



## Effects of lipopolysaccharide (LPS) challenge on antioxidant capability of broiler chickens

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### ABSTRACT

This study was designed to investigate the effects of lipopolysaccharide (LPS) challenge on antioxidant capability (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX; and reduced glutathione to oxidized glutathione, GSH/GSSG) and induce the model of immunological stress in broiler chickens. Day-old, yellow-feathered broilers (140) were randomly assigned to 2 treatments with 7 replicates of 10 chicks each. At day 21, 23, 25 and 27, chicks were injected with LPS and blood samples were collected after 24 h. Chicks (2) from each replicate were killed, and liver and jejunum samples were collected. The activities and gene expression of SOD, CAT, and GPX, were determined. The results showed that LPS challenge reduced serum SOD at day 22 and 26, serum CAT at day 24, 26, and 28, and serum GPX at day 26. LPS challenge decreased liver SOD, liver GPX, liver GSH/GSSG ratio at day 26 and 28. LPS challenge also decreased jejunum SOD at day 26, and jejunum CAT, GPX and GSH/GSSG ratio at day 26 and 28. The expressions of liver CAT, liver GPX1, and jejunum SOD1 were down regulated at day 26. The expressions of jejunum CAT, and jejunum GPX1 were down regulated at day 26. Meanwhile, LPS reduced the expression of liver SOD1 at day 22 and the expression of jejunum GPX1 at day 28. In conclusion, three times of LPS challenge could result in serious oxidative damage and induce immune stress model.

**Key words:** Antioxidant capability, Broiler chicks, Immunological stress, LPS, Oxidative damage

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria, made up from a polysaccharide O-chain and a biologically active lipid-A moiety embedded within the bacterial membrane (Raetz *et al.* 2007). Studies have shown that LPS triggers the rise of reactive oxygen species (ROS) such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide (Bayraktar *et al.* 2015). ROS, which are known to be one of the stimulators of the inflammatory response (Goode and Webster 1993, Zhang *et al.* 2002, Bak *et al.* 2009, Coulibaly *et al.* 2011) can induce oxidative damage (Sebai *et al.* 2008). To counteract ROS-induced damage, cells up regulate the specific defense mechanisms to prevent or repair damage (Hedge *et al.* 2008). Antioxidant enzyme systems, belonging to regulatory mechanisms, will protect against oxidative stress. Meanwhile, its balance becomes necessary, especially during infection or against oxidative stress (Bianca *et al.* 2002, Rigoulet *et al.* 2011, Bozinovski *et al.* 2012).

LPS can induce the inflammatory response of chickens, but literatures are not the same about oxidative damage of LPS challenge frequency on broilers (Koutsos *et al.* 2006,

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Meriwether *et al.* 2010, Selvaraj *et al.* 2010, Shen *et al.* 2010, Rajput *et al.* 2013). Therefore, the present study was designed to analyze how many times LPS challenge could cause significant effects on broiler's antioxidant capability.

### MATERIALS AND METHODS

**Ethics statement:** All animals used in this study were treated following the guidelines for experimental animals established by the Council of China. Animal experiments were approved by the Science Research Department of the Committee of Animal Care, Fujian Agriculture and Forestry University, Fuzhou, China (approval number: FAFUAR1615).

**Animal management, LPS injection, and sample collection:** Day-old yellow-feathered male broilers (140) were purchased from a commercial hatchery and randomly allocated into 2 groups consisting of seven replicates of 10 chicks each. This experiment lasted for 28 days.

LPS was injected as per Koutsos *et al.* (2006). At day 21, 23, 25, and 27, the treatment group received an intra-abdominal injection of 1 mg/kg LPS (*Escherichia coli*, serotype 055:B5, L2880, Sigma-Aldrich, San Luis, MO) dissolved in 0.9% NaCl, while control group received isovolumetric amounts of saline solution (0.9% NaCl).

After 24 h of LPS injection, chicks were weighed from each replicate and then replicate which weighed close to

the average weight was selected. Blood samples were collected from wing vein, and then all chicks were killed by cervical dislocation and manual exsanguination. Liver and jejunum samples were taken from the body cavity, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

*Determination of antioxidant indicators in serum, liver, and jejunum:* Serum samples were separated by centrifugation at  $4000\times g$  for 10 min and stored at  $-20^{\circ}\text{C}$  for this analysis. Liver and jejunal mucosal samples were prepared as previously described (Gao *et al.* 2011). Liver and jejunal mucosal samples were diluted with  $4^{\circ}\text{C}$  PBS containing 137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , and a pH of 7.4, in the ratio 1:9 (wt/vol), then used Ultra-Turrax T10 homogenizer (IKA Laborotechnik, Staufen, Germany) homogenizing at 5,000 rpm for 2 min. The homogenates were then centrifuged ( $4,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ ), and supernatants were divided into small portions and stored at  $-20^{\circ}\text{C}$  for this analysis.

The enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), and reduced glutathione to oxidized glutathione (GSH/GSSG) ratio were analyzed using a diagnostic kit (Nanjing Jiancheng Bioengineering Institute, China). The method and principle were, according to the previous description (Gao *et al.* 2012) and activity was normalized to protein

concentration as determined by Coomassie Blue assay.

SOD activity was measured at 550 nm through UV-1100 spectrophotometer following the reduction of nitrite by a xanthine-xanthine oxidase system which is a superoxide anion generator. CAT activity was determined by incubating in the presence of a known concentration of hydrogen peroxide and then the reaction is quenched with ammonium molybdate. The amount of hydrogen peroxide remaining in the reaction mixture forms a stable coloured complex with ammonium molybdate and the complex is measured at 405 nm through UV-1100 spectrophotometer. GPX was

Table 1. Real-time PCR primer sequences and products

Gene	Primer	Sequences (5'→3')	Product size (bp)
β-actin	Forward	ATCCGGACCCTCCATTGTC	120
	Reverse	AGCCATGCCAATCTCGTCTT	
SOD1	Forward	GGGAGGAGTGGCAGAAGTAG	162
	Reverse	GCTAAACGAGGTCCAGCATT	
CAT	Forward	CATCCATAGCCAGAAGAGGAA	140
	Reverse	GGCGAAATCCATCAGGAA	
GPX1	Forward	AACCAATTCTGGGCACCAG	122
	Reverse	CCGTTACCTCGCACTTCTC	

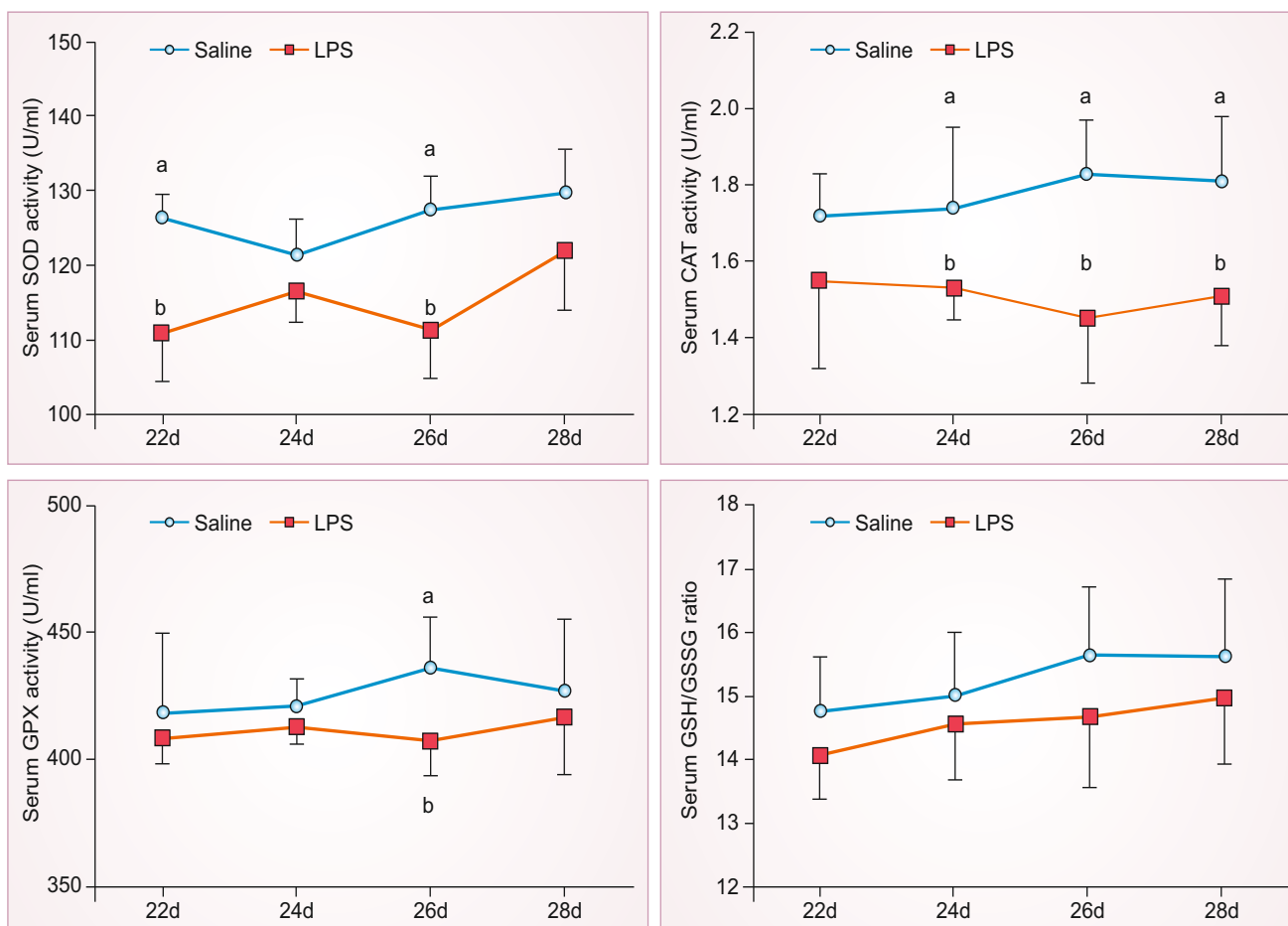


Fig. 1. Effects of LPS on serum SOD, CAT, GPX, and GSH/GSSG ratio at day 22, 24, 26, and 28. Each value is present as mean±SD. Significant differences between the groups are indicated by value<sup>a-b</sup> (P<0.05, t-test).

assayed by the decrease of the GSH, which is reflected by a change in absorbance at 412 nm through UV-1100 spectrophotometer. GSH and GSSG were determined at 412 nm through UV-1100 spectrophotometer by using the 3-Carboxy- 4-nitrophenyl disulfide (DTNB) circular response.

**Quantification of SOD1, CAT, and GPX1:** The method to determine gene expression was generally the same as we previously reported (Gao *et al.* 2015). Total RNA was isolated from broiler chicks' liver and jejunum using RNeasy Pure Tissue Kit according to the instructions of the manufacturer. The RNA quantity and purity were determined at 260/280 nm by NanoDrop 2000 Spectrophotometer and then frozen at  $-80^{\circ}\text{C}$ .

The 20 mL reverse transcription reaction mixture consisted of the following components: 10 mg of total RNA, 0.5 ml of RNase inhibitor, 1 ml of Oligo dT primer (500 mg/ml), 1 ml of random primers, 1 ml of PCR nucleotide mix (10 mM), 1 ml reverse transcriptase, 2 ml of  $\text{MgCl}_2$  (25 mM), 4 ml of  $5\times$  reaction buffer, 6.5 ml of nuclease-free water. The reverse transcription was performed according to the instructions of the manufacturer (A3500, Promega, Madison, Wisconsin, CA). The reverse transcription products (cDNA) were stored at  $-20^{\circ}\text{C}$  for PCR.

To design primers, we used the chicken SOD1, CAT, and GPX1 mRNA GenBank sequence with accession numbers of GGU28407, NM001031215, and HM590226, respectively. Chicken  $\beta$ -actin (GenBank accession number: NM205518) was used as housekeeping gene. The primers (Table 1) were designed using the Primer 5 Software (Molecular Biology Insights, Cascade, CO) and were synthesized by Shanghai Sangon Biotechnology Co. Ltd., China.

Real-time PCR was performed to detect the expression of SOD1, CAT, and GPX1 genes in different cDNA sample using Go Taq<sup>®</sup> qPCR Master Mix (A6001, Promega, Madison, Wisconsin, CA). The reaction mixtures were incubated in an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The program included denaturation step at  $95^{\circ}\text{C}$  for 10 min, 40 cycles at  $95^{\circ}\text{C}$  for 15 sec, and annealing/extension step at  $60^{\circ}\text{C}$  for 1 min. The dissociation curves were analyzed for each PCR reaction to detect and eliminate the possible primer dimer and nonspecific amplification. The expression levels of target genes (SOD1, CAT, and GPX1) were calculated by the  $2^{-\Delta\Delta\text{CT}}$  method, and the data were calibrated as the relative value to the control group. All samples were analyzed in triplicate.

**Statistical analysis:** Statistical analysis of per injection

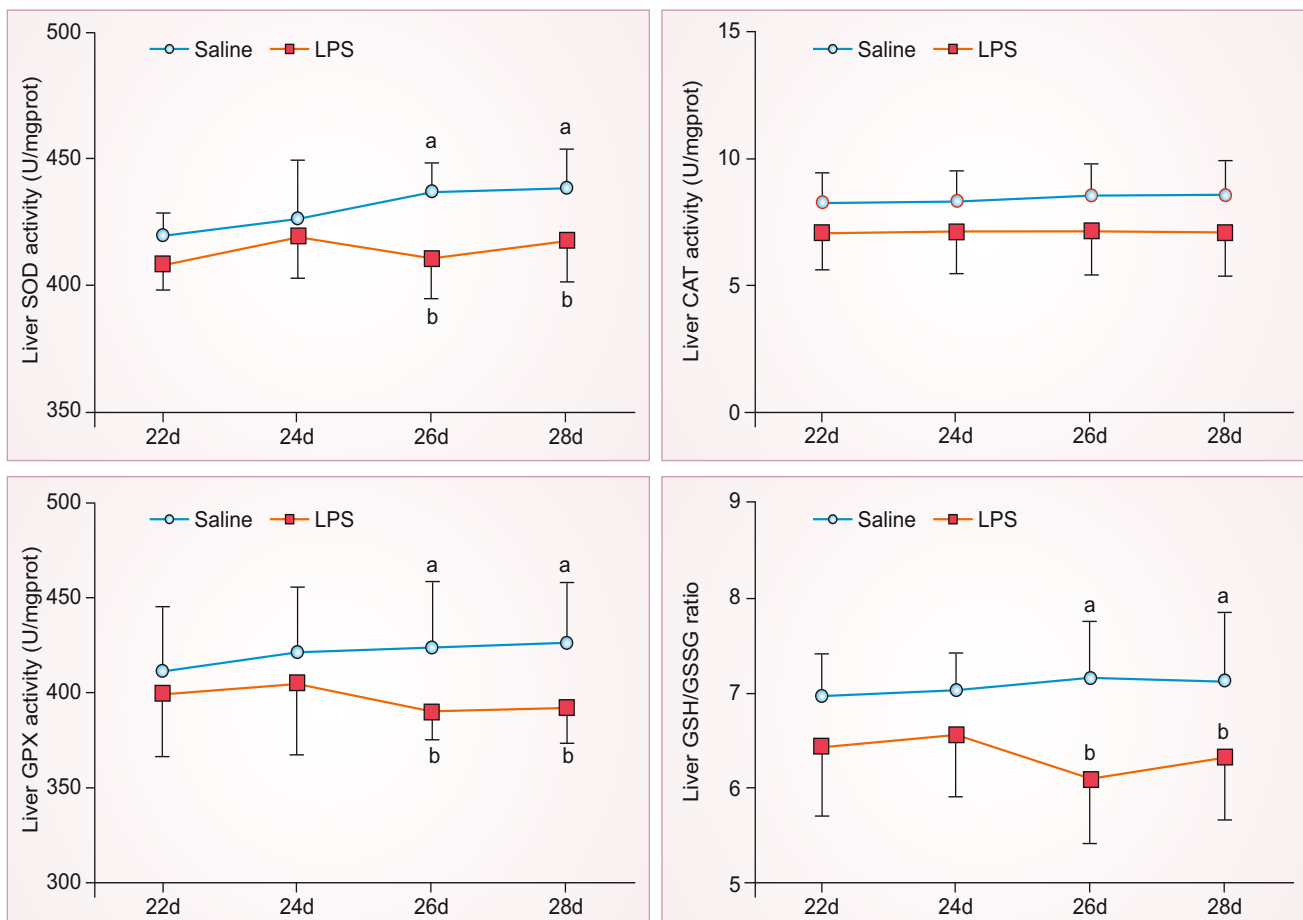


Fig. 2. Effects of LPS on liver SOD, CAT, GPX, and GSH/GSSG ratio at day 22, 24, 26, and 28. Each value is present as mean $\pm$ SD. Significant differences between the groups are indicated by value<sup>a-b</sup> (P<0.05, t-test).

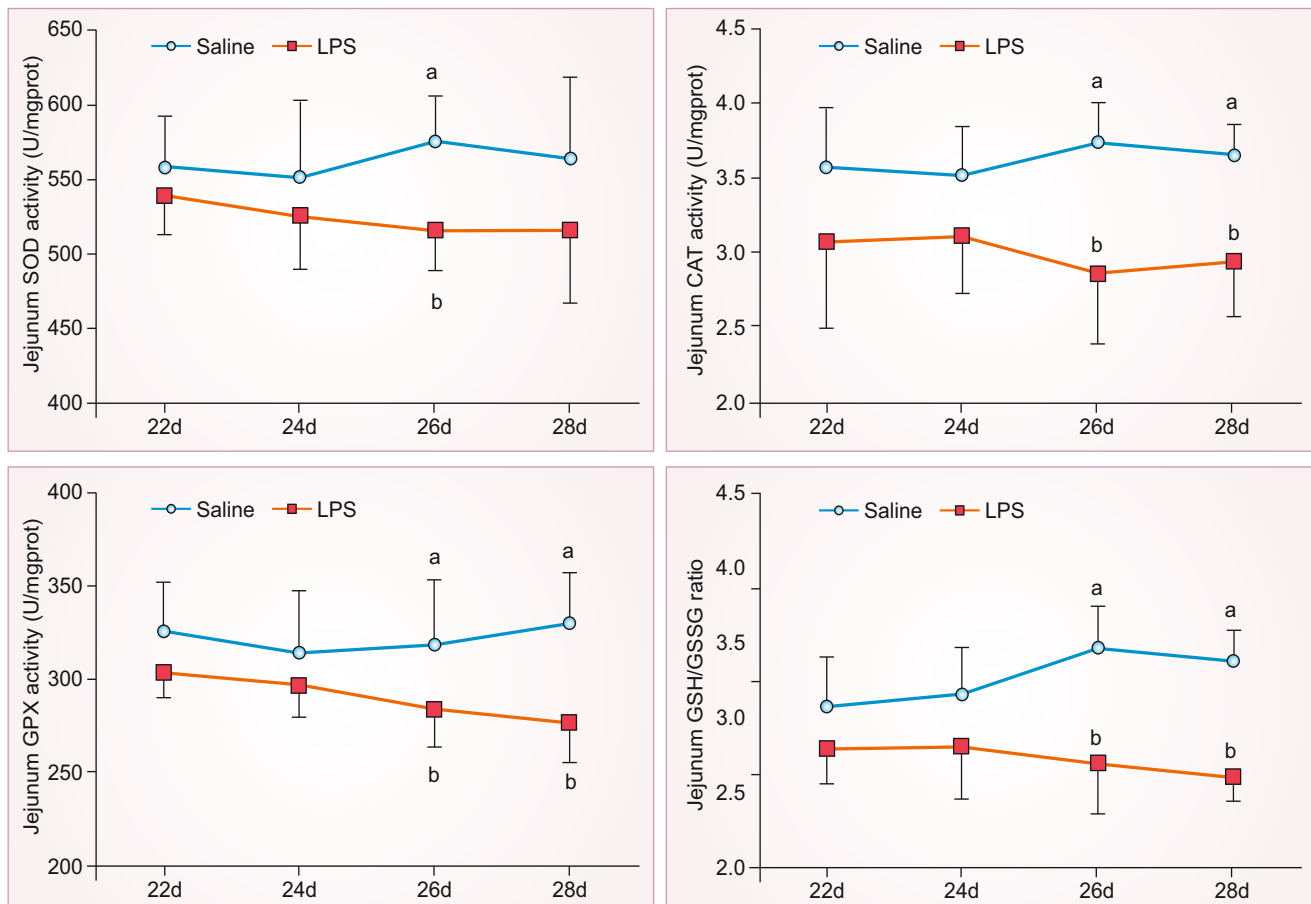


Fig. 3. Effects of LPS on jejunum SOD, CAT, GPX, and GSH/GSSG ratio at day 22, 24, 26, and 28. Each value is present as mean±SD. Significant differences between the groups are indicated by value<sup>a-b</sup> ( $P < 0.05$ , t-test).

data was performed using t-test with SPSS statistical software (v16.0 for windows, SPSS Inc., Chicago). All values were presented as mean±SD. P value less than 0.05 was considered as significant difference, and P value between 0.05 and 0.10 was considered as statistical tendency.

## RESULTS AND DISCUSSION

**Serum antioxidant parameters induced by LPS:** The result of serum antioxidant parameters is shown in Fig. 1. The activity of SOD diminished in response to LPS challenge compared to saline treatment at day 22 ( $P < 0.001$ ) and 26 ( $P < 0.001$ ). The activity of CAT diminished in response to LPS challenge compared to saline treatment at day 24 ( $P = 0.029$ ), 26 ( $P < 0.001$ ), and 28 ( $P = 0.003$ ). In addition, the activity of GPX diminished in response to LPS challenge compared to saline treatment at day 26 ( $P = 0.008$ ). Moreover, the GSH/GSSG ratio was not significantly decreased at day 22 ( $P = 0.106$ ), 24 ( $P = 0.374$ ), 26 ( $P = 0.122$ ), and 28 ( $P = 0.304$ ).

**Liver antioxidant parameters induced by LPS:** The result of liver antioxidant parameters is shown in Fig. 2. The activity of SOD diminished in response to LPS challenge compared to saline treatment at day 26 ( $P = 0.004$ ) and 28 ( $P = 0.029$ ). The activity of CAT was not significantly

decreased at day 22 ( $P = 0.123$ ), 24 ( $P = 0.149$ ), and 26 ( $P = 0.111$ ), but there was a tendency to decrease at day 28 ( $P = 0.089$ ). The activity of GPX diminished in response to LPS challenge compared to saline treatment at day 26 ( $P = 0.033$ ) and 28 ( $P = 0.030$ ). Moreover, LPS challenge reduced the GSH/GSSG ratio at day 26 ( $P = 0.008$ ) and 28 ( $P = 0.048$ ).

**Jejunum antioxidant parameters induced by LPS:** The result of jejunum antioxidant parameters is shown in Fig. 3. The activity of SOD diminished in response to LPS challenge compared to saline treatment at day 26 ( $P = 0.002$ ). The activities of CAT and GPX decreased in response to LPS challenge compared to saline treatment at day 26 ( $P = 0.001$  and  $P = 0.042$ ) and 28 ( $P < 0.001$  and  $P = 0.002$ ). In addition, LPS challenge reduced the GSH/GSSG ratio at day 26 ( $P < 0.001$ ) and 28 ( $P < 0.001$ ).

**LPS challenge reduced the liver relative expression level of antioxidant enzymes mRNA:** The result of the liver relative expression level of antioxidant enzymes mRNA is shown in Fig. 4. The antioxidant enzymes' gene expression analyses of the SOD1, CAT, and GPX1 were performed in liver samples by RT-PCR. The level of SOD1 mRNA decreased in response to LPS challenge by 0.87-fold compared to saline treatment at day 22 ( $P < 0.05$ ). The level of SOD1, CAT and GPX1 mRNA decreased in response to

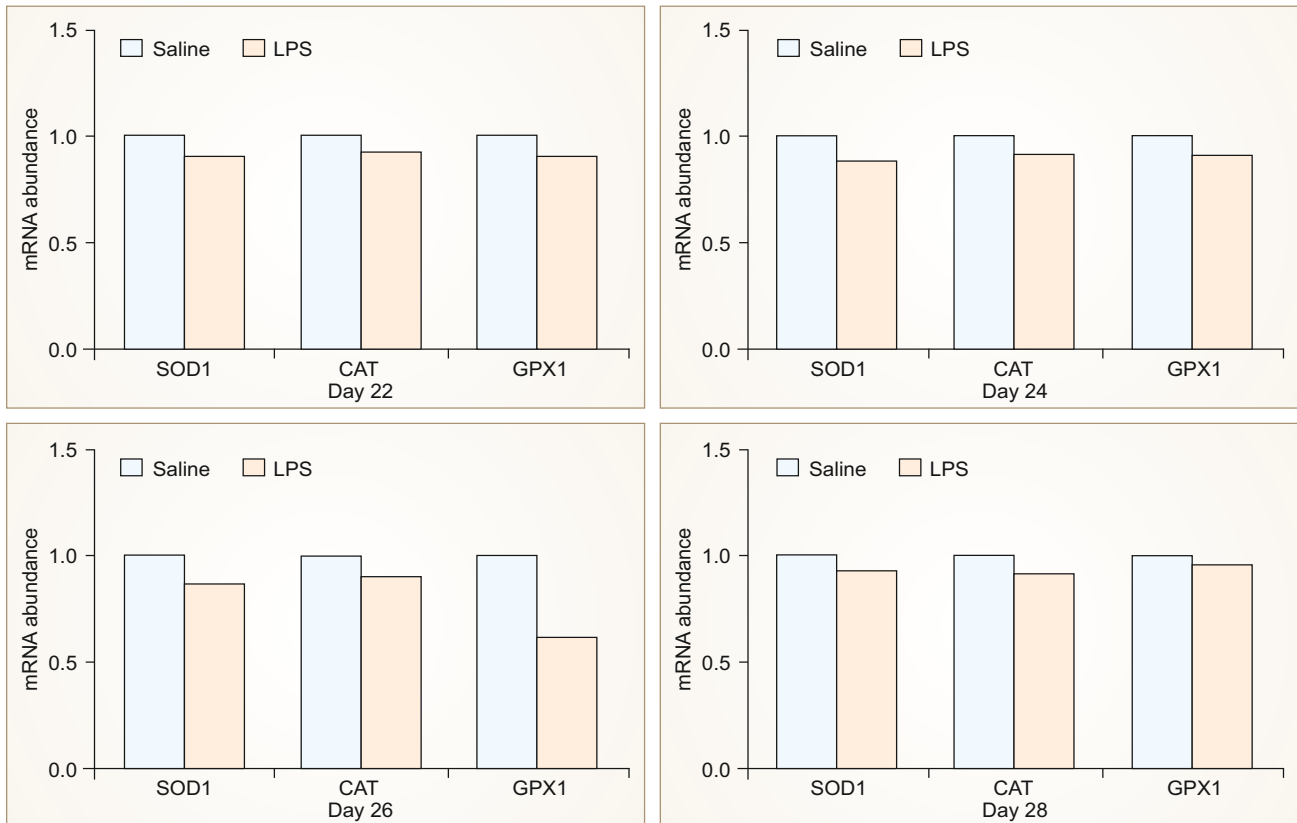


Fig. 4. Effects of LPS on liver relative expression of SOD1, CAT, and GPX1 mRNA at day 22, 24, 26, and 28. The reporter expression in response to saline alone is expressed as 100%. Each value was represent as mean $\pm$ SD. Significant differences between the groups are indicated by asterisk ( $P < 0.05$ , t-test).

LPS challenge by 0.88-, 0.90- and 0.82-fold compared to saline treatment at day 26 ( $P < 0.05$ ) respectively. Moreover, the level of SOD1, CAT and GPX1 mRNA were not significantly decreased at day 24 and 28.

**LPS challenge reduced the jejunum relative expression level of antioxidant enzymes mRNA:** The result of the jejunum relative expression level of antioxidant enzymes mRNA is shown in Fig. 5. The antioxidant enzymes' gene expression analyses of the SOD1, CAT, and GPX1 were performed in jejunum samples by RT-PCR. The level of SOD1, CAT and GPX1 mRNA was not significantly decreased at day 22 and 24. The level of SOD1, CAT and GPX1 mRNA decreased in response to LPS challenge by 0.86-, 0.90- and 0.84-fold compared to saline treatment at day 26 ( $P < 0.05$ ). And then the level of GPX1 also reduced by 0.89-fold in LPS treated animals compared to saline treatment at day 28 ( $P < 0.05$ ).

The focus of this study was to investigate the effects of LPS challenge on broiler's antioxidant capability. The impairment of the antioxidants defense system is a critical step in LPS-stimulated damage. Antioxidant enzymes (SOD, CAT, and GPX) play a central role in the regulation of entry and metabolism in the antioxidant defense system. ROS elimination is a multistage process (Lu *et al.* 2007, Buldak *et al.* 2012). At the beginning, SOD converts  $O_2^-$  into  $H_2O$  (Jaeschke 1995).  $H_2O_2$  may be further converted

into aqua and oxygen by CAT or GPX (Fantel 1996). Briefly, there is a synergistic antioxidant effect among them. But the imbalance between overproduction of ROS molecules and antioxidant system may cause decrease in activity of antioxidant enzymes. The model of LPS has been previously shown to result in the increment of oxidative stress, and early biochemical changes associated with enzyme damage (Lappas *et al.* 2004, Li *et al.* 2013, Martha 2014). In the present study, we reported that multiple injections of LPS can cause imbalance of redox status, which indicated by the decrease in the activities of antioxidant enzymes (SOD, CAT, and GPX). These results were in concordance with the previous study for oxidative stress (Linke *et al.* 2005). Especially three times LPS challenge, the activity of antioxidant enzyme reached significant influence. Buldak *et al.* (2014) also indicated that LPS challenge decreased the SOD and CAT activity, but elevated the GPX activity in the peripheral blood mononuclear cells (Buldak *et al.* 2014). The discrepancy of GPX activity may stem from difference between tissue type and location of its collection.

The result is reinforced through observing GSH/GSSG. Evidence has shown that LPS challenge was characterized by change tissue, circulating antioxidant enzyme's level and antioxidant molecules (Ajuwon *et al.* 2014). Reduced glutathione (GSH) is the major nonprotein thiol in animals, which can prevent LPS-induced damage (Zhao *et al.* 2015).

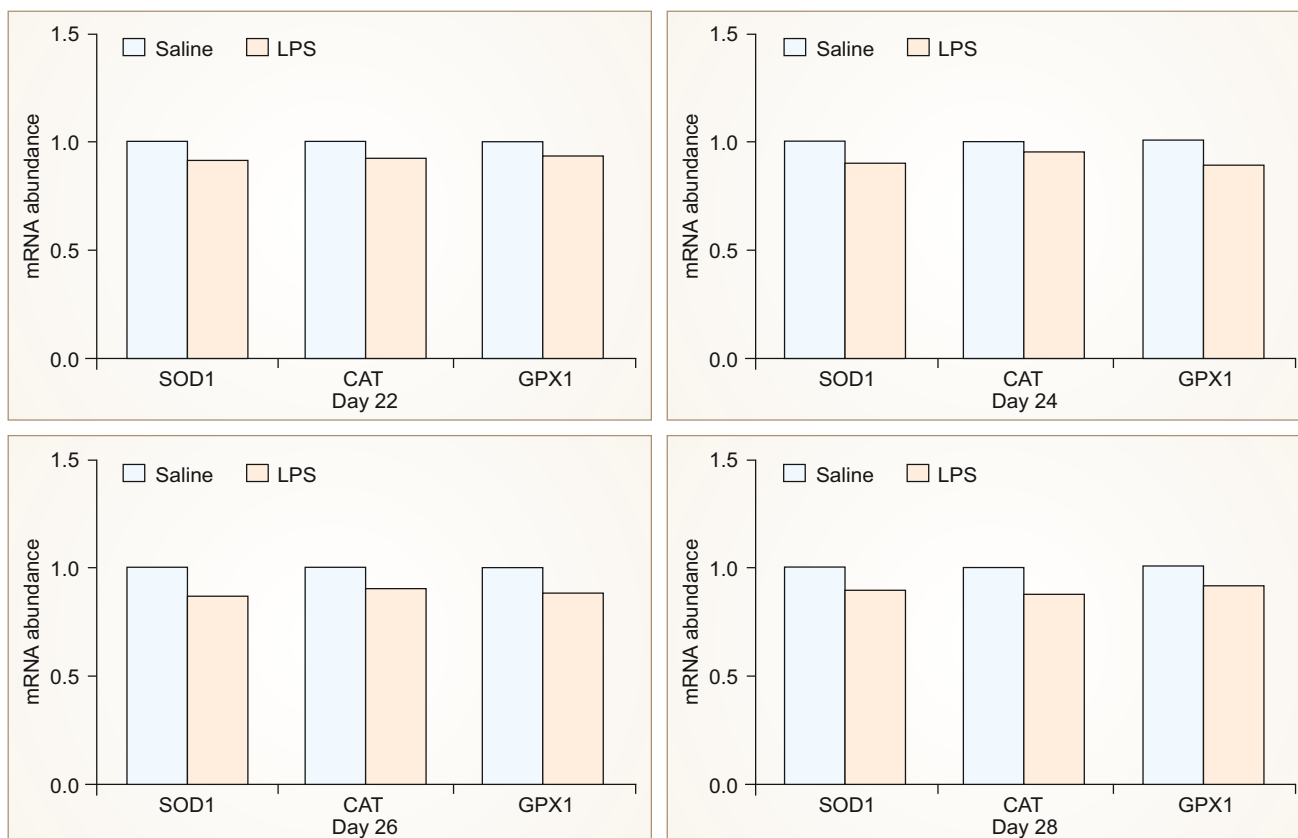


Fig. 5. Effects of LPS on jejunum relative expression of SOD1, CAT, and GPX1 mRNA at day 22, 24, 26, and 28. The reporter expression in response to saline alone is expressed as 100%. Each value is represent as mean±SD. Significant differences between the groups are indicated by asterisk ( $P < 0.05$ , t-test).

Its scavenging and antioxidant properties allow the neutralization of ROS species. Thus, the GSH/GSSG ratio is associated with antioxidant capacity. Our study found significant decrease of GSH/GSSG ratio in the LPS-induced chicks through 3 times of LPS injection. The decreases of GSH synthesis could cause the reduction of antioxidant enzyme's activity. This result was consistent with research in rats (Molinett *et al.* 2015).

In order to deeply observe into the reduction of antioxidant enzyme's activity, studies on gene expression were performed. We also studied the influence of LPS challenge on the gene expression of antioxidant enzyme. Few reports presenting relate antioxidant enzyme genes expression with LPS stimulation. Some authors reports a reduction of SOD1 levels after LPS administration (Pang *et al.* 2001, Munyaka *et al.* 2012, Marimoutou *et al.* 2015, Xu *et al.* 2015). Similar research need to illuminate the occurring processes. In our study, the expression levels of SOD1, CAT, and GPX1 mRNA reduced significantly through three times LPS injection. This may suggest that the gene expression of antioxidant enzyme is response to the activity of antioxidant enzyme by key signaling pathways such as NF- $\kappa$ B and Nrf2 (Kunsch and Medford 1999, Huang *et al.* 2002, Nakamura and Omaye 2009, Marimoutou *et al.* 2015). But the mechanism of signaling pathway need further studies. Our research commonly verifies the effect of LPS challenge on antioxidant capacity

by gene expression and the activity of antioxidant enzyme. Results from the current study showed that 3 times LPS injection caused significant oxidative damage in broilers. But there is no significant oxidative damage in 1 time or 2 times LPS injection. Compared with other studies (Koutsos *et al.* 2006, Rajput *et al.* 2013), this difference may stem from the difference between injection dose and injection period.

The study demonstrated that we can see that 3 doses of LPS have an impact on the level of antioxidant enzymes. These enzymes connects intensely with broiler's homeostasis. Therefore, it will be more meaningful to maintain the balance of the level enzymes for resisting stress and to maintain homeostasis. However, it is worth noting that 4-times LPS injection could only induce partial oxidative damage compared with 3 times LPS injection. This may suggest that chicks could come into being immunologic tolerance more than 3 times LPS injection (Takahashi *et al.* 1994, Balaji *et al.* 2000).

Our study demonstrates that LPS challenge could partially induce serous, hepatic, and jejunal oxidative damage. LPS-induced damage reduced the activity of antioxidant enzymes, and redox status (GSH/GSSG ratio). These effects were achieved by down regulation of antioxidant enzymes mRNA (SOD1, CAT, and GPX1). In addition, this study indicates that LPS-induced oxidative damage was the most significant effect through 3 times of LPS injection.

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