

## Ginseng Stem-Leaf Saponins and Oil Adjuvant Synergistically Promote the Immune Responses to Newcastle Disease in Chickens

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**Abstract:** The aim of the study was to evaluate the adjuvant effect of Ginseng Stem-Leaf Saponins (GSLs) on the immune responses against Newcastle Disease (ND) in chickens. Birds were immunized (i.m.) with ND oil emulsion vaccines containing GSLs (0, 2, 4, 6 and 8 µg/0.2 mL). Blood samples were collected on Weeks 0-3 after immunization for the measurement of Hemagglutination Inhibition (HI) titers and splenocytes were prepared for Lymphocyte Proliferation test. The results showed that serum HI titers were significantly higher in group containing GSLs (2, 4 and 6 µg/0.2 mL) than in control group at Weeks 2 and 3 post immunization ( $p < 0.05$ ). Splenocyte stimulation indexes were significantly enhanced in chickens immunized with ND oil emulsion vaccine containing GSLs (6 µg/0.2 mL) when compared with control group at Week 3 post immunization ( $p < 0.05$ ). No abnormal behaviors and side effects were found in chickens between groups throughout the whole experiment. This suggests that GSLs and oil adjuvant can synergistically promote both humoral and cellular immune responses. Therefore, GSLs could be a promising vaccine additive to improve vaccines against infections in poultry.

**Key words:** Adjuvant, Ginseng stem-leaf saponin, Newcastle disease, vaccine, poultry, oil

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### INTRODUCTION

Newcastle Disease (ND) is one of the most important infectious diseases in poultry production (Alexander, 2001; Giasuddin *et al.*, 2002; Naila *et al.*, 2001). ND is caused by Newcastle Disease Virus (NDV) which is an enveloped RNA virus of the genus *Avulavirus* of the family Paramyxoviridae (Mayo, 2002). The disease can occur in a variety of poultry species and cause clinical signs such as respiratory distress, nervous signs and cessation of egg production resulting in high mortality (Alexander, 2008). Many countries suffered from considerable financial losses during the NDV panzootics (Alexander, 2001). Because of the severity of ND in poultry industry, ND is classified as list A disease in the Office International des Epizooties (OIE) (Alexander, 2000). The recommended policy in case of NDV includes avoiding all exposure to NDV and reducing the risk of transmission. In addition to the biosecurity strategy, ND is commonly prevented through immunization of vaccines (Seal *et al.*, 2000; Hosseini *et al.*, 2011). Attenuated ND vaccines and inactivated ND emulsion vaccines are the two currently available vaccine types in China (Zhen *et al.*, 2010). However, the commercial vaccines are commonly reported to elicit poor immune responses in chickens (Wang, 2010; Shang and Han, 2011). So, a new

approach is needed to enhance the efficacy of ND oil emulsion vaccines to defense against NDV in poultry production.

Ginseng, the root of *Panax ginseng* C.A. Meyer has been used as a conventional herbal medicine for thousands of years in China (Song and Hu, 2009). Among many ingredients in ginseng, saponins or ginsenosides are well investigated for their biological activities and have been regarded as the active components responsible for the major pharmacological effects of ginseng (Kiefer and Pantuso, 2003). Ginseng saponins have shown a wide range of biological activities including anti-stress (Lee *et al.*, 2006), antioxidant (Keum *et al.*, 2000), anti-tumor (Shibata, 2001) and immunomodulatory effects (Rivera *et al.*, 2003b; Sun *et al.*, 2008). The stems and leaves of the plant harbor the saponins (GSLs) similar to those found in the root (Wang *et al.*, 2009). Compared to the saponins from the root, GSLs has a lower cost due to recycled use of the stems and leaves. The previous research has found that the supplement of GSLs in inactivated Foot and Mouth Disease (FMD) oil emulsion vaccine significantly increased the immune responses in a model animal (Song *et al.*, 2009). The present study was designed to investigate the adjuvant effects of GSLs in ND oil emulsion vaccine by measuring serum HI titers and splenocyte proliferation in chickens.

## MATERIALS AND METHODS

**Experimental chickens:** About 1 day old Guangda Meiling native chickens (male) were purchased from the Zhejiang guangda breeder Co., Ltd. (Jiaxing, China). The birds were reared in different groups in separate wire cages with 250 W heat lamps for 10 days. The chickens were fed with commercial feed purchased from Hangzhou Layer Experimental Farm (Hangzhou, China) and given food and water *ad libitum*.

**Antigen, adjuvants and vaccine:** Inactivated (LaSota strain) Newcastle Disease virus antigen and ESSO Marcol 52 oil (MC52) were kindly provided by Hangzhou Jianliang Veterinary Biological Preparations Co., Ltd. Standardized GSLs from Panax ginseng C.A. Meyer was purchased from Hongjiu Ginseng Industry Co., Ltd. (Jilin, China). High Performance Liquid Chromatography (HPLC) analysis showed that GSLs contained 1.86% Rb1, 4.18% Rb2, 3.80% Rc, 10.17% Rd, 19.02% Re and 7.57% Rg1.

Ginseng stem-leaf saponin powder was dissolved in dimethyl sulfoxide at room temperature to form a stock solution (361 mg mL<sup>-1</sup>). To produce ND oil emulsion vaccines containing GSLs (0, 2, 4, 6 and 8 µg/0.2 mL) MC52 oil adjuvant (containing 6% Span 80) with or without GSLs was emulsified in NDV antigen (containing 3% Tween 80) at a ratio of 2:1 (v/v) by using a dispersing device (PLYTRON System PT 1200E, KINEMATICA AG, Switzerland) for 12 min according to the instruction.

**Experimental design:** Sixty birds were randomly divided into 5 groups with 12 birds in each. The animals were intramuscularly (i.m.) immunized once with 0.2 mL ND oil emulsion vaccine containing GSLs (0, 2, 4, 6 and 8 µg) at day 12. Blood samples (0.3-0.6 mL/chicken) were collected from the heart or vein of all chickens before the immunization (0 week) and 1-3 weeks post the immunization. All blood samples were centrifuged at 3500 rpm for 6 min and the serum was harvested and stored at -20°C until analysis. About 3 weeks after immunization, splenocytes were collected from birds immunized with ND oil emulsion vaccines containing GSLs (0 and 6 µg/0.2 mL) for determination of lymphocyte proliferation. Muscle tissues at the sites of injection in chickens immunized with ND oil emulsion vaccines with or without GSLs were harvested for the pathological observations.

**HI test:** The test was performed according to the method as described previously (Alexander, 2008). Briefly, 25 µL of phosphate buffered saline were added to each well of 96 well plates. Serum samples (25 µL) were placed in the first column of plates. Then, twofold serial dilutions followed by passing 25 µL from one column to the next to

the end of 10th column in the plate. Four Hemagglutination Units (HAU) of NDV antigen (25 µL) were added to each well except the 12th column and incubated at 37°C for 40 min. After that 25 µL of 1% rooster erythrocytes suspension was added into each well and allowed to incubate at 37°C for 20 min before hemagglutination activity was observed. Duplicate tests were made to assure the accuracy of results and a positive serum, erythrocytes and NDV antigen were also included as controls. The HI titer was determined by the well of highest serum dilution causing complete inhibition of hemagglutination.

**Splenocyte proliferation assay:** Spleens collected from the NDV-immunized birds under aseptic conditions in Hank's balanced salt solution were minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension. After centrifugation (500×g at 4°C for 10 min), the pelleted cells were washed three times in Hank's balanced salt solution and resuspended in complete medium (RPMI, 1640 supplemented with 0.05 mM2-mercaptoethanol, 100 µg mL<sup>-1</sup> streptomycin, 100 IU mL<sup>-1</sup> penicillin and 10% fetal bovine serum). The viability of the cells was assessed with the trypan blue dye exclusion technique. Splenocytes were seeded into a 96 well flat-bottom microtiter plate (Nunc) at 5.0×10<sup>6</sup> cell mL<sup>-1</sup> in 100 µL of complete medium and Concanavalin A (Con A, final concentration 5 µg mL<sup>-1</sup>), Lipopolysaccharide (LPS final concentration 7.5 µg mL<sup>-1</sup>), NDV antigen (4 HAU) or medium were added to give a final volume of 200 µL. The plates were incubated at 41°C in a humid atmosphere with 5% CO<sub>2</sub> for 68 h. Then, 50 µL of MTT (2 mg mL<sup>-1</sup>) were added to each well. The plates were incubated for an additional 4 h and then were centrifuged (1000×g for 10 min). The untransformed MTT was removed carefully by pipetting. Then, 150 µL of dimethyl sulfoxide were added to dissolve the dark blue crystals. After 15 min shaking, the absorbance was determined in an ELISA reader at 570 nm. The Stimulation Index (SI) was calculated by dividing the absorbance of the mitogen cultures by the absorbance of the non-stimulated cultures.

**Statistical analysis:** Values were expressed as the mean±Standard Deviation (SD). Data analysis was performed with SPSS software (SPSS, Version 11.5, SPSS Inc., Chicago, IL). One-way ANOVA analysis of variance followed by LSD method of multiple comparisons (for between 3 or more groups) or independent-samples of t-test (for between 2 groups) was used to compare the parameters between groups. The p<0.05 were considered significant.

**RESULTS AND DISCUSSION**

**Serum HI titers response:** To investigate the effect of GSLS on the induction of the HI response to ND oil emulsion vaccines in chickens, groups of chickens were immunized (i.m.) once with 0.2 mL ND oil emulsion vaccines containing different GSLS. The NDV-specific HI titers in the sera were measured at 0-3 weeks after immunization using the HI assay. Figure 1 shows that serum HI titers were significantly higher in chickens immunized with 0.2 mL of ND vaccines containing 2, 4 and 6 µg of GSLS than in chickens immunized with the vaccine without GSLS at 2 and 3 weeks post immunization ( $p < 0.05$ ). Highest serum HI titer was found in chickens immunized with ND vaccines plus 6 µg of GSLS.

**Splenocyte responses:** The effect of GSLS and MC52 on splenocyte proliferative responses to Con A, LPS and NDV antigen stimulation are shown in Fig. 2. Chickens immunized with ND oil emulsion vaccine plus GSLS (6 µg/0.2 mL) promoted significantly higher splenocyte proliferative response to LPS and NDV antigen when compared to the chickens injected with ND oil emulsion vaccine alone ( $p < 0.05$ ).

**Experimental observations and pathology:** There were no abnormal behaviors and side effects in chickens between groups throughout the whole experiment. No changes in body weight between the groups at all time points (Fig. 3). No adverse reactions at injection sites were observed in the chickens immunized with ND oil emulsion vaccines with or without GSLS (Fig. 4 and Table 1). Adjuvants are capable of improving or modulating immune responses to vaccines. In the previous study (Song *et al.*, 2009), GSLS

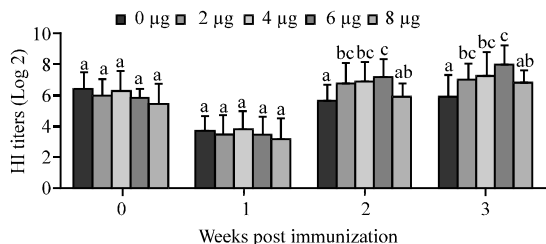


Fig. 1: Newcastle disease virus-specific HI titers in chickens (n = 12) immunized with 0.2 mL of ND oil emulsion vaccines containing GSLS (0, 2, 4, 6 and 8 µg). Blood samples were collected before (0 week) and 1-3 weeks post immunization for measurement of HI titers by a HI test. Data are expressed as means±SD. Bars with different letters are statistically different ( $p < 0.05$ )

was found to exhibit efficient adjuvant effects in a Mouse Model. When the mice were immunized with FMD oil emulsion vaccine containing GSLS, the vaccine elicited significantly higher IgG and IgG subclass responses, level of IFN-γ and IL-5 as well as splenocyte proliferation in

Table 1: Main finding at injection sites in chickens immunized with ND oil emulsion vaccines with or without GSLS

| Adjuvant | Antigen | Main finding |   |   |
|----------|---------|--------------|---|---|
|          |         | ++           | + | - |
| Oil      | NDV     | 7            | 4 | 1 |
| Oil+GSLs | NDV     | 5            | 4 | 3 |

++: Some oil emulsion vaccines; +: A little oil emulsion vaccine; -: No vaccine

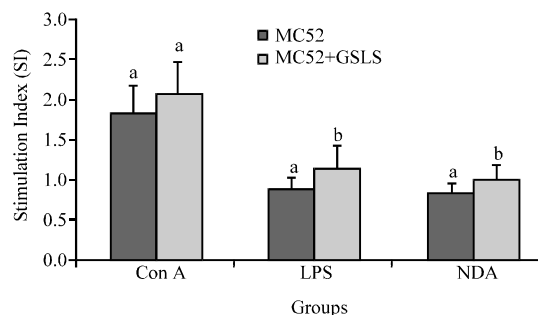


Fig. 2: Splenocyte proliferative responses to Con A, LPS and NDV. Chickens were intramuscularly (i.m.) immunized once with 0.2 mL ND oil emulsion vaccines containing GSLS (6 µg) at days 12. Splenocytes were prepared 3 weeks after immunization and cultured with Con A, LPS and NDV. Splenocyte proliferation was measured by the MTT Method and shown as a Stimulation Index (SI). Data are expressed as means±SD. Bars with different letters are statistically different ( $p < 0.05$ )

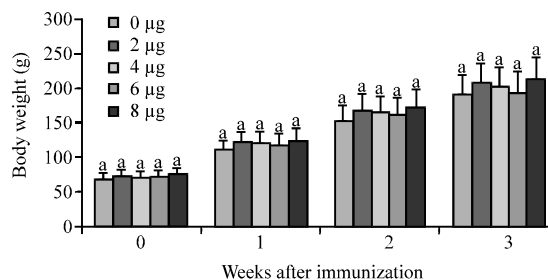


Fig. 3: Body weight in chickens. Body weight of the birds was measured before (0 week) and 1-3 weeks after immunization. Data are expressed as means±SD. No significant difference was found between groups at all weeks ( $p > 0.05$ )

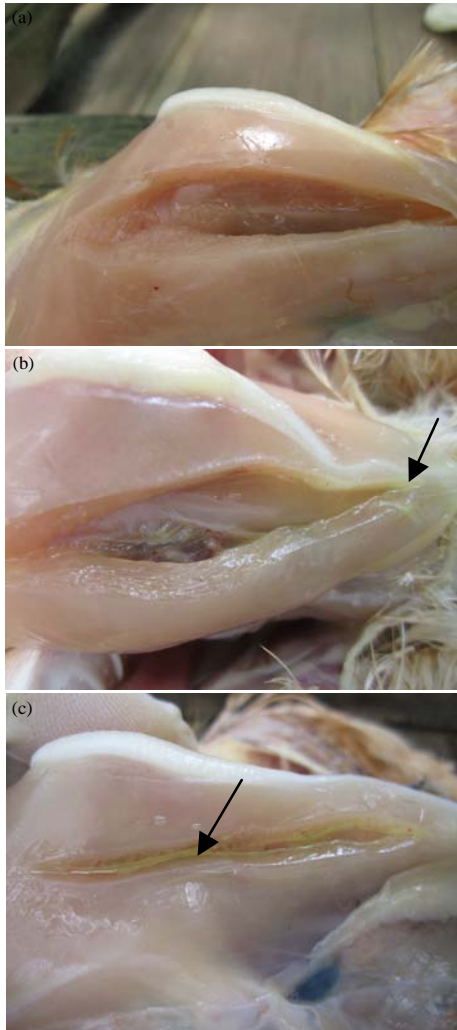


Fig. 4: Pathological finding in the muscle of chickens 3 weeks post inoculation; a) no oil emulsion vaccine; b) only a little oil emulsion vaccine and c) some oil emulsion vaccines. Arrow indicates oil emulsion vaccine

response to LPS and Con A than the vaccine without GSLs. The present study demonstrated that GSLs and oil (MC52) synergistically promoted the immune responses to vaccination against NDV in chickens. Immunization of ND oil emulsion vaccines plus GSLs in chickens induced significantly higher serum HI titers and higher lymphocyte proliferation stimulated with LPS and NDV antigen than ND oil-emulsified vaccine was used alone ( $p < 0.05$ ).

Humoral immune responses play an important role in preventing Newcastle disease virus infection (Seal *et al.*, 2000). HI test is a widely used approach to evaluate the antibody response to ND vaccine and is clearly correlated to the immunity status of chickens

(Alexander, 2008). However, the immunization of currently available ND vaccines in chickens usually elicit poor immune responses (Wang, 2010; Shang and Han, 2011). The present results indicated that supplement of GSLs in ND oil emulsion vaccine enhanced serum HI responses in chickens. When chickens were immunized with ND oil emulsion vaccine containing different concentration of GSLs, the significantly enhanced HI levels were found in the 2, 4 and 6  $\mu\text{g}/0.2\text{ mL}$  of GSLs group as compared with ND vaccine alone. The 6  $\mu\text{g}/0.2\text{ mL}$  of GSLs elicited the highest HI titer. However, in the previous study, the optimal dose of GSLs supplemented in foot and mouth disease vaccine was 10  $\mu\text{g}/0.2\text{ mL}$  (Song *et al.*, 2009). The different optimal dose of GSLs may result from the different antigens used in the vaccines. Figure 1 shows that serum HI titers on Week 0 were higher than those on 1 week post inoculation. The reason for the reduced immune response may attribute to the high level of maternal antibody affecting the immune response to vaccine. The result is in agreement of some other documents reporting high level of maternal antibody interfering with vaccine immunization (Kumar *et al.*, 2000; Rajawat *et al.*, 2008).

The synergism between saponin-like adjuvant and oil for promoting immune responses has been demonstrated in other reports. Gerber reported enhanced immune responses in cats, pigs and guinea pigs immunized with oil-emulsified vaccines containing Quil A (Gerber *et al.*, 1989). Xiao *et al.* (2007a, b) found significant higher immune responses in pigs vaccinated against FMD virus when saponins adjuvants such as extract of *Momordica cochinchinensis* (Lour.) Spreng seeds or Quil A was used in a commercial FMD vaccine than the commercial FMD vaccine was injected alone. Oil emulsion presented well depot effect to co-administered antigen so that antigen was progressively released at the injection site. GSLs possess an immunomodulatory effect. The synergism between GSLs and oil adjuvant may be ascribed to the well depot effect of oil emulsion in combination with the immunomodulatory role of GSLs. The mechanism of immunomodulatory effect of ginseng saponins is still obscure but *in vitro* trials demonstrated that ginseng saponins could improve the antigen presentation of macrophages by promoting the expression of MHC (Major Histocompatibility Complex) Class II (Cao *et al.*, 2011) and stimulate the production of cytokines such as IL-1 $\alpha$  in macrophages (Jang and Shin, 2010). Therefore, ginseng saponins may enhance the immune response possibly by targeting macrophages. Further investigation is needed to assess the specific mechanism.

Lymphocyte-mediated immune response also plays a crucial role in protection against NDV (Seal *et al.*, 2000). The stimulation of lymphocyte proliferation response represented the capacity of inducing an effective T and B-lymphocyte immunity. Data in Fig. 2 shows that lymphocyte proliferative responses to LPS and NDV antigen were increased in the chicken immunized with ND oil emulsion vaccine containing GSLS. Increased lymphocyte response to LPS suggests that B lymphocytes were triggered. Activated B lymphocytes are required to elicit serum antibody production for clonal expansion. The enhanced lymphocyte responses to LPS/NDV stimulation paralleled the augmented NDV-specific HI response in birds immunized with ND oil emulsion vaccine plus GSLS.

Vaccines containing Ginseng Saponins (GS) elicited higher immune responses to vaccination have been demonstrated previously in experimental animals (Rivera *et al.*, 2005), cattles (Hu *et al.*, 2003) and pigs (Rivera *et al.*, 2003a, b). The GS utilized in the previous vaccine formulations was purified from the root of *Panax ginseng* and is limited to use in veterinary vaccines due to its high cost. Compared to ginsenosides in the root of *Panax ginseng* (GS-R), GSLS is cheaper to be supplemented in veterinary vaccines for its recycle use of stems and leaves of ginseng plant. In the present study, GSLS has been demonstrated an adjuvant effect similar to GS-R. HPLC analysis showed that GSLS utilized in this trial contained Rb1, Rb2, Rc, Rd, Re and Rg1. A comparison (Sun *et al.*, 2007) between protopanaxadiols (Rb1, Rb2, Rc, Rd and Rg3) and protopanaxatriols (Rg1, Rg2 and Re) group saponins suggested that Re, Rb1, Rg1, Rg2 and Rg3 in GS-R have potent adjuvant effects and the adjuvant effect of GS is associated with its chemical structure. The adjuvanticity of GSLS in chickens may result from the immunomodulatory effects of its constituents Re, Rb1 and Rg1. The ginsenosides found in ginseng chemically possess a dammarane skeleton which has different sites such as C20 for side sugar chains to attach (Sun *et al.*, 2007). The diverse adjuvant properties of ginsenosides in GSLS might be attributed to the different molecular structures.

In addition to the induction of effective immune response, the toxicity of adjuvants is also a major consideration. Though many saponins such as Quil A were demonstrated have an immunostimulatory effect they were still not allowed to be used as adjuvant in vaccines due to their haemolytic properties. In the previous studies, GS-R(Rg1) or GSLS at the concentration range of 0-500  $\mu\text{g mL}^{-1}$  displayed a slight haemolytic effect while Quil A at 8  $\mu\text{g mL}^{-1}$  exhibited stronger haemolytic activities (Bao, 2008; Sun *et al.*, 2008). In the

present experiment, the highest concentration of GSLS used in chickens is 40  $\mu\text{g mL}^{-1}$  (8  $\mu\text{g}/0.2\text{ mL}$ ) indicating its acceptance as a safe adjuvant. In addition, ginseng as a relatively safe medicine has been used for treatment of human/animal diseases in China for thousands of years (Song and Hu, 2009). In the present study, no chicken death or abnormal behaviors were observed in all the chicken groups. Figure 3 shows that there was no effect on the body weight after immunization. Compared with control group, no adverse reactions at injection site were observed in the chickens immunized with vaccine containing GSLS (Fig. 4 and Table 1). These results indicate that GSLS has very lower or no toxicity to chickens. Though many adjuvants have been demonstrated to possess the adjuvant effect few were licensed to be used for human or veterinary vaccines. The present results indicated that GSLS could be a candidate as a safe adjuvant used in veterinary vaccines.

## CONCLUSION

GSLS can be safely used as an adjuvant for immunization in chickens. Supplement of GSLS in ND oil emulsion vaccine significantly increased the immune responses of birds to NDV vaccination. Therefore, GSLS may be considered as an adjuvant for improvement of vaccines against infections in poultry.

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