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# Adjuvanticity of compound astragalus polysaccharide and sulfated epimedium polysaccharide per os

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ABSTRACT

On the basis of previous researches, compound astragalus polysaccharide (APS) and sulfated epimedium polysaccharide (sEPS) oral liquid (AEO) was prepared. Three hundred and twenty 14-day-old chickens were randomly assigned into eight groups and vaccinated with ND vaccine except for blank control (BC) group, repeated vaccination at 28 days old. At the same time of each vaccination, the chickens in three experimental groups were taken orally with AEO, respectively, at three doses, in two component control groups with APS and sEPS, once a day for three successive days; in injection control group were injected with AEI once, and in vaccination control (VC) and BC groups were not administrated. On days 7, 14, 21, 28 and 35 after the first vaccination, peripheral lymphocyte proliferation, the serum antibody titer, IFN-γ and IL-2 concentrations and on day 35 immune organ index were measured. The results showed that AEO at high and medium doses could significantly promote lymphocyte proliferation and development of immune organ, enhance antibody titer and IFN-γ and IL-2 concentration, which was stronger than actions of AEI and two components. The results confirmed that AEO possessed reliable immunoenhancement and could be exploited into an oral immunopotentiator.



## 1. Introduction

Animal infectious diseases, especially virus diseases, such as Newcastle disease (ND), avian influenza (AI) and infectious bronchitis (IB) and so on, are worldwide concerned as they are highly contagious and are responsible for huge economic losses to domestic animal and poultry production [1]. Up to now, there are not effective drugs to treat these viral infectious diseases, therefore vaccination remains the most effective biomedical approach for the prevention of these diseases [2,3]. Most vaccines, such as killed whole organism and subunit vaccines, generally require the addition of an adjuvant to be effective [4]. However, some commonly used adjuvants, e.g. mineral oil and aluminum hydroxide, have many disadvantages, such as causing pain, inflammation, swelling, necrosis, sterile abscesses in injection site and even inducing systemic reactions [4]. Therefore, research and development of efficient safe adjuvant becomes a hot spot in prevention and control of animal infectious diseases.

A number of studies have shown that many Chinese herbal medicines (CHMs) and their ingredients (CHMIs) possess immune-enhancing properties, whether they are used alone or compound [5–8]. As immunopotentiators, they have some advantages, such as lower side-effects and toxicity, without harmful residues and so on, especially in those immunopotentiators composed of polysaccharides or flavonoids which is characterized by clear ingredients and controllable quality [9,10].

Our previous studies have proved that the immune enhancement of compound CHMIs was stronger than the single and the corresponding compound CHMs [11]. Sulfated modification could further improve the immune enhancement of polysaccharides [12,13]. Consecutive research showed that compound astragalus polysaccharide (APS) and sulfated epimedium polysaccharide (sEPS) injection (AEI) could significantly promote lymphocyte proliferation and enhance serum antibody titer in chickens vaccinated with Newcastle disease (ND) vaccine and avian influenza vaccine [14]. Furthermore, AEI could resist the immunosuppression induced by cyclophosphamide [15]. It has been applied for registration of national new veterinary drug and patent.

In the present study, the compound APS and sEPS (AE) was prepared into oral liquid (AEO). It was taken orally by the chickens vaccinated by ND vaccine taking AEI, APS and sEPS, respectively, as

*Abbreviations:* APS, astragalus polysaccharide; sEPS, sulfated epimedium polysaccharide; AEO, compound astragalus polysaccharide and sulfated epimedium polysaccharide oral liquid; AEI, compound astragalus polysaccharide and sulfated epimedium polysaccharide injection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HI, hemagglutination inhibition; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; PHA, phytohemagglutinin; IFN-γ, interferon-γ; IL-2, interleukin-6; BC, blank control; VC, vaccination control; DMSO, dimethylsulfoxide; ND, Newcastle disease.

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control. The change of peripheral lymphocyte proliferation, serum antibody titer, IFN-γ, IL-2 concentrations and immune organ index after vaccination were measured. The purpose of this research is to validate the immunoenhancement of AE per os and offer theoretical evidences for increasing the dosage form and route of administration of AE and exploiting AE oral liquid.

## 2. Materials and methods

### 2.1. Preparation of drug

APS (lyophilized powder, carbohydrate content of 78.6%), sEPS (lyophilized powder, carbohydrate content of 72.38%) and AEI (carbohydrate concentration of 10 mg mL$^{-1}$) were provided by Key Laboratory of Nanjing Agricultural University. AEO was prepared with APS and sEPS according to a certain proportion based on our previous experiments, and diluted into high (7.5 mg mL$^{-1}$), medium (5.0 mg mL$^{-1}$) and low (2.5 mg mL$^{-1}$) concentration with deionized water. APS and sEPS were diluted into 5.0 mg mL$^{-1}$. All drugs were sterilized and stored at 4 °C for the test.

### 2.2. Vaccine and reagents

Newcastle disease vaccine (La Sota strain, No. 119076) was purchased from Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Science.

RPMI-1640 medium (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 cells, diluting mitogen and culturing cells. Phytohemagglutinin (PHA, Sigma), as the T-cell mitogen, was dissolved with RPMI-1640 medium. Hank's solution, pH was adjusted to 7.4 with 5.6% sodium bicarbonate solution, supplemented with benzylpenicillin 100 IU mL$^{-1}$ and streptomycin 100 IU mL$^{-1}$, was used for diluting blood. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was dissolved into 5 mg mL$^{-1}$ with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4). Sodium heparin was dissolved into 5 mg mL$^{-1}$ with PBS. These reagents were filtered through a 0.22 μm filter. PHA and sodium heparin solution were stored at −20 °C, the others were stored at 4 °C and MTT solution was in dark bottle. Lymphocytes Separation Medium (No. 20121201) was purchased from Tianjin Haoyang Biological manufacture Co. Ltd., dimethylsulfoxide (DMSO) and other chemicals used in experiments were analytical grade.

Chicken Interferon-γ (IFN-γ) ELISA kits and Chicken Interleukin 2 (IL-2) ELISA kits were the products of Shanghai Langdun Biotechnology Inc.

### 2.3. Animals

One-day-old White Roman chickens, 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. The chickens were fed with the commercial starter diet provided by the Feed Factory of Jiangsu Academy of Agricultural Science. All the procedures were performed in strict accordance with internationally accepted principles and Chinese legislation on the use and care of laboratory animals.

### 2.4. Experimental design

Three hundred and twenty 14-day-old chickens were randomly divided into eight groups and vaccinated with ND-IV vaccine except for blank control (BC) group, repeated vaccination at 28 days old. At the same time of each vaccination, the chickens in three experimental groups ($AEO_H$, $AEO_M$, $AEO_L$) were taken orally with 1 mL of AEO at high (7.5 mg mL$^{-1}$), medium (5.0 mg mL$^{-1}$) and low (2.5 mg mL$^{-1}$) concentration, respectively, in two component control groups with 1 mL of APS and sEPS solution, once a day for 3 successive days; in injection control group were once injected with 0.5 mL of AEI, and in vaccination control (VC) and BC groups, not administrated.

### 2.5. Peripheral lymphocytes proliferation assay

On days 7 ($D_7$), 14 ($D_{14}$), 21 ($D_{21}$), 28 ($D_{28}$) and 35 ($D_{35}$) after the first vaccination, the blood (2 mL) samples of four chickens randomly from each group were collected and transferred immediately into aseptic capped tubes with sodium heparin, then diluted with equal volume of Hank's solution and carefully layered on the surface of lymphocytes separation medium. After 20 min of centrifuged at 2000 rpm, a white cloud-like lymphocytes band was collected and washed twice with RPMI-1640 medium without fetal bovine serum. The resulting pellet was re-suspended to $3.0 \times 10^6$ mL$^{-1}$ with RPMI-1640 medium, inoculated into 96-well culture plates, 80 μL per well, then another 20 μL of PHA was added and each sample seeded 4 wells. The plates were incubated at 39.5 °C in a humid atmosphere of 5% $CO_2$. After 44 h of the incubation period, 20 μL of MTT (5 mg mL$^{-1}$) was added into each well, and continued to incubate for 4 h. The supernatant was removed carefully and 100 μL of DMSO was added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of each well was measured by microliter enzyme-linked immunosorbent assay reader (MULTISKAN FC, Thermo) at a wavelength of 570 nm ($A_{570}$ value) as the index of lymphocytes proliferation [16]. At the same time the average lymphocyte proliferation rates of all administration groups were calculated to compare the action strength of every treatment according to the equation: $\text{The average lymphocyte proliferation rate}\,(\%) = (\bar{A}_{\text{administration group}} - \bar{A}_{\text{VC group}})/\bar{A}_{\text{VC group}} \times 100\%$ ($\bar{A}$ was the average value of five time points).

### 2.6. Serum HI antibody assay

On days 7 ($D_7$), 14 ($D_{14}$), 21 ($D_{21}$), 28 ($D_{28}$) and 35 ($D_{35}$) after the first vaccination, the blood samples of eight chickens were collected randomly from each group for examination of serum hemagglutination inhibition (HI) antibody titer by micro-method [17].

### 2.7. Serum IL-2 and IFN-γ assay

On days 7 ($D_7$), 14 ($D_{14}$), 21 ($D_{21}$), 28 ($D_{28}$) and 35 ($D_{35}$) after the first vaccination, the blood samples of six chickens were collected randomly from each group for determining the serum contents of IL-2 and IFN-γ by Enzyme-linked Immunosorbent Assay (ELISA).

### 2.8. Immune organ index assay

On day 35 after the first vaccination, 10 chickens randomly from each group were weighed and sacrificed under anesthesia with ether. The spleen, thymus and bursa of Fabricius were excised surgically and weighed, respectively. The immune organ index was calculated according to the formula: the weight of the immune organ (mg)/body weight (g).