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Research Article

Effect of Chitosan Oligosaccharides on Antioxidant Function, Lymphocyte Cycle and Apoptosis in Ileum Mucosa of Broiler

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Abstract

The purpose of this study was to investigate the effects of chitosan oligosaccharides (COS) on the antioxidative function, lymphocyte cycle and apoptosis of ileum mucosa in broiler. 640 AA broilers were randomly allocated into four groups, which were fed with diets supplemented 0, 200, 350 and 500 mg/kg of COS for six weeks, respectively. The results showed that compared with the control group, the activities of glutathione peroxidase and superoxide dismutase, the ability of total antioxidant capacity and inhibit hydroxy radical as well as the contents of glutathione in the 350 and 500 mg/kg COS groups were significantly increased, while the levels of malonedialdehyde were significantly decreased. The percentages of S and gap 2/mitosis (G_2M) phases and proliferating index of ileum mucosal lymphocytes in the 350 and 500 mg/kg COS groups were increased, but, the percentages of apoptotic ileac lymphocytes were not significantly different compared with the control group. No significant difference in the levels of antioxidant function mentioned above, cell cycle phase distribution and the percentages of apoptotic ileac lymphocyte existed between the 350 and 500 mg/kg COS groups. Conclusion: dietary chitosan oligosaccharides supplements with 350 mg/kg and 500 mg/kg could improve the antioxidant function and accelerate lymphocytes proliferation, but had non-influence on lymphocytes apoptosis in the ileum mucosa of broiler.

Keywords: *Chitosan oligosaccharides, Lymphocyte, Antioxidant function, Cell cycle, Apoptosis*

Kitosan Oligosakkaritlerin Broiler İleum Mukozasında Antioksidan Fonksiyon, Lenfosit Döngüsü ve Apoptozis Üzerine Etkileri

Özet

Bu çalışmanın amacı; kitosan oligosakkaritlerin (COS) broiler ileum mukozasında antioksidan fonksiyon, lenfosit döngüsü ve apoptozise olan etkilerini araştırmaktır. 640 AA broiler rastgele olarak dört gruba ayrılarak sırasıyla 0, 200, 350 ve 500 mg/kg COS içeren diyetle altı hafta süresince beslendi. Kontrol grubu ile karşılaştırıldığında 350 ve 500 mg/kg COS içeren diyetle beslenen gruplarda glutatyon peroksidaz, ve süperoksit dismutaz aktiviteleri, total antioksidan kapasite ile hidroksit radikalini inhibe etme kapasitesi ve glutatyon miktarı anlamlı derecede artarken malondialdehit seviyesi azaldı. S yüzdesi ve gap 2/mitoz (G_2M) fazları ve ileum mukozal lenfositlerinin çoğalma indeksi 350 ve 500 mg/kg COS içeren diyetle beslenen gruplarda artarken, apoptotik iliak lenfositlerin yüzdesi anlamlı derece kontrol grubundan farklılık göstermedi. 350 ve 500 mg/kg COS içeren diyetle beslenen gruplar arasında yukarıda bahsi geçen antioksidan fonksiyon, hücre döngü faz dağılımı ve apoptotik iliak lenfosit yüzdeleri bakımından anlamlı fark tespit edilmedi. Sonuç olarak, diyette kitosan oligosakkaritlerin 350 ve 500 mg/kg oranında ilavesi broilerlerin ileum mukozasında antioksidan fonksiyonu geliştirerek lenfosit çoğalmasını hızlandırırken, lenfositlerde apoptozis üzerine etki etmemektedir.

Anahtar sözcükler: *Kitosan oligosakkaritler, Lenfosit, Antioksidan fonksiyon, Hücre döngüsü, Apoptozis*

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INTRODUCTION

In recent decades, chitosan has been used as one of additives in forage due to its various biological activities, including antitumor [1], antioxidative [2], immunepotentiating [3] and some other health benefits [4], but its high molecular weight and high viscosity may limit its use *in vivo* [5]. Chitosan oligosaccharides (COS) are the depolymerized products of chitosan, having similar or even better biological activities [6]. COS received wide-spread attention because of its solubility [7,8], low toxicity to eukaryotes [4], and immune-enhancing effects [9], along with improvement in health status of human being and animals [10-13].

The antioxidant activity of COS has been demonstrated *in vitro* and *in vivo*. In murine models, COS could reversed the decrease of glutathione (GSH) levels, and catalase (CAT) activity, and the increase of malonedialdehyde (MDA) levels in the liver, lung and kidney from LPS-induced mice [14]. In Alzheimer's disease rat, COS treatment increased hippocampal superoxide dismutase (SOD) activity level [15], and in another *in vitro* study, COS exerted antioxidative effects on pancreatic islet cells in streptozotocin-induced diabetes in rats [16]. HJ Yoon [17] reported that the production of creatinine and MDA was increased and SOD was decreased in the glycerol-induced acute renal failure rats, and COS recovered aforesaid oxidative damage in kidney [17]. COS not only has antioxidative activities in aforementioned disease models, but also has a similar effect in normal animal such as *Penaeus monodon*, in which the total antioxidant status (TAS) and glutathione peroxidase (GSH-Px) activities of digestive gland in the COS diet group were higher than those in the control [18].

It has been shown that COS can affect cell cycle and inhibit tumor growth. In the study of Liu et al.[19], pre-incubation of COS with ECV304 cells for 24 h resulted in the induction of cell cycle arrest in G_1/S+M [19]. Han et al.[20] reported that COS could inhibit the proliferation of human lung cancer line HepG2 cells, and induce their G_2/M phase arrest [20]. In another study, COS significantly inhibited the proliferation of three types of human gastric cancer cells (BGC823 cells, MKN45 cells and SGC7901 cells) after treatment for 48 h and the inhibition rate was positively correlated with the concentration of COS [21]. But, Jiang et al.[22] demonstrated that the proliferation index and the expression of cyclin D1 of normal Schwann cells treated with 0.25, 0.5 and 1.0 mg/mL COS were increased when compared with those of control [22].

Moreover, COS can affect the apoptosis process. It could induce various cells apoptosis, such as human colon cancer cells HT-29 [23], SW480 cells [24], AGS human gastric cancer cells [25], hepatocellular carcinoma cells [26], and human myeloid leukemia HL-60 cells [27]. Besides, 50-200 μg/mL COS treatment reversed the increasing of apoptotic articular chondrocytes by IL-1β-induced and downregulated the expression of Bax and caspase-3, and upregulated the expression of Bcl-2 of chondrocytes [28].

Intestinal tract is the body's structure which contacts with various antigens including bacteria, virus, parasites, sitotoxin and medicines. As part of the intestinal tract, the ileum is the major component of the gastrointestinal tract and its mucosal immune system plays an important role in the intestinal immune function. The lymphocytes of mucosa take part in mucosal immunity, their proliferation and apoptosis are one of the bases of the mucosal immune system operation. Early researches have shown that effects of COS on antioxidant role, cell cycle and apoptosis were mainly focused on tumors or disease models, and information concerning these areas on normal intestine was not available. The aim of this study was to investigate the effects of COS on antioxidant function, lymphocyte cycle and apoptosis in ileum mucosa of broiler by the methods of biochemistry and flow cytometry.

MATERIAL and METHODS

Animals and Diets

Six hundred and forty one-day-old male AA chicken were randomly and equally divided into four groups with eight replicate per group, that is, control group, COS-A group, COS-B group, COS-C group (the degree of deacetylation of COS exceed 95%, the molecular weight of COS was less than 2000 DA, ZTH Tech. Co., Beijing, China). All animal studies were approved by the Animal Ethics Committee of Sichuan Agricultural University (Approval no. 2012-024). Chickens were housed in coops with electrically heated units and provided with water and diet *ad libitum* for 42 days.

In all experiments, the basal diet was a typical corn-soybean diet which was formulated to meet standards of National Research Council [29]. COS was mixed into the corn-soybean basal diet to constitute the experimental diets with 200 mg/kg, 350 mg/kg and 500 mg/kg of COS for COS-A, COS-B and COS-C groups, respectively *(Table 1)*.

Detection of Antioxidant Function in Ileum Mucosa

At 21 and 42 days of age during the experiment, eight broilers of each group were sacrificed and ilea were immediately removed and chilled to 0°C in normal saline (Ileum was defined as the segment before the ileocecal junction equalto the length of the ceca [30]). An approximately 4-cm length of tissue was collected from the middle of ileum. Then, each sample was dissected longitudinally and washed in normal saline. The mucosae were carefully scraped from the inner surface of the each sample. Each sample was weighed, immediately transferred into a centrifuge tube, added nine-volumes of ice-cold 0.85% NaCl solution and homogenized. The homogenized solution was immediately centrifuged at 3500×g for 10

Table 1. *Composition of the experimental diets*

Composition(%)	Control Group	COS-A Group	COS-B Group	COS-C Group
Corn	54.02	54.02	54.02	54.02
Soybean meal	38.19	38.19	38.19	38.19
Soybean oil	3.53	3.53	3.53	3.53
Salt	0.40	0.40	0.40	0.40
Choline chloride	0.15	0.15	0.15	0.15
DL-metionine	0.20	0.20	0.20	0.20
Dicalcium phosphate	1.88	1.88	1.88	1.88
Calcium carbonate	1.20	1.20	1.20	1.20
Multivitamin[1]	0.03	0.03	0.03	0.03
Trace element premix[2]	0.20	0.20	0.20	0.20
COS (mg/kg)	0	200	350	500

Multivitamin[1]: *Vitamin A, 12.500 IU/kg; Vitamin D, 3.000 IU/kg; Vitamin E, 18.75 IU/kg; Vitamin K3, 3 mg/kg; pantothenic acid, 15 mg/kg; folic acid, 1.05 mg/kg; nicotinamide, 30 mg/kg; biotin, 0.14 mg/kg; Trace element premix[2]: $FeSO_4 \cdot H_2O$, 364.7 mg/kg; $CuSO_4 \cdot 5H_2O$, 32 mg/kg; $MnSO_4 \cdot H_2O$, 377.4 mg/kg; $ZnSO_4 \cdot H_2O$, 289.9 mg/kg; $K(IO_3)_2$, 18.4 mg/kg; Na_2SeO_3, 35 mg/kg*

min at 4ºC and the supernatant was preserved for future detection.

According to Bradford method [31], the concentration of total protein in the supernatant of mucosa homogenate was detected. The activities of SOD, GSH-px; and ability of total antioxidant capacity (T-AOC); and ability to inhibit hydroxy radical (IHR), and contents of MDA and GSH in the supernatant were detected by biochemical methods following the instructions of the corresponding reagent kits (SOD: Cat. NO. A001-1; GSH-px: Cat. NO. A005; ability of T-Aoc: Cat. NO. A015; ability to IHR: Cat. NO. A018; MDA: Cat. NO. A003-1; GSH: Cat. NO. A006, All of these kits were purchased from Nanjing Jiancheng Bioengineering Institute of China, Nanjing, China). The absorbance of SOD, GSH-Px, T-AOC, MDA, GSH and IHR was measured at 520, 450, 532, 412, 420 and 550 nm using microtiter plate reader (Thermo, Varioskan Flash, USA), respectively.

Detection of Lymphocytes Cell Cycle of Ileum Mucosa

The mixture of Intra-Epithelial Lymphocytes (IELs) and Lamina Propria Lymphocytes (LPLs) were isolated and detected by Flow Cytometer (FCM). Briefly, at 21 and 42 days of age during the experiment, eight broilers of each group were humanely killed. The ilea were immediately removed and placed in petri dishes containing chilled (4°C) RPMI-1640 (Catalog No. SH4007-13, LOT: MXL0747; Hyclone, Logan, UT, USA). Then ilea were opened longitudinally and washed twice in phosphate buffered saline (PBS) to remove fecal contents. They were transferred to preheated (37ºC) 10 mL glass tube containing 5 mL nutrient (D-Hank's, EDTA, DTT). The glass tubes were incubated at 37°C for 40 min with gentle stirring. The tissue slurry was filtered through a wet 300-mesh nylon mesh in order to remove undigested tissue pieces. The cell suspension was centrifuged for 10 min at 400×g, and supernatant was discarded, and 3 mL of 40% Percoll (Lot: 10036869, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) (4 parts 100% Percoll and 6 parts 10× PBS) was added to the cell pellet, then layered onto 4 mL of 70% Percoll (7 parts 100% Percoll and 3 parts 10×PBS), and centrifuged at 400×g for 30 min. IELs was collected from the two 40%/70% interface areas, combined and washed by centrifugation in supplemented RPMI-1640. The IELs concentration was adjusted to 1.0×10^6 cells/mL with PBS.

Twenty-five mL RPMI-1640 and 60 U/mL of type IV collagenase (Sigma Chemical, St. Louis, MO, USA), 50 µL/mL gentamicin and 1% Fetal calf serum (FCS) were added to a 50 mL tube and prepared for use, then the ileal segments were washed twice with 25 mL RPMI-1640 medium and then transferred to a preparatory 50 mL tube after EDTA treatment (as described in the isolation of IELs). The tubes were incubated horizontally at 37°C for 30 min in a shaking-water bath. The contents of each tube were transferred to petri dishes and 200 µL FCS were added. The ileal mucosa was compressed with a syringe plunger over a plastic mesh. Single cell suspensions containing lamina propria cells were filtered through organdy mesh and then centrifuged 10 min at 2500× g. LPLs were collected and centrifuged in a discontinuous 40/70% Percoll gradient at 600× g for 30 min. Cells collected from the interface were washed and suspended in RPMI-1640 medium with 1% FCS, and then centrifuged at 200× g for 5 min. The cell density was diluted to 1.0×10^6 cells/mL with PBS. Then 1 mL mixture of IELs and LPLs were transferred to a 5-mL centrifuge tube and centrifuged at 200×g for 5 min. The supernatant was discarded, and 1 mL PI staining solution (5 µL/mL propidium iodide, 0.5% Triton X-100, 0.5% RNase, PBS) was added. The cells were gently vortexed and incubated for 20 min at room temperature (25°C) in the dark. 2 mL PBS were added. The cells were re-suspended in 0.5 mL PBS and the cell phases were analyzed by flow cytometry (FACSCalibur, BD, Franklin Lake, NJ, USA).

The proliferating index (PI) was calculated through the following formula:

$$PI = (S+G_2M) \times 100\% / (G_0G_1+S+G_2M)$$

Detection of Lymphocyte Apoptosis of Ileum Mucosa

The aforementioned proper concentration (1.0×10^6 cells/mL) mixture of IELs and LPLs (100 µL) was transferred to a 5-mL flow tube and 5 µL of Annexin V-FITC (Cat. No. 51-65874X, BD Pharmingen, Santiago, CA, USA) and 5 µL of propidium iodode (Cat. No. 51-66211E, BD Pharmingen, Santiago, CA, USA) were added. The samples were slightly vortexed and incubated for 15 min at room temperature (25°C) in the dark. 400 µL PBS was added to each sample and percentages of apoptosis were determined by flow cytometry (FACSCalibur, BD, Franklin Lake, NJ, USA).

Statistical Analysis

The significance of difference between four groups was analyzed by variance analysis, and SPSS 17.0 for Windows was used for statistics calculation. The results were presented as mean± standard deviation (X±S), and a value of P<0.05 was considered significant results data.

RESULTS

The changes of antioxidant function in the ileum mucosa were shown in *Fig. 1*. Compared with the control group, the values of GSH-Px, SOD, GSH, T-AOC and IHR in the COS-B and COS-C groups were significantly increased (P<0.05 or P<0.01), while the levels of MDA in the COS-B and COS-C groups were significantly decreased (P<0.05 or P<0.01). However, no significant changes of antioxidant function in the COS-A group were noted (P>0.05) when compared with the control group except for values of T-AOC and IHR which were significantly increased (P<0.05 or P<0.01). Furthermore, no significant difference in the levels of antioxidant function existed between the COS-B and COS-C groups (P>0.05) *(Fig. 1)*.

As shown in *Table 2*, compared with the control group, the percentages of G_0G_1 phase lymphocytes in the COS-B and COS-C groups were obviously decreased (P<0.05 or P<0.01), while the percentages of G_2M phase, S phase and PI value were increased (P<0.05 or P<0.01) at 21 and 42 days of age except for the percentage of G_2M phase at 21 days of age. However, the percentages of G_0G_1, G_2M and S phase as well as PI value in the COS A group were not significantly different from those in the control group (P>0.05) except for the percentages of S phase (P<0.05). In addition, no significant difference in the percentages of lymphocyte cycle existed between the COS-B and COS-C groups (P>0.05) *(Table 2)*.

As shown in *Table 2*, no significant changes in the percentage of apoptotic lymphocytes in ileum mucosa among four groups were observed (P>0.05).

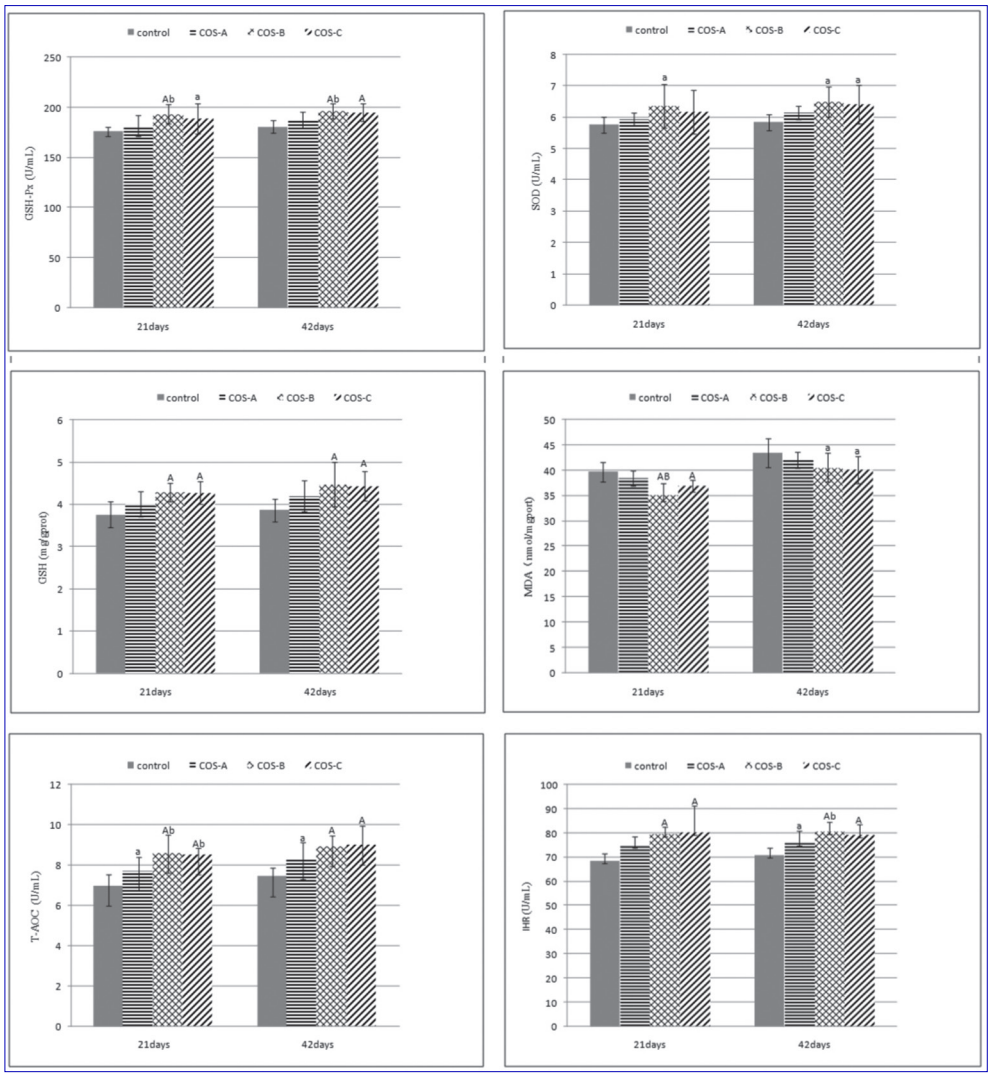

Fig 1. Effects of the GSH-px, SOD activities; GSH and MDA contents; T-AOC and IHR ability in the ileum

Table 2. *Effects of COS on lymphocyte cycle and apoptosis of ileum mucosa*

Time	Group	G_0G_1 Phase (%)	G_2M Phase (%)	S Phase (%)	PI (%)	Apoptosis (%)
21 days	Control	83.05±0.56	7.19±0.6	9.76±0.38	16.95±0.56	7.57±0.58
	COS-A	81.8±5.8	6.32±2.38	12.07±5.21	18.39±5.78	6.91±0.6
	COS-B	$77.55±1.92^{Ab}$	$8.39±0.67^{B}$	$14.06±1.37^{a}$	$22.45±1.92^{Ab}$	6.42±0.81
	COS-C	$77.03±0.29^{ab}$	$8.92±0.27^{b}$	$14.06±0.1^{a}$	$22.97±0.29^{ab}$	7.67±1.61
42 days	Control	85.03±0.31	7.22±0.33	7.75±0.1	14.97±0.31	5.7±0.44
	COS-A	84.24±1.00	7.19±0.17	$8.57±0.85^{a}$	15.76±1.00	5.54±0.87
	COS-B	$83.5±0.43^{A}$	$7.87±0.52^{ab}$	$8.64±0.23^{a}$	$16.50±0.43^{A}$	5.14±0.62
	COS-C	$83.44±0.31^{A}$	$7.99±0.44^{ab}$	$8.58±0.22^{a}$	$16.56±0.31^{A}$	5.4±1.37

Data are presented with the means ± standard deviation (n = 8). $^{a}P<0.05$, $^{A}P<0.01$, compared with the control group; $^{b}P<0.05$, $^{B}P<0.01$, compared with the COS-A group

DISCUSSION

Oxidative stress results when production of ROS exceeds the capacity of cellular antioxidant defenses to remove these toxic species [32], and antioxidant exerts its role in vivo or in food mostly via inhibiting generation of ROS, or scavenging free radicals [33]. Some antioxidant enzymes, such as SOD and CAT, are considered to be the first line of cellular defense against oxidative damage by scavenging free radical [34,35]. Hydroxy radical can cause oxidative stress and GSH is regarded as an early biological marker of oxidative stress [35]. The MDA production induces alteration of membrane fluidity and increase of membrane fragility [36-38]. Early research has shown that LPS could result in oxidative damage, in which the GSH levels and the CAT activity decreased while the MDA levels increased in mice after LPS injection [14]. However, pre-injection 100 mg/kg COS could smooth out the oxidative stress [14]. COS is a potent radical scavenger, which has the high radical scavenging activity [33]. It has been demonstrated that COS exerted antioxidant effects on pancreatic islet cells in streptozotocin-induced diabetes in rats, that is, COS recovered the maladjusted T-AOC and SOD activity as well as MDA levels [39]. In brief, COS can effectively scavenge of ROS [33] and it is a potent therapeutic agent against cancer and antioxidant additive, and has potential for application as a dietary supplement or nutraceutical [40,41]. As part of small intestine, ileum mucosa is one of the important structures that interface with external environments, but it is vulnerable to oxidative damage due to the large workload and high rate oxidative metabolism of intestine which result in abundant ROS [42]. In this study, dietary COS supplements with 350 mg/kg and 500 mg/kg could significantly increase SOD, GSH-Px activities and GSH concentration as well as improve the ability of T-AOC and IHR. These results indicated that COS played an important role in the antioxidant function in the normal ileum mucosa of broilers and could protect animals from oxidative stress. The mechanism of this might be related to the fact that being a potent radical scavenger, COS has effectively scavenging activity of ROS [33].

The cell cycle is a ubiquitous and complex process, and it can be subdivided into G_1, S, G_2 and M phases. The S phase of cell cycle is define as the period during which DNA is replicated; G_2: the period between completion of DNA synthesis and mitosis; M: from prophase to telophase. Our results show that compared with the control group, the percentages of S and G_2M phase and PI index of lymphocytes in the COS-B and COS-C groups were generally increased, suggesting dietary COS supplements with 350 mg/kg and 500 mg/kg could accelerate cell proliferation of lymphocytes in the ileum mucosa. This was accord with previous studies, in which COS enhanced immunity via accelerating T-cell differentiation and maintain T-cell activity [43], and treatment of primary Schwann cells with COS promoted cell proliferation as determined by cell cycle analysis [22]. The cell cycle is controlled by numerous mechanisms ensuring correct cell division, for example, cyclin-dependent kinases (CDK) (a family of serine/threonine protein kinases), which belongs to a family of serine/threonine protein kinases that are activated at specific points of the cell cycle [44]. The three D type cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4, CDK6 and CDK-cyclin D complexes are essential for entry in G_1 [45]. Cyclin A binds with CDK2 and this complex is required during S phase [46]. Previous studies demonstrated that COS could downregulated Cdk-2 and cyclin A of HepG2 cells to inhibited cell proliferation [47], and COS also could increased the cyclin D1 expression of normal neural glia cells [22]. Whether the mechanism of COS affects lymphocytes cycle in the ileum mucosa observed in this study was also related to cyclin-dependent kinases (CDKs), further studies are needed since numerous factors control the progression of the cell cycle.

Early researches have shown that COS has the tumor inhibitory effect, inducing various tumor cells apoptosis [26,27,48-52]. Also, COS had anti-apoptosis effect in IL-1β-induced chondrocytes apoptosis on osteoarthritis model

rats [28]. However, the information about the effects of COS on the cell apoptosis of normal animals is rarely available. In the present study, no significant changes in the lymphocytes apoptosis were observed among the control and three COS groups, suggesting that COS had no effect on the lymphocyte apoptosis of broiler's ileum mucosa. The mechanism of different effects of COS on apoptosis between tumor and normal cells, such as lymphocyte observed in this study may partially related to the different electric fields of different cells. It has reported that altering the electric fields can induce cell apoptosis [53,54], and tumor cells membranes have more negative charges than normal cells [55]. Unlike most polysaccharides, COS has positive charges on surface, and this chemical feature allows COS to bind strongly to negatively charged surfaces and responsible for many of observed biological activities [56,57]. However, the mechanism for this needs further studying, because properties of COS, such as DP (degree of polymerization), DA (degree of acetylation), charge distribution and nature of chemical modification to the molecule strongly influence its observed biological activities [4].

According to the results of the present study and the aforementioned discussion, it is concluded that dietary COS supplement with 350 mg/kg and 500 mg/kg could improve the antioxidant function and accelerated lymphocytes proliferation, but had non-influence on lymphocytes apoptosis in the ileum mucosa of broiler.

Conflict of Interest

The authors declare no conflict of interest.

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