Hyperthermia Affects Immune and Oxidative Stress Indices of Immune Organs of Broilers by Changing the Expressions of ABCG2, SVCT-2 and MCU

Xiuheng Xue
Anhui Agriculture University: Anhui Agricultural University
Chunhuan Ren
Anhui Agriculture University: Anhui Agricultural University
Luping Wang
Anhui Agriculture University: Anhui Agricultural University
Mengzhu Xu Xu
Anhui Agriculture University: Anhui Agricultural University
Caiyun Fan
Anhui Agriculture University: Anhui Agricultural University
Fugui Fang
Anhui Agriculture University: Anhui Agricultural University
Mengling Peng
Anhui Agriculture University: Anhui Agricultural University
Jianbo Cheng
Anhui Agriculture University: Anhui Agricultural University
Juhua Wang (wjhxxh@163.com)
Anhui Agriculture University: Anhui Agricultural University https://orcid.org/0000-0002-1620-760X

Research

Keywords: Broilers, Heat stress, Oxidative stress, Immune, ABCG2, SVCT-2, MCU

Posted Date: October 5th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-936596/v1

Abstract

Background: As global temperatures rise, heat stress has become one of the major environmental stressors in the poultry industry. The purpose of the study was to investigate the effects of heat stress on immune function and oxidative stress, and further reveal the possible mechanisms of oxidative stress induced by heat stress for thymus and spleen of broilers.

Methods: At the age of 28 days, thirty 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); the experience was lasted for 1 week. At the end of the experience, the broilers per group were respectively euthanized and collected some samples, then to be analyzed.

Results: The results showed that the levels of heat shock proteins 70 (HSP70,P 0.01), corticosterone (CORT,P 0.01), the contents of malondialdehyde (MDA, P 0.05), interleukin-6 (IL-6, P 0.01) and tumor necrosis factor-alpha (TNF-α, P 0.01) in serum were significantly higher in heat stress group than that in the control group; The activities of total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) and contents of glutathione (GSH) in heat stress group significantly reduced (P 0.05) in serum. Compared with the control group, the birds subjected to heat stress reduced the weight (P 0.01) and the indices of thymus (P 0.01), the activities of T-AOC (P 0.01) and SOD (P 0.05) of spleen, and levels of IL-10 (P 0.05) and the GSH-PX (P 0.05) in thymus and spleen, and increased the IL-6 content of thymus (P 0.05), the MDA content (P 0.01), and the reactive oxygen species (ROS) levels (P 0.01) in thymus and spleen. Moreover, the expression of immunoglobulin G (IgG) gene in thymus and spleen of heat stressed broiler significantly increased by reverse transcription-polymerase chain reaction (RT-PCR) and real time RT-PCR (qRT-PCR; P 0.05); However, the expression of immunoglobulin M (IgM) gene in spleen significantly increased (P 0.05), and had no significant difference (P 0.05) in thymus of heat-stressed broiler. Furthermore, the relative expression of ATP binding cassette subfamily G member 2 (ABCG2) in thymus and spleen (P 0.05), sodium dependent vitamin C transporter-2 (SVCT-2, P 0.01) and mitochondria calcium uniporter (MCU, P 0.01) mRNA in thymus of heat stressed broilers significantly increased; and the expression of ABCG2 (P 0.05), SVCT-2 (P 0.01) and MCU (P 0.01) protein of thymus and spleen in the heat-stressed broiler increased significantly compared with the control group.

Conclusions: In summary, the study confirmed that heat stress caused oxidative stress to immune organs of broilers, further reduced immune function. Moreover, the potential mechanisms of heat stress-induced oxidative stress for thymus and spleen was further reveal in broilers.

Introduction

The poultry industry is one of the largest among the industrie [1]. Heat stress has become one of the major environmental stressors in the poultry industry resulting in substantial economic loss due to global warming [2]. Heat stress causes increased mortality and reduced feed efficiency, body weight, feed intake

and immunity [3]. Immune system is one of the main targets of heat stress-induced negative effects on the organism [4]. In chickens, the thymus and spleen are significant immunological organs and are respectively the major central lymphoid organ and the peripheral immune organ [5], and both are severally participate in the cellular and humoral immunity [6]. Heat stress could cause immune organ dysfunction and apoptosis [7], and reduce the weight of chicken spleen and thymus [8, 9], then to inhibit their development by inducing the oxidative stress of organs in chickens [10–12]. The cause of heat-induced oxidative stress is mainly intracellular reactive oxygen species (ROS) production changes leading to the modification of the enzyme activity [13]. The hyperthermia could induce oxidative stress and is associated with augmented production of cellular ROS and oxidative damage to immune organ [8]. However, the mechanism of regulating ROS production in thymus and spleen of heat stressed broilers is not still fully understood.

ATP binding cassette (ABC) subfamily G member 2 (ABCG2) is a member of the ABC superfamily of proteins and plays an important role in drug resistance and maintaining cell homeostasis in the stress environment [14]. It has been found that ABCG2 is capable of protecting cells from ROS-mediated cell damage and death [15], and the downregulation of ABCG2 induces the overproduction of ROS to inhibit the production of antioxidants [16, 17]. Moreover, vitamin C (Vc)in cells can eliminate free radicals to alleviate oxidative stress [18]. Sodium dependent Vc transporter (SVCT) is a kind of protein that exists on the membrane and has the function of transporting Vc [19]. SVCT-2 mainly participates in the absorption of Vc to protect tissue from oxidative damage [20]. Thus, we proposed that ROS levels of thymus and spleen may be dependent on the level of SVCT-2 expression in heat stressed broilers. Furthermore, calcium (Ca^{2+}) in cells is the second messenger of information transmission [21], and participates in oxidative stress and apoptosis [22]. The mitochondria calcium uniporter (MCU) is a selective channel that mediates the influx of Ca^{2+} on the inner membrane of mitochondria [23]. The change of MCU activity is closely related to ROS production and oxidative stress [24]. Therefore, we hypothesized that heat stress can change the expression of ABCG2, SVCT-2 and MCU, and further to regulate the production of ROS, then induce the oxidative stress of the thymus and spleen in broilers. Hence, in this study, we investigated that the effects of heat stress on immune and oxidative stress indexes of thymus and spleen, and the relationship between the expression of ABCG2, SVCT-2, MCU and the production of ROS was further analyzed. This will provide a therapeutic target for the later prevention of heat stress in broilers.

Materials And Methods

Broilers and Experimental Design

One-day-old Arbor Acres broilers were obtained from a commercial hatchery in Anhui, and kept in cages with wood shaving litter floor reared under routine commercial management practices. At the end of 28 days, thirty chickens were randomly divided into two groups (the control group and the heat stress group, 15 chickens /group), with 3 replicates per group, and 5 birds per replicate. Namely, the control group, kept at normal temperature conditions (25 ± 2°C; 42–66% RH for 1 week); while the heat stress group, exposed

daily to high ambient temperature (36 ± 2°C; 33–38% RH, 8 h/day for 1 week, other time returned back to the conventional conditions) 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). 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 refer to the article by Wang et al., (2019) [25].

Sample collection and preservation

After 1 week of heat stress, the broilers for the control group and heat stress group were slaughtered, and then the blood samples were respectively collected from the jugular vein in tubes and immediately centrifuged at 3,000 rpm for 15 min to obtain serum, then stored at -20℃. The serum was further used to analyze the heat shock proteins 70 (HSP70), the corticosterone (CORT) and the indices of oxidative stress. The thymus and spleen were respectively collected and weighed, and then were divided into two parts. Part of the sample is quickly put into liquid nitrogen and then stored at -80°C for RNA isolation extract. The other part of the sample was immediately homogenized, and then to determine oxidative stress and immune indexes.

The levels of HSP70 and CORT determination in serum

The levels of HSP70 and CORT concentration in the serum were detected by using enzyme-linked immunoassay (ELISA) kit (Shanghai Fanke Industrial Co., Ltd., Shanghai, China); the procedure was followed as provided by the supplier.

Determination of oxidative stress and immune Indices in serum

After 1 week of heat stress, total antioxidant capacity (T-AOC), the activities of glutathione peroxidase (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 [26]. The levels of interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α) in serum were detected by using a commercial ELISA kit (Nanjing Senberga Biological Technology Institute, Nanjing, China) according to the instructions of the manufacturer.

Measurement of oxidative stress and immune indices for thymus and spleen

The thymus and spleen were immediately collected and homogenized for 2 min in 9 mL of ice-cold saline solution, respectively, and then the homogenates were centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was collected, then the content of MDA, SOD, GSH-PX, T-AOC, H_2O_2 and the levels of IL-6, IL-10 and TNF-α in the supernatant of the thymus and spleen were determined using corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer.

Relative quantitative real-time polymerase chain reaction analysis

Reverse transcription polymerase chain reaction (RT-PCR) and qRT-PCR were performed by referring to previously described methods [25]. Briefly, the total RNA was respectively extracted from thymus and spleen of broilers using OMEGA Total RNA kit(SPECTRIS CO, Egham, Surrey, UK) according to the manufacturer's instructions, and then reverse transcribed into cDNA using Takara prime scripter kit (Takara Bio., Kusatsu, Shiga, Japan) following the instructions of the manufacturer. The cDNA was diluted and stored at -30℃ until gene analysis. The PCR products were examined using 1.5% agarose gel electrophoresis; β-actin was used as the internal standard in the study. The relative levels of IgG, IgM, ABCG2, SVCT-2 and MCU mRNA were respectively determined using CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, USA). The PCR procedure was 95℃ for 10 mins, and then followed by 40 amplification cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 1 min, and extension at 72℃ for 15 s. The melting curve shows a single peak for each PCR product. The relative levels of immunoglobulin G (IgG), immunoglobulin M (IgM), ABCG2, SVCT-2 and MCU mRNA were calculated according to the 2-△△CT method. According to the sequence of β -actin, IgG, IgM, ABCG2, SVCT-2, MCU and housekeeping gene β-actin of chicken in NCBI, the primers were designed by AlleleID6 (PREMIER Biosoft, Canada). The primers were synthesized by Shanghai Jierui biology Co., Ltd., as shown in Table 1.

Table 1
Gene-special primers used in the RT-PCR and qRT-PCR.

Gene	Primer sequence(5'→3')	Product size (bp)
β-actin	Forward: AGACATCAGGGTGTGATGGTTGGT	125
	Reverse: TCCCAGTTGGTGACAATACCGTGT	
IgG	Forward: ATCACGTCAAGGGATGCCCG	168
	Reverse: ACCAGGCACCTCAGTTTGG	
IgM	Forward: CAATGGGATGATGGTGAGG	139
	Reverse: TGAGTGGGACAATGATACG	
ABCG2	Forward: ATTTTCATTGCTCGCTTCTTT	216
	Reverse: GACAGTCTGACATTACTAGCTTTGG	
SVCT-2	Forward: GATTGTCTTGTGCTCCTCCTC	185
	Reverse: GGCTGCTCCATACTGAATAACC	
MCU	Forward: CCTATCTCAGACTCCGTTGG	229
	Reverse: CATCATTCAGCGTGGTTGC	

Protein extraction and Western Blotting

The thymus and spleen were respectively cut into small pieces in the homogenate tube, and then were homogenized for 2 min in 10 times tissue volume of lysate. After the homogenates were cracked on the ice for 30 mins, they were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected as the total protein solution. The protein content of tissue homogenate was measured by a Coomassie brilliant blue staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The procedure was followed as the method provided by the supplier.

Tissue protein samples for the thymus and spleen were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) by electro blotting; After blocking with 5% skimmed milk for 1 h at room temperature, the proteins were labeled with primary antibodies overnight at 4℃; and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody at room temperature for 30 mins; Bound antibodies were detected with ECL Western blot detection reagent (GE Healthcare) and quantified with Fiji/Image J. Relative signal intensities were normalized to β-actin. All the primary antibodies and secondary antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

All the data were analyzed by SPSS software (version 21; IBM, Chicago, USA), and expressed by means ± S.E.M. from 3 replicates. Statistical comparisons were performed using Independent-Samples T Test. A difference with value $P < 0.05$ was considered statistically significant.

Results

The levels of HSP70 and CORT, and oxidative stress and immune indices in serum after heat stress

As shown in Table 2, after 1 week of heat stress, the levels of HSP70 and CORT in serum was significantly higher than that in the control group ($P < 0.01$); The birds had higher the contents of MDA in heat stress group compared with the control group ($P < 0.05$); The activities of T-AOC, 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 levels of IL-6 and TNF-α in heat stress group significantly increased in compared with the control group ($P < 0.01$); while the CAT activity and IL-10 level had no significant difference between the heat stress and the control groups ($P > 0.05$).

Table 2
Effects of heat stress on the levels of HSP70 and CORT, and the oxidative stress and immune indices in serum.

Item[2]	Control group	Heat stress group
Days 28–35		
CORT (ng/mL)	7.43 ± 0.84[a]	10.33 ± 0.64[b]
HSP70 (ng/L)	354.98 ± 14.85[a]	546.27 ± 57.38[b]
MDA (nmol/mL)	7.62 ± 0.47[a]	11.72 ± 0.56[b]
T-AOC (U/mL)	21.34 ± 0.42[a]	15.13 ± 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]
GSH (μmol/L)	9.82 ± 0.47[a]	6.57 ± 0.62[b]
CAT (U/mL)	7.28 ± 0.34	8.16 ± 0.40
IL-6 (ng/L)	20.95 ± 0.42[a]	25.88 ± 1.11[b]
TNF-α (ng/L)	34.67 ± 0.33[a]	40.38 ± 1.58[b]
IL-10 (ng/L)	57.69 ± 2.83	51.63 ± 0.52

[1]Data are expressed as mean ± SEM, n = 3. Values within a row with no common superscripts differ significantly ($P < 0.05$).

[2]Corticosterone (CORT); Heat shock proteins 70 (HSP70); Malondialdehyde (MDA); Total antioxidant capacity (T-AOC); Glutathione peroxidase (GSH-Px); Superoxide dismutase (SOD); Glutathione (GSH); Catalase (CAT); Interleukin-6 (IL-6); Interleukin-10 (IL-10); Tumor necrosis factor-alpha (TNF-α) .

Changes of development and immune indices of thymus and spleen after heat stress

As shown in Table 3, the weight and the indices of thymus in the heat stress group were significantly lower than those in the control group ($P < 0.01$). There was no significant difference in the weight and indices of spleen between the heat stress and the control groups ($P > 0.05$). As shown in Table 4, compared with the control group, the IL-6 contents of thymus in the heat stress group was significantly increased ($P < 0.05$), had no significant change in spleen ($P > 0.05$); The IL-10 levels of thymus and spleen in the heat stress group was both significantly lower ($P < 0.05$); The contents of TNF-α was no significant difference in thymus and spleen ($P > 0.05$).

Table 3
Effects of heat stress on absolute weight and organ index of thymus and spleen of broilers.

Item	Control group	Heat stress group
Absolute thymus weight (g)	5.33 ± 0.32[a]	3.47 ± 0.43[b]
Thymus index	4.89 ± 0.41[a]	3.12 ± 0.29[b]
Absolute weight of spleen (g)	1.69 ± 0.16	1.09 ± 0.09
Spleen index	1.29 ± 0.13	0.75 ± 0.07
Data are expressed as mean ± SEM, n = 3. Values in the same row with different superscripts were significantly different ($P < 0.05$).		

Table 4. Effects of heat stress on the oxidative stress and immune indices in thymus and spleen of broilers.

Item	Thymus		Spleen	
	Control group	Heat stress group	Control group	Heat stress group
MDA (nmol/mg)	4.44 ± 0.12[a]	6.47 ± 0.29[b]	5.74 ± 0.05[a]	6.74 ± 0.14[b]
SOD (U/mg)	446.23 ± 34.23	318.47 ± 36.64	285.43 ± 22.86[a]	162.27 ± 17.87[b]
GSH-PX (U/mg)	8337.93 ± 190.56[a]	6895.81 ±425.18[b]	16488.68 ± 590.16[a]	11262.78 ± 440.96[b]
T-AOC (U/mg	351.01 ± 14.41	323.12 ± 8.61	140.87 ± 2.15[a]	84.47 ± 3.56[b]
H_2O_2 (mmol/g)	80.14 ± 7.71[a]	270.24 ± 19.89[b]	266.77 ± 34.51[a]	633.83 ± 27.17[b]
IL-6 (ng/L)	48.43 ± 2.58[a]	64.65 ± 1.93[b]	63.35 ± 3.31	58.75 ± 11.62
L-10 (ng/L)	245.97 ± 10.24[a]	207.15 ± 5.81[b]	389.10 ± 10.26[a]	316.61 ± 22.53[b]
TNF-α (ng/L)	119.52 ± 3.09	120.21 ± 3.83	142.29 ± 10.14	154.37 ± 2.91

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

Indices of oxidative stress in thymus and spleen of broilers under heat stress

As shown in Table 4, the MDA content and the ROS levels (measured as H_2O_2) of thymus and spleen in heat stress group was significantly higher than that in the control ($P < 0.01$); Compared with the control birds, the activities of T-AOC ($P < 0.01$) and SOD ($P < 0.05$) for spleen in heat stress group were

significantly lower, but they had both no significant change in thymus ($P > 0.05$); Moreover, the GSH-PX content of thymus ($P < 0.05$) and spleen ($P < 0.01$) in heat stress group was significantly lower than that in control group. The results showed that heat stress could cause the increase of lipid peroxides and ROS, the decrease of antioxidant enzymes in thymus and spleen of broilers.

Expression of IgG and IgM mRNA in thymus and spleen of broilers

To understand whether heat stress can induce immune response in the thymus and spleen of broilers or not, the expression of IgG and IgM genes was detected by RT-PCR and RT-qPCR. As shown in Fig. 1A, the expression of IgG and IgM genes in heat stress group significantly increased compared to the control group in thymus and spleen by RT-PCR; The relative expression of IgG and IgM mRNA in heat stress group significantly increased compared to the control group in spleen ($P < 0.05$; Fig. 1B); In the thymus, the expression levels of IgG mRNA in heat stress group was significantly higher than that in the control group ($P < 0.05$); However, the expression level of IgM mRNA had no significant difference ($P > 0.05$; Fig. 1B).

Expression of ABCG2, SVCT-2 and MCU genes in thymus and spleen of broilers under heat stress

To further explore the reasons for the change of ROS levels of thymus and spleen caused by heat stress, the gene expression of several transporters involved in ROS production was examined by RT-PCR and RT-qPCR. As shown in Fig. 2A, the birds exposed to heat stress had higher expression of ABCG2, SVCT-2 and MCU mRNA compared with the control birds in thymus and spleen by RT-PCR analysis. The relative expression of ABCG2 mRNA in heat stress group increased in thymus and spleen ($P < 0.05$) (Fig. 2B). Furthermore, the relative expression of SVCT-2 and MCU mRNA in thymus of heat stressed broilers significantly increased compared with the control birds (Fig. 2C,D) ($P < 0.01$), but it had no significantly change in spleen ($P > 0.05$).

The expression of ABCG2, SVCT-2 and MCU proteins in thymus and spleen of broilers under heat stress by Western Blot

To clarify the reason of ROS production changes in thymus and spleen after heat stress, the expression of several transporters involved in ROS production was examined by Western Blot. As shown in Fig. 3A, the expression of ABCG2, SVCT-2 and MCU proteins were all dramatically increased in thymus and spleen after exposure to heat stress. The expression of ABCG2 ($P < 0.05$; Fig. 3B), SVCT-2 ($P < 0.01$; Fig. 3C) and MCU ($P < 0.01$; Fig. 3D) protein of broiler thymus and spleen in the heat stress group increased significantly compared with the control group. The results suggest that heat stress may mediate oxidative stress in the thymus and spleen of broilers by regulating the expression of ABCG2, SVCT-2 and MCU protein.

Discussion

Environmental temperature plays an important role in poultry industry along with rising of the global temperatures. When the environmental temperature exceeds the upper limit of comfort zone of broilers by 27℃, the imbalance between heat dissipation and heat production of the body will lead to heat stress [27]. Hence, the broiler were exposed at 36℃ for 8 hours per day and lasted for 1 week to mimic a heat wave in the study. The broiler in heat stress group showed shortness of breath, wing spreading, lethargy and increased water consumption at this study. The results are consistent with those reported by Jastrebski et al. (2017) [28]. Previous studies have shown that HSP70 is one of the most conservative proteins in evolution and very stable at heat stress, and can be used as stress indicator protein [29]. Heat stress can significantly increase the expression of HSP70 in broiler cardiomyocytes [30]; moreover, the content of serum CORT is the most important index of heat stress in poultry [31]. Hence, the content of HSP70 and CORT in the serum was used to judge that the broilers had heat stress, and they in heat stress group were significantly higher than that of the control group in the study. In the course of our experiment, the broilers showed a typical heat stress response in the early stages of thermal exposure.

Heat stress is linked to compromised productivity through the change of biochemistry and immunity in blood [32]. It's important to note that immune system is one of the main targets of heat stress-induced negative effects in the organism [4]. The spleen is the biggest peripheral immune organ, and thymus is central lymphoid organs in the immune system of poultry [33]. Hence, we selected spleen and thymus as the target tissues to analyze the mechanism of change of ROS production for heat-stressed birds in the study. Previous studies have confirmed that heat stress affected the absolute weight and organ index of immune organs to a certain extent [11]. In the current study, the absolute weight and index of thymus and spleen all decreased after heat stress; this indicate in the immune system is inhibited to some extent, which is consistent with previous research results [6, 34]. The results showed that heat stress could inhibit the development of thymus and spleen and cause damage to the immune organs.

Cytokines are important information molecules of the immune system in the immune response process of the body [35]. Recent studies have shown that IL-6 and TNF-α, as proinflammatory factors, play important roles in both immune responses and inflammationin, which can activate immune response and inflammation [36, 37]. IL-10 is an important anti-inflammatory cytokine and can inhibit production of inflammatory cytokines by a variety of inflammatory cells [38]. Studies have shown that IL-10 can inhibit the activation, synthesis and release of proinflammatory factors such as IL-6 and TNF-α through various mechanisms, so as to achieve the purpose of inhibiting the occurrence of inflammation [39]. Our present study showed that the levels of IL-6 and TNF-α in serum for heat stressed broilers increased, but the IL-10 level had no significant change. Moreover, heat stress can increase the level of IL-6 and TNF−α, and decrease the level of IL-10 in thymus and spleen of broilers. The results confirmed that heat stress can increase the levels of inflammatory cytokines and decrease the levels of anti-inflammatory cytokines in serum,the thymus, and spleen of broilers.

Besides these cytokines, immunoglobulin also plays an important role in immune regulation. Existing research has demonstrated that IgM and IgG are respectively the immunoglobulin in poultry, which play a leading role in anti-infection [40]. The relative expression of IgM and IgG mRNA in the spleen and thymus

of heat stressed broilers increased significantly in the study. These results agree with previous studies reported by Honda et al. (2015) [40]. This is an indication that heat stress caused infection to the immune organs; further enhanced the immune response of spleen in broilers. Hence, heat stress can lead to inflammatory response and immunoglobulin expression increase in thymus and spleen.

Heat stress affects mainly poultry performance by inducing the oxidative stress [41]. Previous studies had confirmed that MDA content can reflect the degree of oxidative damage [42]. In this study, we found that the MDA content of thymus and spleen increased significantly under heat stress, which indicated that heat stress caused oxidative damage of thymus and spleen in broiler. Existing research has demonstrated that hyperthermia causes oxidative stress damage to the cell by generating augmented ROS [8], while SOD and GSH-PX can remove ROS in the body [43]. In this experiment, the contents of SOD, GSH-PX and T-AOC were decreased, while the ROS in thymus and spleen of broiler increased significantly after heat stress, which indicated that heat stress could reduce the antioxidant capacity and lead to the increase of ROS level in thymus and spleen, and causes oxidative injury in the cell of the thymus and spleen.

To explore the mechanism of change of ROS levels in the thymus and spleen of heat-stressed birds, the expression of several transporters in the thymus and spleen associated with ROS production was detected. ABCG2 often serves as an important marker for cells in oxidative stress [44] and is capable of protecting cells from ROS-mediated cell damage [16]. It has been found that ABCG2 can reduce the ROS levels to alleviate the oxidative stress of cells [15] and improve the content and activity of SOD, the content of glutathione and the activity of glutathione reductase in cells [45]. Our study found that the expression of ABCG2 mRNA and proteins in thymus and spleen of heat stressed broiler was significantly increased. The data showed that ABCG2 expression manifested as a compensatory increase to protect the thymus and spleen from heat-induced oxidative stress by reducing intracellular ROS or exuding other harmful substances under heat stress. However, more in-depth research is needed in the future to articulate the anti-injury mechanism of ABCG2 in heat stress. Moreover, SVCT-2 widely exists in various tissues and organs, which mainly participates in the absorption of vitamin C in the active metabolism tissues in the body, so as to protect these tissues from oxidative damage [20]. Previous studies have indicated that supplementation with vitamin C could mitigate heat-related damage and enhance heat resistance in animals [46]. It has been shown that SVCT-2 gene knockout can reduce the content of Vc in cells and tissues, increase cell oxidative damage and death, and finally lead to embryo death [47]. Our studies found that the expression of SVCT-2 mRNA in thymus increased, while it is no significant different in the spleen of the heat-stressed broiler. However, the expression of SVCT-2 proteins in thymus and spleen of heat stressed broiler was significantly increased. The result indicated that SVCT-2 proteins are stimulated to possibly transfer more Vc and to compensatory to protect against cellular damage of thymus and spleen from the heat-generated stress; however, further research is needed to elucidate the exact mechanism.

Ca^{2+} is a second messenger that mediates cell apoptosis and oxidative stress. A previous study showed that Ca^{2+} dysregulation can give rise to neurodegenerative diseases through oxygenated stress damage

[48]. The MCU has the function of mediating Ca^{2+} influx and plays an important role in the regulation of Ca^{2+} concentration in cells and mitochondria [23]. Since MCU is associated with Ca^{2+} concentration, while mitochondrial Ca^{2+} overload induces a large number of ROS production in cells [49]. Our study showed that the expression of MCU in thymus and spleen of heat stressed broiler was significantly increased compared with the control group. This indicated that under the condition of heat stress, Ca^{2+} overload of mitochondria in thymus and spleen cells of broiler maybe affect ATP synthesis, further stimulated ROS production, and finally led to apoptosis, thus affecting immune function.

Conclusions

In conclusion, chronic heat stress can cause oxidative stress in thymus and spleen of broilers. After heat stress, the body will over-express ABCG2 and SVCT-2 to clear ROS and protect spleen from oxidative damage to a certain extent. However, the decreased expression in thymus may explain that heat stress is more harmful to broiler thymus. While the expression of MCU in the thymus and spleen of broilers increased significantly. The opening of MCU will lead to mitochondrial calcium overload, stimulate ROS production, and lead to apoptosis or necrosis of thymus and spleen cells, which will affect the immune function of broilers.

Abbreviations

CORT:Corticosterone; HSP70:Heat shock proteins 70; MDA:Malondialdehyde; T-AOC:Total antioxidant capacity; GSH-Px:Glutathione peroxidase; SOD:Superoxide dismutase; GSH:Glutathione; CAT:Catalase; ROS:reactive oxygen species;IL-6:Interleukin-6; IL-10:Interleukin-10; TNF-α:Tumor necrosis factor-alpha. IgG:immunoglobulin G; IgM:immunoglobulin M; ABCG2:ATP binding cassette subfamily G member 2; qRT-PCR:Real-time reverse transcription-polymerase chain reaction; RT-PCR: reverse transcription-polymerase chain reaction; MCU:mitochondria calcium uniporter; SVCT-2:sodium dependent vitamin C transporter-2.

Declarations

Acknowledgements

The authors would like to express special appreciation to Dr. Jie Zhou for providing many insightful comments.

Authors' contributions

Xiuheng Xue, Juhua Wang and Fugui Fang conceived and supervised the study; Xiuheng Xue, Juhua Wang and Chunhuan Ren designed the experiments; Luping Wang, Mengzhu Xu and Chunhuan Ren performed experiments; Caiyun Fan and Mengling Peng analyzed the data; Xiuheng Xue, Juhua Wang

and Jianbo Cheng wrote the manuscript and revised the manuscript. The authors read and approved the final manuscript.

Funding

This study was supported by the Open Fund of State Key Laboratory of Tea Plant Biology and Utilization (SKLTOF20200122), and was also supported Key Research and Development Program of Anhui Province (202004a06020006).

Availability of data and materials

The datasets analyzed during the study are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Care and Use Committee of Anhui Agricultural University. The methods were carried out in accordance with the approved guidelines.

Consent for publication

Not applicable.

References

1. Goel A, Ncho CM, Choi YH. Regulation of gene expression in chickens by heat stress. J Anim Sci Biotechnol. 2021;12(1):1-13. https://doi.org/10.1186/s40104-020-00523-5.
2. Nawab A, Ibtisham F, Li GH, Kieser B, Wu J, Liu WC, et al. Heat stress in poultry production: Mitigation strategies to overcome the future challenges facing the global poultry industry. J Therm Biol. 2018;78:131-139. https://doi.org/10.1016/j.jtherbio.2018.08.010.
3. Wasti S, Sah N, Mishra B. Impact of heat stress on poultry health and performances, and potential mitigation strategies. Animals.2020;10(8):1266.https://doi.org/10.3390/ani10081266.
4. Starkie RL, Hargreaves M, Rolland J, Febbraio MA. Heat stress, cytokines, and the immune response to exercise. Brain, Behav Immun. 2005;19(5):404-412. https://doi.org/10.1016/j.bbi.2005.03.005.
5. Cooper MD, Raymond DA, Peterson RD, South MA, Good RA. The functions of the thymus system and the bursa system in the chicken. J Exp Med. 1966;123(1):75-102. https://doi.org/10.1084/jem.123.1.75.
6. Roushdy EM, Zaglool AW, El-Tarabany MS. Effects of chronic thermal stress on growth performance, carcass traits, antioxidant indices and the expression of HSP70, growth hormone and superoxide dismutase genes in two broiler strains. J Therm Biol. 2018;74:337-343. https://doi.org/10.1016/j.jtherbio.2018.04.009.

7. Xu D, Li B, Cao N, Li W, Tian Y, Y Huang. The protective effects of polysaccharide of Atractylodes macrocephala Koidz (PAMK) on the chicken spleen under heat stress via antagonizing apoptosis and restoring the immune function. Oncotarget. 2017;8(41):70394-70405. https://doi.org/10.18632/oncotarget.19709.
8. Ghazi SH, Habibian M, Moeini MM, Abdolmohammadi AR. Effects of different levels of organic and inorganic chromium on growth performance and immunocompetence of broilers under heat stress. Biol Trace Elem Res. 2012;146(3):309-317. https://doi.org/10.1007/s12011-011-9260-1.
9. Habibian M, Ghazi S, Moeini MM, bdolmohammadi A. Effects of dietary selenium and vitamin E on immune response and biological blood parameters of broilers reared under thermo neutral or heat stress conditions. Int J Biometeorol. 2014;58(5):741-752. https://doi.org/10.1007/s00484-013-0654-y.
10. Xu DN, Li WY, Huang YM, He JH, Tian YB. The effect of selenium and polysaccharide of atractylodes macrocephala koidz. (PAMK) on immune response in chicken spleen under heat stress. Biol Trace Elem Res. 2014;160(2):232-237. https://doi.org/10.1007/s12011-014-0056-y.
11. Chand N, Naz S, Khan A, Khan S, Khan RU. Performance traits and immune response of broiler chicks treated with zinc and ascorbic acid supplementation during cyclic heat stress. Int J Biometeorol. 2014;58(10):2153-2157. https://doi.org/10.1007/s00484-014-0815-7.
12. El-Tarabany MS. Impact of temperature-humidity index on egg-laying characteristics and related stress and immunity parameters of Japanese quails. Int J Biometeorol. 2016;60(7): 957-964. https://doi.org/10.1007/s00484-015-1088-5.
13. Akbarian A, Michiels J, Degroote J, Majdeddin M, Golian A, De Smet S. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. J Anim Sci Biotechnol. 2016;7:37. https://doi.org/10.1186/s40104-016-0097-5.
14. Roh YG, Mun MH, Jeong MS, Kim WT, Lee SR, Chung JW, et al. Drug resistance of bladder cancer cells through activation of ABCG2 by FOXM1. Bmb Rep. 2018;51(2):98-103. https://doi.org/10.5483/bmbrep.2018.51.2.222.
15. Shen S, Callaghan D, Juzwik C, Xiong H, Huang P, Zhang W. ABCG2 reduces ROS-mediated toxicity and inflammation: a potential role in Alzheimer's disease. J Neurochem. 2010; 114(6):1590-1604. https://doi.org/10.1111/j.1471-4159.2010.06887.x.
16. Nie S, Huang Y, Shi M, Qian X, Li H, Peng C, et al. Protective role of ABCG2 against oxidative stress in colorectal cancer and its potential underlying mechanism. Oncol Rep. 2018; 40(4):2137-2146. https://doi.org/10.3892/or.2018.6594.
17. Kurokawa H, Ito H, Terasaki M, Matsui H. Hyperthermia enhances photodynamic therapy by regulation of HCP1 and ABCG2 expressions via high level ROS generation. Sci Rep. 2019;9(1):1638. https://doi.org/10.1038/s41598-018-38460-z.
18. Liang WJ, Johnson D, Jarvis SM. Vitamin C transport systems of mammalian cells. Mol Membr Biol. 2001;18(1):87-95. https://doi.org/10.1080/09687680110033774.

19. Daruwala R, Song J, Koh WS, Rumsey SC, Levine M. Cloning and functional characterization of the human sodium-dependent vitamin C transporters HSVCT1 and HSVCT2. FEBS Lett. 1999;460(3):480-484. https://doi.org/10.1016/s0014-5793(99)01393-9.
20. Rajan DP, Huang W, Dutta B, Devoe LD, Leibach FH, Ganapathy V, et al. Human placental sodium-dependent vitamin C transporter (SVCT2): molecular cloning and transport function. Biochem Biophys Res Commun. 1999;262(3):762-768. https://doi.org/10.1006/bbrc.1999.1272.
21. Berridge MJ. Inositol triphosphate and calcium signalling. Nature. 1993;361:315-325. https://doi.org/10.1530/eje.0.130s0004.
22. Ray SK, Fidan M, Nowak MW, Wilford GG, Hogan EL, Banik NL. Oxidative stress and Ca^{2+} influx upregulate calpain and induce apoptosis in PC12 cells. Brain Res. 2000;852(2):326-34. https://doi.org/10.1016/s0006-8993(99)02148-4.
23. De Stefani D, Patron M, Rizzuto R. Structure and function of the mitochondrial calcium uniporter complex. Biochimica Et Biophysica Acta. 2015;1853(9):2006-2011. https://doi.org/10.1016/j.bbamcr.2015.04.008.
24. Peng TI, Jou MJ. Oxidative stress caused by mitochondrial calcium overload. Ann N Y Acad Sci. 2010;1201:183-188. https://doi.org/10.1111/j.1749-6632.2010.05634.x.
25. Wang JH, Xue XH, Liu Q, Zhang SZ, Peng ML, Zhou J, et al. 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. J Therm Biol. 2019;85:102420. https://doi.org/ 10.1016/j.jtherbio.2019.102420.
26. Zhang C, Zhao XH, Yang L, Chen XY, Jiang RS, Jin SH, et al. Resveratrol alleviates heat stress-induced impairment of intestinal morphology, mi-croflora, and barrier integrity in broilers. Poult Sci. 2017;96(12):4325–4332. https://doi.org/10.3382/ps/pex266.
27. Yang Y, Wang X, Zhang M, Feng J. The upper limit temperature of thermoneutral zone estimated by the changes of temperature and respiration rate of the broilers. Scientia Agricultura Sinica. 2019;52(3):550-557. https://doi.org/10.3864/j.issn.0578-1752.2019.03.015.
28. Jastrebski SF, Lamont SJ, Schmidt CJ. Chicken hepatic response to chronic heat stress using integrated transcriptome and metabolomeanalysis. PLoS One. 2017;12(7):e0181900. https://doi.org/10.1371/journal.pone.0181900.
29. Kiang JG, Tsokos GC. Heat Shock Protein 70 kDa Molecular Biology, Biochemistry, and Physiology. Pharmacol Ther. 1998;80(2):183-201. https://doi.org/10.1016/s0163-7258(98)00028-x.
30. Pavlik A, Aneja IS, Lexa J, Al-Zoabi BA. Identification of cerebral neurons and glial cell types inducing heat shock protein Hsp70 following heat stress in the rat. Brain Res. 2003;973(2):179-189. https://doi.org/10.1016/s0006-8993(03)02476-4.
31. Pawar SS, Sajjanar B, Lonkar VD, Kurade NP, Bal SK. Assessing and mitigating the impact of heat stress in poultry. J Anim Vet Adv. 2016;4(6):332-341. https://doi.org/10.14737/journal.aavs/2016/4.6.332.341.

32. Farag MR, Alagawany M. Physiological alterations of poultry to the high environmental temperature. J Therm Biol. 2018;76:101-106. https://doi.org/10.1016/j.jtherbio.2018.07.012.
33. He SP, Yu QF, He YJ, Hu RZ, Xia ST, He JH. Dietary resveratrol supplementation inhibits heat stress-induced high-activated innate immunity and inflammatory response in spleen of yellow-feather broilers. Poult Sci. 2019; 98(12):6378-6387. https://doi.org/10.3382/ps/pez471.
34. Tang J, Chen Z. The protective effect of γ-aminobutyric acid on the development of immune function in chickens under heat stress. J Anim Physiol Anim Nutr (Berl). 2016;100(4):768-77. https://doi.org/10.1111/jpn.12385.
35. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest. 2007;117(5):1175-1183. https://doi.org/10.1172/JCI31537.
36. Lacroix M, Lizotte F, Hivert MF, Geraldes P, Perron P. Calcifediol decreases interleukin-6 secretion by cultured human tropho-blasts from GDM pregnancies. J Endocr Soc. 2019; 3(11):2165–2178. https://doi.org/10.1210/js.2019-00181.
37. Rancourt RC, Ott R, Ziska T, Schellong K, Melchior K, Henrich W, et al. Visceral adipose tissue inflammatory factors (TNF-a, SOCS3) in gestational diabetes (GDM): epigenetics as a clue in GDM pathophysiology. Int J Mol Sci. 2020;21(2):479. https://doi.org/10.3390/ijms21020479.
38. Laffer B, Bauer D, Wasmuth S, Busch M, Jalilvand TV, Thanos S, et al. Loss of IL-10 promotes differentiation of microglia to a M1 phenotype. Front Cell Neurosci. 2019;13:430. https://doi.org/10.3389/fncel.2019.00430.
39. De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med. 1991;174(5):1209-1220. https://doi.org/10.1084/jem.174.5.1209.
40. Honda BTB, Calefi AS, Costola-de-Souza C, Quinteiro-Filho WM, da Silva Fonseca JG, de Paula VF, et al. Effects of heat stress on peripheral T and B lymphocyte profiles and IgG and IgM serum levels in broiler chickens vaccinated for Newcastle disease virus. Poult Sci. 2015;94(10):2375-2381. https://doi.org/10.3382/ps/pev192.
41. Maibam U, Hoodaa OK, Sharmab PS, Upadhyaya RC, Mohanty AK. Differential level of oxidative stress markers in skin tissue of zebu and crossbreed cattle during heat stress. Livest Sci. 2018;207:45-50. https://doi.org/10.1016/j.livsci.2017.11.003.
42. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol. 1990;186:421-431. https://doi.org/10.1016/0076-6879(90)86135-i.
43. Chauhan SS, Celi P, Ponnampalam EN, Leury BJ, Liu F, Dunshea FR. Antioxidant dynamics in the live animal and implications for ruminant health and product (meat/milk) quality:role of vitamin E and selenium. Anim Prod Sci. 2014;54(10):1525-1536. https://doi.org/10.1071/AN14334.
44. Hu J, Li J, Yue X, Wang J, Liu J, Sun L, et al. Expression of the cancer stem cell markers ABCG2 and OCT-4 in right-sided colon cancer predicts recurrence and poor outcomes. Oncotarget. 2017;8(17):28463-28470. https://doi.org/10.18632/oncotarget.15307.

45. Zeng Y, Callaghan D, Xiong H, Yang Z, Huang P, Zhang W. Abcg2 deficiency augments oxidative stress and cognitive deficits in Tg-SwDI transgenic mice. J Neurochem. 2012; 122(2):456-469. https://doi.org/10.1111/j.1471-4159.2012.07783.x.
46. Rafiee F, Mazhari M, Ghoreishi M, Esmaeilipour O. Effect of lemon verbena powder and vitamin C on performance and immunity of heat-stressed broilers. J Anim Physiol Anim Nutr (Berl). 2016;100(5):807-812. https://doi.org/10.1111/jpn.12457.
47. Harrison FE, Dawes SM, Meredith ME, Babaev VR, Li L, May JM. Low vitamin C and increased oxidative stress and cell death in mice that lack the sodium-dependent vitamin C transporter SVCT2. Free Radic Biol Med. 2010;49(5):821-829. https://doi.org/10.1016/j.freeradbiomed.2010.06.008.
48. Penna E, Espino J, De Stefani D, Rizzuto R. The MCU complex in cell death. Cell Calcium. 2018;69:73-80. https://doi.org/10.1016/j.ceca.2017.08.008.
49. Zhang LY, Wang ZC, Lu TF, Meng L, Luo Y, Fu XW, et al. Mitochondrial Ca^{2+} overload leads to mitochondrial oxidative stress and delayed meiotic resumption in mouse oocytes. Front Cell Dev Biol. 2020;8:580876. https://doi.org/10.3389/fcell.2020.580876.

Figures

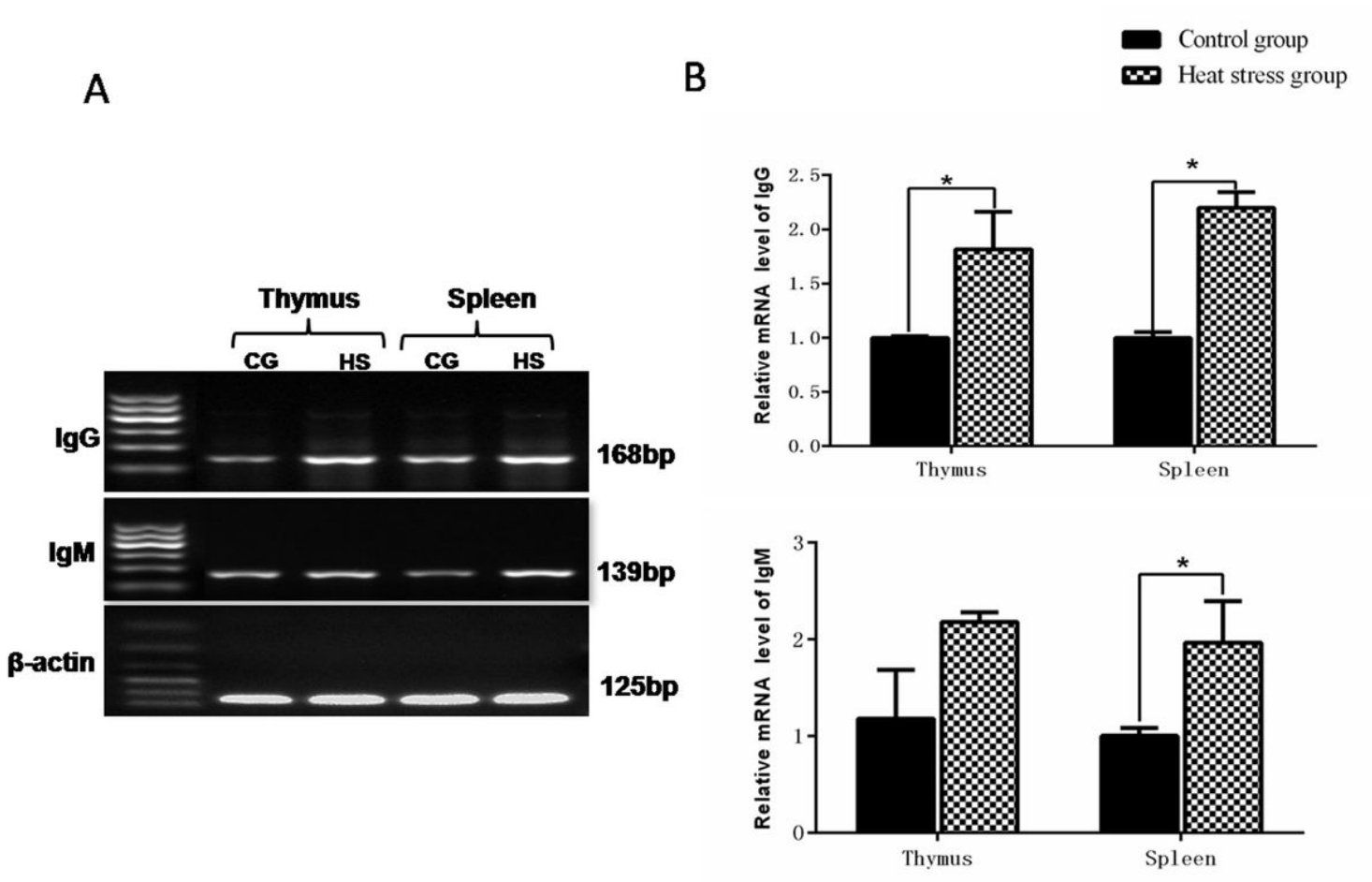

Figure 1

The expression of IgG and IgM mRNA in thymus and spleen by RT-PCR and RT-qPCR analysis. A. Expression of IgG and IgM genes by RT-PCR analysis; B. Relative mRNA expression levels of IgG in thymus and spleen; C. Relative mRNA expression levels of IgM in thymus and spleen. CG, the control group. HS, heat stress group. Note: Compared with the control group, *P < 0.05, ** P < 0.01. Error bars, SEM.

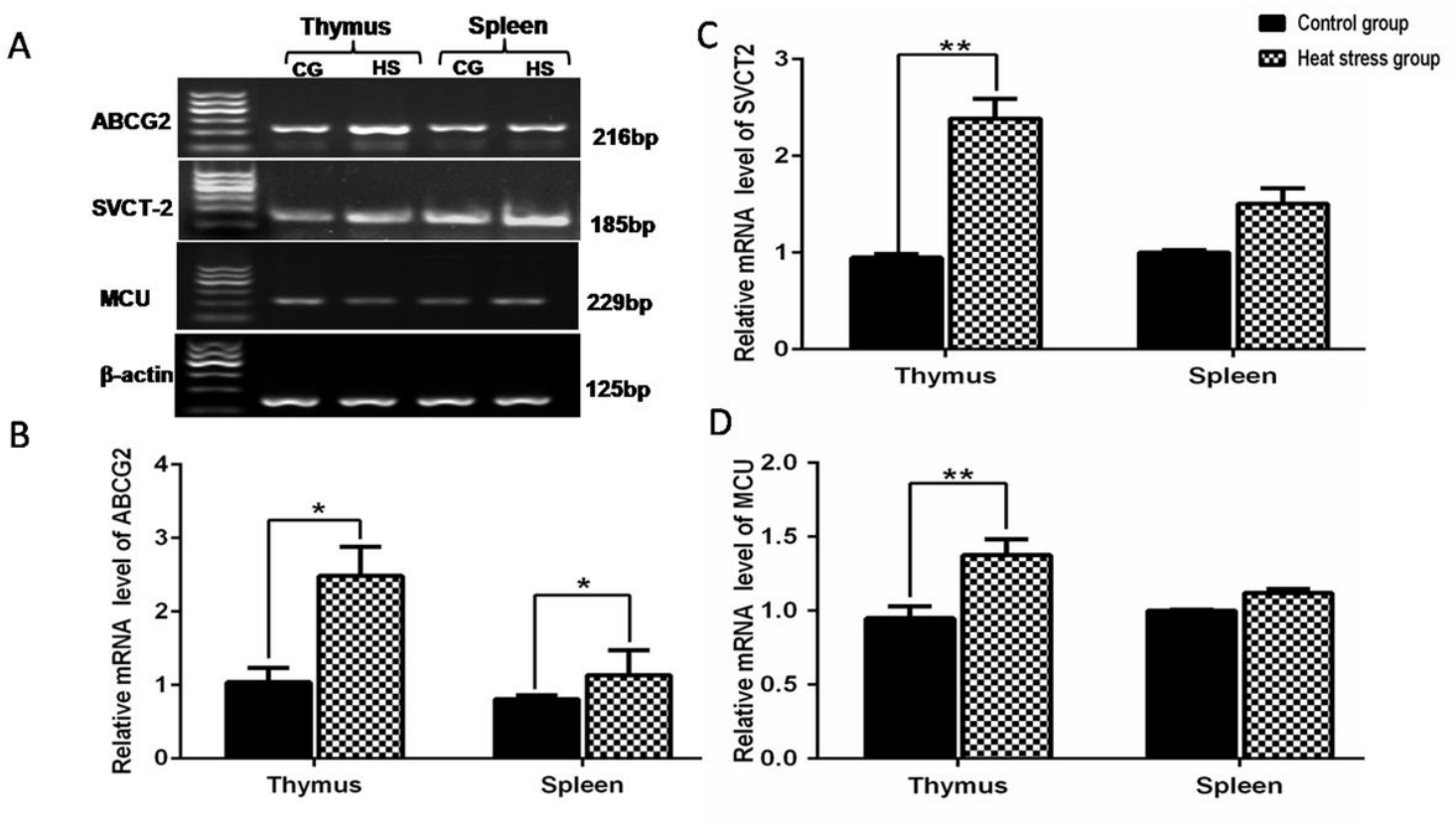

Figure 2

The expression of ABCG2, SVCT-2 and MCU mRNA in thymus and spleen by RT-PCR and RT-qPCR analysis. A. Expression of ABCG2, SVCT-2 and MCU genes by RT-PCR analysis; B. Relative expression levels of ABCG2 mRNA in thymus and spleen; C. Relative expression levels of SVCT-2 mRNA in thymus and spleen. D. Relative expression levels of MCU mRNA in thymus and spleen. CG, the control group. HS, heat stress group. Note: Compared with the control group, *P < 0.05, ** P < 0.01. Error bars, SEM.

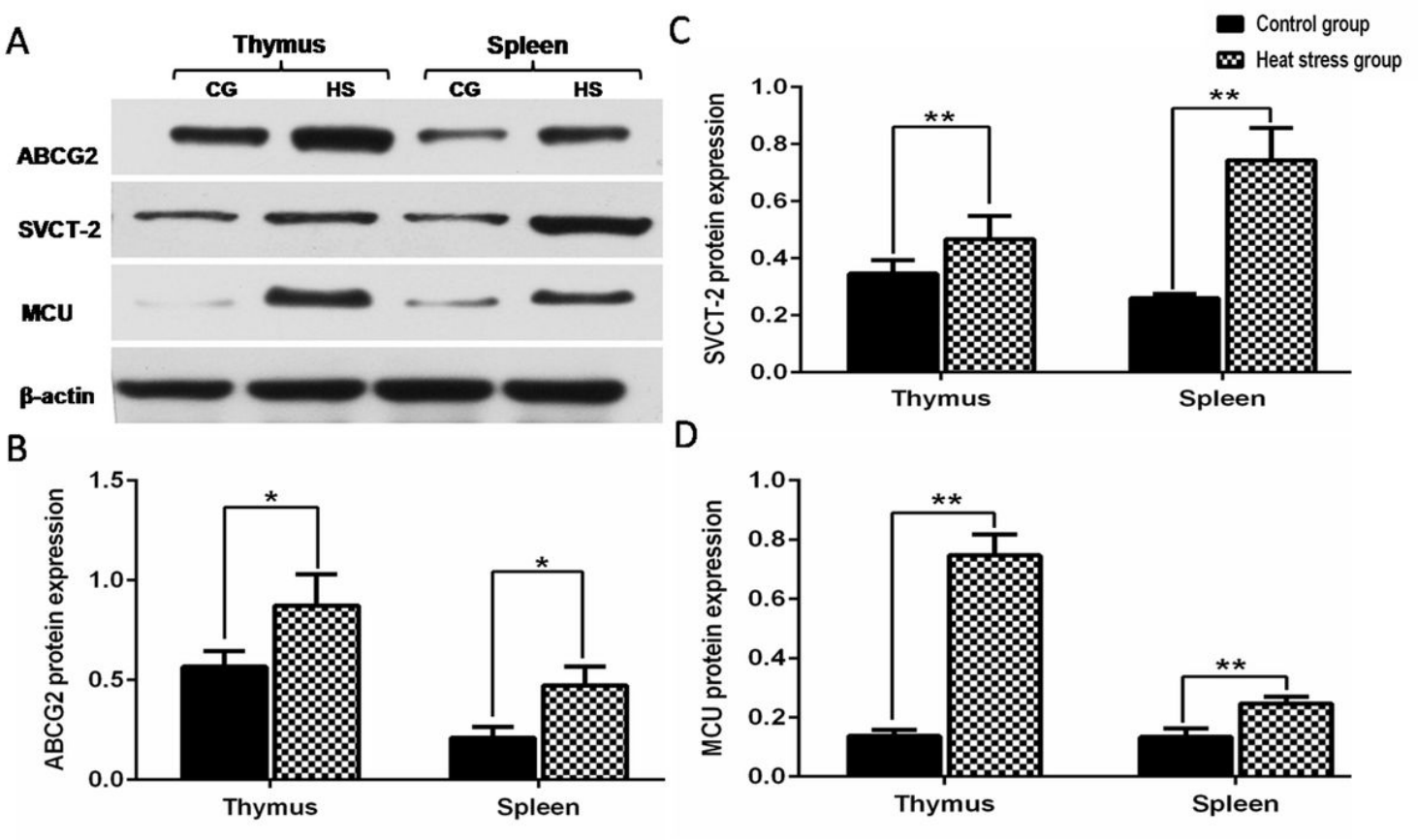

Figure 3

Protein levels of ABCG2, SVCT-2 and MCU in thymus and spleen by Western blots analysis. A. Expression of ABCG2 protein in thymus and spleen; B. Expression of SVCT-2 protein in thymus and spleen; C. Expression of MCU protein in thymus and spleen. CG, the control group. HS, heat stress group. Note: Compared with the control group, *P < 0.05, ** P < 0.01. Error bars, SEM.