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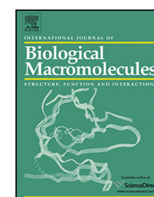

Microemulsion can improve the immune-enhancing activity of propolis flavonoid on immunosuppression and immune response

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ABSTRACT

The objective of the present study was to investigate whether the immune-enhancing activity of propolis flavone (PF) could be improved after PF was made into PF microemulsion (PFM). Two experiments were carried out. In immunosuppression experiment, the immune-enhancing effect of PFM in immunosuppressive chickens was performed. The results showed that PFM at high and medium doses was able to overcome the CTX-induced immunosuppression, significantly increases the immune organ indexes, enhances lymphocyte proliferation and improves the concentrations of IL-2 and IL-6 in serum when compared with PF. In immune response experiment, the adjuvant effect of PFM at three doses and PF were compared on chickens which were immunized intramuscularly with Avian Influenza Recombinant Newcastle Disease Virus bivalent Vaccine. The results showed that PFM at high and medium doses could significantly promote lymphocyte proliferation, enhances antibody titer and the concentrations of IgG and IgM, and its efficacy were significantly better than PF at most time points. These results indicated that PFM could significantly improve the immune-enhancing activity and adjuvanticity of PF, and its high and medium doses possessed the best efficacy. Therefore, the microemulsion could be used as an effective formulation for enhancing the bioavailability of PF.

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1. Introduction

Viral infectious diseases have been widely recognized as serious diseases that harm the breeding industry and cause heavy financial losses. Nowadays, the prevention and control methods to these diseases still largely relied on vaccines. At present, suitable immunopotentiators are widely used for enhancing the efficacy and stability of vaccine besides selecting immunogens [1,2]. In recent years, new generation vaccine has developed rapidly such as synthetic peptide vaccine, genetically engineering vaccine and so on. These vaccines possess great antigenic specificity and low toxicity but poor immunogenicity and unable to induce strong immune reactions, so it is necessary to be used in combination with safe and high-performance immunopotentiators [3]. Because of the disadvantages of the commonly used adjuvants in clinic like aluminum adjuvant, Freund's adjuvant, oil adjuvant and so on [4–6], it is particularly important to develop new immunopotentiators especially from the natural medicine [7]. Studies show that the immunoregulation effect of the traditional Chinese medicine on immune system is versatile. It not only activates T-cell, B-cell, macrophage, natural killer cell and other immunological cells but also activates complement and promotes production of cytokine [8–10].

Propolis is a resinous material that honeybees collect from leaf buds and cracks in the bark of the poplar genus and other tree species [11]. Propolis as a harmless natural product has been used in folk medicines in many regions of the world from ancient times and recently [12]. Recent research has highlighted that propolis exhibits a wide range of biological and pharmacological properties, such as hepatoprotective, antitumor, antioxidative, antimicrobial and anti-inflammatory properties [13–15]. Due to these characteristics, which can bring health benefits, propolis is considered as a functional ingredient and used in food, beverages, cosmetics and medicine for improving health and preventing diseases. Although more than 300 constituents have been identified in propolis during the past few years, these functional properties are mainly ascribed to a few substances: principally polyphenols of flavonoids, phenolic

Abbreviations: PF, propolis flavonoid; PFM, propolis flavonoid microemulsion; RH-40, polyoxyethylene (40) hydrogenated castor oil; CTX, cytoxan; PHA, phytohemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; NC, normal control; AI, avian influenza; ND, Newcastle disease; VC, vaccine control; BC, blank control; IL-2, interleukin-2; IL-6, interleukin-6; IgG, immunoglobulins G; IgM, immunoglobulins M.

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acids and their esters, etc., of which flavonoids in particular are considered as the top contributors [16]. Unfortunately, because of its poor solubility in water, low oxidation stability and bioavailability, and short half-life, its clinical application was limited [17].

Microemulsion has generated considerable interest over the years as potential drug delivery system [18,19]. It is defined as colloidal, optically isotropic, transparent or slightly opalescent formulations of low viscosity consisting of surfactant, co-surfactant, oil and water [20]. Microemulsion offers the advantages of thermodynamic stability, ease of preparation, solubilization capacity of lipophilic, hydrophilic and amphiphilic solutes, improved solubilization and bioavailability of hydrophobic drugs, and in consequence, improved drug delivery [21]. Microemulsion systems have emerged as novel vehicles for drug delivery which allow sustained or controlled release for transdermal, topical, oral, intravenous, parenteral and other administration routes of drugs, improve target specificity and therapeutic activity, and reduce toxicity of drugs [22,23]. Owing to these advantages, they show an enormous potential to be used as delivery vehicles for drugs [24].

Our previous researches demonstrated that PF possessed a better immune enhancement on humoral and cellular immunity in chickens and mice [25,26]. In this study, oil in water (o/w) microemulsion system of propolis flavonoids (PF) was prepared. The immune-enhancing activity of PF microemulsion (PFM) on immunosuppression was evaluated in CTX-induced immunosuppressive chickens, the adjuvanticity was also evaluated for inducing humoral and cellular immunity in chicken against Avian Influenza Recombinant Newcastle Disease Virus bivalent Vaccine (AI–ND vaccine). The main objective of this research was expected to manifest the possibility of microemulsion on further improving the immune-enhancing activity and adjuvanticity of PF.

2. Materials and methods

2.1. Materials

Propolis was purchased from Dahua Traditional Chinese Medicine Company in Nanjing, Jiangsu province. Propolis flavonoids (PF) were prepared in our laboratory. Polyoxyethylene (40) hydrogenated castor oil (RH-40) was purchased from BASF (Berlin, Germany). Cytoxan (CTX) was purchased from Alading Co., Ltd. Lymphocytes separation medium was manufactured by Tianjin Haoyang Biological Co., Ltd. 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), 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 phosphate-buffered saline (PBS, pH 7.2). These reagents were filtered through a 0.22 μm millipore membrane filter. Dimethyl sulfoxide was produced by Zhengxing Chemical Co., Ltd. (Suzhou, China). Ethanol and ethyl acetate were analytical grade and supplied from Kermel Chemical Co., Ltd. (Tianjin, China).

2.2. Vaccine and antigen

Avian Influenza Recombinant Newcastle Disease Virus bivalent Vaccine, Live (rL-H5 Strain, EID_{50}, 6.0) was purchased from Harbin Weike Biotechnology Development Company. ND and AI antigens were provided by Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.3. Preparation of PFM

Based on the results of preliminary experiments, the menstruums were selected with a better solubility of PF. Therefore, ethyl acetate, RH-40, ethanol, deionized water were selected as the oily phase, surfactant, cosurfactant and aqueous phase respectively. Besides, the optimal ratio of surfactant and cosurfactant was set as 2:1 (w/w) by pseudo-ternary phase diagrams. First, PF was added into the mixtures of oil, surfactant and cosurfactant with a certain ratios. Then water was added into the mixture dropwise and stirring for 24 h at 25 °C. Then the solution was filtered by 0.45 μm membrane. The average particle size, zeta potential and PDI of PFM were 16.42 nm, −3.27 mV and 0.202, respectively. Under electron microscope, PFM had a spherical appearance. The diameters of the microemulsion particles observed by transmission electron microscopy were in good agreement with the particle sizes determined as described earlier.

2.4. Experimental design

2.4.1. Experimental animals

One-day-old White Roman chickens (male) purchased from Wugong Poultry Farm were housed in wire cages (40 cm × 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 Shaanxi Chia Tai Co., Ltd. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.4.2. Immunosuppressive experiment

Three hundred one-day-old chickens were randomly assigned into six groups, fifty chickens per group. One group of healthy chickens was used as normal control (NC) group. From days 11 to 13, the other five groups of chickens were intramuscularly injected with CTX (80 mg/kg/d) once a day for 3 successive days to induce immunosuppression, and one group of those CTX-treated chickens was used as a model group (CTX group). At 14-day-old, the chickens in four experimental groups were injected respectively with 1.0 mL of PFM at high (2.0 mg mL^{-1}), medium (1.5 mg mL^{-1}) and low (1.0 mg mL^{-1}) dose, and PF (2.0 mg mL^{-1}), respectively, in NC and CTX groups, with normal saline, once a day for 3 successive days. On days 7, 14, 21 and 28 after the last drug administration, the blood samples of six chickens were collected randomly from each group for determination of peripheral lymphocyte proliferation by MTT assay, and the concentration of IL-2 and IL-6 with enzyme-linked immunosorbent assay (ELISA). On day 28 after the last drug administration, the thymus, spleen and bursa of Fabricius of 10 chickens from each group were excised randomly and weighed to calculate the immune organ index.

2.4.3. Immune response experiment to AI–ND vaccine

Three hundred 14-day-old chickens were randomly assigned into 6 groups and vaccinated with AI–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 five experimental groups were intramuscularly injected respectively with 1.0 mL of PFM at high (2.0 mg mL^{-1}), medium (1.5 mg mL^{-1}) and low (0.5 mg mL^{-1}) dose, and PF at 2.0 mg mL^{-1} respectively, in vaccine control (VC) and BC groups, 1.0 mL of physiological saline. On days 7, 14, 21, 28 and 35 after the first vaccination, the blood samples of six chickens were collected randomly from each group for determination of peripheral lymphocyte proliferation by MTT

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