Therapeutic Efficacy of the Combined Extract of Herbal Medicine Against Infectious Bursal Disease in Chickens

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ABSTRACT
Currently, infectious bursal disease (IBD) is a highly contagious disease leading to huge economic losses in poultry industry. Our objective was to investigate potential therapeutic effects of the combined extracts of Rhizoma Dryopteridis crassirhizomatis and Fructus mume (RDCFM) against IBDV infection. Seventy-two 4-week-old SPF chickens were randomly divided into six groups and inoculated intranasally with 0.2 ml of 10^{2.5} EID50 of IBDV strain CJ801. Twenty-four hours post infection, the birds were orally administered with 400, 200 and 100 mg/kg BW of RDCFM, respectively, 125 mg/kg Astragalus polysaccharide (ASP) and saline water, respectively for 5 days and then monitored daily for 10 days. Finally, the remaining birds were euthanized to collect the sera for detecting antibodies and immune organs for determining the immune organs index as well as virus loads. The herbal extracts improved survival rate and relative body gain rate. Virus loads in bursa of Fabricius in herbal treated groups decreased significantly while higher antibody levels were detected in the three RDCFM groups as compared to those of ASP and infection group. These results implied that chickens administered with 100-200 mg/kg of RDCFM for 5 days could improve protection against IBDV infection and RDCFM may be a promising alternative to ASP and egg yolk antibody.

INTRODUCTION

Rhizoma Dryopteridis crassirhizomatis (RDC) and Fructus mume (FM) have been used in some herbal prescriptions since ancient times in China. In particular, RDC has been widely applied to formulate antiviral granules for severe patients during the severe acute respiratory syndrome (SARS) outbreak in 2003 (Zhao et al., 2007). Furthermore, RDC has been shown to possess anti-cancer properties (Chang et al., 2010) and anti-viral applications (Lee et al., 2008; Min et al., 2001). On the other hand, FM juice has also been shown to inhibit human influenza-A virus infection by inhibiting viral hemagglutinin attachment to host cell surfaces (Yingsakmongkon et al., 2008; Zhao et al., 2007). In recent reports, the combination of FM, Schizandraceae Fructus or Coptidis Rhizoma has shown an effective antimicrobial activity against enterohemorrhagic Escherichia coli infection (Lee and Stein, 2011). The active substances (4-hydroxyxinnamic acid and protocatechuic acid) isolated from FM were confirmed to induce a better protection and improve the immune response for chickens inoculated with infectious bursal disease virus (IBDV) (Ou et al., 2011; Ou et al., 2012; Okwor et al., 2012). However, the combined extracts of RDC and FM haven’t been formulated to apply in poultry industry and its potential therapeutic efficacy on SPF chickens infected with IBDV should be evaluated.

Infectious bursal disease is an acute, highly contagious disease for young chickens and is prevalent in chicken farms throughout the world (Eterradossi and Saif, 2008; Lone et al., 2012). Moreover, IBD leads to severe economic losses due to high morbidity and mortality in an acute form or severe immunosuppression or secondary infections (Muller et al., 2003). However, immunity failure occurs at any time, thereby leading to tremendous difficulties in controlling IBD due to the presence of variant strains (Craft et al., 1990). IBDV vaccine and drug development must be accelerated due to the increasing prevalence of IBDV. Therefore, the cheap RDC was used...
for formulating a prescription with FM in this study and our objective was to evaluate whether RDCFM could provide a better protection against IBDV infection by increasing the immune response in chickens.

**MATERIALS AND METHODS**

**Herbal medicines:** Astragalus polysaccharide (ASP) (batch no. 20110309) was purchased from the Institute of Veterinary Drug and Control, China and the concentration of ASP was determined to be 55.0% using HPLC method (Chen et al., 2003). RDC and FM were purchased from a pharmacy (Xiyanuan Traditional Medicine Hospital, Beijing, China) and authenticated by Professor Jianyong Si, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College.

**Preparation of herb extracts:** A heat reflux method was employed for herb extraction. Briefly, RDC was ground to a powder using a mill and was then mixed with FM at a ratio of 6:4 (w/w). The mixture was soaked with an ethanol/water (75:25, v/v) solution (1:10, w/v) for 24 hours. Finally, the mixture was boiled for 2 hours and filtered to collect the liquid extracts. Subsequently, the solvent was removed under vacuum using a rotary evaporator. The yield of the dried syrup accounted for 23±1% of the dried herbs and the preparation was kept at 4°C until use.

**Quality control of herb extracts:** Citric acid was used to control the FM quality according to the protocol of Chinese Pharmacopoeia while Dryocrassine ABBA was considered as the main active ingredients and quality control reference in RDC. Therefore, the quality control of RDCFM was evaluated by the quantitative determination of citric acid and Dryocrassine ABBA using HPLC and visible spectrophotometry (Gao and Su, 2009; Li et al., 2010).

**Virus:** A classical IBDV strain CJ801 was donated by Prof. Liu Jue, Beijing Institute of Husbandry and Veterinary Research. The LD₅₀ of IBDV CJ801 strain for SPF chickens was determined to be 10⁻²⁴ EID₅₀ in 0.2 ml (Zhou et al., 2010).

**Chickens and experimental design:** All the studies were performed on 4-week-old specific-pathogen-free (SPF) chickens (Vital Bridge Co. Ltd., Beijing, China). The experimental procedures and animal management were approved by the Institutional Animal Care and Use Committee at China Agricultural University. Seventy-two chickens were randomly divided into 6 groups and challenged intranasally with 0.2 ml IBDV CJ801 strain solution except the healthy control group. 24h post challenge, the birds were orally administered with 400 mg/kg (high dosage group), 200 mg/kg (medium dosage group), 100 mg/kg body weight RDCFM (low dosage group), 125 mg/kg body weight ASP (positive control group), the same volume of solvent (infectious control group) and the no-treatment group (healthy control group). Each group was maintained in individual negative pressure isolators. All chickens were provided feed and water ad libitum. The body gain and mortality among all groups were monitored for 10 days. On day 10, the birds were euthanized and sacrificed to collect the serum samples for the detection of antibody and immune organs for determining the virus loads as well as the immune organs index.

**Quantification of IBDV in the bursa of Fabricius:** The total RNA was isolated from the homogenated bursa of Fabricius using the RNasey Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. Total RNA was then reverse transcribed into cDNA and amplified and quantified using the QuantiTect SYBR Green Kit (Qiagen, Germany). β-actin was used as a loading control. The primers were designed to amplify the VP2 gene and β-actin as previously described: IBDV-P1: 5'-AGA AGC ACA CTC TCA GGT CA-3', IBDV-P2: 5'-AGT GTG CTT GAC CTC ACT GT-3'; β-actin-P1: 5'-CAT CAC CAT TGG CAA TGA GAGG-3', β-actin-P2: 5'-GCA AGC AGG AGT ACG ATG AAC-3' (Tham et al., 1995; Khatri and Sharma, 2006). Each reaction was performed in triplicate using the above primers. The real-time PCR procedure was briefly described as follows: an initial incubation for 5 min at 94°C, 40 cycles of 30s at 94°C, 30s at 68°C and 30s at 60°C. The quantification was based on the fluorescence detection by real-time PCR (Bio-Rad, USA). The viral amount in the samples was evaluated by relative quantification.

**Antibody detection:** The antibody levels against IBDV were measured using a commercial test kit (BioChek, Holland). All procedures were performed according to the manufacturer’s instructions. Briefly, 100μl of diluted samples were added into the appropriate wells of IBDV-coated plates. The positive serums and negative samples were also added to the wells. After several washes, the conjugate reagent and substrate reagent were added to react with above samples. Finally, 100μl of stop solution was added to terminate the reactions and the OD values were recorded at 405 nm.

**Statistical analysis:** All data were expressed as the mean ± standard error. The two-tailed one-way ANOVA was employed for all statistical analyses using SPSS 13.0. The results were considered significantly different at the level of P<0.05 or P<0.01.

**RESULTS**

**Determination of citric acid and ABBA in RDCFM extract:** The chromatograms were shown in Fig. 1, including methanol control, positive citric acid control, RDCFM sample and standard curve. The retention time for citric acid was approximately 7.17 min while the calibration curve ranged from 0.2 to 2.4 mg/ml. The citric acid concentration was determined to be 120 mg per gram extract based on the regression equation Y=1E-06 X+0.0072 (R²=0.9997). As for the concentration of Dryocrassine ABBA, the regression equation was A=0.703C⁺0.0436 and OD value was 0.568. Finally, the concentration of Dryocrassine ABBA was 7.459 mg per one gram of the RDCFM extract.
Clinical performance and survival rate: After inoculated with IBDV, the chickens showed classical symptoms, such as decreased feed intake, fluffed feathers and diarrhea. Chickens died on day 3 post challenge and the mortality amounted to the peak on day 4. The remaining birds gradually recovered on day 7 and returned to normal activities on day 10.

The survival rate, relative body gain rate and immune organs index were listed in Table 1. The survival rates were 66.7, 75.0 and 75.0% in the three RDCFM-treated groups, which were higher than those in the ASP and infection groups (58.0 and 50.0%). The relative weight gain rates in drug treatment groups (67.06, 67.92, 62.2 and 64.96%) were significantly higher than that of the infection group (52.88%) (P<0.05).

The RDCFM improved the spleen index in the three groups (3.06, 3.02 and 2.99) while only the bursa index in the 200 mg/kg group (up to 1.41) was higher than those in ASP and infection groups.

Virus loads in bursa of Fabricius: The virus clearance was evaluated by the quantification of the IBDV genome in bursa organs. Based on a standard curve, the relative amount of viral RNA was calculated by log_{10}C_{T} in Fig. 2. The virus loads of the bursal organs in herbal groups were significantly lower than those of the ASP and infection groups (P<0.05). However, there were no significant differences among the three herbal groups.

Antibody detection: The antibody levels for RDCFM groups, ASP group and infection group were shown in Fig. 3. Anti-IBDV antibody levels of the medium- and low-dosage groups were significantly higher (P<0.01) than those of the ASP and infection groups. Additionally, antibody level for the high-dosage group was also significantly higher than that of the ASP group (P<0.05).

DISCUSSION

In the present study, the survival rate amounted to 75% both in the medium and low concentration of RDCFM. Meanwhile, body weight gains, antibody level, virus clearances and immune organ index in the three
intensive labor requirement. Currently, Chinese farmers have been accredited for legal veterinary drugs against IBDV (Kibenge et al., 1988). In this experiment, the protection rate was an objective index to evaluate the efficacy of the combined extracts. A significant survival rate was observed both in the medium group and in the lower herbal-treated group as compared to ASP group, which implied that RDCFM had the therapeutic efficacy against IBDV infection. In the previous reports, spleen and bursa are the target organs of IBDV (Zierenberg et al., 2004). Therefore, the immune organ indexes are used for determining the lesions in spleen and bursa post IBDV infection. Obviously, 200 mg/kg herbal extract was found to have a significant increase of spleen index as well as a good protection of bursa in comparison with ASP and infection groups.

The high levels of antibody responses would induce the significant protection against the virulent IBDV infection (Hsieh et al., 2010). In our experiment, higher antibody levels in the three herbal extract groups were corresponded with the higher body weight gain, the increasing survival rate, lower virus load and better organ indexes post IBDV infection. In this sense, the infected chickens could get protection due to improving birds’ appetite and humoral immunity. In previous reports, chickens administered with ASP were able to get a good protection against IBDV infection due to the increasing erythrocyte-C3b receptor and the immune complex rosette rate (Li et al., 2007). Hence, the combined herbal extracts might involve the protection of B cells in the bursa of Fabricius and induce higher antibody levels post IBDV infection.

Until recently, both the egg yolk antibody and ASP have been accredited for legal veterinary drugs against IBDV in China. Particularly, these substances are complementary approach for vaccine failure, early subclinical infections and the occurrence of the virulent IBDV strains in the field (Kibenge et al., 1988). Nevertheless, the application of egg yolk antibody caused multiple adverse effects, such as the risk of secondary infection and immune depression (Hatta et al., 2008). Furthermore, the egg yolk antibody is not suitable for clinical applications in large poultry flocks due to the intensive labor requirement. Currently, Chinese farmers turn to large amounts of ASP in routine treatments for antiviral activity. As a result, forthcoming viral resistance will be inevitable in the poultry industry. The potential outbreak of new very virulent strains will challenge the health of poultry. Therefore, RDCFM might be an alternative to ASP and egg yolk antibody as a new medicament against IBDV infection.

Based on the evidence presented here, we conclude that RDCFM is a promising medication against IBDV infection by improving antibody level and decreasing virus load in the targeted organs. Further investigations will be performed to understand the mechanism of the herbal extract and expand the potential application of RDCFM against other avian viral diseases.

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