Hydrogen peroxide–induced oxidative stress impairs redox status and damages aerobic metabolism of breast muscle in broilers

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ABSTRACT Oxidative stress has always been a hot topic in poultry science. However, studies concerning the effects of redox status and glucose metabolism induced by hydrogen peroxide (**H_2O_2**) in the breast muscle of broilers have been rarely reported. This study was aimed to evaluate the impact of intraperitoneal injection of H_2O_2 on oxidative damage and glycolysis metabolism of breast muscle in broilers. We also explored the activation of the nuclear factor erythroid 2–related factor 2 (**Nrf2**) signaling pathway to provide possible mechanism of the redox imbalance. Briefly, a total of 320 one-day-old Arbor Acres chicks were randomly divided into 5 treatments with 8 replicates of 8 birds each (noninjected control, 0.75% saline-injected, 2.5, 5.0, and 10.0% H_2O_2-injected treatments). Saline group was intraperitoneally injected with physiological saline (0.75%) and H_2O_2 groups received an intraperitoneal injection of H_2O_2. The dosage of the injection was 1.0 mL/kg BW. All birds in the saline and H_2O_2 groups were injected on days 16 and 37 of the experimental period. At 42 d of age, 40 birds (8 cages per group and one chicken per cage) were selected to be stunned electrically (50 V, alternating current, 400 Hz for 5 s each one), and then immediately slaughtered via exsanguination. The results showed that broilers in the H_2O_2 injection group linearly exhibited higher contents of reactive oxygen species, carbonyl and malondialdehyde, and lower total antioxidant capacity and glutathione peroxidase activities. With the content of H_2O_2 increased, the H_2O_2 groups linearly downregulated the mRNA expressions of GPX, CAT, HMOX1, NQO1, and Nrf2 and its downstream target genes. In addition, H_2O_2 increased serum activities of creatine kinase and lactate dehydrogenase. Meanwhile, in the pectoral muscle, the glycogen content was linearly decreased, and the lactate content was linearly increased in muscle of broilers injected with H_2O_2. In addition, the activities of glycolytic enzymes including pyruvate kinase, hexokinase, and lactate dehydrogenase were linearly increased after exposure to H_2O_2. In conclusion, H_2O_2 injection could impair antioxidant status and enhance anaerobic metabolism of breast muscle in broilers.

Key words: hydrogen peroxide, reactive oxygen species, antioxidant capacity, broiler, glycolysis metabolism, Nrf2 signaling pathway

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INTRODUCTION

In recent years, the development of poultry industry in China has ranked among the top in the world; however, the risk of oxidative stress to broilers is also increasing (Estévez, 2015). The factors that induce oxidative stress in broilers are complicated, including intensive farming, malnutrition, extreme environments, and long-distance transportation (Guo et al., 2020). Previous reports showed that oxidative stress reduces the antioxidant capacity of poultry and increases reactive species, causing the reduction of disease resistance and thereby affecting broiler health (Jiang et al., 2015; Liao et al., 2019; Zhu et al., 2020).

Researchers usually use a single stressor to indirectly cause oxidative stress on studying oxidative damage in poultry. However, the intrinsic incentive of these stressors is different and the treatment methods are different. Therefore, the results of oxidative damage vary and the exact mechanism is difficult to elucidate.

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Hydrogen peroxide (H_2O_2) has been reported to cause oxidative damage by increasing reactive oxygen species (**ROS**) levels (Yin et al., 2015). Previous studies indicated that intraperitoneal injection of H_2O_2 could cause various degrees of oxidative stress, lipid peroxidation and disrupted intestinal permeability, morphology and barrier function in piglets (Celik and Ozkaya, 2002; Yin et al., 2015). Recently, we directly increase ROS levels through intraperitoneal injection of H_2O_2 to establish use the oxidative stress model of in broilers to further explore the impacts of oxidative damage (Chen et al., 2017).

Oxidative stress reflects the imbalance between excessive ROS and redox status (Ahmad et al., 2012). The nuclear factor erythroid 2–related factor 2 (**Nrf2**) plays an important role in maintaining redox balance. When ROS level exceeds the capacity of the antioxidant defense system, the activities of many antioxidant enzymes such as superoxide dismutase (**SOD**), catalase, and glutathione peroxidase (**GSH-Px**) will change (Schieber and Chandel, 2014). Oxidative stress can lead to the ROS-mediated damage of nucleic acids, proteins, and lipids. Antioxidant enzymes are the main antioxidants that can resist excessive ROS attacks (Shacter, 2000). Consequently, under oxidative stress, the defense system changes the regulation and expression of these enzymes (Arsova-Sarafinovska et al., 2009). Oxidative stress can reduce growth and development through systemic hypoxia (Kassahn et al., 2009). Insufficient oxygen supply to muscle, which cannot rely on aerobic respiration, so glycolysis becomes the main source of energy for muscle cells (Shen et al., 2014). The lactic acid produced during glycolysis continues to accumulate in muscle and lead to the decline of muscle pH (Zhang et al., 2017). Previous research in this laboratory found that intraperitoneal injection of H_2O_2 significantly reduced the pH at 24 h post mortem of chicken breast muscles (Chen et al., 2017).

Therefore, the present study was conducted to evaluate the effects of intraperitoneal injection of H_2O_2 on redox status, muscle damage as well as the glycolysis metabolism of broilers. Furthermore, we try to elucidate the mechanism by examining the Nrf2 signaling pathway.

MATERIALS AND METHODS

Preparation of H_2O_2 Solutions

The 30% H_2O_2 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was diluted with 0.75% sodium chloride buffer (saline) into 3 different concentrations (2.5, 5.0, and 10.0%). Concentrations were determined by commercial a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The H_2O_2 injections were kept in a dark and dry place.

Experimental Design, Animals, and Management

All experimental design and procedures involving the use of animals were approved by the Animal Care Committee of Nanjing Agricultural University, Nanjing, P.R. China (GB/T 35892-2018). At 1 d of age, a total of 320 male chicks with a similar body weight (50.12 ± 0.05 g) were selected and randomly allocated to 5 groups with 8 cages (replicates) per group and 8 chickens per cage (110 cm × 60 cm × 50 cm). In total, 40 cages were provided for chickens. In the control group, the chickens were provided with ad libitum access to feed and water; in the saline group, the chickens were injected intraperitoneally with physiological saline buffer (0.75%); in the H_2O_2 groups, the chickens were given an intraperitoneal injection of 2.5, 5.0, or 10.0% H_2O_2, with an injection dosage of 1.0 mL/kg BW. All the birds were fed a common basal feed, formulated according to the nutrient requirements of AA broilers, including a starter diet for 1 to 21 d and a grower diet for 22 to 42 d (Table 1). In the normal control group, birds were not injected H_2O_2 and physiological saline (0.75%). Birds in the saline treatment were intraperitoneally injected with physiological saline (0.75%). Birds in the H_2O_2 treatments were intraperitoneally injected with 2.5, 5.0, or 10.0% H_2O_2. The dosage of the injection was 1.0 mL/kg BW. All birds in the saline and H_2O_2 treatments were injected on day 16 and 37 of the experimental period. For the time and dose of H_2O_2 injections, refer to the method of Chen et al. (2017). Birds were provided with *ad libitum* access to feed and water. The lighting program was 23 h light and 1 h dark throughout the 42 d of the experiment periods. Temperature was kept

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

Item	1–21 d	22–42 d
Ingredients (%)		
Corn	57.61	62.27
Soybean meal	31.00	23.00
Corn gluten meal[1]	3.29	6.00
Soybean oil	3.11	4.00
Limestone	1.20	1.20
Dicalcium phosphate	2.00	2.00
L-lysine	0.34	0.35
DL-methionine	0.15	0.08
Salt	0.30	0.30
Premix[2]	1.00	1.00
Calculated nutrient levels		
ME (MJ/kg)	12.56	13.19
CP (%)	21.10	19.60
Ca (%)	1.00	0.95
Available phosphorus (%)	0.46	0.39
Lysine (%)	1.20	1.05
Methionine (%)	0.50	0.42
Methionine + cysteine (%)	0.85	0.76
Analyzed nutrient levels		
CP (%)	20.83	19.25
Ca (%)	1.02	0.98
Total phosphorus (%)	0.65	0.62

Abbreviations: Ca, calcium; ME, metabolizable energy.
[1]The crude protein (CP) content was 60%. Per kilogram of diet.
[2]Premix provided per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol for vitamin D_3, 2,500 IU; DL-α-tocopheryl acetate for vitamin E, 20 IU; menadione sodium bisulfate, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8.0 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B 12 (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 8.0 mg; Mn (from manganese sulfate), 110 mg; Zn (from zinc sulfate), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

between 32°C and 34°C from days 1 to 3, and then reduced to the ultimate temperature of 20°C at the rate of 2°C to 3°C per week.

Sample Collection and Preparation

At 42 d of age, 40 birds (one chicken per cage totaling 8 animals/treatment) close to average weight of the replicates were selected to be stunned electrically (50 V, alternating current, 400 Hz for 5 s each one), and then immediately slaughtered via exsanguination, necropsied for breast muscle collection. At the location of the left pectoralis major muscle, a slice was transferred to RNAase-free tubes and immediately frozen in liquid nitrogen before storing in ultra-low temperature freezer (−80°C) until further analysis. Blood samples were collected in sterilized centrifuge tubes, shaken gently, and then chilled on ice. The tubes were then centrifuged at 4°C for 15 min at 2,000 g, and serum was stored at −20°C until required for analysis.

Detection of ROS

The levels of ROS were detected by sensitive fluorescent (dichlorofluorescein) probe, and the determination process was operated in accordance with the instructions of the commercial kit (Nanjing Aoqing Bioengineering Institute, Nanjing, China), according to Sang et al. (2012). The oxidated derivatives of fluorescein were determined by fluorescent enzyme labeling instrument. The excitation wavelength was 488 nm and the emission wavelength was 525 nm. The percentage ratio of the average fluorescence value between the experimental group and the control group was calculated as the result of ROS level.

Measurement of Oxidative Parameters

The breast muscle tissue (1 g) stored in liquid nitrogen was homogenized in 9 mL of ice-cold phosphate buffer saline to prepare the tissue homogenate and then centrifuged at 1,500 rpm for 15 min at 4°C, and the supernatant was collected for the assay. The total antioxidant capacity (**T-AOC**, No. A015-1), the total superoxide dismutase (**T-SOD**, No. A001-1) activity, and the total GSH-Px (No. A005) activity were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with manufacturer's instructions.

The content of protein carbonyl was performed using commercial kit A087 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the manufacturer's instructions. After kit usage, absorbance was detected spectrophotometrically at 370 nm. Malondialdehyde (**MDA**) content was determined using the commercial kit A003 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Blood Parameter Measurements

The activities of serum creatine kinase (**CK**) and lactate dehydrogenase (**LDH**) were measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's instructions.

Measurements of Muscle Lactate, Glycogen, and Key Glycolytic Enzymes

Glycogen content measurement was performed in duplicate using a commercial kit A043 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Lactate content was performed spectrophotometrically (530 nm) using a commercial kit A019 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The enzyme activities of hexokinase (**HK**), pyruvate kinase (**PK**), and LDH were evaluated using commercial kits A077, A076, and A020 (Nanjing Jiancheng Bioengineering Institute). The glycolytic potential (**GP**) was calculated in accordance with the formula GP = 2[glycogen] + [lactate] (Monin and Sellier, 1985).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was isolated from the breast muscle using TRIzol reagent (Takara Biotechnology Company Ltd., Dalian, China). The purity and concentration of the RNA were measured using a NanoDrop 1,000 spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA was processed by DNase I (TaKaRa Biotechnology Co. Ltd., Dalian, China) for DNA removal and reverse transcribed to cDNA using the Prime Script RT Master Mix kit (TaKaRa Biotechnology Co. Ltd., Dalian, China). The RT reaction was conducted in a total volume of 20 μL. The RT program is the reaction mixture at 37°C for 15 min and terminated by heating the reaction mixture to 85°C for 5 s followed by cooling it to 4 °C. Real-time PCR was performed using the ABI 7500 Real-Time System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq kit (Takara Biotechnology, Dalian, China). The reaction volume was 20 μL, as recommended by the manufacturer's instructions. The primers were synthesized by Sangon Biotechnology (Shanghai, China) in accordance with the sequences described in GenBank, listed in Table 2. The PCR program is consisted of one cycle at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s, and then it was returned to 95°C for 15 s, kept at 60°C for 1 min, and heated to 95°C for 15 s. Each sample was measured in triplicate, and gene expression was calculated relative to glyceraldehyde-3-phosphate dehydrogenase using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen (2001).

Table 2. The primer sequences used for real-time quantitative PCR analysis.

Gene	GenBank number	Primer sequence (5′→3′)	Product size (bp)
Nrf2	NM_205117.1	Forward: CAGGCCGTCTTGAAGCTCATCTC Reverse: CTTGCCTCTCCTGCGTATATCTCG	179
HMOX1	NM_205344.1	Forward: ACGTCGTTGGCAAGAAGCATCC Reverse: TTGAACTTGGTGGCGTTGGAGAC	181
NQO1	NM_001277621.1	Forward:TCGCCGAGCAGAAGAAGATTGAAG Reverse: GGTGGTGAGTGACAGCATGGC	191
SOD	NM_205064.1	Forward: GGTGACCTCGGCAATGTGACTG Reverse:AATGATGCAGTGTGGTCCGGTAAG	93
CAT	NM_001031215.2	Forward: CACGTATTCAGGCACTGCTGGAC Reverse: ACGAGAAGTGGCTTGCGTGTATG	86
GPx	NM_001277853.2	Forward: AAGTGCTGCTGGTGGTCAACG Reverse: GTTGGTGGCGTTCTCCTGGTG	155
GAPDH	NM_204305.1	Forward: GGTAGTGAAGGCTGCTGCTGATG Reverse: AGTCCACAACACGGTTGCTGTATC	200

Abbreviations: CAT, catalase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; HMOX1, heme oxygenase 1; Nrf2, nuclear factor erythroid two-related factor 2; NQO1, NAD(P)H quinone dehydrogenase 1; SOD, superoxide dismutase.

Statistical Analyses

The data were analyzed with the replicate (each replicate included one broiler) as the experimental unit (n = 8). The differences between the mean values obtained in each treatment were evaluated by the analyses of variance (ANOVA; SPSS 19.0). Differences among the means were tested using Duncan's multiple-range tests. The data were expressed as mean values and standard error, and significance was set at $P < 0.05$.

RESULTS

Redox Status

Compared with the control and the saline treatments, we observed lower T-AOC activities in the breast muscle of the 5.0 and 10.0% H_2O_2 treatment ($P < 0.05$, Table 3) and lower GSH-Px activities in 10.0% H_2O_2 treatment ($P < 0.05$). There was no significant difference in T-SOD activities exposed to H_2O_2 ($P > 0.05$). There was a considerable increase of ROS level exposed to 2.5, 5.0, and 10.0% H_2O_2 ($P < 0.05$), the contents of protein carbonyl and MDA in breast muscle of broilers were increased exposed to 5.0 and 10.0% H_2O_2 ($P < 0.05$). Furthermore, with the increase of H_2O_2 level, the contents of ROS, MDA, and protein carbonyl were increased linearly ($P < 0.05$), the activities of T-AOC and GSH-Px were decreased linearly ($P < 0.05$).

Serum Analysis

As shown in Table 4, the activity of CK in the 10.0% H_2O_2 treatment was higher than that of the other 4 groups ($P < 0.05$). The CK activity increased linearly as the content of H_2O_2 increased ($P < 0.05$). Compared with the control group, the LDH activity in the experimental group had an increasing trend ($P > 0.05$).

Glycolytic Status

As shown in Table 5, the content of glycogen in breast muscle of broilers in the 2.5, 5.0, and 10.0% H_2O_2 groups were lower than that of the control and the saline treatments ($P < 0.05$). Moreover, the lowest content of glycogen was detected in the 10.0% H_2O_2 treatment. The content of lactate and GP in breast muscle of the

Table 3. Effects of intraperitoneal injection of H_2O_2 on the antioxidant activities and the content of oxidative products in the breast muscle of broilers.

	Treatments[1]					*P* Value		
Items	Control	Saline	2.5% H_2O_2	5.0% H_2O_2	10.0% H_2O_2	ANOVA	Linear[2]	Quadratic[2]
ROS generation(% of NC)	100.00 ± 1.23^{d}	97.88 ± 1.92^{d}	109.81 ± 3.11^{c}	129.92 ± 4.04^{b}	138.62 ± 3.14^{a}	<0.001	<0.001	0.717
T-AOC, U/mg of protein	0.26 ± 0.02^{a}	0.22 ± 0.01^{a,b}	0.18 ± 0.02^{b,c}	0.17 ± 0.03^{b,c}	0.15 ± 0.01^{c}	<0.001	<0.001	0.112
T-SOD, U/mg of protein	56.57 ± 3.46	57.33 ± 4.03	55.34 ± 2.11	53.18 ± 2.90	49.18 ± 2.71	0.371	0.055	0.651
GSH-Px, U/mg of protein	14.27 ± 1.96^{a}	14.39 ± 1.93^{a}	10.47 ± 1.84^{a,b}	9.64 ± 1.18^{a,b}	8.27 ± 1.39^{b}	0.044	0.003	0.538
Protein carbonyl, nmol/mg of protein	2.19 ± 0.10^{c}	2.15 ± 0.09^{c}	2.39 ± 0.11^{c}	2.91 ± 0.08^{b}	3.33 ± 0.12^{a}	<0.001	<0.001	0.210
MDA, nmol/mg of protein	0.47 ± 0.02^{c}	0.49 ± 0.01^{b,c}	0.52 ± 0.01^{b}	0.60 ± 0.02^{a}	0.64 ± 0.01^{a}	<0.001	<0.001	0.761

Results are represented as the mean value ± SE (n = 8). The differences between the mean values obtained in each treatment were evaluated by the analyses of variance (ANOVA; SPSS 19.0). Differences among the means were tested using Duncan's multiple-range tests. ^{a,b,c,d}Means in a row without a common superscript letter significantly differ ($P < 0.05$).

Abbreviations: GSH-Px, glutathione peroxidase; MDA, malondialdehyde; ROS, reactive oxygen species; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase.

[1]The control group was the noninjected treatment. The saline group: Birds were injected intraperitoneally with physiological saline buffer (0.75%) with an injection dosage of 1.0 mL/kg BW. The 2.5% H_2O_2, 5.0% H_2O_2 and 10.0% H_2O_2 groups: Birds were given an intraperitoneal injection of 2.5, 5.0 and 10.0% H_2O_2 with an injection dosage of 1.0 mL/kg BW.

[2]Orthogonal polynomial contrast was used to determine linear and quadratic effects of increasing concentrations of H_2O_2 injection.

10.0% H_2O_2 group was higher than that of the control and the saline treatments ($P < 0.05$).

In addition, the activities of HK, PK, and LDH in breast muscle of the 5.0 and 10.0% treatments were higher than those of the control and the saline treatments ($P < 0.05$). Besides, intraperitoneal injection of H_2O_2 linearly decreased the glycogen content, linearly increased the lactate content, GP, and the activities of HK, PK, and LDH ($P < 0.05$).

Nrf2-ARE Signaling Pathway-Related Gene Expressions

As shown in Figure 1, H_2O_2 downregulated the mRNA expression of Nrf2 ($P < 0.05$). In addition, compared with the control and the saline groups, broilers in the 5.0 and 10.0% H_2O_2 groups exhibited significantly lower mRNA expressions of Nrf2, HMOX1, and NQO1 ($P < 0.05$), broilers in the 10.0% H_2O_2 group exhibited significantly lower mRNA expressions of CAT, SOD, and GPx ($P < 0.05$). The mRNA expression of GPx and Nrf2 were decreased linearly in response to the increased H_2O_2 level ($P < 0.05$).

DISCUSSION

It is now widely accepted that broilers are sensitive to oxidative process, and the breast muscle of broilers can be greatly affected by oxidative stress (Min et al., 2008; Xiao et al., 2011). We speculated that the application of H_2O_2 could directly lead to oxidative stress. Thus, we detected the levels of ROS. In the present study, we found increased contents of ROS in breast muscle of broilers after exposed to H_2O_2, which was in accordance with the results of Zheng et al. (2016). The overproduction of ROS is closely related to oxidative stress, which is characterized by significant changes in redox balance and ROS-mediated damage (Valko et al., 2007). Malondialdehyde, the main product of polyunsaturated fatty acid peroxidation, is known as a marker of oxidative stress, and it is overproduced by the increased content of ROS (Del et al., 2005). Reactive oxygen species can lead to protein oxidation by oxidizing the side chains of amino acid residues to ketone or aldehyde derivatives (Fagan et al., 1999). In the present study, we also observed a significant increase in protein carbonyl and MDA content in the breast muscle of broilers exposed to H_2O_2 compared with the control and the saline groups; similar results were reported by Lu et al. (2019).

The occurrence of oxidative stress may be due to the elevation of ROS level and the weakness of antioxidant system (Jayaraj et al., 2006). Through the antioxidant defense system, reactive species and ROS can be effectively removed. The antioxidant defense system is composed of antioxidant enzymes, such as SOD and GSH-Px (Matés et al., 2000). Previous studies have demonstrated that oxidative stress could influence antioxidant system by depleting the activity of antioxidant enzymes (Mohamadin et al., 2009; Delles et al., 2014). In this study, the injection of 10.0% H_2O_2 decreased the activities of T-AOC, T-SOD, and GSH-Px in the breast muscle of broilers. The 10.0% H_2O_2 group exhibited significantly lower mRNA expressions of SOD, CAT, and GPx as compared with the control and the saline groups. The imbalance between ROS production and antioxidant capacity leads to oxidative stress, which can be reached from the impairment of antioxidant defense ability of broilers after 10.0% H_2O_2 injection. Taken together, these results indicated that 10.0% H_2O_2 stimulated the production of ROS, and we assumed that the excessive production of ROS was ascribed to the destroyed antioxidant system and the modified macromolecules including lipids and proteins in the breast muscle of broilers.

It is well known that Nrf2 is a key transcription factor in antioxidant system, which protects the body from oxidative stress (Jaiswal, 2004). The Nrf2 pathway plays an important role in defending cellular redox status, a lot of antioxidants have been found to protect cells through Nrf2 signaling pathway (Zhang et al., 2018). Wang et al. (2017a) observed an increase in the level of ROS and a significant decrease in the mRNA expression of Nrf2 in cells treated with aflatoxin B1. Similarly, in this study, the overproduction of ROS caused by H_2O_2 injection significantly reduced the mRNA expression of Nrf2. To prevent ROS accumulation, the organism can form a complex antioxidant defense system, in which Nrf2 is an important redox-sensitive transcription factor (Khor et al., 2011). Nuclear factor erythroid 2–related factor 2 has been proven to encode the transcription of phase II detoxification enzymes, which consists of HO-1, NQO1, SOD, CAT, and GPx (Bai et al., 2016). HO-

Table 4. Effects of intraperitoneal injection of H_2O_2 on activities of serum CK and LDH in broiler chickens.

	Treatments[1]					*P* Value		
Items	Control	Saline	2.5% H_2O_2	5.0% H_2O_2	10.0% H_2O_2	ANOVA	Linear[2]	Quadratic[2]
CK, U/mL	2.18 ± 0.17[b]	2.29 ± 0.19[b]	2.56 ± 0.24[b]	2.49 ± 0.21[b]	3.28 ± 0.18[a]	0.004	0.001	0.539
LDH, U/mL	2.54 ± 0.29	2.77 ± 0.17	2.34 ± 0.23	2.93 ± 0.32	3.28 ± 0.34	0.172	0.070	0.414

Results are represented as the mean value ± SE (n = 8). The differences between the mean values obtained in each treatment were evaluated by the analyses of variance (ANOVA; SPSS 19.0). Differences among the means were tested using Duncan's multiple-range tests. [a,b]Means in a row without a common superscript letter significantly differ ($P < 0.05$).

Abbreviations: CK, creatine kinase; LDH, lactate dehydrogenase.

[1]The control group was the noninjected treatment. The saline group: Birds were injected intraperitoneally with physiological saline buffer (0.75%) with an injection dosage of 1.0 mL/kg BW. The 2.5% H_2O_2, 5.0% H_2O_2 and 10.0% H_2O_2 groups: Birds were given an intraperitoneal injection of 2.5, 5.0 and 10.0% H_2O_2 with an injection dosage of 1.0 mL/kg BW.

[2]Orthogonal polynomial contrast was used to determine linear and quadratic effects of increasing concentrations of H_2O_2 injection.

Table 5. Effects of intraperitoneal injection of H_2O_2 on the contents of glycogen, lactate and key muscle glycolytic enzymes in breast muscle of broiler chickens.

Items	Treatments[1]					ANOVA	*P* Value	
	Control	Saline	2.5% H_2O_2	5.0% H_2O_2	10.0% H_2O_2		Linear[2]	Quadratic[2]
Glycogen, μmol/g	4.46 ± 0.07^{a}	4.47 ± 0.05^{a}	3.72 ± 0.03^{b}	3.70 ± 0.05^{b}	3.41 ± 0.04^{c}	<0.001	<0.001	0.007
Lactate, μmol/g	149.83 ± 12.95^{b}	154.12 ± 3.85^{b}	140.27 ± 9.48^{b}	164.56 ± 4.90^{a,b}	184.24 ± 11.61^{a}	0.022	0.013	0.162
GP, μmol/g	158.75 ± 13.04^{b}	163.06 ± 3.89^{a,b}	147.69 ± 9.50^{b}	171.95 ± 4.89^{a,b}	191.06 ± 11.58^{a}	0.029	0.021	0.151
HK, U/g of protein	13.11 ± 0.85^{b}	12.79 ± 0.54^{b}	13.54 ± 0.81^{b}	17.07 ± 1.62^{a}	18.51 ± 1.54^{a}	<0.001	<0.001	0.525
PK, U/g of protein	8.04 ± 0.62^{c}	8.14 ± 0.49^{c}	8.71 ± 0.53^{b,c}	10.66 ± 1.03^{a,b}	11.22 ± 0.94^{a}	0.011	0.001	0.933
LDH, U/mg of protein	3.14 ± 0.16^{c}	3.31 ± 0.11^{c}	3.83 ± 0.12^{b}	3.74 ± 0.15^{b}	4.43 ± 0.16^{a}	<0.001	<0.001	0.642

Results are represented as the mean value ± SE (n = 8). The differences between the mean values obtained in each treatment were evaluated by the analyses of variance (ANOVA; SPSS 19.0). Differences among the means were tested using Duncan's multiple-range tests. ^{a,b,c}Means in a row without a common superscript letter significantly differ ($P < 0.05$).

Abbreviations: GP, glycolytic potential, 2[glycogen] + [lactate]; HK, hexokinase; LDH, lactate dehydrogenase; PK, pyruvate kinase.

[1]The control group was the noninjected treatment. The saline group: Birds were injected intraperitoneally with physiological saline buffer (0.75%) with an injection dosage of 1.0 mL/kg BW. The 2.5% H_2O_2, 5.0% H_2O_2 and 10.0% H_2O_2 groups: Birds were given an intraperitoneal injection of 2.5, 5.0 and 10.0% H_2O_2 with an injection dosage of 1.0 mL/kg BW.

[2]Orthogonal polynomial contrast was used to determine linear and quadratic effects of increasing concentrations of H_2O_2 injection.

1 is the rate-limiting enzyme in heme degradation process, which can catalyze the formation of carbon monoxide, bilirubin, and ferrous iron, thus protecting tissues from oxidative damage (Chiu et al., 2002). NQO1 is an inducible enzyme that catalyzes the double electron reduction and detoxification of quinone and its derivatives (Zhang et al., 2008). It has been reported that florfenicol can induce oxidative stress in broilers through inhibiting Nrf2 pathway and downregulating mRNA expressions of Nrf2 (Han et al., 2020). This study

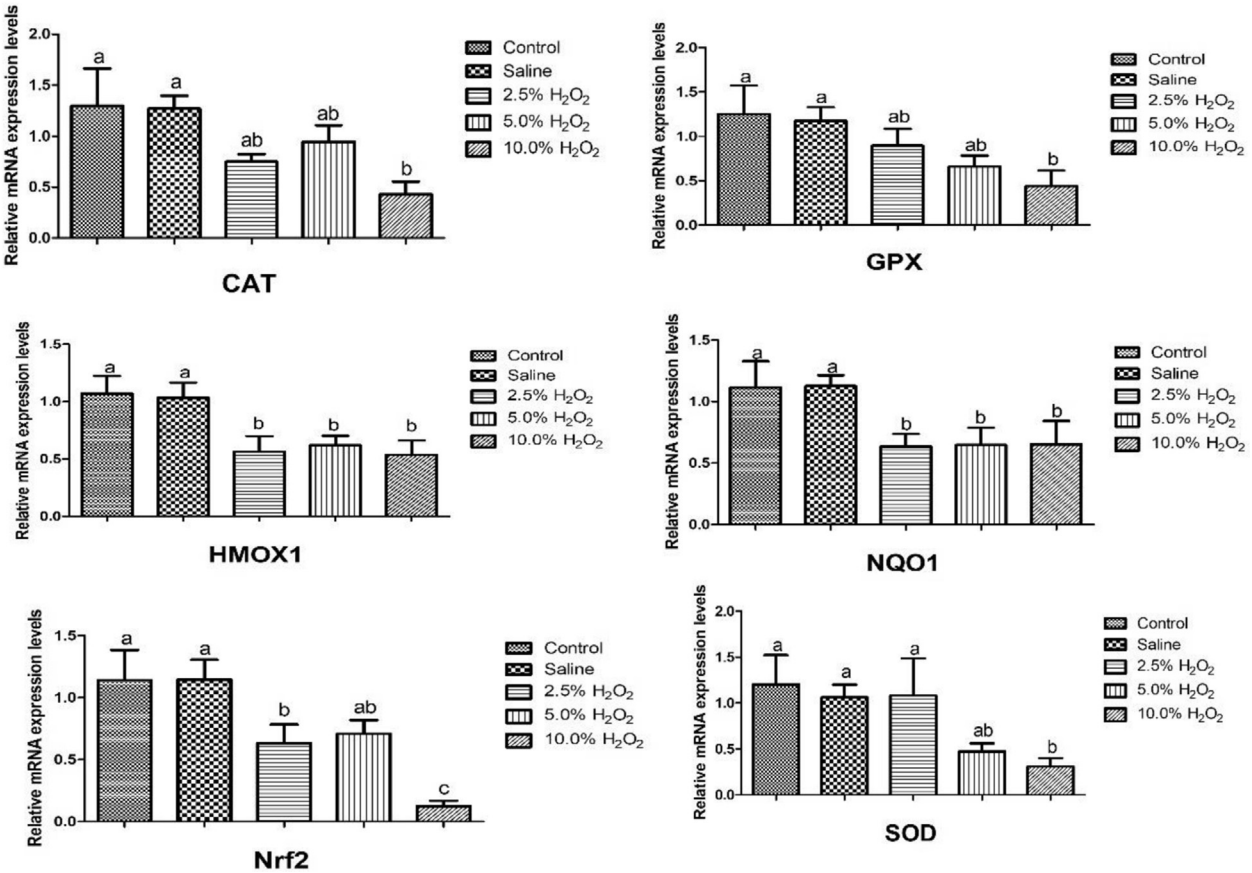

Figure 1. Effects of H_2O_2 on the relative mRNA expressions of Nrf2 and antioxidative capacity–related genes of breast muscle in broilers. The control group was the noninjected treatment. The saline group: Birds were injected intraperitoneally with physiological saline buffer (0.75%) with an injection dosage of 1.0 mL/kg BW. The 2.5% H_2O_2, 5.0% H_2O_2 and 10.0% H_2O_2 groups: Birds were given an intraperitoneal injection of 2.5, 5.0, and 10.0% H_2O_2 with an injection dosage of 1.0 mL/kg BW. Data are means ± SE of 8 replicates of one bird per cage (n = 8). The differences between the mean values obtained in each treatment were evaluated by the analyses of variance (ANOVA; SPSS 19.0). Differences among the means were tested using Duncan's multiple-range tests. ^{a,b,c}Means in a row without a common superscript letter significantly differ ($P < 0.05$). Abbreviations: CAT, catalase; GPx, glutathione peroxidase; HMOX1, heme oxygenase 1; Nrf2, nuclear factor erythroid two-related factor 2; NQO1, NAD(P)H quinone dehydrogenase 1; SOD, superoxide dismutase.

confirmed and expanded this view. The findings of the present study specifically indicated that excessive ROS significantly reduced Nrf2 mRNA expression level in the breast muscle of broilers. These results may explain the adverse effects of ROS on antioxidative capacity in broilers.

In this study, the activity of serum CK gradually increased with the doses of H_2O_2. Although the activity of LDH did not increase in the serum, but the activity of LDH in the muscle increased, under high concentration of H_2O_2. Over the time course studied, the activities of the CK and LDH in serum were highest at the dose of 10.0% H_2O_2. Skeletal muscle is the main source of CK and LDH compared with other organs and tissues, and these enzymes can be easily released into blood under stressful situations (Yu et al., 2009). Evidence from animal studies suggests that the changes of CK and LDH activities can be used as indicators of muscle cell damage and muscle fatigue (Krasnodębska-Depta and Koncicki, 2000; Yu et al., 2007). In the present study, the activities of CK and LDH clearly increased as the dose of H_2O_2 increased. Xie et al. (2015) reached a similar conclusion, suggesting that chronic heat stress may result in disturbance of blood metabolites, and cause tissue damage as reflected by the increased plasma LDH and CK activities. This finding indicated that the exposure to H_2O_2 is detrimental to physiological status of breast muscle of broilers.

We used contents of glycogen and lactate in muscle to evaluate early postmortem muscle energy metabolism in this study. With the doses of H_2O_2 injection increase, muscle glycogen was continuously consumed, which was consistent with the findings of Wang et al. (2017b). It is suggested that injection of H_2O_2 can cause stress to broilers, which further contribute to the acceleration of metabolism and induce the depletion of muscle glycogen (Fidan et al., 2015). Meanwhile, the concentrations of lactate increased significantly after exposure to 5.0 and 10.0% H_2O_2 compared with the control and saline groups. In line with Ryu et al. (2005), muscle with lower glycogen content and and higher lactate concentrations exhibits faster glycolytic rate, which is probably ascribed to the almost all fast-twitch glycolytic fibers (type IIb fiber) in the breast muscle of broilers (Cong et al., 2017); therefore, the energy production mainly depends on the glycolytic metabolism.

The glycolytic rate is regulated by several key enzymes. Hexokinase is the first enzyme in this pathway, which converts glucose to glucose 6-phosphate. The other 2 key enzymes are PK and LDH, which are involved in the terminal steps of the pathway, converting phosphoenolpyruvate to pyruvate and pyruvate to lactate under anaerobic conditions, respectively (Scheffler and Gerrard, 2007). In the present study, oxidative stress significantly enhanced the activities of HK, PK, and LDH in the 5.0 and 10.0% H_2O_2 groups compared with the control and the saline groups, suggesting that the glucose getting into the tricarboxylic acid cycle was limited, and the intracellular energy production mainly depended on anaerobic glycolysis, which was in agreement with the report by Lu et al. (2017). Furthermore, it was reported that the breast muscles of oxidative-stressed broilers increased lipid and protein oxidation, decreased SERCA activity, and sped the pH drop indicating that oxidative stress could cause PSE-like conditions in broilers (Zhang et al., 2011).

CONCLUSIONS

In conclusion, the results in this study indicated that H_2O_2 injection could induce the overproduction of ROS, and ROS negatively affects the antioxidant status and the expression of antioxidant genes, leading to oxidative stress and cell damage of myocytes in the pectoral muscle. The molecular mechanisms of the oxidative damage might be ascribed to the protein carbonylation and lipid peroxidation, which causes anaerobic glycolytic metabolism of broilers. In addition, the model of oxidative stress was established by intraperitoneal injection of H_2O_2, the harmful dose of H_2O_2 was 10.0%.

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DISCLOSURES

None of the authors have any conflicts of interest to declare.

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