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The preparation optimization and immune effect of epimedium polysaccharide-propolis flavone liposome

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ABSTRACT

The preparation conditions of epimedium polysaccharide-propolis flavone liposome (EPL) were optimized by response surface methodology taking entrapment rates of epimedium polysaccharide and propolis flavone as indexes. The immunoenhancement of EPL prepared with optimized condition was determined taking epimedium polysaccharide-propolis flavone suspension (EPS) and epimedium polysaccharide-propolis flavone watery solution (EPW) as control. The results showed that the optimized preparation condition was as follows: the ratio of drug to lipid was 14:1, the ratio of soybean phospholipid to cholesterol was 6:1, and the ultrasonic time was 19 min. EPL could significantly promote the proliferation of T and B lymphocytes singly or synergistically with PHA or LPS, mRNA expression of IL-2 and IL-6 and secretion of IgG and IgM as compared with EPS and EPW. These results indicated that liposome could significantly improve the immunoenhancement of epimedium polysaccharide-propolis flavone immunopotentiator (EPI) and would be as the suitable dosage form of EPI.

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1. Introduction

All drugs should be made into suitable form which was called as the dosage form for clinical use (Li, Qiao, & Yan, 2008). Although the efficacy of drug mainly depends on the drug itself, the dosage form also plays an important role under certain conditions (Gu & Peng, 1987). For instance, the suitable dosage forms can ensure the efficacy and stability of the drug, change the property of drug, regulate the release of drug, reduce or eliminate the toxic side effect. Moreover, some dosage forms have the positioning or targeting action (Chen, 2006). The different dosage forms might lead to different efficacy for same medicine (Yao, Fu, & Jiang, 2002).

The liposome is a new material for dosage form. It is artificially prepared membranous vesicles composed of natural phospholipids and cholesterol. Its structure is similar to cell membrane with hydrophilicity and lipophilicity and suitable for use as the carrier of drugs (Li, Braiteh, & Kurzrock, 2005). It has the action of targeting and slow releasing, reducing toxicity, improving bioavailability and so on (Wang, Qiu, & Xu, 2004). Therefore, it is attracting more and more attention in medical domain. Many researches indicated that the actions of some Chinese herbal medicinal ingredients were obviously enhanced after they are encapsulated with liposome (Deng, 1996; Lee, Chung, & Lee, 2008; Liu & Guo, 1999).

Herba epimedii, a commonly used Chinese herbal medicine, could promote the specific and non-specific immune function of human and animal (Tian, 2010). Its active ingredients include polysaccharide, flavonoid glycosides, terpenoids and alkaloid, especially the polysaccharide playing an important role (Nada, Miodrag, Aleksandar, & Zvezdana, 2006). As one kind of new-type immunopotentiator, *propolis* has been applied widely, and obtains marked achievements. The propolis as immunopotentiator has the following advantages, such as higher protection rate, longer immune protection period, producing immunity earlier and so on (Shen, 1989). Its main active component is propolis flavone (PF). Our previous researches demonstrated that EP and PF possessed synergistically immunologic enhancement and antiviral effect, the efficacy of EP–PF prescription (named EP–PF immunopotentiator, EPI) was better than those of other three prescriptions (Fan et al., 2010, 2011), and had been applied for national new

Abbreviations: EP, epimedium polysaccharide; PF, propolis flavone; EPI, epimedium polysaccharide-propolis flavone immunopotentiator; EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PHA, phytohemagglutinin; CC, cell control; PHAC, PHA control; LPS, lipopolysaccharide; LPSC, LPS control; DMSO, dimethyl sulfoxide; IL-2, interleukin-2; IL-6, interleukin-10; Ig, immunoglobulin.

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veterinary drug and invent patent in China as a new immunopotentiator.

In the present research, EPI was made into three dosage forms, EPI liposome (EPL), EPI suspension (EPS) and EPI watery solution (EPW). The preparation conditions of EPL were optimized by response surface method. EPL was prepared with the optimized condition so as to obtain the highest entrapment rates of EP and PF. The effects of EPL on T and B lymphocytes proliferation, IL-2 and IL-6 mRNA expression of T lymphocyte, IgG and IgM secretion of B lymphocyte were determined and compared with EPS and EPW. The purpose of this research was to select the optimum preparation condition of EPL, probe into the probability of liposome on improving the immune enhancement of EPI, and select a suitable dosage form for this immunopotentiator.

2. Materials and methods

2.1. EPS, EPW and reagents

Epimedium polysaccharide (net content of 71.23%) and propolis flavone (net content of 70.99%) were provided in our laboratory. EP–PF suspension (EPS) and EP–PF watery solution (EPW) were also prepared in our laboratory, they were diluted into five working concentrations (15.625–0.977 $\mu g\,mL^{-1}$) in two-fold serial dilution with RPMI-1640 containing 10% fetal bovine serum based on the previous determination result of safe concentration (Fan et al., 2011). When the endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU mL^{-1}) (Veterinary Pharmacopoeia commission of the People's Republic of China, 2006), they were stored at 4 °C for the test.

Soybean phospholipid (No. 20100728) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Cholesterol (No. 20100908) was purchased from Anhui Tianqi Chemical Technology Co., Ltd. Protamine (Sigma, P4380) was dissolved by physiological saline to 10 $mg\,mL^{-1}$. Lymphocyte separation medium (No. 100218) was the product of Shanghai Huajing Biology Inc. RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 10% fetal bovine serum was used for washing and re-suspending cells, diluting mitogen and culturing the cells. Phytohemagglutinin (PHA, Sigma, No. L-8754), as a T-cell mitogen, was dissolved into 0.1 $mg\,mL^{-1}$ with RPMI-1640. Lipopolysaccharide (LPS, Sigma, No. L2880), as a B-cell mitogen, was dissolved into 0.05 $mg\,mL^{-1}$ with RPMI-1640. Hanks' solution was used for diluting blood. 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 (CMF) phosphate-buffered saline (PBS, pH 7.2). These reagents were filtered through a 0.22 μm millipore membrane filter. PHA solution was stored at −20 °C, MTT solution, at 4 °C in dark bottles, RPMI-1640 were stored at 4 °C. Dimethyl sulfoxide (DMSO, No. 20090519) was produced by Kemiou Institute of Chemical Engineering in Tianjin. RNAiso Plus, DEPC and SYBR Green Real-time PCR Master Mix were supplied by TakaRa Biochnology Co. Ltd. Other chemicals used in experiments were analytical grade.

2.2. Optimization of EPL preparation conditions by response surface method

2.2.1. Box–Behnken design of the preparation conditions

Base on the single-factor experiments and our previous researches, the ratio of drug to lipid, ratio of soybean phospholipid to cholesterol and ultrasonic time were mainly effective factors on entrapment rate of EPL. Therefore these three factors at three levels were used respectively and seventeen reactive conditions were designed according to Box–Behnken central composite design. These three factors at three levels were as follows: the ratio of drug to lipid (10:1, 15:1 and 20:1, w/w); the ratio of soybean phospholipid to cholesterol (4:1, 6:1 and 8:1, w/w); ultrasonic time (10, 20 and 30 min).

2.2.2. Preparation of EPL

PF, soybean phospholipid, cholesterol and tocopherol were dissolved in ethanol–chloroform solution (1:1, v/v), and poured into round bottom flask. The solution was evaporated to dryness in 40 °C water bath by rotary evaporator (Model RE-52A, Yarong Biochemical Instrument Manufacturer, Shanghai City), finally a dry film was formed in the sidewall. EP and sucrose was dissolved in phosphate buffered saline (PBS, pH 7.2), and then poured into the round bottom flask, joggled until the film was completely dissolved at room temperature (25 °C). The suspension was dealt with ultrasonication in ice bath for 20 min. Then, crude suspension was filtered through 0.8 μm, 0.45 μm and 0.22 μm millipore membrane filters successively (Zhang, Anyarambhatla, & Ma, 2005).

Under the transmission electron microscope, the particles of EPL presented spherical or nearly spherical shape with uniform size whose diameters were less than 200 nm. EPL was diluted into five working concentrations (15.625–0.977 $\mu g\,mL^{-1}$) in two-fold serial dilution with RPMI-1640 containing 10% fetal bovine serum, sterilized and stored at 4 °C for the test.

2.2.3. Entrapment rate assay of EPL

The entrapment rate of EPL was assayed by protamine method according to reference (Yu, Zhang, & Zheng, 2003). The entrapment rate (ER) was calculated according to the formula: $ER = 1\text{-}C_f/(C_e + C_f) \times 100\%$ (C_f: the content of free drug; C_e: the content of encapsulated drug) (Yu et al., 2003). The contents of EP and PF (the content of free drug, C_f) were determined respectively by vitriol–phenol method and rutin method (Gunter, Gunter, Jarkowski, & Rosier, 1982; Meng & Chen, 1990).

2.2.4. Confirmatory test

According to the optimum preparation condition optimized by response surface method, five verification experiments were carried out, the entrapment rates of EP and PF were determined in order to observe whether the experimental results were consistent with regression model or not.

2.3. T lymphocyte proliferation assay

The blood samples were collected from non-immune White Roman chickens (male) at 60-day old (provided by Tangquan Poultry Farm) and transferred immediately into aseptic capped tubes containing sodium heparin, then diluted with an equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After centrifugation at $800 \times g$ for 20 min, the lymphocytes was collected and washed twice with RPMI-1640 without fetal bovine serum. The resulting pellet was re-suspended and diluted to 5×10^6 mL^{-1} with RPMI-1640 after the cell viability was assessed by trypan blue exclusion. The solution was divided into two parts, one part was added with PHA, and respectively incubated into 96-well culture plates, 100 μL per well. Then, EPL, EPS and EPW at series of concentrations were added, in cell control group and PHA control group, RPMI-1640 and PHA respectively, 100 μL per well, four wells each concentration. The final concentration of PHA reached to 20 $\mu g\,mL^{-1}$. The plates were incubated in a humid atmosphere with 5% CO_2 (Revco, Co., USA) at 39.5 °C for 48 h. Briefly, 20 μL of MTT (5 $mg\,mL^{-1}$) was added into each well at 4 h before the end of incubation. Then the plates were centrifuged at $1000 \times g$ for 10 min at room temperature. The supernatant was removed carefully and 100 μL of DMSO was added into each well. The plates were shaken for 5 min to dissolve the crystals

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