Effects of dietary chitosan oligosaccharides on oxidative stress and inflammation response in liver and spleen of yellow-feather broilers exposed to high ambient temperature

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ABSTRACT
Heat stress jeopardised animal health by inducing oxidative stress and inflammation. This study was done to investigate the effects of chitosan oligosaccharides (COS) on oxidative stress and inflammation response in liver and spleen of yellow-feather broilers under high ambient temperature. A total of 144 35-day-old female Chinese indigenous yellow-feather broilers (body weight 450.21 ± 10.05 g) were randomly allocated into 3 dietary treatments with 6 replication pens, each pen had 8 broilers. The treatments were: Control, basal diet; COS100, basal diet with 100 mg/kg COS; COS200, basal diet with 200 mg/kg COS. During day 22 to 42 (57 to 77 days of age), broilers in the COS200 group had higher \( p < 0.05 \) ADFI than broilers in the Control group. Broilers in the COS200 group had lower \( p < 0.05 \) serum alanine aminotransferase, aspartate aminotransferase, and tumour necrosis factor-\( \alpha \) levels, and spleen malondialdehyde (MDA) content, but had higher \( p < 0.05 \) liver superoxide dismutase activity and serum interleukin-10 level than broilers in the Control group. Broilers in the COS200 group had lower \( p < 0.05 \) serum alanine aminotransferase, aspartate aminotransferase, and tumour necrosis factor-\( \alpha \) levels, and spleen malondialdehyde (MDA) content, but had higher \( p < 0.05 \) liver superoxide dismutase activity and serum interleukin-10 level than broilers in the Control group. Broilers in the COS100 and COS200 groups had lower \( p < 0.05 \) serum and liver MDA content, and serum interleukin-1\( \beta \) level, but had higher \( p < 0.05 \) serum glutathione peroxidase activity than broilers in the Control group. In conclusion, dietary COS supplementation can alleviate heat stress-induced oxidative stress and inflammation response in liver and spleen by decreasing lipid peroxidation, increasing anti-oxidant enzyme activities and IL-10 level.

HIGHLIGHTS
- Dietary COS decreased serum ALT, AST, IL-1\( \beta \), TNF-\( \alpha \) and MDA.
- Dietary COS increased serum GSH-Px and IL-10, liver SOD, and spleen GSH-Px.

Introduction
High ambient temperature jeopardised animal performance and health, which made great economic losses to global animal production (Attia et al. 2011). With global warming, heat stress became a widely attention issue for animal industry, particularly in sub-tropical and tropical regions. Broilers were extremely sensitive to high ambient temperature due to feather covered, lack of sweat glands, and higher metabolic activity (Liu et al. 2014). Previous studies indicated that heat stress had negative effects on performance, health, and well-beings of broilers (Lara and Rostagno 2013). Moreover, heat stress resulted in oxidative stress (Ando et al. 1997), which, in turn, inducing adverse effects on DNA, protein, and lipids, and finally led to tissue damage, inflammatory disease, and some other diseases (Zhang et al. 2018; Cheng et al. 2019a; Cheng et al. 2019b). Heat stress also induced inflammation response (Jang et al. 2014; Ohtsu et al. 2015; Wang et al. 2018). Oxidative stress and inflammation response were closely related, previous studies reported that heat stress can induce oxidative and inflammation stress in broilers (Jang et al. 2014; Song et al. 2017). Both oxidative stress and inflammation response can activate tissue apoptosis (Xia et al. 2014; Jiang et al. 2018), therefore, suppressing oxidative stress and inhibiting inflammation response were important for preventing tissue damage under heat stress. Liver was the primary detoxified and metabolic organ, the oxidative stress and excessive inflammation response can lead to liver disorder (Sakka 2007; Abdel-Misih and Bloomston 2010; Sun and Karin 2013). Spleen...
was the peripheral lymphoid organ and regulating immune response by changing cytokines expression (Abdul-Careem et al. 2007; Coble et al. 2011; Ohtsu et al. 2015). Former studies reported that heat stress-induced spleen dysplasia, accompanied with oxidative stress, apoptosis, and changed splenic cytokines expression (Ohtsu et al. 2015; Zhang et al. 2018). Therefore, suppressing oxidative stress and inhibiting inflammation response were important for maintain liver and spleen function under heat stress. Nutritional manipulation was one of the effective methods for deletion or adjustment of high ambient temperature effects on broilers, especially the anti-oxidant supplementation (Alishah et al. 2013; Nourozi et al. 2013). Previously, we reported that dietary chitosan oligosaccharides (COS) supplementation can alleviate liver and spleen oxidative stress and inflammation response in heat-stressed rats (Lan et al. 2019), as well as reducing lipid peroxidation and increasing antioxidative capacity in H2O2-challenged rats (Lan et al. 2019). The COS, was the degraded products of chitosan or chitin (Zou et al. 2016), had numerous biological activities, including anti-oxidant (Li et al. 2017), anti-inflammatory (Xiong et al. 2015), immune stimulating (Li et al. 2019), and free radical scavenging capacity (Je et al. 2004). It was unknown whether dietary COS supplementation can attenuate oxidative stress and inflammation response of broilers under high ambient temperature, therefore, the purpose of this study was to investigate the effects of COS on oxidative stress and inflammation response in liver and spleen of broilers under high ambient temperature.

**Materials and methods**

**Experiment design and dietary treatments**

A total of 200 1-day-old female Chinese indigenous yellow-feather broilers were purchased from local breeding company (Zhanjiang, Guangdong province, China) and raised at a recommended environment condition from 1 to 34 days of age with commercial feed. At day of 35, a total of 14,435-day-old broilers (body weight 450.21 ± 10.05 g) were selected and randomly divided into 3 treatments with 6 replication pens, each pen had 8 broilers. The treatments were: Control, basal diet without COS; COS100, basal diet with 100 mg/kg COS; COS200, basal diet with 200 mg/kg COS. 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 purchased from Jiangsu Xinrui Biotechnology Co., Ltd. (HPLC purity 95%, deacetylation degree over 95% and average molecular weight below 32 kDa).

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

| Ingredients          | Content (g/kg) |
|----------------------|----------------|
| Corn                 | 699.5          |
| Soybean              | 221.0          |
| Soybean oil          | 27.0           |
| Calcium hydrogen phosphate | 17.0       |
| Shell power          | 19.3           |
| Salt                 | 3.5            |
| Methionine           | 1.0            |
| Lysine               | 0.5            |
| Zeolite powder       | 8.0            |
| Vitamin premixa      | 1.6            |
| Mineral premixb      | 1.6            |

_Calculated value_

| Metabolic energy, MJ/kg | 12.65 |
|-------------------------|-------|
| Crude protein, %        | 162.9 |
| Calcium, %              | 11.8  |
| Total phosphorus, %     | 6.2   |
| Available phosphorus, % | 4.1   |
| Methionine, %           | 3.6   |
| Lysine, %               | 8.7   |
| Methionine + Cystine, % | 6.4   |
| Threonine               | 6.6   |
| Isoleucine              | 7.0   |
| Valine                  | 6.4   |
| Tryptophan              | 1.8   |
| Arginine                | 10.8  |

_Provided per kilogram of complete diet: 12,800IU trans-retinol; 1,600U cholecalciferol; 60IU α-tocopheryl acetate; 1.6 mg menadione; 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, 3.2 mg pyridoxine; 0.03 mg cyanocobalamin._

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

**Experimental conditions**

Broilers of each replication pen were assigned in battery pens (124 cm length × 64 cm width × 40 cm height). Artificial light provided 23 hour per day by fluorescent lights and had free access to feed and water. The indoor ambient temperature and relative humidity were recorded daily at 08:00, 12:00, and 18:00 daily, and average temperature and relative humidity were calculated and presented in Figure 1.

**Performance parameters**

On day 1 (35 days of age), 21 (56 days of age), 42 (77 days of age), and 56 (91 days of age), body weight (BW) recorded on pen basis. Feed intake (FI) recorded on pen basis every week. The average daily gain (ADG), average daily feed intake (ADF), and feed conversion ratio (FCR) were calculated.

**Sample preparation**

On day 56 (91 days of age), after 12-hour fast, 6 broilers per treatment (1 broiler from each replication pen) randomly selected, blood samples were collected from the brachial vein and centrifuged at
3,000 g \times 10\text{mins} at 4\,^\circ\text{C} to obtain serum. The serum samples stored at \(-20\,^\circ\text{C}\) until analysis. Then the broilers were euthanized by cervical dislocation, liver and spleen were collected and flushed with ice-cold phosphate-buffered saline (PBS), quick freezing in liquid nitrogen, and frozen at \(-80\,^\circ\text{C}\) until analysis.

**Serum parameters**

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were measured with commercial enzyme immunoassay kits (Cusabio Biotech. Co., Ltd., Wuhan, Hubei, China) according to the manufacturers’ instructions. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT), the levels of malondialdehyde (MDA), interleukin-1\(\beta\) (IL-1\(\beta\)), interleukin-10 (IL-10), and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) were measured with corresponding commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturers’ instruction.

**Oxidative status and cytokines**

One gram of liver and spleen samples were homogenised at a ratio of 1: 9 (weight/volume) with ice-cold PBS. Homogenate was centrifuged at 3,000 g \times 15 mins at 4\,^\circ\text{C} to obtain supernatant, and the analysis conducted immediately. The supernatant protein concentration was determined by the Bradford method. The SOD, GSH-Px, and CAT activities, and MDA, IL-1\(\beta\), IL-10, and TNF-\(\alpha\) levels were measured with corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturers’ instruction.

**Statistical analysis**

The pen was used as the experiment unit and all data were analysed with SAS 2003 (v. 9.1, SAS Institute Inc., Cary, NC). Data was analysed using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test to analyse differences among treatments. Orthogonal polynomial contrasts were conducted to analyse the linear and quadratic effects of dietary COS supplementation level. \(p < .05\) was considered significant, and \(p < .10\) was considered as a trend.

**Results**

**Growth performance**

During day 1 to 21 (35 to 56 days of age), day 43 to 56 (78 to 91 days of age), and day 1 to 56 (35 to 91 days of age), no significant differences were observed in ADG, ADFI or G:F among treatments (Table 2). During day 22 to 42 (57 to 77 days of age), there were linear \((p < .05)\) improvement in ADFI and quadratic \((p < .05)\) improvement in ADG with COS supplementation. In addition, broilers in the COS200 group had higher \((p < .05)\) ADG than broilers in the COS100 group. Broilers in the COS200 group had higher \((p < .05)\) ADFI than broilers in the Control group.

**Liver function**

There were linear \((p < .05)\) decreasing in serum ALT and AST levels with COS supplementation (Figure 2), and dietary 200 mg/kg COS supplementation significantly decreased \((p < .05)\) ALT and AST levels compared with the Control group.
**Table 2.** Effects of chitosan oligosaccharides on growth performance of broilers under high ambient temperature.

| Itema | Control | COS100 | COS200 | SEMb | Linear | Quadratic |
|-------|---------|--------|--------|------|--------|-----------|
| Day 1–21 | 35–56 days of age |       |        |      |        |           |
| ADG, g | 25.12 | 24.09 | 22.32 | 1.20 | 0.118 | 0.800     |
| ADFI, g | 81.93 | 82.27 | 79.33 | 1.82 | 0.329 | 0.474     |
| G:F   | 0.31  | 0.29  | 0.28  | 0.02 | 0.300 | 0.968     |
| Day 22–42 | 57–77 days of age |       |        |      |        |           |
| ADG, g | 18.92c,d | 16.05c | 20.90c | 0.98 | 0.172 | 0.006     |
| ADFI, g | 73.66d | 86.52c | 100.03d | 7.55 | 0.026 | 0.972     |
| G:F   | 0.26  | 0.20  | 0.22  | 0.02 | 0.182 | 0.141     |
| Day 43–56 | 78–91 days of age |       |        |      |        |           |
| ADG, g | 13.70 | 14.29 | 15.70 | 1.52 | 0.367 | 0.825     |
| ADFI, g | 112.96 | 97.91 | 104.95 | 12.27 | 0.316 | 0.693     |
| G:F   | 0.13  | 0.19  | 0.19  | 0.03 | 0.136 | 0.939     |
| Day 1–56 | 35–91 days of age |       |        |      |        |           |
| ADG, g | 19.25 | 18.14 | 19.64 | 0.77 | 0.720 | 0.185     |
| ADFI, g | 89.52c | 88.90 | 91.44 | 4.47 | 0.766 | 0.777     |
| G:F   | 0.21  | 0.21  | 0.22  | 0.01 | 0.619 | 0.458     |

*aADG: average daily gain; ADFI: average daily feed intake; G:F: Gain:Feed ratio; COS: chitosan oligosaccharides; Control group, basal diet; COS100 group, basal diet with 100 mg/kg COS; COS200 group, basal diet with 200 mg/kg COS.

*bSEM: standard error of mean.

c,dWithin the same row with different superscripts differ (p < .05).

**Anti-oxidant status**

No significant differences were observed in serum SOD and CAT activities among treatments (Figure 3). There were linear (p < .05) decreasing in serum MDA content, and linear (p < .05) improvement in serum GSH-Px activity with COS supplementation. Dietary 100 mg/kg and 200 mg/kg COS supplementation significantly decreased (p < .05) MDA content and increased (p < .05) GSH-Px activity compared with the Control group.

No significant differences were observed in liver GSH-Px and CAT activities among treatments (Figure 4). There were linear (p < .05) decreasing in liver MDA content, and linear (p < .05) improvement in liver SOD activity with COS supplementation. Dietary 100 mg/kg and 200 mg/kg COS supplementation significantly decreased (p < .05) MDA content and dietary 200 mg/kg COS supplementation significantly increased (p < .05) SOD activity compared with the Control group.

No significant differences were observed in spleen SOD and CAT activities among treatments (Figure 5). There was linear (p < .05) decreasing in liver MDA content and a linear increasing trend (p < .10) in spleen GSH-Px activity with COS supplementation. Dietary 200 mg/kg COS supplementation significantly decreased (p < .05) MDA content compared with the Control group.

**Inflammatory cytokines**

There were linear (p < .05) decreasing in serum IL-1β and TNF-α levels, and linear (p < .05) improvement in serum IL-10 level with COS supplementation (Figure 6). Dietary 100 mg/kg and 200 mg/kg COS supplementation significantly decreased (p < .05) IL-1β level, and dietary 200 mg/kg COS significantly increased (p < .05) IL-10 and decreased TNF-α level compared with the Control group.

No significant differences were observed in liver and spleen IL-1β and TNF-α levels, but a linear improving trend (p < .10) in liver and spleen IL-10 with COS supplementation (Figures 7 and 8, respectively).

**Discussion**

The main purpose of poultry production was to maintain the broilers’ health status, improve production performance and immune response (Abdel-Wareth et al. 2019). Our study demonstrated that dietary COS supplementation can improve ADG, ADFI, anti-oxidant capacity and alleviate inflammation response in serum, liver and spleen of broilers under high ambient temperature.

The COS, the functional oligosaccharides, was beneficial to broilers’ growth performance by increasing BW gain, FI and FCR (Huang et al. 2005; Li et al. 2007; Li et al. 2019). Moreover, dietary oligosaccharides exhibited better FI, BW gain, and FCR in heat-stressed broilers (Cheng et al. 2018; Cheng et al. 2019c). Similarly, in this study, dietary COS supplementation improved ADG and ADFI during day 22 to 42 (57 to 77 days of age). The improvement ADG may due to the enhancement of nutrient digestibility, intestinal integrity, anti-oxidant capacity and immunity (Huang et al. 2005; Li et al. 2007; Zhou et al. 2009; Li et al. 2019). It was believed that ambient temperature above 30°C is sufficient to induce heat stress for broilers, resulting in physiological disturbance and poor growth performance (Quinteiro-Filho et al. 2010). In this study, the broilers reared under ambient temperature above 30°C, dietary COS supplementation improved growth performance, suggesting that COS can attenuate the heat stress and simultaneously better growth performance. However, the ADG, ADFI, and G:F did not differ among the treatments during day 1 to 21 (35 to 56 days of age), day 22 to 42 (57 to 91 days of age), or day 1 to 56 (78 to 91 days of age). Similarly, Tufan et al. (2015) indicated that dietary COS supplementation had no effects on ADG, ADFI or G:F in Japanese quails. While, other studies reported that dietary COS supplementation had beneficial effects on ADG, ADFI or G:F in broilers (Li et al. 2007; Li et al. 2019). The inconsistent results may be associated with the stress condition, the different deacetylation
In health status, AST and ALT enzymes were normally expressed in liver, when liver damaged, AST and ALT enzymes released into the blood (Kim et al. 2015), the elevated serum ALT and AST levels were served as marker of liver damage (Dufour et al. 2000). Heat stress was known to induce liver damage (Zeng et al. 2014), and increased serum AST and ALT levels (Tessari et al. 2010; Cheng et al. 2019b). As expected, dietary COS supplementation alleviated liver damage by decreasing serum AST and ALT levels under high ambient temperature. Other studies also reported that dietary COS supplementation alleviated liver damage by decreasing serum AST and ALT levels in D-galactose-induced subacute aging mice (Kong et al. 2018) and carbon tetrachloride-induced mice (Miao et al. 2007). These results suggested that dietary COS supplementation can attenuate the high ambient temperature-induced liver damage.

Exposure to heat stress resulted in reactive oxygen species (ROS) overproduction and disturbing the balance between ROS production and antioxidant defense system via increasing lipid peroxidation and depletion of antioxidant enzymes (Azad et al. 2010; Yang et al. 2010; Liu et al. 2014), eventually contributed to oxidative stress and damage (Hall et al. 2010; Sahin et al. 2010). The MDA, the end product of lipid peroxidation, served as the marker of oxidative stress (Cheng et al. 2017). Previous studies indicated that MDA content was increased and anti-oxidant enzymes were decreased in heat-stressed broilers (Cheng et al. 2018; Cheng et al. 2019c). In this study, dietary COS supplementation decreased MDA content in serum, liver and spleen. Consistent with our results, former studies also demonstrated that dietary COS

Figure 3. Effects of chitosan oligosaccharides (COS) on anti-oxidant status in serum of yellow-feather broilers under high ambient temperature. Control group, basal diet; COS100 group, basal diet with 100 mg/kg COS; COS200 group, basal diet with 200 mg/kg COS. MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; CAT: catalase. Values are mean ± standard error of mean. The values have different superscript letters are different ($p < .05$).
supplementation decreased MDA content in jejunum and ileum mucosa (Li et al. 2017; Li et al. 2019). In the process of anti-oxidant defense functions, liver was the major organ for synthesising the anti-oxidant enzymes. Yang et al. (2010) indicated that acute heat stress-induced overproduction of ROS, increased MDA content, SOD, GSH-Px and CAT activity in liver. Seven et al. (2009) indicated that heat stress increased liver MDA content and CAT activity, but decreased GSH-Px activity. Zhang et al. (2018) indicated that heat stress-induced higher spleen MDA content and lower T-AOC, T-SOD, and CAT activities. In this study, dietary COS supplementation linearly increased serum GSH-Px activity, liver SOD activity, as well as a linearly increasing trend of spleen GSH-Px activity. The improvement of anti-oxidant enzymes and decreased MDA content in liver and spleen, indicating that the anti-oxidant capacity of liver and spleen were enhanced by COS supplementation under high ambient temperature. Similar results also reported in rat, which demonstrated that COS can prevent different stress-induced oxidative stress in liver and spleen (Qiao et al. 2011; Lan et al. 2019; Lan et al. 2019). The COS can work as anti-oxidant due to its anti-oxidant capacity and ROS scavenging property (Zou et al. 2016), the improved anti-oxidant capacity in this study is primarily due to the anti-oxidant capacity of COS.

Inflammation was induced by the innate immune system in response to tissue damage or invade pathogens. In response to heat stress, the levels of pro-inflammatory cytokines increased, which may result in haemorrhage and necrosis in liver and spleen (Jang et al. 2014; Ohtsu et al. 2015). Former studies indicated that the pro-inflammatory cytokines production were associated with the overproduction of ROS under heat stress (Jang et al. 2014). In this study, dietary

Figure 4. Effects of chitosan oligosaccharides (COS) on anti-oxidant status in liver of yellow-feather broilers under high ambient temperature. Control group, basal diet; COS100 group, basal diet with 100 mg/kg COS; COS200 group, basal diet with 200 mg/kg COS. MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; CAT: catalase. Values are mean ± standard error of mean. The values have different superscript letters are different \((p < .05)\).
COS supplementation linearly decreased serum IL-1β and TNF-α levels, linearly increased IL-10 level, and a linearly increasing trend in liver and spleen IL-10 level. Bits of cytokines may serve as cell-signaling molecules, which activated nuclear factor kappa B signal pathway, hence promoting the production of cytokines (Xu

Figure 5. Effects of chitosan oligosaccharides (COS) on anti-oxidant status in spleen of yellow-feather broilers under high ambient temperature. Control group, basal diet; COS100 group, basal diet with 100 mg/kg COS; COS200 group, basal diet with 200 mg/kg COS. MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; CAT: catalase. Values are mean ± standard error of mean. The values have different superscript letters are different (p < .05).

Figure 6. Effects of chitosan oligosaccharides (COS) on inflammatory cytokines in serum of yellow-feather broilers under high ambient temperature. Control group, basal diet; COS100 group, basal diet with 100 mg/kg COS; COS200 group, basal diet with 200 mg/kg COS. IL-1β: interleukin-1β; IL-10: interleukin-10; TNF-α: tumour necrosis factor-α. Values are mean ± standard error of mean. The values have different superscript letters are different (p < .05).
et al. 2018). Our results indicated that high ambient temperature-induced inflammation was repressed with COS supplementation. However, the results were not always inconsistent, Lan et al. (2019) indicated that dietary COS supplementation increased IL-10 and decreased IL-1β concentration in the liver of heat-stressed rats (Lan et al. 2019). Lan et al. (2019) also reported that dietary COS supplementation had no significant effects on inflammatory cytokines concentration in the liver and spleen of H2O2-challenged rat. Qiao et al. (2011) demonstrated that dietary COS supplementation decreased serum IL-1β and TNF-α levels in LPS-challenged mice. The inconsistent results indicated that the effects of COS on inflammation response were related to many factors, including species, stressor types, and environmental condition.

**Conclusions**

In conclusion, supplementation COS at 200 mg/kg of diet alleviates oxidative stress and inflammation response in broilers exposed to the high ambient temperature. These promising effects may exert through decreased MDA content, increasing SOD and GSH-Px activities, and IL-10 level in vital organs.

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**Ethical approval**

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

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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