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Optimization on preparation condition of epimedium polysaccharide liposome and evaluation of its adjuvant activity

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ABSTRACT

The aim of this strategy was to investigate whether the adjuvant activity of epimedium polysaccharide (EPS) could be further enhanced after encapsulated with liposome. In preparation of EPS liposome (EPSL) test, an orthogonal L_9 (3^4) test design was used to optimize the preparation condition of EPSL. In adjuvant activity test, 350 14-day-old chickens were randomly assigned to 7 groups and vaccinated with Newcastle disease (ND) vaccine. Simultaneously, the chickens in experimental groups were injected with EPSL at three doses, EPS and blank liposome, respectively. The activity of lymphocytes proliferation, titer of serum antibody and concentrations of cytokines were determined. Results showed that the optimal preparation condition of EPSL was that ratio of drug to lipid, ratio of soybean phospholipid to cholesterol, ultrasonic time, and water bath temperature were 1:30, 4:1, 10 min and 40 °C, respectively. EPSL could significantly enhance the immune response of ND vaccine and promote cytokines secretion, and its high dose possessed the best efficacy. These findings indicated that liposome encapsulation could significantly improve the adjuvant activity of EPS.

1. Introduction

The aim of vaccination is to generate a strong immune response providing long term protection against infection. However, many vaccines, such as inactivated vaccines, killed whole organism or subunit vaccines, could not attend to ideal immune effect. These vaccines generally require the addition of adjuvants to improve the efficacy [1,2]. Recent years, the development of new adjuvant for vaccines has become an expanding field of research for generating stronger vaccines capable of inducing protective and long-lasting immunity [1]. Various categories of adjuvant including mineral salts, emulsions, polysaccharides, Chinese herbal medicinal ingredients, cytokines, microorganism-derived adjuvant and so on, have different advantages and disadvantages [3]. Chinese herbal medicinal ingredients, as a kind of novel adjuvant, possess the characteristic of few side effects, lower toxicity, non systemic stimulation, which is consistent with the request of food safety for people.

However, when the Chinese herbal medicinal ingredients are administrated, it is difficult to sustain high concentration in plasma and the targeting is bad [4]. Liposome is artificially prepared membranous vesicles composing essential of naturally occurring phospholipids and is excellent carrier system for both hydrophilic and hydrophobic drugs, which gives them an advantage as a blood dosing and delivery method [5–7]. Additionally, liposome is known to be effective as immune adjuvant and vaccine carriers [8–12]. Plenty of researchers confirmed that after the Chinese herbal medicinal ingredients are encapsulated with liposome, their pharmaco-dynamic efficacy were obviously increased [13–16].

Epimedium, a famous traditional herbal medicine, used for centuries in China, as a herbal to tonify the kidney, invigorate *yang*, and strengthen muscles and bones. The active ingredients of epimedium include polysaccharide, flavonoid glycosides, ignans, terpenoids, alkaloid magnoflorine and so on, especially the polysaccharide playing an important role [17]. Our previous researches demonstrated that epimedium polysaccharide (EPS) was a potent adjuvanticity [18–20]. Whether the adjuvanticity of EPS can be further enhanced after encapsulated with liposome?

In this study, firstly, the preparation condition of EPS liposome (EPSL) was optimized by orthogonal test design. Then, EPSL was injected in chicken at the same time with inoculating Newcastle disease (ND) vaccine. The effects of EPSL on the lymphocytes proliferation, antibody titers and concentrations of four kinds of cytokines were observed. The aim of this strategy is to investigate

Abbreviations: EPS, epimedium polysaccharide; EPSL, epimedium polysaccharide liposome; BC, blank control; BL, blank liposome; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ND, Newcastle disease; PBS, phosphate buffered saline; PHA, phytohemagglutinin; VC, vaccine control; IFN-γ, interferon-γ; IL, interleukin.

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whether adjuvant formulations of EPS and liposome can further enhance or modulate the immune response against a given antigen compared with the adjuvant alone.

2. Materials and methods

2.1. EPS and reagents

Epimedium was purchased from Dahua Chinese traditional medicine company in Nanjing, Jiangsu province. EPS was prepared in our laboratory.

Soybean phospholipid (No. 20090728) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Cholesterol (No. 20090908) 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. 090218) was manufactured by 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. 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 at 4 °C. Dimethyl sulfoxide (DMSO, No. 20090519) was produced by Kemiou Institute of Chemical Engineering in Tianjing. Other chemicals used in experiments were analytical grade.

2.2. Vaccine

ND vaccine (La Sota strain, No. 091118) was bought from Beijing Veterinary Bio-drug Company.

2.3. Preparation of EPSL

The mixture of soybean phospholipid, cholesterol (2:1, w/w) and tocopherol was 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. The EPS dissolved with PBS (pH 7.2) was poured into the round bottom flask and joggled until the film was completely dissolved at 30 °C in water bath. The suspension was homogenized with ultrasonication in ice bath for 20 min. Ultimately, the solution was filtered with 0.8 μm, 0.45 μm and 0.22 μm millipore membrane successively [21]. The particle diameter of liposome was determined with transmission electron microscope and the average particle size was 200 nm. In animal experiment, EPSL was diluted into high (1.00 mg mL^{-1}), medium (0.75 mg mL^{-1}) or low (0.50 mg mL^{-1}) dose with physiological saline. The endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU mL^{-1}).

2.3.1. Encapsulation efficiency and drug-loading rate of EPSL assay

Half of 1 mL of EPSL was mixed with 0.1 mL of protamine solution (10 mg mL^{-1}). After 3 min, 3 mL of physiological saline was added. After adequate mixing, this suspension was centrifuged at 3000 × g at the room temperature for 30 min. Part of the supernatant (2 mL) was used to assay the content of EPS by vitriol–phenol method, called the content of free drug. The precipitation remained in centrifuge tube was dissolved by 0.6 mL of Triton X-100 and 2.6 mL of physiological saline, the content of EPS in this solution was determined and called the content of encapsulated drug. The formula to calculate liposome encapsulation efficiency was EN % = $(1 - C_f/C_t)$, C_f: the content of free drug, C_t: the total content of drug. The formula to calculate the drug-loading rate was = $(W_T - W_F)/W_P \times 100\%$, W_T: the total weight of the EPS in the solution, W_F: the weight of the free EPS, W_P: the total weight of soybean phospholipid and cholesterol [22,23].

2.3.2. Single-factor test of EPSL preparation

Preparation of EPSL was the same as Section 2.3. Effects of six single factors (the ratio of chloroform to ethanol, water bath temperature, the ultrasonic time, ratio of drug to lipid, ratio of soybean phospholipid to cholesterol and the hydration media) on the encapsulation efficiency and drug-loading rate of EPSL were observed.

2.3.3. Orthogonal test of preparation of EPSL

The single-factor tests found that the four factors, ratio of drug to lipid (w/w) (A), ratio of soybean phospholipid to cholesterol (w/w) (B), ultrasonic time/min (C), water bath temperature/°C (D), were the mainly effective factors on encapsulation efficiency and drug-loading rate of EPSL. Therefore, an orthogonal L_9 (3^4) test design was used for optimizing the preparation condition of EPSL. Three levels per factor were used, respectively. Nine reacting conditions were designed according to orthogonal test as L_9 (3^4) (Table 1). Due to two evaluating indexes used in the experiment, the comprehensive index was applied to evaluate the optimal preparation condition of EPSL. The comprehensive index = (encapsulation

Table 1
Analysis of L_9 (3^4) test results.

No.	Factors				Determination index		General index
	A	B	C	D	Encapsulation efficiency (%)	Drug-loading rate (%)	
1	1:20	4:1	10	30	75.49	4.71	1.09
2	1:20	2:1	20	40	74.95	4.47	1.07
3	1:20	1:1	30	50	70.55	3.70	0.98
4	1:30	4:1	20	50	75.22	4.79	1.10
5	1:30	2:1	30	30	68.10	4.85	1.02
6	1:30	1:1	10	40	76.84	5.71	1.17
7	1:40	4:1	30	40	66.33	3.71	0.94
8	1:40	2:1	10	50	60.04	3.54	0.86
9	1:40	1:1	20	30	54.39	3.14	0.77
K_1	1.047	1.043	1.040	0.960			
K_2	1.097	0.983	0.980	1.060			
K_3	0.857	0.973	0.980	0.980			
R	0.240	0.070	0.060	0.100			

A: ratio of drug to lipid (w/w); B: ratio of soybean phospholipid to cholesterol (w/w); C: ultrasonic time/min; D: water bath temperature/°C; "R" refers to the result of extreme analysis.