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Effects of sulfated polysaccharides and their prescriptions on immune response of ND vaccine in chicken

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ABSTRACT

In order to select the sulfated polysaccharide prescription (sPSP) with the best immunoenhancement, 270 14-day-old chickens were averagely divided into nine groups and vaccinated with ND-IV vaccine. The chickens in seven experimental groups were intramuscularly injected with three sulfated polysaccharides and their four prescriptions (sPSPs). The serum antibody and peripheral lymphocyte proliferation were measured. On day 23 after vaccination, all chickens except for Negative control group were challenged with Newcastle disease virus. The morbidity, mortality and immune protective rate were calculated and their serum antibody titers were determined. The results showed that all sulfated polysaccharides and their prescriptions could obviously or significantly enhance the immune response of ND vaccine in vaccinated chicken and increase the immune protective rate in challenged chicken. sPSP1 possessed the best efficacy, stronger than its components as well, and would be expected as a candidate of new-type immune adjuvant.

1. Introduction

Although the strict preventive program has been taken in the farms, some animal infectious diseases, especially virus diseases, are still hard to control due to the genic variation of microorganisms, the occurrence of immunosuppressive diseases, or the inferior quality or improper conservation and transportation of vaccines. It is considered that the application of vaccine with immunopotentiator could improve the efficacy of vaccination. However, the common adjuvants (e.g. oil-emulsion and aluminium) ordinarily result in side effect, strong local stimulation and carcinogenesis, or failure to increase immunogenicity of weak antigen and so on (Talarico et al., 2005). Therefore, it becomes so urgent to study and develop a new-type adjuvant with high efficiency and low toxicity (Long & Wu, 1996; Ma, Xiang, & Wang, 2007).

Some Chinese herbal medicines and their ingredients, especially polysaccharides, were used in animals and chicken after or before vaccination, resulting in the decrease of the incidence of infectious diseases and the increase in animal immunity response (Hu, 1997; Wang, Hu, Kong, et al., 2005; Wang, Hu, Sun, et al., 2005). Our previous researches confirmed that the sulfated modification could enhance the adjuvant activity of astragalus polysaccharide and lentinan for ND vaccine (Guo et al., 2009; Huang, Hu, et al., 2008; Huang, Wang, et al., 2008) and the antiviral activity of epimedium polysaccharide and astragalus polysaccharide in vitro (Huang, Hu, et al., 2008; Huang, Wang, et al., 2008; Lu, Wang, Hu, Huang, & Wang, 2008) and selected out three sulfated polysaccharide, sulfated epimedium polysaccharide (sEPS), sulfated astragalus polysaccharide (sAPS) and sulfated lentinan (sLNT), with better immunoenhancement. The authors also compared the immunoenhancement of single Chinese herbal medicinal ingredients (CHMIs) with compound CHMIs (cCHMIs) and confirmed that some cCHMIs had stronger adjuvant activity than the single (Wang, Hu, Kong, et al., 2005; Wang, Hu, Sun, et al., 2005).

In present experiments, four sulfated polysaccharide prescriptions (sPSPs) were prepared with sEPS, sAPS and sLNT. The effects of four prescriptions (sPSPs) and their components on immune response of ND vaccine were compared by determination of antibody titers, peripheral lymphocyte proliferation and protective rate in chicken. The purpose of this research is to probe into whether sulfated polysaccharide (sPS) possessed synergetic immune-enhancing action and select the best sPSP and offer theoretical evidence for developing new-type immune adjuvant.

Abbreviations: sPS, sulfated polysaccharide; sPSP, sulfated polysaccharide prescription; sAPS, sulfated astragalus polysaccharide; sEPS, sulfated epimedium polysaccharide; sLNT, sulfated lentinan; CSA, chlorsulfonic acid; Pyr, pyridine; SPF, specified-pathogens free; NDV, Newcastle disease virus; DMSO, dimethyl sulfoxide; CMF, calcium and magnesium-free; PBS, phosphate-buffered saline; DS, the degree of sulfate group; HI, hemagglutination inhibition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ConA, concanavalin A.

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2. Materials and methods

2.1. Extraction and purification of polysaccharides

Three polysaccharides, APS, EPS and LNT, were prepared in our laboratory. Briefly, *Astragali Radix*, *Epimedium* and *Lentinula edodes* were decocted 3 times with distilled water and condensed into decoction corresponding 1 g materia medica mL^{-1}, respectively. Then three total polysaccharides were extracted by ethanol precipitation that is ethanol was added into above-mentioned decoction to make ethanol concentration reach to 75% (v/v). They were purified, respectively by Sevag's method (Qin, Huang, & Xu, 2002) and active carbon adsorption, then chromatography with macroporous adsorption resins D101 and ADS-7 column and sephadex column (Zhao, 1994) in turn to remove protein, pigment and other impurity. The polysaccharide contents (w/w) of APS, EPS and LNT were 44.00%, 20.59% and 91.00%, respectively.

2.2. Sulfated modification of polysaccharides

Three sulfated polysaccharides, sAPS, sEPS and sLNT, were prepared by chlorosulfonic acid–pyridine method and the modified conditions were based on our preparative experiment. sAPS was prepared as follows in brief: the mixture of chlorosulfonic acid and pyridine with a ratio of 1:6 (v/v) was prepared in ice bath. Then, APS was added and stirred for 1 h at 95 °C. The compound was dissolved in 100 mL ice-cold distilled water, cooled to room temperature, neutralized with saturated NaOH solution and precipitated with 95% ethanol. The sediment was re-dissolved with distilled water and dialyzed in dialysis sack against tap water for 48 h and distilled water for 12 h, then lyophilized to obtain sAPS. The preparations of sEPS and sLNT used the same method except the ratio of chlorosulfonic acid and pyridine being 1:8 and 1:4 and the reaction temperature was 80 °C and 60 °C, respectively. Their degrees of substitution (DS) were determined by Antonopoulos' method (Zhang, Li, & Fan, 2002). The DS of sAPS, sEPS, and sLNT were 1.545, 0.696 and 0.980, respectively.

2.3. Preparation of sulfated polysaccharide and prescriptions

Four sulfated polysaccharide prescriptions were composed. sPSP1 consisted of sEPS and sAPS, sPSP2, sEPS, sAPS and sLNT, sPSP3, sAPS and sLNT, sPSP4, sEPS and sLNT according to a certain proportion. Each sPSP and sulfated polysaccharide was diluted with deionized water (Key laboratory of Nanjing Agricultural University) into 24 mg mL^{-1} according to our previous researches (Guo et al., 2009; Huang, Hu, et al., 2008; Huang, Wang, et al., 2008). The diluted preparation was sterilized by pasteurization and detected for endotoxin by pyrogen tests (Lu et al., 2008). When the endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU mL^{-1}), they were stored at 4 °C for the test.

2.4. Reagents

Alsever's solution was obtained by dissolution of glucose 2.05 g, sodium citrate 0.80 g and sodium chloride 0.42 g in 100 mL of deionized water, regulating pH to 7.2 with 0.1% citric acid and filtering through a 0.22 μm syringe filter, stored at 4 °C. Rooster erythrocytes suspension was prepared by separating 5 mL of blood in 20 mL of Alsever's solution, washing 3 times and diluting into 1% with physiological saline. It was stored at 4 °C (valid for 1 week).

RPMI-1640 (Gibco) supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 10% fetal bovine serum, was used for washing and re-suspending the cells, diluting the mitogen and culturing the cells. Concanavalin A (ConA, Sigma), as the T-cell mitogen, was dissolved into 2.5 μg mL^{-1} with RPMI-1640. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4). These reagents were filtered through a 0.22 μm syringe filter. ConA solution was stored at −20 °C, MTT solution at 4 °C in dark bottle. Lymphocytes separation medium (Ficoll–Hypaque, ρ: 1.077 ± 0.002, No. 20001220) was the product of Hengxing Biostix Shanghai Inc. Dimethyl sulfoxide (DMSO) and dehydrated alcohol were produced by Zhengxing Institute of Chemical Engineering in Suzhou, chlrosulfonic acid and pyridin, Sinopharm Chemical Reagent Co. Ltd. Other chemical used in experiment was analytical grade.

2.5. Vaccine and virus

ND vaccine (LaSota strain, No. 081220) was offered by Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Science. ND virus (NDV, $F_{48}E_9$ strain) was supplied by China institute of Veterinary Drug Control. NDV was propagated with 10-day-old specific pathogen-free (SPF) chicken embryos and used for challenge experiment.

2.6. Animals

One-day-old White Roman chickens (male) purchased from Tangquan Poultry Farm were housed in wire cages (100 cm × 60 cm × 40 cm) in air-conditioned room at 37 °C and lighted for 24 h per day at the beginning of pretrial period. The temperature was gradually declined to the room temperature and the light time to 12 h per day, which were kept constant in the following days. Chickens were fed with the commercial starter diet provided by the Feed Factory of Jiangsu Academy of Agricultural Science.

2.7. Experimental design

Two hundred and seventy 14-day-old chickens, their average ND-HI antibody titer of maternal antibody was 3.0 $\log_2$ and the average body weight was 130 g, were randomly divided into nine groups averagely. The chickens in Groups 1–9 were vaccinated with 0.5 mL of ND-IV vaccine by intranasal and intraocular routes, repeated vaccination at 28-day-old. At the same time of the first vaccination, the chickens in seven experimental groups were intramuscularly injected, respectively with 0.5 mL of sAPS, sEPS, sLNT, sPSP1, sPSP2, sPSP3, and sPSP4 at concentration of 24 mg mL^{-1}, in Positive and Negative control groups, 0.5 mL of physiological saline. Before (D_0) and on days 7 (D_7), 14 (D_{14}) and 21 (D_{21}) after first vaccination, 6 chickens were sampled randomly from each group for determination of serum hemagglutination inhibition (HI) antibody titer by micro-method. On days D_{14} and D_{21}, 4 chickens were sampled randomly from each group for examination of peripheral lymphocyte proliferation by MTT assay. On day 23 after first vaccination, the chickens except for Negative control group were challenged with 0.2 mL of NDV at 10^5 embryo infectious dose 50 (EID_{50}) (Darrell, Kapczynski, Daniel, & King, 2005) by intramuscular injection. The clinical symptom and mortality of chickens were observed per day. On day 14 after challenge (D_{14}), the morbidity, mortality and immune protective rate in every groups were calculated according to the formula: morbidity (%) = (the number of chickens dead and showing clinical symptoms on D_{14}/the number of sample) × 100%, mortality (%) = (the number of dead chicken within 14 days after challenge/the number of sample) × 100%, immune protective rate (%) = (the number of chickens without clinical symptoms on D_{14}/the number of sample) × 100%.

Before the challenge (D_0) and D_{14}, 6 chickens were sampled randomly from each group for determination of serum HI antibody titer.

2.8. Sample collection and assay

2.8.1. Serum HI antibody assay

Blood samples (0.5 mL per chick) were drawn into Eppendorf tubes from the brachial vein of chicken and allowed to clot at 37 °C for 2 h. The serum was separated by centrifugation and stored at −20 °C for HI antibody assay. Briefly, after the serum was inactivated at 56 °C for 30 min, twofold serial dilution were made in a 96-well V-shaped bottom microtiter plate containing 50 μl of CMF-PBS in each well, then 50 μl of NDV antigen (4 HA units) was added into all the wells except the last row as the controls. Serum dilutions ranged from 1:2 to 1:2048. The plate was incubated at 37 °C for 10 min, then 50 μl of 1% rooster erythrocytes suspension was added into each well and continued to incubate for 30 min. A positive serum, a negative serum, erythrocytes, and antigens were also included as controls. The highest dilution of serum caused complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI (Thekisoe, Mbati, & Bisschop, 2004; Xu, 1998).

2.8.2. Peripheral lymphocyte proliferation assay

Blood samples (2 mL per chicken) were collected by cardiopuncture and transferred immediately into aseptic capped tubes with sodium heparin, then diluted with an equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After 20 min of centrifugation at 800 × *g*, a white cloud-like lymphocytes' band was collected and washed twice with RPMI 1640 media without fetal bovine serum. The resulting pellet was re-suspended to 2.5×10^6 mL^{-1} with RPMI 1640 media and incubated in 96-well culture plates, 80 μl per well, each sample seeded 6 wells. Then another 20 μl of ConA was added into per well. The plates were incubated at 39.5 °C for 48 h in a humid atmosphere of 5% CO_2. After 44 h of the incubation period, 20 μl of MTT (5 μg mL^{-1}) was added into each well, and the plates were re-incubated for 4 h. Then the plates were centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant was removed carefully and 100 μl of DMSO were added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance at a wave length of 570 nm (A_{570} value) of lymphocyte cells in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model DG-3022, East China Vacuum Tube Manufacturer). The mean A_{570} values were used as the indicator of peripheral T lymphocyte proliferation (Bao, 1998; Thiagarajan, Ram, & Bansal, 1992; Wang & Li, 2002).

2.9. Statistical analysis

Data expressed as the mean ± S.D. Duncan's multiple range test was used to determine the difference among groups. Differences between means were considered significant at $p < 0.05$.

3. Results

3.1. The changes of antibody titer

As seen in Table 1, on day 7 after the first vaccination, the antibody titer in sPSP1 group was the highest and significantly higher than those in other groups except for sAPS group. The antibody titers in sLNT, sEPS and sPSP2 groups were significantly higher than those in two control groups ($p < 0.05$). On day 14, the antibody titer in sPSP1 group was significantly higher than those in other groups except sAPS, and the antibody titer in sAPS was significantly higher than those of sLNT and three other sPSP groups, the antibody titer in sEPS group was significantly higher than those of sPSP2, sPSP3, sPSP4 and two control groups, and the antibody titer in all adjuvant groups except sPSP2 and sPSP4 group were significantly higher than those of two control groups ($p < 0.05$). On day 21, the antibody titers in sPSP1, sAPS and sEPS were significantly higher than those of other groups ($p < 0.05$).

Table 1
The change of ND antibody titer in every group ($\log_2$).

Group	D_0	D_7	D_{14}	D_{21}
sLNT	3.0 ± 0.6^{a}	5.7 ± 0.5^{bc}	6.2 ± 0.4^{cd}	6.3 ± 0.5^{b}
sAPS	3.0 ± 0.6^{a}	6.5 ± 0.5^{a}	7.2 ± 0.4^{ab}	7.3 ± 0.5^{a}
sEPS	3.0 ± 0.6^{a}	5.8 ± 0.4^{b}	6.7 ± 0.5^{bc}	7.0 ± 0.6^{a}
sPSP1	3.0 ± 0.6^{a}	6.7 ± 0.5^{a}	7.3 ± 0.5^{a}	7.5 ± 0.5^{a}
sPSP2	3.0 ± 0.6^{a}	5.3 ± 0.5^{bcd}	6.0 ± 0.0^{de}	6.3 ± 0.5^{b}
sPSP3	3.0 ± 0.6^{a}	5.0 ± 0.0^{de}	5.8 ± 0.5^{de}	6.0 ± 0.6^{b}
sPSP4	3.0 ± 0.6^{a}	5.2 ± 0.4^{cde}	5.7 ± 0.5^{def}	5.7 ± 0.5^{b}
Positive control	3.0 ± 0.6^{a}	4.7 ± 0.5^{e}	5.2 ± 0.4^{f}	5.8 ± 0.4^{b}
Negative control	3.0 ± 0.6^{a}	4.2 ± 0.3^{e}	5.0 ± 0.5^{f}	5.7 ± 0.5^{b}

a–f: Data within a column without the same superscripts differ significantly ($p < 0.05$).

3.2. The changes of lymphocyte proliferation

The changes of A_{570} values in every group are shown in Fig. 1. On day 14 after the first vaccination, the A_{570} values in all adjuvant groups were significantly higher than those of two control groups, and the A_{570} value in sPSP1 group was the highest and significantly higher than those in other groups, and the A_{570} value in sAPS and sEPS groups were higher than those in sLNT and other three sPSP groups ($p < 0.05$); on day 21, the A_{570} values in all adjuvant groups were significantly higher than those in two control groups, and the A_{570} values in sPSP1, sEPS and sAPS groups were significantly higher than those in sLNT and other three sPSP groups, and the A_{570} in sLNT and sPSP3 groups were higher than those in sPSP2, sPSP4 and two control groups ($p < 0.05$); the A_{570} values of four sPSP groups were significantly raised on day 14 than those on day 21 ($p < 0.05$).

3.3. Protective effects

As seen in Table 2, the morbidity in sPSP1 group was significantly lower than that in positive control group ($p < 0.05$), and in sAPS and sEPS groups was lower than those in other groups. The mortality in positive control group was the highest, and higher 23 in percentage that in sPSP1. The protective rate in sPSP1 was the highest in four sPSP groups and significantly higher than that in positive control ($p < 0.05$), and in sAPS and sEPS groups were higher than those in the rest groups.

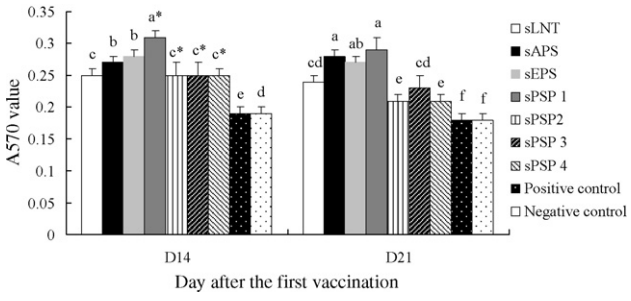

Fig. 1. The changes of lymphocyte proliferation on days 14 and 21 after the first vaccination (A_{570} value). a–f: Bars without the same superscripts differ significantly on the same day ($p < 0.05$). *In same group, the difference is significant in comparison with D_{21} ($p < 0.05$).

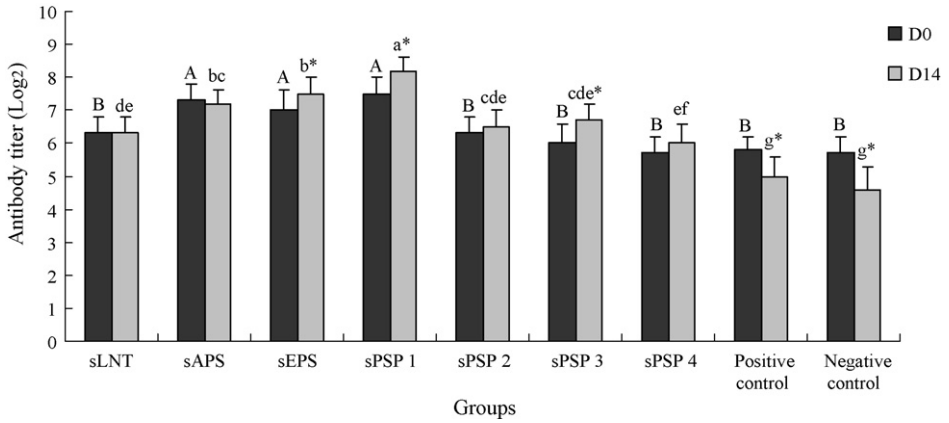

Fig. 2. The changes of antibody titer before challenge (D_0) and on day 14 after challenge (D_{14}) (log_2). A–B: Bars without the same superscripts differ significantly on D_0 ($p < 0.05$). a–g: Bars without the same superscripts differ significantly on D_{14} ($p < 0.05$). *In same group, the difference is significant in comparison with D_0 ($p < 0.05$).

3.4. The changes of antibody titer after challenge

The changes of antibody titer are shown in Fig. 2. Before challenge (D_0), the antibody titers in sPSP1, sAPS and sEPS group were significantly higher than those of other groups ($p < 0.05$); on day 14 after challenge with NDV, the antibody titer in sPSP1 group was the highest and significantly higher than those in rest groups, and in sEPS group was significantly bigger than those in other adjuvant groups except group sAPS ($p < 0.05$); the antibody titers of sEPS, sPSP1 and sPSP3 groups were significantly raised and the antibody titers of two control groups were significantly lower in comparison with before challenge ($p < 0.05$).

4. Discussion

The results of immune response test revealed that the antibody titers of sPSP1 and sAPS adjuvant groups at most time points after vaccination were significantly higher than those of other two sPS groups and three sPSP groups, and in sPSP1 group was the highest. The A_{570} values in sPSP1, sAPS and sEPS groups on days 7 and 21 were significantly larger than those in sLNT and three other sPSP groups, and in sPSP1 group was the largest. These indicated that sPSP could significantly enhance the serum antibody titer and promote the lymphocyte proliferation in chickens vaccinated ND vaccine as compared with single sulfated polysaccharide, and sPSP1 had the best effect.

The immune protection test showed that the protective rates in sPSP1, sAPS and sEPS groups were higher than those in other adjuvant groups, and in sPSP1 group was the highest, which proved that sPSP1 possessed better curative effect for ND in comparison with single sulfated polysaccharide. Besides, the antibody titers in sPSP1 group on day 14 after challenge were significantly higher than those in rest group, which indicated that sPSP1 could accelerate the antibody production in challenged chicken.

Table 2
The immune protective effects in every group.

Group	Morbidity (%)	Mortality (%)	Protective rate (%)
sLNT	30.0^{ab}	26.7^{ab}	70.0^{abc}
sAPS	20.0^{abc}	13.3^{abc}	80.0^{ab}
sEPS	20.0^{abc}	16.7^{abc}	80.0^{ab}
sPSP1	16.7^{bc}	13.3^{abc}	83.3^{a}
sPSP2	36.7^{ab}	30.0^{ab}	63.3^{abc}
sPSP3	30.0^{ab}	23.3^{ab}	70.0^{abc}
sPSP4	36.7^{ab}	30.0^{ab}	63.3^{abc}
Positive control	43.3^{a}	36.7^{a}	56.7^{bc}
Negative control	0^{bc}	0^{bc}	–

a–c: Data within a column without the same superscripts differ significantly ($p < 0.05$).

The changes of antibody titer and lymphocytes proliferation, respectively reflected the state of the humoral immunity and cellular immunity in animal organism. This research confirmed that single sulfated polysaccharide and their prescriptions could improve the humoral and cellular immunity of chickens, thus protecting chickens from attacking of NDV. The level of ND antibody titer is proportional to the livability of chickens challenged by NDV. If the antibody titer is higher, the infection degree of chicken to NDV will be less serious (Xue et al., 2005). Merz, Scheid, and Choppin (1981) also reported that both humoral and cellular immunity played important roles in the host's defense against infectious disease. Many other researches also found that sAPS and sLNT could improve humoral immunity (Guo et al., 2009; Huang, Hu, et al., 2008; Huang, Wang, et al., 2008); T lymphocyte also plays an important role in enhancing immune function of organism. It can recognize and present antigen, especially T_H cells and T_S cells can regulate the proliferation and differentiation of B cell (Adair, McNeilly, & Mconnel, 1991; Minato, Kawakami, Nomura, Tsuchida, & Mizuno, 2004). The researches confirmed that NDV vaccine strains were able to induce measurable levels of NDV specific cell-mediated immunity within the 1st week and both vaccinated groups differed ($p < 0.05$) from the unvaccinated controls (Rauwa et al., 2009, 2010). Although this research did not determine the inducing action of NDV vaccine, the results revealed that the A_{570} values in sPS and sPSP groups were significantly higher than those in two control groups singly vaccinated with NDV vaccine. This indicated that sPS and sPSP could significantly promote T lymphocytes proliferation thus enhancing chicken immune function. Many researches confirmed that Chinese herbal polysaccharides could enhance the activity of T lymphocyte, promote the excretion of some cytokines, such as interleukin-1 (IL-1), IL-2, IFN and TNF-α (Wang, Hu, Kong, et al., 2005; Wang, Hu, Sun, et al., 2005; Wang et al., 2006). Maybe, this also was also one of immune-enhancing mechanisms of sPS and sPSP.

Our previous researches found that the activity of compound Chinese herbal medicinal ingredients had stronger adjuvant action in comparison with the single (Wang et al., 2006; Yang et al., 2008). This research also confirmed that the effect of sPSP1 was better than that of sAPS and sEPS in enhancing antibody titters and promoting lymphocyte proliferation. The good efficacy of sPSP1 would be basing on synergistic effects of their components of sAPS and sEPS. But not all sPSPs are like this, such as sPSP3 and sPSP4. Maybe it is due to the occurrence of toxicity or conflict among components. therefore it should optimize the sulfated polysaccharide prescrip-

tion with better bioactivity and the lower toxicity (Lin et al., 2009; Zhao, Xu, & Xu, 2007).

This research results confirmed that four sPSPs and their three components could enhance the immune response and immune protection rate of ND vaccine in chicken, in which sPSP1 possessed the best efficacy, stronger than its components as well and it would be expected as a candidate of new-type immunologic adjuvant.

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References

Adair, B., McNeilly, F., & Mconnel, C. (1991). Effects of chicken anemia on lymphokine production and lymphocyte transformation in experimentally infected chickens. *Avian Disease*, *35*, 783–792.

Bao, E. D. (1998). Effect of Se-V_E compound on nature killer (NK) cell activities and lymphocyte transformation rate of chicken. *Journal of Nanjing Agricultural University*, *3*, 89–93.

Darrell, R., Kapczynski, Daniel, J., & King. (2005). Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine*, *23*, 3424–3433.

Guo, Z., Hu, Y., Wang, D., Ma, X., Zhao, X., Zhao, B., et al. (2009). Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. *Vaccine*, *27*, 660–665.

Hu, Y. (1997). Progress in the study of immunopharmacology of Chinese herbal medicine. *Chinese Journal of Immunology*, *13*, 96–98.

Huang, X., Hu, Y., Zhao, X., Yu, L., Wang, J., Zhang, F., et al. (2008). Sulfated modification can enhance the adjuvanticity activity of astragalus polysaccharide for ND vaccine. *Carbohydrate Polymers*, *73*, 303–308.

Huang, X., Wang, D., Hu, Y., Lu, Y., Guo, Z., Kong, X., et al. (2008). Effect of sulfated astragalus polysaccharide on cellular infectivity of infectious bursal disease virus. *International Journal of Biological Macromolecules*, *3*, 166–171.

Lin, L., Liu, J., Zhang, Y., Duan, C., Lin, C., & Fu, J. (2009). Evaluation of different combinations of components of Chinese formulation Shuangshentongguan by using AUC values. *Chinese Acta Pharmaceutica Sinica*, *44*, 1029–1033.

Long, X., & Wu, Y. (1996). The actuality and current in research of immune adjuvant. *Chinese Journal of Veterinary Drug*, *1*, 41–43.

Lu, Y., Wang, D., Hu, Y., Huang, X., & Wang, J. (2008). Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV. *Carbohydrate Polymers*, *71*, 180–186.

Ma, J., Xiang, S., & Wang, Z. (2007). The research and development of immunity actuality of Chinaese Herbal Medicine. *Chinese Journal of Veterinary Drug*, *10*, 44–46.

Merz, D., Scheid, A., & Choppin, P. (1981). Immunological studies of the functions of paramyxovirus glycoprotein. *Virology*, *28*, 208–221.

Minato, K., Kawakami, S., Nomura, K., Tsuchida, H., & Mizuno, M. (2004). An exo 1,3-glucanase synthesized de novo degrades lentinan during storage of *Lentinule edodes* and diminishes immunomodulating activity of the mushroom. *Carbohydrate Polymers*, *56*, 279–286.

Qin, C., Huang, K., & Xu, H. (2002). Isolation and characterization of a novel polysaccharide from the mucus of the loach, Misgurnus anguillicaudatus. *Carbohydrate Polymers*, *49*, 367–371.

Rauwa, F., Gardinb, Y., Palyac, V., Borma, S., Gonzea, M., Lemairea, S., et al. (2009). Humoral, cell-mediated and mucosal immunity induced by oculonasal vaccination of one-day-old SPF and conventional layer chicks with two different live Newcastle disease vaccines. *Vaccine*, *27*, 3631–3642.

Rauwa, F., Gardinb, Y., Palyac, V., Anbaria, S., Lemairea, S., Boschmansa, M., et al. (2010). Improved vaccination against Newcastle disease by an in ovo recombinant HVT-ND combined with an adjuvanted live vaccine at day-old. *Vaccine*, *28*, 823–833.

Talarico, L., Pujol, C., Zibetti, R., Faria, P., Noseda, M., Duarte, M., et al. (2005). The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell. *Antiviral Research*, *66*, 103–110.

Thekisoe, M. O., Mbati, P. A., & Bisschop, S. P. R. (2004). Different approaches to the vaccination of free ranging village chickens against Newcastle disease in Qwa-Qwa, South Africa. *Veterinary Microbiology*, *101*, 23–30.

Thiagarajan, D., Ram, G. C., & Bansal, M. P. (1992). Optimum conditions for in vitro chicken IL-2 production and its in vivo role in Newcastle disease vaccinated chickens. *Veterinary Immunology and Immunopathology*, *36*, 945–955.

Wang, D., Hu, Y., Kong, X., Sun, J., Zhang, B., & Liu, J. (2005). Effects of Chinese herbal medicine ingredients on proliferation of splenic lymphocyte cultured in chichen. *Acta Veterinaria Et Zootetechnica Sinica*, *11*, 1210–1214.

Wang, D., Hu, Y., Sun, J., Kong, X., Zhang, B., & Liu, J. (2005). Comparative study on adjuvanticity of compound Chinese herbal medicinal ingredients. *Vaccine*, *23*, 3704–3708.

Wang, D., Li, X., Xu, L., Hu, Y., Zhang, B., & Liu, J. (2006). Immunologic synergism with IL-2 and effects of cCHMIs on mRNA expression of IL-2 and IFN-γ in Chicken peripheral T lymphocyte. *Vaccine*, *24*, 7109–7114.

Wang, Y., & Li, X. (2002). Effect of astragaloside IV on T, B lymphocyte proliferation and peritoneal macrophage function in mice. *Acta Pharmaceutica Sinica*, *3*, 263–266.

Xu, W. (Ed.). (1998). *Veterinary virology*. (pp. 222–225). Beijing: Chinese Agricultural Press.

Xue, F., Wu, S., Tang, Y., Zhang, P., Ding, H., Gao, S., et al. (2005). The relative experiment on antibody level of ND and AI and immune protect of chicken. *Chinese Journal of Veterinary Medicine*, *9*, 23–25.

Yang, L., Hu, Y., Xue, J., Wang, F., Wang, D., Kong, X., et al. (2008). Compound Chinese herbal medicinal ingredients can enhance immune response and efficacy of RHD vaccine in rabbit. *Vaccine*, *26*, 4451–4455.

Zhang, H., Li, B., & Fan, J. (2002). Study on gravimetric analysis of hydrochloric acid hydrolysis and barium sulfate precipitation in sulfated polysaccharides. *Food Science*, *23*, 107–111.

Zhao, F., Xu, L., & Xu, L. (2007). Trains of thoughts and methods in studying substantial basis effects of Chinese medicinal compounds. *Chinese Journal of Integrative Medicine*, *27*, 80–82.

Zhao, Y. (Ed.). (1994). *Theory and application of biochemistry technology*. (pp. 40–189). Wuhan: Wuhan University Press.