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Immunoregulatory and antitumor activity of schizophyllan under ultrasonic treatment

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ABSTRACT

Aim of this study was to investigate the effect of ultrasonic treatment on the biological activities of schizophyllan (SPG) from *Schizophyllum commune*. The immunoregulatory and antitumor activity *in vitro* and *in vivo* of SPG and ultrasonic-treated SPG (USPG) were evaluated by splenic lymphocytes, macrophages RAW264.7 and human breast carcinoma T-47D cells. Compared with SPG, USPG fractions had small molecular weight and narrow distribution. Meantime, more enhancement of NO production in macrophages RAW264.7, lymphocytes proliferation rates, IL-2 and TNF-α level from spleen lymphocytes and T-47D cells inhibition rates were observed in USPG fractions groups. This result indicated that the immune-enhancing and antitumor activity of SPG was significantly improved after ultrasonic treatment. USPG60 exhibited the highest biological activity in this study. In conclusion, application of ultrasonic technology on SPG preparation is an efficient approach to get high biological polysaccharide, and USPG60 might be a potential functional component for immunoregulatory and cancer treatment.

1. Introduction

Ultrasound has been explored in the food industry for many years. Various areas have been shown to be of great potential for future development, e.g. microorganisms and enzymes inactivation, crystallization, drying, degassing, extraction, filtration, homogenization, meat tenderization, oxidation, sterilization, etc. [1,2]. Comparing with other methods, the emerging ultrasonic method has advantage of saving energy and time, reducing not only the consumption of organic solvents, but also the waste produced by chemical reaction on the environment and so on [3,4]. Ultrasonic degradation is a great physical method for producing homologous series of lower molecular weight of organic matter, and has attracted more interests of researchers in recent years [5–7]. Many reports exposed extensively the changes of physicochemical property and chemical structure and degradation kinetics of ultrasonic-treated polysaccharides. Moreover, it was proved to be an effective and favorable tool to improve the bioactivities of polysaccharides [1,8–12].

Schizophyllan (SPG) is a fungus polysaccharide produced by fermentation from filamentous fungi *Schizophyllum commune* [13]. Native SPG had great biological activities and had been used as immunomodulator, anticancer drug and bioactive cosmetics ingredient in the drugs and functional foods. Such as, it had been registered as an anticancer drug in Japan for its great clinical effectiveness in patients with lung cervical and gastric cancers [14]. However, SPG application in pharmaceuticals and cosmetics industries has been limited for his high molecular weight and viscosity [15]. In our previous study, ultrasonic technology was used to process SPG and three ultrasonic-treated SGP (USPG) fractions with small molecular weight and low viscosity were obtained [15]. Whereas, the biological activities of these USPG samples are still unclear.

This study focused on the functions of USPG fractions with different molecular weights on immune and antitumor systems. The biological activities of USPG fractions were evaluated *in vitro* and *in vivo* by splenic lymphocytes, macrophages and tumor cells. The final aim of this research was to investigate the efficient ability of ultrasonic technology on the biological activities of SPG, and find a potential functional polysaccharide component. Meantime, this research could provide the theoretical basis for the further

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experiment *in vivo* to study the biological activities of SPG and USPG fractions.

2. Materials and methods

2.1. Preparation of SPG and USPG fractions

The strain of *Schizophyllum commune* Fr. ACCC51174 used in this study was obtained from the Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences. SPG and USPG fractions samples were produced according to the method described by Zhong et al. [15,16]. SPG sample after ultrasonic treatment was separated and purified by alcohol fractional precipitation technology with final alcohol concentration was 40%, 60% and 80%, respectively. Three USPG fractions were obtained finally and USPG40, USPG60 and USPG80 were used as names for fractions 0–40%, 40–60% and 60–80%, respectively. Lucid ganoderma polysaccharide (LGP) which used as the positive control in this study was produced in our lab. Total sugar (TS) and total protein (TP) contents were 90.54% and 3.22%, respectively.

2.2. Chemicals and regents

3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Roswell Park Memorial Institute (RPMI) 1640 medium, Griess reagent and bovine serum albumin (BSA) were all purchased from Sigma Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Raw murine macrophages (RAW 264.7) and human breast carcinoma T-47D cells were obtained from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China) and American Type Cell Culture (USA), respectively. Dimethyl sulfoxide (DMSO) was produced by Zhengxing Chemical Co. Ltd. (Suzhou, China). All other chemicals and solvents used in this experiment were of reagent grade unless otherwise specified.

2.3. Chemical compositions and general structure analysis of SPG and USPG fractions

The TS and TP content of SPG and USPG fractions were colorimetrically measured by the reaction with phenol in the presence of sulfuric acid at 486 nm and by the Brandford assay using BSA as the standard [17,18], respectively. The glucose and BSA were used as standard material, respectively.

The average molecular weight and distribution determination of SPG and USPG fractions were determined with High Performance Liquid Chromatography (HPLC), RI detector and the multiangle laser light scattering method using a chromatographic column (OCpak SD-822 M ZQ) eluted with 0.1 M $NaNO_3$ at a flow rate of 0.6 mL/min. The Astra software was used to analyze the data. Monosaccharide analysis of test sample was performed by ion chromatography [19].

2.4. Determination of NO production in macrophages RAW264.7

The NO production test was used following the method described by Yao et al. with little modifications [20]. Macrophages RAW264.7 were resuspended in RPMI 1640 medium (5×10^5 cells/mL) containing various concentration polysaccharides (25–400 μg/mL). Negative control group only used the culture medium without polysaccharide. Cells were incubated at 37 °C in 5% CO_2 for 24 h, and 50 μL supernatant was pipetted from the medium and added into the 96-well plate and four wells each concentration. Supernatant mixed with an equal volume of Griess reagent (50 μL) and incubated for 15 min at room temperature. The absorbance at 540 nm was measured in an enzyme-linked immune sorbent assay (ELISA) reader (Bio-Tek MQX200, USA) and NO production of test sample was calculated with reference to a standard curve obtained with $NaNO_2$ (6.25–100 μM).

2.5. Isolation and preparation of splenic lymphocytes

ICR mice were killed and spleens were removed in sterile condition immediately. Spleens were mashed (70 μm pore mesh) and erythrocytes were depleted with ammonium chloride buffer solution. Splenic Lymphocytes were washed with RPMI and resuspended in Red Blood Cell Lysing buffer Hybri-Max™ (sigma), incubated for 3 min, and then washed twice with RPMI. The Pellets were resuspended to 5×10^6 cells/mL with RPMI 1640 medium containing 10% FBS.

2.6. Determination of splenic lymphocytes proliferation

Above prepared splenic lymphocyte solution (100 μL per well) were placed in the 96-well culture plates. 100 μL of SPG and USPG fractions solution with different concentrations were added into wells and four wells each concentration. Negative control group was treated by 100 μL culture medium. The mixture was incubated at 37 °C with 5% CO_2 for 12 h, 24 h and 48 h, respectively, and 20 μL of solution containing 5 mg/mL of MTT was added into each well and incubated for 4 h. Subsequently, the plates were centrifuged ($1400 \times g$, 5 min) and the untransformed MTT was removed by pipetting. DMSO (200 μL) solution (192 L DMSO with 8 L 1 M HCl) was added to each well and the plates were shaken for 5 min to dissolve the crystals completely. The absorbance at 570 nm of cells in each well was evaluated using the ELISA (Bio-Tek MQX200, USA). A_{570} value was used as index of lymphocyte proliferation, and lymphocyte proliferation rate was calculated as follows:

$$\text{Proliferation rate } (\%) = \frac{A_{\text{test group}} - A_{\text{negative group}}}{A_{\text{negative group}}} \times 100$$

2.7. Determination of IL-2 and TNF-α level

Lymphocytes stimulated by SPG and USPG fractions with different concentrations (50, 150 and 300 μg/mL) were collected as in section 2.6. The incubation time of lymphocytes cells was 24 h. Culture supernatants were collected and the concentrations of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) were assayed by ELLSA kit (BD Biosciences Pharmingen, San Diego, CA, USA).

2.8. Determination of inhibition activity on T-47D cells

The inhibition activity against T-47D cells proliferation *in vitro* of SPG and USPG fractions was measured using the colorimetric MTT assay as described previously [21]. Briefly, cells were seeds (2.5×10^4 cells/mL, 200 μL) in culture medium of RPMI 1640 medium supplemented with 10% FBS in 96-well plate. After incubating the cells at 37 °C in a 5% carbon dioxide incubator for 24 h, cells were treated with SGP, SPG and USPG fractions solutions with different concentrations (final concentrations of polysaccharides was 25, 50, 100, 200 and 400 μg/mL, respectively) and incubated for additional 24 h at the same condition. Negative control group was treated by the culture medium. 20 μL MTS-reagent was added to each well and cells were incubated for another 3 h. Absorbance was measured at 490 nm by the microplate ELISA reader. Antitumor activity of polysaccharide was described by the inhibition rate (IR) as follows:

$$\text{Inhibition rate } (\%) = \frac{A_{\text{test group}} - A_{\text{negative group}}}{A_{\text{negative group}}} \times 100$$

Table 1
Chemical composition and structure of *SPG and USPG fractions.*

	SPG	USPG40	USPG60	USPG80
TS (%)	92.33 ± 1.55	93.15 ± 2.74	90.56 ± 2.48	88.77 ± 3.33
TP (%)	2.55 ± 0.11	2.77 ± 0.07	2.57 ± 0.09	3.78 ± 0.15
Mw (Da)	7.38×10^6	5.78×10^6	6.99×10^5	4.89×10^4
Mw/Mn	1.80	1.45	1.41	1.35
Glucose (%)	89.26 ± 2.54	90.77 ± 3.79	86.56 ± 2.88	82.17 ± 1.33

Values are mean of three-independent experiments.

2.9. Preparation of serum samples containing polysaccharides

8-Week-old male Sprague-Dawley rats ($n = 30$, body weight 190–210 g) were obtained from Vital River Lab Animal Technology Co., Ltd. (Beijing, China). All rats were housed individually in stainless steel cages under controlled environmental conditions (23 ± 2 °C, 55 ± 5% relative humidity, and 12 h light/dark cycle), and acclimatized for one week before starting the experiment. During the experiment the rats were provided with commercial standard pellet diet and tap water ad libitum. Then the rats were divided into five groups (6 rats each group), one normal control group and 4 experimental groups (treated by SPG and 3 USPG fractions, respectively). The rats of 4 experimental groups were treated with polysaccharides samples (1 g/kg/day) by oral gavage for 7 days, and those in normal group were given the same volume of normal saline alone. The rats were fasted overnight and then sacrificed by cervical dislocation. Blood was collected in sterilized polystyrene tubes without the anticoagulant, and the serum was immediately separated by centrifugation at 1000 × *g* and room temperature (23 ± 2 °C) for 10 min. The serum samples of normal control group and experimental groups were kept at −20 ± 1 °C until assayed.

The effects of serum samples on splenic lymphocytes proliferation and releases of IL-2 and TNF-α level were investigated to study the immunoregulatory activity of SGP and USPG fractions *in vivo*. Lymphocyte proliferation effect and production of IL-2 and TNF-α level were assayed by MTT assay and ELLSA kit, respectively.

2.10. Statistical analysis

Data were expressed as the means ± standard deviations (SD) of three or more replicate determinations. Analysis of variance (ANOVA) was performed with the software Microcal Origin 8.0 (Microcal Software, Inc., Northampton, USA). P-values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Chemical compositions and molecular weights of SPG and USPG fractions

The Chemical compositions and molecular weights of SPG and USPG fractions were measured and data were showed in Table 1. All polysaccharides samples had high carbohydrate contents (TS content >88%) and low TP contents (<4%). Glucose was the only monosaccharide of SPG and USPG fractions, and their contents in samples were ranged from 82.17% to 90.77%. Natural SPG had higher molecular weight (7.38×10^6 Da) and wider molecular distribution (1.80). Compared with SPG, the molecular weight and molecular distributions of USPG fractions decreased significantly ($p < 0.05$). USPG80 had smallest molecular weight (4.89×10^4 Da).

3.2. Immunoregulatory and antitumor activities of SPG and USGP fractions in vitro

3.2.1. NO production in macrophages RAW264.7

Fig. 1 illustrated the effects of SPG and USGP fractions with different concentrations on NO production in macrophages RAW264.7. Compared with negative control, positive control produced low NO level, and highest NO production was below 20 μM at 100 μg/mL. No significant NO level was observed in the SPG group ($p > 0.05$). Whereas, three USPG fractions stimulated powerfully macrophages RAW264.7 to produce NO in the medium experimental dose (25–400 μg/mL). NO production was enhanced at high polysaccharide concentration, and the highest NO productions of USPG fractions were 81.91 μM (USPG40, 100 μg/mL), 84.71 μM (USPG60, 50 μg/mL) and 72.92 μM (USPG80, 100 μg/mL), respectively.

3.2.2. Splenic lymphocyte proliferation

The splenic lymphocyte proliferation effects of SPG and USPG fractions (150 μg/mL) with different incubation time were investigated by measuring cell viability using the MTT assay (Fig. 2). The

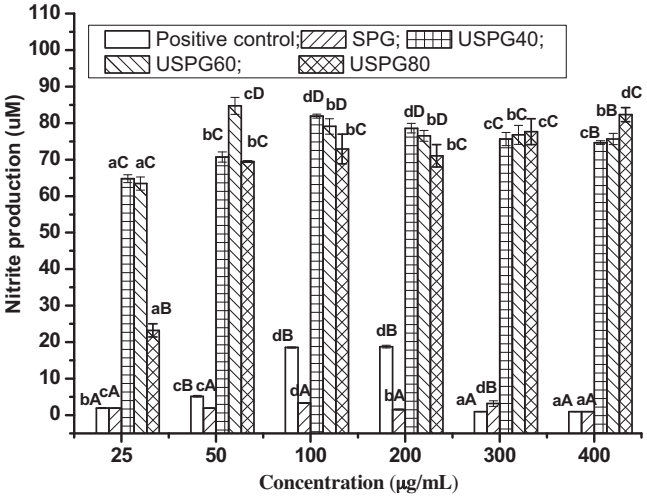

Fig. 1. Stimulation effects of SPG and USPG fractions on NO production in macrophages RAW264.7. [a–d]: Same polysaccharides sample with different concentration, bars without the same superscripts differ significantly ($p < 0.05$). [A–D]: Different polysaccharides sample with same concentration, bars without the same superscripts differ significantly ($p < 0.05$).

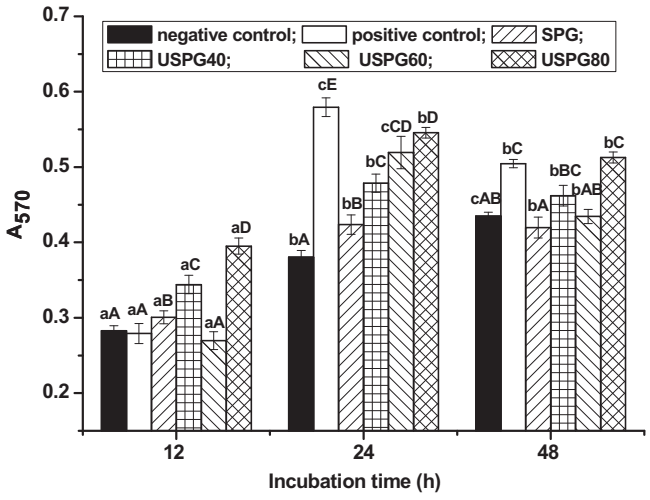

Fig. 2. Proliferation effects of SPG and USPG fractions with different incubation time on splenic lymphocyte (sample concentration was 150 μg/mL). [a–c]: Same sample group with different incubation time, bars without the same superscripts differ significantly ($p < 0.05$). [A–D]: Different sample group with same incubation time, bars without the same superscripts differ significantly ($p < 0.05$).

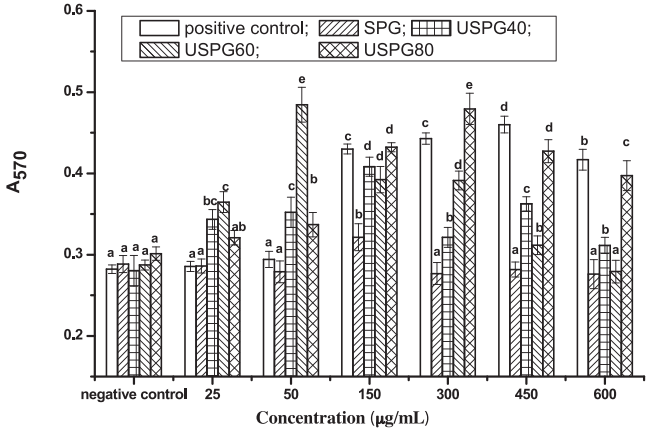

Fig. 3. Proliferation effects of SPG and USPG fractions with different concentration on splenic lymphocyte (incubation time was 24 h). [a–d]: Same sample group with different concentration, bars without the same superscripts differ significantly ($p < 0.05$).

A_{570} value of negative control increased significantly when incubation time enhanced from 12 h (0.283) to 24 h (0.380) ($p < 0.05$). No significant increase of A_{570} value was observed when incubation time was further extended to 48 h ($p > 0.05$). Similar enhancements of A_{570} values were observed in SPG and USPG fractions groups, and polysaccharide groups had higher A_{570} values than negative control group at the same incubation time ($p < 0.05$). Compared with negative group, each polysaccharide group had enhancement of A_{570} value in different degrees with varied incubation time, and highest A_{570} value was obtained at the incubation time for 24 h. Therefore, 24 h was selected as the incubation time in the followed experiments.

Fig. 3 showed the effects of polysaccharides concentrations on the splenic lymphocyte proliferation. Lymphocyte cells were incubated for 24 h with medium culture containing different concentration of polysaccharides (25–600 μg/mL). Compared with negative control group (0 μg/mL), the A_{570} values of positive control group at 150–600 μg/mL, SPG group at 150 μg/mL, USPG40 group at 25–600 μg/mL, USPG60 group at 25–450 μg/mL and USPG80 group at 50–600 μg/mL were increased significantly, respectively ($p < 0.05$). However, these A_{570} values decreased in different degree at higher polysaccharides concentration. USPG60 and USPG80 groups had higher A_{570} values than SPG and USPG40 groups at the same concentration.

3.2.3. Release of IL-2 and TNF-α level

Figs. 4 and 5 showed the release of IL-2 and TNF-α level from splenic lymphocyte in each test group. At 50–300 μg/mL, the IL-2 level of USPG60 group was higher than that of SPG and other USPG fractions groups. High concentration could stimulate the release of IL-2, and highest IL-2 level was obtained in USPG60 group at 300 μg/mL (180 pg/mL). The releases of TNF-α level of SPG and USPG fractions groups had the similar change trends, and USPG60 group had highest TNF-α level with the concentration of 300 μg/mL (166 pg/mL).

3.2.4. Antitumor activity

The antitumor activity effects of SPG and USGP fractions on T-47D cells were exhibited in Fig. 6. All test groups had great T-47D cells inhibition effects, and these inhibition rates increased in a concentration dependent manner. USPG fractions had higher T-47D cell inhibition rates than SPG, and USPG60 had highest cell inhibitions rate (about 32%) at 400 μg/mL. For positive control group, it has more inhibition rate (about 45%) than USPG fractions in the experiment concentrations.

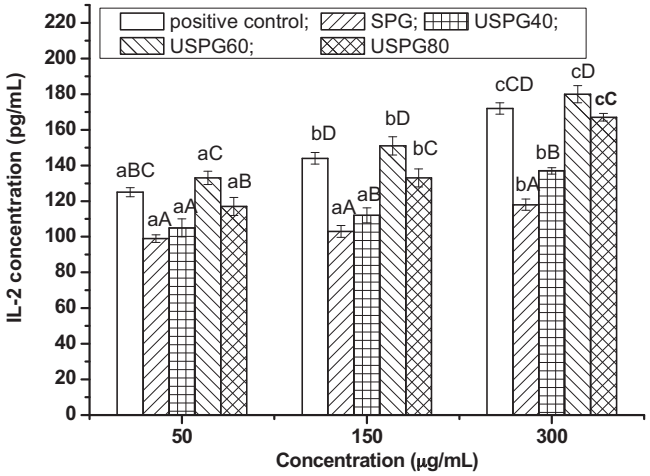

Fig. 4. Release effects of SPG and USPG fractions with different concentrations on IL-2 level in splenic lymphocyte cells. [a–c]: Same sample group with different incubation time, bars without the same superscripts differ significantly ($p < 0.05$). [A–D]: Different sample group with same incubation time, bars without the same superscripts differ significantly ($p < 0.05$).

3.3. Immunoregulatory activities of SPG and USGP fractions in vivo

3.3.1. Splenic lymphocyte proliferation

The splenic lymphocyte proliferation effects of serum samples containing SPG and USPG fractions were investigated using the MTT assay. As shown in Fig. 3, compared with the negative control group, A_{570} values of serum samples containing SPG and USPG fractions groups all increased significantly ($p < 0.05$). Serum concentration had significant effect on the A_{570} values, and highest A_{570} value of each polysaccharide group was achieved at the serum concentration of 20%. The A_{570} values of polysaccharides groups had no significant differences at low splenic concentration (10%), however, the differences enhanced significantly with the increasing of concentration ($p < 0.05$). USPG60 had the highest A_{570} value at the concentrations of 20% (Fig. 7).

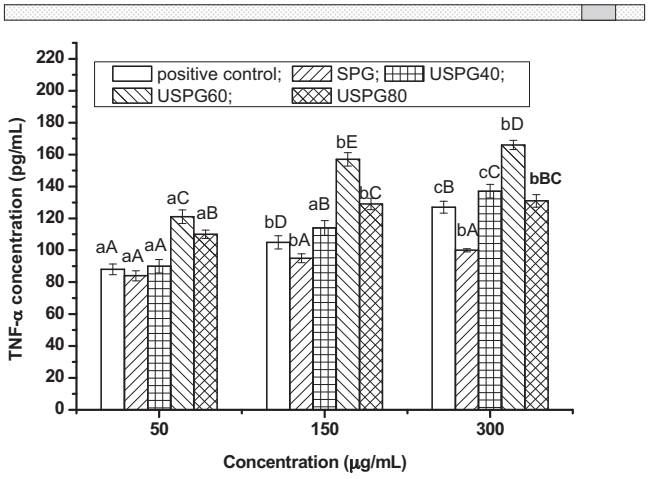

Fig. 5. Release effects of SPG and USPG fractions with different concentrations on TNF-α level in splenic lymphocyte cells. [a–c]: Same sample group with different incubation time, bars without the same superscripts differ significantly ($p < 0.05$). [A–E]: Different sample group with same incubation time, bars without the same superscripts differ significantly ($p < 0.05$).

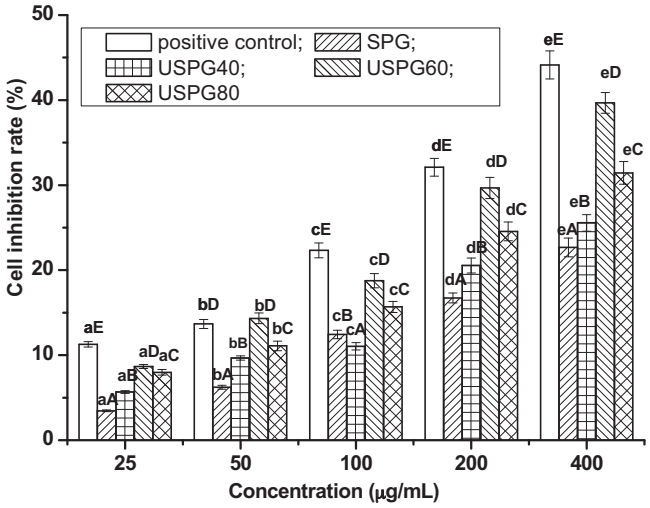

Fig. 6. Antitumor activities of SPG and USPG fractions with different concentrations. [a–e]: Same polysaccharides sample with different concentration, bars without the same superscripts differ significantly ($p < 0.05$). [A–E]: Different polysaccharides sample with same concentration, bars without the same superscripts differ significantly ($p < 0.05$).

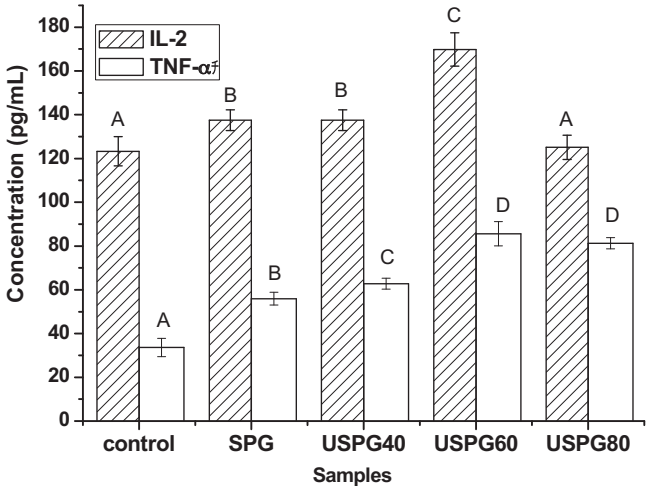

Fig. 8. Release effects of serum samples containing SPG and USPG fractions on IL-2 and TNF-α level. [A–D]: Bars without the same superscripts differ significantly ($p < 0.05$).

3.3.2. Releases of IL-2 and TNF-a level

Fig. 8 showed the levels of IL-2 and TNF-α in serum samples containing SPG and USPG fractions. The experimental groups had higher levels of IL-2 and TNF-α, which indicated that both SPG and USPG stimulated the releases of IL-2 and TNF-α level. Furthermore, USPG60 group showed the highest productions of IL-2 (169.85 pg/mL) and TNF-α (85.62 pg/mL) than SPG and other USPG groups, which further indicated the biological ability of USPG60 on promoting cytokines secretion was stronger than other fractions.

4. Discussions

As we know, ultrasonic technology could decrease molecular weight of SPG, and three USPG fractions with small molecular weights were obtained after ultrasonic treatment and various final ethanol concentrations in this study. The molecular weights of these USPG fractions were 5.78×10^6 Da (USPG40), 6.99×10^5 Da (USPG60) and 4.89×10^4 Da (USPG80), respectively (Table 1). In addition, only glucose was measured in SPG and USPG fractions by monosaccharides analysis, which was similar with previous reports [22].

Native SPG has great immunity and antitumor activity, but that of USPG fractions remain unclear. Therefore, the immunoregulatory and antitumor activity of SPG and USPG fractions were investigated to evaluate the potent application of ultrasonic technology. Because the experiment *in vitro* takes relatively short time and its conditions are easily controlled, which is suitable for initial screening of functional samples and used in this study [23,24].

Macrophages play a major role in host defense as part of the nonspecific defense (innate immunity) and specific defense (adaptive immunity) systems. Macrophages are the first cells to recognize infectious agents and are central to cell-mediated and humoral immunity. Activated macrophages increase production of NO and cytokines, which function as macrophage-mediated cytotoxicity and mediators of the immune system [25]. NO is an important intra- and inter-cellular regulatory signaling molecule that acts in many tissues to organize a diverse range of physiological process [20]. In this study, the stimulation effects of SPG and USPG fractions on NO production in macrophages RAW264.7 were examined. Compared with SPG, USPG fractions (25–400 μg/mL) stimulate significantly the NO production, and NO level in USPG60 group was higher than that in other USPG fractions groups (Fig. 1).

Lymphocytes are the important element for immunologic response in organism, and proliferation of lymphocyte is the most immediate index of reflecting organic cellular immunity [23,24,26]. The determination of the changes of lymphocytes proliferation stimulated by test sample is a great method to study the biological activity of samples. In general, A_{570} value was used as an index of lymphocytes proliferation, and more lymphocytes proliferation effects were obtained with higher A_{570} values. The lymphocyte proliferation rates (LPR) of SPG and USPG fractions with different concentrations were calculated and data were showed in Table 2. SPG had weak proliferation effect and LPR was only about 11% in a dose of 150 μg/mL. Compared with SPG, USPG fractions had more proliferation effects and presented different stimulation effects with various concentrations. The highest LPR in test groups were 45.69% (USPG40, 150 μg/mL), 68.67% (USPG60, 50 μg/mL) and 59.11% (USPG60, 300 μg/mL), respectively.

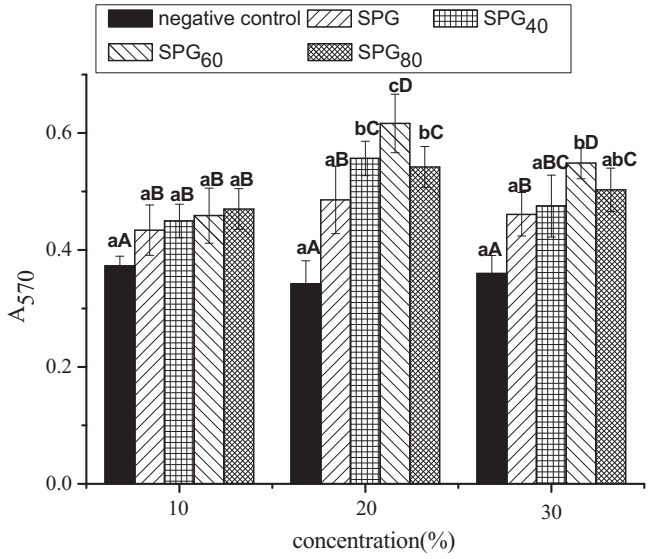

Fig. 7. Proliferation effects of serum samples containing SPG and USPG fractions on splenic lymphocyte. [a–c]: Same serum sample with different concentration, bars without the same superscripts differ significantly ($p < 0.05$). [A–D]: Different serum sample with same concentration, bars without the same superscripts differ significantly ($p < 0.05$).

Table 2
Splenic lymphocyte proliferation rates of SPG and USPG fractions (%).

Concentrations	Positive control	SPG	USPG40	USPG60	USPG80
25	–	–	22.56 ± 0.87^{bC}	26.94 ± 1.17^{cB}	6.44 ± 0.30^{aA}
50	4.22 ± 0.02^{aA}	–	25.66 ± 1.14^{cD}	68.67 ± 2.58^{dD}	11.82 ± 0.56^{bB}
150	52.41 ± 1.03^{dC}	11.42 ± 0.44^{a}	45.69 ± 2.03^{cF}	36.58 ± 1.64^{bC}	43.48 ± 1.05^{cD}
300	56.95 ± 1.14^{cD}	–	14.65 ± 0.59^{aB}	36.20 ± 1.25^{bC}	59.11 ± 0.26^{cE}
450	63.04 ± 2.05^{dE}	–	29.36 ± 1.08^{bE}	8.46 ± 0.39^{aA}	41.85 ± 1.82^{cD}
600	47.73 ± 2.13^{cB}	–	11.16 ± 0.41^{aA}	–	31.83 ± 1.47^{bC}

[a–d]: Different sample groups with the same concentrations, bars without the same superscripts differ significantly ($p < 0.05$).
[A–F]: Same sample group with the different concentrations, bars without the same superscripts differ significantly ($p < 0.05$).
–: No significant lymphocyte proliferation rate was measured compared with negative control group.

The levels of two cytokines (IL-2 and TNF-α) were measured to further investigate the immunoregulatory activities of SPG and USPG fractions. IL-2 and TNF-α are the cytokines produced by the activation and play the important role in immunomodulation. IL-2 is a critical component in activation and differentiation of T lymphocyte as well as the regulation of the immune system. It is belong to the Th1 cells and mediate cellular function. TNF-α is also a significant regulator of the inflammatory and immune response and is exceedingly important for killing and degrading tumor cells [27]. Therefore, the changes of IL-2 and TNF-α production from splenic lymphocytes can be regarded as the important symbols for immunity function. The releases of IL-2 and TNF-α level in SPG and USPG fractions groups significant increased compared with negative control, which indicated that SPG and USPG fractions had promoted significantly splenic lymphocyte's secretion of both IL-2 and TNF-α. Moreover, USPG60 showed the significant higher IL-2 and TNF-α production than other polysaccharides samples. It indicated that the ability of USPG60 on promoting cytokines secretion was stronger than other polysaccharides.

SPG and USPG fractions distinctly inhibited proliferation of T-47D cells in a dose-dependent manner in this study. USPG fractions had higher tumor cell inhibition rates than SPG at the same concentrations and presented a definite dose-efficacy relationship. USPG60 had highest cell inhibition rates.

Furthermore, the immunoregulatory activities of SPG and USPG fractions *in vivo* were investigated further in this study. The serum samples of rats treated with SPG and USPG fractions by oral gavage were collected. These serum samples containing SPG and USPG fractions enhanced significantly the splenic lymphocytes proliferation and releases of IL-2 and TNF-α level, which also showed that SPG and USPG fractions had great immunoregulatory activities *in vivo*. USPG fractions groups had higher biological activities than SPG, and USPG60 had highest lymphocytes proliferation and releases of IL-2 and TNF-α level. This result was similar with the results obtained *in vitro* measurements.

Therefore, the results of the above study showed that USPG fractions possessed the significant immunoregulatory and antitumor properties *in vitro* and *in vivo*. Similar results were also observed in previous reports, which ultrasonic technology is an effective and favorable tool to improve the bioactivities of polysaccharides [10–12]. Furthermore, USPG60 exhibited higher biological activities than native SPG and other USPG fractions. It was well documented that the molecular weight of polysaccharide had significant effect on its bioactivity. In our previous study, high molecular weight and viscosity could limit the industrial application of SPG. USPG fractions with different Mw presented various viscoelastic behaviors, higher viscosity of polysaccharide sample was obtained at bigger Mw. USPG40 and USPG60 presented typical viscoelastic behaviors while USPG80 exhibited viscous responses over the entire accessible frequency range [15]. USPG60 had moderate Mw (about 10^5 Da) and viscosity compared with other 2 USPG fractions, which was considered the reason for its higher biological activities. Other structure and conformational characteristics of these USPG fractions could be studied in the future to explain the relationships of structure and biological activity and bioactivity mechanisms of USPG.

5. Conclusion

In conclusion, *in vitro* and *in vivo*, compared with SPG, USPG fractions had higher NO production in macrophages RAW264.7, lymphocytes proliferation rates, IL-2 and TNF-α levels from spleen lymphocytes and T-47D cells inhibition rates. These results indicated that ultrasonic treatment enhanced the immunological and antitumor activities of native SPG, which was similar with other reports. In addition, USPG60 exhibited higher immunoregulatory and antitumor activity than other USPG fractions. It could also to the conclusion that use of ultrasonic treatment technology on SPG preparation is an efficient approach to get greater biological activity polysaccharides, and USPG60 might be a potential functional component for immunoregulatory and cancer treatment.

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References

[1] X. Yu, C. Zhou, H. Yang, X. Huang, H. Ma, X. Qin, J. Hu, Carbohydr. Polym. 117 (2015) 650–656.
[2] H. Ma, L. Huang, J. Jia, R. He, L. Luo, W. Zhu, Ultrason. Sonochem. 18 (2011) 419–424.
[3] M. Kuijpers, M. Kemmere, J. Keurentjes, Ultrasonics 40 (2002) 675–678.
[4] T. Poinot, K. Benyahia, A. Govin, T. Jeanmaire, P. Grosseau, Construct. Build. Mater. 47 (2013) 1046–1052.
[5] P. Pfefferkorn, J. Beister, A. Hild, H. Thielking, W.-M. Kulicke, Cellulose 10 (2003) 27–36.
[6] D. Goodwin, D. Picout, S. Ross-Murphy, S. Holland, L. Martini, M. Lawrence, Carbohydr. Polym. 83 (2011) 843–851.
[7] A.V. Mohod, P.R. Gogate, Ultrason. Sonochem. 18 (2011) 727–734.
[8] S. Baxter, S. Zivanovic, J. Weiss, Food Hydrocoll. 19 (2005) 821–830.
[9] G. Cravotto, S. Tagliapietra, B. Robaldo, M. Trotta, Ultrason. Sonochem. 12 (2005) 95–98.
[10] W. Cheng, J. Chen, D. Liu, X. Ye, F. Ke, Carbohydr. Polym. 81 (2010) 707–711.
[11] J. Xiao, Z. Liang, X. Hu, Y. Xiao, N. Fang, W. Wan, J. Immunol. 21 (2004) 51–53, 58.
[12] Z.-Y. Zhu, W. Pang, Y.-Y. Li, X.-R. Ge, L.-J. Chen, X.-C. Liu, Q. Lv, G.-L. Dong, A.-J. Liu, Y. Zhang, Carbohydr. Polym. 114 (2014) 12–20.
[13] U. Rau, Biopolymers (2002).
[14] Y. Ding, S.S. Alkan, G. Baschang, J. Defaye, Carbohydr. Res. 328 (2000) 71–76.
[15] K. Zhong, Q. Zhang, L. Tong, L. Liu, X. Zhou, S. Zhou, Ultrason. Sonochem. 23 (2015) 75–80.
[16] K. Zhong, L. Liu, L. Tong, X. Zhong, Q. Wang, S. Zhou, Int. J. Biol. Macromol. 62 (2013) 13–17.
[17] M. Dubois, K.A. Gilles, J.K. Hamilton, P. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[18] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[19] S. Zhou, X. Liu, Y. Guo, Q. Wang, D. Peng, L. Cao, Carbohydr. Polym. 81 (2010) 784–789.
[20] Y. Yao, Z. Shi, G. Ren, Int. J. Mol. Sci. 15 (2014) 19307–19318.
[21] O.S. Vishchuk, S.P. Ermakova, T.N. Zvyagintseva, Carbohydr. Res. 346 (2011) 2769–2776.

[22] N. Sutivisedsak, T.D. Leathers, K.M. Bischoff, M.S. Nunnally, S.W. Peterson, Enzyme Microb. Technol. 52 (2013) 203–210.
[23] Y. Fan, Y. Hu, D. Wang, J. Liu, J. Zhang, X. Zhao, X. Liu, C. Liu, J. Yuan, S. Ruan, Carbohydr. Polym. 88 (2012) 68–74.
[24] Y. Fan, X. Ma, J. Zhang, L. Ma, Y. Gao, W. Zhang, X. Song, W. Hou, C. Guo, D. Tong, Carbohydr. Polym. 119 (2015) 219–227.
[25] S. Moncada, R. Palmer, E. Higgs, Pharmacol. Rev. 43 (1991) 109–142.
[26] W. Pan, X. Liu, Y. Wang, Res. Spec. Local Prod. 1 (2011) 16–19.
[27] Y. Huang, C. Jiang, Y. Hu, X. Zhao, C. Shi, Y. Yu, C. Liu, Y. Tao, H. Pan, Y. Feng, Carbohydr. Polym. 96 (2013) 516–521.