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Liposome and epimedium polysaccharide-propolis flavone can synergistically enhance immune effect of vaccine

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ABSTRACT

Three preparations of epimedium polysaccharide-propolis flavone immunopotentiator (EPI), EPI liposome, EPI suspension and EPI watery solution were prepared. In immune response test, their adjuvanticities were compared in 14-day-old chickens vaccinated with Newcastle disease (ND) vaccine. In immune protection test, the effects of the three preparations on Newcastle disease virus (NDV) infection were compared in chickens vaccinated with ND vaccine then challenged with NDV. The results displayed that EPI liposome could enhance the antibody titer, T lymphocyte proliferation and the concentrations of interferon-γ and interleukin-6, when compared with the other two preparations. In EPI liposome group, the antibody titers, lymphocyte proliferation and protective rate were the highest, while the mortality and morbidity were the lowest, in comparison with the other groups. These results indicated that liposome could enhance the immune effect of EPI on ND vaccine and would be expected as the suitable dosage form of this immunopotentiator.

1. Introduction

Immunopotentiator, also called immunologic adjuvant, refers to one class of substance which could non-specifically enhance the specific immune response of animal after they are injected *in vivo* precedingly or simultaneously with antigen. According to the mechanism of action they could be divided into storage type and centre type. The former can protect the antigen by lengthening the persistent time of antigen within organism thus form the durative immune stimulation and the high effective immune reaction, such as aluminum hydroxide, oil emulsion, aluminum phosphate and so on. The latter can directly stimulate or activate the activity cell of immune system together with the antigenic substance, such as bacterium components, endotoxin, liposome, Chinese herbal medicine and so on [1].

Liposomes for delivery of various drugs have been studied extensively, and drug formulations including liposomes have been marketed. Therapeutic agents can be encapsulated in the liposomes, linked covalently to the liposomal surface, or associated externally to the lipid bilayer [2]. It also has the action of adjuvant. For instance, it can induce B cell to enhance the antibody titer, improve immunologic memory ability and heighten the cellular immunologic response. As the carrier of hapten it can induce the specific immune response, enhance the stability of subunit antigen and prolong the storage life [3]. Liposome can also provide slow release of an encapsulated drug, resulting in sustained exposure to organism and enhanced efficacy [4]. For example, liposome could improve the anti-tumor efficacy of paclitaxel [5], the cistanche deserticola polysaccharide liposome could significantly enhance the hemagglutination inhibition (HI) antibody titer, lymphocyte proliferation and the concentrations of interleukin (IL)-2, IL-4 and interferon (IFN)-γ of broiler chicken after vaccinated with Newcastle disease (ND) vaccine [6]. The actions of some Chinese herbal medicinal ingredients (CHMIs) were obviously enhanced after they had been encapsulated with liposome [7,8].

Our previous researches found that two compound CHMIs, epimedium polysaccharide plus propolis flavone (EP–PF) and astragalus polysaccharide plus ginsenoside had better immune-enhancing effect. In addition, two compound CHMIs could promote

Abbreviations: CHMIs, Chinese herbal medicinal ingredients; EP, epimedium polysaccharide; PF, propolis flavone; EPI, epimedium polysaccharide-propolis flavone immunopotentiator; ND, Newcastle disease; NDV, Newcastle disease virus; DMSO, dimethyl sulfoxide; HI, hemagglutination inhibition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-liumbromide; PBS, phosphate buffered saline; PHA, phytohemagglutinin; NA, non-adjuvant control; BC, blank control; IFN, interferon; IL, interleukin.

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mRNA expression of IL-2, IL-10 and IFN-γ of peripheral T lymphocyte in chicken and rabbit [9,10]. The recent researches confirmed that EP and PF possessed synergistically immunologic enhancement and antiviral effect, and the efficacy of EP–PF (named EP–PF immunopotentiator, EPI)was better than those of epimedium flavone plus PF, epimedium flavone plus propolis extracts, and EP plus propolis extracts [11,12]. Therefore, EPI had been applied for national new veterinary drug and invent patent in China and will be expected to exploit into a new immunopotentiator.

In the present research, EPI was made into three different types of adjuvants, EPI liposome, EPI suspension and EPI watery solution, according to different dispersion system [13]. Their immune-enhancing actions for ND vaccine were compared. The purpose of this research is to investigate the synergistic immune-enhancing action of liposome adjuvant and EPI, probe into the probability of liposome further to raise the adjuvanticity of EPI and screen out a suitable preparing method for this immunopotentiator.

2. Materials and methods

2.1. Reagents

Soybean phospholipid was purchased from Taiwei Pharmaceutical Co., Ltd. (Shanghai, Chinese). Cholesterol and Triton X-100 were supplied from Merck (Darmstadt, Germany). Protamine (Sigma Chemical Co.) was dissolved into 10 mg mL^{-1} with physiological saline. RPMI-1640 (GIBCO) supplemented with 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 10% fetal bovine serum was used for cultivating the cells. Phytohemagglutinin (PHA, Sigma Chemical Co.) was dissolved into 0.1 mg mL^{-1} with RPMI-1640. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium-free phosphate-buffered saline (PBS, pH 7.2). Ethanol, glycerine, polyethylene glycols-400 and other chemicals used in experiments were analytical grade and supplied by Guoyao Co., Ltd. (Shanghai, Chinese).

2.2. Vaccine and virus

ND vaccine (La Sota strain, No. 091118) was bought from Biodrug Company of Veterinary, Beijing City. ND virus (NDV, $F_{48}E_9$ strain) was supplied by China institute of veterinary drug control and propagated with 10-day-old specific pathogen-free chicken embryos and used for challenge experiment.

2.3. Preparation of the three EPIs

2.3.1. Preparation of EPI liposome

EPI liposome was prepared by the film-extrusion method [14]. PF (net content of 70.99%), soybean phospholipid, cholesterol and tocopherol were dissolved with ethanol-chloroform solution (1:1, v/v) in round bottom flask, of rotary evaporator (Model RE-52A, Yarong Biochemical Instrument Manufacturer, Shanghai City), evaporated in 40 °C water bath to make solution form into dry film on the sidewall of the flask. EP (net content of 71.23%) and sucrose were dissolved in PBS, poured into the flask, joggled till the film was completely dissolved at room temperature (25 °C). The suspension was filtered through 0.8 μm, 0.45 μm and 0.22 μm millipore membrane filters in turn for degerming, subpackaged into vials under aseptic conditions, lyophilized for 24 h in vacuum freeze-drying machine (Model LGJ-25, Dongxing Machinery Industry Co., Ltd., Shamen City), and stored at room temperature.

2.3.2. Morphologic observation of EPI liposome

The appearance of EPI liposome was observed by naked eye. Then EPI liposome was diluted with PBS, added on copper screen and covered with carbon. The ultramicro morphous of liposome was observed under transmission electron microscope (Model H-7650, Hitachi, High-Technologies Co., Ltd.).

2.3.3. Entrapment rate assay of EPI liposome

Fifteen milligram of EPI liposome was diluted into 1.5 mg mL^{-1} with physiological saline. One hundred microliter of the suspension was added with 100 μL of protamine solution, mixed, added with 3 mL of physiological saline, mixed again and centrifuged at 2000 × g for 20 min. The contents of EP and PF (the content of free drug, C_f) in the 2 mL of supernatant were determined by vitriol–phenol method and rutin method, respectively [15,16]. The precipitate was dissolved with 0.6 mL of Triton X-100 and 2.6 mL of physiological saline and the contents of EP and PF (the content of encapsulated drug, C_e) were determined respectively by the above mentioned method. The entrapment rate (ER) was calculated according to the formula: $ER = 1 - C_f/(C_e + C_f) \times 100$ [17].

2.3.4. Preparation of EPI suspension

Seventy-five milligram of EP was dissolved in 100 mL of 1% sodium carboxymethyl cellulose solution and 75 mg of PF was grinded into fine powder in a mortar. Then EP solution was slowly added into the mortar and grinded continuously till PF was dispersed evenly preparing into 1.5 mg mL^{-1} (the total net content of EP and PF), subpackaged into vials, sterilized by boiling sterilization (100 °C, 30 min) and stored at room temperature.

2.3.5. Preparation of EPI watery solution

Seventy-five milligram of EP was dissolved with 85 mL of water for injection (Key laboratory of Nanjing Agricultural University), and 75 mg of PF was dissolved in 15 mL ethanol plus appropriate amount of glycerine and polyethylene glycol 400, then the PF solution was slowly added into EP solution, mixed evenly preparing into 1.5 mg mL^{-1} (the total net content of EP and PF). Finally the EPI watery solution was sterilized by boiling sterilization (100 °C, 30 min) and stored at room temperature.

EPI liposome was diluted into 1.5 mg mL^{-1} (the total net content of EP and PF). The contents of endotoxin of EPI liposome, EPI suspension and EPI watery solution were determined. When the contents were up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU mL^{-1}), they were used for clinical trial.

2.4. Animals

One-day-old White Roman chickens (male) purchased from Tangquan Poultry Farm were housed in wire cages (60 cm × 100 cm) in air-conditioned rooms at 37 °C and lighted for 24 h 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 constantly in the following days. Chickens were fed with the commercial starter diet provided by the feed factory of Jiangsu Academy of Agricultural Science.

2.5. Experimental design

2.5.1. Immune response test

Two hundred and fifty 14-day-old chickens were randomly assigned into 5 groups and vaccinated with ND vaccine except for blank control (BC) group, repeated vaccination at 28-day-old. At the same time of the first vaccination, the chickens in three adjuvant groups were intramuscularly injected with 0.5 mL of three dosage forms of EPI, respectively. The non-adjuvant control (NA) and BC groups were intramuscularly injected with 0.5 mL of physiological saline. Before vaccination and on days 7, 14, 21 and 28 after the first vaccination, blood was sampled for determination of serum HI antibody titer and lymphocyte proliferation. On days 14, 21 and 28