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Immune regulation of avian influenza vaccine in hens using *Hypericum perforatum* L. methanol extraction

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Abstract

Hypericum perforatum L. has been widely used for centuries as a medicinal herb. In present study, the effect of *Hypericum perforatum* extract (HPE) as a dietary supplement on humoral regulation to influenza vaccine in hens was investigated. Chicks were immunized with reassortant avian influenza (AI) virus H5 subtype vaccine, inactivated (H5N1, Re-5+Re-4 strain) administered intramuscularly on day 20 of age (primary vaccination) followed by a boosted 20 days after the first vaccination. Chicks received the basal soybean meal-corn diet supplemented with 0 (control), 250, 500, and 1,000 mg kg^{-1} HPE for 7 days starting on the day of each vaccination. Antibody concentration against AI was measured by enzyme-linked immunosorbent assay (ELISA) method and antibody titer within a few days of vaccination detected by hemagglutination inhibition (HI) test. Diet supplementation with HPE at 500 mg kg^{-1} was associated with increased antibody level to H5 subtype AI by 9.82% after the first vaccination and by 30.63% after the boost ($P \leq 0.05$). Furthermore, HPE also increased Re-4 polyclonal H5 subtype AI antibody titer by 11.27% after first immunization and 27.40% after second immunization ($P \leq 0.05$), respectively. While, Re-5 polyclonal H5 subtype AI antibody titer in 500 mg kg^{-1} HPE addition level of HPE were higher than control group after 1st vaccination and the 2nd vaccination, especially increased by 7.70% and 16.44% in antibody peaks ($P \leq 0.05$), respectively. HPE administered as a dietary supplement in the peri-immunization period augmented the humoral immunity of hens to the influenza vaccine. These findings suggest that HPE may be useful in the poultry industry.

Keywords: Avian influenza; ELISA; Hens; HI; *Hypericum perforatum* extract; Immunity.
Abbreviations: AF-after first time immunization (primary vaccination), eg: 10d AF means 10 days after the first time immunization; AS-after second time immunization (secondary vaccination), eg: 7d AS means 7 days after the second time immunization; AI- avian influenza; ELISA- enzyme-linked immunosorbent assay; HI- hemagglutination inhibition.

Introduction

Hypericum perforatum L is a popular over-the-counter dietary supplement and herbal remedy that has been used traditionally as an anti-inflammatory, healing agent and an antidepressant for treatment of mild to relieve depression and widely distributed in Europe, Asia, Northern Africa and naturalized in the USA (Brolis et al., 1998; Barnes et al., 2001; Greeson et al., 2001; Wang et al., 2001). Patterns of secretory structure and their relation to hypericin content in *H. perforatum* had investigated in detail by Lu et al. (2001). It is unknown namely what biologically active compounds of *H. perforatum* produce neutropic effects until now, but its well known that the chemical composition of the herb contains flavonoids, anthracene derivatives, phloroglucinols, tanning agents, hyperoside, rutin, etc and much of antioxodant activities was implicated in flavonoids (Hansen et al., 1999; Butterweck et al., 2000; Ganzera et al., 2002; Jurgenliemk and Nahrstedt, 2002; Zou et al., 2005; Kurkin and Pravdivtseva, 2007). Hypericin and pseudohypericin were showed to possess marked antiretroviral activity (Lavie et al., 1989), and Schempp et al. (2003) suggested the biologically active products obtained from *H. perforatum* exhibited the immunotropic properties.

Avian influenza (AI) virus cause serious disease in a wide variety of birds and mammals, numerous vaccines against AI had been developed and shown to be efficacious for prevention the disease experimentally (Perdue et al., 1999; Dinapoli et al., 2007), however, highly pathogenic AI virus outbreaks in commercial poultry has been increasing rather than being controlled and eradicated such as the world on the brink of a global outbreak of influenza in 1918 and 2003 (Oxford, 2000; Phillips and Killingray, 2002; Choi et al., 2004; Oxford and Lambkin, 2006). These haunting memories had caused concern about the ongoing outbreaking of AI in world, especially in Asia. In China, traditional herbal medicines had been used to treat influenza infection for 2000 years (Mori et al., 1999). These herbal medicines had been used clinically for various diseases and the use of dietary supplements derived from plants had accelerated in the world in recent years (Cowan, 1999; Celep et al., 2011; Chatterjee et al., 2011), such as *Geranium sanguineum* (Serkedjieva and Hay, 1998); *Fagopyrum esculentum* (Kim et al., 2010); the roots of *Scutellaria baicalensis* (Nagai et al., 1995); *Phyllanthus amatus* (Thyagarajan et al., 1988; Zulkaliph et al., 2011); *Maclura cochinchinensis* (Bunyapraphatsara et al., 2000); the marine diatom *Haslea ostrearia* (Berge et al., 1999). Thus, several hundred herb species have potential as novel antiviral agents had been studied (Jassim and Naji, 2003). The medicinal herb *H. perforatum* was interested to observe that it showed wonderful antioxidant activity *in vitro* (Conforti et al., 2002; Masuda et al., 2003). An extract of *H. perforatum* has been reported that increase the levels of serotonin and the serotonin

possesses a protective effect against oxidative damage in neuronal cells (Park et al., 2002). Nevertheless, nowadays research on this plant has mainly focussed on its antidepressant activity (Bombardelli and Morazzoni, 1995). Despite the studies on the antioxidant activity and bioactive components of *H. perforatum* are detailed, information of *H. perforatum* extract on the immune response of AI vaccine, especially in hens, has hitherto not been reported. Therefore, in our previous study, we investigated the effects of several levels of *H. perforatum* extract added to layers' diets on the level of antibody to H5 subtype AI influenza and filtered out the optimum supplemented level in enhancing the ability to prevent AI influenza.

Results

The measurement value of antibody level in different treatments

In the supplemental 500 mg HPE kg^{-1} treatment, antibody level was significantly higher than different levels of HPE supplement on day 21AS. The antibody level of four treatments was insignificant difference when compared to the control treatment at the 7d AS, 14d AS and 30d AS. Compared with the level of supplemental 1,000 mg HPE kg^{-1}, in which the antibody level was increased initially and then decrease before secondary vaccination, this phenomenon was contrary to other treatments (Fig. 1). Also, values of antibody level of supplemental 500 mg HPE kg^{-1} at 10d AF was 11.54% higher than control treatment and 31.03% higher than control treatment at 21d AS ($P \leq 0.05$), respectively (Table 2). At the 20d AF, antibody level of the 1,000 (mg kg^{-1}) HPE supplement treatment was the highest (20% higher than control group). The antibody level of all the treatments peaked at 21d AS and the measurement of 500 mg kg^{-1} HPE reached the highest.

The measurement value of Re-4 and Re-5 strain antibody titer level in different treatments

(Fig. 2) and (Fig. 3) showed the Re-4 and Re-5 antibody titer in different treatments at different testing time. On day 7d AS, both the Re-4 and Re-5 antibody titer under 500 mg HPE kg^{-1} were significantly higher than other treatments. In details, comparing with the control, in which the Re-4 antibody titer of 500 mg kg^{-1} supplement level was 11.27%, 21.63%, 27.40 % and 1.32 % higher ($P \leq 0.05$) at 10d AF, 7d AS, 14d AS and 30d AS, respectively (Table 3). Re-5 antibody titer level at the 500 HPE supplement treatment always the highest compared to other treatments (7.69% higher at 10d AF and 33.33% higher at 7d AS, respectively. $P \leq 0.05$).

The optimum level of HPE to immune response

Antibody level peaked 10 days after primary vaccination (10d AF) in different treatments except 1,000 mg kg^{-1} supplement which peaked 20 d later (20d AF). After the 2nd vaccination, the level peaked 21 days at 250 mg kg^{-1} supplement, 14 d at 500 mg kg^{-1} supplement and 7 d at 1,000 mg kg^{-1} supplement, respectively (Fig. 2 and Fig. 3). The Pearson correlation coefficients revealed Re-4 strain antibody titer is significantly related to the Re-5 strain antibody titer ($P \leq 0.01$) (Table 4). Overall, compared to control treatment, the supplemented with 500 mg kg^{-1} of HPE treatment increased Re-4 strain antibody titer for 11.27 % after the primary vaccination and 27.40 % after the second vaccination in antibody peaks ($P \leq 0.05$), respectively. The percentage of increasing yield of Re-4 strain antibody titer at supplemented of 250 and 1,000 mg kg^{-1} treatments had overall lower values than the supplemented with 500 mg kg^{-1} treatment. Similarly, the Re-5 strain antibody titer at supplemented with 500 mg kg^{-1} treatment was increased for 7.69 % at primary vaccination and 16.44 % at the second vaccination in antibody peaks, respectively.

Table 1. Nutrient content of the basal diet (%).

Nutrient	Compsition (%)
Yellow Corn	59.20
Dehulled soybean meal	26.20
Fish meal	2.50
Soybean oil	1.00
Calcium bicarbonate	1.10
Shell powder	3.50
Stone powder	5.20
Common salt	0.30
Premix [1]	1.00
Total	100.00

The premix contained per kilogram of diet: vitamin A, 10,000 IU; vitamin D_3, 1,500 IU; vitamin E, 20 IU; vitamin K, 2.3 mg; vitamin B_1, 1.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; choline chloride, 250 mg; folic acid, 0.5 mg; biotin, 220 µg; zinc, 90 mg; iron, 24 mg; iodine, 0.6 mg; selenium, 0.3 mg.

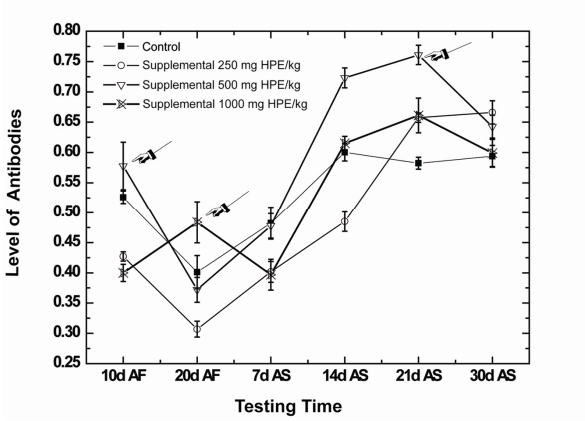

Fig 1. Changes of serum antibodies level to avian influenza virus of H5 subtype in layers by ELISA test. The points with the fingers stand for significantly sign ($P < 0.05$).

Discussion

In our study, the main effective ingredient of *Hypericum perforatum* extract (HPE) is hypericin, which had been widely used throughout the history of folk medicine (Kubin et al., 2005). As a dianthrone derivative, Brockmann et al. (1939) first detailed reported this chemical abstracts and hypericin is proving to medicinal applications because the activities of antidepressive, antitumor, antineoplastic and also, the antiviral (Brockmoller et al., 1997; Pince et al., 2000; Miskovsky, 2002; Saw et al., 2008; Prodan et al., 2010). Colds and the flu are serious threats to health in world which are caused by viruses, and the treatment on chemical or biochemical agents is mainly on numerous vaccines (Wang et al., 2006). In our study, we confirmed the phenomenon that enzyme-linked immunosorbent assay (ELISA) showed HPE supplement treatments were different from control group (Fig. 2). The peak got one week later and the antibody level was higher and last longer, especially in the treatment of 500 mg kg^{-1}. Thus, a HPE with hypericin content of 0.3% in the present experiment was found effective in advancing the immune respondence of AI. Serum

Table 2. Antibody levels as affected by different levels of HPE supplement under different tested time.

Testing time	Antibody level			
	HPE supplement level (mg/kg)			
	0	250	500	1000
10d AF	(0 %)	(−17.31 %)	(**11.54 %**)	(−23.08 %)
20d AF	(0 %)	(−22.50 %)	(−7.50 %)	(**20.00 %**)
7d AS	(0 %)	(−16.67 %)	(0 %)	(−16.67 %)
14d AS	(0 %)	(−16.67 %)	(20.00 %)	(3.33 %)
21d AS	(0 %)	(13.79 %)	(31.03 %)	(13.79 %)
30d AS	(0 %)	(13.56 %)	(8.47 %)	(1.69 %)

Values in parenthesis presented in each row mean the percentage increase or decrease as compared with control group (HPE supplement at 0 mg kg^{-1} case) and the maximum calculated value which was significantly high is in **bold** fonts ($P < 0.05$).

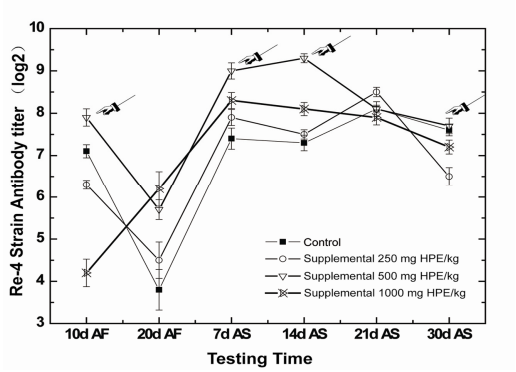

Fig 2. Changes of antibody titer level to avian influenza virus of H5 subtype (Re-4) in layers. The points with the fingers stand for significantly sign ($P < 0.05$).

antibody titer results showed the same phenomenon according with Ellis et al. (2004) that there was high correlation between Re-4 strain and Re-5 strain and HPE had similar effect on Re-4 and Re-5 strain. For world's poultry industry, the poultry consumption had increased greatly over years (Givens et al., 2011). The technology of feed ingredient suppliers in poultry had been applied for nearly two decades, like the commercial application of enzymes as a feed additive to enhance nutrient digestibility and alleviate environmental burdens by reducing P excretion in the excreta (Williams, 1999; Choct, 2006). Nowadays, poultry rations are made up of three groupings of ingredients: premixed (consists of vitamins, mineral, essential amino acids), protein and grain as an energy source. Sometimes prophylactic such as coccidiostat was added. The most prominent grain used worldwide is corn with wheat, triticale. There's less or no widely use feed additives though many reports had already showed stimulation of immune system by adding some natural products, like honeybee products, propolis (Giurgea et al., 1983; Blonska et al., 2004; Khalil, 2006), *Mosla scabra* (Yu et al., 2010), *astragalosides* polysaccharide, *Isatis* root polysaccharide, Chinese *angelica* polysaccharide (Kong et al., 2004). HPE supplement used in our study suggested that it could enhance cellular immunity and stronger as the Chinese herbal medical ingredients mentioned above. Thus, HPE supplement could be potential feed additive in purpose to improve humoral benefits of AI vaccination and get marketed commercial eventually. Mast and Goddeeris (1999) and Ben-Shira (2003) demonstrated that the immune function is attained during the first 2 weeks of life in neonatal chickens, functional maturation occurred at the first week post-hatch and the second week. Thus, it is suggested that chicken within 2 weeks aged used in our experiments is suitable and could produce normal immune responses.

Material and methods

Animals

Eight hundreds female chicks (one day old) obtained from commercial rearing farm were divided into 4 treatments in a completely randomized design and the diets were based on soybean corn (Table 1) regarding the Council procedure 1994 (NRC, 1994). *Hypericum perforatum* extract (HPE) content of the diet were 0 (control), 250, 500, and 1,000 mg kg^{-1} of diet. The chick treatments were manually feed the water and kept in an experimental house.

Experimental design

The HPE was feed after the first and second immunization lasted for 7 d, after weakly immunization program only include vaccination against AI virus by intramuscular injection. Blood samples were collected 10 and 20 d post vaccination and 7, 14, 21, 30 d after the 2nd immunization, from each treatment with randomly 20 chicks under wing vein (0.5-1.0 mL) and stored at 4 °C until being submitted to use, starting on the day 10 and continued to day 53 after primary immunization.

Preparation of HPE

Most clinical trial used standard HPE contain 0.3% hypericin, the dosage was around of 700 mg day^{-1}, because of extracting by ethanol, there are a few water insoluble and it is necessary to slightly enlarged dosage with feeding water. The HPE was provided by Shaanxi Hongda Industry Co., Ltd (China).

Table 3. Antibody titers as affected by different levels of HPE supplement under different tested time.

Testing time	Detection items			
	HPE supplement level (mg/kg)			
	(1) Re-4 strain antibody titer			
	0	250	500	1000
10d AF	(0 %)	(−11.27 %)	(**11.27 %**)	(−40.85 %)
20d AF	(0 %)	(18.42 %)	(50.00 %)	(63.16 %)
7d AS	(0 %)	(6.76 %)	(**21.62 %**)	(12.16 %)
14d AS	(0 %)	(2.74 %)	(**27.40 %**)	(10.96 %)
21d AS	(0 %)	(4.94 %)	(0 %)	(−2.47 %)
30d AS	(0 %)	(−14.47 %)	(**1.32 %**)	(−5.26 %)
	(2) Re-5 strain antibody titer			
	0	250	500	1000
10d AF	(0 %)	(1.54 %)	(**7.69 %**)	(−30.77 %)
20d AF	(0 %)	(−21.74 %)	(39.13 %)	(8.70 %)
7d AS	(0 %)	(9.80 %)	(**33.33 %**)	(5.88 %)
14d AS	(0 %)	(−1.37 %)	(16.44 %)	(6.85 %)
21d AS	(0 %)	(11.11 %)	(11.11 %)	(−4.76 %)
30d AS	(0 %)	(−1.49 %)	(7.46 %)	(−2.99 %)

Values in parenthesis presented in each row mean the percentage increase or decrease as compared with control group (HPE supplement at 0 mg kg^{-1} case) and the maximum calculated value which was significantly high is in bold fonts ($P < 0.05$).

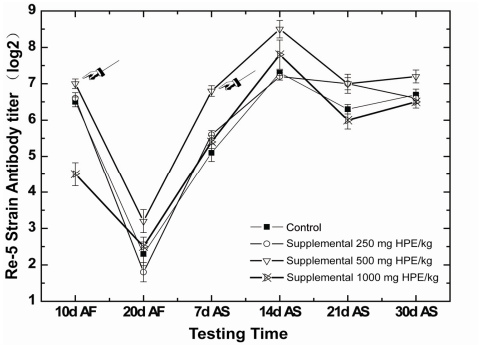

Fig 3. Changes of antibody titer level to avian influenza virus of H5 subtype (Re-5) in layers. The points with the fingers stand for significantly sign ($P < 0.05$).

Table 4. The Pearson correlation coefficients of H5 subtype Re-4 and Re-5 strain antibody titer.

	Testing time	H5 Re-5 strain antibody titer
H5 subtype Re-4 strain antibody titer	10d AF	0.621 **
	20d AF	0.504 **
	7d AS	0.413 **
	14d AS	0.443 **
	21d AS	0.484 **
	30d AS	0.663 **

**$P < 0.01$

Influenza vaccine

The hens were immunized against diseases with the reassortant avian influenza (AI) virus H5 subtype vaccine, inactivated (H5N1, Re-5+Re-4 strain) and H5 standardization antigen, H5 Positive serum and negative serum were provided by Qingdao Yeboi Bioengineering Co., Ltd (China).

Immune monitoring

Experiment of ELISA

The avian influenza (AI) virus H5 subtype antibody ELISA test kit was provided by Shenzhen Lvshiyuan Biotechnology Co., Ltd (China), and ELISA procedure was showed as followed: Influenza virus serum was 100 times diluted and added 100 μL diluted sample to enzyme plate and incubated for 30 min and then decanted. The plate was rinsed 5 times with cleaning solution and immediately added enzyme conjugate, same procedure. After shaking off the liquid, 100 μL of substrate was added and incubated for another 15min. Finally, 100 μL of stop solution was added and homogeneous mixing. At this time, the blue color product changed to yellow during the incubation. The absorbance of the solution at 650 nm against water blank was measured by using a dual-wavelength spectrophotometer, the OD value was convicted of positive when greater than 0.2 and negative lower than 0.2.

Experiment of HI

Antibody against H5 avian influenza virus in the sera was determined by serum hemagglutination inhibition (HI) titer

using H5 standardization antigen. Before HI test we determined the antibody titers (antigens correction) by hematocyte agglutination (HA) test first, each sample seeded four wells in 96-micotter plate, antibody titers were expressed as the highest dilution which could inhibit agglutination. Antibody titer to log2 for the unit and samples were consider positive if titers were ≥ 4 and negative when ≤ 2, at the 3 case it will be considered as suspicious.

Statistical Analysis

Data were analyzed by SPSS version 17.0 for windows (SPSS Inc., Chicago, IL). The correlation between different variables was examined using the Pearson correlation. Data were expressed as mean ± SD, were compared by Duncan's range test and the presented data as percentages differences were considered significant at $P \leq 0.05$.

Conclusion

The results indicated that supplemented with 500 mg kg^{-1} of HPE could advance the immune respondence and strengthen the immune effect. Antibiotics had been extensively used to improve immunity system in animal and human life, but its more and more side effects are found. It is possible taking HPE as immunologic adjuvant that associated with certain doses. Hypericin could be a potential therapeutic in treatment of AI and necessary to the poultry industry. Basic information gained from this investigation may be helpful to agricultural economists and poultry industries.

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