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Immunomodulatory effects of *Caulerpa racemosa* var peltata polysaccharide and its selenizing product on T lymphocytes and NK cells in mice

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A polysaccharide, CrvpPS, was isolated from Caulerpa racemosa var peltata. It was reacted with nano-selenium in distilled water containing ascorbic acid (Vit C) to form a stable CrvpPS-nano-Se complex. The immunomodulatory effects of CrvpPS and CrvpPS-nano-Se on T lymphocytes subgroups and NK cells in mice were investigated. After intragastric administration for 10 days separately, both CrvpPS and CrvpPS-nano-Se showed significant stimulatory functions to thymus gland of mice. Moreover, the CrvpPS-nano-Se induced the percentage of CD_3^+, $CD_3^+CD_4^+$, NK cells and the CD_4^+/CD_8^+ value to increase significantly (P<0.05) when analyzed by flow cytometry, which is better than the CrvpPS, sucrose-nano-Se, and even the positive drug levamisole.

Caulerpa racemosa var peltata polysaccharides (CrvpPS), nano-Se, T lymphocyte subgroups, natural killer cells, immunomodulation, flow cytometric analysis

Seaweeds are low cryptogam grown in ocean water and contain many bioactive compounds. For examples, the seaweed polysaccharides have been reported to show various pharmacological actions, such as antiviral, antitumor, antioxidative, anticoagulant activities, lowering blood glucose, controlling blood lipid and improving body immunity[1]. *Caulerpa racemosa* var peltata, distributed widely in South China Sea, is a tropical and semi-tropical green seaweed belonging to Chlorophyta Caulerpaceae. This seaweed contains a large amount of polysaccharides with immunomodulatory effects on T lymphocyte subgroup and natural killer cell in mice[2]. Selenium is a necessary trace element with strong antioxidative and immunoenhancing properties for mammals[3], and these activities will be greatly enhanced while side effects minimized if the selenium changes into nano-selenium particles[4,5]. There are some natural or synthetic polysaccahride-selenium compounds with various bioactivities[6]. In this study, CrvpPS, the polysaccahride isolated from *Caulerpa racemosa* var peltata, was selenized with nano-selenium to form CrvpPS-nano-Se for investigating its immunomodulatory effects on mouse peripheral T lymphocyte subgroups (CD_3^+, CD_4^+, CD_8^+) and natural killer cells (NK cells) isolated from mouse spleen, respectively.

1 Materials and methods

1.1 Reagents

Rat IgG_1-FITC, IgG_1-PE, IgG_1-PE-Cy_5, rat anti-mouse monoclonal antibody CD_3-PE, CD_4-PE-Cy_5, CD_8-FITC, NK-PE (human monoclonal antibody is CD_{16+56}-PE), red blood cell disruption solution, and fixation fluid. All of these reagents were purchased from BD BioSciences Pharmingen (CA, USA). Hydrocortisone Injection (25 mg/5 mL) and Levamisole (50 mg) were obtained from

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Yangzhou Pharmaceutical Co., Ltd., Yangzhou, China. and Hainan Drug Manufactory, Hainan, China, respectively.

1.2 Seaweed and polysaccharide isolation

Seaweed, *Caulerpa racemosa* var peltata, was collected from the South China Sea, Zhanjiang, and identified by the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China. The seaweed polysaccharide, CrvpPS, was obtained by water extraction and ethanol precipitation according to the method described in a previous paper[2].

1.3 CrvpPS-nano-Se and sucrose-nano-Se preparation

CrvpPS (1 g / 18 mL distilled water) reacted with Vit C solution (557.7 μg/mL, 1 mL) and SeO_2 solution (100 μg/mL, 1 mL) at room temperature for more than 24 h until the complex became stable. Sucrose-nano-Se was prepared with sucrose (0.2 g / 20 mL water) and nano-Se (5 μg/mL), while the CrvpPS-nano-Se system was a mixture of CrvpPS (5 mg/mL) and nano-Se (5 μg/mL). These sample solutions were autoclaved and stored at 4 ℃ till use.

1.4 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of tested samples

The CrvpPS, CrvpPS-nano-Se and sucrose-nano-Se solutions were dropped separately on clean cover slips, air dried, and coated with gold *in vacuo*. Their surface morphology was observed using an XL-30 scanning electron microscope (Philips, UK). These samples spread on copper grides respectively. After air drying, they were observed under a TECNAI-10 transmission electron microscope (Philips, UK).

1.5 Laser light scattering detection

The solutions of CrvpPS, CrvpPS-nano-Se and sucrose-nano-Se were filled in sample cells of the BI 9000AT Laser Light Scattering Goniometer (Brookhaven, USA) separately for the measurement of the particle diameters using the Dynamic Light Scattering software at the laser wavelength of 678 nm, angle of incidence 90°, and constant temperature of 25℃.

1.6 Animals and groups

Healthy female Kunming mice, 6－8 weeks old and (20±2) g, were purchased from the Guangdong Animal Studies Center (Certificate: SCXK (Yue) 2003-0002, No.: 2005A012). Forty-eight mice were divided randomly into 6 groups, namely (1) untreated 8 mice with saline at the dose of 0.4 mL/d were used as the normal control group, and the other 40 mice were daily hypodermically injected with hydrocortisone (20 $mg \cdot kg^{-1} \cdot d^{-1}$) for 7 consecutive days to induce weak immune functions which were mock mice. The treatment groups included (2) Mock group with saline at the dose of 0.4 mL/d; (3) CrvpPS group with CrvpPS solution at the dose of 100 $mg \cdot kg^{-1} \cdot d^{-1}$, 0.4 mL/d; (4) Sucrose-nano-Se group with sucrose-nano-Se solution and the dose of Se was 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$ (0.4 mL/d); (5) CrvpPS-nano-Se group with CrvpPS-nano-Se solution (polysaccharide 100 mg / kg·d and Se 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$), 0.4 mL/d; (6) Levamisole group with levamisole solution (25 $mg \cdot kg^{-1} \cdot d^{-1}$), 0.4 mL/d. During the experiment, all mice were kept under normal laboratory conditions.

After the experiment, all the mice were sacrificed by cervical dislocation and blood was immediately collected in the test tube containing 2 μL heparin from the left ventricle for flow cytomitric analysis. The thymus glands and spleens were removed from the bodies, and weighed for organ index analysis[7]. Furthermore, splenic lymphocytes were collected for NK cells measurement according to this method: spleens were minced and placed on gauze at 200 meshes. They were then washed with PBS to remove cell debris and tissue pieces. Then the suspension was centrifuged at 1000 r/min for 10 min. The cell pellet was re-suspended in 1 mL PBS for testing.

1.7 Sample preparation and flow cytometric detection

Each blood and splenic NK cell sample was divided into two test tubes. Ten μL of 3 antibodies against CD_3, CD_4 and CD_8 was added into tube 1, while fluorescence IgG (rat IgG_1-FITC, IgG_1-PE and IgG_1-PE-Cy_5) was added to tube 2 as the respective controls. After incubation at room temperature in darkness for 15 min, the samples were loaded into the Q-prep, an automatic sample preparation equipment, for hemolysis and cell fixation. Cell suspension was made in 0.3 mL PBS for analysis of percentages of positive CD_3, CD_4 and CD_8 cells in the blood and positive NK cells in spleens using an ELITE Flow Cytometric System (Beckman-Coulter, America) at 488 nm. All data obtained were analyzed on computer.

1.8 Statistical analysis

Data were analyzed with the statistical package of the

SPSS 12.0. Descriptive data were given as mean ± standard deviation (SD). Comparison between two groups was performed with *t*-test and covariance analysis, and $P<0.05$ was considered as statistically significant.

2 Results

2.1 Morphology of CrvpPS and CrvpPS-nano-Se samples

Under the SEM, the CrvpPS was dispersed flocculate with irregular appearance, and the average diameter was about 1－2 μm (Figure 1(a)). After reacting with nano-Se, the particles changed to irregular cotton-like spherites with the diameter diminished to about 1 μm (Figure 1(b)). Moreover, the nano-Se appeared as regular and fine globular particles of 100 nm in diameter and was absorbed on the surface of CrvpPS molecule.

Under the TEM, CrvpPS was irregular in shape and size, appearing to be cotton-like and distributed in groups with diameter of more than 1 μm (Figure 2(a)). In the sucrose-nano-Se solution, only the nano-Se particles (no more than 100 nm in diameter) were regular spheres and distributed homogeneously (Figure 2(b)). In the CrvpPS-nano-Se solution, the nano-Se particles were spheres, similar to those in sucrose-nano-Se (Figure 2(c)). Furthermore, some large regular spheres of about 500 nm in diameter were distributed around nano-Se (Figure 2(d)), which might be the package or absorption phase of CrvpPS and selenium.

2.2 Particle diameter of CrvpPS and nano-Se in laser light scattering detection

Figure 3 shows the particle diameter of CrvpPS and nano-Se using laser light scattering detection. The CrvpPS at the mass concentration of 0.5% appeared as large molecules with a wide diameter distribution ranging from 500 to 1700 nm (Figure 3(a)). The nano-Se connected with 1% sugar as the surface modifier had very uniform particle diameters, and most of them were about 47.1 nm (Figure 3(b)). There were two peaks shown in the CrvpPS-nano-Se solution. The first one was nano-Se at 83.3 nm and the second one was CrvpPS in a narrow range of around 1032.0 nm (Figure 3(c)). This revealed that in the CrvpPS-nanoa-Se solution, both the CrvpPS and selenium had changed their properties and the size of CrvpPS became more uniform.

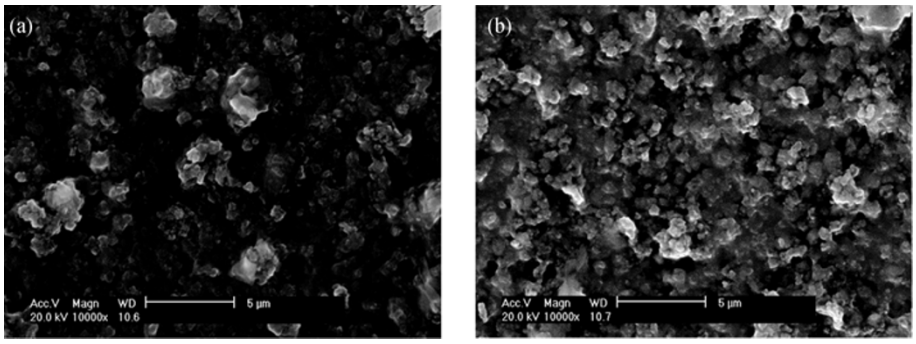

Figure 1 SEM graphs of CrvpPS (a) and CrvpPS-nano-Se (b) (×10000).

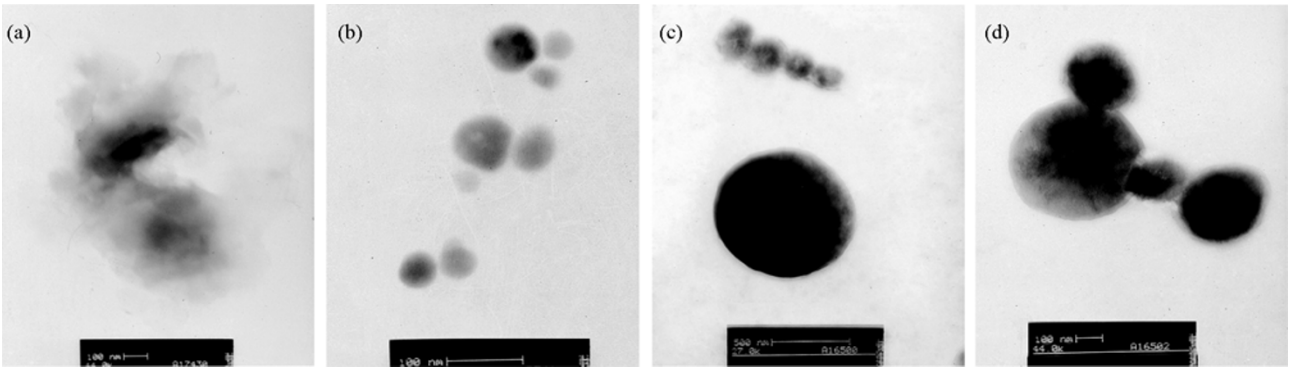

Figure 2 TEM graphs of CrvpPS (a), sucrose-nano-Se (b) and CrvpPS-nano-Se ((c) and (d)).

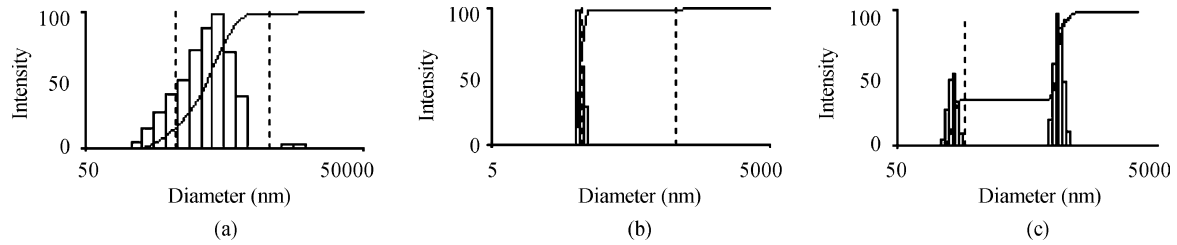

Figure 3 The particle diameter distribution of 0.5% CrvpPS (a), 1% sucrose-nano-Se (b) and 0.5% CrvpPS-nano-Se (c).

2.3 Influence of CrvpPS and CrvpPS-nano-Se on immune organ index of mouse

The results in Table 1 do not show any body weight change in all treatment groups after intragastric administration with different solutions. Compared with normal mice in the control group, mice in the five treatment groups decreased in spleen index and thymus index (P<0.05), especially the mice in the mock group showed the lowest indexes at 27.09 and 12.87, respectively (Table 1). Based on this level, the mice administered with CrvpPS, sucrose-nano-Se and CrvpPS-nano-Se showed nonstatistically significant increase in spleen indexes, with an exception for those treated with levamisole. However, the thymus indexes increased significantly in all groups, suggesting that the CrvpPS and CrvpPS-nano-Se had stronger stimulatory effects on thymus than on spleen.

2.4 Stimulatory effects on T lymphocyte subgroups (CD_3^+, CD_4^+, CD_8^+) and NK cells

Mock mice treated with CrvpPS, sucrose-nano-Se and CrvpPS-nano-Se showed that the levels of CD_3^+ and $CD_3^+CD_4^+$ lymphocytes increased significantly (P<0.05) and even exceeded the levels of those in the normal mice group (Table 2). The percentages of both CD_3^+ lymphocytes and $CD_3^+CD_4^+$ lymphocytes in the CrvpPS-nano-Se group were higher than those in the CrvpPS and

Table 1 Immune organ index of mice in different groups

Groups	n	Body weight (g)	Spleen weight (g)	Spleen index	Thymus weight (g)	Thymus index
Normal (control)	8	24.23±2.34	96.40±11.82	39.96±5.10	69.64±12.23	28.77±4.28
Mock	8	23.05±2.30	62.68±13.03	27.09±4.18 a)	29.74±5.14	12.87±1.50 a)
CrvpPS	8	22.89±1.33	70.61±11.90	30.81±4.54 a)	41.25±5.59	18.20±2.73 a,b)
sucrose-nano-Se	8	23.73±1.15	67.03±7.21	28.23±2.45 a)	44.19±8.33	18.74±4.09 a,b)
CrvpPS-nano-Se	8	25.50±3.02	78.95±11.30	30.95±2.38 a)	48.16±6.39	19.06±3.05 a,b)
Levamisole	8	24.58±2.43	82.21±12.39	33.40±3.14 a,b)	54.05±7.02	22.01±1.86 a,b)
F		1.612	9.181	11.864	24.278	23.617
P		0.178	0.000	0.000	0.000	0.000

a) denotes P<0.05 compared with the normal control group; b) P<0.05 compared with the mock group.

Table 2 Percentage of CD_3^+, CD_4^+, CD_8^+ in blood and spleen NK cells of mice in different groups

Groups	n	CD_3^+	$CD_3^+CD_4^+$	$CD_3^+CD_8^+$	CD_4^+/CD_8^+	NK
Normal (control)	8	34.69±3.48	25.44±4.40	9.11±1.47	2.87±0.76	3.55±0.56
Mock	8	22.53±6.98 a)	14.28±4.40 a)	7.21±2.67	2.06±0.39	2.44±0.49 a)
CrvpPS	8	53.80±5.49 a,b)	42.20±5.98 a,b)	11.13±0.96	3.84±0.79◇	8.39±1.26 a)
sucrose-nano-Se	8	45.84±2.79 a,b,c)	35.31±4.41 a,b)	9.95±1.72	3.65±0.79◇	5.59±0.64 a,b,c)
CrvpPS-nano-Se	8	68.08±5.01 a,b,c,d)	58.00±6.21 a b,c,d)	10.90±2.09	5.61±1.81 a,b)	12.34±2.90 a,b,d)
Levamisole	8	48.90±4.55 a,b,e)	42.86±4.07 a,b,d,e)	5.79±0.88 a,c,d,e)	7.53±1.08 a,b,c,d)	8.69±2.52 a,b)
F		81.998	74.249	11.832	29.659	37.556
P		0.000	0.000	0.000	0.000	0.000

a) Compared with the mean of the normal group: P<0.05. b) Compared with the mean of the mock group: P<0.05. c) Compared with the mean of the CrvpPS group: P<0.05. d) Compared with the mean of the sucrose-nano-Se group: P<0.05. e) Compared with the mean of the CrvpPS-nano-Se group: P<0.05.

sucrose-nano-Se group (P<0.05), reaching the values of 68% and 58%, respectively, indicating that the CrvpPS, nano-Se and sucrose-nano-Se possessed positive adjusting on mouse T CD_3^+ and CD_4^+ lymphocyte subgroup, with the CrvpPS-nano-Se possessing the strongest effect (Figure 4).

At the same time, the percentage of spleen NK cells in different groups changed notablly (as listed in Table 2). Compared with the normal group, the ratio of NK cells in mock mice was depressed, but recovered (P<0.05) after administration with CrvpPS, sucrose-nano-Se, CrvpPS-nano-Se and levamisole. Among these solutions, CrvpPS-nano-Se had stronger stimulatory effect on NK cells than those from CrvpPS or sucrose-nano-Se and even the levamisole. These results demonstrated that the combination of CrvpPS and nano-Se is beneficial to the regulative action on T lymphocyte subgroup and NK cells in mice.

3 Discussion

It is well documented that particulate nano-selenium has favourable bioactivities and low toxicity, but it aggregates easily into an inactive form. Modification of the particulate surface by chemicals can prevent nano-Se particles from aggregation. Sucrose can act as surface modifier which controls the generation speed and also the diameter of nano-Se particles, subsequently forming a stable system[8]. The amino and hydroxyl groups of polysaccharide can also modify the surface appearance and particle diameter as well as stabilize the nano-Se particles[9]. When H_2SeO_3 is dissolved in the acidic polysaccharide solution, Se(Ⅳ) is reduced to Se^0, in the form of nano-Se. These nano-Se particles are then coated and adsorbed by the polysaccharide to form the polysaccharide-nano-Se liquid system, which is stable at least for six months, and is difficult to disintergrate by conventional methods[9].

In this study, the appearance of particulate CrvpPS, nano-Se and CrvpPS-nano-Se, as demonstrated by both SEM and TEM, is similar to those reported by other groups[9]. In the sucrose-nano-Se system, the nano-Se particles appeared as regular globes with uniform diameter of about 47.1 nm. The CrvpPS-nano-Se system has similar globular particles which are further stabilized by hydrogen bonds between CrvpPS and nano-Se, and packed by polysaccharide emulsion.

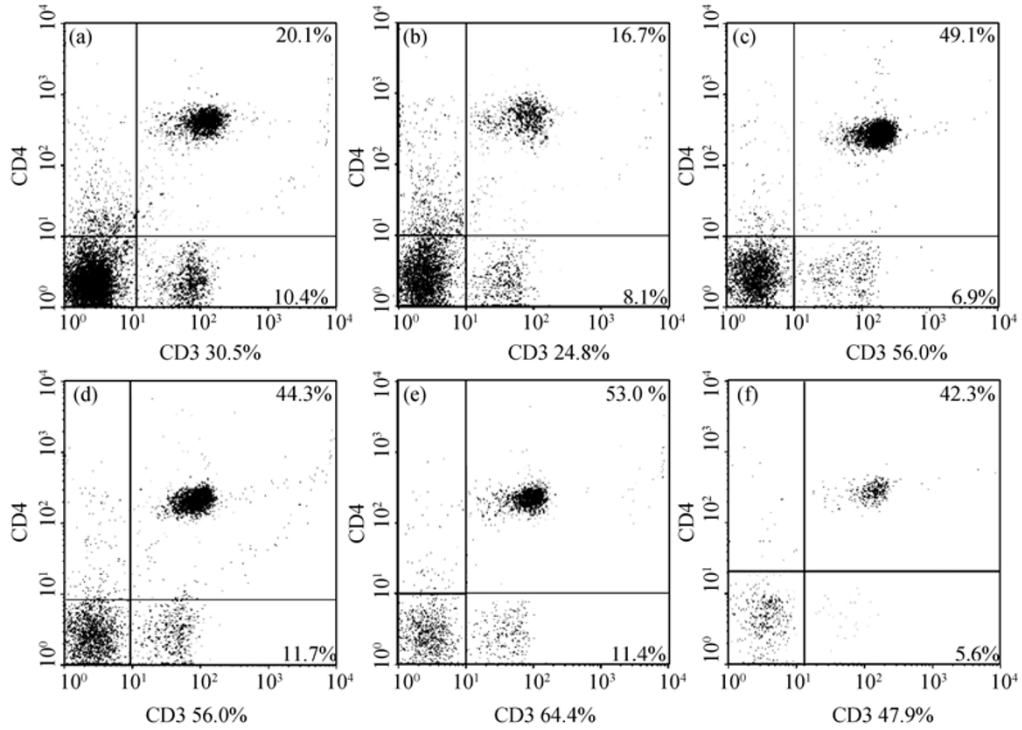

Figure 4 Two-way fluorescent light scatter plots of $CD_3^+CD_4^+$ in blood of different groups. (a) Normal mice group; (b) Mock group; (c) CrvpPS group; (d) sucrose-nano-Se group; (e) CrvpPS-nano-Se group; (f) levamisole group. In these figures, the cells distributed in the left area of Y-axis are CD_3^- while in the right area are CD_3^+ cells. The cells distributed below the X-axis are the CD_4^-, and the uppers are CD_4^+ cells. Cells in the right upper area are $CD_3^+CD_4^+$ cells.

Polysaccharides are not only the structural support to the organism, but also important components of many endogenous bioactive molecules in the immune system[10]. Selenium, a trace element incorporated as selenocysteine in the glutathione peroxidase, plays an important role in antioxidantion[11], immune competence[12], and production of immunoglobulins and antibodies. It has been reported that Se deficiency impairs the immune function, decreases T lymphocyte proliferation, and suppresses NK cells and phagocyte activities. A synergistic combination of selenium and polysaccharide to form a selenized polysaccharide is a transform from inorganic selenium to organic selenium that can be easily absorbed to improve human immune functions[13–15].

In this study, all the CrvpPS, CrvpPS-nano-Se and sucrose-nano-Se showed similar stimulatory effect on mouse thymus but not on the spleens. The thymus index increased significantly when compared with that of the mock mice. However, there was no evidence for synergy between CrvpPS and nano-Se on mouse lymphoid organs, e.g., thymus and spleen. This may be due to insufficient time for drug treatment in this experiment.

It has been reported that Se-Lentinan, a seleno-polysaccharide isolated from a mushroom, could increase the proliferation of CD_4^+ lymphocytes and the CD_4^+/CD_8^+ ratio in an immunodeficient mouse model[16]. In addition to sharing similar stimulatory activities on CD_4^+ lymphocytes, k-selenocarrageenan, a seaweed seleno-polysaccharide, also enhanced both humoral (IgG and IgA) and cellular (macrophage phagocytosis) immunity in patients under chemotherapy[17].

In this study, both the percentage of CD_3^+, and $CD_3^+CD_4^+$ lymphocyte subgroups, and the ratio of CD_4^+/CD_8^+ in the mock mice decreased significantly when compared with the controls. Treatment with CrvpPS, sucrose-nano-Se, CrvpPS-nano-Se or levamisole could recover lymphocyte proliferative activities, with CrvpPS-nano-Se being the most potent immunostimulant in which CrvpPS and nano-Se acted in synergy to elevate the CD_4^+/CD_8^+ ratio and also improved the defensive ability of host against pathogens[13,16]. The mechanism mediating these immunomodulatory functions is not clear, so further studies are required to determine the possible action(s) of individual and a combination of these two components.

Natural killer (NK) cells are a subpopulation of lymphocytes in the innate immune system that plays important roles in host defense against infectious pathogens and tumor growth. Both polysaccharide and selenium were able to stimulate NK cell numbers and functions, for examples, the nano red elemental selenium could inhibit the growth of HepG2 cells *in vitro* or reduce the tumor mass in the tumor-bearing mice[18]. Selenium polysaccharide (SePS) isolated from Spirulina showed higher efficacy with synergy between selenium and polysaccharide to improve tumor killing activity of NK cells in mice receiving daily SePS at dose of 50 mg/kg and 100 mg/kg[13]. Although no synergy was demonstrated between CrvpPS and nano-Se in the present study, the various combinations of Se and CrvpPS did show target-specific immune funtions: the CrvpPS-nano-Se increased the thymus index, T cell subpopulations and NK cells; and the CrvpPS-nano-Se stimulated CD_3^+ and $CD_3^+CD_4^+$ lymphocytes. The CrvpPS-nano-Se is therefore an ideal natural immunomodulator from the seaweed with low toxicity, and it deserves further study for the development as a potential therapeutic agent.

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