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The comparison of immune-enhancing activity of sulfated polysaccharidses from *Tremella* and *Condonpsis pilosula*

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ABSTRACT

Based on our previous research, four sulfated polysaccharide (sPSs) from *Tremella* and *Condonpsis pilosula*, $sTPS_{tp}$, $sTPS_{70c}$, $sCPPS_{tp}$ and $sCPPS_{50c}$, were prepared and their effects on splenic lymphocytes proliferation in vitro and the immune response of ND vaccine in chicken were compared taking the unmodified polysaccharide (uPS) TPS_{tp} as control. The results showed that four sPSs could significantly or numerically stimulate splenic lymphocyte proliferation singly or synergistically with LPS in vitro, $sTPS_{70c}$ and $sCPPS_{tp}$ demonstrated better effect; promote peripheral lymphocytes proliferation and enhance serum HI antibody titer in chickens vaccinated with ND vaccine, the actions of sPSs were stronger than that of uPS, and $sTPS_{70c}$ at medium dosage presented the best efficacy. These indicated that sulfation modification could improve the immune-enhancing activity of TPS and CPPS, $sTPS_{70c}$ possessed the strongest activity and would be expected as a component of new-type immunopotentiator.

1. Introduction

The application of an immunopotentiator, an agent in stimulating immune response, has a very important significance in improving the immune function, and thereby enhancing resistance for infectious diseases in animal production (Deng, 2006; He & Xi, 2002). However, most of the commonly used immunopotentiators are chemically synthesized, potentially having hidden dangers in the drug residues (Liu, Wang, & Tian, 2010). As the food safety and security arouse public concern, the use of traditional herbal medicines has demonstrated some advantages (Belska et al., 2010; Yang, Zhao, Li, Wang, & Lv, 2008). Many studies have shown that polysaccharide is an important component of traditional Chinese medicine, which not only has antiviral effect, but also the immune-enhancing effect through working on the regulation of the lymphocytes, cytokines, antibody level and the neuroendocrine-immune network, consequently resulting in a widespread influence on specific immunity, non-specific immunity, cellular immunity and humoral immunity (Guo, Zhang, Yan, & Tong, 2008; Lee et al., 2003; Yuan et al., 2008).

The recent studies have shown that sulfation modification is a widely used method to enhance the biological activities of polysaccharides by reforming its structure, especially in antiviral and immune-enhancing effects (Chaidedgumjorn et al., 2002; Guo et al., 2009). Ma, Guo, Wang, Hu, and Shen (2010) reported that sulfated polysaccharides (sPSs) and their prescriptions could significantly enhance the immune response of ND vaccine in vaccinated chicken and increase the immune protective rate in challenged chickens.

In our previous research, the active site and sulfation modification of *Tremella* polysaccharide (TPS) were investigated. The results indicated that sulfation modification could significantly improve the antiviral activity of TPS (Zhao et al., 2011), and $sTPS_{tp}$ and $sTPS_{70c}$ possessed better antiviral and immune-enhancing activity. The same investigation was conducted in *Condonpsis pilosula* polysaccharide (CPPS), and $sCPPS_{tp}$ and $sCPPS_{50c}$ were picked out.

In this research, the effects of $sTPS_{tp}$, $sTPS_{70c}$, $sCPPS_{tp}$ and $sCPPS_{50c}$ on chicken splenic lymphocytes proliferation in vitro and the immune response of ND vaccine were compared taking the unmodified polysaccharide (uPS) TPS_{tp} as control. The objective of this study was to confirm whether sulfation modification could enhance the immune-enhancing activity of TPS and CPPS, and select the best sPS and its optimal dosage for developing a new-type immunopotentiator.

Abbreviations: sPS, sulfated polysaccharide; TPS, tremella polysaccharide; sTPSs, sulfated tremella polysaccharide; sCPPSs, sulfated condonpsis pilosula polysaccharide; uPS, unmodified polysaccharide; CSA, chlorsulfonic acid; Pyr, pyridine; LPS, lipopolysaccharide; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; DS, degree of sulfation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

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2. Materials and methods

2.1. Drug and vaccine

Tremella was the product of Fujian Gutian of China, standard No. GB11675-2006. *Condnopsis pilosula* was the product of Zhangjiagang Green Chinese Traditional Medicinal Electuary Co., Ltd., standard No. Y20060235. Newcastle (ND) vaccine (Lasota strain, No. 081328) was purchased from Nanjing Tianbang Bio-industry Co., Ltd.

2.2. Preparation of polysaccharide

2.2.1. Extraction and purification of polysaccharides

Four crude polysaccharide, crude total tremella polysaccharide (TPS_{tc}), crude total codonopsis pilosula ($CPPS_{tc}$), fractional tremella polysaccharide TPS_{70c} and fractional codonopsis pilosula polysaccharide $CPPS_{50c}$ were extracted by water decoction and alcohol precipitation method (Fang & Ding, 2007). $CPPS_{tc}$ was purified by removing the protein and pigment using Sevage's method (Qin, Huang, & Xu, 2002), active carbon adsorption and through Sephadex G-75 column (Zhang et al., 2005). TPS_{tc} was purified through column chromatography of DEAE-Sepharose Fast-Flow and Sephadex G-200 (Masuko et al., 2005).

The eluate was dialyzed in a dialysis sack against distilled water for 24 h and then lyophilized to get the purified $CPPS_{tp}$ and TPS_{tp}. The polysaccharide contents of TPS_{tc}, TPS_{70c}, $CPPS_{tc}$, $CPPS_{50c}$, $CPPS_{tp}$ and TPS_{tp} were 60.49%, 86.23%, 95.1%, 96.7%, 67.2% and 77.94%, respectively, measured by the phenol-sulfuric acid method (Ren & Wen, 2006).

2.2.2. Sulfation modification of polysaccharides

Four sulfated polysaccharides, $sTPS_{tp}$, $sTPS_{70c}$, $sCPPS_{tp}$ and $sCPPS_{50c}$, were prepared using chlorosulfonic acid–pyridine method (Lu, Wang, Hu, Huang, & Wang, 2008; Yang et al., 2010). In brief, TPS_{tp} and TPS_{70c} were resuspended in N,N-dimethylformamide (DMF) and added into three-necked flask filled with CAS–Pyr of 1:6 (v/v) sulfating reagent in ice bath, the mixture was stirred for 1.5 h at 80 °C, then cooled to room temperature, pH was adjusted to 7–8 with saturated NaOH solution, and 3-fold volume of dehydrated alcohol was added. The precipitation was redissolved with water, dialyzed in dialysis sack against tap water for 48 h and distilled water for 24 h in turn, and dried in vacuum freeze-drying machine (Model LGJ-25, Dongxing Machinery Industry Co. Ltd. Shamen City) to obtain $sTPS_{tp}$ and $sTPS_{70c}$. The preparations of $sCPPS_{tp}$ and $sCPPS_{50c}$ used the same method except the reaction time was 3 h.

The degrees of substitution (DS) were determined by barium chloride-gelatin assay. The DS of $sTPS_{tp}$, $sTPS_{70c}$, $sCPPS_{tp}$ and $sCPPS_{50c}$ were 1.09, 0.73, 1.27 and 1.36, respectively. Their polysaccharide contents were 32%, 48%, 29.88% and 28.98%, respectively.

For *in vitro* test, $sTPS_{tp}$, $sTPS_{70c}$, $sCPPS_{tp}$, $sCPPS_{50c}$ and unmodified TPS_{tp} were diluted into 2 mg mL^{-1} with tri-distilled water. For *in vivo* test, they were diluted into 2 mg mL^{-1}, 1 mg mL^{-1} and 0.5 mg mL^{-1} with tri-distilled water. The diluted solutions were sterilized by pasteurization and detected for content of endotoxin (less than 0.5 EU mL^{-1}) by pyrogen tests (Veterinary Pharmacopeia commission of the People's Republic of China, 2000), and stored at 4 °C until tested (Wang et al., 2006).

2.3. In vitro test (splenic lymphocyte proliferation)

Firstly the safe concentrations of four sPS_s and unmodified TPS_{tp} for chicken embryo fibroblast (CEF) were measured by the MTT assay (Liu, Yang, & Xiao, 2004). The results showed that the A_{570} values of $sCPPS_{50c}$ and TPS_{tp} at 25 μg mL^{-1}, $sCPPS_{tp}$ at 12.5 μg mL^{-1}, $sTPS_{70c}$ and $sTPS_{tp}$ at 6.25 μg mL^{-1} group were not significantly lower than those of corresponding cell control groups. Therefore these concentrations could be considered as their maximal safe concentration. In order to make the comparison at the same level, their maximal safe concentrations were supposed as 6.25 μg mL^{-1}.

Four sPSs and unmodified TPS_{tp} were dissolved with RPMI-1640 media from 6.25 μg mL^{-1} to 0.391 μg mL^{-1}. The preparation of splenic lymphocytes was performed as previously reported (Abula et al., 2011; Zhai, Li, Wang, Wang, & Hu, 2011). Briefly, spleens cells from adult cocks were counted and adjusted to 1×10^7 mL^{-1} and incubated into 96-well cultureplates, divided into two parts. One part was added with LPS (final concentration reaching to 10 μg mL^{-1}), and each concentration of polysaccharides respectively, 100 μL per well. Then in polysaccharide groups four sPS_s and unmodified TPS_{tp} at series of concentrations were added, in cell control group and LPS control group, RPMI 1640 and LPS, respectively, 100 μL per well, four wells each concentration.

The plates were incubated at 39.5 °C in a humid atmosphere of 5% CO_2 (CO_2 incubator, American Revco Company). After 44 h of the incubation period, 30 μL of MTT (5 μg mL^{-1}) was added into each well, and the plates were continued to incubate for 4 h. 100 μL of DMSO was added into each well and the plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cell in each well was measured by microliter enzyme-linked immunosorbent assay reader (DG-3022, East China Vacuum Tube Manufacturer) at a wave length of 570 nm (A_{570} value) as the index of splenic lymphocytes proliferation. In order to compare the strength of lymphocytes proliferation, the highest proliferation rate was calculated according to the equation (Wei et al., 2009; Guo et al., 2012): The highest proliferation rate = (the highest $\bar{A}_{570}$ value of polysaccharide group-$\bar{A}_{570}$ value of cell or LPS control group)/($\bar{A}_{570}$ value of cell or LPS control group) × 100%. (The $\bar{A}_{570}$ value was the average value of five concentration groups of polysaccharide or four wells of control group).

2.4. In vivo test

2.4.1. Experimental animals

One-day-old White Roman chickens (male, purchased from Tangquan Poultry Farm) were housed in wire cages (80 cm × 100 cm) in air-conditioned rooms at 37 °C with the light period of 24 h at the beginning of pretrial period. The temperature was gradually reduced to the room temperature and the light time to 12 h per day, which were kept constant in the subsequent days. The chickens were fed with the commercial starter diet provided by the feed factory of Jiangsu Academy of Agricultural Sciences.

2.4.2. Experimental design

A total of 476 White Roman chickens (male, 14-day-old) with 3.1 log 2 of the average ND-HI titer of maternal antibody, were randomly assigned into seventeen groups. The chickens except blank control (BC) group were vaccinated with ND vaccines by nose- and eye-dropping method, the repeated vaccination was at 28 days old. At the same time of the first vaccination, the chickens in fifteen polysaccharide groups were intramuscularly injected respectively with $sTPS_{tp}$, $sTPS_{70c}$, $sCPPS_{tp}$, $sCPPS_{50c}$ and TPS_{tp} at high (2 mg mL^{-1}), medium (1 mg mL^{-1}) and low (0.5 mg mL^{-1}) dosage, in the non-adjuvant (NA) and BC group, with 0.5 mL of physiological saline, once a day for three successive days.

On days 7 (D_7), 14 (D_{14}), 21 (D_{21}) and 28 (D_{28}) after the first vaccination, the blood samples (2 mL per chicken) of four chickens randomly from each group were collected from heart

Table 1
The splenic lymphocyte proliferation of every group in single stimulation of polysaccharide (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sTPS_{tp}$	$sTPS_{70c}$	$sCPPS_{tp}$	$sCPPS_{50c}$	TPS_{tp}
6.25	0.397 ± 0.014^{abc}	0.320 ± 0.027^{a}	0.368 ± 0.010^{ab}	0.407 ± 0.019^{a}	0.123 ± 0.002^{c}
3.125	0.428 ± 0.002^{a}	0.268 ± 0.015^{bc}	0.393 ± 0.005^{a}	0.413 ± 0.018^{a}	0.144 ± 0.003^{a}
1.563	0.340 ± 0.012^{bcd}	0.286 ± 0.018^{ab}	0.381 ± 0.009^{ab}	0.400 ± 0.005^{ab}	0.138 ± 0.002^{ab}
0.781	0.408 ± 0.008^{ab}	0.307 ± 0.016^{ab}	0.354 ± 0.014^{b}	0.379 ± 0.013^{ab}	0.132 ± 0.003^{bc}
0.391	0.358 ± 0.015^{abcd}	0.228 ± 0.009^{c}	0.311 ± 0.017^{c}	0.370 ± 0.011^{ab}	0.134 ± 0.001^{b}
Cell control	0.297 ± 0.028^{d}	0.219 ± 0.008^{c}	0.252 ± 0.001^{d}	0.297 ± 0.028^{c}	0.130 ± 0.003^{bc}

[a–d] Data within a column without the same superscripts differ significantly ($p < 0.05$).

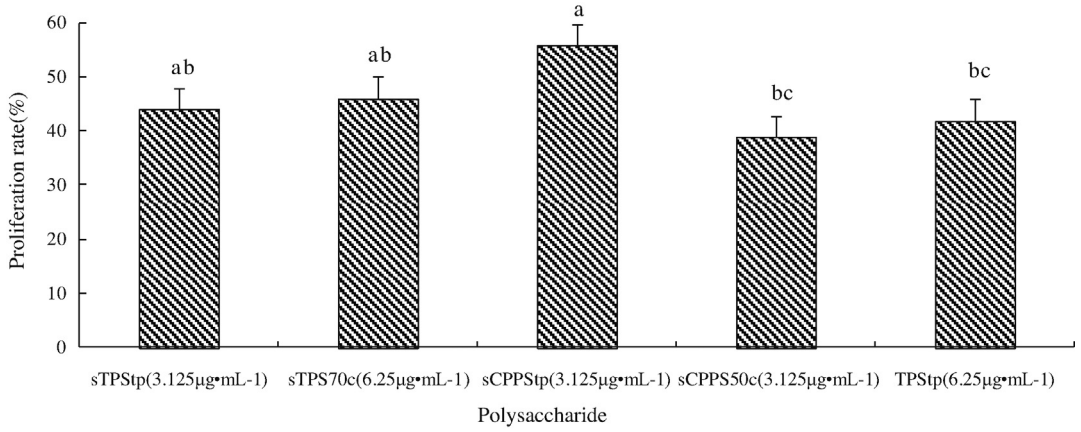

Fig. 1. The highest lymphocyte proliferation rates of every group in single stimulation of polysaccharide. [a–c]Bars without the same superscripts differ significantly ($p < 0.05$).

for determination of peripheral lymphocytes proliferation by MTT assay, six chickens randomly from each group were sampled for examination of serum hemagglutination inhibition (HI) antibody titer by micro-method (Thekisoe, Mbati, & Bisschop, 2004; Wang et al., 2010).

2.4.3. Assay of peripheral lymphocyte proliferation

The method was as described by Guo et al. (2009) and Ma et al. (2010). The absorbance of cell in each well was measured by microliter enzyme-linked immunosorbent assay reader at a wave length of 570 nm (A_{570} value) as the index of peripheral blood lymphocytes proliferation.

2.4.4. Serum HI antibody

The methods for determination of 4 HA units and serum HI antibody were performed as previously reported (Kong, Hu, Rui, Wang, & Li, 2004; The Luong Nguyen et al., 2012).

2.5. Statistical analysis

Data are expressed as means ± SE. Duncan's multiple range test was used to determine the difference among groups. Significant differences were considered at $P < 0.05$.

3. Results

3.1. The splenic lymphocyte proliferation in single stimulation of polysaccharide

The A_{570} values of each group are listed in Table 1. The A_{570} values of $sTPS_{tp}$ at 3.125–6.25 and 0.781 $\mu g\,mL^{-1}$, $sTPS_{70c}$ at 6.25 and 0.781–1.563 $\mu g\,mL^{-1}$, $sCPPS_{tp}$ and $sCPPS_{50c}$ at 0.391–6.25 $\mu g\,mL^{-1}$ and TPS_{tp} at 3.125 $\mu g\,mL^{-1}$ groups were significantly higher than those of corresponding cell control group ($P < 0.05$).

The highest lymphocyte proliferation rates are illustrated in Fig. 1. During the single stimulation, the proliferation rate in $sCPPS_{tp}$ at 3.125 $\mu g\,mL^{-1}$ group was the highest (55.95%) and significantly higher than that in unmodified TPS_{tp} and $sCPPS_{50c}$ group ($P < 0.05$), The next was $sTPS_{70c}$ at 6.25 $\mu g\,mL^{-1}$ group (46.12%) being higher than those in $sTPS_{tp}$, $sCPPS_{50c}$ and TPS_{tp} groups ($P > 0.05$).

3.2. The splenic lymphocyte proliferation in synergistical stimulation of polysaccharide with LPS

The A_{570} values of each group are listed in Table 2. The A_{570} values of $sTPS_{tp}$, $sCPPS_{50c}$ and TPS_{tp} at 6.25 $\mu g\,mL^{-1}$, $sTPS_{70c}$ at 6.25 and 0.781 $\mu g\,mL^{-1}$ and $sCPPS_{tp}$ at 3.125–6.25 $\mu g\,mL^{-1}$ groups were

Table 2
The splenic lymphocyte proliferation of every group in synergistically stimulation of polysaccharide with LPS (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sTPS_{tp}$	$sTPS_{70c}$	$sCPPS_{tp}$	$sCPPS_{50c}$	TPS_{tp}
6.25	0.404 ± 0.011^{a}	0.307 ± 0.016^{ab}	0.385 ± 0.024^{a}	0.416 ± 0.012^{a}	0.262 ± 0.005^{a}
3.125	0.372 ± 0.020^{ab}	0.278 ± 0.012^{bc}	0.363 ± 0.014^{ab}	0.390 ± 0.026^{ab}	0.265 ± 0.003^{ab}
1.563	0.357 ± 0.014^{abc}	0.276 ± 0.013^{bc}	0.357 ± 0.007^{abc}	0.376 ± 0.023^{abc}	0.285 ± 0.006^{ab}
0.781	0.354 ± 0.011^{abc}	0.341 ± 0.007^{a}	0.332 ± 0.017^{abc}	0.378 ± 0.010^{abc}	0.285 ± 0.010^{b}
0.391	0.321 ± 0.006^{bc}	0.231 ± 0.011^{de}	0.328 ± 0.028^{bc}	0.341 ± 0.027^{abc}	0.275 ± 0.007^{b}
LPS control	0.332 ± 0.007^{bc}	0.258 ± 0.010^{cd}	0.304 ± 0.005^{c}	0.332 ± 0.007^{bc}	0.258 ± 0.010^{b}

[a–e] Data within a column without the same superscripts differ significantly ($p < 0.05$).

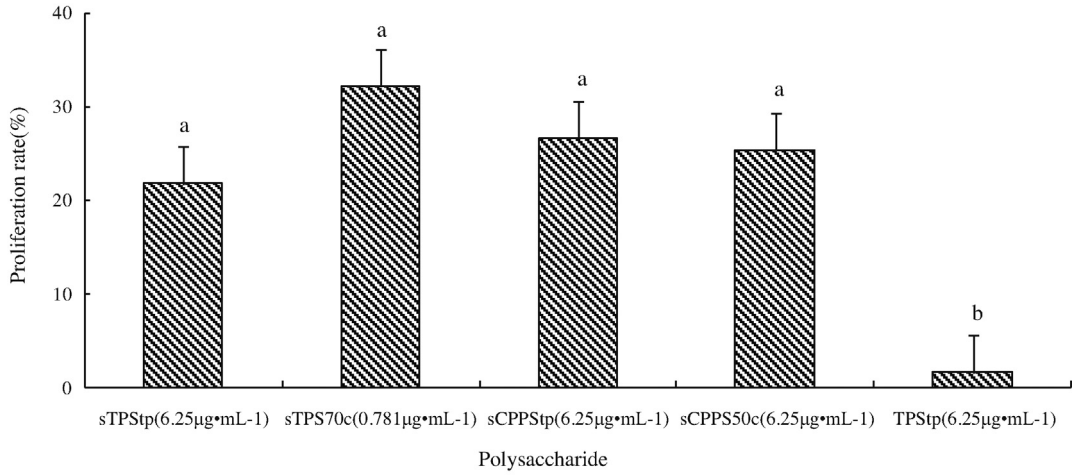

Fig. 2. The highest splenic lymphocyte proliferation rates of every group in synergistically stimulation of polysaccharide with LPS. [a–b] Bars without the same superscripts differ significantly ($p < 0.05$).

significantly higher than those of corresponding cell control group ($P < 0.05$).

The highest lymphocyte proliferation rates are illustrated in Fig. 2. The proliferation rates in $sTPS_{70c}$ at 0.781 μg mL^{-1}, $sCPPS_{tp}$, $sCPPS_{50c}$ and $sTPS_{tp}$, at 6.25 μg mL^{-1} groups were significantly higher than that of unmodified TPS_{tp} group ($P < 0.05$). The lymphocyte proliferation rate of $sTPS_{70c}$ was the highest (32.17%), the next were $sCPPS_{tp}$ (26.64%) and $sCPPS_{50c}$ (25.3%).

3.3. The changes of peripheral blood lymphocyte proliferation in vivo test

Changes of lymphocyte proliferations of each group are listed in Table 3. The A_{570} values in all sPSs groups at all time points after the first vaccination were higher than those in corresponding NA group, in $sCPPS_{tpM}$, $sCPPS_{50cM}$ and $sTPS_{tpM}$ groups at four time points, $sCPPS_{50cL}$ group on D_7–D_{21}, $sCPPS_{tpH}$ group on D_7 and D_{21}, $sTPS_{tpL}$ group on D_{14} and D_{28}, $sTPS_{70cH}$, $sTPS_{70cM}$ and $sTPS_{70cL}$ group on D_{21} and D_{28}, $sCPPS_{tpL}$ and $sCPPS_{50cH}$ group on D_{14} were significantly higher than those in corresponding NA group ($P < 0.05$).

The A_{570} values in $sCPPS_{tpM}$ on D_7, $sCPPS_{50cM}$ on D_{14} and $sTPS_{70cM}$ groups on D_{21} and D_{28} were the highest. At the first three points they were significantly higher than those in TPS_{tp} at high, middle and low dosage groups, on D_{28} in $sTPS_{70cM}$ group were significantly higher than that in TPS_{tp} at high dosage group ($P < 0.05$).

3.4. The changes of serum antibody titer

The antibody titers of each group are listed in Table 4. At all time points after the first vaccination, the serum antibody titers in all sPS_s groups were higher than those in corresponding NA group, in $sTPS_{70cM}$ group at four time points, $sCPPS_{tpM}$ and $sTPS_{tpM}$ groups on D_7 and D_{14}, $sTPS_{tpL}$ group on D_{14} and D_{21} were significantly higher than those in corresponding NA group ($P < 0.05$).

The serum antibody titers in $sTPS_{70cM}$ group on D_7, D_{21} and D_{28} and $sTPS_{70cH}$ group on D_{14} were the highest, and $sTPS_{70cM}$ at four time points were significantly higher than those in TPS_{tp} at high, middle and low dosage groups ($P < 0.05$).

4. Discussion

Polysaccharide could promote the proliferation and differentiation of immune cells, and stimulate the number of lymphocytes especially T and B lymphocytes, and enhance the cellular immune function and humoral immune function of animal (Fu, 2003). The lymphocyte proliferation is the most direct index of the cellular immunity (Li, Santoso, & Lo, 2007). The results of *in vitro* test showed that during single stimulation, the A_{570} values of four sPSs

Table 3
The lymphocyte proliferation of every group in test in *vivo* (A_{570} value).

Groups	D_7	D_{14}	D_{21}	D_{28}
$sCPPS_{tpH}$	0.285 ± 0.023^{a}	0.232 ± 0.011^{cdef}	0.277 ± 0.003^{abc}	0.255 ± 0.007^{abcde}
$sCPPS_{tpM}$	0.287 ± 0.009^{a}	0.287 ± 0.011^{a}	0.291 ± 0.006^{abc}	0.275 ± 0.010^{abc}
$sCPPS_{tpL}$	0.254 ± 0.024^{abcde}	0.281 ± 0.002^{ab}	0.249 ± 0.005^{cd}	0.227 ± 0.008^{bcde}
$sCPPS_{50cH}$	0.258 ± 0.003^{abcd}	0.253 ± 0.009^{bcd}	0.250 ± 0.009^{cd}	0.271 ± 0.023^{abcd}
$sCPPS_{50cM}$	0.280 ± 0.009^{a}	0.303 ± 0.011^{a}	0.292 ± 0.019^{abc}	0.279 ± 0.024^{ab}
$sCPPS_{50cL}$	0.274 ± 0.016^{a}	0.246 ± 0.033^{cd}	0.280 ± 0.013^{abc}	0.261 ± 0.029^{abcd}
$sTPS_{tpH}$	0.257 ± 0.016^{abcde}	0.220 ± 0.007^{defg}	0.221 ± 0.001^{de}	0.255 ± 0.012^{abcde}
$sTPS_{tpM}$	0.273 ± 0.001^{a}	0.256 ± 0.008^{bc}	0.297 ± 0.001^{ab}	0.277 ± 0.014^{ab}
$sTPS_{tpL}$	0.264 ± 0.012^{abc}	0.279 ± 0.007^{ab}	0.251 ± 0.030^{bcd}	0.273 ± 0.008^{abc}
$sTPS_{70cH}$	0.245 ± 0.002^{abcde}	0.242 ± 0.002^{cde}	0.297 ± 0.002^{ab}	0.273 ± 0.006^{abc}
$sTPS_{70cM}$	0.267 ± 0.026^{ab}	0.239 ± 0.009^{cde}	0.313 ± 0.013^{a}	0.319 ± 0.045^{a}
$sTPS_{70cL}$	0.267 ± 0.005^{ab}	0.227 ± 0.001^{cdefg}	0.290 ± 0.007^{abc}	0.291 ± 0.011^{ab}
TPS_{tpH}	0.208 ± 0.031^{de}	0.195 ± 0.005^{gh}	0.251 ± 0.001^{bcd}	0.211 ± 0.008^{cde}
TPS_{tpM}	0.218 ± 0.023^{bcde}	0.201 ± 0.000^{fgh}	0.255 ± 0.004^{bcd}	0.265 ± 0.002^{abcd}
TPS_{tpL}	0.211 ± 0.006^{cde}	0.229 ± 0.015^{cdef}	0.256 ± 0.030^{bcd}	0.266 ± 0.017^{abcd}
NA	0.214 ± 0.005^{bcde}	0.213 ± 0.003^{efg}	0.220 ± 0.010^{de}	0.207 ± 0.006^{de}
BC	0.204 ± 0.008^{e}	0.171 ± 0.002^{h}	0.193 ± 0.002^{e}	0.196 ± 0.008^{e}

[a–h] Data within a column without the same superscripts differ significantly ($p < 0.05$).

Table 4
The variation of HI antibody titer of every group in test in *vivo* (Log_2).

Groups	D_7	D_{14}	D_{21}	D_{28}
$sCPPS_{tpH}$	4.5 ± 0.50^{def}	5.5 ± 0.34^{cde}	6.67 ± 0.49^{abcd}	6.67 ± 0.56^{ab}
$sCPPS_{tpM}$	5.75 ± 0.25^{ab}	6.25 ± 0.25^{bcd}	6.25 ± 025^{bcd}	7.20 ± 0.37^{ab}
$sCPPS_{tpL}$	4.2 ± 0.20^{ef}	5.83 ± 0.17^{bcde}	6.20 ± 0.73^{bcd}	6.80 ± 0.20^{ab}
$sCPPS_{50cH}$	4.2 ± 0.20^{ef}	6.33 ± 0.33^{abcd}	5.67 ± 0.33^{d}	6.60 ± 0.40^{b}
$sCPPS_{50cM}$	5.4 ± 0.24^{bcd}	5.60 ± 0.24^{cde}	7.33 ± 0.33^{abc}	6.40 ± 0.24^{b}
$sCPPS_{50cL}$	4.25 ± 0.25^{ef}	6.67 ± 0.33^{abc}	7.5 ± 0.34^{ab}	6.60 ± 0.40^{b}
$sTPS_{tpH}$	5.2 ± 0.20^{bcde}	5.00 ± 0.41^{e}	7.5 ± 0.29^{ab}	7.20 ± 0.37^{ab}
$sTPS_{tpM}$	5.5 ± 0.29^{bc}	6.60 ± 0.24^{abc}	7.5 ± 0.50^{ab}	7.33 ± 0.21^{ab}
$sTPS_{tpL}$	4.33 ± 0.33^{ef}	6.20 ± 0.37^{bcd}	7.67 ± 0.21^{a}	7.33 ± 0.61^{ab}
$sTPS_{70cH}$	3.8 ± 0.58^{f}	7.40 ± 0.40^{a}	6.17 ± 0.17^{bcd}	7.00 ± 0.58^{ab}
$sTPS_{70cM}$	6.5 ± 0.29^{a}	6.83 ± 0.17^{ab}	7.80 ± 0.20^{a}	8.00 ± 0.00^{a}
$sTPS_{70cL}$	4.25 ± 0.25^{ef}	6.67 ± 0.42^{abc}	6.83 ± 0.40^{abcd}	7.00 ± 0.00^{ab}
TPS_{tpH}	4.33 ± 0.21^{ef}	4.83 ± 0.40^{e}	6.00 ± 0.71^{cd}	6.00 ± 0.45^{b}
TPS_{tpM}	4.00 ± 0.26^{f}	5.40 ± 0.51^{de}	6.25 ± 0.25^{bcd}	6.60 ± 0.40^{b}
TPS_{tpL}	4.75 ± 0.25^{cdef}	5.33 ± 0.49^{de}	6.33 ± 0.33^{bcd}	6.50 ± 0.29^{b}
NA	4.00 ± 0.37^{f}	5.60 ± 0.24^{efg}	6.17 ± 0.31^{bcd}	6.17 ± 0.31^{b}
BC	2.60 ± 0.24^{g}	2.50 ± 0.22^{h}	2.17 ± 0.17^{e}	1.67 ± 0.21^{c}

[a–h] Data within a column without the same superscripts differ significantly ($p < 0.05$).

at 3–5 concentration groups and TPS_{tp} only at 3.125 μg mL^{-1} group were significantly higher than those of corresponding cell control group, the proliferation rate of $sCPPS_{tp}$ at 3.125 μg mL^{-1} group was the highest, the second was $sTPS_{70c}$ group, both were significantly or numerically higher than that of unmodified TPS_{tp} group. During synergistic stimulation with LPS, the A_{570} values of five polysaccharides at 6.25 μg mL^{-1} group, $sTPS_{70c}$ at 0.781 μg mL^{-1} group, $sCPPS_{tp}$ at 3.125 μg mL^{-1} group were significantly higher than those of corresponding LPS control group and the lymphocyte proliferation rate of $sTPS_{70c}$ at 0.781 μg mL^{-1} group was the highest, the next were other three sPSs groups and four sPSs groups were significantly higher than that of unmodified TPS_{tp} group. This confirmed that the activities of $sCPPS_{tp}$ and $sTPS_{70c}$ were significantly stronger than that of unmodified TPS_{tp} in enhancing cellular immunity singly or synergistically with LPS, and sulfation modification could improve the immune-enhancing activity of CPPS or TPS.

During single stimulation of polysaccharides, TPS_{tp} and $sTPS_{70c}$ groups at the maximum concentration (6.25 μg mL^{-1}), $sCPPS_{tp}$ $sTPS_{tp}$, and $sCPPS_{50c}$ groups at the higher concentration (3.125 μg mL^{-1}) presented highest lymphocyte proliferation effect and the effect decreased along with the decrease of concentration. This indicated that the activities of sulfated polysaccharide were related to the dose at a certain extent. Chen et al. (2007) reported that along with increase of concentration, the effect of sulfated *Bletilla striata* polysaccharide on B lymphocyte proliferation was first increased, then descreased and the highest lymphocyte proliferation rate was at 100 mg L^{-1}. Yang, Jia, Shang, Mei, and Zhao (2001) reported that *astragalus* polysaccharide and their modifiers possessed direct promoting effects on active splenocyte proliferation, which had good relationship with dose. These were in accordance with our results.

Under normal physiological conditions, the number of T-lymphocytes in the blood of animals and the antibody level produced by activated B lymphocytes are important indicators to evaluate the cellular immunity and humoral immunity (Li, 2007). The level of the antibody titer reflects the humoral immunity of organism directly. The experimental results demonstrated that the antibody titers in $sTPS_{70cM}$ group at all time points after vaccination, $sCPPS_{tpM}$ group on D_7, $sCPPS_{50cL}$, $sTPS_{tpM}$, $sTPS_{70cH}$, $sTPS_{70cL}$ on D_{14} and $sTPS_{tpL}$ group on D_{21} were significantly higher than that of TPS_{tp} at high, middle and low dosage group, which implied that the actions of sPSs were stronger than that of uPSs in enhancing the humoral immunity response of ND vaccine, and the antibody titers in $sTPS_{70cM}$ group appeared early, rose quickly and sustained the higher level for longer time. This confirmed that $sTPS_{70cM}$ had significantly enhanced the humoral immunity in chickens, and could improve the immune efficacy of vaccine.

In five polysaccharides groups, the total number of antibody titers which were significantly higher than that of NA group at four time points were arranged as $sTPS_{70cM}$ (4) > $sCPPS_{tpM}$, $sTPS_{tpM}$ and $sTPS_{tpL}$ (2) > $sCPPS_{50cM}$, $sTPS_{tpM}$, $sCPPS_{50cH}$, $sCPPS_{50cL}$, $sTPS_{70cH}$ and $sTPS_{70cL}$ (1). In $sTPS_{70cM}$ group the average antibody titer of peak value at four time points was 7.28 and higher than TPS_{tp} group, this also confirmed that the action of modified polysaccharides was stronger than the unmodified polysaccharide. Huang et al. (2008) reported that the immune-enhancing efficacy of sulfated APS was much better than that of APS, and $sAPS_{60}$ could significantly enhance antibody titer in chicken vaccinated with ND vaccine. The Luong Nguyen et al. (2012) also confirmed that sulfation modification could enhance the immune-enhancing activity of *Auricularia auricula* polysaccharide and their sulfated modifiers could enhance the humoral immunity response of ND vaccine. These were in accordance to our results.

Cellular immunity mediated by T-lymphocyte is an important immunologic reaction (Lei et al., 2006). The experimental results demonstrated that the A_{570} values in $sCPPS_{tpM}$, $sCPPS_{50cM}$ groups on D_7 and D_{14} after vaccination, $sCPPS_{tpH}$, $sCPPS_{50cL}$ and $sTPS_{tpM}$ groups on D_7, $sCPPS_{tpL}$ and $sTPS_{tpL}$ group on D_{14} and $sTPS_{70cM}$ group on D_{21} were significantly higher than that of TPS_{tp} at high, middle and low dosage group, which implied that the actions of sPSs were stronger than that of uPSs in enhancing the cellular immunity response of ND vaccine.

In five polysaccharides groups the total numbers of the A_{570} values which were significantly higher than that of NA group at four time points were arranged as $sCPPS_{tpM}$, $sCPPS_{50cM}$ and $sTPS_{tpM}$ (4) > $sCPPS_{50cL}$ (3) > $sCPPS_{tpH}$, $sTPS_{tpL}$, $sCPPS_{tpM}$, $sTPS_{70cH}$ and $sTPS_{70cL}$ (2) > $sCPPS_{tpL}$ and $sTPS_{70cM}$. In $sCPPS_{50cM}$ group the average A_{570} values at four time points was higher than that in TPS_{tp} group, which also confirmed that the immune-enhancing effects of modified polysaccharides were stronger than that of unmodified polysaccharide. Abula et al. (2011) reported that fractional *Siberian solomonseal rhizome* polysaccharide $SRPS_{50}$ could significantly promote lymphocyte proliferation thus improving the immune function of chickens. Wang et al. (2010) confirmed that sulfated *lycium barbarum* polysaccharide (LBPS) could significantly promote lymphocytes proliferation and enhance serum antibody titer, which indicated that sulfation modification could enhance the immune-enhancing activity of LBPS. These were in accordance to our results.

The present study found that adjuvant activity of all polysaccharides has a certain dose-effect and time-effect relationship. In

enhancing the antibody titer, the effects of $sTPS_{70cM}$, $sCPPS_{tpM}$ and $sTPS_{tpM}$ at middle dosage were better. The antibody peak in $sTPS_{70cM}$ group was the highest, the next was $sTPS_{tpL}$. $sTPS_{70cM}$ group at four time points keep the best immune effect, and $sCPPS_{tpM}$ and $sTPS_{tpM}$ groups were better in early stage. In promoting T-lymphocyte proliferation, the effects of $sCPPS_{tpM}$, $sCPPS_{50cM}$ and $sTPS_{tpM}$ at middle dosage were better. The A_{570} peak in $sCPPS_{50cM}$ group was the highest, the next was $sTPS_{70cM}$. $sCPPS_{50cM}$ group at four time points keep the best immune effect. These results indicated that $sTPS_{70cM}$ and $sCPPS_{50cM}$ at middle dosage could significantly promote the immune efficacy of ND vaccine.

In conclusion, sulfation modification could enhance the immune-enhancing activity of CPPS and TPS, $sTPS_{70c}$ presented the strongest activity and would be expected as a component of new-type immunopotentiator.

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