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Effect of sulfated yeast beta-glucan on cyclophosphamide-induced immunosuppression in chickens

Mi Wang, Xiaoyang Wang, Lifang Zhang, Ruile Yang, Chenzhong Fei, Keyu Zhang, Chunmei Wang, Yingchun Liu, Feiqun Xue*

Key Laboratory of Veterinary Chemical Drugs and Pharmaceutics, Ministry of Agriculture, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, PR China

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ABSTRACT

Immunosuppression is a condition that causes large economic losses in the poultry industry. To investigate the effect of sulfated yeast beta-glucan on immunosuppression, two hundred and fifty 11-day-old chickens were randomly assigned to five groups, and except for the normal control group, injected with cyclophosphamide once a day for 3 successive days. At 14 days of age, sulfated yeast beta-glucan from *Saccharomyces cerevisiae* (sGSC) was orally administered at three doses to the chickens in three experimental groups for 14 days. On days 7 and 14 after the first sGSC dose, serum cytokine concentrations and peripheral lymphocyte proliferation were measured. Gut microbiota, organ index, and histopathological changes in the bursa were investigated on day 14. The results demonstrated that at 4 mg/kg, sGSC could significantly enhance the bursa index and IFN-γ and IL-6 concentrations, decrease TGF-β1 concentration, and promote lymphocyte proliferation; it could effectively decrease histopathological changes in the bursa and improve gut *Bifidobacterium* and *Lactobacillus* populations in cecal digesta of chickens compared with the model control group. This indicated that sGSC could effectively alleviate immunosuppression and regulate the beneficial microbiota in the gut.

1. Introduction

Immunosuppression is a temporary or permanent immune disorder that is currently endangering the poultry industry. Because of damage to the immune system, affected organisms become more sensitive to pathogens [1,2]. In poultry, many factors, such as infection and stress, cause immunosuppression. Infections can interfere with the acquired immune system of chickens [3–6], whereas stress due to improper feeding and mismanagement can affect their innate immune system [7]. Immunosuppression increases morbidity and mortality rates in the poultry industry as a result of increased susceptibility to secondary infections, poor feed conversion, and reduced response of the protective system to commonly used vaccines [8]. Immunosuppression results in great economic loss to the poultry industry. Therefore, it is necessary to find an immunopotentiator that could reduce the harmful side effects and that is useful for enhancing the immunity and productivity of chickens.

Beta-glucans from fungi, plants, some bacteria and sea weeds are a heterogeneous group of glucose polymers [9]. They have different solubility, primary structure, molecular weight, branching and polymer charge. Which influence the biological activities of beta-glucans [10–12]. Beta-glucans regulate immune activity by activating various immune cells. Sulfated modification could change their physicochemical parameters and affect their biological activities. Our previous investigations demonstrated that sulfated modification of yeast beta-glucan significantly improved the immune efficacy of Newcastle disease vaccine. It promoted lymphocyte proliferation, enhanced antibody titer in serum and improved serum IL-2 and IFN-γ concentrations in chickens [13]. Sulfated yeast beta-glucan also significantly enhanced Hemagglutination inhibition (HI) antibody titer, and promoted the production of IL-2, IFN-γ, IL-4, and IL-6 in mice inoculated with inactivated H5N1 vaccine. These results indicated that sulfated yeast beta-glucan has an excellent adjuvant effect on H5N1 vaccine in mice [14]. Oral administration of sulfated yeast beta-glucan improved serum Catalase (CAT) and Phospholipid hydroperoxide glutathione peroxidase (GSH-Px)activities and decreased Malondialdehyde (MDA) level in the mice. It significantly improved spleen and thymus indexes and effectively enhanced the percentage of $CD4^+$ T cells, decreased the percentage of $CD8^+$ T cells, and elevated` the $CD4^+/CD8^+$ ratio [15]. Previous studies were mainly focus on the effect of immune activities of sulfated

* Corresponding author.
E-mail address: fqxue@shvri.ac.cn (F. Xue).

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beta-glucan from *Saccharomyces cerevisiae* (sGSC) on healthy animals. However, the effect of sGSC on the immunomodulatory activity in immunosuppressed chickens has scarcely been investigated.

Polysaccharides have multiple effects on the body. They have a protective effect on the gastric mucosa and their digestion in the gastrointestinal tract could affect gastrointestinal motility. They might alter gut microbiota and affect the intestinal mucosal immune system. Gut microbiota affects many aspects of the host's physiology and is associated with the health status, metabolic phenotype, nutrient absorption or production, and the development and regulation of the immune system of the host [16–22]. Different sources of glucan have varying impacts on the populations of bacteria in the gut. Previous studies have indicated that oat and barley β-glucans increased the number of beneficial bacteria in pigs and in older healthy human volunteers, respectively [23–25]. Mulberry leaf polysaccharides inhibited gut *Escherichia coli* and promoted gut *Lactobacilli* and *Bifidobacteria* in early weaned pigs [26]. However, the effect of sGSC on the gut microbiota of immunosuppressed chickens has scarcely been investigated.

Therefore, the purpose of the present study was to examine the effect of oral administration of sGSC on the immune action and gut microbiotain chickens intramuscularly injected with cyclophosphamide. The effect of sGSC was evaluated based on body weight, organ index, peripheral lymphocyte proliferation, cytokine production, gut microbiota, and histopathological changes in the chickens.

2. Materials and methods

2.1. Reagents

Cyclophosphamide was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, Jiangsu, China). RPMI-1640 was obtained from Gibco (USA). Concanavalin A (Con A) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthia-zol-2-yl)-2,5-dipheny-ltetrazoliumbromide (MTT) was obtained from Amresco Co. *Bifidobacterium* BS medium and *Lactobacillus* MRS medium were obtained from Hangzhou Baisi Biotechnology Co., Ltd. in Zhejiang. IFN-γ, IL-6 and TGF-β1 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Shanghai Enzyme-linked Biotechnology Co.,Ltd. (Shanghai,China). All chemicals used in experiments were analytical grade.

2.2. Preparation of sulfated glucan

Methods for preparing sGSC and determining carbohydrate contents, molecular weights, and sulfation degrees were as described in our previous report [15]. The carbohydrate content, molecular weight, and sulfation degree of sGSC were 78.24%, 12.9 kDa, and 0.16, respectively.

2.3. Experimental design

Two hundred and fifty 11-day-old male Sanhuang chickens were randomly divided into five groups of 50 chickens each. The feed was made in our lab without any drugs. Food and water were freely consumed, and chickens were housed in a room maintained with a 12/12 h light/dark cycle. All of the animal experiments were conducted with the approval of the Animal Use and Care Committee at Chinese Academy of Agricultural Sciences Shanghai Veterinary Research Institute. One group was used as the normal control (NC) group. On days 11–13, the other four groups were subjected to immunosuppression by intramuscularly administration of cyclophosphamide (8 mg/kg/day); one of these groups of cyclophosphamide treated chickens was used as model control (MC) group. On days 14–28, the other three groups of cyclophosphamide-treated chickens were orally administered with 2, 4, and 8 mg/kg of sGSC ($sGSC_L$, $sGSC_M$ and $sGSC_H$ groups, respectively). On days 21 and 28, the blood from six chickens randomly selected each group was sampled to determine IFN-γ, IL-6, and TGF-β1 concentrations by ELISA and lymphocyte proliferation using the MTT method. On days 28, the thymus, spleen, bursa, and cecum of 10 chickens randomly selected from each group were weighed to calculate the immune organ index and gut microbiota and observe histopathological changes. The organ indices (mg/g) were calculated by the weight (mg) of thymus, spleen, orbursa/body weight (g).

2.4. Lymphocyte proliferation assay

The peripheral blood lymphocytes of chickens were collected as described in the previous paper [14]. Cells were diluted to $2.5 \times 10^6\ mL^{-1}$ and inoculated in 96-well culture plates, 100 μL per cell, and 20 μL Con A was then added. MTT was added into each well after a 44-h incubation, and the plates were reincubated for 4 h. DMSO (100 μL) was added to each well. The absorbance at 570 nm (A_{570} value) was measured.

2.5. Cytokine secretion assay

IFN-γ, IL-6, and TGF-β1 concentrations were assayed using a chicken ELISA kit according to the manufacturer's instructions. Chicken serum samples($n = 6$) were diluted at 1:4,thenincubated in 96-well microtiter plates at 37°Cin the incubator. The cytokine concentration was calculated by standard curve.

2.6. Gut Lactobacilli and Bifidobacterium population

The collected cecal contents were diluted in sterile water (1 g/9 mL), homogenized using glass beads, and serially diluted. The $1{:}10^6$dilution was then spread onto *Lactobacilli* MRS agar and *Bifidobacterium*-selective medium agar to isolate *Lactobacillus* and *Bifidobacterium*, respectively. The agar plates were incubated for 48 h at 37 °C in an anaerobic incubator (RuskinnBugBox M, Bridgend, UK), then the number of colonies was counted [26,27]. Colony forming units were transformed to Log_{10}.

2.7. Microscopic examination of the organ

The tissues of bursa were fixed with formaldehyde solution(10%) for > 15 days, then embedded in paraffin. The tissues were cut approximately 4-μm-thick tissue sections, then stained with hematoxylin and eosin for histopathological examination.

2.8. Statistical analysis

Data were analyzed with SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used for analyzing the difference among groups. Values were expressed as means ± S.D. P values of < 0.05 were considered statistically significant.

3. Results

3.1. Effect of sGSC on the body weight of chickens

Changes in the body weight and daily weight gain are listed in Fig. 1 and Fig. 2, respectively. Daily weight gain was significantly lower in the MC group than in the NC group ($P < 0.05$).

3.2. Effect of sGSC on the immune organ index of chickens

Changes in immune organ index are listed in Fig. 3. The bursa index was significantly smaller in the MC group than in the NC and $sGSC_M$ groups ($P < 0.05$) and significantly larger in the $sGSC_M$ group than in the NC, $sGSC_L$, and $sGSC_H$ groups ($P < 0.05$).

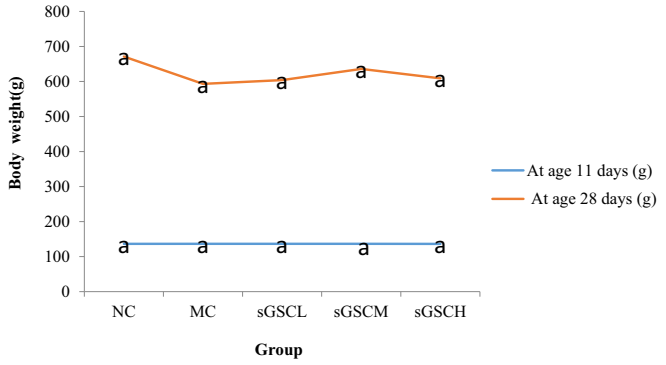

Fig. 1. Effect of sGSC on body weight in every group (g). The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. [a]Lines with the same superscripts don't differ significantly ($P > 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M- 4 mg/kg and H-8 mg/kg.

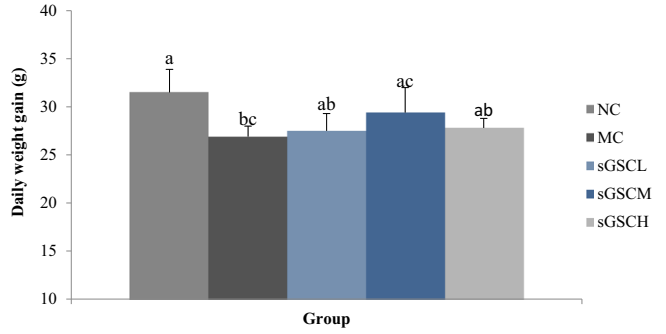

Fig. 2. Effect of sGSC on daily weight gain in every group (g). The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. [a-c]Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M- 4 mg/kg and H-8 mg/kg.

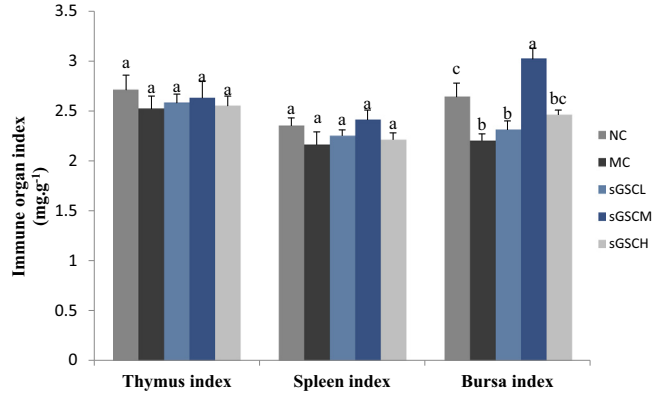

Fig. 3. Effect of sGSC on immune organ index in every group (mg/g). The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. [a-c]Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M- 4 mg/kg and H-8 mg/kg.

3.3. Effect of sGSC on gut Bifidobacterium and Lactobacillus population

Changes in gut microbiota population are listed in Fig. 4. The *Bifidobacterium* population was significantly smaller in the MC group than in the NC and all sGSC groups ($P < 0.05$). The *Lactobacillus* population was significantly larger in all sGSC groups than in the MC group ($P < 0.05$)and the population in the $sGSC_M$ group had a tendency to be

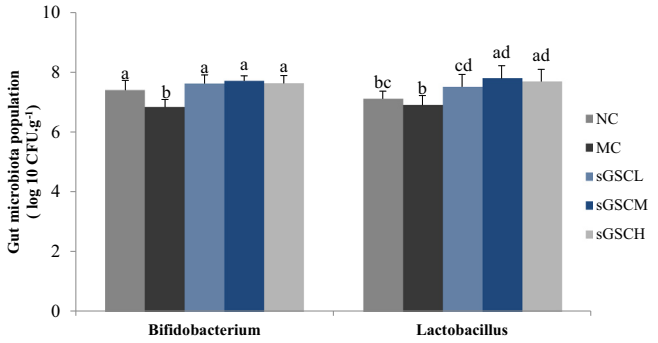

Fig. 4. Gut *Bifidobacterium* and *Lactobacillus* population (log10 CFU/g)in cecaldigesta of chickens in every group. The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. [a-d]Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M- 4 mg/kg and H-8 mg/kg.

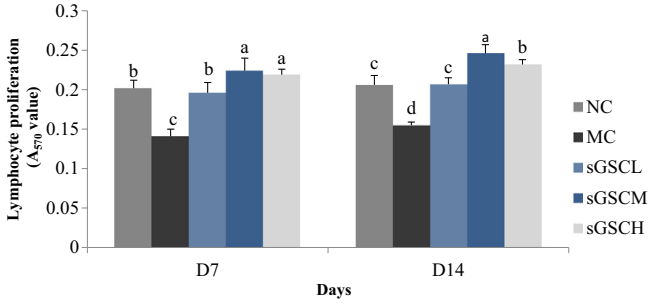

Fig. 5. Effect of sGSC on peripheral lymphocyte proliferation (A_{570} values) in every group. The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. [a–d]Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M-4 mg/kg and H-8 mg/kg.

higher than in the $sGSC_L$ and $sGSC_H$ groups ($P > 0.05$).

3.4. Effect of sGSC administration on peripherallymphocyte proliferation

Changes in A_{570} values are listed in Fig. 5. A_{570}values on days 7 and 14 were significantly lower in the MC group than in the NC and all sGSC groups ($P < 0.05$). On day 7, the A_{570} value was significantly higher in the $sGSC_M$ group than in the NC and $sGSC_L$ groups ($P < 0.05$). On day 14, the A_{570} value was significantly higher in the $sGSC_M$ group than in the NC, $sGSC_L$, and $sGSC_H$ groups ($P < 0.05$).

3.5. Effect of sGSC on cytokine content

Changes in IFN-γ concentrations after administering sGSC on days 7 and 14 are listed in Fig. 6. IFN-γ concentrations were significantly lower in the MC group than in the NC, $sGSC_M$, and $sGSC_H$ groups ($P < 0.05$) and significantly higher in the $sGSC_M$ group than in the $sGSC_L$ group ($P < 0.05$)on day 7. IFN-γ concentrations were significantly lower in the MC group than in the $sGSC_M$ and $sGSC_H$ groups ($P < 0.05$) and significantly higher in the $sGSC_M$ group than in the NC, $sGSC_L$, and $_sGSC_H$groups ($P < 0.05$)on day 14.

Changes inIL-6 concentrations after administering sGSC on days 7and 14 are listed in Fig. 7. IL-6 concentrations were significantly lower in the MC group than in the NC, $sGSC_M$, and $sGSC_H$ groups ($P < 0.05$) and significantly higher in the $sGSC_M$ group than in the $sGSC_L$ group ($P < 0.05$)on day 7. IL-6 concentrations were significantly lower in the MC group than in the $sGSC_M$ and $sGSC_H$ groups ($P < 0.05$) and significantly higher in the $sGSC_M$ group than in the NC, $sGSC_L$, and $_sGSC_H$

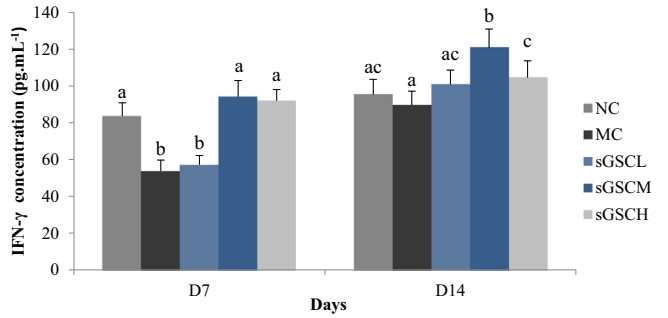

Fig. 6. The changes of IFN-γ concentration in every group (pg·mL^{-1}). The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. $^{a-c}$Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M-4 mg/kg and H-8 mg/kg.

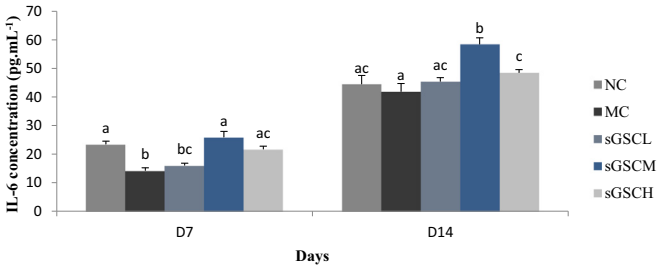

Fig. 7. The changes of IL-6 concentration in every group (pg·mL^{-1}). The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. $^{a-c}$Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M- 4 mg/kg and H-8 mg/kg.

groups ($P < 0.05$)on day 14.

Changes in TGF-β1 concentrations after administering sGSC on days 7 and 14 are listed in Fig. 8. TGF-β1 concentration was significantly higher in the MC group than in the NC and all sGSC groups ($P < 0.05$) and significantly lower in the $sGSC_M$ group than in the $sGSC_L$and $sGSC_H$ groups ($P < 0.05$) on day 7. TGF-β1 concentration was significantly higher in the MC group than in the other groups ($P < 0.05$) and significantly lower in the $sGSC_M$ group than in the NC, $sGSC_L$, and $sGSC_M$ groups ($P < 0.05$) on day 14.

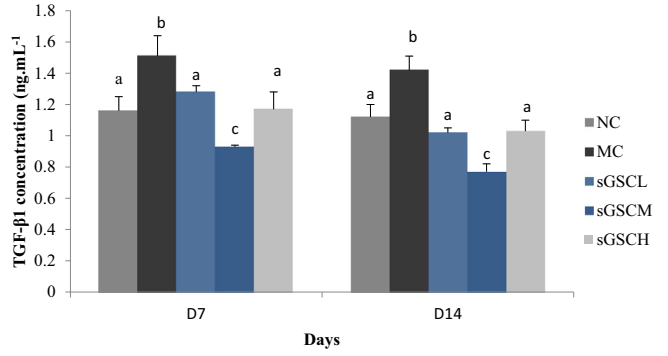

Fig. 8. The changes of TGF-β1 concentration in every group(ng·mL^{-1}). The data are expressed as the mean ± S.D. Significant differences were considered at $P < 0.05$. $^{a-c}$Bars without the same superscripts differ significantly ($P < 0.05$). sGSC, sulfated Beta-glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M- 4 mg/kg and H-8 mg/kg.

3.6. Histology of organs

In chickens treated with cyclophosphamide, inflammation was observed in the bursa (Fig. 9). The histopathological changes were effectively eliminated after sGSC was orally administered. The bursa of chickens treated with cyclophosphamide alone revealed focal inflammation in the bursal mucosa and some epithelial cells dropped (Fig. 9.b). Chickens administered $sGSC_M$ and $sGSC_H$ showed the typical bursa with well-constituted bursal mucosa, and there was macrophages in the lymphoid tissue (Fig. 9 d and e).

4. Discussion

Cyclophosphamide is an immunosuppressive agent that is widely used in organ transplantation and for the treatment of several types of cancer and various autoimmune disorders. It can inhibit both humoral and cellular immunity, and its immunosuppressive action in mammals and birds is well known [28]. Therefore, cyclophosphamide was used to establish the chicken immunosuppressive model and evaluate the immune-enhancing effect of sGSC in this experiment. Our experimental results showed that daily weight gain, bursa index, gut *Bifidobacterium* population,A_{570}value, and IFN-γ and IL-6 concentrations were significantly lower in the MC group than in the NC group. TGF-β1 concentration was significantly higher in the MC group than in the NC group. This indicates that the immune functions of chickens are significantly inhibited by cyclophosphamide and that the immunosuppressive model was successfully established.

Cyclophosphamide is a cytotoxic alkylating agent with a broad spectrum of activity against a variety of diseases. Administration of cyclophosphamide causes hematopoietic depression, gastrointestinal symptoms, hemorrhagic cystitis, and alopecia [29]. Immunosuppression is a major side effect of long-term cyclophosphamide therapy. Body weight, relative weights of the spleen, bursal and thymus, B cell and T cell proliferation, and NK cell activity decrease after cyclophosphamide administration [30,31]. The present results confirmed that sGSC could prevent decline of daily weight gain and the bursa index induced by cyclophosphamide. The bursa index was significantly higher in the $sGSC_M$ group than in the $sGSC_L$ and $sGSC_H$ groups. This indicates that at a suitable dose, sGSC may promote growth in chickens, inhibit the atrophy of immune organs in cyclophosphamide-induced immunosuppression. According to previous reports, some polysaccharides of Chinese herbal medicine significantly improved the immune organ index of chickens and inhibit their atrophy [1,32].

Polysaccharides contribute to the proliferation of beneficial bacteria and improve microbial diversity in the gut [19]. In previous results, glycosidic linkage type was shown to possibly affect the profiles of intestinal microbial communities. Intact β-glucans (from oat) increased “good bacteria” in pigs [23,24], and barley β-glucan in the diet increased “good bacteria” in older healthy human volunteers [25]. The type and amount of polysaccharide affect the intestinal microbial community *in vivo*. Since butyrate-producing bacteria are selectively favored by high levels of β-glucan, its presence in the diet may improve microbial diversity [33]. According to our results, the *Bifidobacterium* and *Lactobacillus* populations are significantly smaller in the MC group than in any sGSC group. This indicates that at medium levels, sGSC can significantly promote the proliferation of *Bifidobacterium* and *Lactobacillus*. It is possible that the polysaccharides themselves are more easily used by bacteria such as *Lactobacillus* to produce large amounts of short-chain fatty acids, which can rapidly reduce the pH of gut environment. The reduction would suppress the growth of pH-sensitive *E. coli* [34,35]. However, the effects of sGSC on the production of intestinal short-chain fatty acids and the intestinal environment need further investigate. The result of the present study is consistent with that of Mulberry leaf polysaccharides. The polysaccharides at 0.9 g/kg could significantly inhibit gut *E. coli* and promote gut *Lactobacilli* and *Bifidobacteria* [26].

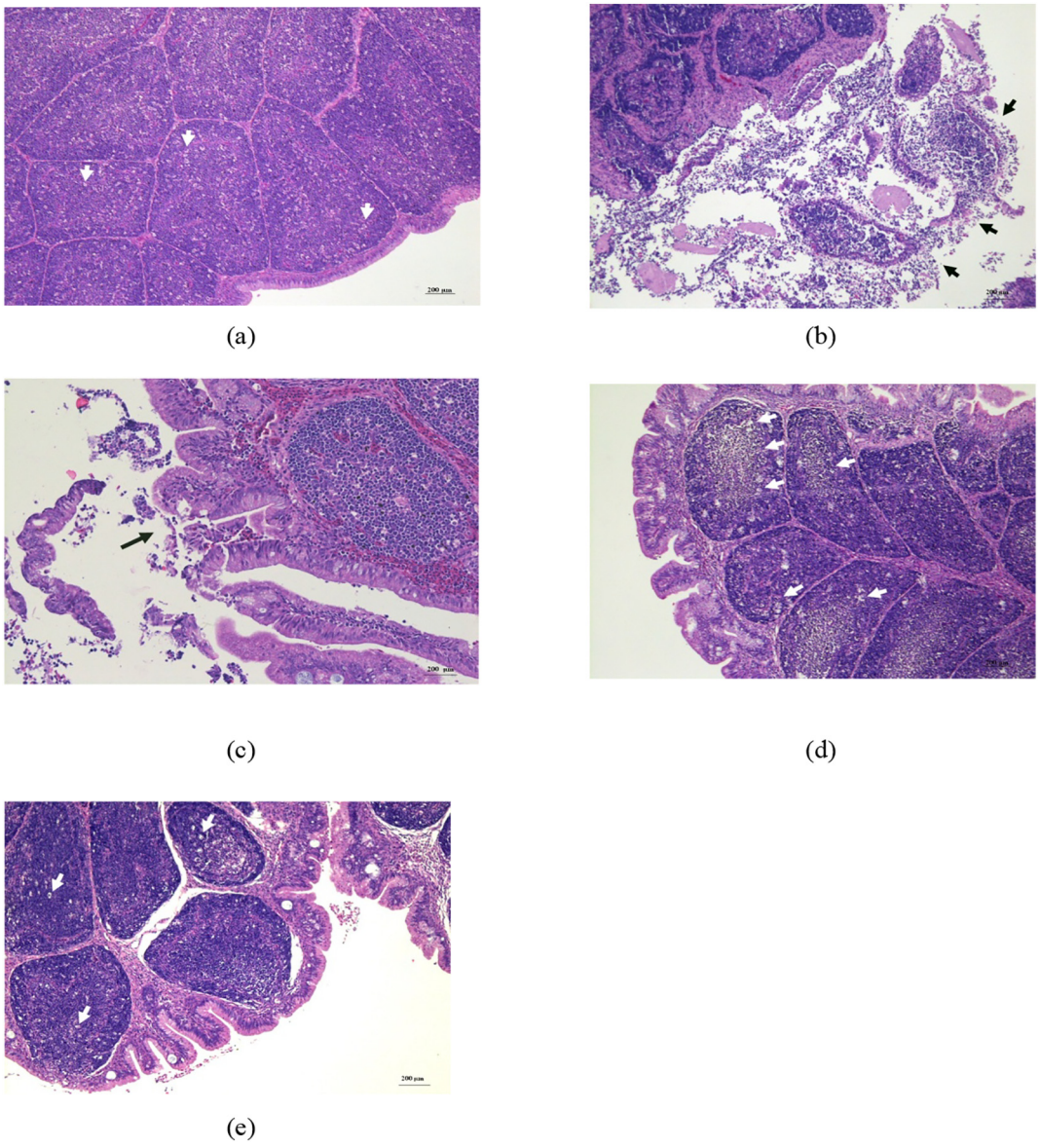

Fig. 9. Histopathological changes in the bursa. (**a**) NC, (**b**) MC, (**c**) $sGSC_L$, (**d**) $sGSC_M$, (**e**) $sGSC_H$ (Hematoxylin–eosin HE-stain, magnification 200 ×). sGSC-sulfated glucan from *Saccharomyces cerevisiae*. L-2 mg/kg; M-4 mg/kg, and H-8 mg/kg. (**a**) The mucosa of the bursa was normal, and more macrophages were observed in the lymphoid tissue. (**b**) Focal inflammation was observed in the bursal mucosa, and some epithelial cells dropped. (**c**) The mucous membrane of the bursa was mildly inflamed, and a small amount of epithelial cells were shed. (**d**, **e**) The bursal mucosa was normal, and more macrophages were observed in the lymphoid tissue. White arrows indicated macrophages, and black arrows indicated focal inflammation.

Cell-mediated immunity can exert anti-infection and anti-tumor activity, helping lymphocytes to produce antibodies by sensitizing them against the corresponding antigen [32]. Our results confirm that A_{570} values in the $sGSC_L$, $sGSC_M$, and $sGSC_H$ groups are significantly higher than those in the MC group at days 7 and 14, indicating that sGSC can significantly promote the activation potential of T cells in cyclophosphamide-induced immunosuppressed chickens and enhance the immune response. It may be that sGSC promoted the recovery of spleen in chicken with immunosuppression, and then enhanced lymphocyte activity. Many studies have also proved that Chinese herbal medicine polysaccharides could withstand the immunosuppression induced by cyclophosphamide and significantly promote lymphocyte proliferation [1,32].

Previous studies have indicated that polysaccharides might stimulate cytokine production. As a major immunoregulatory molecule, it induced an effective immune response to bacterial and exogenous infectious diseases. IFN-γ can mediate cellular immunity [36–38]. IL-6 can mediate humoral immunity. It was one of the important immune and inflammatory mediators to regulate cell function, including proliferation and differentiation of B cells and T cells [39,40]. TGF-β1 mainly played an immunosuppressive role in the animal immune system [38]. Changes in serum IFN-γ, IL-6 and TGF-β1 concentrations were detected in our study. The results showed that administering sGSC could prevent cyclophosphamide-induced decline of IFN-γ and IL-6 and improve TGF-β1 concentration. The effects especially in the $sGSC_M$ group were superior to those in the $sGSC_L$ and $sGSC_H$ groups. This indicated that sGSC can avoid immunosuppression and enhance IFN-γ and IL-6 secretion and decrease TGF-β1 secretion. It was reported that some polysaccharides or compound polysaccharides also could overcame cyclophosphamide-induced immunosuppression and significantly increase IFN-γ and IL-2 concentrations [1,17–19]. The cytokines were secreted by Helper T cells. Which could be divided intoTh1-related cytokines (IFN-γ) and Th2-related cytokines (IL-6) [41]. It suggested that both Th1 and Th2 immune responses were stimulated by sGSC, and

the best level is the medium level. The mechanism of the elevation of IFN-γ and IL-6 concentration might be that the medium sGSC stimulated the activity of corresponding Helper T cells, and the mechanism of the effect of TGF-β1 concentration was unknown and needed further study.

The bursa of chickens treated with cyclophosphamide alone induced focal inflammation and epithelial cells dropped. Chickens administered 4 mg/kg and 8 mg/kg sGSC showed the well-constituted bursal mucosa, and there was macrophages in the lymphoid tissue. The improving effects of sGSC on immunosuppression in cyclophosphamide -treated chickens may be due to the recovery of the bursa-dependent lymphoid system and stimulation of immunocompetent B cells for morphological reconstitution of the bursa of cyclophosphamide-treated chickens.

At 4 mg/kg, sGSC could effectively alleviate immunosuppression and regulate gut microbiota. Study of the histology of bursa showed that at this dose, sGSC completely eliminated inflammation of the bursa. Previous results have suggested that sugarcane extract also has reconstituting effects in terms of functionality and morphology on the bursa-dependent immune system in immunosuppression chickens [31].

In conclusion, the results of the present study shows that sGSC could alleviate the immunosuppression induced by cyclophosphamide. It could significantly change the concentration of cytokines, promote the functionality of the immune system, decrease lesions, and increase the “good” gut bacteria. It is therefore an effective immunopotentiator and regulator of gut microbiota.

Acknowledgments

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