

Chitosan oligosaccharides alleviate acute heat stress-induced oxidative damage by activating ERK1/2-mediated HO-1 and GSH-Px gene expression in breast muscle of broilers

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ABSTRACT The purpose of this study was to evaluate the effects of chitosan oligosaccharides (COS) on acute heat stress (AHS) induced poor meat quality by alleviating oxidative damage through mitogen-activated protein kinase-nuclear factor-erythroid 2-related factor 2-antioxidant responsive element (MAPK-Nrf2-ARE) signaling pathway. A total of 108 thirty-five-day-old Chinese indigenous broilers (Luhua chicken) was used for this 42-d experiment. The broilers were randomly allocated to 3 treatments: control group (CON), AHS group, and AHS with 400 mg/kg COS supplementation (AHS-C) group. Both CON and AHS groups given the basal diet, and the AHS-C group given the basal diet with 400 mg/kg COS supplementation. On d 42, broilers in the AHS and AHS-C groups treated with AHS (increasing temperature from 24 to 34°C in 2-h and held for another 2-h), and the CON group under normal temperature (24°C). AHS exposure elevated ($P < 0.05$) body temperature (rectal, comb, eyelids, and feet) of broilers, increased ($P < 0.05$) breast muscle lightness (L^*), drip loss, shear force, hydrogen

peroxide (H_2O_2) scavenging activity, reactive oxygen species (ROS) production, malondialdehyde (MDA) content, and catalase (CAT) activity, however, decreased ($P < 0.05$) pH_{45min}, pH_{24h}, redness (a^*), and relative expression of heme oxygenase-1 (HO-1). Compared to the AHS group, dietary COS supplementation increased ($P < 0.05$) breast muscle pH_{45min}, pH_{24h}, and a^* , H_2O_2 scavenging activity, as well as relative expression of HO-1 and glutathione peroxidase (GSH-Px), however, decreased ($P < 0.05$) drip loss, shear force, superoxide anion free radicals ($O_2^{\bullet-}$) scavenging activity, ROS production, and MDA content. It was concluded that AHS impaired meat quality, which may be related to oxidative damage, as evidenced by increasing ROS production, MDA content, and decreasing the relative expression of HO-1. Dietary COS supplementation could effectively elevate the meat quality of broilers exposed to AHS via decreasing ROS production, activating the Nrf2 pathway, and Nrf2-mediated HO-1 and GSH-Px gene expression.

Key words: chitosan oligosaccharides, acute heat stress, meat quality, antioxidant status, MAPK-Nrf2-ARE signaling pathway

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INTRODUCTION

Nowadays, the consumption of poultry meat sustainable growth worldwide, owing to its nutritive value, economical price, and no religious obstacles (Devatkal et al., 2019; Petracci et al., 2019). Besides the increasing demands, consumers also paid more and more attention to the meat quality, such as color, flavor, juiciness, and tenderness (Pieterse et al., 2019). With the

modern poultry breeds selected of rapid growth rate and increasing global warming, heat stress became the most stressful environmental stressors affecting their health, productivity, and meat quality worldwide (Varasteh et al., 2015; Lan et al., 2020b). Numerous studies demonstrated that either chronic or acute heat stress impaired meat quality by accelerating muscle postmortem glycolysis and overproduction of reactive oxygen species (ROS), resulting in rapid pH decline, oxidative damage, poor meat color, water holding capacity (WHC), and texture (Wang et al., 2017; Zaboli et al., 2019; Lan et al., 2020b). Muscle antioxidant status was one of the most important factors determine the meat quality, which not only influence flavor, but also shorten shelf life (Zhang et al., 2017; Rocchetti et al., 2020). Heat stress induced breast

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MATERIALS AND METHODS

Animals, Diets, Sample Collection, and Preparation

muscle oxidative damage mainly by damaging the antioxidant system. The mitogen-activated protein kinase-nuclear factor-erythroid 2-related factor 2-antioxidant responsive element (MAPK-Nrf2-ARE) signaling pathway involved in regulating the antioxidant status in muscle (Xu et al., 2018). Nrf2 was a redox-sensitive transcription factor regulating ARE, which regulated the expression of Nrf2-mediated phase II detoxifying enzyme genes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and heme oxygenase-1 (HO-1) (Zhang et al., 2019). As effective stimulus of Nrf2, both heat stress and nutritional intervention could restore Nrf2 level, then regulated Nrf2-mediated phase II detoxifying enzyme gene expression, and change the antioxidant defense capacity (Sahin et al., 2016; Lu et al., 2019; Hu et al., 2020). Our previous studies demonstrated that the link between the poor meat quality and breast muscle oxidative damage in broilers exposed to chronic heat stress. Dietary chitosan oligosaccharides (COS) supplementation could alleviate chronic heat stress induced antioxidative damage in breast muscle, evidenced by decreasing MDA level, and increasing SOD and GSH-Px activity, which were paralleled with better meat quality (Chang et al., 2020). It also reported that COS could alleviate oxidative damage by hydrogen peroxide (H_2O_2) via activating Nrf2 pathway and upregulating the relative expression of HO-1, and enhanced antioxidant enzyme activities (Lan et al., 2020a; Lan et al., 2021). However, few was known about whether MAPK-Nrf2-ARE-mediated antioxidant defense system and antioxidant enzymes were integrated in the response of COS on AHS-induced oxidative damage in broilers, and finally led to poor meat quality. To further illustrated the effects of COS on AHS-induced poor meat quality, oxidative status and the mechanisms involved, this study was to evaluate the effects of dietary COS supplementation on meat quality, oxidative status, and the expression of MAPK-Nrf2-ARE signaling pathway related genes in breast muscle of broilers exposed to AHS.

A total of 200 one-day-old female Chinese indigenous chicken (Luhua chicken) was provided by a commercial hatchery (Maoming Wannong Qinye Co. Ltd, Guangdong, China). All broilers were fed the same starter diet from d 1 to 35, and on d 35, 108 broilers with similar body weight were selected and then randomly allocated to 3 treatments with 6 replicates of 6 broilers each: control (CON) group, AHS group, and AHS and dietary 400 mg/kg COS supplementation (AHS-C) group. Both the CON and AHS groups given the basal diet, and the AHS-C group given the basal diet with 400 mg/kg COS supplementation. From experimental d 1 to 41, the temperature and humidity in all groups were maintained at 24°C and 65%, respectively. On d 42, the broilers in the CON group still maintained at 24°C, while the broilers in the AHS and AHS-C groups the temperature started increasing at 08:00 with an increase of 10°C in 2-h and kept at 34°C for 2-h (Figure. 1). Broilers of each replication were assigned in battery pens (124 cm length × 64 cm width × 40 cm height). The basal diet (Table 1) was formulated to meet or exceed the nutrient requirement of the Feeding Standard of Chicken, China (NY/T 33-2004). COS was provided by Huizhou Changlong Biotechnology Co., Ltd. (HPLC purity 95%, deacetylation degree over 95% and average molecular weight was 1200Da). The feed was provided in mash form, and the supplementation of COS to the basal diet at the expense of corn. After AHS exposure, immediately following sample collection, 18 broilers (n = 6/group) with similar body weight were selected and the entire right breast muscle was collected and stored at 4°C for the measurement of meat quality. Samples from the left breast muscle were collected to determine the level of ROS and antioxidant capacity immediately, and about 1-g sample was collected, quick freezing in liquid nitrogen and stored at -80°C for

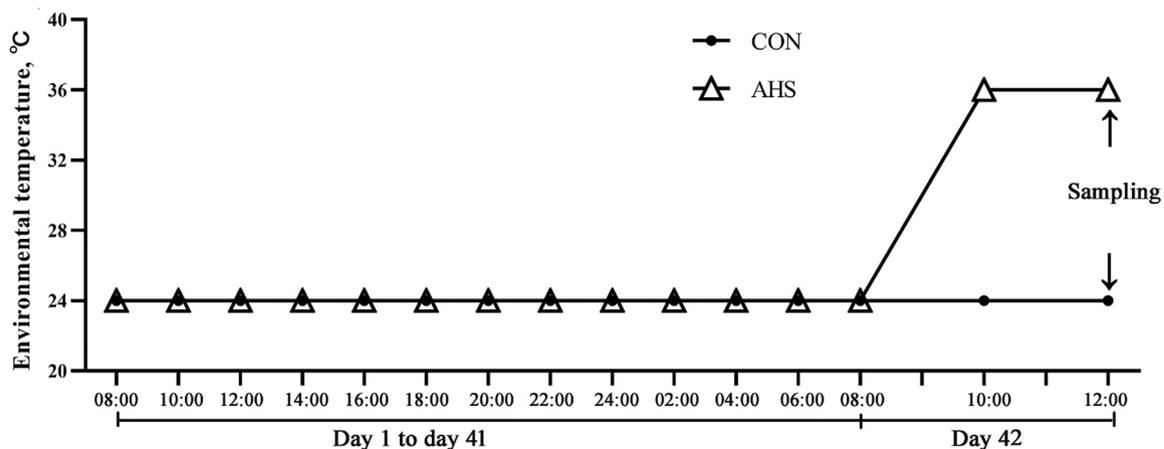


Figure 1. Illustration of acute heat stress treatment. During the experimental period, the temperature in the control (CON) group was maintained at 24°C. On d 42, the temperature in the acute heat stress (AHS) and AHS-C groups the temperature started increasing at 08:00 with an increase of 10°C in 2-h and kept at 34°C for 2-h.

Table 1. Basal diet composition (as-fed basis).

Ingredients	Content, %
Corn	69.95
Soybean	22.10
Soybean oil	2.70
Calcium hydrogen phosphate	1.70
Shell power	1.93
Salt	0.35
Met	0.10
Lys	0.05
Zeolite powder	0.80
Vitamin premix ¹	0.16
Mineral premix ²	0.16
Total	100.00
Nutrient level	
Metabolic energy, MJ/kg	12.65
Crude protein, %	16.29
Calcium, %	1.18
Total phosphorus, %	0.62
Available phosphorus, %	0.41
Met, %	0.36
Lys, %	0.87
Met + Cys, %	0.64

¹Provided per kilogram of complete diet: 12,800 IU Vitamin A, 1,600 IU Vitamin D₃, 60 IU Vitamin E, 1.6 mg Vitamin K₃, 0.12 mg Biotin, 50 mg Choline, 1.2 mg Folic acid, 32 mg Nicotinic acid, 16 mg Pantothenic acid, 4.8 mg Riboflavin, 2.4 mg Thiamine (VB₁), 3.2 mg Vitamin B₆, and 0.03 mg Vitamin B₁₂.

²Provided per kilogram of diet: Mg, 79 mg as manganese oxide; Zn, 60 mg as zinc oxide; Cu 100 mg as copper sulfate; Fe, 120 mg as iron sulfate; I, 0.96 mg as potassium iodine; Co, 0.16 mg as cobalt sulfate and Se, 0.24 mg as sodium selenite.

mRNA expression. The experimental protocol used in this study followed the Animal Care and Use Committee of Guangdong Ocean University (SYXK-2018-0147).

Meat Quality Measurement

Both at 45 min ($\text{pH}_{45\text{min}}$) and 24h ($\text{pH}_{24\text{h}}$) postmortem, muscle pH was measured using a pH-meter. Each sample was measured at three different locations and calculate the average value as result. Muscle color were measured using a CR410 Chroma Meter (Konica Minolta Sensing Inc., Osaka, Japan) at both 45 min and 24 h postmortem. When storing the breast muscle sample at 4°C for 24 h, cooking loss, drip loss, and WHC were measured following the methods described by Dai et al. (2018). After cooking loss measurement, the samples were cut into strips parallel to the muscle fibers then shear force was measured by using a CILM3 digital meat tenderness meter (Northeast Agricultural University, Harbin, China).

Free Radical Scavenging Capacity Measurement

Commercial kits were used for the measurements of 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) ($\text{ABTS}^{\bullet+}$), superoxide anion free radicals ($\text{O}_2^{\bullet-}$), and H_2O_2 scavenging activity. The methods were following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Reactive Oxygen Species Measurement

ROS measurements were using an ROS-measurement kit (Nanjing Jiancheng Bioengineering Institute) followed the methods described by Song et al. (2012). Briefly, the fresh samples were used to prepare single-cell suspensions for flow cytometry. Then the flow cytometer (FC500 MCL/MPL, Beckman Coulter Inc., CA) with the excitation at 488 nm and emission at 525 nm were used to monitor the formation of 2',7'-dichloro-fluorescein (DCF). ROS generation was quantified by the mean fluorescence intensity of the CON group.

Antioxidant Status Measurement

One gram breast muscle sample was homogenized in 9 mL ice-cold phosphate buffer saline (PBS), and centrifuged at 3000 *g* at 4°C for 10 min, then collected the supernatant for further assay. The bicinchoninic acid (BCA) method was used to measure the protein concentration. Commercial kits were used for the measurements of total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and malondialdehyde (MDA) following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Real-Time Quantitative PCR

Total mRNA extraction, cDNA reverse transcription, and quantitative real-time polymerase chain reaction (RT-PCR) analysis were followed the methods described in our previous research (Lan et al., 2014). The primers were designed by Primer premier 5.0 software according to the sequences described in GenBank and shown in Table 2. The PCR reactions were performed in triplicate, and the results were normalized to β -actin mRNA expression. The relative mRNA expression was calculated by $2^{-\Delta\Delta\text{Ct}}$ method.

Statistical Analyses

The replication pen as used as the experiment unit and performed with SAS 2003 (v. 9.1, SAS Institute Inc., Cary, NC). Differences of the rectal and body surface temperature between before and after AHS exposure were tested using Tukey's test, and Duncan's multiple-range tests were used to analyze the differences among the means. The results were expressed as mean \pm standard error, and significance was set at $P < 0.05$.

RESULTS

Temperature Profile

The results of the rectal and surface temperature (comb, eyelids, and feet) in broilers challenged by AHS were shown in Figure 2. As expected, after AHS exposure, rectal and comb temperature increased ($P < 0.05$)

Table 2. The primer sequences.

Gene ¹	Accession NO.	Primer sequence (5' to 3')	Product size (bp)
β -actin	NM_205518.1	F: ATCCGGACCCTCCATTGTC R: AGCCATGCCAATCTCGTCTT	120
ERK1/2	NM_204150.1	F: AGCAAGCTTTAGCCCATCCA R: CCTTCGGCAAGTCATCCAAT	108
JNK	NM_205095.1	F: AGCAGCCTCGATGCCTTGAC R: CAAGCAATTCAGGCCCAATG	110
p38 MAPK	XM_001232615.2	F: TGTGTTCAACCCTGCCAAGT R: GCCCCGAAGAATCTGGT T	149
Nrf2	NM_205117.1	F: ATCACGAGCCCTGAAACCAA R: GGCTGCAAAATGCTGGAAAA	143
HO-1	XM_205344.1	F: ACTTCTATGGCAGCAACT R: AATAGCGGG GTAGGC	129
SOD	NM_205064.1	F: TTGTCTGATGGAGATCATGGCTTC R: TGCTTGCCTTCAGGATTAAGTGG	98
GSH-Px	NM_000581.4	F: GATGAGATCCTGAGAGTGGTGGAC R: TCATCAGGTAAGGTGGGCACAA	116
CAT	NM_001031215.2	F: TACGGTTCCTCACTGTTGCTG R: TGGATGAAGGATGGAAACAAC	177

¹Abbreviations: CAT, catalase; Cu/Zn-SOD, Cu/Zn-superoxide dismutase; ERK1/2, extracellular-signal regulated kinase; GSH-Px, glutathione peroxidase; HO-1, heme oxygenase 1; JNK, c-Jun N-terminal kinase; Nrf2, nuclear factor-erythroid 2-related factor 2; p38 MAPK, p38 mitogen-activated protein kinase.

in both AHS and AHS-C groups, and eyelids temperature increased ($P < 0.05$) in AHS group. No significant differences were observed in feet temperature after AHS exposure.

Meat Quality

Table 3 shows the effects of COS on meat quality after AHS exposure. AHS exposure decreased ($P < 0.05$) breast muscle pH_{45min}, pH_{24h}, redness (a^*), however, increased ($P < 0.05$) lightness (L^*), drip loss, and shear force when compared to the CON group. Compared to the AHS group, dietary COS supplementation increased ($P < 0.05$) breast muscle pH_{45min}, pH_{24h}, a^* , however, decreased ($P < 0.05$) cooking loss and shear force.

Free Radical Scavenging Capacity

The ABTS^{•+}, H₂O₂, and O₂^{•-} radical scavenging capacities of breast muscle were shown in Figure 3. The H₂O₂ radical scavenging capacity in AHS and AHS-C groups were higher ($P < 0.05$) than that of CON group at 45 min postmortem, whereas at 24 h postmortem, the H₂O₂ radical scavenging capacity in the AHS-C group was higher ($P < 0.05$) than both CON and AHS groups. The O₂^{•-} radical scavenging capacity in the CON and AHS groups were higher ($P < 0.05$) than both AHS-C group at both 45 min and 24 h postmortem. No significant differences were observed in ABTS^{•+} scavenging capacity among the treatments.

ROS Generation and Antioxidant Status

The effects of AHS exposure on the ROS generation and antioxidant capacities of breast muscle were shown in Figures 4 and 5, respectively. Figure 4 demonstrated that there was higher ($P < 0.05$) ROS and MDA content (Figure 5A) of breast muscle in AHS group when

compared to the CON group. Dietary COS supplementation decreased ($P < 0.05$) ROS and MDA content when compared to the AHS group. In addition, CAT activity in the AHS and AHS-C groups were increased ($P < 0.05$) when compared to the CON group at 24 h postmortem. No significant differences were observed in T-SOD or GSH-Px activity among treatments.

Gene Expression of MAPK-Nrf2-ARE Signaling Pathway

The relative expression of MAPK-Nrf2-ARE signaling pathway in breast muscle were demonstrated in Figure 6. The relative expression of extracellular-signal regulated kinase1/2 (ERK1/2) in the AHS-C group was increased ($P < 0.05$) when compared to the CON group, the relative expression of HO-1 in the AHS group was decreased ($P < 0.05$) when compared to the CON and AHS-C groups, the relative expression of GSH-Px in the AHS-C group was increased ($P < 0.05$) when compared to the CON and AHS groups. No significant differences were observed in the relative expression of p38 MAPK (p38 mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase), Nrf2, CAT, or SOD.

DISCUSSION

Temperature was the general indicator of the heat load status, could partly reflect the broilers' thermal balance and response of heat stress (Farag and Alagawany, 2018). Heat stress was generally associated with high body temperature (Xie et al., 2015; Zhang et al., 2018; He et al., 2019). In this study, broilers subjected to AHS significantly increased rectal, comb and eyelids temperature, suggested the physiological response and validated model of heat stress in broilers. Former literatures 216-217 indicated that the body temperature of pigs, cattle, and cows would exceed their thermoneutral

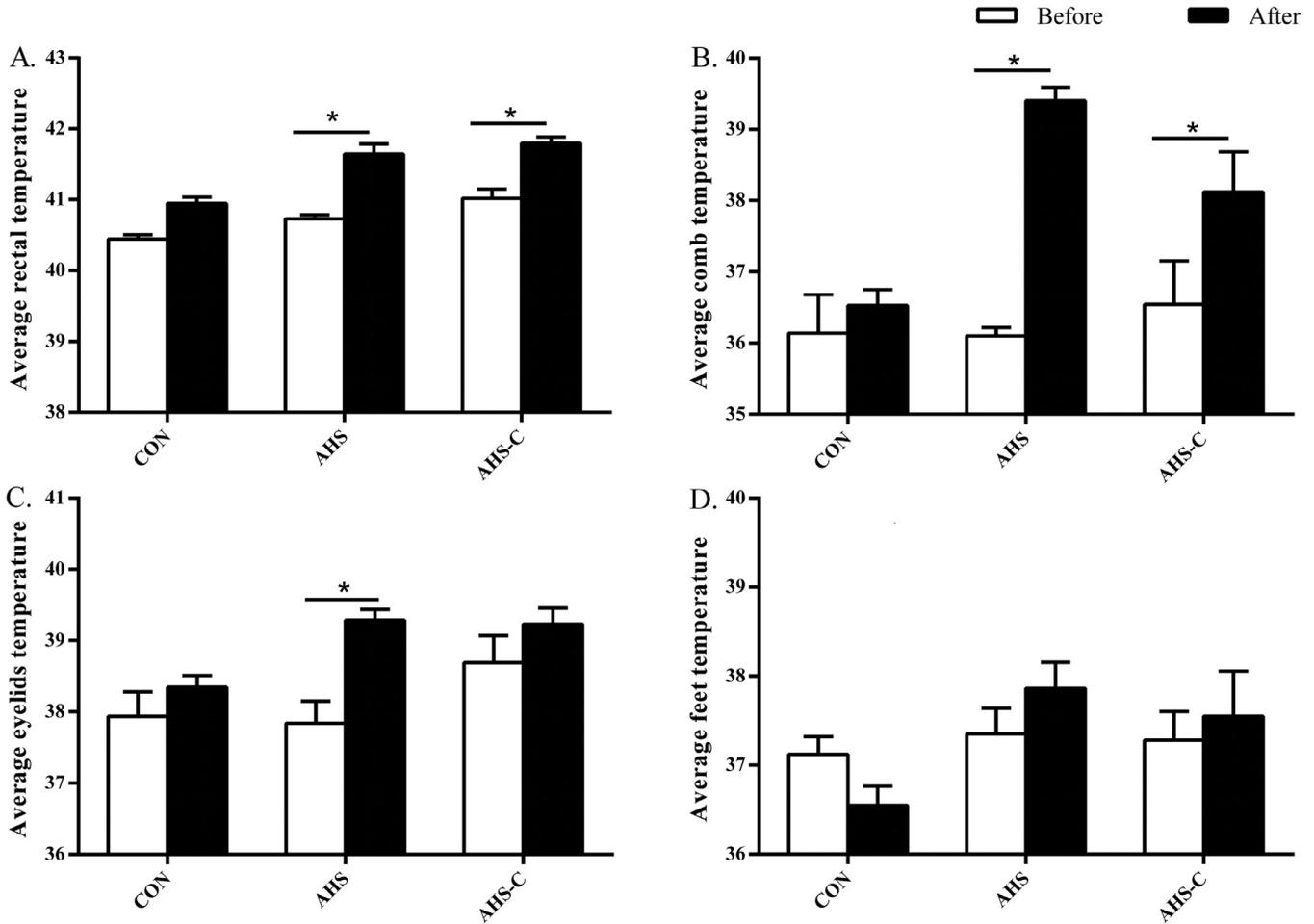


Figure 2. Effects of acute heat stress exposure on rectal and body surface temperature in broilers. (A) average rectal temperature; (B) average comb temperature; (C) average eyelids temperature; (D) average feet temperature. Before, before acute heat stress exposure; After, after acute heat stress exposure. Results are presented as mean \pm standard error (n = 6). * Means significant different according to Tukey's test ($P < 0.05$). Abbreviations: AHS, basal diet + acute heat stress challenge; AHS-C, 400 mg/kg COS + acute heat stress challenge; CON, basal diet + normal temperature; COS, chitosan oligosaccharides.

zone when challenged by heat stress (Bernabucci et al., 2010; Yu et al., 2010).

Nowadays, consumers pay more and more attention to the quality of poultry meat. Meat quality is described as the sum of all meat quality characteristics, including pH, meat color, WHC, and shear force. It was widely accepted that heat stress could impair meat quality and might lead to pale, soft, exudative (PSE)-like syndrome

Table 3. Effects of COS on the meat quality in broilers after acute heat stress exposure.

Item ¹	CON	AHS	AHS-C
pH _{45min}	6.30 \pm 0.06 ^a	6.19 \pm 0.06 ^b	6.24 \pm 0.12 ^a
pH _{24h}	5.85 \pm 0.06 ^a	5.69 \pm 0.12 ^b	5.79 \pm 0.05 ^a
L* (Lightness)	51.24 \pm 1.27 ^a	54.38 \pm 1.78 ^b	52.42 \pm 1.00 ^{ab}
a* (Redness)	5.13 \pm 0.18 ^a	4.69 \pm 0.17 ^b	5.15 \pm 0.16 ^a
b* (Yellowness)	14.97 \pm 0.54	15.21 \pm 0.38	14.96 \pm 0.27
Cooking loss, %	12.92 \pm 0.60	16.92 \pm 1.05	12.87 \pm 0.73
Drip loss, %	1.63 \pm 0.47 ^a	2.10 \pm 0.65 ^b	2.04 \pm 0.52 ^a
Shear force, N	14.61 \pm 0.27 ^a	18.46 \pm 0.61 ^b	15.18 \pm 0.62 ^a

Abbreviations: CON, basal diet + normal temperature; AHS, basal diet + acute heat stress challenge; AHS-C, 400 mg/kg COS+ acute heat stress challenge; COS, chitosan oligosaccharides.

¹Results are presented as mean \pm standard error (n = 6).

^{a,b}Values in the same row with different letters are significant different ($P < 0.05$).

of poultry meat, as evidenced by low WHC, soft texture, and light appearance (Carvalho et al., 2017). In this study, AHS exposure decreased pH_{45min}, pH_{24h}, and a*, whereas increased L*, drip loss and shear force, which is consistent with former studies (Wang et al., 2009; Dai et al., 2018). Meat color directly affected by pH and protein denaturation in the muscle, the higher L* and lower a* were attributed to the denaturation of sarcoplasmic proteins, which elevated light scattering in the muscle (Bendall and Wismer-Pedersen, 1962; Barbut et al., 2008). Oxidative damage was regarded as the major reason for poor meat quality of heat-stressed broilers, and numerous nutritional manipulation managements were used to alleviating heat stress-induced poor meat quality (Kanani et al., 2017; Zhang et al., 2017; Zhuang et al., 2017). In this study, dietary COS supplementation increased pH_{45min}, pH_{24h}, and a*, whereas decreased drip loss and shear force in broilers exposed to AHS. Our previous study also indicated that dietary COS supplementation was an effective nutritional intervention way to attenuate chronic heat stress induced poor meat quality by improving b*, pH_{24h}, and cooking loss (Chang et al., 2020). Postmortem pH was classified as one of the most important characteristic of

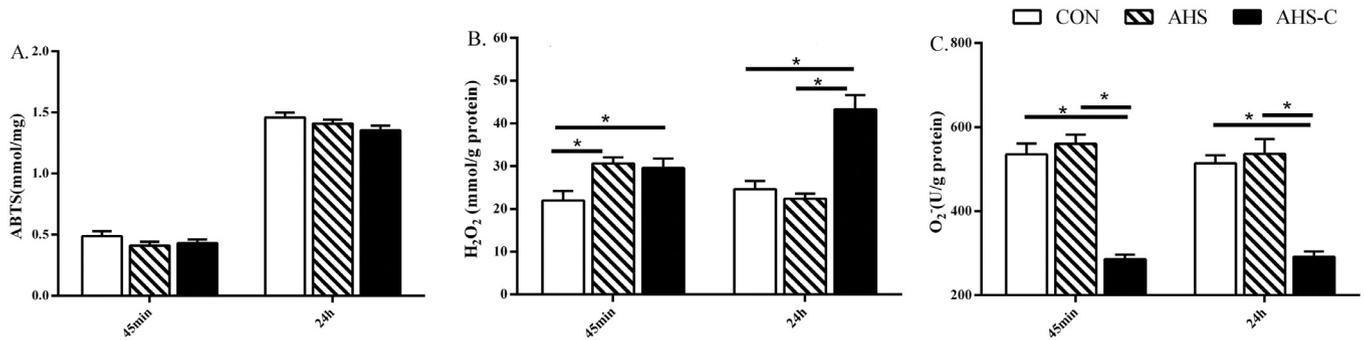


Figure 3. Effects of COS on free radical scavenging activity of breast muscle in broilers after acute heat stress exposure. (A) ABTS^{•+} scavenging activity; (B) H₂O₂ scavenging activity; (C) O₂^{•-} scavenging activity. Results are presented as mean ± standard error (n = 6). * Means significant different (*P* < 0.05). Abbreviations: AHS, basal diet + acute heat stress challenge; AHS-C, 400 mg/kg COS + acute heat stress challenge; ABTS^{•+}, 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate); CON, basal diet + normal temperature; COS, chitosan oligosaccharides; H₂O₂, hydrogen peroxide; O₂^{•-}, superoxide anion free radicals.

meat quality, which directly reflected the postmortem glycolysis (Zhang et al., 2012). Literatures concluded that chronic heat stress induced a lower postmortem pH with variation in meat color, drip loss, cooking loss and shear force via affecting muscle protein denaturing (Young et al., 2004; Wang et al., 2009; Gonzalez-Rivas et al., 2020). It was accepted that oxygen supply was removed postmortem, and the variation of postmortem pH was affected only by lactic acid accumulation, suggested that COS supplementation could alleviate AHS induced poor meat quality by slow-down pH decline.

AHS exposure increased broilers' body temperature, accompanied with increasing content of ROS and MDA compared to the CON group, which suggested that AHS induced oxidative damage. AHS affected the metabolic characteristics and induced oxidative damage to skeletal muscle in broilers, which was closely related to the overproduction of ROS (Azad et al., 2010). The increased level of ROS induced oxidative damage and triggered lipid peroxidation. In this study, the increased ROS and

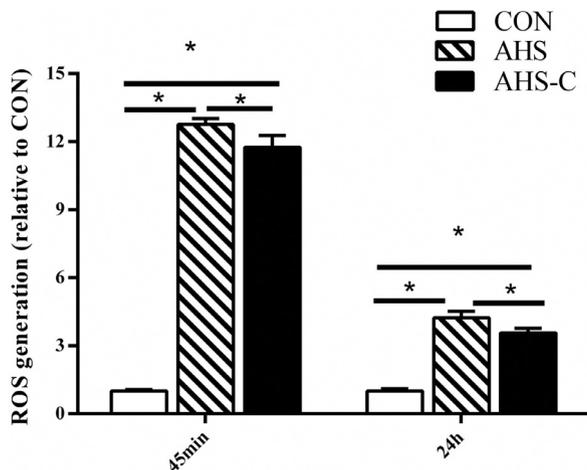


Figure 4. Effects of COS on ROS levels of breast muscle in broilers after acute heat stress exposure. Results are presented as mean ± standard error (n = 6). * Means significant different (*P* < 0.05). Abbreviations: 45 min, 45-minute postmortem; 24 h, 24-hour postmortem; AHS, basal diet + acute heat stress challenge; AHS-C, 400 mg/kg COS + acute heat stress challenge; CON, basal diet + normal temperature; COS, chitosan oligosaccharides; ROS, reactive oxygen species.

MDA level of breast muscle in broilers after AHS exposure, suggested the oxidative damage in breast muscle. Similar results also reported by Zhang et al. (2015) and Lu et al. (2019), who indicated that there were increased ROS and MDA level in chronic heat stress exposure broilers. Wang et al. (2009) and Mujahid et al. (2009) also indicated that broilers exposed to AHS significantly increased MDA content in breast or skeletal muscle. AHS exposure increased MDA and ROS level, suggested the imbalance of antioxidant system. In this study, T-SOD, GSH-Px, and CAT activities in breast muscle were also measured to evaluate the response of antioxidant enzymatic scavenging system. Compared with the CON group, AHS exposure significantly increased CAT activity at 24 h postmortem, but had no effects on T-SOD or GSH-Px activity. These results were in consistent with Dai et al. (2018), who indicated that AHS exposure had no significant differences in CAT, GSH-Px, or T-AOC activity in breast muscle. Xie et al. (2015) also reported that neither acute or chronic heat stress had no significant effects on plasma MDA, protein carbonyl content, or T-SOD activity. These differences might due to different exposure temperature, last time, or tissue-specific. While, Chang et al. (2020) demonstrated that dietary COS could decrease MDA content, and increase SOD and GSH-Px activity in breast muscle of broilers under chronic heat stress. In this study, dietary COS supplementation increased the CAT activity, as well as decreased the ROS and MDA level in breast muscle. These results in consistent with the results of Liu et al. (2009), who reported that dietary COS supplementation alleviated H₂O₂-induced increasing in ROS and MDA level. Zhang et al. (2019) also indicated that COS reduced the ROS production induced by doxorubicin. The decreased ROS and MDA level, as well as the increased CAT activity in the AHS-C group, suggested that dietary COS supplementation could partly alleviate the oxidative damage induced by AHS. However, there was no significant difference in the CAT activity between the AHS and AHS-C groups, which suggested that the positive effects of COS were not related to improve anti-oxidant enzymes directly.

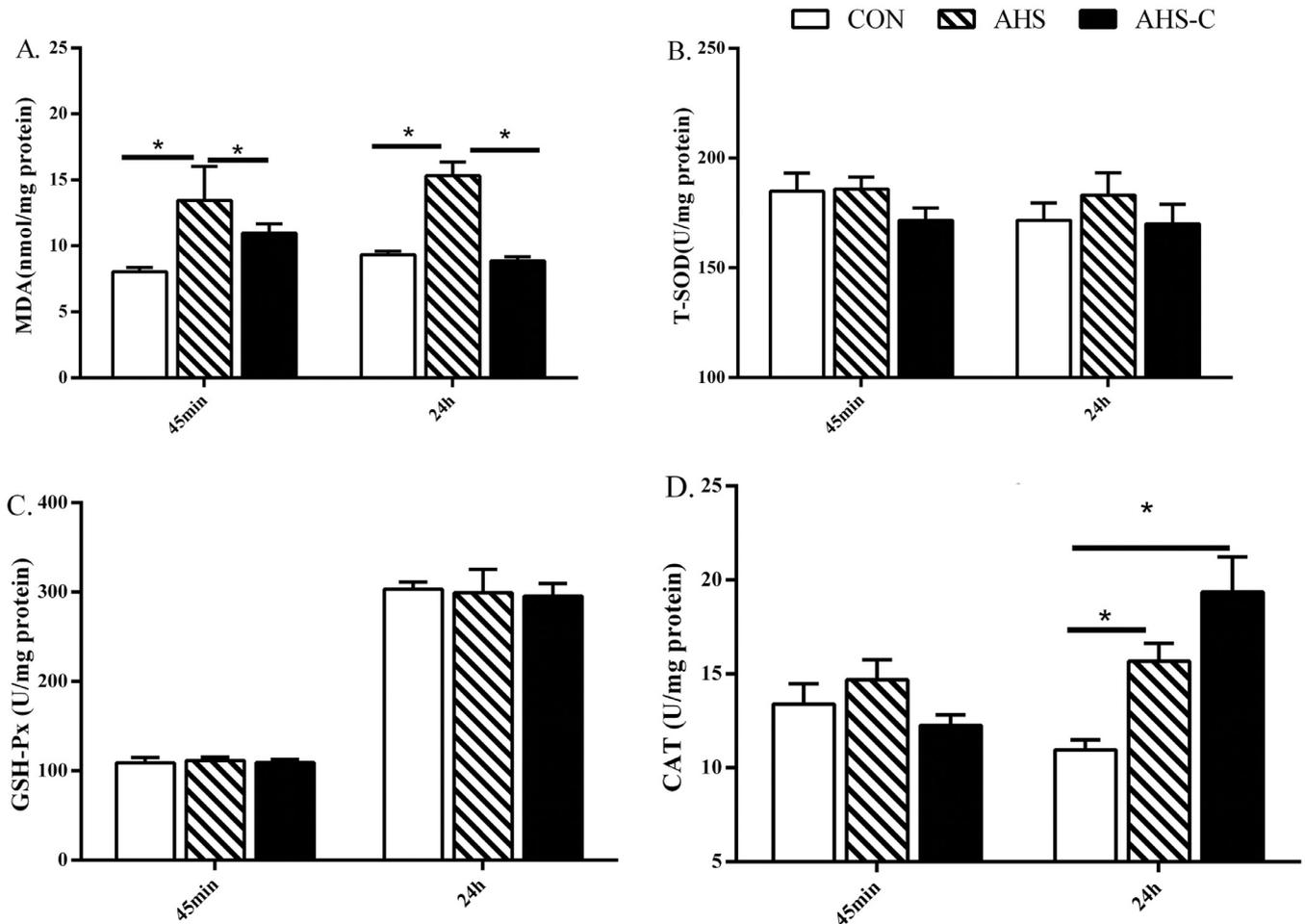


Figure 5. Effects of COS on antioxidant capacity of breast muscle in broilers after acute heat stress exposure. (A) MDA; (B) T-SOD; (C) GSH-Px; (D) CAT. Results are presented as mean \pm standard error ($n = 6$). * Means significant different ($P < 0.05$). Abbreviations: 45 min, 45-minute postmortem; 24 h, 24-hour postmortem; AHS, basal diet + acute heat stress challenge; AHS-C, 400 mg/kg COS + acute heat stress challenge; CON, basal diet + normal temperature; CAT, catalase; COS, chitosan oligosaccharides; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; T-SOD, total superoxide dismutase.

As former mentioned, AHS induced ROS overproduction and lipid peroxidation, the increased H_2O_2 radical scavenging activity in AHS group might associated with higher ROS production, to combat with oxidative damage, an endogenous mechanism developed. Similar, Cramer et al. (2018) indicated that there were increased DPPH radical scavenging activity in breast muscle of broilers under chronic heat stress. However, Wan et al. (2018) reported that chronic heat stress decreased ABTS $^{\bullet+}$, $O_2^{\bullet-}$, and H_2O_2 radical scavenging activity, but had no effects on DPPH radical scavenging activity in breast muscle. In this study, no significant differences were observed in ABTS $^{\bullet+}$ or $O_2^{\bullet-}$ radical scavenging activity. It notable that COS had excellent antioxidant and radical scavenging capacity both in vitro and in vivo (Lan et al., 2020a; Lan et al., 2021). Our former studies indicated that dietary COS supplementation enhanced DPPH, ABTS $^{\bullet+}$, $O_2^{\bullet-}$, and $OH^{\bullet-}$ radical scavenging activity in jejunum mucosa of rats under H_2O_2 exposure (Lan et al., 2021). However, in this study, dietary COS supplementation significantly increased H_2O_2 radical scavenging activity in AHS group, decreased $O_2^{\bullet-}$ radical scavenging activity in AHS group at both 45 min and 24 h postmortem. H_2O_2

and $O_2^{\bullet-}$ radical scavenging activity displayed differences to COS supplementation. These results, we cannot exclude the possibility of COS capacities. We speculated that dietary COS supplementation alleviating AHS was not enhancing free radical scavenging activities directly, but might be associate with decreased ROS production and CAT activity.

Oxidative stress usually activates certain signaling pathways, including MAPK-Nrf2-ARE signaling pathways (Zhang et al., 2020). Nrf2 was a redox-sensitive nuclear transcription factor that regulating ARE transcription, HO-1, CAT, GSH-Px, and SOD were key enzymes regulated by Nrf2 (Zhang et al., 2019). MAPK was critical ubiquitous intracellular kinase for the nuclear transcription of Nrf2, former studies indicated that MAPK signaling pathway could modulate Nrf2-ARE signaling pathway (Xu et al., 2018; Zhang et al., 2018; Hu et al., 2020). Former literatures reported that electrical stunning up-regulated the relative expression of ERK1/2, p38 MAPK, JNK1, JNK2, Nrf2, and SOD (Xu et al., 2018). In this study, no significant differences were observed in the relative expression of ERK1/2, p38 MAPK or JNK between the CON and AHS groups. While, dietary COS supplementation increased the

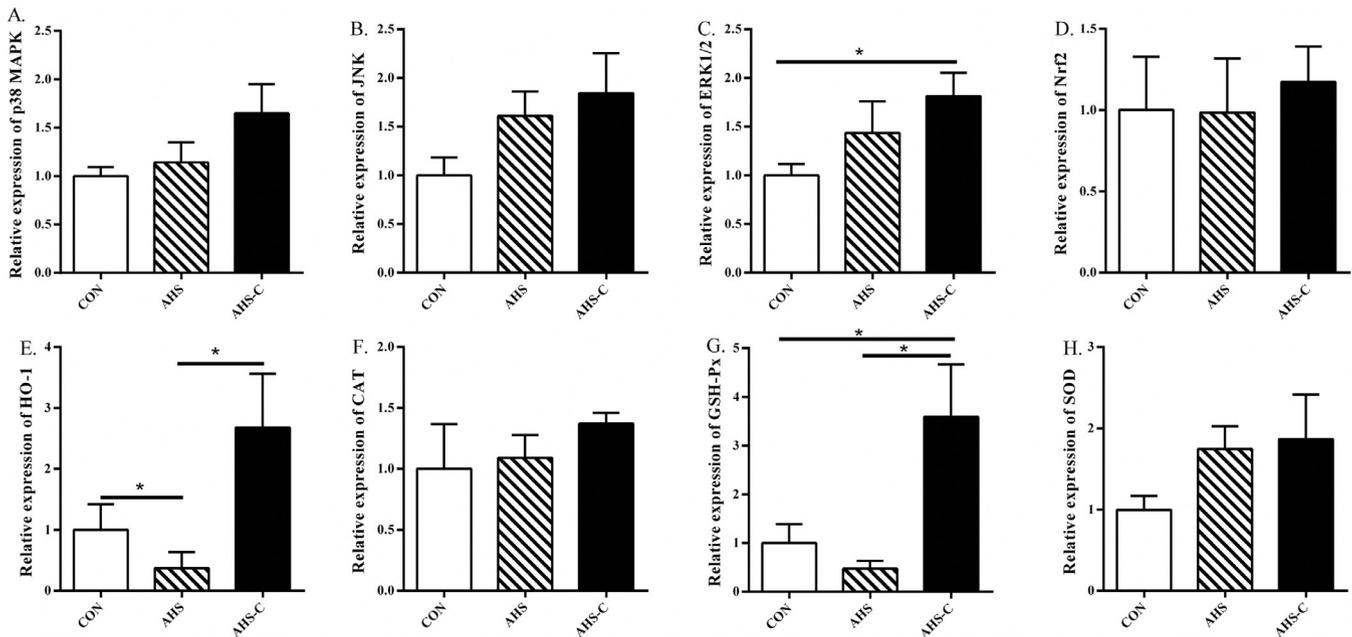


Figure 6. Effects of COS on gene expression of breast muscle in broilers after acute heat stress exposure. (A) p38 MAPK; (B) JNK; (C) ERK1/2; (D) Nrf2; (E) HO-1; (F) CAT; (G) GSH-Px; (H) SOD. Results are presented as mean \pm standard error ($n = 6$). * Means significant different ($P < 0.05$). Abbreviations: AHS, basal diet + acute heat stress challenge; AHS-C, 400 mg/kg COS + acute heat stress challenge; CAT, catalase; CON, basal diet + normal temperature; COS, chitosan oligosaccharides; ERK1/2, extracellular-signal regulated kinase; GSH-Px, glutathione peroxidase; HO-1, heme oxygenase 1; JNK, c-Jun N-terminal kinase; Nrf2, nuclear factor-erythroid 2-related factor 2; p38 MAPK, p38 mitogen-activated protein kinase; SOD, superoxide dismutase.

relative gene expression of ERK1/2. Former literatures reported that heat stress mediated oxidative damage via Nrf2-ARE signaling pathway, chronic heat stress down-regulated the relative gene expression of Nrf2, HO-1, Cu/Zn SOD, CAT, and GSH-Px in liver of broilers, as well as Nrf2 in breast muscle (Zhang et al., 2018; Hu et al., 2020). The results not always in consistent, Zhang et al. (2019) indicated that chronic heat stress had no significant differences in the relative expression of Nrf2, keap1, NQO-1, or HO-1 in breast muscle. In this study, we found that AHS exposure had no significant differences in the relative expression of Nrf2, CAT, GSH-Px, or SOD, but downregulated the relative expression of HO-1. When compared to the AHS group, COS supplementation significantly increased the relative expression of HO-1 and GSH-Px, which suggested that the Nrf2-ARE signaling pathway could be activated by dietary COS supplementation. Based on these results, we speculated that COS may attenuate oxidative stress via ERK1/2 activated of HO-1 and GSH-Px gene expression. Similar results also reported in cell models (Hyung et al., 2016; Zhang et al., 2020).

It was concluded that AHS impaired meat quality by decreasing $\text{pH}_{45\text{min}}$, $\text{pH}_{24\text{h}}$, a^* , and increasing L^* , drip loss, and share force, which may be related to oxidative damage, as evidenced by increasing ROS production, MDA content, and decreasing the relative expression of HO-1. Dietary COS supplementation could effectively elevate the meat quality of broilers exposed to AHS via decreasing ROS production, activating the Nrf2 pathway and Nrf2-mediated HO-1 and GSH-Px gene expression.

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DISCLOSURES

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

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