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Original Article

Water soluble chitosans shows anti-cancer effect in mouse H22 liver cancer by enhancing the immune response

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Abstract: Water soluble chitosan (WSC), with low molecular weight, has many special biological, chemical, and physical properties, such as antifungal activity, antibacterial activity, and antitumor activity. In the current study, we examined its antitumor activity in mouse liver cancer H22-bearing mice. Here, oral water-soluble chitosan was administered to reduce the tumor growth and final tumor weight in liver tumor H22-bearing mice. The results suggested that oral administration of WSC could significantly reduce the tumor size and tumor weight of H22-bearing mouse. WSC also increased $CD3^+$ and $CD4^+$ T cell number, CD4/CD8 ratio and NK cells compared with control group. Some chemokines and cytokines, such as IL-1β, IL-2 and TNF-α levels were also increased compared with the control group. The results indicate that oral administration of WSC can enhance the immunity against tumor proliferation in vivo, and therefore oral administration of WSC may be a potential and promising adjuvant therapy against liver cancer.

Keywords: Water-soluble chitosan, antitumor activity, interleukins, liver cancer, macrophage

Introduction

Chitosan, (1⟶4)-2-amino-2-deoxy-β-d-glucan, is a natural polymer generally obtained by full or partial deacetylation of chitin [1]. Generally, chitosan was divided into three subgroups based on their molecular weights, low-molecular-weight (average molecular weight, 40,000 Da), medium-molecular-weight (average molecular weight, 480,000 Da) and high-molecular-weight (average molecular weight, 650,000 Da) [2].

Water soluble chitosan (WSC), with low molecular weight, has many special biological, chemical, and physical properties, such as antifungal, antibacterial, antitumor, and immunity activities, which are different from ordinary chitosan [3]. WSC can be prepared by either chemical or enzymatic hydrolysis. The chemical approach has some defects, e.g. harsh hydrolytic conditions, low yields, and chemical modifications of glucose rings. However, enzymatic methods possess advantages such as mild reaction conditions, high specificity, no glucose ring modifications, and mass production of chitooligosaccharides.

Recently some studies reported that a high-molecular-weight chitosan prevented the adverse effects (myelotoxicity, gastrointestinal toxicity, immunocompetent organic toxicity, and reduction of body weight) induced by the administration of cancer chemotherapeutic drugs, including 5-fluorouracil, cisplatin, and doxorubicin, without interfering with the antitumor activities of these drugs [4-6], but it had no direct antitumor activity in sarcoma 180-bearing mice [4]. Furthermore, high-molecular-weight chitosans exhibit myriad biological actions, namely, hypocholesterolemic, antimicrobial, wound healing and anti-obesity properties [7-10]. There is little doubt that such properties would influence absorption in the human intestine because

most animal intestines, especially human gastrointestinal tract, do not possess enzymes such as chitinase and chitosanase, which directly degrade the β-glucosidic linkage in chitosan [11]. Low-molecular-weight chitosans, obtained by chemical or enzyme hydrolysis of high-molecular-weight chitosan, have lower viscosity and are soluble in water. Subsequently, they seem to be readily absorbed in vivo. A low-molecular-weight chitosan was shown to reduce blood glucose and serum triglyceride levels in obese diabetic KK-Ay mice [12]. It was reported that oligochitosans such as N-acetylchitohexaose and chitohexaose increase NK activity in tumor-bearing mice [13], and that oligochitosans (molecular weight: 1-3 kDa and 3-5 kDa) prevent oxidative stress in mice [14]. In some clinical studies, a high-molecular-weight chitosan was used for the prevention of hypercholesterolemia, diabetes, obesity, and cancer [15, 16]. Although it was suggested that water-soluble chitosans may have antitumor activities in clinical use, such effects are as yet unproven. In this study, we examined the antitumor activities and mechanisms of action of low molecular water-soluble chitosans in liver cancer H22-bearing mice.

Materials and methods

Materials

Raw chitosan from shrimp shells was obtained from Weifang Sea source Biological Products Co., Ltd. The molecular weight (MW) and degree of deacetylation were 3×10^4 Da and 95%, respectively. The crude cellulase, derived from Trichoderma viride, was a product of the company or institute. Chitosan was dissolved in 2% (v/v) aqueous acetic acid (HAc) to a concentration of 5% (w/w) and the pH was adjusted to 5.5 using 1 M NaOH. A 1 mg mass of cellulase was added into a reactor containing 100 mL of chitosan solution and then maintained in a thermostatic water bath at 50°C for 4 h. The hydrolysates were incubated at 95°C for 5 min to denature the enzyme, neutralized with 1 M NaOH, filtered, concentrated to 20% (w/v), and precipitated with 6 volumes of absolute ethanol. The precipitate was then filtered through a pre-weighed Whatman GF/A filter, dried at 70°C for 2 h, and finally crushed. The WSC was dissolved in distilled water to make a solution at a concentration of 1%.

Cells

The liver cancer H22 cells were used to evaluate the antitumor effects of WSC through immune function. The H22 cells were maintained in DMEM and RPMI 1640 supplemented with 100 g/L FBS, penicillin (1×10^5 U/L), streptomycin (100 mg/L) and amphotericin B (0.25 mg/L).

Animals

Thirty male ICR strain mice (6 weeks old) were obtained from Wei Tong Lihua animal production company, Beijing China. These mice were housed in a room maintained at 25 ± 1°C with 60% relative humidity and provided with free access to laboratory standard diet and water. The room lights were on for 12 h/d starting at 07:00 am. Mice were treated according to the ethics guidelines of No. 401 Hospital of Chinese People's Liberation Army. The experimental protocol was approved by the Animal Studies Committee of No. 401 Hospital of Chinese People's Liberation Army. The mouse were randomly divided into three groups, each contains 10 mice. All mouse received the liver H22 cell xenograft, group only receiving water was regarded as control, and the other two groups received two different dosage of chitosan treatment respectively.

Treatment of liver cancer H22-bearing mice

Solid-type H22 liver cancer xenograft was prepared by subcutaneous transplantation of 2×10^6 cells into the backs of mice on Day 0. WSC were dissolved in distilled water at a concentration of 10 or 30 g/L. The solution was administered orally at two dosages of 100 or 200 mg/kg daily for 14 days, starting 12 h after the implantation of tumor cells. Control mice were also given distilled water on the same schedule.

The tumor volume was determined by direct measurement with calipers and calculated as follows: [length (mm) × width $(mm)^2$]/2 every 2 or 3 d starting 5 d after the tumor implantation. On d 14, blood was obtained by venous puncture in mice under diethyl ether anesthesia. Subsequently, the tumor, epididymal adipose tissue, spleen, and thymus were removed and analyzed for evaluation of antitumor activity and immunocompetent functions. Blood sam-

ples were chilled in test tubes containing heparin, and the numbers of leukocytes and RBC were measured using a Coulter Counter. The hemoglobin concentration in the blood was determined using Hemoglobin-Test kits (Wako Pure Chemical Industries, USA).

Cytotoxicity against H22 cells (in vitro)

H22 cells were placed in DMEM supplemented with 100 g/L FBS at 1×10^4 cells/well in 96-well culture plates. After the cells were cultured overnight, the medium was changed to fresh DMEM with 100 g/L FBS, and the cells were exposed to the indicated amounts of soluble chitosans for 24 h. After the incubation period, the cytotoxicity against H22 cells was assessed using the Cell Counting kit (CCK8 assay, Sigma Aldrich, Germany).

Isolation of splenic lymphocytes or liver lymphocytes

Splenic and liver lymphocytes were isolated using methods described previously [17, 18]. Briefly, the fresh splenic and liver segments were added to 45 mL of HBSS supplemented with 50 g/L FBS, penicillin (1×10^5 U/L), streptomycin (100 mg/L), and amphotericin (0.25 mg/L) and shaken at 150 rotations per min and 37°C for 45 min. The resultant cell suspension was collected and passed through a glass-wool column to remove cell debris and sticky cells, and was then subjected to Percoll (Pharmacia) gradient centrifugation. Lymphocytes were isolated at the interphase between the 440 and 700 g/L Percoll solutions.

Preparation of BCECF-labeled H22 cells

Loading BCECF into H22 cells was carried out using a modification of the method described previously [17, 19, 20]. Briefly, 3 μmol/L BCECF-AM was added to the H22 cell suspension (1×10^9 cells/L) in RPMI 1640 medium supplemented with 100 g/L FBS and 1 mmol/L EDTA; the cells were incubated for 30 min at 37°C with gentle agitation in a water bath. After the incubation period, the cells were then washed twice with RPMI 1640 medium supplemented with 100 g/L FBS.

Flow cytometry and ELISA

Assay of CD3, CD4, CD8, and NK cell level in whole blood kin mouse were performed on a flow cytometer (FACS Calibur, BD Company, USA) by commercial biomarkers on cell surface. The levels of IL-2 and TNF-α were determined by the commercial ELISA kit (R&D systems, USA).

Cytotoxic activity of liver and splenic lymphocytes against H22 cells (in vitro)

Isolated liver or splenic lymphocytes were placed in RPMI 1640 medium containing 100 g/L FBS at 5×10^5 cells in 96-well culture plates and exposed to the indicated amounts of various chitosans for 24 h. After the incubation period, the liver or splenic lymphocytes were washed twice with fresh RPMI 1640 medium containing 100 g/L FBS. BCECF-labeled H22 cells (target cells; 5×10^3 cells) were added to the effector cells and incubated with them for 2 h; then these cell mixtures were centrifuged at 500 × g for 10 min. The fluorescence intensity of the supernatant was measured by fluorimetry (FP-777, JASCO) with excitation at 500 nm and emission at 540 nm. The total fluorescence intensity of the target cells (BCECF-labeled H22 cells) was determined after solubilizing the cells by adding 2.5 g/L Triton X-100. The specific cytotoxic activity was calculated as follows: % specific cytotoxicity = (total fluorescence intensity of target cell plus experimental group liver or splenic lymphocytes-fluorescence intensity of spontaneous release)/(total fluorescence intensity of target cells plus control group-fluorescence intensity of spontaneous release) × 100.

Statistical analysis

All statistical analyses were performed with the SPSS 19.0 (Chicago, IL, USA). Quantitative values are expressed as means ± SD. Differences among groups were analyzed by one-way ANOVA or independent-sample-t test, and Fisher's least significant test (LSD-t) was used for multiple comparison. Differences were considered statistically significant at $P < 0.05$.

Results

Antitumor activities

Chitosan significantly reduced the tumor volume and final tumor weight at 100 and 200 mg/kg at dose dependent manner (**Figure 1** and **Table 1**). No severe loss of body weight was found by oral administration of chitosan.

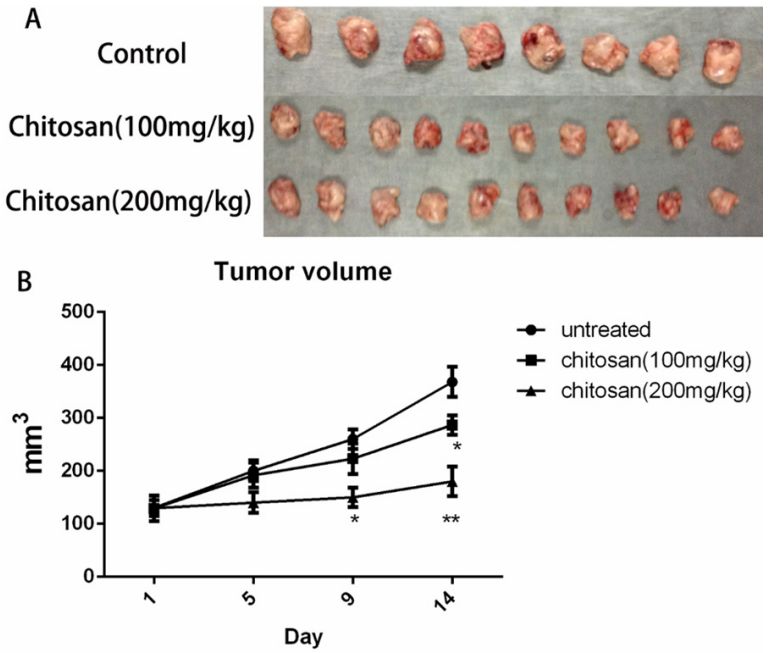

Figure 1. The effect of chitosans on proliferation of H22 liver cancer in vivo. A. Representative picture of tumors. Two mouse died in control group, while all the mouse were alive at day 14 in WSC-treated group; B. Bar graph showed the change of tumor volume. *Indicates the significant differences ($P < 0.05$). **Means $P < 0.01$.

Table 1. Effects of oral administration of chitosans on final tumor weight in H22-bearing mice

Group	Dosage	Tumor weight (g) (X ± SD)	Inhibition rate
Control (n = 10)		5.78 ± 1.11	
Chitosan (n = 10)	100 mg/kg	4.91 ± 1.39	15.1%
Chitosan (n = 10)	200 mg/kg	3.60 ± 0.50**	37.7%

Note: **, $P < 0.01$, compared with control group.

Table 2. Effects of oral administration of various chitosans on the weights of body, tissue, and blood counts in liver cancer H22-bearing mice

	Control	WSC (100 mg/kg)	WSC (200 mg/kg)
Final body Weight (g)	37.2 ± 2.3	34.4 ± 2.1	34.8 ± 1.9*
Spleen (mg)	231.2 ± 39.5	154.3 ± 22.6*	135.6 ± 11.9**
Leukocytes (10^3/L)	2.68 ± 0.47	2.74 ± 0.77	3.54 ± 0.98*
Monocytes (10^3/L)	0.30 ± 0.12	1.26 ± 0.59**	1.45 ± 0.61**
RBC (10^4/L)	7.03 ± 0.64	7.61 ± 0.13*	7.91 ± 0.15*
Hemoglobin (g/L)	104.60 ± 8.82	114.00 ± 7.48	121.00 ± 8.22*

*, $P < 0.05$, **, $P < 0.05$, compared with control group.

Body and tissue weights and blood count

The oral administration of chitosan (100 or 200 mg/kg) had no effect on body weight, adipose tissue, or thymus in H22 bearing mice (data not shown). The weight of the spleen (231.2 ± 39.5 mg, n = 10) in H22-bearing mice was significantly greater than that of normal mice (97.2 ± 4.83 mg, n = 10). The increase in spleen weight in sarcoma H22-bearing mice was significantly reduced (to the normal level) by the oral administration of chitosan at 200 mg/kg. By analyzing the blood cell numbers (leukocytes and RBC) and blood hemoglobin concentration, we found chitosan could significantly increase the cell number of lymphocytes, monocytes and neutrophils in **Table 2**).

Effect of WSC on immune-related molecules

Effect of WSC on CD3, CD4, and CD8 levels in liver tissues are shown in **Figure 2**. WSC increased CD3 (**Figure 2A**) and CD4 (**Figure 2B**) levels in the WSC groups (both low and high dosage) compared with the control group ($P < 0.05$). However, there were not any significant differences in CD8 level among the three groups ($P > 0.05$; **Figure 2C**). Thus, the CD4/CD8 ratio for the experimental group was higher than that of the control group ($P < 0.05$; **Figure 2D**). In addition, Oral administration of WSC (both low and high dosage) increased TNFα (**Figure 3A**), IL-2 (**Figure 3B**) and NK cell number (**Figure 3C**) levels compared with the control group ($P < 0.05$).

Cytotoxic activity against H22 cells (in vitro)

Chitosan did not show direct cytotoxic activity to H22 cell in vitro (cytotoxic activity was below 10%, data not shown), however, it enhanced the lymphocyte to inhibit the proliferation of

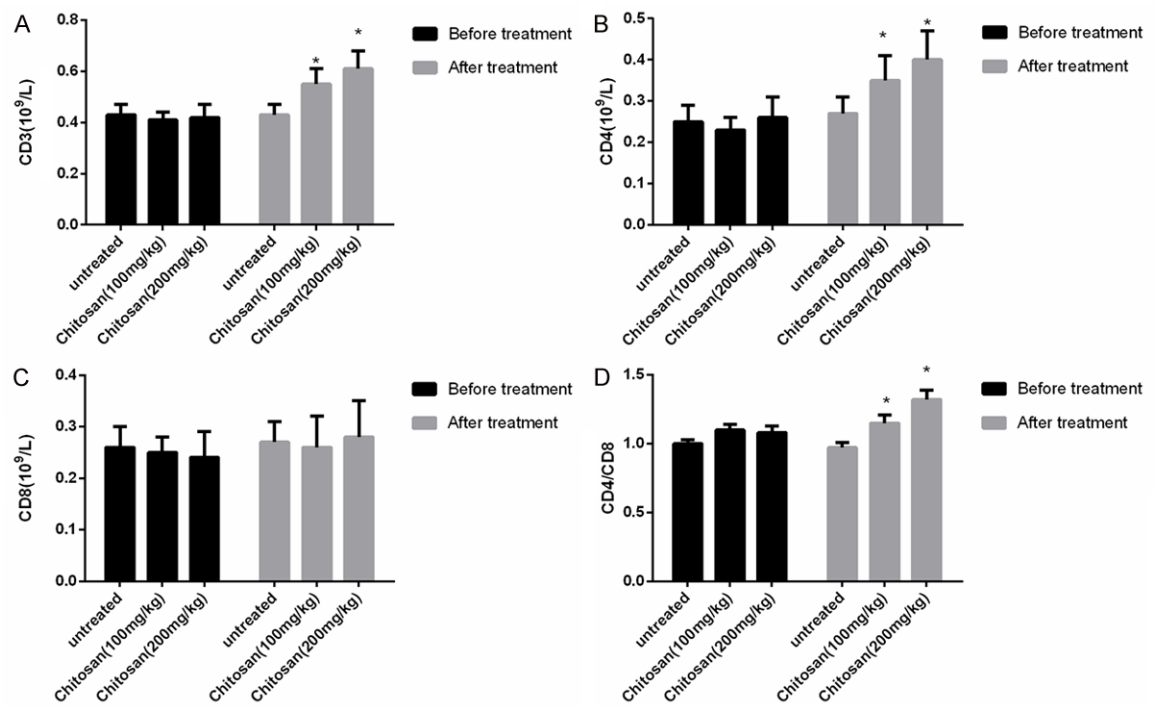

Figure 2. Effect of chitosan treatment on CD3 (A), CD4 (B), CD8 (C) and CD4/CD8 (D) levels in peripheral blood of mouse within the same treatment stage (before or after treatment). *Indicate the significant differences ($P < 0.05$).

H22 cells. We examined the cytotoxic activity against H22 cells by liver or splenic lymphocytes treated with chitosans. Treatment of lymphocytes with chitosan (1-50 μg/mL) enhanced their cytotoxic activity against H22 cells compared with that of untreated lymphocytes (**Figure 4**).

Discussion

Although it was suggested that clinical treatment with water-soluble chitosan with a low molecular weight may have antitumor effects by modulating the immune system, these effects have not been confirmed. The main aim of this study was to clarify this issue. Liver cancer cells are generally used as the first choice for evaluating the antitumor effects of WSC, and they have a high response to immune system, therefore, in this study, we used the mouse liver cancer H22 cells to evaluate the antitumor effects of WSC through immune function. In this study, the antitumor activities of water-soluble chitosans in H22-bearing mice were examined, and the results demonstrated that the water-soluble chitosans with a low molecular weight had a promising antitumor activity.

It was shown clinically that the increase in natural killer (NK) cell activity was significantly higher in the group treated with oligochitoisan than in the placebo group, 6 and 9 h after administration of oligochitosan or placebo in a crossover double-blind test [21]. Seo et al. [22] reported that the synergism between the effects of interferon (IFN)-γ and water-soluble chitosan on nitric oxide (NO) synthesis and tumoricidal activity was dependent mainly on the increased secretion of tumor necrosis factor-α induced by water-soluble chitosan. Shibata et al. [23] reported that C57BL/6 mice pretreated with monoclonal antibodies against mouse IFN-γ or NK.1.1 had a markedly decreased level of alveolar macrophage priming after injection of chitin particles (10 μm). They suggested that the alveolar macrophage priming mechanism of chitin was due to direct activation of macrophages by IFN-γ, which is produced by NK1.1+ and $CD4^+$ T cells in the spleen [23]. That partially explains the mechanism of the anti-cancer effect of chitosan.

Immunological studies showed that the incidence of cancer development and treatment were associated with immune suppression and immune defects [24, 25]. In the body of the anti-tumor immune system, cell immune mediated by T cell which dominantly destroys the tumor [26, 27]. WSC was reported to have both

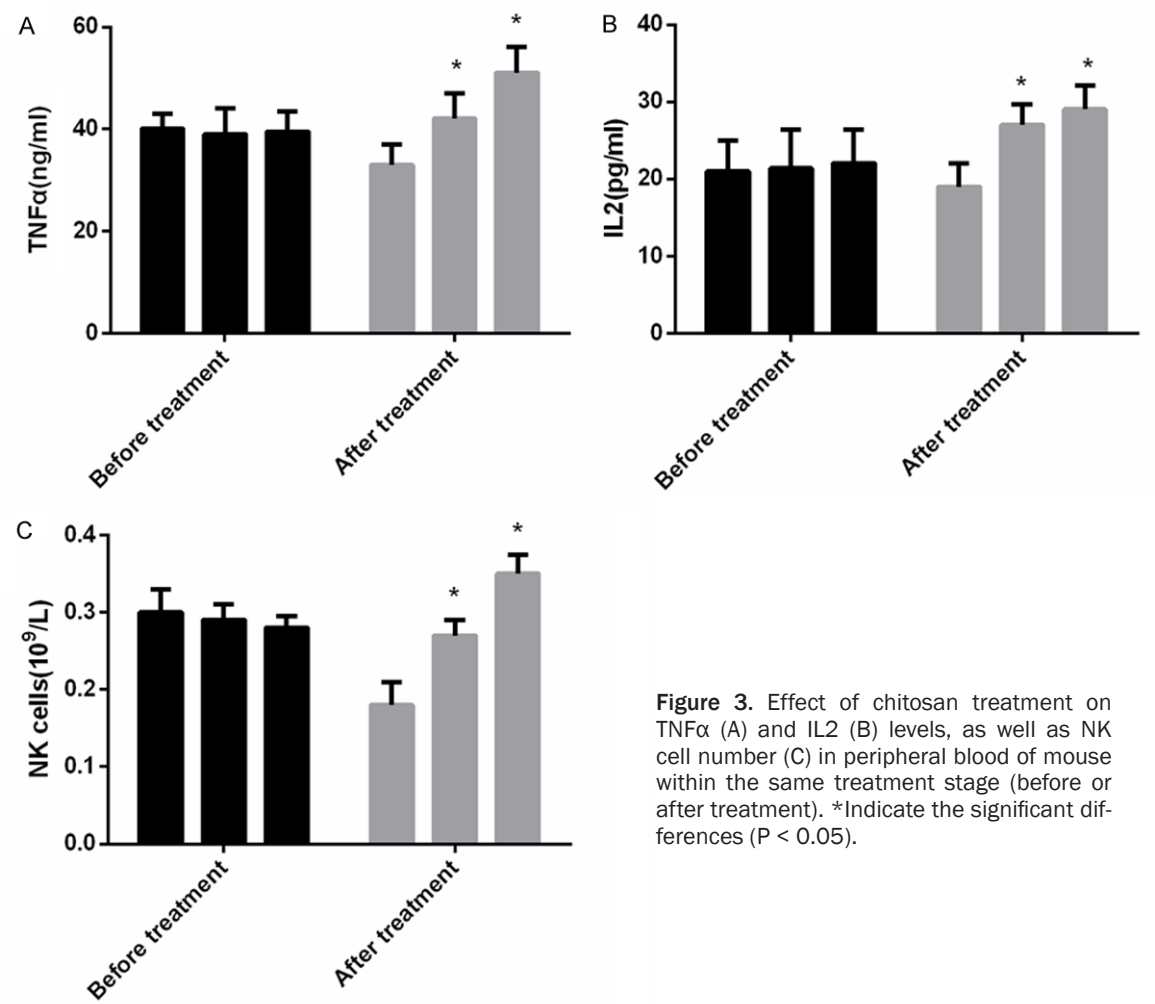

Figure 3. Effect of chitosan treatment on TNFα (A) and IL2 (B) levels, as well as NK cell number (C) in peripheral blood of mouse within the same treatment stage (before or after treatment). *Indicate the significant differences ($P < 0.05$).

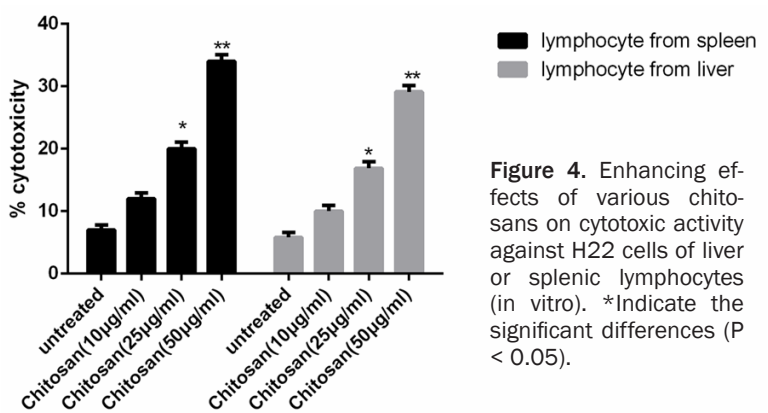

Figure 4. Enhancing effects of various chitosans on cytotoxic activity against H22 cells of liver or splenic lymphocytes (in vitro). *Indicate the significant differences ($P < 0.05$).

antitumor and immunity activities and therefore may be used as an adjuvant therapy for tumor [1]. We examined the antitumor activities and mechanisms of action of low molecular water-soluble chitosans in liver cancer H22-bearing mice through some *vitro* and *vivo* experiments. The data indicated that CD4 subgroups in the mouse blood decreased, while CD8 subgroups relatively increased, and CD4/CD8 ratio decreased. CD4/CD8 ratio is a direct reflection of the body's cellular immune function, and the decrease in CD4/CD8 ratio is mainly due to immunodeficiency and cancer, etc. NK cells play an important role in immune surveillance and anti-tumor immunity. IL-2 and TNF-α play important role in the innate immune response and specific immune response, which may related to each other. Through the analysis of above indexes, the immune status of H22 liver cancer mouse can be evaluated. Our results indicate that oral administration of WSC could

increase CD3, CD4, CD4/CD8 ration, NK cells, IL-2, and TNF-α levels in whole blood of the mouse. Therefore, oral administration of WSC may be used as an adjuvant therapy for liver cancer.

In this study, low-molecular-weight water-soluble chitosan enhanced the cytotoxic activity against tumor cells compared with untreated lymphocyte in both *vitro* and *vivo* experiments. Splenic lymphocytes treated with the WSC in vitro enhanced the cytotoxic activity against tumor cells. These findings suggest that water-soluble chitosan with a low molecular weight may act as an immunomodulator in the intact immune systems of animals. They further suggest that the antitumor activity of low-molecular-weight water-soluble chitosans might be due in part to an enhancement of the cytotoxic activity of macrophage against tumors. Furthermore, it seems that low-molecular-weight water-soluble chitosans may induce the activation of macrophages through the production of cytokines such as IFN-γ, IL-12, and IL-18 from the lymphocytes; consequently, their chitosans may have antitumor activity. Experiments are now in progress to clarify the activation of macrophages through the alteration of immune function in spleen and liver by treatment with water-soluble chitosans in tumor-bearing mice.

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Disclosure of conflict of interest

None.

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