Effects of selenide chitosan sulfate on glutathione system in hepatocytes and specific pathogen-free chickens

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ABSTRACT This study aimed to investigate the effects of selenide chitosan sulfate (Se-CTS-S) on glutathione (GSH) system in hepatocytes and chickens. Chitosan, sodium selenite (Na2SeO3), selenide chitosan, chitosan sulfate (CTS-S), and Se-CTS-S were added to the culture medium and the basal diets; glutathione peroxidase (GSH-Px) activity, GSH content, total antioxidant capacity (T-AOC), and mRNA levels of cellular GPx (GPx-1) and phospholipid hydroperoxide GPx (GPx-4) in vivo and in vitro were determined. The results showed that Se-CTS-S increased (P < 0.05) GPx-1 and GPx-4 mRNA levels in hepatocytes and livers, and GSH-Px activity, GSH content, and T-AOC in the medium, hepatocytes, plasma, and livers compared with the control and chitosan treatments. Compared with CTS-S, Se-CTS-S treatments increased (P < 0.05) GPx-1 and GPx-4 mRNA levels in hepatocytes and livers, and GSH-Px activity, GSH content, and T-AOC capacity in the medium, hepatocytes, and livers. Compared with Na2SeO3 and CTS-Se, Se-CTS-S increased (P < 0.05) GPx-1 mRNA levels in hepatocytes and livers, GPx-4 mRNA levels in hepatocytes and livers, GSH-Px activity in the medium, hepatocytes, and livers, GSH contents in plasma and livers, and T-AOC in the medium, plasma, and livers. Thus, Se-CTS-S showed better biological activity that mainly benefited from the synergistic effects of Se and sulfate on GSH system.

Key words: selenide chitosan sulfate, glutathione, glutathione peroxidase, hepatocyte, chicken

INTRODUCTION

Chitosan (CTS), the generic term for a family of linear amino polysaccharides, is an N-acetylated product of chitin with properties of nontoxicity, biocompatibility, biodegradability, being environmentally friendly, and involving low cost (Sahariah and Måsson, 2017; Adhikari and Yadav, 2018). CTS is involved in a variety of biological functions including immune enhancement, anti-aging, anti-oxidation, anti-cancer, anti-rheumatism, and antimicrobial functions (Jan et al., 2012; Smith et al., 2014; Younes and Rinaudo, 2015). Therefore, it is highly suitable for use in food, animal production, textile, environment, and medical and pharmaceutical applications (Suh et al., 2016; Wang et al., 2018).

In a CTS molecule, there are 2 active groups: hydroxyl and amidogen, which are an ideal carrier of a variety of trace elements and chemical groups. Chemical modification of CTS can improve its solubility and enhance its biological effects (Fan et al., 2012; Qin et al., 2015). Especially in solution, the hydroxyl and amidogen groups of CTS are protonated, the resultant soluble polysaccharide is positively charged (cationic), and CTS molecules are easy to combine or chelate with sulfonic acid groups and multiple trace element ions (Patel et al., 2012; Pires et al., 2013; Xing et al., 2015). When CTS binds to them, these CTS complexes show better bioavailability, biological activity, and lower toxicity (Battin and Brumaghim, 2009; Wang et al., 2012; Qin et al., 2015; Zhai et al., 2017).

Selenide chitosan sulfate (Se-CTS-S) is a product of CTS modified by selenization and sulfation. We believe that Se-CTS-S might have excellent biological activity. To date, there are no studies that indicate the biological
activity of Se-CTS-S, and we do not know whether it can play the synergistic role of Se and sulfate. Glutathione peroxidase (GSH-Px), an important peroxide-degrading enzyme, can catalyze the conversion of reduced glutathione (GSH) to oxidized GSH, reduce toxic peroxides to non-toxic hydroxyl compounds, and thereby protect cell membrane structure and function from peroxide interference and damage (Wu et al., 2010; Gan et al., 2014; Bakhshalinejad et al., 2018). Nowadays, GSH system is one of the most important indicators commonly used for evaluating the biological activity of Se compounds and polysaccharide sulfates (Chen et al., 2017; Wang et al., 2017; Bakhshalinejad et al., 2018). Therefore, the purpose of this study was to investigate the effects of Se-CTS-S on GSH system and compare them with those of CTS, sodium selenite (Na$_2$SeO$_3$), selenide chitosan (Se-CTS), and chitosan sulfate (CTS-S) in vitro and in vivo.

**MATERIALS AND METHODS**

**CTS-S, Se-CTS, and Se-CTS-S Preparation**

CTS-S, Se-CTS, and Se-CTS-S were synthesized with CTS (molecular weight ≤ 5,000 g/mol) with a deacetylation degree of 90.5% (Qingdao BZ Oligo Co., Ltd., Qingdao, China), a sulfonated reagent (Chengdu Laibao Technology Co., Ltd., Chengdu, China), and (or) Na$_2$SeO$_3$ (analytical grade >98.5%) solution. Se content was detected by hydride generation atomic fluorescence spectrometry (Pires et al., 2013) and sulfate content was detected by the gelatin–BaC$_2$O$_4$ method (Dodgson and Price, 1962). Se content was 30 to 40 mg/g and sulfate content was 200 to 250 mg/g in Se-CTS-S.

**In Vitro Experiment**

White Leghorn hens (aged 60 D) were used to obtain hepatocytes by the collagenase perfusion method and cultured by serum-free culture method (Wu et al., 2010). Hepatocyte monolayers were treated with CTS, CTS-S, Na$_2$SeO$_3$, Se-CTS, and Se-CTS-S in 6-well plates, respectively. Group 1 served as a control that was treated without Se, sulfate, and CTS; group 2 was treated with 10 mg/L of CTS; groups 3 and 4 were treated with 1.5 μmol/L of Se as Na$_2$SeO$_3$ and Se-CTS, respectively; group 5 was treated with 3 mg/L of sulfate as CTS-S; and group 6 was treated with 1.5 μmol/L of Se and 3 mg/L of sulfate as Se-CTS-S. Se, sulfate, and CTS content in the culture medium are shown in Table 1. Each treatment was done in 6 wells. After 24 h of incubation, the culture medium was collected for assay of GSH-Px activity, GSH content, and total antioxidant capacity (T-AOC); hepatocytes were harvested and stored in liquid nitrogen for assay of GSH-Px activity, GSH content, T-AOC, and the mRNA levels of cellular Gpx (GPx-1) and phospholipid hydroperoxide Gpx (GPx-4).

**In Vivo Experiment**

Feeding experiment was carried out at the Laboratory Animal Center, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Qingdao Agricultural University. A total of 2 hundred and forty 7-day-old specific pathogen-free male White Leghorn chickens with initial body weight of 43.0 ± 2.0 g were purchased from the Institute of Animal Husbandry and Veterinary Medicine, Shandong Academy of Agricultural Sciences and randomly allocated to 6 experimental treatments with 4 replicate pens, with each replication containing 10 chickens. Group 1 served as a control and was fed a basal diet; group 2 was fed the basal diet supplemented with 30 mg CTS per kg; groups 3 and 4 were fed the basal diet supplemented with 0.3 mg Se from Na$_2$SeO$_3$ (analytical grade >98.5%) and Se-CTS per kg, respectively; group 5 was fed the basal diet supplemented with 6.0 mg sulfate from CTS-S per kg; and group 6 was fed the basal diet supplemented with 36.3 mg Se-CTS-S per kg. The basal diet was a corn-soybean meal-based diet, which was formulated to approximately meet the nutrient requirements for chickens. The basal diet formulation and approximate composition are shown in Table 2. Over the entire experimental period of 28 D, chickens were housed in a closed and ventilated building and provided with continuous light; water and feed were provided ad libitum. On days 14 and 28, blood samples were drawn from the heart, and plasma was collected and stored at −20°C for assay of GSH-Px activity, GSH content, and T-AOC; 12 chickens from each group (3 chickens per pen) were selected and euthanized with dimethyl ether, and livers were removed and stored in liquid nitrogen for assay of GSH-Px activity, GSH content, T-AOC, and the mRNA levels of GPx-1 and GPx-4.

**Laboratory Assay**

GSH-Px activity, GSH content, and T-AOC in the medium, hepatocytes, plasma, and livers were determined according to the instructions of GPx assay kit (colorimetric method), GSH assay kit (microenzyme labeling method), and T-AOC assay kit (ABTS method) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

| Table 1. Se, sulfate, and CTS content in the culture medium. |
|-------------------|---------|--------|---------|--------|--------|---------|
| Items             | Control | CTS    | Na$_2$SeO$_3$ | CTS-Se | CTS-S  | Se-CTS-S |
| Se content (μmol/L) | 0       | 0      | 1.5     | 1.5    | 0      | 1.5      |
| Sulfate content (mg/L) | 0      | 0      | 0       | 0      | 3      | 3        |
| CTS content (mg/L)  | 0      | 10     | 0       | 10     | 10     | 10       |

Abbreviations: CTS, chitosan; CTS-S, chitosan sulfate; CTS-Se, selenide chitosan; Na$_2$SeO$_3$, sodium selenite; Se-CTS-S, selenide chitosan sulfate.
Table 2. Formulation and approximate composition of experimental diets.

| Ingredient (%) | Value |
|----------------|-------|
| Maize          | 66.15 |
| Soybean meal   | 27.0  |
| Fish meal      | 3.30  |
| Dicalcium phosphate | 1.20 |
| Limestone      | 1.00  |
| Salt (NaCl)    | 0.25  |
| DL-Methionine  | 0.10  |
| Mineral premix  | 0.50 |
| Vitamin premix  | 0.50 |
| Total          | 100   |

Chemical composition

- Gross energy (kcal/kg): 2,843
- CP (% (DM)): 19.0
- Calcium (%): 0.91
- Available phosphorus (%): 0.41
- Lysine (%): 1.03
- Methionine (%): 0.44
- Methionine + cysteine (%): 0.78

1Mineral premix supplied the following per kilogram of complete feed: ZnSO4 80 mg; MnSO4 100 mg; FeSO4 80 mg; CuSO4 6 mg; KI 0.35 mg; and CoCl2 0.4 mg.

2Vitamin premix supplied the following per kilogram of complete feed:
- vitamin A: 4,000 IU
- vitamin B1: 1.8 mg
- vitamin B2: 3.6 mg
- vitamin B12: 0.01 mg
- vitamin D3: 800 IU
- vitamin E: 10 IU
- vitamin K: 0.5 mg
- biotin: 0.15 mg
- choline: 1,300 mg
- folacin: 0.55 mg/kg
- niacin: 30 mg
- and pantothenic acid: 10 mg.

The mRNA levels of GPx-1 and GPx-4 in hepatocytes and livers were determined by quantitative real-time reverse transcription PCR (Chen et al., 2017). Total RNA was extracted from the hepatocytes and livers with TRIzol reagent (Sangon Biotech [Shanghai] Co., Ltd., China), and then reversely transcribed in 40 μL of reaction mixture according to the manufacturer’s instructions (Sangon Biotech [Shanghai] Co., Ltd.), and stored in liquid nitrogen. Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA) was used to design specific primers for GPx-1, GPx-4, and β-actin (Chen et al., 2018; Su et al., 2018; Zhang et al., 2018) (Table 3). Quantitative real-time reverse transcription PCR was performed using SYBR Premix Ex Taq II (Sangon Biotech [Shanghai] Co., Ltd.) with 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The ratio of GPx-1 and GPx-4 mRNA levels to that of the β-actin internal control was used for statistical comparison of the different treatments.

**Statistical Analyses**

Data were expressed as means ± SD analyzed by using one-way ANOVA to compare means and the multivariate analysis of the GLM procedure of IBM SPSS 22.0 for Windows statistical software package (IBM Corporation, Armonk, NY). The least significant difference and Dunnett’s T3 tests were used to determine differences between means. The culture well and the pen were respectively defined as the experimental unit for statistical analysis and all calculations were generated based on well and pen averages. Differences were considered significant at $P < 0.05$ for all tests.

**RESULTS**

**GSH Content, GPx Activity, and T-AOC in Culture Medium and Hepatocytes**

The effects of CTS, sulfate, and Se on GSH content in vitro are shown in Figure 1A. Compared with the control, CTS, and CTS-S groups, Na2SeO3, CTS-Se, and Se-CTS-S supplementation increased GSH content in the culture medium and hepatocytes ($P < 0.05$). No significant effects on GSH content in the culture medium and hepatocytes were observed among the Se treatments (Na2SeO3, CTS-Se, and Se-CTS-S) and among the control, CTS, and CTS-S groups ($P > 0.05$).

The effects of CTS, sulfate, and Se on GPx activity and T-AOC in vitro are shown in Figures 1B and 1C. Compared with the control group, CTS supplementation increased GPx activity and T-AOC in the culture medium and hepatocytes ($P < 0.05$). Compared with the control group, CTS supplementation increased GPx activity and T-AOC in hepatocytes, and CTS-S supplementation increased GPx activity and T-AOC in the culture medium and hepatocytes ($P < 0.05$). Compared with the control group, CTS-S supplementation increased GPx activity and T-AOC in the culture medium and hepatocytes ($P < 0.05$).

**GSH Content, GPx Activity, and T-AOC in Plasma**

The effects of dietary CTS, sulfate, and Se supplementation on GSH content in plasma are shown in Figure 2A. Compared with the control, CTS, and CTS-S groups, Na2SeO3, CTS-Se, and Se-CTS-S supplementation increased GSH content in plasma ($P < 0.05$). Compared with the control group, dietary CTS

| Ingredient (%) | Value |
|----------------|-------|
| Maize          | 66.15 |
| Soybean meal   | 27.0  |
| Fish meal      | 3.30  |
| Dicalcium phosphate | 1.20 |
| Limestone      | 1.00  |
| Salt (NaCl)    | 0.25  |
| DL-Methionine  | 0.10  |
| Mineral premix  | 0.50 |
| Vitamin premix  | 0.50 |
| Total          | 100   |

Chemical composition

- Gross energy (kcal/kg): 2,843
- CP (% (DM)): 19.0
- Calcium (%): 0.91
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1Mineral premix supplied the following per kilogram of complete feed: ZnSO4 80 mg; MnSO4 100 mg; FeSO4 80 mg; CuSO4 6 mg; KI 0.35 mg; and CoCl2 0.4 mg.

2Vitamin premix supplied the following per kilogram of complete feed:
- vitamin A: 4,000 IU
- vitamin B1: 1.8 mg
- vitamin B2: 3.6 mg
- vitamin B12: 0.01 mg
- vitamin D3: 800 IU
- vitamin E: 10 IU
- vitamin K: 0.5 mg
- biotin: 0.15 mg
- choline: 1,300 mg
- folacin: 0.55 mg/kg
- niacin: 30 mg
- and pantothenic acid: 10 mg.

The mRNA levels of GPx-1 and GPx-4 in hepatocytes and livers were determined by quantitative real-time reverse transcription PCR (Chen et al., 2017). Total RNA was extracted from the hepatocytes and livers with TRIzol reagent (Sangon Biotech [Shanghai] Co., Ltd., China), and then reversely transcribed in 40 μL of reaction mixture according to the manufacturer’s instructions (Sangon Biotech [Shanghai] Co., Ltd.), and stored in liquid nitrogen. Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA) was used to design specific primers for GPx-1, GPx-4, and β-actin (Chen et al., 2018; Su et al., 2018; Zhang et al., 2018) (Table 3). Quantitative real-time reverse transcription PCR was performed using SYBR Premix Ex Taq II (Sangon Biotech [Shanghai] Co., Ltd.) with 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The ratio of GPx-1 and GPx-4 mRNA levels to that of the β-actin internal control was used for statistical comparison of the different treatments.

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**RESULTS**

**GSH Content, GPx Activity, and T-AOC in Culture Medium and Hepatocytes**

The effects of CTS, sulfate, and Se on GSH content in vitro are shown in Figure 1A. Compared with the control, CTS, and CTS-S groups, Na2SeO3, CTS-Se, and Se-CTS-S supplementation increased GSH content in the culture medium and hepatocytes ($P < 0.05$). No significant effects on GSH content in the culture medium and hepatocytes were observed among the Se treatments (Na2SeO3, CTS-Se, and Se-CTS-S) and among the control, CTS, and CTS-S groups ($P > 0.05$).

The effects of CTS, sulfate, and Se on GPx activity and T-AOC in vitro are shown in Figures 1B and 1C. Compared with the control group, CTS supplementation increased GPx activity and T-AOC in the culture medium and hepatocytes ($P < 0.05$). Compared with the control group, CTS supplementation increased GPx activity and T-AOC in hepatocytes, and CTS-S supplementation increased GPx activity and T-AOC in the culture medium and hepatocytes ($P < 0.05$). Compared with the control group, CTS-S supplementation increased GPx activity and T-AOC in the culture medium and hepatocytes ($P < 0.05$).

**GSH Content, GPx Activity, and T-AOC in Plasma**

The effects of dietary CTS, sulfate, and Se supplementation on GSH content in plasma are shown in Figure 2A. Compared with the control, CTS, and CTS-S groups, Na2SeO3, CTS-Se, and Se-CTS-S supplementation increased GSH content in plasma ($P < 0.05$). Compared with the control group, dietary CTS
supplementation increased GSH content in plasma (on day 14) \( (P < 0.05) \), and dietary CTS-S supplementation increased GSH content in plasma (on days 14 and 28) \( (P < 0.05) \). Compared with the \( \text{Na}_2\text{SeO}_3 \) and CTS-Se groups, dietary Se-CTS-S supplementation increased GSH content in plasma (on days 14 and 28) \( (P < 0.05) \).

The effects of dietary CTS, sulfate, and Se supplementation on GSH-Px activity in plasma are shown in Figure 2B. Compared with the control group, dietary CTS supplementation increased GSH-Px activity in plasma (on day 14) \( (P < 0.05) \), and dietary CTS-S, \( \text{Na}_2\text{SeO}_3 \), CTS-Se, and Se-CTS-S supplementation increased GSH-Px activity in plasma (on days 14 and 28) \( (P < 0.05) \). Compared with the CTS group, dietary \( \text{Na}_2\text{SeO}_3 \) and CTS-S supplementation increased GSH-Px activity in plasma (on day 28) \( (P < 0.05) \), and dietary CTS-Se and Se-CTS-S supplementation increased GSH-Px activity in plasma (on days 14 and 28) \( (P < 0.05) \). Compared with the CTS-S group, dietary \( \text{Na}_2\text{SeO}_3 \) supplementation increased GSH-Px activity in livers (on day 28) \( (P < 0.05) \), and dietary CTS-Se and Se-CTS-S supplementation increased GSH-Px activity in livers (on days 14 and 28) \( (P < 0.05) \).

The effects of dietary CTS, sulfate, and Se supplementation on GSH content in plasma are shown in Figure 3A. Dietary \( \text{Na}_2\text{SeO}_3 \) and Se-CTS-S supplementation increased GSH content in plasma (on day 28) \( (P < 0.05) \), and dietary Se-CTS-S supplementation increased GSH content in livers (on day 28) \( (P < 0.05) \), and dietary CTS-Se and Se-CTS-S supplementation increased GSH content in livers (on days 14 and 28) \( (P < 0.05) \). Compared with the control group, dietary CTS supplementation increased GSH content in livers (on day 14) \( (P < 0.05) \), and dietary CTS-S, \( \text{Na}_2\text{SeO}_3 \), CTS-Se, and Se-CTS-S supplementation increased T-AOC in plasma (on day 28) compared to the control group \( (P < 0.05) \), dietary CTS-Se supplementation increased T-AOC in plasma (on day 28) compared with the CTS and CTS-S group \( (P < 0.05) \), and dietary Se-CTS-S supplementation increased T-AOC in plasma (on day 28) compared with the CTS, \( \text{Na}_2\text{SeO}_3 \), and CTS-S group \( (P < 0.05) \).

### GSH Content, GSH-Px Activity, and T-AOC in Livers

The effects of dietary CTS, sulfate, and Se supplementation on GSH content in livers are shown in Figure 3A. Compared with the control group, dietary CTS supplementation increased GSH content in livers (on day 14) \( (P < 0.05) \), and dietary CTS-S, \( \text{Na}_2\text{SeO}_3 \), CTS-Se, and Se-CTS-S supplementation increased GSH content in livers (on days 14 and 28) \( (P < 0.05) \). Compared with the CTS group, dietary \( \text{Na}_2\text{SeO}_3 \) and CTS-S supplementation increased GSH content in livers (on day 28) \( (P < 0.05) \), and dietary CTS-Se and Se-CTS-S supplementation increased GSH content in livers (on days 14 and 28) \( (P < 0.05) \). Compared with the CTS-S group, dietary \( \text{Na}_2\text{SeO}_3 \) supplementation increased GSH content in livers (on day 28) \( (P < 0.05) \), and dietary CTS-Se and Se-CTS-S supplementation increased GSH content in livers (on days 14 and 28) \( (P < 0.05) \). Compared with the Na\(_2\)SeO\(_3\) group, dietary CTS-Se
supplementation increased GSH content in livers (on day 28) \((P < 0.05)\), and dietary Se-CTS-S supplementation increased GSH content in livers (on days 14 and 28) \((P < 0.05)\). Compared with the CTS-Se group, dietary Se-CTS-S supplementation increased GSH content in livers (on day 28) \((P < 0.05)\).

The effects of dietary CTS, sulfate, and Se supplementation on GSH-Px activity in livers are shown in Figure 3B. Compared with the control group, dietary CTS supplementation increased GSH-Px activity in livers (on day 14) \((P < 0.05)\), and dietary CTS-S, Na\(_2\)SeO\(_3\), CTS-Se, and Se-CTS-S supplementation increased GSH-Px activity in livers (on days 14 and 28) \((P < 0.05)\). Compared with the CTS group, dietary CTS-Se and Se-CTS-S supplementation increased GSH-Px activity in livers (on days 14 and 28) \((P < 0.05)\), and dietary CTS-S, Na\(_2\)SeO\(_3\), CTS-Se, and Se-CTS-S supplementation increased T-AOC in livers (on day 28) \((P < 0.05)\). Compared with the Na\(_2\)SeO\(_3\) and CTS-Se group, dietary Se-CTS-S supplementation increased T-AOC in livers (on day 28) \((P < 0.05)\).

**mRNA Levels of GPx-1 and GPx-4 in Hepatocytes and Livers**

The effects of dietary CTS, sulfate, and Se supplementation on the mRNA levels of GPx-1 in hepatocytes and livers are shown in Figure 4A. Compared with the control group, CTS and CTS-S supplementation increased the mRNA levels of GPx-1 in hepatocytes \((P < 0.05)\). Compared with the control, CTS, and CTS-S groups, Na\(_2\)SeO\(_3\), CTS-Se, and Se-CTS-S supplementation increased the mRNA levels of GPx-1 in hepatocytes \((P < 0.05)\). Compared with the Na\(_2\)SeO\(_3\) and CTS-Se group, dietary Se-CTS-S supplementation increased the mRNA levels of GPx-1 in hepatocytes and livers (on days 14 and 28) \((P < 0.05)\). Compared with the Na\(_2\)SeO\(_3\) group, CTS-Se supplementation decreased the mRNA levels of GPx-1 in livers (on...
day 14) \((P < 0.05)\), and Se-CTS-S supplementation increased the mRNA levels of GPx-1 in hepatocytes and livers (on days 14 and 28) \((P < 0.05)\). Compared with the CTS-Se group, Se-CTS-S supplementation increased the mRNA levels of GPx-1 in hepatocytes and livers (on days 14 and 28) \((P < 0.05)\).

The effects of CTS, sulfate, and Se supplementation on the mRNA levels of GPx-4 in hepatocytes and livers are shown in Figure 4B. Compared with the control, CTS, and CTS-S groups, \(\text{Na}_2\text{SeO}_3\), CTS-Se, and Se-CTS-S supplementation increased the mRNA levels of GPx-4 in hepatocytes and livers (on days 14 and 28). Compared with the \(\text{Na}_2\text{SeO}_3\) group, Se-CTS-S supplementation increased the mRNA levels of GPx-4 in hepatocytes (on day 28) \((P < 0.05)\). Compared with the CTS-Se group, Se-CTS-S supplementation increased the mRNA levels of GPx-4 in hepatocytes \((P < 0.05)\).
DISCUSSION

The scavenging effects of 3 kinds of CTS with different molecular weights and their sulfate derivatives on O$_2^-$ and -OH were studied. The results showed that the scavenging ability of CTS-S to O$_2^-$ was significantly enhanced after sulfation, and CTS-S could achieve the effect of scavenging O$_2^-$ at a very low mass concentration (0.1 g/L) (≥90%). This shows that the sulfated product of CTS is a highly active scavenger of O$_2^-$ (Xing et al., 2015). The antioxidant activity of CTS-S may be the reason for increasing the relative mRNA level of GPx-1 in hepatocytes. Accumulating evidence indicated the antioxidant effects of CTS on indices of oxidative stress by scavenging free radicals or preventing oxidative damage (Ngo and Kim, 2014; Ngo et al., 2015; Wang et al., 2016; Kim et al., 2018), and the protective and repair effects of sulfate (sulfated polysaccharides) on damaged cells caused by oxidative stress (Liu et al., 2014; Shao et al., 2014; Deng et al., 2015; Bai et al., 2017). Similarly, the present study confirmed the antioxidant effects of CTS and CTS-S in vivo and in vitro. Moreover, we found that sulfate supplementation from CTS-S treatment enabled better antioxidant activity than CTS treatments both in vivo and in vitro. These results suggested that sulfate in the CTS-S molecule played an important role in enhancing the antioxidant function in vitro and in vivo, and CTS became a more efficient compound after modification of sulfation (Battin and Brumaghim, 2009; Wang et al., 2012).

It is noteworthy that the antioxidant properties are one of the most important characteristics of Se compounds, especially regulating effects on antioxidant enzymes (Khan et al., 2018; Zoidis et al., 2018; Yang et al., 2019). Compared with the control and CTS treatments, Se supplementation from different Se treatments (Na$_2$SeO$_3$, Se-CTS, and Se-CTS-S) indicated higher GSH-Px activity, GSH content, T-AOC, and mRNA levels of GPx-1 and GPx-4 in vitro and in vivo. These results further demonstrated that Se supplementation could improve the antioxidant function in vitro and in vivo (Wu et al., 2010; Fan et al., 2012; Chen et al., 2017; Yang et al., 2019). Moreover, the antioxidant activity of CTS was significantly higher following Se modification and Se-CTS was a more efficient compound (Qin et al., 2015).

The present study showed that Se-CTS treatments exhibited similar antioxidant activity as Na$_2$SeO$_3$, except that Se-CTS had higher GSH content in livers (day 28) and T-AOC in the medium, and lower GSH-Px activity in plasma (day 14) and GPx-1 mRNA level in livers (day 14) than those of Na$_2$SeO$_3$ treatments. In contrast, Qin et al., (2015) reported that Se-CTS increased blood GSH-Px activity compared to the control and Na$_2$SeO$_3$ treatments, and its biological activity was better than Na$_2$SeO$_3$ in mice. Bai et al., (2017) reported that Se nanoparticles-loaded CTS microspheres displayed powerful antioxidant activities, and demonstrated similar efficacy in increasing GSH-Px activity in the blood and liver of mice compared with Na$_2$SeO$_3$. We believe that this difference may be explained by the statement that different forms of Se-CTS may exhibit different biological activities in different samples and animals.

Previous studies have investigated the chemical mechanisms for antioxidant behavior and demonstrated that sulfate and Se compounds could ameliorate oxidative damage by utilizing multiple complex antioxidant mechanisms (Battin and Brumaghim, 2009). In the present study, Se supplementation from different Se treatments (Na$_2$SeO$_3$, Se-CTS, and Se-CTS-S) exhibited better antioxidant activities than CTS treatments, especially increasing GSH content, GSH-Px activity, T-AOC, and GPx-1 and GPx-4 mRNA levels in vitro and in vivo. These results suggested that the biological ability of Se, especially the antioxidant activity, was more pronounced than that of sulfate. This could be explained by the theory that the antioxidant activities of sulfate and Se might be quite distinct as a result of utilizing different antioxidant mechanisms; sulfate itself does not primarily exhibit GSH-Px activity; moreover, as Se-dependent antioxidant enzymes, their expressions, activity, and effectors (GSH) of GPx were mainly regulated by Se rather than by sulfate (Battin and Brumaghim, 2009; Wu et al., 2010; Chen et al., 2017; Yang et al., 2019).

The present study suggested that Se-CTS-S treatments exhibited higher antioxidant activity by increasing GSH content, GSH-Px activity, T-AOC, and GPx-1 and GPx-4 mRNA levels than CTS-S and Se-CTS treatments in vivo and in vitro. We considered that both selenite and sulfate may synergistically play an important role in enhancing antioxidant function in vitro and in vivo when CTS became a more efficient compound (Se-CTS-S) after modification of selenization and sulfation.

In summary, Se-CTS-S supplementation could improve antioxidant function by activating the GSH system, increasing GPx-1 and GPx-4 mRNA expressions, GSH-Px activity, and GSH produce, and then enhancing T-AOC of hepatocytes and chickens. Moreover, Se-CTS-S showed better biological activity, which mainly benefits from the synergistic effects of selenite and sulfate on enhancing antioxidant function.

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