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Research paper

Improved immune responses to a bivalent vaccine of Newcastle disease and avian influenza in chickens by ginseng stem-leaf saponins

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ABSTRACT

Our previous investigation demonstrated that ginseng stem-leaf saponins (GSLS) derived from the stems and leaves of *Panax ginseng* C.A. Meyer promoted humoral and gut mucosal immunity in chickens vaccinated with live infectious bursa disease vaccine. The present study was designed to evaluate the effect of GSLS on the immune response to a bivalent inactive vaccine of Newcastle disease (ND) and avian influenza (AI) in chickens immunosuppressed by cyclophosphamide (Cy). One hundred and sixty-eight specific-pathogen-free (SPF) chickens were randomly divided into 7 groups, each containing 24 birds. Chickens in groups 3–7 received intramuscular injection of Cy at 100 mg/kg BW for 3 days to induce immunosuppression. Groups 1 and 2 were injected with saline solution in the same way as groups 3–7. Following injection of Cy, groups 4–7 were orally administrated GSLS (2.5, 5 and 10 mg/kg BW) or astragalus polysaccharide (APS) (200 mg/L) in drinking water for 7 days; groups 1–3 were not medicated and served as control birds. After administration of GSLS or APS, groups 2–7 were subcutaneously injected with a bivalent inactive vaccine of ND and AI. After that, serum was sampled for detecting antibody titers by HI, spleen was collected for lymphocyte proliferation assay, and duodenum tissues were collected for measurement of IgA-secreting (IgA+) cells and intestinal intraepithelial lymphocytes (iIELs). The results showed that injection of Cy significantly suppressed immunity in chickens; oral administration of GSLS before immunization recovered splenocyte proliferation induced by ConA and LPS, and the numbers of IgA+ cells and iIELs as well as the specific antibody response to a bivalent inactive vaccine of ND and AIin immunosuppressed chickens treated with Cy. Therefore, GSLS may be the potential agent to improve vaccination in immunosuppressed chickens.

1. Introduction

Immunosuppression is a state of immunity dysfunction and is endangering poultry industry nowadays (Fan et al., 2013). Under intensive poultry-rearing conditions, stress due to large population, inappropriate management, environmental ammonia, foodborne mycotoxins, and suboptimal nutrition pose a considerable challenge for the innate immune system (Thachil et al., 2014). More importantly, infectious diseases such as Marek's disease, infectious bursal disease, chicken infectious anemia, hydropericardium syndrome, avian leukosis, hemorrhagic enteritis and avian pneumovirus can interfere with the acquired immune system (Balamurugan and Kataria, 2006; Bhatt et al., 2013; Hoerr, 2010; Tan et al., 2015). When immunosuppression occurs, poultry industry undergoes heavy morbidity and mortality as well as severe economic loss of production, resulting from more susceptibility to secondary infections and sub-optimal response to vaccinations (Shini et al., 2010). Among the diseases, Newcastle disease (ND) and avian influenza (AI) are two leading devastating diseases. ND is highly contagious and affects many species of birds causing significant economic losses to the poultry industry (Fentie et al., 2014; Zhang et al., 2014); AI is not only the disease that just damages poultry, but also has become a life-threatening disease to human being since the first identified cases of human high-pathogenicity H5N1 avian influenza in Hong Kong in 1997 (Sims et al., 2003). At present, a mandatory vaccination program is enforced for ND and AI in China. As reviewed by Peyre et al. (2009), outbreaks of the diseases frequently take place following vaccination due to the immune

Abbreviations: AI, avian influenza; AIV, avian influenza virus; APS, astragalus polysaccharide; BW, body weight; Cy, cyclophosphamide; DAB, 3,3′-diaminobenzidine; GSLS, ginseng stem-leaf saponins; HBSS, Hank's balance salt solution; HE, hematoxylin–eosin; HI, Hemagglutination inhibition; IBD, infectious bursal disease; IgA+ cells, IgA-secreting cells; iIELs, intestinal intraepithelial lymphocytes; MTT, thiazolyl blue tetrazolium bromide; ND, Newcastle disease; NDV, Newcastle disease virus; sIgA, secretory IgA; SPF, specific-pathogen-free.

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failure caused by immunosuppression and other numerous factors. Therefore, there is an urgent need to improve the immunization with currently available vaccines so as to effectively protect the host from infections.

Saponins isolated from the stem-leaf of *Panax ginseng* C.A. Meyer (GSLS) has been reported to be useful to improve vaccination. Zhai et al. observed that administration of GSLS in drinking water produced significantly better vaccination against Newcastle disease, avian influenza and infectious bursal disease in chickens (Zhai et al., 2011a, 2011b, 2014). However, no research has been found regarding the effect of GSLS on the immune responses in immunosuppressive birds. The present study was designed to investigate the oral administration of GSLS for its effect on the immune responses induced by a bivalent inactive vaccine of Newcastle disease virus (NDV) and avian influenza virus (AIV) in chickens immunosuppressed by cyclophosphamide (Cy) which is often used to elicit suppression in both humoral and cellular immunity in animal models (de Jonge et al., 2005). As astragalus polysaccharide (APS) is one of the most intensively immunostimultors (Kallon et al., 2013; Zahran et al., 2014), it served as a positive control in this study.

2. Materials and methods

2.1. Ginseng stem-leaf saponin (GSLS) and astragalus polysaccharide (APS)

Standardized ginseng stem-leaf saponins from *Panax ginseng* C.A. Meyer (GSLS) were purchased from Hongjiu Ginseng Industry Co. Ltd. (Jilin, China) and contained the saponins Rg1 (6.0%), Re (16%), Rb1 (1.4%), Rb2 (3%), Rc (2.5%), and Rd (8%) as determined by HPLC analysis. Duo Tang Bao (APS), containing more than 70% astragalus polysaccharide, was purchased from Beijing Centre Biology Co. Ltd. (Beijing, China). 200 mg APS was dissolved in 1 L water to make the final APS solution according to the manufacturer's instruction.

2.2. Chickens

One day old specific-pathogen-free (SPF) White Leghorn chickens were obtained from Zhejiang Shennong Stock Breeding Inc. (Ningbo, China). The birds were reared in separated units with at 14:10 light:dark photoperiod. Temperature in the house was set at 35 °C for the first 3 d and was reduced by 3 °C each consecutive week until it reached 26 °C. Feed and water were supplied ad libitum. All animals received humane care according to the criteria outlined in the Zhejiang University Committee on Animal Care and Use.

2.3. Vaccines

Bivalent inactive vaccine of Newcastle disease virus (NDV, Strain La Sota) and avian influenza virus (AIV, H9N2) was the product of Qingdao Yebio Bioengineering Co. Ltd. (Qingdao, China). The vaccine includes inactivated Newcastle disease virus (NDV, Strain La Sota) and inactivated orthomyxoviridae type A influenza virus (AIV, Subtype H9/Chicken/Shanghai/1/98, Stain F) as well as oil adjuvant.

2.4. Reagents

Cyclophosphamide (Cy) was purchased from Tonghua Maoxiang Pharmaceutical Co. Ltd. (Tonghua, China). The antigen and positive control sera used for the ND virus-specific and AI virus-specific hemagglutination inhibition (HI) test were purchased from Qingdao Yebio Bioengineering Co. Ltd. (Qingdao, China). Concanavalin A (ConA), lipopolysaccharides (LPS, *Escherichia coli* 0127:B8) and thiazolyl blue tetrazolium bromide (MTT) for lymphocyte proliferation assay and rabbit-anti-goat IgG (whole molecule)-peroxidase and 3,3′-diaminobenzidine (DAB) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). For the analysis of IgA+ cells, goat-anti-chicken IgA was purchased from Bethyl Laboratories Inc. (Montgomery, TX, USA).

Table 1
Schedule for the treatments of different groups.

Group	*n*	Cy injection	GSLS or APS administration		Vaccination
			GSLS (mg/kg BW)	APS	
1	24				
2	24				+
3	24	+			+
4	24	+	2.5		+
5	24	+	5		+
6	24	+	10		+
7	24	+		+	+

2.5. Experimental design

After 2 weeks of acclimatization, 168 SPF chickens were randomly divided into 7 groups, each consisting of 12 male and 12 female animals. Chickens in groups 3–7 received intramuscular injection of Cy at 100 mg/kg BW for 3 days to induce immunosuppression. Groups 1 and 2 were injected with saline solution in the same way as groups 3–7. Groups 4–7 were orally administrated GSLS (2.5, 5 and 10 mg/kg BW) or APS (200 mg/L) (APS served as a positive control in this study as it is the one of mostly studied immunomodulators derived from medicinal herbs) in drinking water for 7 days; groups 1–3 were not medicated and served as control birds. After that, groups 2–7 were subcutaneously inoculated at the neck with a bivalent inactive vaccine of NDV and AIV (Table 1). From 6 chickens randomly selected in each group before and 1, 2, and 3 weeks after immunization, blood samples were collected for measurement of NDV- and AIV-specific HI titers; splenocytes were prepared for lymphocyte proliferative assay; three pieces of duodenum tissues (1 cm in length) were sampled and fixed immediately in 10% (v/v) neutral buffered formalin.

2.6. Hemagglutination inhibition (HI)

Serum NDV- and AIV-specific HI titers were determined as described by Zhai et al. (2011a). Briefly, a 2-fold serial dilution of serum (1:2 to 1:2048) was placed in the V-shaped bottom 96-well microtiter plate containing 25 μL of PBS in each well, pH 7.2–7.4. Shortly afterwards, 25 μL of virus antigen (NDV or AIV, 4 HA units) was added per well and incubated at 37 °C for 40 min with the exception of the last row, which served as the control wells. Next, a 25-μL quantity of 1% rooster erythrocyte suspension in PBS was added to each well, and then the plate was reincubated at 37 °C for 15 min. All samples were tested in triplicate, and positive and negative serum controls were included on each plate. The HI titer was defined as the reciprocal of the last dilution of serum causing complete inhibition of hemagglutination. The data were expressed as the log 2 of the highest dilution that exhibited HI.

2.7. Lymphocyte proliferation assay

The test was performed as described by Li et al. (2009). Briefly, spleens collected into Hank's balance salt solution (HBSS) were minced and passed through a fine steel mesh to obtain the cell suspension under aseptic conditions. The cells were then washed in HBSS three times by centrifugation (500 × *g* at 4 °C for 10 min), and cultured in RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin

and 10% heat inactivated fetal calf serum (FCS). Cell viability was assessed with trypan blue exclusion stain and the cell suspension was adjusted to 1.0×10^7/mL. Mitogen-induced stimulation was performed by incubating equal volumes (100 μL) of lymphocyte suspension with either ConA (40 μg/mL) or LPS (35 μg/mL) in each well for the MTT assay. RPMI 1640 medium (100 μL) was added to the cell suspension in control wells. Cells were added in triplicate, and incubated in a 5% CO_2 at 40 °C for 44 h. After that, 50 μL MTT (2 mg/mL) was added to each well and incubated for an additional 4 h. Media and untransformed MTT were then removed by centrifuging at 1000 × *g* for 10 min, followed by careful inverting and blotting of the plate. Add 150 μL of acidic dimethyl sulfoxide (DMSO, 0.04 N HCl) to each well and mix thoroughly by slightly shaking to solubilize the MTT formazan. The mean optical density (OD) was read using a test wavelength of 570 nm. The stimulation index (SI) was calculated based on the formula: SI = OD value of mitogen-stimulated cells divided by OD value of non-stimulated cells.

2.8. Analysis of IgA+ cells

The IgA+ cells were identified with the histochemical staining method described by Zhai et al. (2011b). Briefly, the fixed intestinal tissues were first embedded in paraffin, serially cut into 6 μm in thickness and mounted on poly-lysine-coated glass slides. The slides were then passed through graded alcohols to phosphate-buffered saline (PBS; pH 7.4, 0.01 M). Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 30 min. The sections were then incubated with citrate-buffered solution (pH 6.0, 0.01 M) at 95 °C for 20 min, to enhance antigen retrieval. After 3 washes, the sections were treated with 5% FCS in PBS to reduce non-specific background binding at room temperature for 30 min. The sections were then incubated sequentially with primary (goat-anti-chicken IgA antibody, 1:1000) and secondary antibody (rabbit-anti-goat IgG conjugated HRP, 1:250) at 37 °C for 1 h each. Sections were washed 3 times in PBS between each incubation stage. For control purposes, primary antibody was replaced PBS. After that, the sections were stained for the peroxidase using 0.05 M Tris–HCl buffer (pH 7.6) containing 0.05% DAB and 0.03% H_2O_2. After immunohistochemical staining, the sections were slightly counterstained with hematoxylin.

2.9. Analysis of iIELs

The fixed samples of duodenum were embedded in paraffin and serially cut into 6 μm in thickness and mounted on glass slides. After hematoxylin–eosin (HE) staining, the sections were mounted. The number of intestinal intraepithelial lymphocytes (iIELs) was counted at 10 different fields of intestinal villi in each section for the statistical analysis of the data.

2.10. Statistical analysis

Data analysis was performed with SPSS software (version 19.0, SPSS Inc., Chicago, IL). One-way ANOVA with Duncan post hoc test was used for multiple comparisons between groups. Values were expressed as the mean ± standard error of the mean (S.E.M.). *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Lymphocyte proliferation

The lymphocyte proliferative responses to ConA and LPS are shown in Fig. 1. Before treatment, no significant difference in stimulation indexes (SI) was found between groups. However, injection of Cy significantly inhibited both ConA and LPS induced lymphocyte proliferation (group 3) when compared with the control birds treated with saline (groups 1 and 2) ($P < 0.05$). Both ConA and LPS induced lymphocyte proliferative responses in chickens administered GSLS (groups 4–6) or APS were significantly higher than in group 3 and even restored to the levels similar to the control (group 2). In GSLS treated birds, highest SI values were observed in the birds administered GSLS at 5 mg/kg BW, which did not significantly differ from those in APS-treated birds.

3.2. Intestinal IgA+ cells

Changes of the numbers of IgA+ cells in duodenum are shown in Fig. 2. The IgA+ cells were morphologically round with nuclei surrounded by a ring of yellow-brown cytoplasm dyed with DAB (Fig. 2A). Before treatment, no significant difference was found between groups. After injection of Cy, the number of IgA+ cells (group 3) significantly decreased when compared with the control birds treated with saline (group 2) ($P < 0.05$). However, the number of IgA+ cells in Cy-treated chickens significantly increased after administration of GSLS (groups 4–6) or APS (group 7) when compared with Cy-treated chickens without medication (group 3) ($P < 0.05$). In GSLS-treated chickens, birds administered GSLS at 5 mg/kg BW were found to have the highest number of IgA+ cells (group 5).

3.3. Intestinal intraepithelial lymphocytes (iIELs)

Changes of the numbers of iIELs in duodenum are shown in Fig. 3. iIELs are small round cells with densely stained nuclei, located within the epithelial cell layer in duodenum (Fig. 3A). Before treatment, no significant difference was found between groups. After injection of Cy, the amount of iIELs (group 3) significantly decreased when compared with the control birds treated with saline (group 2) ($P < 0.05$). However, the number of iIELs Cy-treated chickens significantly increased after administration of GSLS (groups 4–6) or APS (group 7) when compared with Cy-treated chickens without medication (group 3) ($P < 0.05$). In GSLS-treated chickens, birds administered GSLS at 5 mg/kg BW were found to have the highest number of iIELs (group 5).

3.4. HI responses induced by a bivalent inactive vaccine of NDV and AIV

Anti-NDV and anti-AIV HI responses were detected by HI test as previously described and the data are summarized in Fig. 4. Prior to immunization, no specific HI titers were detected in any group. Following immunization, the HI titers elicited by vaccination with a bivalent inactive vaccine of NDV and AIV progressively increased. After injection of Cy, both NDV- and AIV-HI titers were significantly decreased when compared with the control (group 2) ($P < 0.05$). Notably, NDV- and AI-HI titers in chickens administered GSLS (groups 4–6) or APS (group 7) were significantly higher than in group 3 ($P < 0.05$) and even restored to the levels similar to the normal control (group 2) at week 3 post vaccination ($P > 0.05$). In GSLS-treated birds, highest HI titers were observed in the birds administered GSLS at 5 mg/kg BW, which did not significantly differ from those in APS-treated birds.

4. Discussion

Immunomodulatory effects of ginseng stem-leaf saponins (GSLS) on immune-inhibited chickens were demonstrated in the present study. After oral administration of GSLS in drinking water, the spleen lymphocyte proliferation induced by ConA and LPS, the numbers of IgA+ cells and iIELs in the duodenum as well as serum

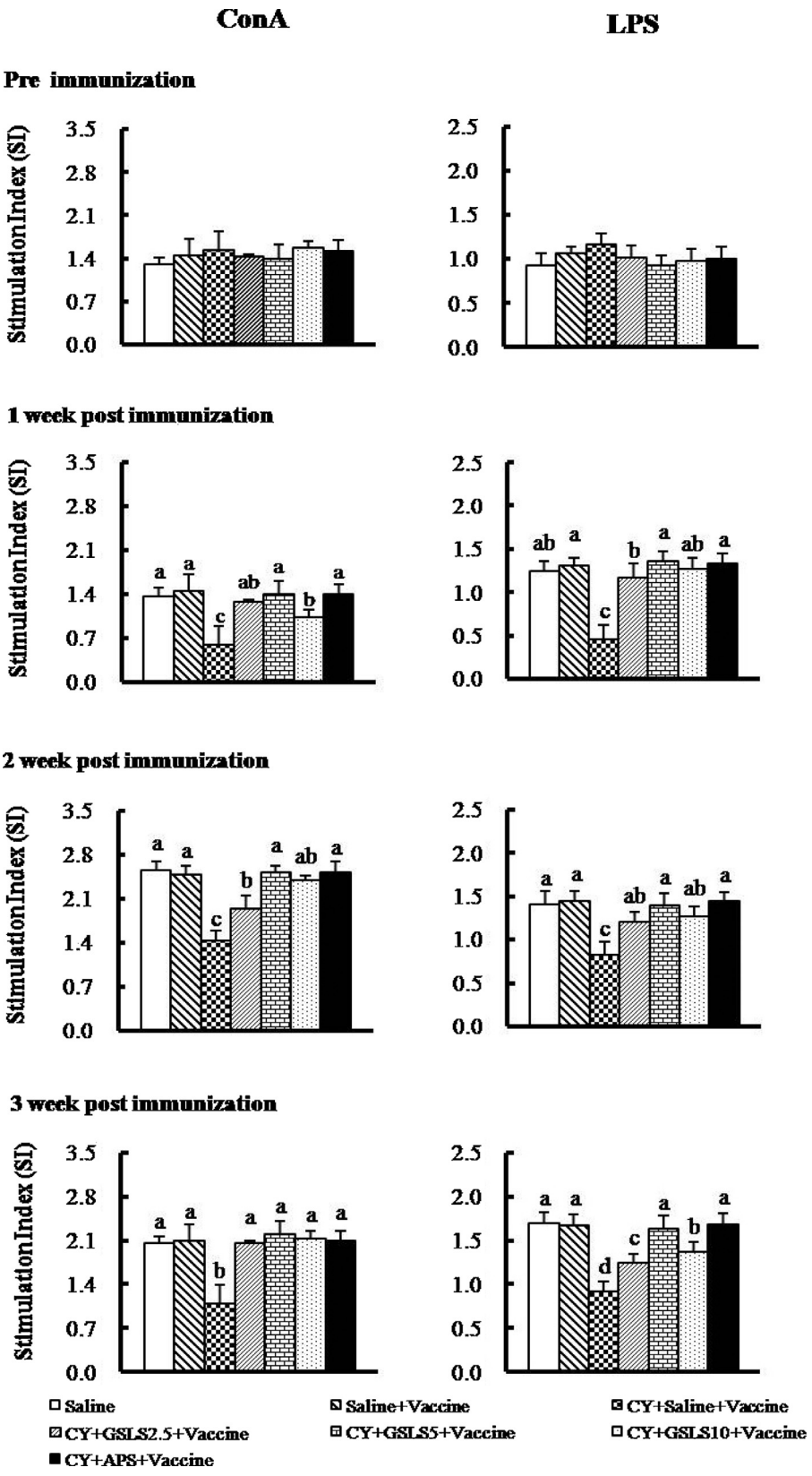

Fig. 1. Splenocyte proliferative responses to ConA and LPS. Chickens were i.m. injected with Cy at 100 mg/kg for 3 days to induce immunosuppression, and those without injection of Cy were used as control. After that, the birds were orally administered APS or GSLS (0, 2.5, 5 and 10 mg/kg) in drinking water for 7 days and then vaccinated with a bivalent inactive vaccine of NDV and AIV. Six chickens were randomly euthanized before and 1, 2 and 3 weeks after immunization for splenocyte collection. Splenocyte proliferation was measured by the MTT method as described in the text, and the results are shown as stimulation indexes (SI). The values are represented as mean ± S.E. Bars with different letters are statistically different ($P < 0.05$).

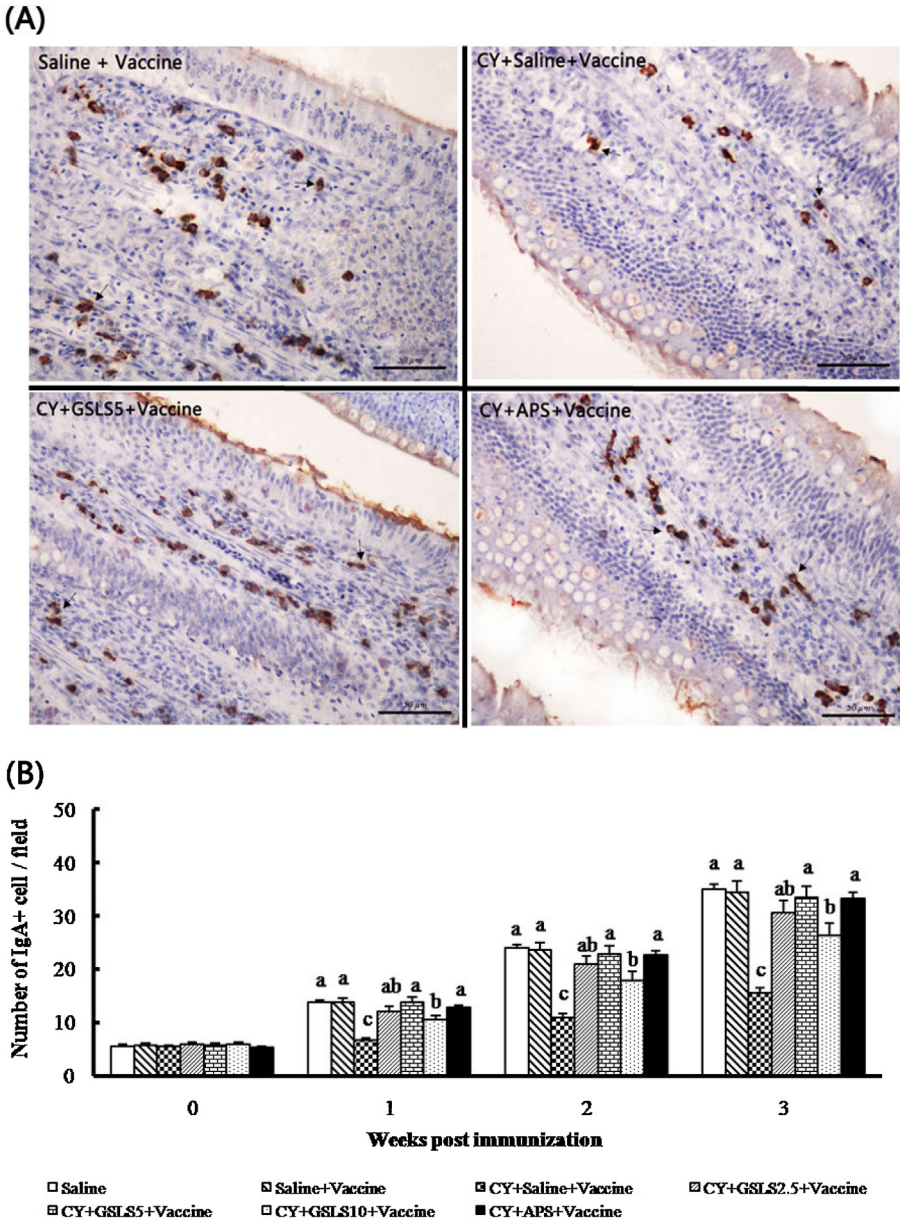

Fig. 2. Immunoglobulin A+ cells in the mucosa of duodenum. Chickens were i.m. injected with Cy at 100 mg/kg for 3 days to induce immunosuppression, and those without injection of Cy were used as control. After that, the birds were orally administered APS or GSLS (0, 2.5, 5 and 10 mg/kg) in drinking water for 7 days and then vaccinated with a bivalent inactive vaccine of NDV and AIV. Six chickens were randomly euthanized before and 1, 2 and 3 weeks after immunization for intestinal tissue samples collection. The samples were fixed in 10% neutral buffered formalin (v/v) for immunohistochemical staining. (A) Immunoglobulin A+ cells (arrows) in the mucosa of duodenum in the control group (upper left), Cy group (upper right), GSLS 5 group (lower left) and APS group (lower right) at week 3 postimmunization. The IgA+ cells are visible as yellow-brown dots distributed in the lamina propria or around the intestinal crypts, immunohistochemical staining (400×), scale bar 50 μm. (B) The number of IgA+ cells in the mucosa of the duodenum. The values are represented as mean ± S.E. Bars with different letters are statistically different ($P < 0.05$).

antibody response to a bivalent inactive vaccine of ND and AI were significantly increased in chickens immunosuppressed by Cy, being similar to the levels in chickens orally administered APS.

GSLS represents a structurally diverse class of saponins deriving from the discarding part of ginseng (stems and leaves) and has a variety of biological functions such as immunomodulatory effects (Zhai et al., 2011a, 2011b). To investigate its effect on the immune suppressed animals, chickens treated with Cy were served as a model. Cy is an alkylating agent widely used as antineoplastic drugs for its therapeutic effects on a variety of cancers and disorders (Perini et al., 2007; Shien et al., 2014), but high dose or long-term administration may endanger both humoral and cellular immunity, thus causing immunosuppression and even death. Therefore, it is often used to induce immunosuppression in animal models (de Jonge et al., 2005; Varalakshmi and John, 2014). APS, on the other side, possess positive immunostimulatory effects in vaccination programs against Newcastle disease (ND), avian influenza (AI) and infectious bursal disease (IBD) by stimulating cell proliferation, inducing the expression of surface antigens on lymphocytes, affecting the expression of cytokines, and promoting the production of antibodies (Huang et al., 2008; Kallon et al., 2013; Sun et al., 2013a, 2013b). Thus APS was used as a positive control in this study.

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses (Park, 2014). Different mitogens stimulate different lymphocyte subtypes. T lymphocytes are responsive to ConA; B lymphocytes are

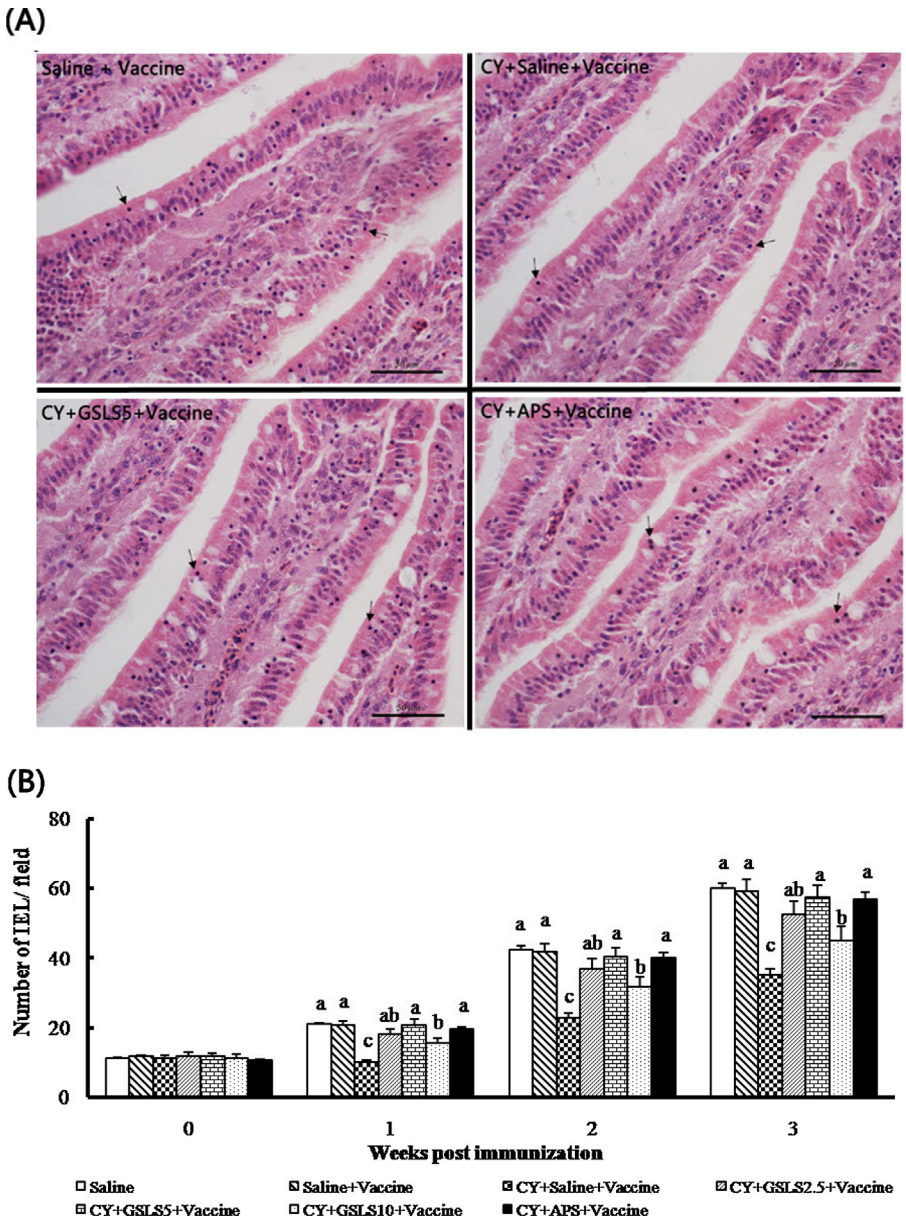

Fig. 3. Intestinal intraepithelial lymphocytes (iIELs) in the mucosa of duodenum. Chickens were i.m. injected with Cy at 100 mg/kg for 3 days to induce immunosuppression, and those without injection of Cy were used as control. After that, the birds were orally administered APS or GSLS (0, 2.5, 5 and 10 mg/kg) in drinking water for 7 days and then vaccinated with a bivalent inactive vaccine of NDV and AIV. Six chickens were randomly euthanized before and 1, 2 and 3 weeks after immunization for intestinal tissue samples collection. The samples were fixed in 10% neutral buffered formalin (v/v) for immunohistochemical staining. (A) Intestinal intraepithelial lymphocytes (iIELs; arrows) in the mucosa of duodenum in the control group (upper left), Cy group (upper right), GSLS 5 group (lower left) and APS group (lower right) at week 3 postimmunization. The iIELs are small round cells with densely stained nuclei, and lie within the epithelial cell layer in the duodenum, hematoxylin–eosin staining (400×), scale bar 50 μm. (B) The number of iIELs in the mucosa of the duodenum. The values are represented as mean ± S.E. Bars with different letters are statistically different ($P < 0.05$).

responsive to LPS (Ren et al., 2014). In this study, proliferative responses to either ConA or LPS of the lymphocytes isolated from chickens treated with Cy were significantly suppressed (Fig. 1). After administration of GSLS or APS, the lymphocyte proliferative responses to either ConA or LPS were completely recovered (Fig. 1). However, it is difficult to know that the altered proliferative responses were due to changes in the number or in responsiveness of T and B cells in the spleen based on our present data. It is clear that GSLS has a positive effect but whether the activities of B and T cells are restored or on the number of cells that can respond should be further studied using flow cytometry analysis.

The increased lymphocyte proliferative responses paralleled the enhanced gut mucosal immunity observed in this study. The intestines, as the largest lymphoepithelial organ, contain various types of antibody producing cells, of which the IgA-secreting (IgA+) cells, residing in the intestinal lamina propria, are of remarkable dominance in the intestinal mucosal immunity as approximately 80% of all IgA+ cells locate in the gut mucosa (Mestecky et al., 1999; Mora and von Andrian, 2008). The discovery of secretory IgA (sIgA) dominates humoral mucosal immunity, corresponding with IgG dominates systemic immune system, was early evidence for the distinct nature of mucosal immunology (Blutt et al., 2012; Macpherson et al., 2008). SIgA serves as the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities (Mantis et al., 2011). Despite of the irreplaceable role of sIgA in mucosal

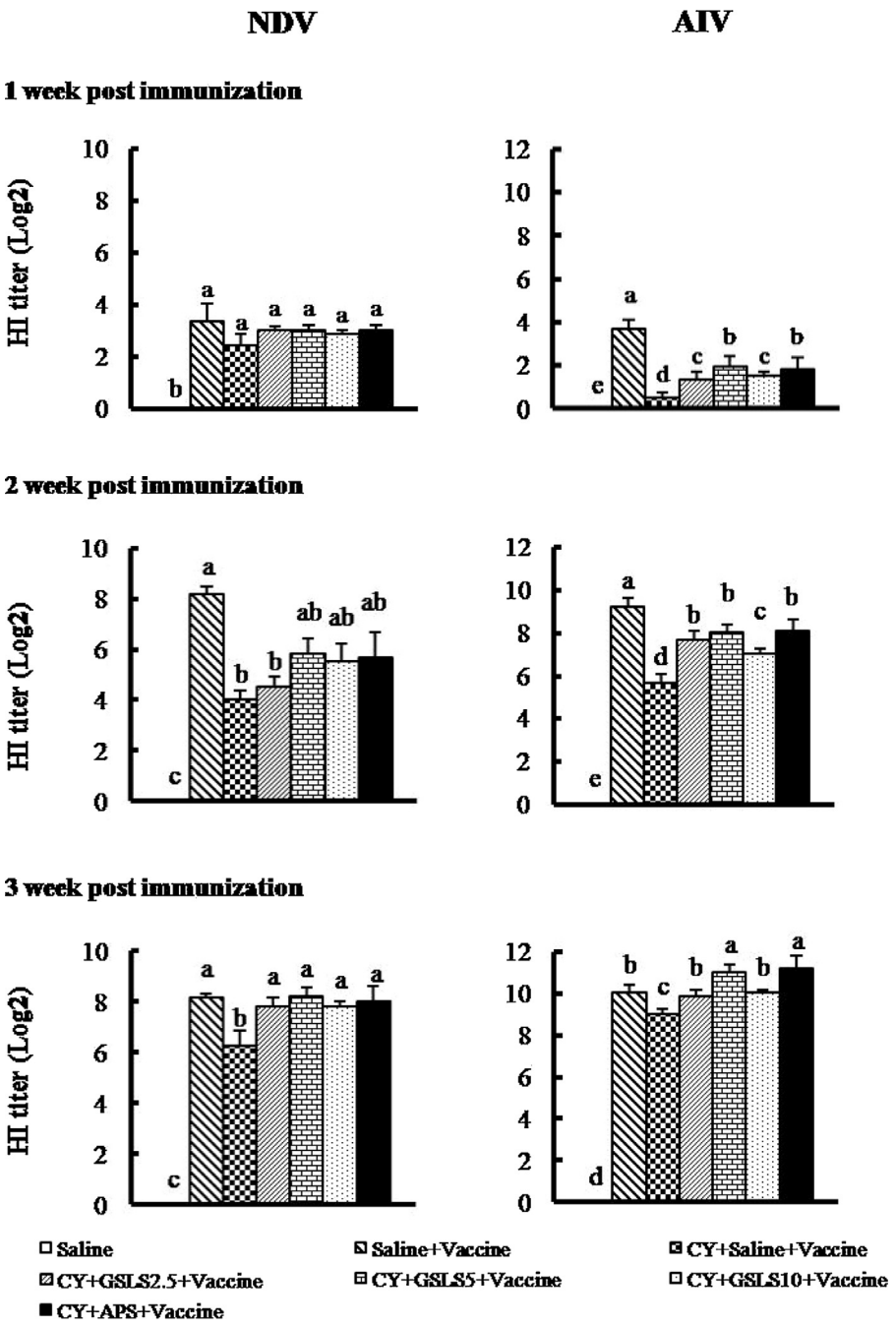

Fig. 4. Antibody responses to a bivalent inactive vaccine of NDV and AIV were measured by HI titers. Chickens were i.m. injected with Cy at 100 mg/kg for 3 days to induce immunosuppression, and those without injection of Cy were used as control. After that, the birds were orally administered APS or GSLS (0, 2.5, 5 and 10 mg/kg) in drinking water for 7 days and then vaccinated with a bivalent inactive vaccine of NDV and AIV. Blood samples were collected before and 1, 2 and 3 weeks after immunization for analysis of NDV and AIV specific HI titers. The values are represented as mean ± S.E. Bars with different letters are statistically different ($P < 0.05$).

immunity, it is hard to evaluate it directly, whereas IgA+ cells, which are closely correlated with sIgA and the location is relatively fixed, can be counted instead of amounting sIgA as a parameter of intestinal mucosal immunity (Kang and Kudsk, 2007). Intestinal intraepithelial lymphocytes (iIELs), on the other hand, locate at the basolateral side of the epithelial layer, predominantly composed by NK cells (not antigen experienced) and gamma delta cells (function unknown) (Lillehoj and Chung, 1992; Gobel et al., 2001; Bucy et al., 1988), are the first cells in the immune system to encounter pathogens that have invaded through the gastrointestinal tract (Befus et al., 1986; Lillehoj, 1989). The majority of iIELs contain abundant cytoplasmic granules for cytotoxic activity including NK activity and spontaneous cytotoxicity, and they can express effector cytokines, such as interferon-γ, interleukin-2 (IL-2), IL-4 and IL-12, hence establishing an immune surveillance and guarding the safety of mucosa integrity (Kumar and Das, 1996; Chai and Lillehoj, 1988; Cheroutre et al., 2011; Gobel et al., 2001; Hong et al., 2006; Kapczynski, 2008; Myers and Schat, 1990). Therefore, measuring the number of iIELs can be used as an important marker for intestine mucosal immunity, evidenced by the iIELs counts in histology sections have been long accepted as a conventional method for quantifying mucosal immune response (Ferguson and Ziegler, 1986; Sun et al., 2013a, 2013b; Wang et al., 2009). Because our previous studies showed that oral administration of GSLS promoted

both the numbers of IgA+ cells and iIELs in the duodenum, jejunum and ileum of the chickens, of which the most obvious results are found in duodenum, thus we measured the quantity of IgA+ plasma cells and iIELs in the duodenum to assess the effect of GSLS on the mucosal immunity in immunosuppressive chickens. In the present study, we observed that injection of Cy significantly decreased the numbers of IgA+ cells and iIELs in the duodenum while administration of GSLS and APS restored the numbers of the cells, indicating the intestinal mucosal immune responses were enhanced by oral administration of the herbal extracts (Figs. 2 and 3).

Humoral immunity plays a vital role in poultry against NDV and AIV infections by the secretion of specific antibodies. Vaccines able to elicit ample antibody response can provoke an effective protection against diseases by reducing virus shedding and raising the threshold for infection and transmission, and hence control of outbreaks of NDV or AIV in poultry, as well as humans (Ge et al., 2007; Park et al., 2006). Our previous studies showed that GSLS enhanced the immune response induced by a monovalent vaccine of NDV or AIV (Zhai et al., 2011a, 2011b). In this study, we vaccinated chickens by performing one injection of a bivalent inactive vaccine of NDV and AIV instead of two injections of the monovalent vaccines. Results showed that oral administration of GSLS effectively promoted the recovery of both NDV- and AIV-specific antibody titers in Cy-treated birds and GSLS at 5 mg/kg BW was more effective than the other doses, inducing titers similar to APS group (Fig. 4).

Taken together, the present study demonstrated that oral administration of GSLS enhanced the immune responses in immunosuppressive chickens. When oral administration of GSLS in drinking water was performed before immunization of a bivalent vaccine of ND and AI, NDV- and AIV-specific antibody titers, splenocyte proliferation induced by ConA and LPS, as well as the numbers of IgA+ cells and iIELs were recovered in immunosuppressed chickens treated with Cy. Therefore, GSLS may have the potential agent to improve vaccination in immunosuppressed chickens.

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