Combined adjuvant effect of ginseng stem-leaf saponins and selenium on immune responses to a live bivalent vaccine of Newcastle disease virus and infectious bronchitis virus in chickens

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ABSTRACT Vaccination with a live bivalent vaccine of Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) is a routine practice in poultry industry in China. This study was designed to evaluate ginseng stem-leaf saponins (GSLS) in combination with selenium (Se) for their adjuvant effect on the immune response to vaccination against NDV and IBV in chickens. A live bivalent vaccine of NDV and IBV was diluted in saline solution containing GSLS or Se or both and used to immunize chickens via a intraocular-and-intranasal route. Results showed that GSLS promoted significantly higher NDV- and IBV-specific antibody responses with the highest antibody response detected in GSLS-Se group. The increased antibody was capable of neutralizing NDV and IBV. In addition, GSLS-Se enhanced lymphocyte proliferation and production of IFN-γ and IL-4. More importantly GSLS-Se was found to promote early production and prolong the duration of the antibody responses. In order to improve the efficacy of vaccination in chicken flocks, the diluent containing GSLS-Se deserves further studies to evaluate its effect on other chicken vaccines.

Key words: ginseng stem-leaf saponins, selenium, Newcastle disease, infectious bronchitis

2019 Poultry Science 98:3548–3556
http://dx.doi.org/10.3382/ps/pez207

INTRODUCTION

Newcastle disease (**ND**) is a highly contagious poultry disease caused by Newcastle disease virus (**NDV**). The disease is characterized by nervous, respiratory, enteric, and reproductive infections, and the agent has been classified by the World Organization for Animal Health (**OIE**) as a notable disease-causing virus (Hoffmann et al., 2009). Similarly, infectious bronchitis (**IB**) is an acute and highly contagious viral respiratory disease caused by infectious bronchitis virus (**IBV**) characterized by wet eyes, swollen face, respiratory disease, tracheal and kidney lesions, reduced weight gain in broilers, decreased production, and poor egg quality in layers (Brown and Bevins, 2017; Shirvani, et al., 2018). These 2 diseases can have destructive economic effects on the world domestic poultry production, and outbreaks have been commonly reported in recent years (Pitangui Mendonca et al., 2009; Umar, 2017). Vaccination with a live bivalent vaccine of NDV and IBV is one of the most common approaches to control the diseases. However, poor immune responses to vaccination have been reported and cause incomplete protection (Kapczynski et al., 2013; Jordan, 2017; Rauw et al., 2017; El-Dabae et al., 2018).

Adjuvants are known to improve the efficacy of vaccines (Meunier et al., 2016). Our previous studies have showed that saponins extracted from the stem-leaf of *Panax ginseng* C.A. Meyer (**GSLS**) added an adjuvant effect to vaccine. Zhai et al. (2011b, 2014) observed that oral administration of GSLS has capacity to improve vaccination against ND, avian influenza, and infectious bursal disease in chicken. Yu et al. (2015b) found that oral administration of GSLS had capacity to recover the chickens' immunity with oxidative stress by showing increased antibody response to NDV and avian influenza virus (**AIV**), splenocyte proliferation, and intestinal IgA+ cells and ilELs. Selenium (**Se**) is an essential trace element for animals and plays a crucial role in immunity. Studies have suggested a close relation between selenium and the immunity in chickens (Wang et al., 2018a,b). We hypothesized that combination of GSLS and Se could boost the immune responses and improved the immunization. However, no report has been found regarding the combined effect of GSLS and Se on vaccination. As a live vaccine is usually supplied in a form of lyophilized powder, it needs to be resuspended in a saline solution before use. In this study, we used a solution containing GSLS and Se as a diluent to resuspend a live bivalent vaccine of NDV and IBV and evaluate the adjuvant effect of the diluent on the immune responses in chickens.

Received January 9, 2019.
Accepted March 24, 2019.
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The appropriate scientific section for this paper: Immunology, Health and Disease

MATERIALS AND METHODS

Animals

One-day-old yellow chickens (female) and fertile eggs were purchased from Ningbo Zhenning Animal Husbandry Co., Ltd. (Ningbo, China). Eggs were hatched at 39°C in incubator. Chickens were housed in separated cages and given access to feed and water ad libitum. The room was controlled at 35°C for the first 3 D and gradually reduced to 26°C. All the chickens were treated according to the Zhejiang University Committee on Animal Care and Use.

Vaccines and Virus

Live bivalent vaccine of NDV (Strain La Sota), IBV (Strain H120), ND virus (Strain La Sota), and IB virus (Strain H120) for neutralization test were purchased from Zhejiang CEVA EBVAC Biotech Co., Ltd. (Hangzhou, China).

Ginseng Saponins

Standardized GSLS was a product of Hongjiu Ginseng Industry Co. Ltd, (Jilin, China) containing Re (16.36%), Rd (9.0%), Rg1 (6.0%), Rb2 (3.8%), Rc (3.7%), Rb1 (2.4%), and Rf (0.1%) according to HPLC analysis.

Reagents

The antigen and positive control serum for NDV-specific hemagglutination inhibition (**HI**) test were purchased from Qingdao Yebio Biological Engineering Co., Ltd. (Qingdao, China). The IBV antibody test kit was purchased from IDEXX Laboratories Inc. (Westbrook, ME). Chicken gamma interferon (**IFN-γ**) and IL-4 test kit were purchased from Shanghai Lengton Bioscience Co., Ltd. (Shanghai, China). Chicken lymphocyte separation medium was purchased from Beijing Solarbio Science & Technology CO. Ltd. (Beijing, China). Se in the form of sodium selenium, concanavalin A (**Con A**), lipopolysaccharides (**LPS**), and thiazolyl blue tetrazolium bromide (**MTT**) were the products of Sigma-Aldrich Inc. (St. Louis, MO).

Experiment Design

Experiment 1 To evaluate the effect of GSLS and/or Se on the vaccine antibody response, 70 chickens at 2 days old were randomly divided into 5 groups with 14 birds in each and received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV in saline solution (50 μL) with or without GSLS (5 μg), Se (25 μg), or GSLS (5 μg) + Se (25 μg). Blood samples were collected at 10, 14, and 18 D post immunization for tests of NDV-specific HI titers and IBV-specific antibody titers.

Experiment 2 To observe the changes of maternal or vaccine-induced serum antibody responses, 45 chickens at 12 days old were randomly divided into 3 groups with 15 birds in each and immunized with the same method as Experiment 1. Every 3 D post immunization, blood samples were collected for NDV-specific HI titers and IBV-specific antibody titers tests.

Experiment 3 To evaluate serum cytokines, neutralizing antibodies, and lymphocyte proliferative responses, 45 chickens at 12 days old were randomly divided into 3 groups and immunized with the same method as Experiment 1. At 48 h post immunization, blood samples were collected for measurement of serum IFN-γ and IL-4. At 2 wk post immunization, blood was sampled for isolation of lymphocytes to test lymphocyte proliferation and neutralizing antibodies.

Experiment 4 The trial was carried out on the Hangzhou Layer Experimental Farm in the suburb of Hangzhou and immunization method was according to the routine protocol used on the farm. A total of 500 chickens at 8 days old received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV in saline solution (50 μL) containing GSLS-Se, whereas other 500 chickens were immunized in the same way but the vaccine in saline solution alone and served as a control group. The chickens in both groups were boosted with an inactivated vaccine of NDV and IBV after 2 wk. Blood samples were collected from 50 chickens randomly selected from each group at 2 and 3 wk post first immunization to test specific antibody responses.

Hemagglutination Inhibition Test

Serum NDV-specific HI titers were determined as previously described (Zhai et al., 2011a). Briefly, a 2-fold serial dilution of serum samples (1:2 to 1:1,024) was made in a V-shaped bottom 96-well microtiter plate containing 25 μL saline each well. Afterwards, 4 hemagglutination units (**HAU**) of NDV antigen were added to each well. After incubation at 37°C for 30 min, 25 μL of 1% rooster erythrocyte suspension was added and incubated at 37°C for another 30 min. All samples were tested repeatedly, and each plate included positive and negative serum controls. HI titers were defined as the reciprocal of the final dilution of serum that results in complete inhibition of hemagglutination. Data were expressed as log 2 of the highest dilution of HI.

Serum IBV-Specific Antibody Assay

Serum IBV-specific antibodies were tested by ELISA kits according to the manufacturers' instructions. The relative level of antibody titer in optical density of the sample was determined by calculating the ratio of sample to positive (**S/P**) as (mean of sample optical density

− mean of negative control optical density)/(mean of positive control optical density − mean of negative control optical density). The endpoint titer was calculated with the following formula: log10 titer = 1.09 (log 10 S/P) + 3.36 (Flock Check program, IDEXX).

Peripheral Blood Lymphocyte Proliferation Test

The test was performed as previously described (Li et al., 2009). Briefly, peripheral blood lymphocytes were isolated by chicken lymphocyte separation medium. Microtiter plate wells containing 5.0×10^6/mL peripheral blood lymphocytes added in triplicate in 100 μL RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat inactivated fetal bovine serum were cultured in 5% CO_2 at 40°C. LPS at 20 μg/mL, ConA at 20 μg/mL, or NDV antigen at 4 HA in the well was used as a stimulator. Wells without mitogen were used as controls. After 20 h, 20 μL MTT (5 mg/mL) was added to each well and incubated for another 4 h. The plates were centrifuged (1,000 × g for 10 min), and the supernatant was carefully removed. Approximately 150 μl of acidic dimethyl sulfoxide (0.04 N HCl) was added to each well and mixed thoroughly by slightly shaking to solubilize the MTT formazan. The average optical density (**OD**) was read at 570 nm. The stimulation index (**SI**) was calculated based on the following formula: SI = (OD value of mitogen-stimulated cells)/(OD value of non-stimulated cells).

Cytokine Assay

The concentrations of IFN-γ and IL-4 were determined by specific ELISA kits from Shanghai Lengton Bioscience Co. Ltd. (Shanghai, China) according to the manufacturer's instructions.

Neutralizing Antibody Assay

To test NDV-specific neutralizing antibody, serum samples (in 1:4 to 1:1,024 dilution) were prepared in EP tubes. The diluted serum was mixed with an equal volume of 10^2 50% tissue culture infective dose (**TCID50**) of the NDV Lasota strain, followed by incubation for 1 h at 37°C. Neutralizing antibody titers were estimated by inoculating chicken embryo fibroblasts (prepared from 10-day-old fertile eggs) with incubated virus in serum prepared above. Standard positive serum was used as a positive control. After 144 h, the number of wells with cytopathic effect was recorded for each group of experimental cells, half of which was calculated according to the Reed-Muench method (PD50). Virus neutralizing (**VN**) titers of the serum was calculated and expressed as mean ± SEM.

To test IBV-specific neutralizing antibody, serum samples (in 1:4 to 1:1,024 dilution) were prepared in EP tubes. The diluted serum was mixed with an equal volume of 10^2 50% tracheal organ culture infective dose **(TOCID50)** of the IBV H120 strain, followed by incubation for 1 h at 37°C. Neutralizing antibody titers were estimated by inoculating Tracheal Organ Culture of Chicken Embryo (**TOC**, prepared from 20-day-old fertile eggs) with incubated virus serum prepared above. IBV hyper immune serum was used as a positive control. After 144 h, the number of wells with live TOC was recorded for each group of experimental tissues, half of which was calculated according to the Reed-Muench method (PD50). VN titers of the serum were calculated and expressed as mean ± SEM.

Statistical Analysis

Data analysis was performed with SPSS 20 software (version 20.0, SPSS Inc., Chicago, IL). ANOVA with LSD test was performed for multiple comparisons between groups. P value less than 0.05 was considered significantly different.

RESULT

GSLS and Se Synergistically Enhance the Antibody Response

Figure 1 shows that significantly higher NDV- and IBV-specific antibody responses were detected in birds immunized with vaccine diluted in physiological saline solution (**PSS**) containing GSLS and GSLS in combination with Se than the birds with PSS alone. However, the highest antibody response was found in birds when saline solution GSLS in combination with Se was used as vaccine diluent. Therefore, the solution containing GSLS and Se was used as a vaccine diluent in the following experiments.

GSLS-Se Promotes Early Production and Prolongs Duration of Antibody Responses

Serum NDV-specific HI titer ≥4 and IBV-specific antibody titer ≥396 are believed to have protective effect on the chickens challenged by NDV and IBV, respectively. The present study showed that GSLS-Se promoted early production of antibody responses. After immunization, NDV-specific HI titer was ≥4 in group with GSLS-Se + Vaccine but ≤4 in group with Vaccine alone on day 9 (Figure 2a); IBV-specific antibody titer was ≥396 in group with GSLS-Se + Vaccine but ≤396 in group with Vaccine alone on day 15 (Figure 2b). In addition, GSLS-Se prolonged duration of antibody responses. NDV-specific HI titer ≥4 lasted from day 9 to day 24 in group with GSLS-Se + Vaccine but lasted from day 12 to 15 in group with Vaccine alone (Figure 2a); IBV-specific antibody titer ≥396 in group with GSLS-Se + Vaccine lasted from day 15 to 27 but ≥396 in group with Vaccine alone lasted day 18 to 21 (Figure 2b).

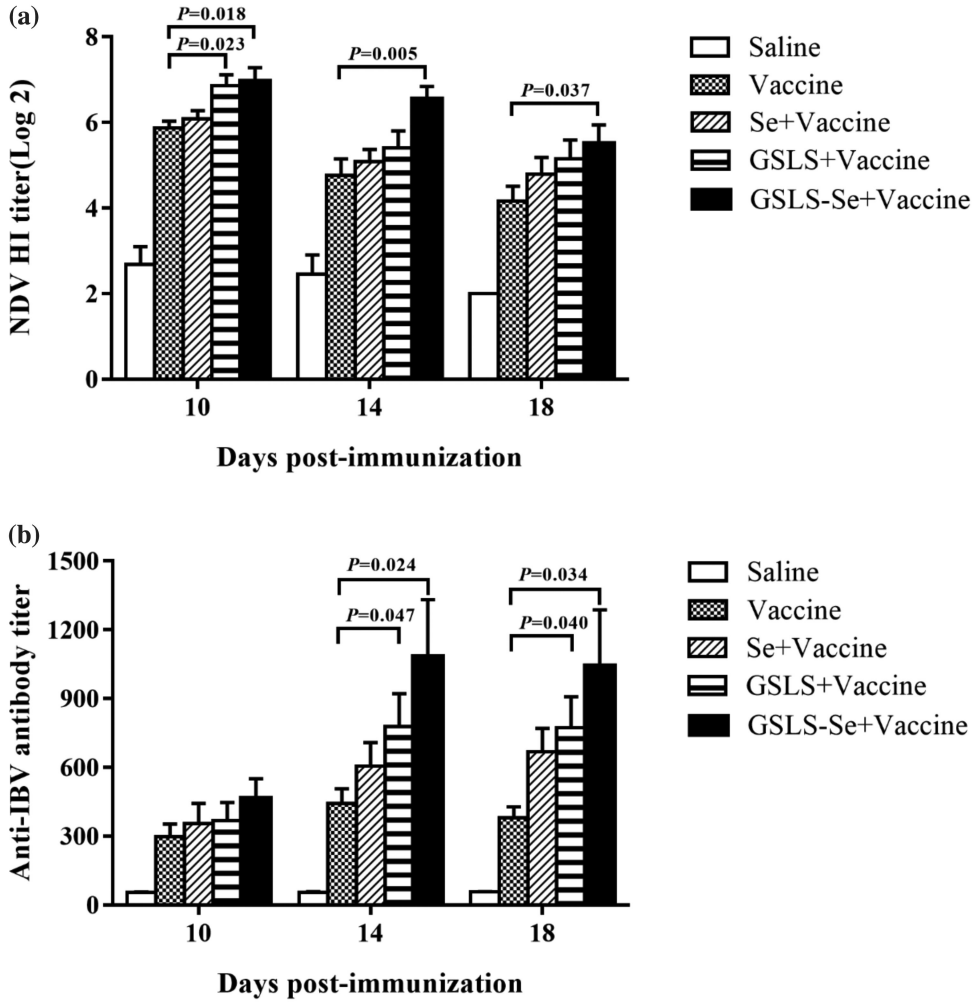

Figure 1. Effect of GSLS and/or Se on the antibody response to vaccination against NDV and IBV. Chickens (n = 14/group) received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV in saline solution (50 μL) containing GSLS (5 μg), Se (25 μg), or GSLS (5 μg) + Se (25 μg). Chickens receiving saline solution only served as a control. Blood samples were collected at 10, 14, and 18 D post immunization for analysis of NDV-specific HI (a) and IBV-specific antibody (ELISA) (b). Data are represented as mean ± SE.

GSLS-Se Enhances Lymphocyte Proliferation and Cytokine Production in Blood

Figure 3 shows that lymphocyte proliferative responses to LPS, Con A, and NDV antigen were significantly higher in group with GSLS-Se + Vaccine than in group with Vaccine alone. Significantly higher levels of IFN-γ and IL-4 in blood were detected in group with GSLS-Se + Vaccine than in group with Vaccine alone (Figure 4).

GSLS-Se Increases Neutralizing Antibodies

Figure 5 shows chickens that received a live bivalent vaccine of NDV and IBV in PSS with GSLS-Se had significantly higher NDV/IBV neutralizing antibodies than the chickens without GSLS-Se.

GSLS-Se Enhances NDV- and IBV-specific Antibody Responses in a Commercial Chicken Flock

When GSLS-Se was used in a commercial chicken flock, significantly higher NDV- and IBV-specific antibody responses were also detected in group with GSLS-Se + Vaccine than group with Vaccine alone as shown in Figure 6.

DISCUSSION

Combined adjuvant effects of GSLS and Se on the immune response to a live vaccine in chickens was demonstrated in the present study. When chickens were administered an intraocular-and-intranasal immunization of a live bivalent vaccine of NDV and IBV resuspended in PSS containing GSLS and Se, the chickens were induced significantly higher NDV- and IBV-specific antibody responses (Figures 1, 5, and 6), lymphocyte

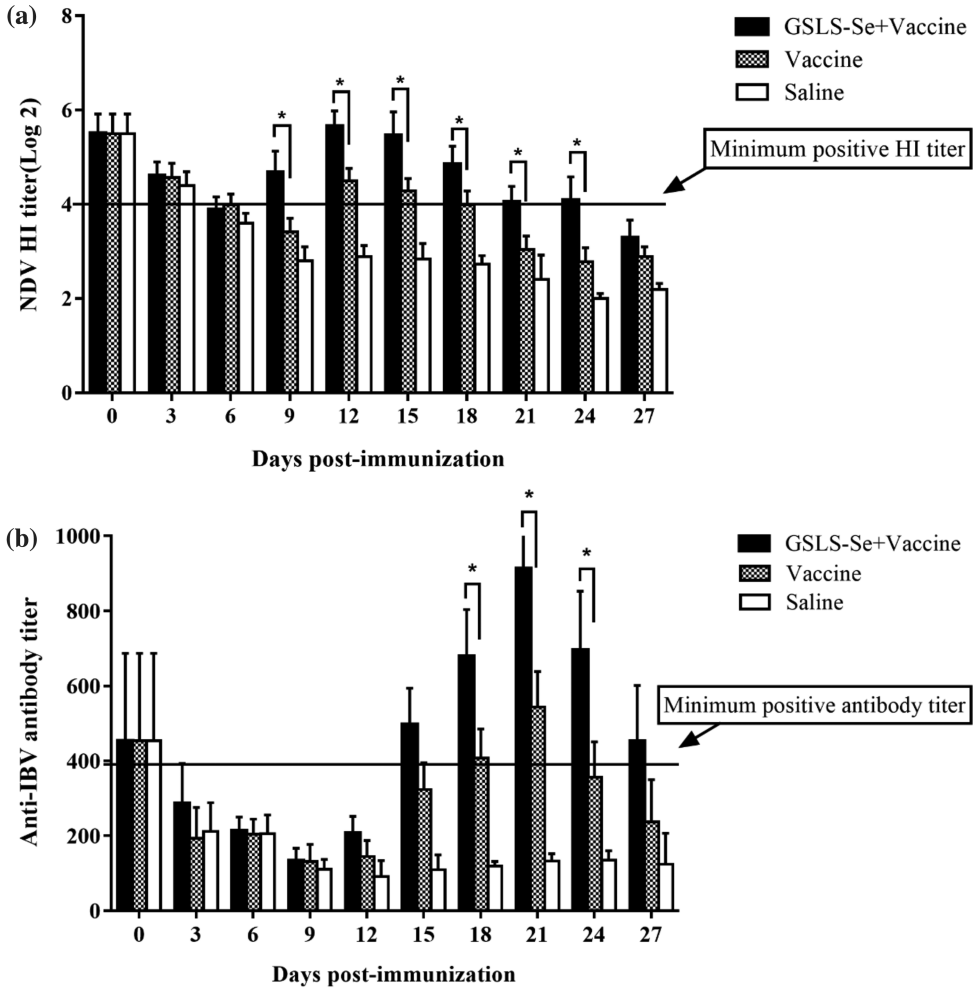

Figure 2. Changes of serum NDV- and IBV-specific antibody titers. Chickens (n = 15/group) received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV in saline solution (50 μL) with or without GSLS-Se. Chickens receiving saline solution served as a control. Blood samples were collected before and every 3 D post immunization for analysis of NDV-specific HI (a) and IBV-specific antibody (ELISA) (b). Data are represented as mean ± SE.

proliferation (Figure 3), and production of IFN-γ and IL-4 (Figure 4) when compared to the vaccine resuspended in PSS alone. More importantly, an early produced and prolonged antibody responses to the vaccine resuspended in PSS with GSLS-Se were also observed (Figure 2).

Newcastle disease and infectious bronchitis are 2 important poultry diseases that cause huge amounts of economic losses in the world (Chen et al., 2013; Bello et al., 2018). To control these diseases on commercial chicken farms in China, a live bivalent vaccine of NDV and IBV is usually used for vaccination. The vaccine is generally supplied in form of lyophilized powder and diluted in PSS before use. But feeble immunity has been frequently found in chicken flocks, leading to poor immune protection. For examples, Berhanu et al. (2010) reported sporadic outbreaks of NDV in Malaysia, remaining a constant threat to commercial poultry in spite of routine vaccination programs; Pitangui Mendonca et al., (2009) observed that periodical outbreaks of IBV in densely populated poultry regions in Brazil where routine vaccination against IBV was carried out. Therefore, searching for a practical approach to improve the efficacy of vaccination is needed.

The use of GSLS to enhance immune responses to vaccination has been reported previously. Kong et al. (2004) found that subcutaneous injection of ginseng saponins promoted lymphocyte proliferation and antibody titer in chickens after immunized with ND vaccine. Zhai et al. (2011a) reported enhanced NDV-specific antibody and lymphocyte proliferative responses in chickens after oral administration of GSLS. Yu et al. (2015a,b) observed that oral administration of GSLS had capacity of recovery of the immunity in chickens with immune suppression induced by cyclophosphamide, showing increased antibody responses to vaccination against NDV and AIV, splenocyte proliferation, intestinal IgA+ cells, and ilELs. Ni et al. (2016) found attenuated pseudorabies virus (**aPrV**) vaccine diluted in PSS containing GSLS and thimerosal instead of PSS alone induced a significantly higher immune response. El-Dabae et al. (2018) discovered that subcutaneous injection NDV vaccine together with ginseng saponins in chickens was effective to protect

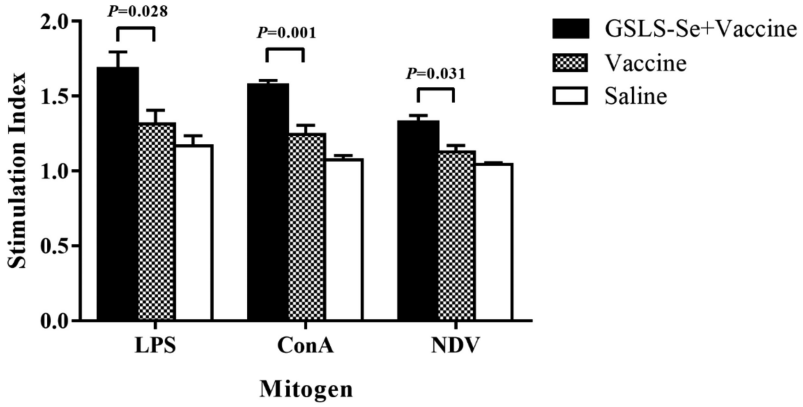

Figure 3. Peripheral blood lymphocyte proliferation in response to LPS, Con A, or NDV. Chickens (n = 15/group) received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV in saline solution (50 μL) with or without GSLS-Se. Chickens receiving saline solution only served as a control. Lymphocytes were isolated from peripheral blood collected 2 wk post immunization and stimulated with LPS, Con A, or inactivated NDV antigen in vitro. Lymphocyte proliferation was measured by the MTT method as described in the text and shown as a stimulation index (SI). Data are represented as mean ± SE.

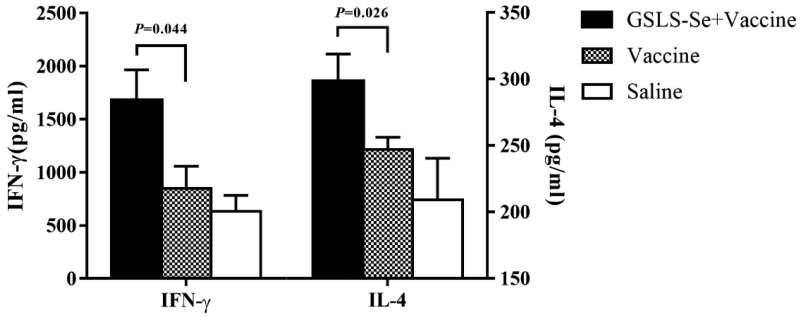

Figure 4. Serum IFN-γ and IL-4. Chickens (n = 15/group) received intraocular-and-nasal immunization with a live bivalent vaccine of NDV and IBV (50 μL) with or without GSLS-Se. Chickens receiving saline solution only served as a control. Blood samples were collected at 2 wk post immunization for IFN-γ and IL-4 tests by ELISA. Data are represented as mean ± SE.

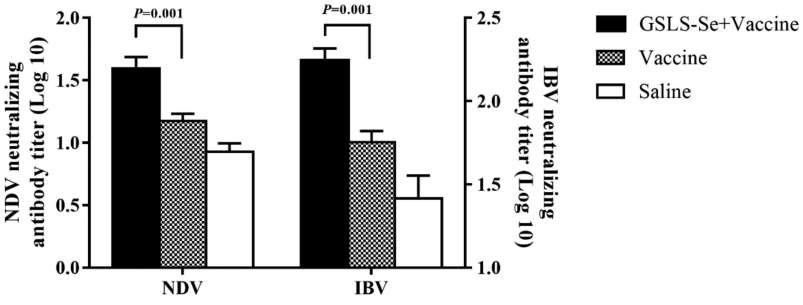

Figure 5. Serum ND and IB virus neutralization. Chickens (n = 15/group) received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV (50 μL) with or without GSLS-Se. Chickens receiving saline solution only served as a control. Blood samples were collected at 2 wk post immunization for ND virus neutralization assay by CEF and IB virus neutralization assay by Tracheal Organ Culture. Data are represented as mean ± SE.

chickens against an Egyptian field NDV strain and had capacity to reduce the shedding of the virus. Selenium is an essential element and usually used as a dietary supplement for growth and productivity in poultry. Singh et al. (2006) observed chickens receiving supplements of selenium together with vitamin E enhanced significantly higher HI antibody titers and heavier spleen and bursa after immunizing against NDV vaccine. Lee et al. (2014) observed in ovo injection of Se with experimental necrotic enteritis in broiler chickens showed significantly lower intestinal lesions and higher levels of transcripts for IL-1β and IL-6. Rao et al. (2016) reported that supplement of organic selenium in diet had positive effect on anti-oxidant, performance, and immune responses in broilers in tropical summer. Hu et al. (2018) found that a dietary supplement of Se diminished aflatoxin B_1 (**AFB1**)-induced immune toxicity in chicken's bursa of Fabricius by alleviating oxidative damage and cell cycle arrest.

In the present study, we used a PSS containing GSLS and/or Se as a diluent instead of PSS alone to

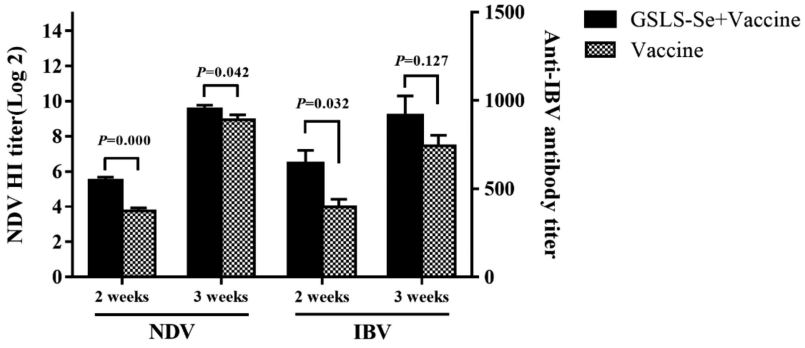

Figure 6. Enhanced antibody responses to vaccination against NDV and IBV in a commercial chicken flock. Chickens (n = 500/group) received intraocular-and-intranasal immunization with a live bivalent vaccine of NDV and IBV (50 μL) with or without GSLS-Se and a booster immunization was administered with an inactivated vaccine of NDV and IBV after 2 wk. Blood samples were collected from 50 chickens randomly selected in each group at 2 and 3 wk post booster immunization for NDV-specific titers by HI and IBV-specific antibody titers by ELISA. Data are represented as mean ± SE.

resuspend a lyophilized powder of a live vaccine of NDV and IBV and immunized chickens with the vaccine via a intraocular-and-intranasal route. Significantly increased antibody response was found in chickens when GSLS or GSLS-Se was used in the diluent and the highest antibody responses were found when the diluent contained both GSLS and Se (Figure 1). The enhanced immune responses may result from the combined effects of GSLS and Se. Figure 5 shows that GSLS-Se enhanced antibody was capable of neutralizing virus.

Maternally derived antibodies (**MDA**) provide early protection from disease. They can be found in the progeny of vaccinated chickens as a consequence of routine vaccination campaigns and are naturally passed from the hens to the chicks through the egg yolk (Sakaguchi et al., 1998; Bertran et al., 2018). MDA gradually declines and reaches a low level due to natural degradation of proteins 2 or 3 wk after chicks are hatched (Hamal et al., 2006). A similar change of MDA was found in chickens without immunization in the present study (Figure 2).

According to the manufacturers' instructions, the minimum protective HI titer to NDV and antibody titer to IBV are 1:4 and 1:396, respectively. Chickens with HI or antibody titers lower than the minimum are susceptible to infections of NDV and IBV. Figure 2 shows MDA declined below the minimum at 18 days old for HI titer to NDV while 15 days old for antibody titer to IBV after hatched chickens. An incubation period is needed for a vaccine to induce a protective immune response. Ganapathy et al. (2006) reported NDV HI antibody titers in commercial chickens began to rise after 14 D post immunizing with live vaccine of NDV via a intraocular-and-intraoral route. To induce the immune responses higher than the minimum protective antibody titers, it took 12 D for NDV-specific HI titers and 18 D for IBV-specific antibody titer when the vaccine was resuspended in PSS while it took only 9 D for NDV and 15 D for IBV when the vaccine was resuspended in a solution containing GSLS-Se (Figure 2), indicating that GSLS-Se promoted early production of the vaccine-induced antibodies and shortened the incubation period.

Lymphocytes are required for antibody production, and clone expansion of lymphocytes takes place in response to mitogen stimulation. Lymphocyte proliferation is often used to evaluate the lymphocytes in humoral and cellular immune responses (Chaplin, 2006). There are T and B subsets of lymphocytes, and response of different subsets depends on the mitogen used. T cells are responsive to Con A, whereas B cells are responsive to LPS (Tizard, 2009). In this study, proliferative responses to Con A or LPS of lymphocytes isolated from chickens immunized with vaccine resuspended in PSS with GSLS-Se were significantly increased when compared with the group immunized with vaccine resuspended in PSS alone (Figure 3), indicating that both T and B lymphocytes were activated. The increased cell proliferative response to NDV stimulation paralleled the elevated serum NDV-specific antibody in the group using PSS containing GSLS-Se as a diluent.

T cells are often dominated by IFN-γ classified as T helper 1 response (**Th1**), whereas B cells are dominated by IL-4 classified as T helper 2 response (**Th2**) (Ferrick et al., 1995). In the present study, significantly increased serum IFN-γ and IL-4 were observed in chickens immunized with vaccine in PSS containing GSLS-Se when compared with chickens immunized with vaccine in PSS without GSLS-Se (Figure 4), suggesting that GSLS-Se stimulate both Th1 and Th2 immune responses.

To evaluate PSS containing GSLS-Se as a vaccine diluent in use on chicken farms, a clinical trial was carried out in a commercial chicken flock on Hangzhou Layer Experimental Farm. As the traditional attenuated bivalent vaccine of NDV and IBV induced a short duration of antibody responses shown in Figure 2, a boost injection was administered with an inactivated vaccine of NDV and IBV 2 wk after the primary immunization based on the protocol used on this farm. The result in Figure 6 shows that both NDV- and

IBV-specific antibody titers significantly enhanced in chickens with GSLS-Se when compared to the chickens without GSLS-Se.

In conclusion, a live vaccine of NDV and IBV diluted in a PSS containing GSLS-Se induced significantly higher NDV- and IBV-specific antibody and lymphocyte proliferative responses, and production of IFN-γ and IL-4 than the vaccine diluted in PSS alone. Besides, GSLS-Se promoted early production and prolonged duration of antibody responses. Therefore, the diluent containing GSLS-Se deserves further studies to evaluate its effect on other chicken vaccines.

ACKNOWLEDGMENTS

This study was supported by the project (No. 31172356) of the National Natural Scientific Foundation of China. The authors are thankful to the students in the Laboratory of Traditional Chinese Veterinary Medicine (TCVM Lab) for their assistance in the experiment.

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