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Effects of duration of thermal stress on growth performance, serum oxidative stress indices, the expression and localization of ABCG2 and mitochondria ROS production of skeletal muscle, small intestine and immune organs in broilers

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ABSTRACT

The purpose of the current study was to investigate that effect of duration of thermal stress on growth performance, oxidative stress indices in serum, the expression and localization of ABCG2, and mitochondria ROS production in skeletal muscle, small intestine and immune organs, and then to further reveal correlations between indicators. At 28 days of age, sixty broilers were randomly divided into the control group (25 ± 2 °C; 24 h/day) and the heat stress group (36 ± 2 °C; 8 h/day lasted for 1 week or 2 weeks). Fifteen broilers per group were respectively euthanized, and some samples were respectively collected from the control and the heat stress groups at the end of the 1st week or the 2nd week of heat stress. A typical heat stress response has been observed at this temperature. Compared with the control group, the birds subjected to heat stress at the end of the 1st week reduced ($P < 0.05$) body weight (BW), average daily feed intake (ADFI), average daily gain (ADG), the activity of serum antioxidant enzyme and content of glutathione (GSH), while increased ($P < 0.05$) feed conversion ratio (FCR), serum corticosterone and malondialdehyde (MDA) levels. However, when the heat stress lasted for the end of the 2nd week, there was no significant difference ($P > 0.05$) in ADFI, ADG, FCR and serum contents of corticosterone, MDA and GSH. Regardless of duration of thermal stress, the localization of ABCG2 protein had no change. Moreover, heat stress also did not affect ($P > 0.05$) the IOD of the ABCG2 positive portion and the expression of the ABCG2 mRNA in the pectorales, crureus, duodenum, jejunum, ileum and spleen, while significantly increased ($P < 0.05$) the corresponding tissues ROS production at the end of the 1st week of heat stress. In contrast, at the end of the 2nd week of heat stress, IOD of the ABCG2 positive portion and the expression of the ABCG2 mRNA in heat stress group significantly increased ($P < 0.05$), while the corresponding tissues ROS production had no difference ($P > 0.05$) compared to the control group. Collectively, duration of thermal stress affects growth performance, serum oxidative stress indices, and the expression of ABCG2 and the ROS production of broiler tissues in a time-dependent manner. There is a negative correlation between the expression of ABCG2 and the ROS production in the corresponding tissues under heat stress.

1. Introduction

With the rise of the global temperatures, heat stress has become the main environmental factor in poultry production since it impacted the welfare and caused critical economic losses (Aamir et al., 2018). It has also been reported that whether acute or chronic thermal stress significantly hinders the growth performance of broilers (Roushdy et al., 2018), even induces the pathological changes of the poultry body or death (Akbarian et al., 2016). Physiologically, the reduction of the growth rate may relate to reduced feed intake, suppressed intestines nutrient transporters and immune response by injuring intestines and immune organ (Habashy et al., 2017; Yi et al., 2017). Meanwhile, heat

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stress also impaired skeletal muscle growth and caused changes in muscle metabolism and redox balance (Ganesan et al., 2017a, 2017b; Volodina et al., 2017; Ganesan et al., 2018a, 2018b). Some studies have verified that thermal stress affects poultry performance mainly by inducing the oxidative stress (Zhang et al., 2012; El-Tarabany, 2016), and the oxidative stress damage of broilers induced by high ambient temperature has been reported by some studies (Lin et al., 2006; Mujahid et al., 2007, 2009; Attia et al., 2016; Alhenaky et al., 2017; Maibam et al., 2018). Furthermore, many studies suggest that the cause of heat-induced oxidative stress is mainly intracellular reactive oxygen species (ROS) production changes leading to the modification of the enzyme activity and gene expression profiles (Lin et al., 2006; Mujahid et al., 2007, 2009; Azad et al., 2010; Akbarian et al., 2016), and broiler chickens subjected to heat stress have distinct time-dependent physiological responses (Mujahid et al., 2009; Azad et al., 2010) and the obvious genetic variations (Cedraz et al., 2017). At present, there were a relatively few reports about the comparative effects of the duration of thermal stress on oxidative stress (Azad et al., 2010)and the levels of gene expression (Zaglool et al., 2019). Azad et al. (2010) reported that mitochondrial ROS production for the heat-stressed group broiler chicken was increased after 3, 5, and 9 days of heat exposure and returned to original levels at day 14, and speculated the animals had possibly acclimatized to environmental heat stress. However, the mechanism of regulating ROS production under the duration of chronic heat stress is still unclear.

ATP binding cassette (ABC) subfamily G member 2 (ABCG2) is a member of the ABC superfamily of proteins and widely distributes in a variety of normal tissues, such as small intestine, liver, and placenta, as well as many cancer tissues (Maliepaard et al., 2001; Young et al., 2003; Sarkadi et al., 2004). It plays an important role in drug resistance and maintaining cell homeostasis in the stress environment (Roh et al., 2018). ABCG2 can actively excrete a variety of toxic substrates and tumor chemotherapy drugs to protect the cells and tissues against various xenobiotics (Hasanabady and Kalalinia, 2016; Zhang et al., 2016). In addition, it has also been reported that a hypoxic stress environment can induce high expression of ABCG2, and not only in stem cells cultured in vitro, but in a physiologic hypoxic cell environment in vivo (He et al., 2018). Previous studies have shown that ABCG2 is capable of protecting cells from ROS-mediated cell damage and death (Shen et al., 2010), and the downregulation of ABCG2 induces the overproduction of ROS inhibiting the production of antioxidants (Nie et al., 2018; Kurokawa et al., 2019). Moreover, existing studies confirmed that some gene changes are related to heat stress of broilers (Lei et al., 2013). In especial, some studies had reported that heat stroke can affect vital organs of broiler chickens, such as the changes of gene expression in muscle tissue (Li et al., 2011), lower relative weights of thymus and spleen (Ghazi et al., 2012) and lesions and dysfunction of small intestinal (Yi et al., 2017). However, the underlying mechanism of these changes in broilers caused by heat stress remains unclear. Hence, it may be hypothesized that duration of heat stress can affect the expression and localization of ABCG2 and tissue mitochondria ROS production, and increased expression of the ABCG2 can relieve the heat-induced oxidative stress in skeletal muscle, small intestine, immune organs of broilers by reducing ROS generation, and then maintain the stability of growth performance and oxidative stress indices of broiler chicken. In present trial, the effects of duration of thermal stress on growth performance and serum oxidative stress indices were explored. Moreover, the association between the ABCG2 expression and the ROS generation were analyzed under different heat stress weeks.

2. Materials and method

2.1. Ethical permission

The experimental procedures were approved by the Animal Care and Use Committee of Anhui Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The methods were carried out in accordance with the approved guidelines.

2.2. Broilers and experimental design

One-day-old Arbor Acres chicks (initial body weight; 44 ± 2 g) were obtained from a commercial hatchery in Anhui, and kept in cages with wood shaving litter floor reared under routine commercial management practices. At 28 days of age, sixty chickens were randomly divided into two groups (30 chickens/group), with 6 replicates per group, and 5 birds per replicate. The two groups were: the control group, kept at normal temperature conditions (25 ± 2 °C; 24 h/day for 1 week or 2 weeks; 42–66% RH); the heat stress group, exposed daily to high ambient temperature (36 ± 2 °C; 8 h/day, other time returned back to the conventional conditions; 33–38% RH) to mimic an environmental heat wave in an environmentally-controlled chamber. The heat exposure protocol was conducted for 1 week (from 28 to 35 days) and 2 weeks (from 28 to 42 days). The 25 °C is considered as a normal temperature for chickens at this age (Alhenaky et al., 2017). The chickens were kept under constant light throughout the experiment, with feed and water being provided ad-libitum. Ingredients and chemical composition of the basal diet are presented in Table 1. The models of heat exposure were created to simulation the heat waves that occur in nature.

2.3. Growth performance

Individual body weight (BW) was recorded at 28, 35 and 42 days of age. The feed intakes and feed conversion ratio (g feed/g gain, FCR) were calculated at weekly intervals from 28 to 42 days. The residual feed in the troughs was collected and weighed, and feed consumption was calculated by subtracting weekly residual feed from the offered feed. The average daily feed intake (ADFI), average daily gain (ADG), and FCR were calculated in the experiment.

2.4. Blood sampling

Blood was respectively collected twice for the control group and

Table 1
Ingredients and composition of the basal diets (as-fed basis, %, unless otherwise indicated).

Item	Starter diet (0–21 d)	Grower diet (22–42 d)
Ingredient		
Corn	62.5	65.5
Soybean meal	21.0	18.0
Bran	3.0	3.0
Fish meal	3.0	3.0
Corn protein powder	5.0	5.0
Yeast powder	2.0	2.0
$CaHPO_4$	1.2	1.2
Limestone powder	1.0	1.0
NaCl	0.3	0.3
Premix[a]	1.0	1.0
Total	100	100
Nutritional level		
Metabolisable energy (MJ/kg)	12.57	13.28
Crude protein	22.03	20.19
Coarse fiber	4.29	5.94
Calcium	1.10	0.96
Available phosphorus	0.45	0.38
Methionine	0.50	0.45
Lysine	1.20	1.05

[a] Premix provided per kg of diet: vitamin A 8000 IU, VD3 1228 IU, VE 15 IU, VK3 3.0 mg, VB1 1.3 mg, VB_2 3.1 mg, VB_6 1.2 mg, calcium pantothenate 13.4 mg, choline chloride 500 mg, biotin 0.11 mg, niacin 25 mg, folic acid 0.68 mg, VB_{12} 0.03 mg, Fe 120 mg, Cu 10 mg, Zn 130 mg, Mn 100 mg, I 0.3 mg, Se 0.3 mg.

heat stress group at day 35 and day 42 of the experiment. A total of 30 blood samples were collected from 30 birds, 15 from each treatment, every time. Blood samples were collected from the jugular vein in tubes and immediately centrifuged at 3000 rpm for 15 min to obtain the serum. The serum was further used to analyze the corticosterone hormone and the indices of oxidative stress.

2.5. Serum corticosterone determination

Corticosterone concentration in the serum was measured using an enzyme-linked immunoassay commercial ELISA kit (Corticosterone ELISA kit; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the procedure was followed as provided by the supplier.

2.6. Indices of oxidative stress determination

After 1 and 2 weeks of heat stress treatment, total antioxidant capacity (T-AOC), the activities of the glutathione reductase (GR), glutathione per-oxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), and the contents of the glutathione (GSH) and malondialdehyde (MDA) were determined using corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer as previously described (Zhang et al., 2017).

2.7. Tissue sampling

Fifteen broilers per treatment (3 replicates per group, and 5 birds per replicate) at the end of the 1st or 2nd week of heat exposure were respectively euthanized in the control and heat stress groups, and the skeletal muscle (pectorales and crureus), the small intestine (duodenum, jejunum and ileum) and immune organs (spleen and thymus) were respectively collected, and divided into three parts. The first part of each tissue sample was fixed with 4% paraformaldehyde (PFA) and further used for the immunohistochemistry analysis. The second part was immediately frozen in liquid nitrogen and then stored at −80 °C for RNA isolation extract. The third part was immediately collected, then homogenized and used to isolate mitochondria, then to determine ROS production.

2.8. Immunohistochemistry analysis

After the tissues were fixed, they were routinely dehydrated and embedded in paraffin, and serially sliced to 5 μm thick slices on a microtome (Felles Instruments Inc., Shanghai, China). Immunohistochemistry analysis was performed according to previously described methods (Yeboah et al., 2008) and slightly modified, in brief, sections were deparaffinised in 100% xylene (Sinopharm Chemical Reagent Co. LTD, Shanghai, China) and rehydrated in a graded series of ethanol (Xiangruixin Chemical Technology Co. LTD, Tianjin, China). After being washed twice with phosphate buffer saline (PBS) for 10 min each time, the sections were placed in a microwave at high power (maximum output 1100 W) for 1 min then at 30% power for 10 min in 10 mM citrate buffer solution (Sigma-Aldrich, St Louis, MO, USA; pH = 6.0) to unmask antibody binding sites. After being cooled for 20 min, the slides were washed respectively twice with water and PBS for 5 min each, then incubated to block endogenous peroxidase activity of tissues in endogenous enzyme blocking solution (Qingdao Jisskang biotechnology Co., Ltd, Qingdao, China) for 30 min. After being washed twice with PBS for 10 min each time, the slides were incubated in 2.0% goat serum (Gibco, Carlsbad, CA, USA) in PBS for 10 min to block non-specific binding sites. Then the sections were incubated in rabbit anti-ABCG2 primary polyclonal antibodies (1:100 dilution; Bioss Biological Technology, Beijing, China) for 18 h at room temperature. After being rinsed two times with PBS, the slides were incubated for 30 min with goat anti-rabbit IgG-biotinylated secondary antibodies (1:100 dilution; Bioss Biological Technology, Beijing, China). After being rinsed one time with PBS, the slides were incubated with streptavidin-horse-radish peroxidase (HRP) 30 min. Before being incubated with diaminobenzidine (DAB) in DAB buffer for 8 min, the slides were respectively rinsed one time with PBS and deionized water. The immunohistochemical staining method and procedure were carried out strictly according to the manufacturer's instructions, and PBS was used instead of the primary antibody as the negative controls. Slides were counterstained with haemotoxylin stain (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), dehydrated in ethanol, cleared in xylene compound and mounted with permount (Suyi Chemical Reagent Co., Ltd, Shanghai, China). All the images were photographed using a fluorescence microscope and (Olympus, Japan). The cumulative optical density (IOD) of the positive portion in the images was measured using Image J software analysis, and the high IDO value indicates increased ABCG2 expression.

2.9. Quantitative real-time polymerase chain reaction analysis

Total RNA were respectively extracted from the pectorals, crureus, duodenum, jejunum, ileum, spleen and thymus using OMEGA Total RNA kitII(SPECTRIS CO, Egham, Surrey, UK) according to the manufacturer's instructions. Reverse transcription was performed using the Takara Prime Script RT reagent Kit (Takara Bio, Kusatsu, Shiga, Japan) following the instructions of the manufacturer. The cDNA was diluted ten times with sterile water and stored at −30 °C until gene analysis. The relative levels of ABCG2 mRNA were determined using an ABI Step-One Plus real-time PCR system (Applied Biosystems, Foster, CA, USA). Reactions were performed in a 10 μL mixture including 1 μL of diluted cDNA template, 0.6 μL of the forward and reverse primers, 5 μL of SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany), and 3.4 μL of ddH_2O. The PCR protocol was 95 °C for 10 min, followed by 40 amplification cycles of denaturation at 94 °C for 5 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s. The melting curve showed a single peak for each PCR product. Relative mRNA level of ABCG2 was calculated according to $2^{-\triangle\triangle}$CT method (Liu et al., 2012). The target ABCG2 and housekeeping gene β-actin were designed as previously described (Guo et al., 2016; Bai et al., 2017). The PCR primers for target and housekeeping (β-actin) genes are showed in Table 2.

2.10. Isolation of mitochondria and H_2O_2 production assay

After the birds in the control and heat stress groups were killed respectively at the end of the 1st week and 2nd week of heat exposure, fresh skeletal muscle, small intestine and immune organs were

Table 2
Gene-special primers for ABCG2 and β-actin used in the qPCR.

Gene	Primer sequence (5′→3′)	Tm (°C)	Product size (bp)
ABCG2	Forward: ATTTTCATTGCTCGCTTCTTT Reverse: GACAGTCTGACATTACTAGCTTTGG	60	216
β-actin	Forward: AGACATCAGGGTGTGATGGTTGGT Reverse: TCCCAGTTGGTGACAATACCGTGT	60	125

immediately collected, then homogenized to separate several kinds of tissue mitochondria using the tissue mitochondrial isolation kit produced by Shanghai Beyotime biotechnology Co., Ltd (Shanghai, China) following the manufacturer's instructions as described previously (Chen et al., 2016). All procedures were carried out at 4 °C or on ice to minimize mitochondrial injury.

Assay of mitochondrial H_2O_2 generation rates was referred to the previously described method (Azad et al., 2010). H_2O_2 generation rates were determined fluorometrically by measurement of oxidation of 10-acetyl-3,7-dihydroxyphe -noxazine coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase. Mitochondria (0.35 mg protein/mL) were incubated in a standard incubation buffer (80 mM KCl, 5 mM K_2HPO_4, 50 mM HEPES, 1 mM EGTA, 5 mM $MgCl_2$, and 0.1% (wt./vol) BSA, pH 7.2, at 38 °C) containing 50 μM amplex red, 6 U/mL horseradish peroxidase and 30 U/mL superoxide dismutase. After addition of 4 mM succinate, the H_2O_2 generation rates were determined by computer-controlled spectrofluorimeter (RF-5301 PC, Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 539 and 590 nm, respectively. The assay was carried out with appropriate correction for background and use of a standard curve.

2.11. Statistical analysis

All data analyses were performed using Statistical Package for the Social Sciences (SPSS) software (version 19; SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SEM from 3 replicates. Statistical comparisons were performed using one-way ANOVA and verified by the post-hoc Newman-Keuls test. A difference with value $P < 0.05$ was considered statistically significant.

3. Results

3.1. Growth performance

Table 3 showed after 1 week of heat stress, compared with the control birds, BW, ADG and ADFI of birds were lower ($P < 0.05$), while FCR in heat stress group was higher ($P < 0.05$). After 2 weeks of heat stress, there was no significant difference ($P > 0.05$) in FCR, ADG and ADFI. However, final body weight in heat stress group was significantly lower than those of the control group ($P < 0.05$). The results indicated that heat stress affects broiler performance in a time-dependent manner and the reduced growth performance of birds mainly occurs in the early stage of heat stress.

Table 3
The effects of duration of thermal stress on broiler performance.

Item [a]	Control group	Heat stress group
Initial BW (g), day 28	1317.44 ± 92.38	1308.53 ± 91.98
BW (g), day 35	1784.17 ± 83.26[a]	1592.87 ± 71.23[b]
Final BW (g), day 42	2272.11 ± 67.38[a]	2079.46 ± 77.29[b]
28–35 d		
ADFI (g/bird)	139.29 ± 56.15[a]	114.51 ± 32.33[b]
ADG (g/bird)	66.69 ± 0.60[a]	40.62 ± 0.97[b]
FCR (g/g)	2.08 ± 0.43[a]	2.81 ± 0.64[b]
36–42 d		
ADFI (g/bird)	136.52 ± 40.24	139.60 ± 37.06
ADG (g/bird)	69.68 ± 0.93	69.52 ± 0.60
FCR (g/g)	1.96 ± 0.51	2.01 ± 0.47

Data are expressed as mean ± SEM, n = 3. In the same row, values with different superscripts are significantly different ($P < 0.05$), while with same superscripts were insignificantly different ($P > 0.05$).

[a] Body weight (BW); Average daily feed intake (ADFI); Average daily gain (ADG); Feed conversion ratio (FCR).

Table 4
The changes in serum corticosterone levels and oxidative stress indices for broilers under different heat stress weeks.

Item[a]	Control group	Heat stress group
Day 35		
Corticosterone (ng/mL)	7.43 ± 0.84[a]	10.33 ± 0.64[b]
T-AOC (U/mL)	21.34 ± 0.42[a]	15.13 ± 0.57[b]
GR (U/L)	33.42 ± 0.44[a]	17.48 ± 0.57[b]
GSH-Px (U/mL)	237.56 ± 1.83[a]	210.32 ± 1.87[b]
SOD (U/mL)	14.76 ± 0.73[a]	11.22 ± 0.67[b]
CAT (U/mL)	7.28 ± 0.34[a]	8.16 ± 0.40[a]
GSH (μmol/L)	9.82 ± 0.47[a]	6.57 ± 0.62[b]
MDA (nmol/mL)	7.62 ± 0.47[a]	11.72 ± 0.56[b]
Day 42		
Corticosterone (ng/mL)	8.24 ± 0.12[a]	9.75 ± 0.48[a]
T-AOC (U/mL)	19.44 ± 0.51[a]	26.48 ± 0.76[b]
GR (U/L)	28.79 ± 0.66[a]	36.25 ± 0.53[b]
GSH-Px (U/mL)	207.81 ± 0.93[a]	233.76 ± 0.68[b]
SOD (U/mL)	12.49 ± 0.61[a]	16.32 ± 0.42[b]
CAT (U/mL)	8.37 ± 0.24[a]	11.56 ± 0.62[b]
GSH (μ mol/L)	11.42 ± 0.72[a]	10.53 ± 0.43[a]
MDA (nmol/mL)	8.69 ± 0.84[a]	9.12 ± 0.69[a]

Data are expressed as mean ± SEM, n = 3. Values in the same row with different superscripts were significantly different ($P < 0.05$), while with same superscripts were insignificantly different ($P > 0.05$).

[a] Total antioxidant capacity (T-AOC); Glutathione reductase (GR); Glutathione peroxidase (GSH-Px); Superoxide dismutase (SOD); Catalase (CAT); Glutathione (GSH); Methane dicarboxylic aldehyde (MDA).

3.2. Serum corticosterone levels and indices of oxidative stress

As shown in Table 4, after 1 week of heat exposure, the birds had higher serum corticosterone levels and the contents of MDA in heat stress group compared with the control group ($P < 0.05$); The activities of T-AOC, GR, GSH-Px and SOD and contents of GSH in heat stress group were significantly lower ($P < 0.05$) than those in the control. Moreover, the CAT activity had no significant difference between the heat stress and the control groups ($P > 0.05$). After 2 weeks of heat stress, serum corticosterone levels and the contents of MDA and GSH in heat stress group had no significant difference ($P > 0.05$), however, the activities of T-AOC, GR, GSH-Px, CAT and SOD in heat stress group significantly increased in compared with the control ($P > 0.05$). The results indicated that the antioxidant enzyme activity decreases in the initial stage of heat exposure, while broilers gradually produces a compensatory changes for indices of oxidative stress with duration of heat stress.

3.3. Immunohistochemical analysis of ABCG2 protein in pectorales and crureus

To reveal effect of duration of heat stress on ABCG2 protein in skeletal muscle, the localization and expression of ABCG2 in pectorales and crureus were analyzed by immunohistochemistry after 1 and 2 weeks of heat stress. As shown in Fig. 1A, ABCG2 protein localized on the myolemma and perimysium regardless of skeletal muscle type and duration of heat stress. However, IOD of the ABCG2 positive portion changed in a time-dependent manner. After 1 week of heat stress, the IOD of the ABCG2 positive portion had no significant difference in the pectorales and crureus between the control group and heat stress group (Fig. 1B and C; $P > 0.05$). However, the IOD of the ABCG2 positive portion for the pectorales ($P < 0.01$) and crureus ($P < 0.05$) in heat stress group were respectively higher than those of the control group after 2 weeks of heat stress (Fig. 1B and C). The results indicated that duration of heat stress does not influence the localization of ABCG2 in skeletal muscle, but changes the expression of ABCG2 in a time-dependent manner.

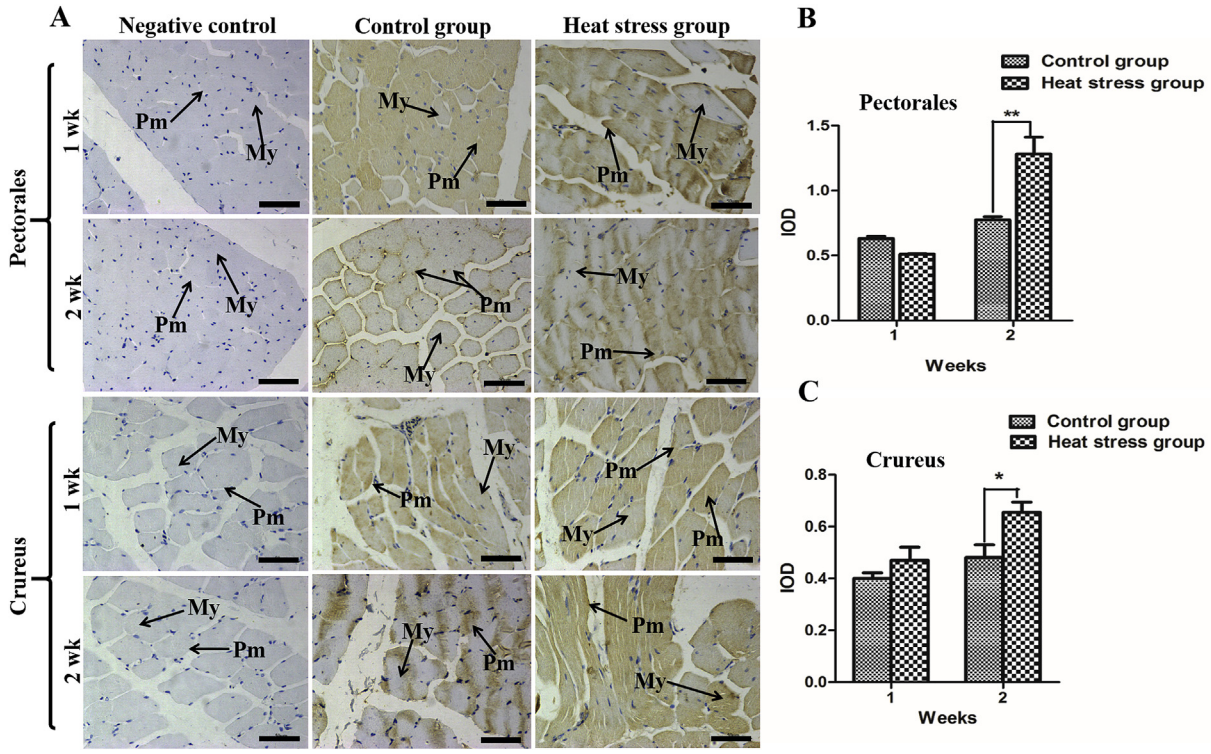

Fig. 1. Localisation and expression of ABCG2 in skeletal muscle of broilers under heat stress. A, Immunohistochemical representative images of ABCG2 in the pectorales and crureus of broiler under different heat stress weeks. ABCG2 is present in the myolemma (My) and perimysium (Pm). Immunohistochemical slices were observed under the microscope; the nuclei were stained by hematoxylin. B, The IOD of the ABCG2 positive portion from broiler pectorals (n = 15). C, The IOD of the ABCG2 positive portion from broiler crureus (n = 15). *, P < 0.05. **, P < 0.01. Error bars, SEM. The amount of positive reaction is represented by the shade of yellow stained in the images. The cumulative optical density (IOD) of the positive portion in the images was measured using Image J software analysis, and the high IDO value indicates increased ABCG2 expression. Scale bars represent 50 μm.

3.4. Immunohistochemical analysis of ABCG2 protein in duodenum, jejunum and ileum

To examine effect of duration of heat stress on ABCG2 protein in the small intestine, the localization and expression of ABCG2 in duodenum, jejunum and ileum were analyzed. The ABCG2 protein was mainly present in the apical membranes and basolateral membranes of the duodenum, jejunum and ileum epithelial cells regardless of the position of small intestine and duration of heat stress (Fig. 2A). Compared with the control group, there was all no significant difference in the IOD of the ABCG2 positive portion for the duodenum, jejunum and ileum in heat stress group after 1 week of heat stress (Fig. 2B, C and D; P > 0.05). However, the IOD of the ABCG2 positive portion of the duodenum (P < 0.01), jejunum (P < 0.01) and ileum (P < 0.05) in heat stress group was significantly higher than that in the control group after 2 weeks of heat stress (Fig. 2B, C and D). The data showed that duration of heat stress does not influence the localization of ABCG2 in the small intestine, but changes the expression of ABCG2 in a time-dependent manner.

3.5. Immunohistochemical analysis of ABCG2 protein in thymus and spleen

The localization and expression of ABCG2 in thymus and spleen under heat stress were also analyzed using immunohistochemistry. The results showed that ABCG2 is mainly enriched in the cortex of thymus and white myeloid of spleen regardless of duration of heat stress (Fig. 3A). Compared with the control group, the IOD of thymus ABCG2 positive portion in heat stress group was significantly increased after 1 week (P < 0.05) or 2 weeks (P < 0.01) of heat stress (Fig. 3B). However, whether 1 week or 2 weeks, there was no significant difference in the IOD of spleen ABCG2 positive portion between the control group and heat stress group (Fig. 3C; *P* > 0.05). The results confirmed that duration of heat stress does not influence the localization of ABCG2 in thymus or spleen, but thymus are more sensitive than spleen to express ABCG2 under heat stress.

3.6. Expression of ABCG2 mRNA and mitochondria ROS production in pectorales and crureus of broilers under heat stress

To further verify the association between expression of ABCG2 mRNA and the mitochondria ROS production in the skeletal muscles of broilers under heat stress, qPCR and mitochondria H_2O_2 production were tested. As shown in Fig. 4A and B, the results showed that the expression of the ABCG2 mRNA in heat stress group significantly increased compared to the control group in pectorals (Fig. 4A; P < 0.01) and crureus (Fig. 4B; P < 0.05) at the end of the 2nd week of heat stress; However, there was no significant difference at the end of the 1st week of heat stress (Fig. 4A and B; P > 0.05). The mitochondria ROS production in heat stress group were higher than those of the control group from pectorales (Fig. 4C; P < 0.01) and crureus (Fig. 4D; P < 0.01) after 1 week of heat stress, while after 2 weeks of heat stress, it had no significant differences between the control and heat stress groups (Fig. 4C and D; P > 0.05). These findings indicated that the expression of the ABCG2 mRNA is negatively correlated with mitochondria ROS production in pectorales and crureus of broilers. Due to low expression of the ABCG2, the skeletal muscle are more vulnerable to ROS damage after 1 week of heat stress.

3.7. Expression of ABCG2 mRNA and mitochondria ROS production in duodenum, jejunum and ileum of broilers under heat stress

To analyze the correlation between expression of the ABCG2 mRNA

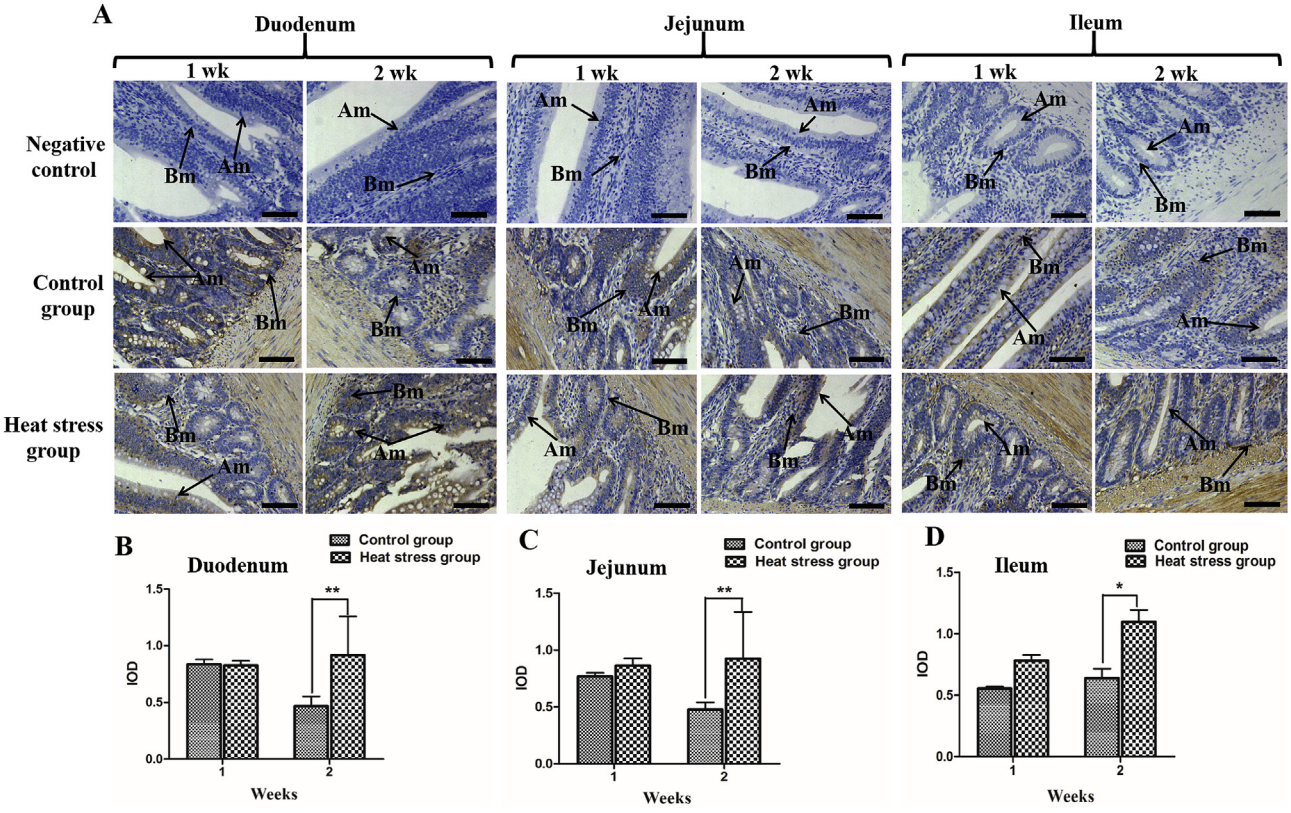

Fig. 2. Localisation and expression of ABCG2 in the small intestine of broilers under heat stress. A, Immunohistochemical representative images of ABCG2 in the duodenum, jejunum and ileum of broilers under different heat stress weeks. ABCG2 is present in the apical membranes (Am) and basolateral membranes (Bm) of the duodenum, jejunum and ileum cells. Immunohistochemical slices were observed under the microscope; the nuclei were stained by hematoxylin. B, The IOD of the ABCG2 positive portion in broiler duodenum (n = 15). C, The IOD of the ABCG2 positive portion in broiler jejunum (n = 15). D, The IOD of the ABCG2 positive portion in broiler ileum (n = 15). *, P < 0.05. **, P < 0.01. Error bars, SEM. The amount of positive reaction is represented by the shade of yellow stained in the images. The cumulative optical density (IOD) of the positive portion in the images was measured using Image J software analysis, and the high IDO value indicates increased ABCG2 expression. Scale bars = 50 μm.

and mitochondria ROS production in small intestine under heat stress, expression of the ABCG2 mRNA and mitochondria ROS production in duodenum, jejunum and ileum were studied. The expression of ABCG2 mRNA in heat stress group significantly increased compared to the control group in duodenum and jejunum (Fig. 5A and B; P < 0.01), but it had no significant difference in ileum after 2 weeks of heat stress (Fig. 5C; P > 0.05); Moreover, after 1 week of heat stress, there were all no differences for duodenum, jejunum and ileum between the control group and heat stress group (Fig. 5A, B and C; P > 0.05). Compared with the control group, mitochondria ROS production of duodenum (Fig. 5D; P < 0.01), jejunum (Fig. 5E; P < 0.01) and ileum (Fig. 5F; P < 0.01) in heat stress group were higher after 1 week of heat stress, however, when heat exposure continues to the end of 2 weeks, no significant difference existed for the mitochondria ROS production of duodenum, jejunum and ileum between the control and heat stress groups (Fig. 5D, E and F; P > 0.05). It's similar to the results of skeletal muscle, and the expression of ABCG2 is negatively correlated with mitochondria ROS production.

3.8. Expression of ABCG2 mRNA and mitochondria ROS production in thymus and spleen of broilers under heat stress

To confirm the correlation between expression of ABCG2 mRNA and mitochondria ROS production in immune organs under heat stress, the expression of the ABCG2 mRNA and the mitochondria ROS production in thymus and spleen were tested. As shown in Fig. 6A, the expression of ABCG2 gene in the thymus were significantly higher in heat stress group compared to the control group at the end of 1st week ($P < 0.01$) and 2nd week (P < 0.05) of heat stress, while significant difference was not observed for the mitochondria ROS production between the control and heat stress groups (Fig. 6C; P > 0.05). In contrast, the heat stress has no significant effect on the expression of the ABCG2 mRNA in spleen regardless of duration of heat stress (Fig. 6B; P > 0.05), however, mitochondria ROS production in heat stress group were higher than the control group after 1 week (P < 0.05) and 2 weeks (P < 0.01) of heat stress (Fig. 6D). These results indicated that there is a correlation between the expression of the ABCG2 mRNA and mitochondria ROS production in immune organs.

4. Discussions

In the current study, broiler chicken were exposed at 36 °C for 8 h per day and lasted for 1 week or 2 weeks to mimic a heat wave. This yields a rectal temperature of 43.2 °C within 2 h of the onset of heat stress. This body temperature can induce a heat stress response in chicken (Jastrebski et al., 2017). In the course of our experiment, broiler chicken showed a typical heat stress response in the early stages of thermal exposure, such as increased panting, lethargy, wing spreading and increased water consumption at this temperature. The results are consistent with those reported by Jastrebski et al. (2017). In general, broilers have the most rapid growth and more sensitive gene expression to high ambient temperature from 21 days to 42 days of age (Roushdy et al., 2018), hence, the broilers from 28 days to 42 days of age were selected as the research object in our study. Moreover, to exclude the effect of nutritional status on ABCG2 expression, a pair fed group was once set up in our preliminary test, but there was no

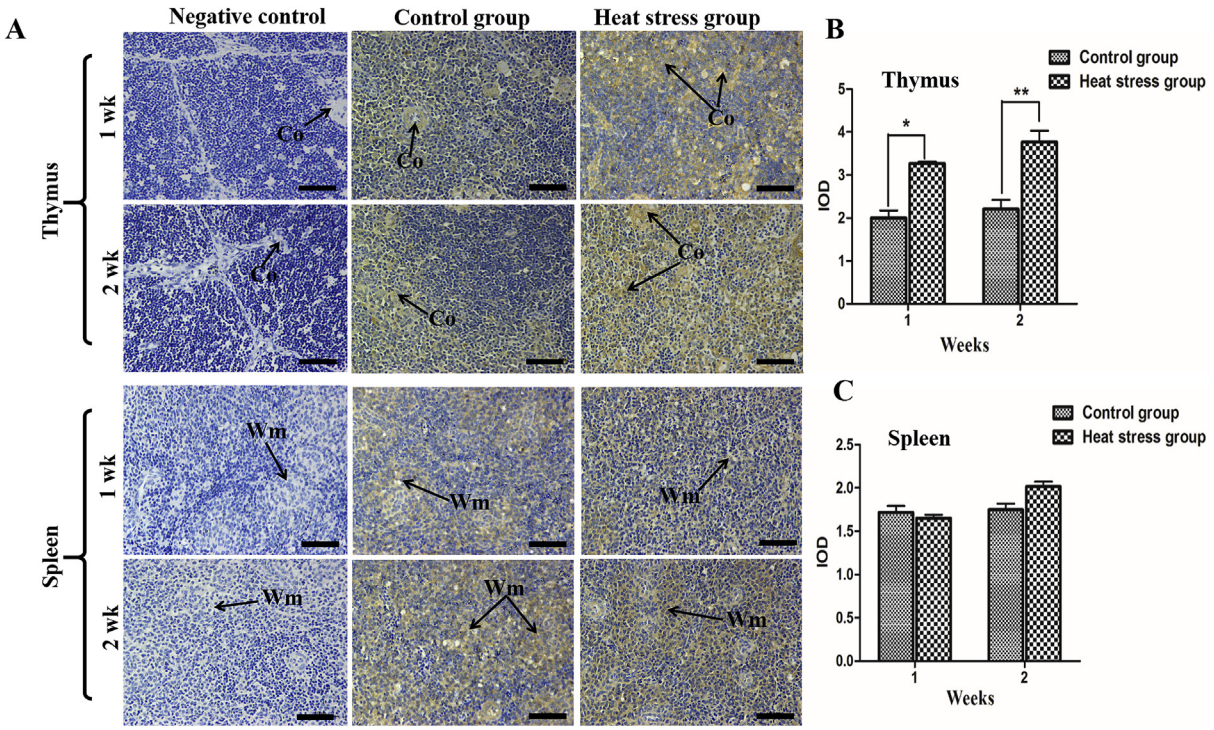

Fig. 3. Iocalisation and expression of ABCG2 in the immune organs of broilers under heat stress. A, Immunohistochemical representative images of ABCG2 in the thymus and spleen of broilers under different heat stress weeks. ABCG2 was present in the cortex (Co) of thymic and white myeloid (Wm) of the spleen. Immunohistochemical images were observed under the microscope; the nuclei were stained by hematoxylin. B, The IOD of the ABCG2 positive portion from broiler thymus (n = 15). C, The IOD of the ABCG2 positive portion from broiler spleen (n = 15). *, P < 0.05. **, P < 0.01. Error bars, SEM. The amount of positive reaction is represented by the shade of yellow stained in the images. The cumulative optical density (IOD) of the positive portion in the images was measured using Image J software analysis, and the high IDO value indicates increased ABCG2 expression. Scale bars = 50 μm.

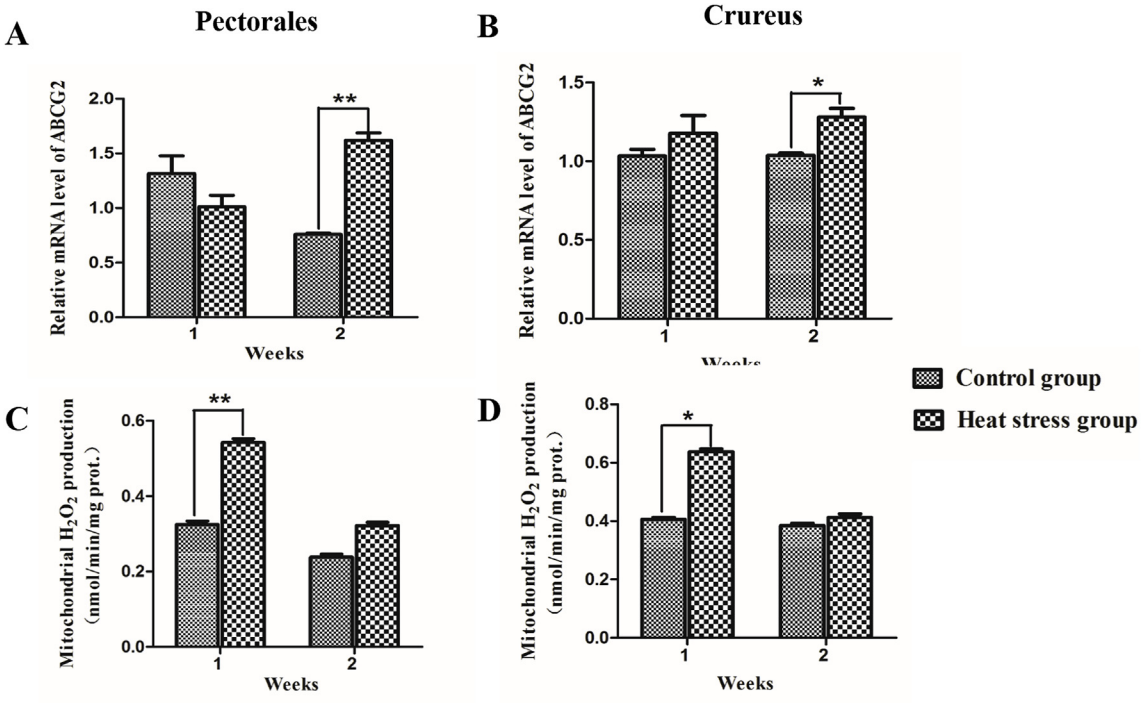

Fig. 4. The expression of the ABCG2 mRNA and mitochondrial ROS (measured as H_2O_2) production in skeletal muscle of broilers under different heat stress weeks. A and B show effects of duration of thermal stress on the expression of the ABCG2 mRNA in pectorals and crureus, respectively. C and D show mitochondrial ROS production in pectorals and crureus, respectively. *, P < 0.05. **, P < 0.01. Error bars, SEM.

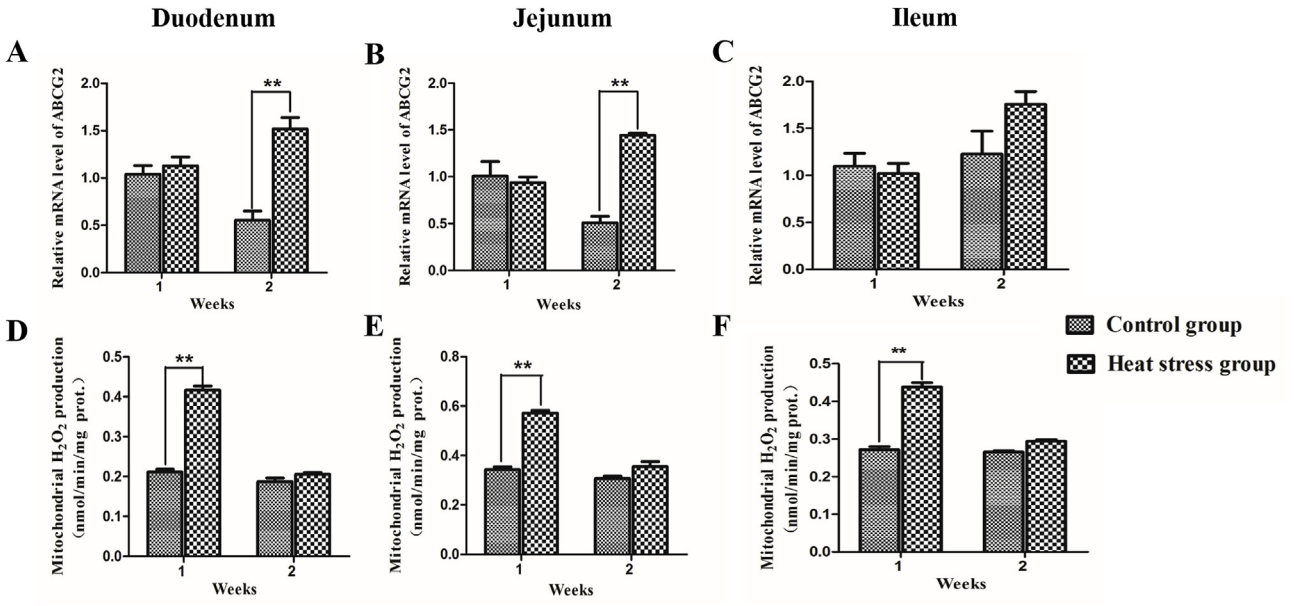

Fig. 5. The expression of the ABCG2 mRNA and mitochondrial ROS (measured as H_2O_2) production in the small intestine under different heat stress weeks. A, B and C indicate that effects of duration of thermal stress on the expression of the ABCG2 mRNA in duodenum, jejunum and ileum, respectively. D, E and F show mitochondrial ROS production of duodenum, jejunum and ileum, respectively. *, $P < 0.05$. **, $P < 0.01$. Error bars, SEM.

significant difference between the control and the paired groups for the growth performance and indicators of oxidative stress in serum. This indicated that the changes of function in broilers is only caused by heat stress. Therefore, the heat stress and the control groups were designed referring to previous studies in this study (Mujahid et al., 2007, 2009; Alhenaky et al., 2017; Jastrebski et al., 2017; Roushdy et al., 2018). Previous researchers reported that heat stress hindered the growth performance of birds by decreasing feed intake and body weight gain (Alhenaky et al., 2017; Roushdy et al., 2018); Moreover, strain, categories and duration of thermal stress can also induce the changes in chicken growth performance (Roushdy et al., 2018; Zaglool et al., 2019). Our present study showed that duration of thermal stress produces a different effect on broiler performance. At the end of the 1st week of heat stress, broiler BW reduced due to reductions in both feed consumption and the efficiency of feed utilization. However, the performance changes were no longer observed at the end of the 2nd week of heat stress, possibly because the animals had acclimatized to environmental heat stress. An increase in serum corticosterone levels is one of the most important results of the activation of the stress system (Alhenaky et al., 2017). In the study the heat stress increased serum

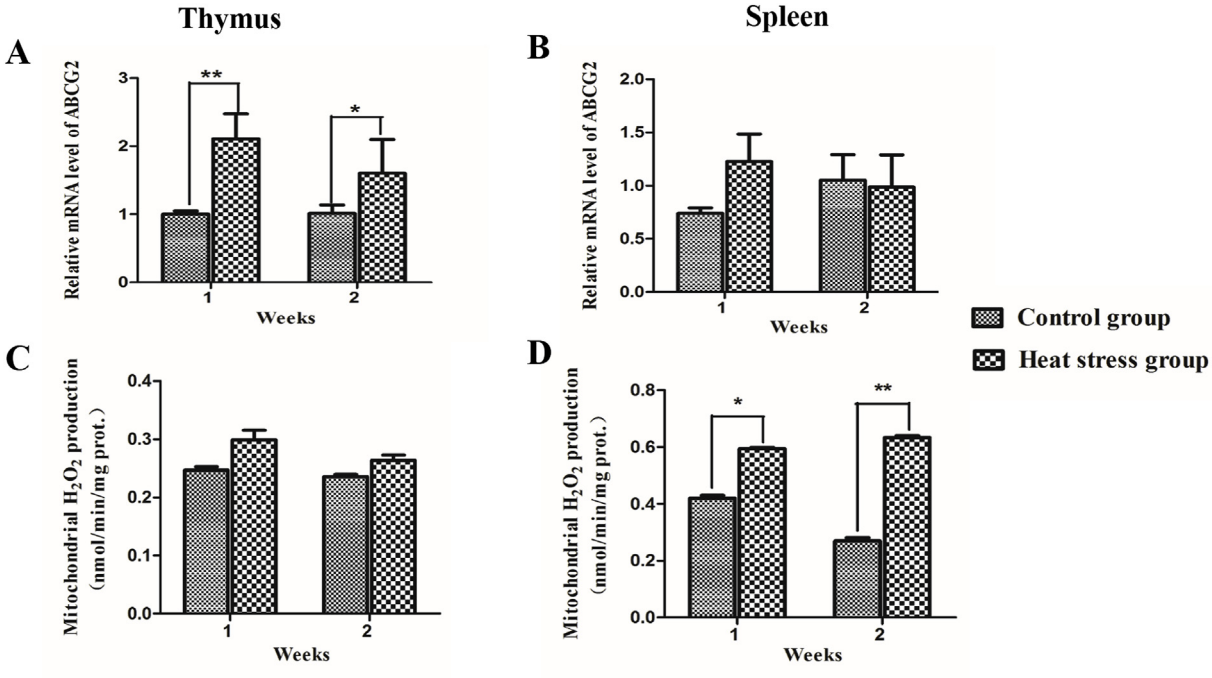

Fig. 6. The expression of the ABCG2 mRNA and mitochondrial ROS (measured as H_2O_2) production in the immune organs under different heat stress weeks. A and B indicate effects of duration of thermal stress on the expression of ABCG2 mRNA in thymus and spleen, respectively. C and D show mitochondrial ROS production of thymus and spleen, respectively.*, $P < 0.05$. **, $P < 0.01$. Error bars, SEM.

corticosterone levels at the end of the 1st week; however, when the heat stress lasted up to the end of the 2nd week, no significant change was reported. In the current experiment, the changes in growth performance of heat-stressed broiler had a correlation with the corticosterone levels. Previous studies reported similar results that increased corticosterone levels caused a decrease in both feed intake and growth performance (Quinteiro-Filho et al., 2012; Alhenaky et al., 2017). Moreover, the oxidative stress damage in broilers induced by high ambient temperature has been reported in some studies (Lin et al., 2006; Mujahid et al., 2007, 2009; Attia et al., 2016; Alhenaky et al., 2017; Maibam et al., 2018), and even thermal stress affects poultry performance mainly by inducing the oxidative stress (Zhang et al., 2012). In the current trial, we detected the effect of heat stress on oxidative stress factors in the serum. The results showed that heat stress significantly reduced the activity of T-AOC, GR, GSH-Px and SOD and contents of GSH after 1 week, in contrast, after 2 weeks of heat exposure, the indices of oxidative stress manifested as a compensatory increase, while the contents of MDA and GSH gradually reverted to normal levels. With duration of heat exposure, the increased activity of the antioxidant enzyme possibly acclimates the organism to continuous thermal challenge in broilers.

Existing research has demonstrated that hyperthermia could induce oxidative stress and is associated with augmented production of cellular ROS and oxidative damage to skeletal muscle (Mujahid et al., 2007, 2009; Ganesan et al., 2017a,b; Ganesan et al., 2018a,b), small intestine (Alhenaky et al., 2017; Yi et al., 2017; Zhang et al., 2017; Al-Zghoul et al., 2019) and immune organ (Ghazi et al., 2012). The skeletal muscle is considered one of the main factors determining the growth rate of animals (Habashy et al., 2017). Some studies have also shown that the intestinal tract is also affected one of the primary organs by heatstroke (Yi et al., 2017). Heat stress induces a subsequent reduction of the intestinal absorption and gastrointestinal injuries (Söderholm and Perdue, 2001; Burkholder et al., 2008). Moreover, spleen and thymus are significant immunological organs and participate in the cellular and humoral immunity (Roushdy et al., 2018). Hence, we selected skeletal muscle, small intestine and immune organs as the target tissues and focused on the mechanism of change of ROS production for heat-stressed birds.

ABCG2 often serves as an important marker for a variety of stem cells in oxidative stress (Ding et al., 2010; Hu et al., 2017) and is capable of protecting cells from ROS-mediated cell damage (Shen et al., 2010; Nie et al., 2018). Our study found that the expression of ABCG2 changed with the extension of heat stress time and various organs in broilers. First, we examined the localization of ABCG2 in skeletal muscle, small intestine and immune organs of broilers with or without heat stress treatment. The results showed that ABCG2 protein was mainly enriched in the myolemma and perimysium of pectorales and crureus, the apical membrane and basolateral membrane of intestinal epithelial cells, as well as the thymic cortex and spleen white myeloid cells regardless of duration of heat stress. These results are the same as previous studies on the distribution of ABCG2 protein (Sarkadi et al., 2004). At the same time, the IOD of the ABCG2 positive portion has no significant effect on pectorales, crureus, duodenum, jejunum, ileum and spleen expect for the thymus at the end of the 1st week of heat stress, but at the end of the 2nd week of heat stress, expect for spleen, there were significant differences in other tissues between the control and heat stress groups. Hence, our results showed that duration of heat stress does not influence the location of ABCG2, but changes expression of ABCG2 in skeletal muscle, small intestine and immune organs in a time-dependent manner.

In the study, after 1 week of heat stress, the expression of ABCG2 mRNA was all no significant difference with or without heat exposure in skeletal muscle, small intestine. On the contrary, mitochondria ROS production of corresponding tissue increased for heat stress group compared to the control group. However, with significant increase of the ABCG2 mRNA expression for pectorales, crureus, duodenum, jejunum and ileum in heat stress group, mitochondria ROS production for corresponding tissues have no significant difference after 2 weeks of heat stress. Moreover, regardless of duration of heat stress, the expression of ABCG2 mRNA in the thymus has a significant increase, while in the spleen, there is no significant difference between the heat stress and control groups, but mitochondria ROS production for corresponding tissues shows the opposite effects. So far, the effects of animal heat stress on ABCG2 expression and the relevant mechanism have not been reported. In this study, we demonstrated for the first time that a hyperthermia regulates the expression of ABCG2 in a time-dependent manner and then changes cellular ROS production to regulate the oxidative stress state of vital tissues. Previous studies have also found that ABCG2 can reduce the production of cellular ROS and intracellular inflammatory responses by excreting harmful substances from the cells, and increase the antioxidant capacity of cells under oxidative stress conditions (Shen et al., 2010; Nie et al., 2018), meanwhile, recent studies have shown that ABCG2 expression downregulated in the cancer cells treated by hyperthermia, but the ROS production increased (Kurokawa et al., 2019). Our results are similar to the previous studies, and confirmed that the expression of the ABCG2 was negatively correlated with corresponding tissue mitochondria ROS production. Hence, our results demonstrated that duration of heat stress affects ABCG2 expression and tissue mitochondria ROS production, serum corticosterone levels and indices of oxidative stress in a time-dependent manner, in turn, influences growth performance of broiler. Moreover, we proposed that ABCG2 protects the tissues from heat-induced oxidative stress by reducing intracellular ROS or exuding other harmful substances under heat stress. However, more systematic and in-depth research is needed in the future to articulate the anti-injury mechanism of ABCG2 in heat stress. So our findings may provide a new way to alleviate heat stress using ABCG2 activator in poultry production.

5. Conclusion

The present study demonstrated that duration of thermal stress affects growth performance, antioxidant enzyme activities, serum corticosterone levels and the contents of MDA in a time-dependent manner. The increased ABCG2 expression can inhibit the mitochondria ROS production, and relieves heat-induced oxidative stress damage in skeletal muscle, small intestinal and thymus to maintain stable growth performance of broiler chicken. The damage of heat stress to the body mainly occurs in an early stage of heat stress.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.102420.

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