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Ophiopogon polysaccharide liposome can enhance the non-specific and specific immune response in chickens

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ABSTRACT

The purpose of this study is to investigate the immune-enhancing activity of ophiopogon polysaccharide liposome (OPL). In non-specific immune response experiment, the phagocytosis and cytokines secretion of peritoneal macrophages in vitro and in vivo were performed. In specific immune response experiment, the activity of OPL was measured on chickens which were vaccinated with Newcastle disease (ND) vaccine and then challenged with ND virus at 49-old-day. The results showed that OPL could significantly promote the phagocytosis of macrophages and induce the secretion of IL-2 and IL-6 in vitro; OPL at high and medium doses could significantly improve the phagocytosic index, promote lymphocyte proliferation, increase the proportion of T lymphocyte subpopulations ($CD4^+$ and $CD8^+$), enhance antibody titer and improve the protective rate in vivo. Moreover, its efficacy was significantly better than ophiopogon polysaccharide (OP). These results indicated that the immune-enhancing activity of OP was significantly improved after encapsulated with liposome.

1. Introduction

Animal epidemics have serious and extensive impact on breeding industry in our country, which for instance, Newcastle disease, Avian influenza, porcine reproductive and respiratory syndrome and etc. often happen and bring the business a great loss. These diseases most belong to viral infectious diseases. Nowadays, vaccination is still the principal means for the prevention and control of viral infectious diseases owing to the lack of specific treatment. However, most vaccines especially the genetically engineering vaccine can get satisfactory effect with the help of immunopotentiator, which play an essential role in improving the effect of vaccine (Zhao et al., 2014). While the chemical immunopotentiator we are using in clinic presently have some disadvantages such as easy to cumulate, single target, great side effects and so on (Harandi, Medaglini, Shattock, & Working Group convened by EUROPRISE, 2010; Ma, Guo, Wang, Hu, & Shen, 2010).

Polysaccharide is one of the four basic substances constituting of the life, which has various biological activities, such as immunomodulation, antiviral, antitumor, antioxidation, anti-inflammation and so on (Kouakou et al., 2013). In numerous biological activities of polysaccharides, its immunomodulatory effect was the most remarkable. So the polysaccharide from Chinese medicine is also called immune activity polysaccharide (Li, Nie, Xie, & Li, 2014; Zhang et al., 2013). As a natural macromolecule active material, polysaccharide can not only activate the immune cells, improve the level of antibodies, promote the release of cytokines and activate complement, but also play important roles in virus control and cancer therapy (Schepetkin et al., 2013; Sheu, Lyu, Lee, & Cheng, 2013). In addition, polysaccharide will not produce significant side effects when it regulates the immune function of body, it is the ideal immunopotentiator in clinical application.

Ophiopogonis japonicus (*Radix Ophiopogonis Japonici*), as a traditional Chinese medicine, its use could be dated back more than 2000 years ago and be recorded in Shen Nong's Materia Medica.

Abbreviations: OP, ophiopogon polysaccharide; OPL, ophiopogon polysaccharide liposome; ND, Newcastle disease; IL, interleukin; PHA, phytohemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; NDV, Newcastle disease virus; VC, vaccine control; BC, blank control; BL, blank liposome; CC, cell control.

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Ophiopogon polysaccharide (OP), as the main active constituents of ophiopogonis japonicus, possesses stronger immunologic enhancement activity, such as enhancing the phagocytic function of macrophages and improving the humoral and cellular immunity (Lin et al., 2010; Xiong, Li, Huang, Lu, & Hou, 2011; Wang, Sun, Zhang, Chen, & Liu, 2012). Xu et al. reported that Crude Ophiopogon polysaccharide prepared by water extract-alcohol precipitation was separated and purified using ultrafiltration, DEAE Sepharose FF and Sephadex G-25 column chromatography, then its structure was studied by complete hydrolysis, periodate oxidation, Smith degradation, methylation analysis, ^{1}H NMR and ^{13}C NMR analysis. The results showed that OP was a water-soluble β-D-fructosan, containing a backbone composed of Fruf (2 → l), and a branch of Fruf (2 → 6) Fruf (2 → per average 2.8 of main chain residues). The molecular weight was 5000. The reducing terminal of OP connects to α-D Glc, and the molar ratio of Fru and Glc is approximately 35:1 (Xu et al., 2005).

However, because of its high-hydrophilicity and about 2 nm molecular sizes, the half-time of OP is short, OP is quickly excreted through kidney directly, which leads to its less distribution in the target tissue and instability in organism. All of the unsatisfactory pharmacokinetic characters above severely limited the clinical application of OP (Lin et al., 2010). Therefore, in order to improve the bioavailability and exert better pharmacological function, it is particularly important to develop a new type of OP preparation or choose a high-efficiency carrier for improving the pharmacokinetic characters of OP.

Liposome, as one of the important research contents in drug delivery system, has received growing emphasis because of its advantages such as lengthening drug effect, improving stability, reducing toxicity and so on (Ohnishi et al., 2013). Nowadays, the study of liposome on traditional Chinese medicine has become a hotspot. Our preliminary research had proved that epimedium polysaccharide and astragalus polysaccharide could significantly improve their immune enhancement activity and lengthen drug effect after being prepared into liposome (Fan et al., 2012; Gao et al., 2012). The polysaccharide consists of more than ten monosaccharide molecules or monosaccharide derivatives which are connected by glycosidic bond, and different kinds of plant polysaccharides have similar structure. Therefore, we speculate that OP liposome (OPL) could also achieve better immune-enhancing activity and overcome the disadvantages of metabolizing quickly in the body as compared with OP.

Thus, in the present study, the activity of OPL on enhancing non-specific immune response was evaluated by peritoneal macrophages in vitro and vivo, the activity on enhancing specific immune response was also evaluated for inducing humoral and cellular immunity against Newcastle disease (ND) vaccine. The purpose of this study was to investigate whether liposome could further improve the immune-enhancing activity of OP, and screen out a new-type preparation for OP.

2. Materials and methods

2.1. Materials

Ophiopogon polysaccharide (OP, net content of 98.8%) was prepared in our laboratory according the literature (Xu et al., 2005). Soybean phospholipid (no. 20130304) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Cholesterol (no. 20130916) was purchased from Anhui Tianqi Chemical Technology Co., Ltd. Mouse-anti-chicken monoclonal antibodies (anti-CD80-FITC and anti-CD86-PE) were supplied by BioLegend Inc. (USA). RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin, 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-diphenyltetrazo-lium 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 (DMSO) was produced by Zhengxing Chemical Co. Ltd. (Suzhou, China).

2.2. Preparation and characterization of OPL

OPL was prepared by using the reverse-phase evaporation method. First, cholesterol and soybean phospholipid were dissolved with chloroform, then PBS contained OP (4 mg mL^{-1}) was injected into the solution of chloroform. The mixture was homogenized through using the ultrasound in the ice bath to form stable water in oil type (W/O) emulsion. Second, the emulsion was evaporated to form a colloid using a rotary vacuum evaporation, and then 20 mL of PBS was added to hydrate for 15 min. Third, the resulting mixture was homogenized again by using ultrasonic for 20 min to form the well-proportioned liposome. Finally, the liposome was filtered using 0.45 and 0.22 μm millipore membrane successively. The entrapment rate of OPL was assayed by protamine and vitriol–phenol methods according to reference (Gunter, Gunter, Jarkowski, & Rosier, 1982). The entrapment rate of OPL was 64.95%, and the average particle size, zeta potential and PDI was 245.3 nm, −4.56 mV and 0.326, respectively. The article size distribution spectrum and transmission electron microscopy of OPL is showed in Supplementary data. For the test in vitro, OPL was dissolved into five concentrations in two-fold serial dilution with RPMI-1640. In vivo, it was diluted into three doses with PBS. The endotoxin amount was up to the standard of Chinese Veterinary Pharmacopeia (less than 0.5 EU mL^{-1}), they were stored at 4 °C for the experiment.

2.3. Experimental animals

One-day-old White Roman chickens (male) 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. Activity of OPL on non-specific immune response

2.4.1. Isolation and culture of peritoneal macrophages

Macrophages were isolated by peritoneal lavage method with minor modifications according to previous report (Sánchez-Fidalgo et al., 2013). Chickens (14-old-day) were injected intraperitoneally with 2 mL of 6% starch–broth medium. Two days later, the peritoneal cavity was washed with 20 mL of PBS, and the macrophages in peritoneal fluid were collected. After centrifugation at 2000 rpm for 10 min, the cells were collected and washed twice with PBS. Then the cells were resuspended in RPMI-1640 and seeded in culture plates (1×10^{6} cells mL^{-1}) for 2 h at 37.5 °C in a humid atmosphere with 5% CO_2. Non-adherent cells were removed by lightly washing twice with RPMI-1640, and the adherent cells were macrophages. Cell viability measured by trypan blue exclusive assay was never below 95%.

2.4.2. Activity of OPL on phagocytosis of macrophages in vitro

The phagocytosis of macrophages was measured by neutral red uptake (Chen, Zhang, Shen, & Wang, 2010). After cultured with OPL or OP for 48 h, 100 μL of neutral red solution (0.075%, w/w) was added and incubated for 4 h. After discarding supernatant, the cells were washed with PBS twice to remove the neutral red which wasn't phagocytized by Macrophages. Then DMSO (100 μL well^{-1}) was added to lyse Macrophages. The absorbance at 490 nm was assayed by a microplate reader.

2.4.3. Determination of IL-2 and IL-6 levels

The macrophages were inoculated into 96-well culture plates. After being treated with or without drugs for 48 h, the plates were centrifuged at 1000 × *g* for 10 min, and the supernatant was collected for determining the contents of IL-2 and IL-6 by ELISA kit (Biosamite Biotechnology Co. Ltd. Shanghai, China), respectively, according to the manufacturer's instructions.

2.4.4. Activity of OPL on phagocytic index in vivo

The phagocytic index of the macrophages was measured by carbon clearance according to the procedure of previous description (Wang, Wang, Li, Shi, & Zhong, 2009). Ninety 14-day-old chickens were randomly assigned into six groups. The chickens in five experimental groups were intramuscularly injected with 1.0 mL of OPL at high (4.0 mg mL^{-1}), medium (2.0 mg mL^{-1}) and low (1.0 mg mL^{-1}) dose, OP (4.0 mg mL^{-1}) and BL, respectively, in BC group, with physiological saline, once a day for 3 successive days. On days 7 and 14 after the last drug administration, six chickens were chosen randomly from each group and intravenously injected with diluted India ink at 1 mL/100 g body weight. Blood samples were collected at 2 min (T_2) and 10 min (T_{10}) from the brachial vein, and then 20 μL of blood was mixed with 2 mL 0.1% Na_2CO_3. The absorbance at 600 nm was measured on a UV–visible spectrophotometer. The liver and the spleen were weighed, and the phagocytic index was calculated as follows: $K = (\lg OD_2 - \lg OD_{10})/(T_{10} - T_2)$. Phagocytic index = $K^{1/3} \times M_{\text{body weight}}/(M_{\text{liver weight}} + M_{\text{spleen weight}})$.

2.5. Activity of OPL on specific immune response

Three hundred 14-day-old chickens were randomly divided into seven groups and vaccinated with ND vaccine (La Sota strain, no. 130920, Bio-drug Company of Veterinary, Beijing City) except 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 with 1.0 mL of OPL at high (4.0 mg mL^{-1}), medium (2.0 mg mL^{-1}) and low (1.0 mg mL^{-1}) dose, OP (4.0 mg mL^{-1}) and BL, 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 measuring peripheral lymphocyte proliferation by MTT method, $CD4^+$ and $CD8^+$ T cells distribution by flow cytometry, and serum hemagglutination inhibition (HI) antibody titer by micro-method. On day 35 after the first vaccination, the chickens except for BC group were challenged with 0.5 mL of NDV (F48E9 strain, supplied by China institute of veterinary drug control) with 10 LD_{50} by intramuscular injection. The pathogenic and dead statuses of chickens were examined daily for 15 successive days after challenged. The mortality, morbidity and protective rate in each group was calculated according to the formula: mortality (%) = the number of dead chicken up to day15 after challenge (D_{15})/the number of sample × 100%, morbidity (%) = the number of chickens dead and showing clinical symptoms on D_{15}/the number of sample × 100%, protective rate (%) = the number of chickens without clinical symptoms on D_{15}/the number of sample × 100%.

2.6. Lymphocytes proliferation assay

Lymphocytes proliferation was performed as previously described (Zhao et al., 2011). Briefly, blood samples were collected from heart, and then carefully layered on the surface of lymphocyte separation medium. After centrifugated, lymphocytes were collected and washed twice with RPMI-1640. Then were diluted into 2.5×10^6 mL^{-1} and added into 96-well plates with 80 μL well^{-1}, another 20 μL of PHA was added. The plates were incubated in a humid atmosphere of 5% CO_2 at 37.5 °C. After a culture for 44 h, 20 μL of MTT (5 μg mL^{-1}) was added, and the plates were re-incubated for 4 h. Then the supernatant was removed and 100 μL of DMSO were added and shaken for 5 min. The absorbance at 570 nm (A_{570} value) was measured as the index of peripheral T lymphocytes proliferation.

2.7. Serum HI antibody assay

Blood sample were collected, put into Eppendorf tubes and allowed to clot at 37 °C for 2 h. Serum was separated and inactivated complements for assaying HI antibody. The method was referred to the previous report (Zhao et al., 2011). The mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI.

2.8. Measurement of $CD4^+$ and $CD8^+$ T cells

T lymphocytes were collected according to the method in 2.5. Then 50 μL of lymphocytes were added to Falcon tubes. After added 2 μL of monoclonal antibodies (anti-$CD4^+$-FITC and anti-$CD8^+$-PE), the mixture was incubated in the dark at 4 °C for 30 min. After centrifugation (2000 × *g*, at 4 °C for 5 min), the cells were washed with PBS, and resuspended in 0.5 mL of PBS. The percentages of $CD4^+$ and $CD8^+$ T cells were measured by flow cytometry (Yang, Chen, Sun, Ye, & Fang, 2007).

2.9. Statistical analysis

Data are expressed as the mean ± S.D. Duncan's multiple range test was used to determine the differences among groups with the software SPSS 19.0. χ^2-Test was used to analyze the difference of the mortality, morbidity and protective rate. Significant differences were considered as $P < 0.05$.

3. Results

3.1. Non-specific immune response experiment

3.1.1. The effect of OPL on phagocytosis of macrophages

The effect of OPL on phagocytosis of neutral red is illustrated in Fig. 1. At 125–7.813 μg mL^{-1}, the A_{490} values in OPL and OP groups were significantly higher than those in BL and CC group ($P < 0.05$). The A_{490} values in OPL at 125, 31.25 and 7.813 μg mL^{-1} groups were significantly higher than those in all OP groups ($P < 0.05$).

3.1.2. The effect of OPL on IL-2 secretion

The effect of OPL on the release of IL-2 from mecrophages is shown in Fig. 2. The IL-2 concentrations in OPL at 125–15.625 μg mL^{-1} and OP at 125–31.25 μg mL^{-1} groups were significantly higher than those in BL and CC groups ($P < 0.05$). The IL-6 concentrations in OPL were significantly higher than those in OP at 125–31.25 μg mL^{-1} groups ($P < 0.05$).

3.1.3. The effect of OPL on IL-6 secretion

The effect of OPL on the release of IL-6 from mecrophages is shown in Fig. 3. The IL-6 contents in OPL at five concentrations and

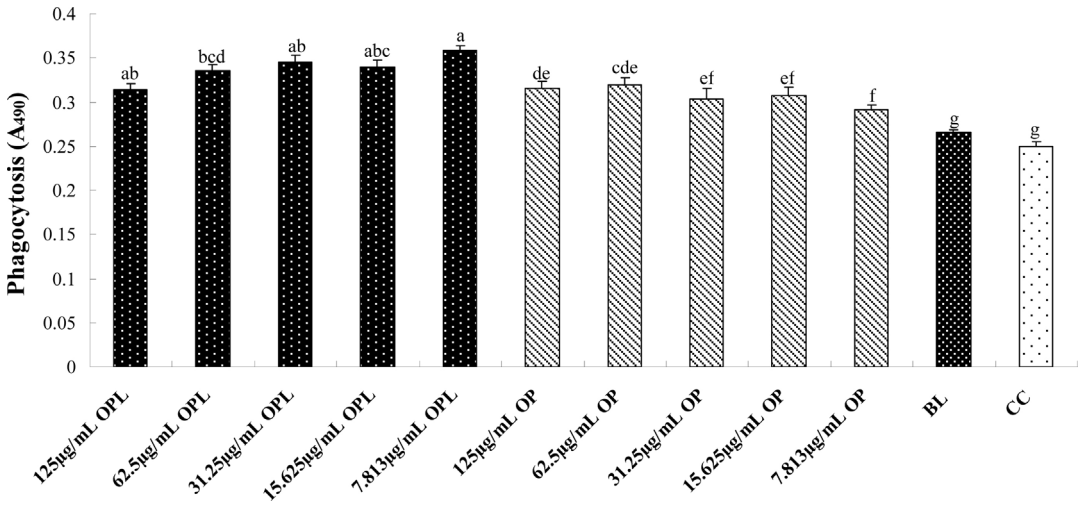

Fig. 1. The effect of OPL on phagocytosis of macrophages in vitro. Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

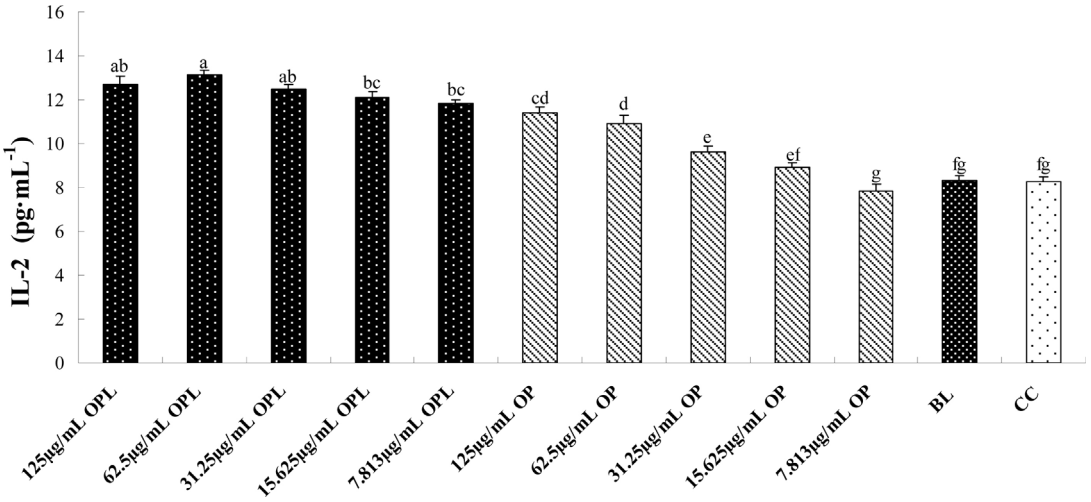

Fig. 2. Effect of OPL on IL-2 secretion of mecrophages. Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

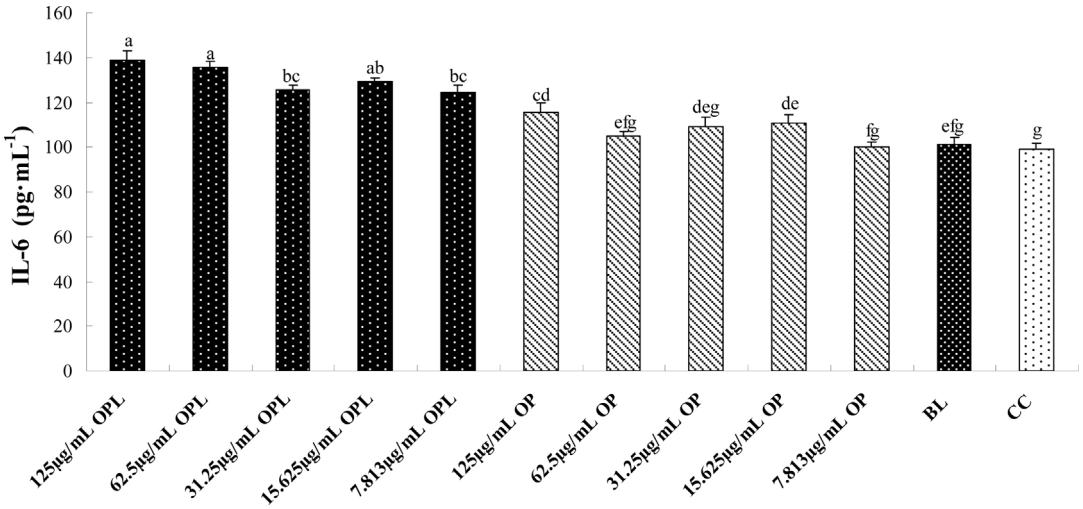

Fig. 3. Effect of OPL on the IL-6 secretion of mecrophages. Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

OP at 125 μg mL^{-1} groups were significantly higher than those in BL and CC groups ($P<0.05$). The IL-2 contents in OPL at five concentration groups were significantly higher than those in OP at 62.5–7.813 μg mL^{-1} groups ($P<0.05$).

3.1.4. The effect of OPL on phagocytic index in vivo

The effect of OPL on phagocytic index is illustrated in Fig. 4. At all time points after the last drug administration, the phagocytic index in OPL_H, OPL_M and OP groups were significantly higher than those in BL and BC groups ($P<0.05$). Besides, the phagocytic index in OPL_H and OPL_M groups were significantly higher than those in OP group on days 7 and 14 ($P<0.05$). There were no significant differences between OPL_H and OPL_M groups at two time points ($P>0.05$).

3.2. Specific immune response experiment

3.2.1. The dynamic changes of lymphocytes proliferation

The A_{570} values in each group are showed in Fig. 5. At all time points after the first vaccination, the A_{570} values in OPL_H and OPL_M groups were significantly higher than those in OPL_L, BL, VC and BC groups ($P<0.05$). On day 7, the A_{570} value in OP group was the highest and significantly higher than those in OPL_M, OPL_L, BL, VC and BC groups ($P<0.05$). On days 14–35, the A_{570} values in OPL_H group were significantly higher than those in OP group ($P<0.05$). On days 21–35, the A_{570} values in OPL_M group were significantly higher than those in OP group ($P<0.05$). On days 7–28, there were no significant differences between OPL_H and OPL_M groups ($P>0.05$).

3.2.2. The dynamic changes of antibody titer

The antibody titers in every group are illustrated in Fig. 6. At all time points after the first vaccination, the titers in OPL_H and OPL_M groups were significantly higher than those in VC and BC groups ($P<0.05$). On days 14–35, the titers in OPL_M group was the highest and significantly higher than those in OPL_L, OP and BL groups ($P<0.05$). On days 21–35, the titers in OPL_H group were significantly higher than those in OP group ($P<0.05$).

3.2.3. The dynamic changes of $CD4^+$ T cells

The dynamic changes of $CD4^+$ T cells are showed in Fig. 7A. On days 14–35 after the first vaccination, the proportion of $CD4^+$ T cells in OPL_H and OPL_M groups were significantly higher than those in OP, BL, VC and BC groups ($P<0.05$). On days 14–35, the proportion of $CD4^+$ T cells in OP group was higher than those in BL, VC and BC groups, and the differences were significant on days 14 and 21 ($P<0.05$).

3.2.4. The dynamic changes of $CD8^+$ T cells

The dynamic changes of $CD8^+$ T cells are showed in Fig. 7B. On days 14–35 after the first vaccination, the proportion of $CD8^+$ T cells in OPL_H and OPL_M groups were significantly higher than those in BL, VC and BC groups ($P<0.05$). On days 14–35, the proportion of $CD8^+$ T cells in OPL_H and OPL_M groups were higher than those in OP group, and the differences were significant on days 21, 28 and 35 ($P<0.05$). On days 14–35, the proportion of $CD4^+$ T cells in OP group was higher than those in BL, VC and BC groups, and the differences were significant on days 14 ($P<0.05$).

3.2.5. Protective effects

The results are showed in Fig. 8. The mortalities and morbidities in three OPL and OP groups were lower than those in BL and VC groups, and the protective rates were higher than those in BL and VC group. The mortality in OPL_H group was the lowest and significantly lower than those in OP, BL and VC groups, and in OPL_M group was significantly lower than those in BL and VC groups ($P<0.05$). The morbidities in OPL_H and OPL_M groups were significantly lower than those in OP, BL and VC groups ($P<0.05$). The protective rates in OPL_H and OPL_M groups were significantly higher than those in OP, BL and VC groups ($P<0.05$).

4. Discussion

The immune system containing non-specific and specific immune is the body's ultimate defense against various diseases. Macrophage is not only an important part of natural resistance but also an indispensable part of specific immunity in animal. It has many functions such as phagocytic and damaging effect, antigen-presenting ability, synthesizing and secreting various kinds of active factors (Konno, Maricato, Konno, Mariano, & Lopes, 2009). It is greatly significant to measure the activity of macrophages for evaluating the immune function of the organism. Therefore, in this experiment, we investigated the effect of OPL on activating macrophages, so as to explore the activity of OPL on enhancing the non-specific immune response.

Phagocytosis of macrophages is an important factor of maintaining the homeostasis, a key link of nonspecific immunity and the foundation of producing specific immune response (Joosten & Ottenhoff, 2008). On the surface of activated macrophages, there are mannose receptors, glucan receptors, polysaccharide receptors and antigen presenting related molecule which all have ties to phagocytic activity. When foreign matter (such as bacteria, microbial debris, and other antigenicity substance) invade the organism, by macrophage, they will quickly be phagocytized and eliminated, and then the antigen presented to T helper cells to activate specificity cellular immunity. The treatment of macrophage will greatly enhance the immunogenicity of the most antigens and further stimulate satisfactory immune response (Santanu, Subhankari, & Somenath, 2010; Sánchez-Fidalgo et al., 2013). Therefore, it's greatly significant to measuring the phagocytosis of macrophages for evaluating the non-specific immune function of the organism. In this study, in order to reflect the phagocytic activity of macrophages, the phagocytic index was measured by neutral red uptake in vitro and carbon clearance test in vivo. The results showed that OPL could significantly improve the phagocytic activity of macrophages in vitro and vivo as compared with OP, which demonstrated that the immuno-enhancement effect of OP were significantly improved after encapsulated with liposome. Yu also proved that gypenosides liposome could significantly the phagocytosis activity of the peritoneal macrophages in vitro as compared with gypenosides (Yu et al., 2014).

In non-specific immune system, the cytokines secreted by macrophages also play an important role in immune response. IL-2 is able to induce the differentiation and proliferation of natural killer cells and promote the production of immunoglobulins (Nhiem et al., 2010). IL-6 could activate lymphocytes, promote the generation of antibody, and influence the specific immune response (Lin et al., 2010). In order to further investigate the effects of OPL on macrophages, IL-2 and IL-6 were measured. In the present study, the results showed that OPL could enhance the secretion of cytokines in macrophages, and its effect was significantly better than that of OP, suggesting that OPL may enhance non-specific and specific immune function by means of increasing the secretion of cytokines. Many researches also confirmed that Chinese herbal medicines could significantly improve the secretion of cytokines in vitro and vivo after being encapsulated by liposome (Yu et al., 2013; Yuan et al., 2013).

Specific immune response includes cellular immunity and humoral immunity. In present study, the activity of OPL on specific immune was carried out against Newcastle disease vaccine. T lymphocytes proliferation is a main participants in specific immune response and commonly used for detecting the immune-enhancing

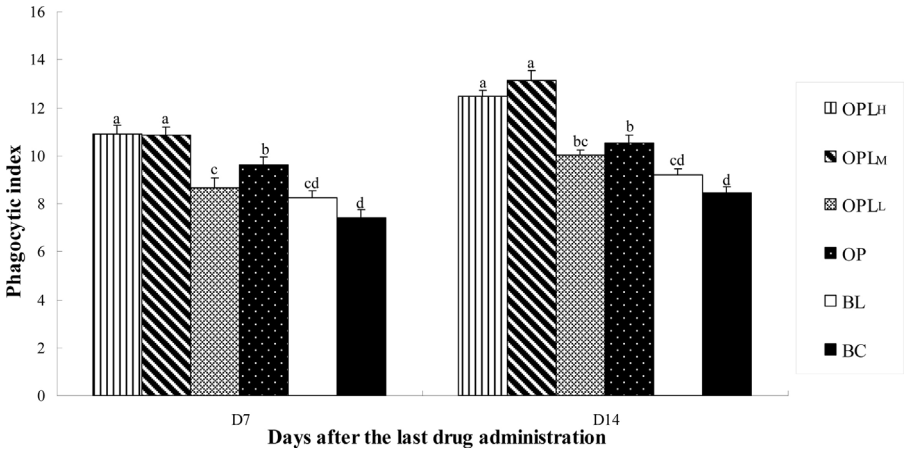

Fig. 4. Effect of OPL on phagocytic index in vivo. Bars in the same day without the same superscripts differ significantly ($P<0.05$). H, high dose; M, medium dose; L, low dose.

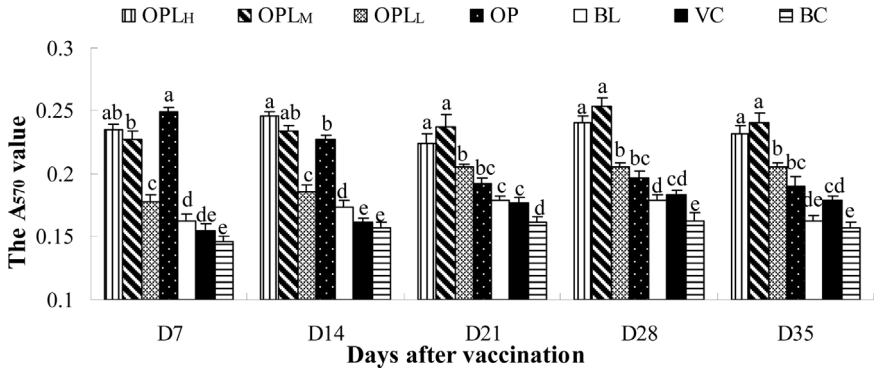

Fig. 5. The dynamic changes of lymphocytes proliferation in each group (A_{570} value). Bars in the same day without the same superscripts differ significantly ($P<0.05$). H, high dose; M, medium dose; L, low dose.

activity of drugs, it's also an important index that reflects the function of cellular immunity (Li, Santoso, & Lo, 2007). Results in Fig. 5 showed that OPL at high and medium doses could significantly promote lymphocytes proliferation at most time points after the first vaccination, and its effect was significantly better than that of OP.

To further observe the effect of OPL on the cellular immunity, the counts of $CD4^+$ and $CD8^+$ T lymphocytes subpopulations were determined. $CD4^+$ T cells differentiated to two effector phenotypes, T helper type 1 (Th1) and T helper type 2 (Th2). Th1 could promote cell-mediated immune response, and Th2 is able to promote humoral or allergic response (Constant & Bottomly, 1997; Mosmann & Coffman, 1989). $CD8^+$ T cells mediate pathogen clearance, which could recognize and kill the infected host cells (Deborah & Cytolytic, 2010). $CD4^+$ and $CD8^+$ T cells are key links of immunoregulation of organism. So the number of $CD4^+$ and $CD8^+$ T cell can be used to evaluate the immune state of body. If the counts of $CD4^+$ T and $CD8^+$ T cells increase, the humoral and cellular immune function also will be enhanced (Zhang & Bevan, 2011). The results showed the proportion of $CD4^+$ and $CD8^+$ T lymphocyte subpopulations in OPL_H and OPL_M groups were significantly higher than those in OP group at most time points, which indicated that helper T lymphocytes were activated by OPL, and the efficacy of OPL on increasing the proportion of $CD4^+$ T and $CD8^+$ T cells was better than that of OP.

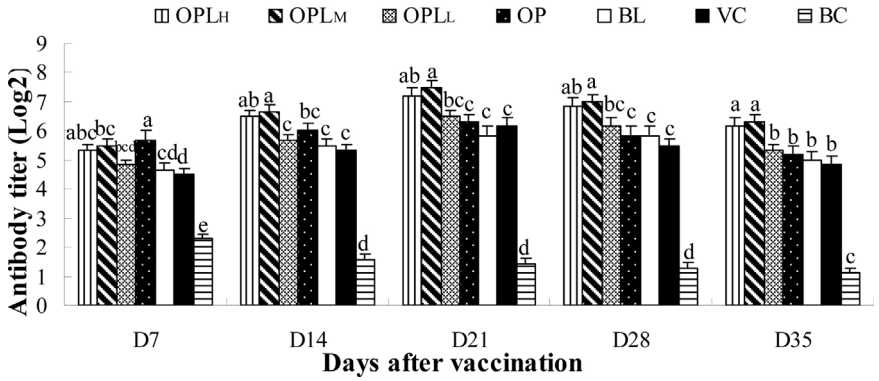

Fig. 6. The dynamic changes of antibody titer in each group ($\log_2$). Bars in the same day without the same superscripts differ significantly ($P<0.05$). H, high dose; M, medium dose; L, low dose.

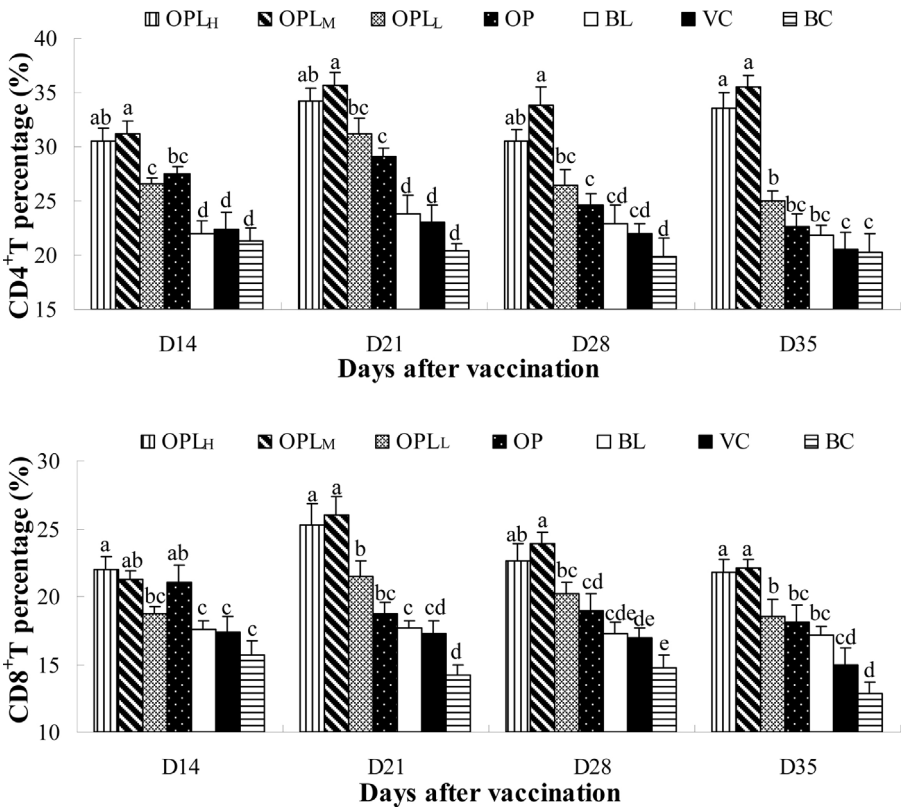

Fig. 7. The dynamic changes of $CD4^+$ and $CD8^+$ T cells in each group. (A) The dynamic changes of $CD4^+$ T cells; (B) the dynamic changes of $CD8^+$ T cells. Bars in the same day without the same superscripts differ significantly ($P < 0.05$). H, high dose; M, medium dose; L, low dose.

Humoral immunity mediated by B lymphocytes, is an important specific immune reaction of body against infection and tumors. The changes of antibody titer reflect the situation of humoral immunity in animal (Hadden, 2003; Irie, Watarai, & Kodama, 2003). A variety of antibodies could induce effector cells to recognize and kill pathogens and tumor cells. Our results showed that OPL at high and medium doses were capable of enhancing humoral immunity by increasing the levels of ND antibody titers, and the effect was significantly better than OP (Fig. 6). These results implied that OPL has significant activity on enhancing humoral immunity.

In order to further investigate the effect of OPL on specific immune response, the chickens were challenged with NDV at 49-day-old, and the protective effects were determined. As shown in Fig. 8, in OPL at high and medium dose groups, the morbidities were significantly lower and the protective rates were significantly higher in comparison with OP group. These results suggested that OPL at suitable dose could significantly enhance the immune efficacy of ND vaccine, decrease the mortality and morbidity, and improve the protective effect. The reasons may be that OPL possessed better effect on enhancing the cellular and humoral immunity, it could make higher lymphocyte proliferation, antibody titer and the proportion of $CD4^+$ and $CD8^+$ T lymphocyte subpopulations maintain a longer time, thus reduced the incidence of ND. The results also indicated that the dosage could be an important factor in development of new-type immunopotentiator. The dose-effect and time-effect relationships were also confirmed in our previous researches (Fan et al., 2010; Guo et al., 2009).

Based on the comparison between OPL and OP, it could be found that the immune-enhancing activity of OPL was superior to that of OP. The reasons may be that the liposome is easy to be taken in by cells because its constituents also are the fundamental components of cell membrane, and its structure is similar to

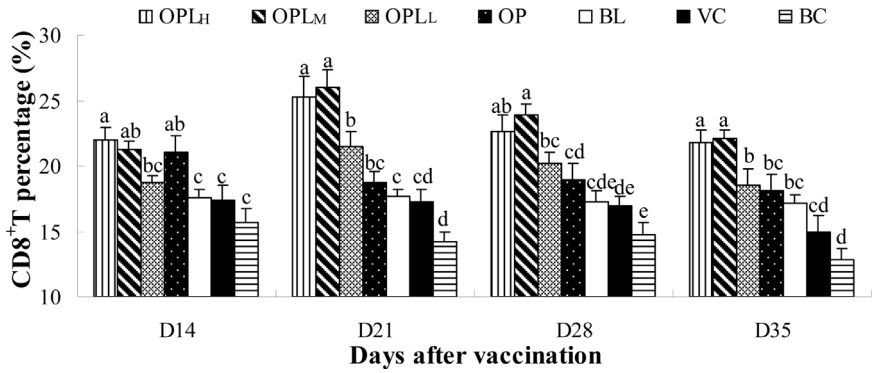

Fig. 8. The mortality, morbidity and protective rate in each group. Bars in the same day without the same superscripts differ significantly ($P < 0.05$). H, high dose; M, medium dose; L, low dose.

cell membrane (Elenkov & Chrousos, 1999). Therefore, OP encapsulated by liposome is easy to be absorbed by cell. Many other researches also proved that the efficacy of drug encapsulated with liposome was significantly better than non-encapsulated drug (Faikoh, Hong, & Hu, 2014; Yücel, Değim, & Yilmaz, 2013). Although the exact mechanism of OPL is unknown, based on the results in this study, we speculated that the immune-enhancing effect of OPL may be attributed to its capacity to modulate improve non-specific and specific immune response, finally the efficacy of OP was improved. Especially in regards to specific immune, OPL could activate T lymphocyte and its subpopulations ($CD4^+$ and $CD8^+$), and improve the antibody titer. To promote the clinical application of OPL, further studies on the mechanism of action are in progress.

5. Conclusions

In this study, the results showed that OPL not only could significantly promote the cykotines secretion of macrophages in vitro and enhance the phagocytosis of macrophages in vitro and vivo, but also enhance lymphocyte proliferation and antibody titer, and improve the protective effect against NDV. OPL possesses the immune-enhancing activity in regulating non-specific and specific immune response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.048.

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