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Epimedium polysaccharide and propolis flavone can synergistically inhibit the cellular infectivity of NDV and improve the curative effect of ND in chicken

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ABSTRACT

Four prescriptions, epimedium flavone plus propolis flavone (EF-PF), epimedium flavone plus propolis extracts (EF-PE), epimedium polysaccharide plus propolis flavone (EP-PF) and epimedium polysaccharide plus propolis extracts (EP-PE), were prepared and their antiviral effects were compared. In test in vitro, the four prescriptions within safety concentration scope and Newcastle disease virus (NDV) were added into cultured chick embryo fibroblast (CEF) in three modes, pre-, post-adding drug and simultaneous-adding drug and virus after being mixed, the cellular A_{570} values were determined by MTT method and the highest virus inhibitory rates were calculated to compare the antiviral activity of four prescriptions. In test in vivo, three hundred 21-day-old chickens were randomly divided into 6 groups and challenged with NDV except for blank control group. After 24 h the chickens in four prescription groups were injected with corresponding drugs respectively, in virus control and blank control groups, with physiological saline, once a day for three successive days. On days 3, 7 and 14 after challenge, the serum antibody titer was determined. On day 15 after challenge, the mortality, morbidity and cure rate in every group were counted. The results showed that the most of A_{570} values in EP-PF group were numberly or significantly larger than those of the corresponding virus control group and the highest virus inhibitory rates of EP-PF at optimal concentration group were the highest among four prescription groups in three drug-adding modes, which confirmed that EP-PF could significantly inhibit the infectivity of NDV to CEF, its action was stronger than those of other three prescriptions; in EP-PF group, the antibody titers and cure rate were the highest and the mortality and morbidity were lowest presenting numberly or significantly differences in comparison with other three prescription groups. These results indicated that epimedium polysaccharide and propolis flavone possessed synergistical action, EP-PF prescription could significantly inhibit the cellular infectivity of NDV, improve the curative effect of ND in chicken and would be expected to exploit into a new-type antiviral drug.

1. Introduction

Animal infectious diseases, especially the viral diseases, are worldwide concerned as they usually cause a great loss in domestic animal and poultry industry [1]. Because virus has unique biological characteristics and pathogenesis, there are no effective treatment methods for viral diseases [2]. Vaccination is the most common preventive method, but some infectious diseases are still difficult to control because the efficacies of some vaccines are severely decreased when a virus circulation does not have a good match with vaccine strains due to antigenic drift or inaccurate epidemiological predictions [3], or some vaccines with inferior quality, improper conservation and transportation problems [4]. Some cytokines, such as medical exogenous IFN and IL-2, can inhibit viral replication and improve cellular immunity, but they still stay in the laboratory research or clinical trial stage because of high cost [5]. A few chemical drugs have been used, but their clinical effects are not satisfied and there are obvious negative effects such as drug residues, drug tolerance, high recurrence rate, environmental pollution and so on [6]. Therefore it becomes so urgent to study and develop new-type antiviral drugs with high efficiency and low toxicity.

Abbreviations: BC, blank control; CEF, chick embryo fibroblast; CHM, Chinese herbal medicine; CHMI, Chinese herbal medicinal ingredient; DMSO, dimethyl sulfoxide; EF, epimedium flavone; EF-PE, epimedium flavone plus propolis extracts; EF-PF, epimedium flavone plus propolis flavone; EP, epimedium polysaccharide; EP-PE, epimedium polysaccharide plus propolis extracts; EP-PF, epimedium polysaccharide plus propolis flavone; MEM, Eagle's minimum essential medium; MM, maintenance medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDV, Newcastle disease virus; PBS, phosphate buffered saline; PE, propolis extracts; PF, propolis flavone; VC, virus control.

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In recent years, it has been proved that many Chinese herbal medicines (CHMs) and their ingredients (CHMIs) possess antiviral effect [6,7]. Some CHMs or their extracts have been developed into antibacterial and antiviral agents [8]. For example, "Tamiflu" is an accepted effective drug for H_1N_1 influenza and bird flu. Its raw material is *Illicium verum Hook*.f. of CHMs [9]. The pharmacological effects of CHM are based on their chemical constituents. Polysaccharide and flavone are two classes of the most important active ingredients and can enhance immune function of organism and inhibit the virus infection [10–12]. In previous researches, it was confirmed that epimedium polysaccharide (EP), epimedium flavone (EF), propolis flavone (PF) and propolis extracts (PE) could inhibit the cellular infectivity of some viruses [13,14]. The further researches showed that some compound CHMIs (cCHMIs) had stronger effect in comparison with the single ones [15].

In present research, four prescriptions, epimedium polysaccharide plus propolis flavone (EP-PF), epimedium polysaccharide plus propolis extracts (EP-PE), epimedium flavone plus propolis flavone (EF-PF) and epimedium flavone plus propolis extracts (EF-PE), were prepared and their antiviral effects were compared by determination of the effect on cellular infectivity of Newcastle disease virus (NDV) and on curative effect of Newcastle disease (ND) by artificial challenge. The purpose of this research is to observe the antiviral action of cCHMIs, search for optimal combination of CHMI, select out the best prescription and offer theoretical evidences for development of new-type antiviral drugs.

2. Materials and methods

2.1. Preparation of prescriptions

Epimedium polysaccharide (EP, net content of 71.23%), epimedium flavone (EF, net content of 60.89%), propolis flavone (PF, net content of 70.99%) and propolis extracts (PE, PF content of 50.33%) were provided in our laboratory. Four prescriptions, EF-PF, EF-PE, EP-PF and EP-PE were prepared according to a certain proportion based on our previous experiments. They were diluted into 1.5 mg mL^{-1} (net content) with deionized water (Key Laboratory of Nanjing Agricultural University), sterilized and stored at 4 °C. For test in vitro, they were diluted into nine working concentrations (250–0.977 μg mL^{-1}) in two-fold serial dilutions with MM containing 2% fetal bovine serum. For test in vivo, the endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia [16].

2.2. Reagents

Eagle's minimum essential medium (MEM) (Gibco) supplemented with penicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 5% fetal bovine serum was called growth medium and used for culturing the cells, 2% fetal bovine serum, maintenance medium (MM) was used for diluting the polysaccharides and maintaining the cells. Hank's solution was used for washing the chick embryo tissue shiver and cells. Trypsin (Amresco-0858) was dissolved with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4) into the concentration of 0.25%. MTT (Amresco Co.), 3-(4,5-dimethyithiazol- 2-yl)-2,5-diphenyltetrazolium bromide was dissolved with CMF-PBS (pH 7.4) into 5 mg mL^{-1}. These reagents were filtered through a 0.22 μm millipore membrane filter. MEM and MM were stored at 4 °C; trypsin solution, at −20 °C; MTT solution, at 4 °C in dark bottles. Dimethyl sulfoxide (DMSO, No. 30072418) was the production of Chemical Agent Company of Chinese Medicine Groups, Nanjing. Other chemicals used in the experiment were analytical grade.

2.3. Virus

ND vaccine (La Sota strain IV, No. 090820) was bought from Beijing Veterinary Bio-drug Company. $TCID_{50}$ of the virus liquid was 1×10^{-8} by Reed-Mueeh assay [17]. It was diluted into 10^{-6} (100 $TCID_{50}$) with 2% MEM in vitro. ND virus (NDV, $F_{48}E_9$ strain) supplied by China Institute of Veterinary Drug Control was propagated with 10-day-old specific pathogen-free (SPF) chicken embryo and used for challenge experiment.

2.4. Determination of test in vitro

2.4.1. Cytotoxicity analysis

CEF were prepared with 10-day-old specific pathogen-free (SPF) chicken embryo (Nanjing Pharmaceutical & Apparatus Factory of China Animal Husbandry Industry Company). The cells was diluted into 1×10^6 mL^{-1} with 5% MEM and inoculated into 96-well culture plates at 38.5 °C in a humid atmosphere of 5% CO_2 for use.

When CEF grew into monolayer after cultivation about 24 h, in four prescription groups, EF-PF, EF-PE, EP-PF and EP-PE at series of concentrations were added into the plates respectively, four wells for each concentration. At the same time, cell control group (only adding MM) and blank group (no cell) were designed. After cultivation for 68 h at 38.5 °C in a humid atmosphere of 5% CO_2, 30 μL of MTT was added into each well, after continuously incubated for 4 h, the supernatant was removed and 100 μL of DMSO was added. The plates were shaken for about 5 min to dissolve the crystals completely. The absorbance at 570 nm (A_{570} value) of each well was measured by microliter enzyme-linked immunosorbent assay reader (Model DG-3022, East China Vacuum Tube Manufacturer) [18,19].

2.4.2. Antiviral assays

According to the result of safety concentration test, the prescriptions were diluted into 5 concentrations from 15.625 to 0.977 μg mL^{-1} with MM respectively. When CEF grew into monolayer, the prescriptions and NDV solution were added in three drug-adding modes respectively [20].

Pre-adding drug. Prescription solution was added into CEF plate firstly, 100 μL per well and four wells per concentration. After incubated for 4 h at 38.5 °C, the prescription solution was removed, the cell was washed twice with Hanks' solution and the virus solution was added.

Simultaneous-adding drug and virus. The prescription solution and virus solution were mixed and incubated for 2 h at 4 °C, then added into CEF plate, four wells per concentration.

Post-adding drug. The virus solution was added into CEF plate firstly, after incubated for 2 h at 38.5 °C, the virus solution was removed, the cells were washed twice with Hank's solution, prescription solutions were added, four wells per concentration.

At the same time, the NDV control group (only adding virus), cell control group (only adding MM) and blank group (no cell) were designed. All the plates were placed into 5% CO_2 incubator at 38.5 °C. When the NDV control group appeared obviously cytopathic effect (72 h), the CEF livingness (A_{570} value) was measured by the MTT method, The virus inhibitory rate was calculated based on the formula [21]: Virus inhibitory rate $= (\bar{A}_{\text{prescription+virus}} - \bar{A}_{\text{virus control}})/(\bar{A}_{\text{cell control}} - \bar{A}_{\text{virus control}}) \times 100\%$. The A_{570} values and virus inhibitory rate were considered as the indicator of antiviral activity.

2.5. Determination of test in vivo

2.5.1. Animals

One-day-old White Roman chickens (male) purchased from Tangquan Poultry Farm were housed in wire cages (60 cm ×