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Preparation and optimization of ophiopogon polysaccharide liposome and its activity on Kupffer cells

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ABSTRACT

The purpose of this study was to prepare and optimize ophiopogon polysaccharide liposome (OPL), and to improve the immune-enhancing activity of ophiopogon polysaccharide (OP). OPL was prepared and optimized using the methods of reverse-phase evaporation and response surface methodology. The property was evaluated with particle size, zeta potential, and morphology. The results showed that the optimum preparation conditions were: soybean phosphatide to OP ratio of 9.5:1, soybean phospholipid to cholesterol ratio of 8:1, and chloroform to phosphate-buffered saline ratio of 3:1. Subsequently, the immune-enhancing activity of OPL on Kupffer cells (KCs) was performed. The results showed that OPL could significantly promote the phagocytosis of KCs, induce the secretion of nitric oxide, induced nitric oxide synthase, IL-6 and IL-12, and improve the expression of CD80 and CD86 compared with OP at 125–7.813 $\mu g\, mL^{-1}$. These results indicated that the immune-enhancing activity of OP was significantly improved after encapsulated with liposome. Therefore, liposome would be expected to exploit into a new-type preparation of OP.

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1. Introduction

Ophiopogonis japonicus (Radix Ophiopogonis Japonici), as a traditional Chinese medicine, its use could be dated back more than 2000 years ago and be recorded in Shen Nong's Materia Medica written in the Han dynasty. It possesses the special functions on promoting the secretion of fluid, nourishing yin, moisturizing the lung, and clearing away the heart-fire (Zhang et al., 2012). Ophiopogon polysaccharide (OP), as the main active constituents of *O. japonicus*, possesses stronger immunologic enhancement activity, such as enhancing the phagocytic function of macrophages and improving the humoral and cellular immunity (Lin et al., 2010; Xiong et al., 2011; Wang et al., 2012). However, because of its high-hydrophilicity and about 2 nm molecular sizes, the half-time of OP is short, OP is quickly excreted through kidney directly, which leads to its less distribution in the target tissue and instability in organism. All of the unsatisfactory pharmacokinetic characters above severely limited the clinical application of OP (Lin et al., 2010). Therefore, in order to improve the bioavailability and exert better pharmacological function, it is particularly important to develop a new-type of OP preparation or choose a high-efficiency carrier for improving the pharmacokinetic characters of OP.

Liposome, as one of the important research contents in drug delivery system, has received growing emphasis because of its advantages such as, lengthening drug effect, improving stability, reducing toxicity, and so on (Patil and Jadhav, 2014). Nowadays, the study of liposome on traditional Chinese medicine has become a hotspot. Our preliminary research had proved that the immune enhancement activity and drug effect of epimedium polysaccharide and astragalus polysaccharide were significantly improved and lengthened after being prepared into liposome (Gao et al., 2012; Fan et al., 2012). The polysaccharide consists of more than ten monosaccharide molecules or monosaccharide derivatives which are connected by glycosidic bond, and different kinds of plant polysaccharides have similar structure. Therefore, we speculate that OP liposome (OPL) could also achieve better immune-enhancing activity and overcome the disadvantages of metabolizing quickly in the body compared with OP.

Abbreviations: OPL, ophiopogon polysaccharide liposome; OP, ophiopogon polysaccharide; KCs, Kupffer cells; RSM, response surface methodology; IL, interleukin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; MTT, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PDI, polydispersity index; TEM, transmission electron microscopy; BL, blank liposome; NO, nitric oxide; iNOS, induced nitric oxide synthase; CC, cell control.

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Studies show that when common drug liposome (unmodified liposome) enters the blood circulation, instead of releasing into the circulatory system directly, it is mainly absorbed by mononuclear macrophage in reticuloendothelial system, especially the Kupffer cells (KCs) in liver, and change the distribution of encapsulated drugs in vivo, finally make most of the drugs accumulate in liver (60–90%), then activate the autoimmunity functions of the organism (Allen and Cullis, 2013; Scarpa et al., 2011). Therefore, after being prepared into common liposome and getting into the body, OP is mainly taken by KCs.

KCs are the largest amount of macrophages in the body (Wynn et al., 2013). At present, KCs have been involved in the pathogenesis of various liver diseases, such as, viral hepatitis, alcoholic liver disease, steatohepatitis, liver transplantation, liver fibrosis, and so on. They are recognized as important modulators which control the liver's response to injury and to repair (Stienstra et al., 2010; Yang et al., 2013). So the current studies of KCs main focus on the relationship between KCs and various liver diseases. However, as the important part of innate immune cells, the role of KCs in other aspects has not been extensively examined so far. Especially, after drug liposomes for non-treatment of liver diseases entered the body, they were almost absorbed by KCs, and the drug is how to activate KCs, and activated KCs is how to boot the whole immune system of body, there are little reports at home and abroad. In addition, there is also little information on the effect of OP on KCs.

Thus, in the present study we choose KCs as cell model, and investigate the immunomodulatory effects of OPL on KCs in vitro. Firstly, OPL was prepared by using the reverse-phase evaporation method, and the preparation conditions were optimized through response surface methodology (RSM). Then, the activity of OPL on KCs was measured, including detecting the production of nitric oxide (NO) and induced nitric oxide synthase (iNOS), the phagocytosis, the secretion of interleukin IL-6 and IL-12, and the expression of costimulatory molecules CD80 and CD86. The purpose of this research was to improve the immune-enhancing activity of OP through the combination of liposome and the immune function of OP.

2. Materials and methods

2.1. Materials

Ophiopogon polysaccharide (OP, net content of 98.8%) was provided in our laboratory. Cholesterol (No. 20130304) was purchased from Anhui Tianqi Chemical Technology Co., Ltd. Soybean phospholipid (No. 20130916) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Goat anti-mouse monoclonal anti-CD80-FITC and anti-CD86-PE antibodies were supplied by BioLegend Inc. (USA). Percoll (P-8370) was purchased from Pharmacia Co., Ltd. RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin and streptomycin, and 10% fetal bovine serum (Hyclone, USA) was used for washing cells, diluting mitogen, and culturing the cells. Lipopolysaccharide (LPS, Sigma, No. L2880) was dissolved into 0.05 mg mL^{-1} with RPMI-1640. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co., Ltd.) was dissolved into 5 mg mL^{-1} with phosphate-buffered saline (PBS, pH 7.2). These reagents were filtered through a 0.22 μm millipore membrane filter. Dimethyl sulfoxide (DMSO) was produced by Kemiou Institute of Chemical Engineering in Tianjin, China.

2.2. Optimizing the preparation conditions of OPL

2.2.1. Preparation of OPL

OPL was prepared by using the reverse-phase evaporation method. Firstly, cholesterol and soybean phospholipid were dissolved with chloroform, then PBS contained OP (4 mg mL^{-1}) was injected into the solution of chloroform. The mixture was homogenized through using the ultrasound in the ice bath to form stable water in oil type (W/O) emulsion. Secondly, the emulsion was evaporated to form a colloid using a rotary vacuum evaporation, and then 20 mL of PBS was added to hydrate for 15 min. Thirdly, the resulting mixture was homogenized again by using ultrasonic for 20 min to form the well-proportioned liposome. Finally, the liposome was filtered using 0.45 and 0.22 μm millipore membrane successively. The entrapment rate of OPL was assayed by protamine and vitriol–phenol methods according to reference (Gunter et al., 1982; Guo et al., 2010).

2.2.2. Box–Behnken design of the preparation conditions

Base on the previous researches, we found that the ratio of drug to soybean phospholipids, ratio of soybean phospholipids to cholesterol, and ratio of chloroform to PBS were the mainly factors on influencing the entrapment rate of OPL. Therefore, the three factors mentioned above at three levels were used and seventeen experiments were adopted according to the Box–Behnken design. The three level-three variables were as follows: the ratio of drug to soybean phospholipids (8:1, 10:1, and 12:1, w/w); the ratio of soybean phospholipid to cholesterol (6:1, 8:1, and 10:1, w/w); the ratio of chloroform to PBS (2:1, 3:1, and 4:1, v/v). Experimental data was analyzed by the Design-Expert program, and the three-dimensional response surfaces were also built.

2.3. Characterization of OPL

2.3.1. Particle size and zeta potential

The particle size, zeta potential, and distribution (characterized by polydispersity index, PDI) of OPL were measured at 25 °C by Malvern laser particle size analyzer (Nano-ZS, Malvern, UK). Particle size data were expressed as the intensity-weighted distribution.

2.3.2. Morphology observation

The morphology of OPL was examined by transmission electron microscopy (TEM, H-600 II, Hitachi, Japan). One drop of diluted sample was dyed by 2% phosphotungstic acid, and then fixed on the copper grid coated with carbon film, followed by drying at 25 °C before examining under the TEM.

2.4. Activity of OPL in vitro

2.4.1. Culture of KCs

The rats were anaesthetized by diethyl ether. The KCs were isolated from the liver according the method of type IV collagenase digestion (Kitani et al., 2011). Firstly, the portal vein of mouse was inserted with a catheter, and then the liver was perfused in situ with 10 mL of PBS (37 °C, 3 mL/min) in a non-recirculation method to drain away the red cells. Secondly, the liver was transferred immediately into culture dish, and cut into 1–2 mm^3 pieces. Thirdly, the liver tissues were digested with 10 mL of RPMI-1640 containing 0.2% type IV collagenase at 37 °C for 30 min. Finally, the liver homogenate was filtered through a sterile 200-mesh nylon screen, and the filtrate was centrifuged at 300 × *g* for 5 min. KCs were further separated and purified by the methods of two-step (70% and 30%, v/v) percoll gradient and selective adherence to plastic (Liu et al., 2012; Yang et al., 2013). Briefly, the cells were resuspended, then suspension was overlaid on a two-step (70% and 30%, v/v) percoll gradient and centrifuged for 15 min at 2000 × *g*. The cells between the two percoll layers were collected, then seeded into six-well culture plates and incubated for 2 h. Non-adherent cells were removed by lightly

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