



Salidroside liposome formulation enhances the activity of dendritic cells and immune responses



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ABSTRACT

Salidroside, the important composition, of *Rhodiola rosea* L. has been reported to have various pharmacological properties. Liposome is known to be effective as drug carriers and immune adjuvant. Therefore, the aim of this study is to investigate immunological adjuvant activity of salidroside liposome. Here we reported the preparation, the effect on DCs in vitro and the immune response in vivo. The immunological adjuvant activity of salidroside liposome formulation was compared with that of salidroside and liposome. The result showed that salidroside liposome formulation not only could promote the maturation of DCs, the stimulation of DCs on MLR proliferation and the antigen presenting ability, but also induced the sustained cellular immune and humoral immune response. Overall, the results showed that salidroside liposome formulation had the potential to act as effective sustained release vaccine delivery systems.

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1. Introduction

With the development of molecular biology, genomics, proteomics and protein engineering, a vast range of potential vaccine antigens for a host of animal's most serious diseases have been identified [1]. However, experiences inform us that vaccines based on recombinant proteins and synthetic peptides lack the immunogenicity of the whole [2]. There is a pressing need to identify powerful adjuvants which can safely enhance these weak responses with a minimum of undesirable side effects in order to fully realize the potential benefits of recombinant antigen-based vaccines [1]. Despite major advances in our understanding of vaccine adjuvants, both old and new vaccines seem likely to depend on aluminum salts. However, these adjuvants can lead to serious adverse effects [3]. Thus, development of immunological adjuvants can be seen as a balancing act, achieving maximum enhancement of immune responses (adjuvant activity) with minimal detrimental side effects to the host (reactogenicity) [1]. Traditional Chinese medicine immuno-modulators are paving their way as a safe alternative [4,5]. These modulators can be administered along with the vaccine to elicit a faster and stronger immune response.

Rhodiola rosea L. (Crassulaceae, syn. *Sedum rhodiola* - DC. *Sedum rosea* - (L.) Scopcop) has a long history as a valuable medicinal plant having appeared in the *Materia Medica* of a number of Asian and European countries [6]. Studies showed that the pharmacological effects of *Rhodiola rosea* and its extracts were manifold, including adaptogenic and stress-protective effects, antioxidant effect, stimulating effect on the central nervous system including effects on cognitive functions such as attention, memory and learning, anti-fatigue effect, endocrine activity normalizing, etc. [7,8]. Salidroside is one of the main important compositions, which is extracted from *Rhodiola rosea* L., and has been reported to have various pharmacological properties including anti-aging, anticancer, anti-inflammation, hepatoprotective, and antioxidative effects [4,9,10].

Liposome is a synthetic bilayer membrane vesicle with phosphorus and has good affinity on cell membrane [11]. A major achievement in liposome medical application is the ability to load a sufficient amount of drug needed to achieve therapeutic efficacy. Additionally, liposome is known to be effective as immune adjuvant and vaccine carriers [12,13]. Therefore, it will be of great importance that traditional Chinese medicine immuno-modulator is encapsulated with liposome and used as formulation of adjuvant.

In the present research, salidroside was encapsulated with liposome and the immunological adjuvant activity was observed. The aim of the strategy was to investigate the impact of encapsulating an antigen (ovalbumin, OVA) and an adjuvant (Salidroside) in liposomes on the immune response.

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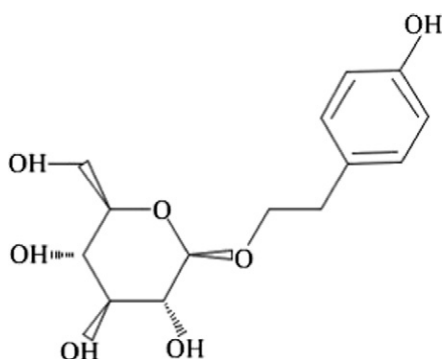
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2. Materials and methods

2.1. Preparation of salidroside liposome

The salidroside (with purity of 98% HPLC, Mol.wt: 300.01, basic structure



purchased from Alading Co., Ltd., No.10338-51-9) liposomes were prepared by ammonium sulfate gradient method [14]. Briefly, soybean phospholipid (manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd., No. 20110728), cholesterol (purchased from Anhui Tianqi Chemical Technology Co., Ltd., No. 20110908) and tween-80 (6: 1: 1, w:w:w) were dissolved into ethanol. The solution was injected into 0.26 mol/L $(\text{NH}_4)_2\text{SO}_4$ solution in 43 °C water bath with the speed of 3 mL/min. Then, ethanol was removed by rotary vacuum evaporation under lipid transition temperature. The blank liposomes were dialyzed with the bag filter in PBS for 24 h to remove $(\text{NH}_4)_2\text{SO}_4$ in external environment of liposomes, which could form the pH gradient between inside and outside of the blank liposomes. Loading of salidroside was driven by the pH gradient generated from the ammonium sulfate within the liposomes. Salidroside solution was added into the blank liposome suspension at drug: lipid = 1:20 (w/w), followed by incubation at 50 °C to monitor the uptake of the drug. The resulting mixture was homogenized with ultrasonic cleaner for 30 min to form the small single-chamber liposomes. Ultimately, the solution was filtered with 0.45 μm and 0.22 μm millipore membrane successively. The entrapment efficiency of salidroside liposomes was 95.42%. The endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 ED/mL).

2.2. Analysis of salidroside liposomes on dendritic cells (DCs)

2.2.1. Generation of bone marrow-derived DCs

Primary bone marrow DCs were obtained from mouse bone marrow precursors as previously described [15,16]. In brief, the femurs and tibiae of ICR mouse (4–6 weeks old) were removed and isolated from surrounding muscle tissue. Intact bones were washed twice in PBS. Bone marrow cells were flushed from the femur and tibiae of ICR mouse and treated with ACK lysis buffer (Sigma) to lyse erythrocytes. DCs were cultured at a starting concentration of $2.0 \times 10^6/\text{mL}$ in round-bottomed 12-well plates with RPMI-1640 supplemented with GM-CSF, rmlL-4, 10% FCS, 2 mL per well. Cells were cultured in a humidified chamber at 37 °C and 5% atmospheric CO_2 . After incubation for 24 h, the medium with non-adherent cells was replaced with fresh medium. The culture medium was removed and replenished with an equal volume of fresh medium every two days. On the 7th day, the suspended and loosely adherent cells were collected as immature DCs (CD11c + cells were about 85%) and non-adherent DCs were positively purified with microbead-conjugated anti-CD11c mAb by MACS columns (Miltenyi Biotec, Auburn, CA).

2.2.2. Flow cytometry

The effect of salidroside liposomes on the phenotype of DCs was examined using flow cytometry. The immature DCs were collected as

in Section 2.2.1. On the 7th day of incubation, different concentrations (60, 15, 3.75 $\mu\text{g}/\text{mL}$) of salidroside liposomes, serum-free RPMI-1640 (GIBCO) or 5 $\mu\text{g}/\text{mL}$ LPS were added into DCs incubated for another 48 h. The mature DCs were collected and added to Falcon tubes. After centrifugation ($1500 \times g$ at 4 °C for 5 min), the DCs were stained with FITC-conjugated anti-mouse CD80 (Peprotech), PE-conjugated anti-mouse CD86 (Peprotech) and FITC-conjugated anti-mouse MHC class II (Peprotech) Abs respectively. The mixture was incubated at 4 °C for 30 min in the dark. Following washed with PBS twice, DCs were resuspended in 0.5 mL 1% paraformaldehyde. The percentages of CD80, CD86 and MHC class II were assessed by flow cytometry.

2.2.3. Analysis of salidroside liposomes on DCs stimulating proliferation of allogenic mixed leukocyte reaction (MLR)

The immature DCs were collected as in Section 2.2.1. On the 7th day of incubation, different concentrations (60, 30, 15, 7.5, 3.75 $\mu\text{g}/\text{mL}$) of salidroside liposomes, serum-free RPMI-1640 or 5 $\mu\text{g}/\text{mL}$ LPS were added into DCs incubated for another 48 h. After 30 min treatment with 50 $\mu\text{g}/\text{mL}$ mitomycin C at 37 °C, cells were washed with PBS twice, and then resuspended in complete RPMI-1640 at a concentration of $5 \times 10^5/\text{mL}$. Spleens from C57BL/6 mouse were harvested with sterility and gently mashed by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). After the red blood cells were lysed, the splenocytes were washed twice and resuspended in complete RPMI-1640. Splenocytes ($1.0 \times 10^6/\text{mL}$) were cultured in 96-well plates in a volume of 100 $\mu\text{L}/\text{well}$. The mature DCs were added into each well, four wells each group. The cultures were incubated at 37 °C and 5% CO_2 for 72 h. In the last 4 h of incubation, MTT (Amresco, 5 mg/mL, 30 $\mu\text{L}/\text{well}$) was added into each well. Then supernatant was discarded before DMSO (100 $\mu\text{L}/\text{well}$) was added. Finally, A_{570} was tested as the index of DCs stimulating the proliferation of MLR.

2.2.4. Ability of antigen presentation assessment of salidroside liposomes on DCs

OVA solution mixed with alum adjuvant, each mouse was immunized subcutaneous with 100 μg OVA. On the 7th, 14th day, the mice were separately boosted. Three days after the last immunization, the responding cells were collected as “Section 2.2.3”. Lymphocytes were resuspended in complete RPMI-1640 at a concentration of $1.0 \times 10^6/\text{mL}$, supplemented with OVA (100 $\mu\text{g}/\text{mL}$), in round-bottomed 96-well plates. The DCs were treated as in Section 2.2.3. DCs having been treated with mitomycin C were added into lymphocytes, four wells each group. The cultures were incubated at 37 °C and 5% CO_2 for 72 h. In the last 4 h of incubation, MTT (5 mg/mL, 30 $\mu\text{L}/\text{well}$) was added into each well. Thereafter, supernatant was discarded before DMSO (100 $\mu\text{L}/\text{well}$) was added. Finally, A_{570} was tested as the index of ability of antigen presentation of DCs.

2.3. Animal treatment protocol

The same quantity of female and male C57BL/6 mice (4-week-old) was purchased from Comparative Medicine Centre of Yangzhou University and acclimatized for 7 days prior to use. The mice were maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of $50 \pm 10\%$, and a 12/12 h light-dark cycle with free access to food and water. Each mouse was used once and treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.4. Immunization

Mice were injected subcutaneously in the dorsal skinfold on day 0, with a boost on 1 week and 2 week, with 0.5 ml each formulation containing of OVA (100 μg) and salidroside liposomes (0.8 mg), salidroside (1.6 mg), alum adjuvant and blank liposome, respectively. On 1, 2, 3, 4, 5 and 6 weeks after the first vaccination, mice were euthanized and the

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