mg; Lodine, 0.35 mg.

[b] Provided with nutrient levels per kilogram diet: Metaboliazble energy (ME) was a calculated value, other nutrient levels were measured values.

568 **Table 2.** Primer sequences (5' to 3') used for the detection of mRNA specific for HSP70,
569 HSP90, NF-κB, EGF and β-actin.

Gene	Accession number	Primer sequences (5' to 3')	Product size (bp)	Annealing temperature (°C)
***HSP*70**	EU747335.1	F: TGTGTCCATCCTTACCATTGAG R: GCTTGTGCTTACCCTTGAACTC	134	59
***HSP*90**	DQ267486.1	F: TCAGACTTGATAACGGTGAACCT R: TGTCTTCTCCTCCTTCTCCTCTT	233	59
NF-κB p65	D13721.1	F: GTTCGGGTCTCTTTGGTGAC R: GTTGAAGGGGTTGTTATTGGTG	213	58
EGF	AY588969.1	F: AGGCATGGATCGTCGGGTTATA R: CCCAGTCAGAAAGCCAAAGGTG	221	60
β-actin	L08165.1	F: AGCCAACAGAGAGAAGATGACAC R: CATCACCAGAGTCCATCACAATA	134	59

570 F = forward primer; R = reverse primer.
571

572 **Table 3.** Comparison of jejunal and ileal mucosa epithelial morphology, the quantity of GCs
573 and IELs during every 100 villous epithelium cells in black-boned chickens

Items	[a]Treatments			P_1-value	P_2-value
	NT	HT	HT+Res400		
Jejunum					
Villus height (μm)	464.30	335.50	393.67	0.025	0.035
Crypt depth (μm)	127.07	162.93	143.23	0.014	0.046
Villus height/Crypt depth	3.69	2.10	2.75	0.020	0.013
GCs/100 villus epithelial cells	39.67	26.33	30.67	0.046	0.020
IELs/100 villus epithelial cells	113.00	89.00	104.67	0.029	0.031
Ileum					
Villus height (μm)	341.90	257.40	296.70	0.005	0.047
Crypt depth (μm)	118.57	152.83	130.83	0.006	0.101
Villus height/Crypt depth	2.89	1.82	2.27	0.006	0.064
GCs/100 villus epithelial cells	34.33	21.00	29.00	0.017	0.014
IELs/100 villus epithelial cells	132.33	95.67	114.33	0.032	0.113

574 [a] Subjected black-boned chickens were fed a basal diet under normal temperature (NT) or high temperature
575 (HT) environment, or fed a basal diet supplemented with 400 mg/kg resveratrol under HT environment
576 (HT+Res400) for 15 days (n=6). P_1-value was used to evaluate the environment effect between NT and HT
577 treated without resveratrol, P_2-value to evaluate the resveratrol effect between HT and HT treated with 400
578 mg/kg resveratrol.
579

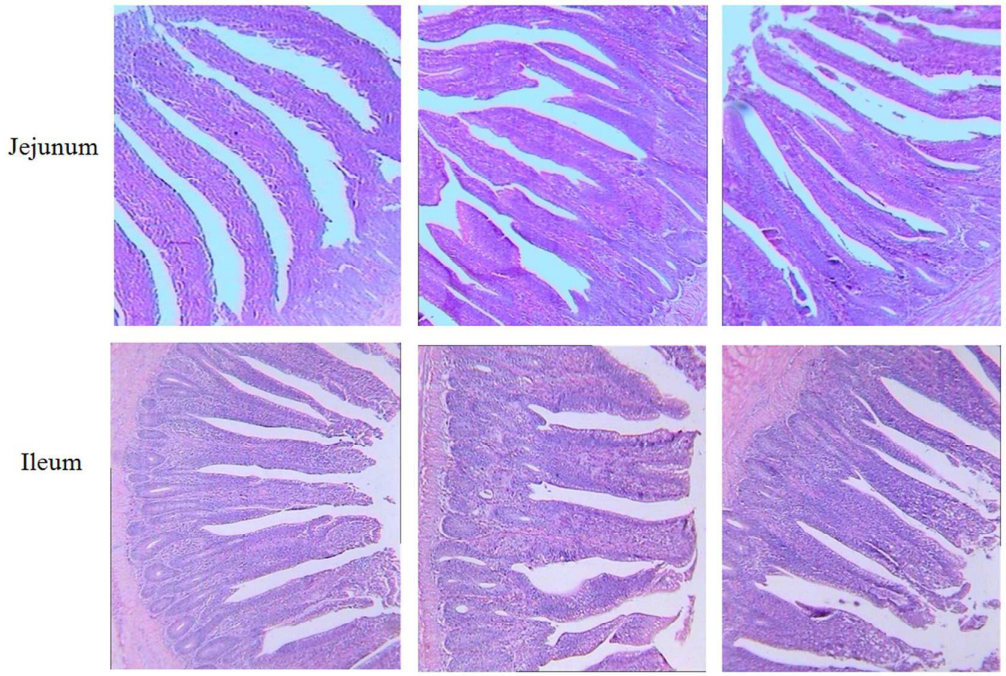
580 **Table 4.** The integral optical density of HSP70, HSP90, NF-κB and EGF protein expression
581 in jejunal villus

Items	[a]Treatments			P_1-value	P_2-value
	NT	HT	HT+Res400		
HSP70	301.29	622.63	536.28	<0.001	0.043
HSP90	404.69	708.71	487.36	0.023	0.014
NF-κB	375.88	1579.40	867.86	0.003	0.005
EGF	298.68	157.34	188.68	0.004	0.027

582 [a] Subjected black-boned chickens were fed a basal diet under normal temperature (NT) or high temperature
583 (HT) environment, or fed a basal diet supplemented with 400 mg/kg resveratrol under HT environment
584 (HT+Res400) for 15 days (n=6). P_1-value was used to evaluate the environment effect between NT and HT
585 treated without resveratrol, P_2-value to evaluate the resveratrol effect between HT and HT treated with 400
586 mg/kg resveratrol.

587

588 **Fig. 1**



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591 **Fig. 2**

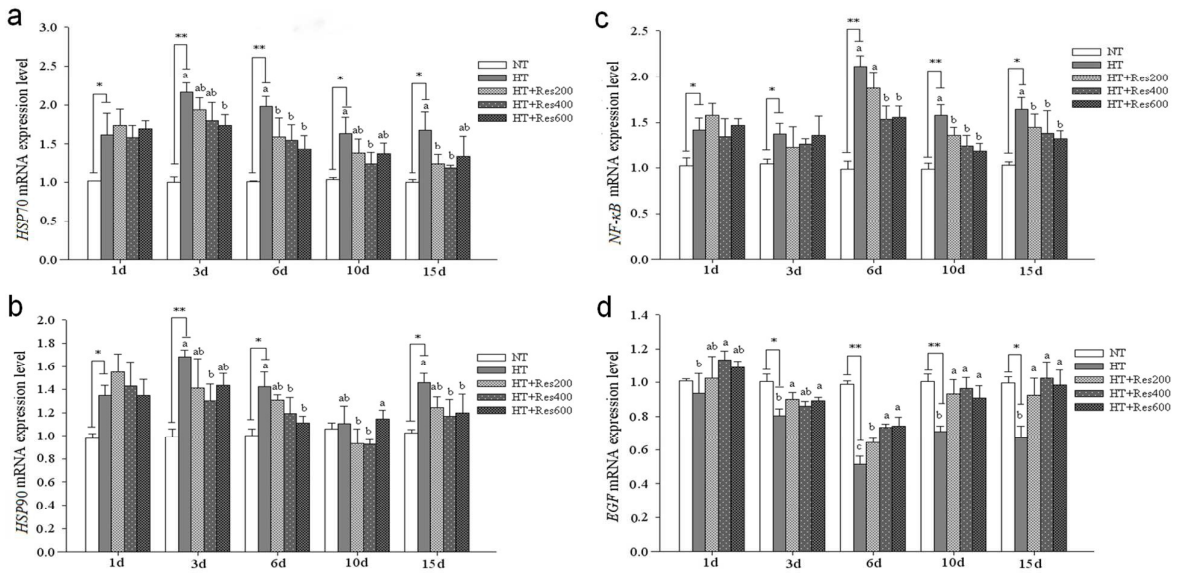

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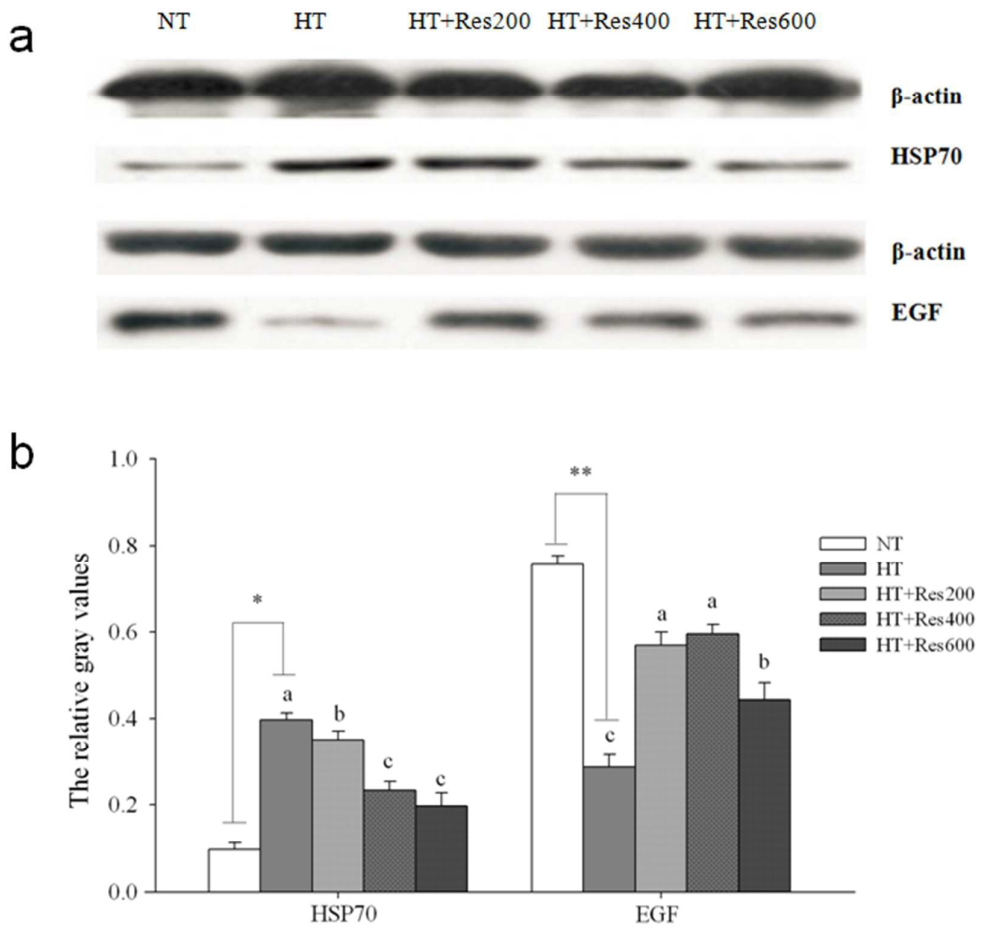
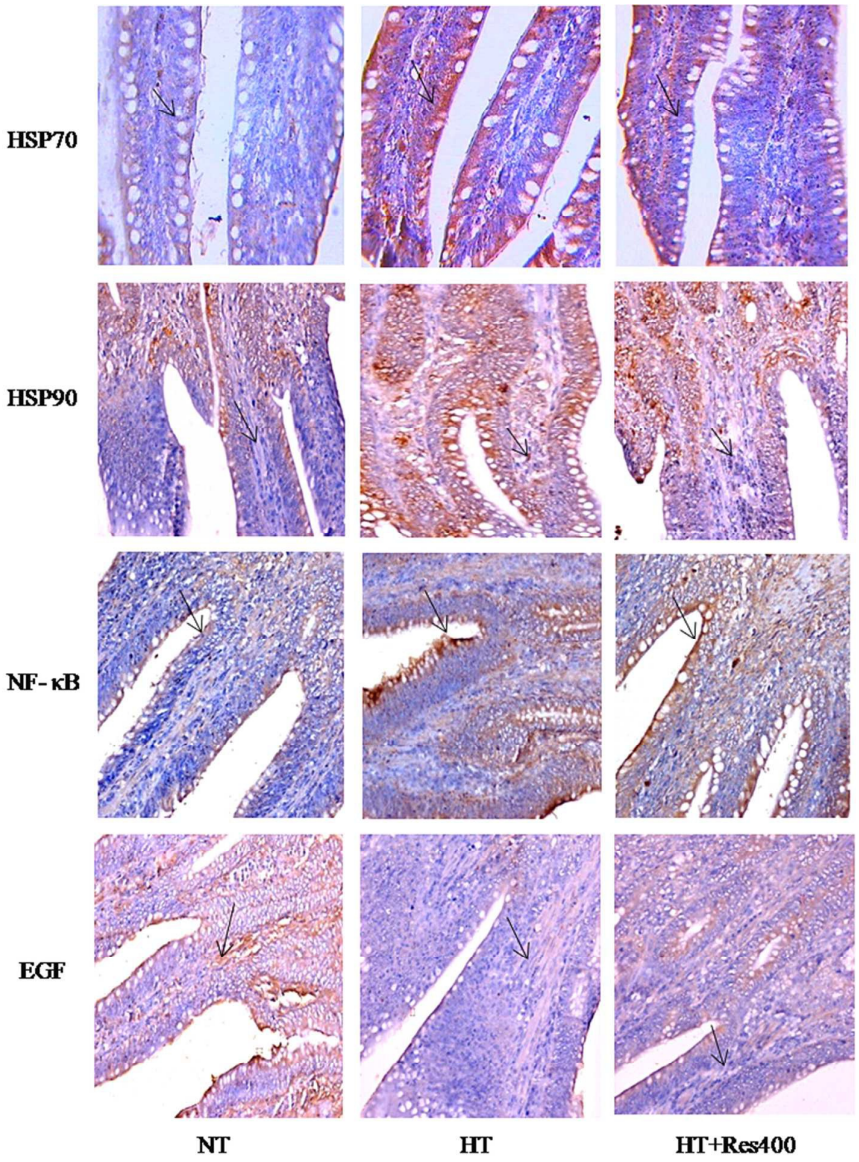
Fig. 3



597 **Fig. 4**



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Graphical abstract