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RESEARCH ARTICLE

Enhanced immune response to foot-and-mouth disease vaccine by oral administration of ginseng stem-leaf saponins

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ABSTRACT

Vaccination is an important approach to the control of foot-and-mouth disease (FMD). This study evaluated the effect of oral administration of ginseng stem–leaf saponins (GSLS) on the immune response to FMD vaccine and the gut mucosal immunity in mice. In experiment 1, mice were orally administered GSLS or not treated as a control. The animals were then immunized twice with FMD vaccine. Blood was sampled weekly within five weeks after the boost immunization for measurement of serum IgG and the isotypes. In experiment 2, mice were orally administrated GSLS or not treated as a control. After that, splenocytes were prepared from sacrificed mice for lymphocyte proliferation assay and intestinal tissues were sampled for immunohistochemistry and histological examination. The results showed that oral administration of GSLS significantly enhanced serum IgG and the isotype responses to FMD vaccine as well as the number of intestinal intraepithelial lymphocytes (IELs) and immunoglobulin A (IgA)+ cells. Therefore, GSLS may be a potent oral adjuvant and deserve further study to improve vaccination in susceptible animals.

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KEYWORDS
Foot-and-mouth disease; ginseng saponins; mucosal immunity; oral adjuvant

Introduction

Foot-and-mouth disease (FMD) is an acute febrile contagious disease caused by FMD virus[1]. The disease affects cloven-hoofed animals such as cattle, pigs, sheep, goats, buffalo, yaks and wild ruminants[2–4]. Rats and mice are susceptible under experimental conditions[5,6]. The virus exists in the form of seven different serotypes: A, O, C, Asia1, SAT1, SAT2 and SAT3, but a large number of subtypes have evolved within each serotype[7,8]. FMD is considered to be the most important economic animal disease in the world. Direct losses include reduction in milk and meat. Because there is no specific treatment for FMD at the present, vaccination has become a routine practice in many countries[3,9,10]. However, currently available FMD vaccines have been reported to induce weak immune responses in swine[11–14]. Therefore, searching for a proper approach to improve the efficacy of vaccination remains an interesting topic.

Saponins are chemically a class of natural products which are constructed of an aglycone (triterpene or steroid) linked to one or more sugars. The saponins isolated from the stem and leaf of *Panax ginseng* C. A. Meyer (GSLS) have been reported to be useful to improve vaccination in chickens. It has been found that administration of GSLS in drinking water caused significantly enhanced gut mucosal immunity and better vaccination against Newcastle disease, avian influenza and infectious bursal disease in both normal and immunosuppressive birds[15–17]. However, no reports have been found regarding the effect of oral administration of GSLS on the immune responses in mammals. This study was designed to investigate the effect of oral administration of GSLS on the immune responses induced by FMD vaccine as well as the intestinal mucosal immunity.

Materials and methods

Animals

Female ICR mice (five weeks old) weighing 18–22 g were purchased from Shanghai Slack Laboratory Animal Co. Ltd. (Shanghai, China). The animals were kept in hygienically controlled environment with a temperature of 24 ± 1 °C, humidity of 50 ± 10% and a 12/12 h light/dark cycle, and acclimatized for one week prior to use. Feed and water were provided *ad libitum*. 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. The researchers received ethical training from the Zhejiang University ethics committee.

Reagents

Standardized ginseng stem-and-leaf saponins (GSLS) were purchased from Hongjiu Ginseng Industry Co. Ltd. (Jilin, China). Based on analysis by high-performance liquid chromatography (HPLC), GSLS contained Rb1 (1.4%), Rb2 (3.0%), Rc (2.5%), Rd (8.0%), Re (12.0%) and Rg1 (6.0%). FMDV type O

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vaccine was from Lanzhou Veterinary Research Institute (Lanzhou, China).

Experimental design

Experiment 1. Twenty-four mice were randomly divided into four groups with six mice in each. In groups 2 to 4, each animal received daily intake of 0.05, 0.5 or 5 mg of GSLS in water for four days. Group 1 was not treated with GSLS and served as a control (Table 1). After that, all mice received twice subcutaneous injections of FMDV vaccine at two-week intervals. Blood samples were weekly collected in five weeks after the booster immunization for analysis of IgG and the IgG subclasses.

Experiment 2. Based on the results from experiment 1, we hypothesized that oral administration of GSLS may have activated nonspecific immune response before specific response was induced. We therefore designed this experiment to investigate the effect of oral administration on the nonspecific immune response. Forty mice were randomly divided into two groups and orally administrated saline solution or GSLS (0.5 mg) for four days. On zero, one, two, three weeks after the treatment, five mice were sacrificed in each group, splenocytes were prepared for lymphocyte proliferation test and intestinal tissues were sampled for analysis of immunoglobulin A (IgA) positive cells and intestinal intraepithelial lymphocytes (IELs) (Table 2).

Evaluation of FMDV-specific IgG and isotypes

Serum IgG, IgG isotypes and IgG titers were analyzed by an indirect double antibody sandwich enzyme-linked immunosorbent assay as previously described[18]. A total of five polyvinyl 96-well microtiter plates were coated with 50 μl rabbit anti-FMDV serotype O antibody (LVRI, Lanzhou, China) diluted in 0.05 M carbonate/bicarbonate buffer (1:1000), pH 9.6, and incubated overnight at 4 °C. After washing with phosphate buffer saline containing 0.05% tween-20 (PBST), the wells were blocked with 5% skimmed milk and incubated at 37 °C for 2 h. Thereafter, 50 μl FMDV type O antigen (LVRI, Lanzhou, China) (1:10 dilution) was added and incubated at 4 °C for 2 h. Following washing, 50 μl of serum (diluted serially for IgG or diluted 1:40 for isotype analysis in PBS 5% skimmed milk) was added to each well and incubated at 37 °C for 1 h. Plates were then washed five times in PBST. For IgG titer detection, 50 μl of goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (1:800) was added to the wells and incubated at 37 °C for 1 h. Plates were washed again with PBST. Each well was added 50 μl 3,3′,5,5′-tetramethyl benzidine solution (100 μg/ml of 0.1 M citrate-phosphate, pH 5.0) and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The optical density (OD) of the plate was read by an automatic ELISA plate reader at 450 nm. For IgG isotypes, 50 μl of HRP-conjugated goat anti-mouse IgG1 or IgG2a or IgG2b or IgG3 (1:1000) was added to corresponding plate and then incubated for 1 h at 37 °C.

Splenocyte proliferation assay

The collected spleens (Table 2 for the number of spleens for each sampling) were kept in Hank's balanced salt solution (HBSS, Sigma, St. Louis, MO). The organ was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. To lyse contaminated erythrocytes, 0.16 M NH_4Cl in 0.01 M Tris-HCl (pH 7.2) was added to the suspension. After centrifugation (380×*g* at 4 °C for 10 min), the pelleted cells were washed three times in HBSS and resuspended in complete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal bovine serum (FBS)). Cells were counted with a hemocytometer by trypan blue dye exclusion method. Cell viability exceeded 95%.

Splenocyte proliferation was assayed as described previously with modification[19]. Briefly, splenocytes were seeded into a 96-well flat-bottom microtiter plate (Nunc, Rochester, NY) at 5.0×10^6 cells/ml in 100 μl complete medium. Thereafter, Con A (final concentration 5 μg/ml), lipopolysaccharide (LPS) (8 μg/ml), FMDV antigen (200 μg/ml) or medium were added giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2 for two or five days. All the tests were carried out in triplicate. The cell proliferation was evaluated using MTT method. Briefly, 50 μl of MTT solution (2 mg/ml) was added to each well 4 h before the end of incubation. The plates were centrifuged (1400×*g*, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 150 μl of a dimethyl sulfoxide (DMSO) working solution (192 μl DMSO with 8 μl 1 M HCl) was added, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen cultures/the absorbance value for non-stimulated cultures.

Table 2. Design of experiment 2.

Group	No. of mice	Daily intake (mg)/mouse of GSLS	No. of mice sacrificed each week[a]			
			0	1 wk	2 wks	3 wks
1	20	0.5	5	5	5	5
2	20	–	5	5	5	5

[a]The number of mice weekly sacrificed after GSLS administration for intestinal tissue sampling.

Table 1. Design of experiment 1.

Group	No. of mice	Daily intake (mg)/mouse of GSLS	Vaccine	Blood sampling
1	6	–	Immunized twice at 2-week intervals	Weekly sampled after vaccination for analysis of IgG and IgG isotypses
2	6	0.05		
3	6	0.5		
4	6	5		

Histological examination for IELs

The fixed tissue samples were embedded in paraffin and serially sectioned at a thickness of 6 μm. After hematoxylin–eosin (HE) staining, the sections were sealed with a coverslip. Epithelial cells and lymphocytes in five different fields of intestinal villi of each mouse were counted (magnification ×400) for the statistical analysis.

Immunohistochemical staining for IgA + cells

The IgA + plasma cells were identified with the immunohistochemical staining method as previously described[19]. The samples (20 mice/group) embedded in paraffin were serially cut into 6 μm-thicknesses and mounted on poly-lysine-coated glass slides. Endogenous peroxidase was inhibited with 3% H_2O_2 in methanol for 15 min, and then rinsed in PBS. The sections were incubated in 0.01 M citrate-buffered solution (pH 6.0) at 95 °C for 20 min for antigen retrieval, then allowed to cool at room temperature and rinsed in PBS. To block nonspecific binding, the sections were treated with 5% fetal calf serum (HyClone Laboratories, Inc., Logan, UT) in PBS at room temperature for 30 min. The excessive solution was shaken off. The sections were incubated with goat anti-mouse IgA antibody (1:1000) (NB7501, Novus biological, Inc., Littleton, CO) at 4 °C over night, and then washed in PBS, followed by incubation with rabbit anti-goat IgG conjugated HRP (1:500) (NB7352, Novus biological, Inc., Littleton, CO) at 37 °C for 1 h. After the sections were rinsed with PBS for 15 min, the reactions were made visible with metal-enhanced diaminobenzidine (DAB) (AMRESCO LCC, Cleveland, OH). All incubations were performed in a moist chamber. Control staining was carried out simultaneously in which the first antibody was replaced with PBS. No specific staining was found in the control. The magnification of ×400 was used to perform IgA + cell counting.

Statistical analysis

The sections were observed under a light microscope (Nikon, Shizuoka, Japan). Five different regions were randomly selected in each section and the images were captured with a video camera connected to a computer. The number of IELs per 100 epithelial cells and the relative area of IgA + cells in five different microscope fields of intestinal villi were counted using Nikon NIS element BR 2.30 software (Nikon, Shizuoka, Japan). Data were expressed as means ± standard deviations (SD). Duncan's test was used to compare the parameters between groups by using SPSS 20. p Values of less than 0.05 were considered statistically significant.

Results

Effect of oral administration of GSLS on the humoral immune responses

To investigate the effect of oral administration of GSLS on the humoral immune responses, mice were orally administrated GSLS at a dose of 0.05, 0.5 or 5 mg. The other animals were orally administered saline solution only and served as a control. After that, all animals were subcutaneous (SC) injected twice with the FMD vaccine at two-week intervals. Blood samples were weekly collected until five weeks post boost immunization to measure FMDV-specific IgG and the isotypes. The results Figure 1 showed that serum IgG level changed time-dependently with the highest IgG response recorded at three weeks post boost immunization, and the OD values for IgG in groups 1 to 4 were 0.58 ± 0.06, 0.73 ± 0.18, 1.14 ± 0.18 and 0.90 ± 0.12, respectively; oral administration of GSLS enhanced the IgG response to FMD vaccination and the highest IgG levels were found in mice administered 0.5 mg of GSLS. Figure 2 indicated significantly higher IgG isotypes IgG1 (1.04 ± 0.03 versus 0.69 ± 0.16), IgG2a (0.63 ± 0.08 versus

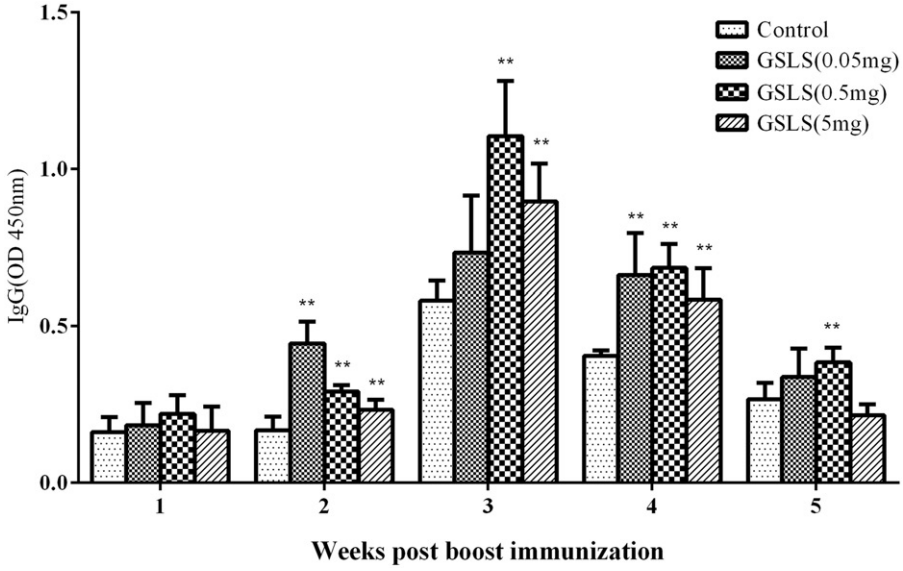

Figure 1. FMDV-specific IgG response. Mice (n = 6/group) were orally administrated GSLS (0.05, 0.5 and 5 mg) or saline solution. After that, the animals were SC injected twice with the FMD vaccine at 2-week intervals. Blood samples were weekly collected during five weeks post the boost immunization for measurement of serum IgG by an indirect double antibody sandwich ELISA. Data are presented as mean ± SD. Values with ** are considered significantly different when compared with the control group ($p < 0.05$).

0.39 ± 0.06), IgG2b (0.59 ± 0.02 versus 0.35 ± 0.02) and IgG3 (0.15 ± 0.01 versus 0.13 ± 0.01) in mice of 0.5 mg GSLS than in the control group. Although they were statistically different between GSLS group and the control, the IgG3 was scarecely induced in both groups as found in our previous study.

Effect of oral administration of GSLS on splenocyte proliferation

To investigate the effect of oral administration GSLS on splenocyte proliferation, mice were orally administered 0.5 mg of GSLS or saline solution. After that, splenocytes were prepared from mice sacrificed at different time points to determine the proliferative response. The results are shown in Figure 3. Figure 3 showed that splenocyte proliferation progressively increased during two weeks after GSLS administration and then began to decline. At one and two weeks post GSLS administration, SI of the splenocyte proliferative responses induced by Con A in GSLS group (2.43 ± 0.36 and 4.51 ± 0.53) were significantly higher than the control (1.71 ± 0.09 and 3.35 ± 0.11) (Figure 3(a)); SIs of the splenocyte proliferative responses induced by LPS in GSLS group (1.77 ± 0.31 and 3.77 ± 0.36) were significantly higher than the control (1.26 ± 0.15 and 2.65 ± 0.18) (Figure 3(b)).

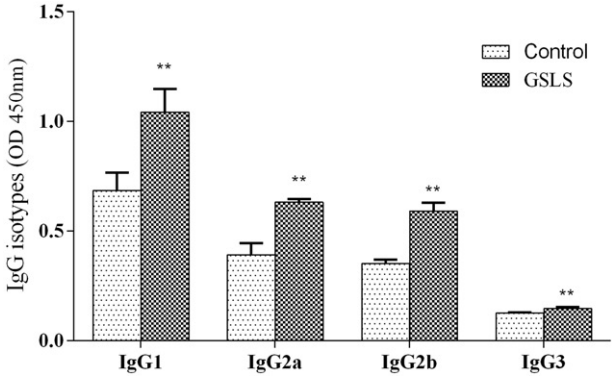

Figure 2. FMDV-specific IgG isotypes. Mice ($n = 6$/group) were orally administrated GSLS (0.5 mg) or saline solution. After that, the animals were s.c. injected twice with the FMD vaccine at two-week intervals. Blood samples were collected three weeks after the boost immunization for measurement of serum IgG isotypes by an indirect ELISA. Data are expressed as mean ± SD. Values with ** are considered significantly different when compared with the control group ($p < 0.05$).

Effect of oral administration of GSLS on the intestinal mucosal immunity

To investigate the effect of oral administration of GSLS on the intestinal mucosal immunity, duodenum samples were collected from above mice at different time points for analysis of IELs and IgA + cells. The results are shown in Figures 4 and 5. Figures 4 and 5 show that the number of IELs and IgA + cells changed time-dependently. Both types of cells reached the peak at one week post GSLS administration and then declined. During 1 to 3 weeks post GSLS administration, the means of IELs/100 epithelial cells in GSLS group were 23.74 ± 2.93, 21.31 ± 3.66 and 20.09 ± 1.61, respectively, which were significantly more than the control group (17.97 ± 3051, 16.08 ± 1.89 and 16.91 ± 1.56) (Figure 4(a)); the means of IgA + cells/mm2 in GSLS group were 689.60 ± 120.56, 590.33 ± 77.46 and 530.90 ± 36.59, respectively, which were significantly more than the control group (431.21 ± 55.47, 456.75 ± 9.44 and 484.96 ± 5.43) (Figure 5(a)).

Discussion

This study demonstrated that the saponins extracted from the stem and leaf of *Panax ginseng* C. A. Mayer (GSLS) have immunomodulatory properties in mice. After oral administration of GSLS, the serum antibody response to FMD vaccine antigen, the spleen lymphocyte proliferation induced by Con A and LPS as well as the numbers of IgA + cells and IELs in the duodenum were significantly increased.

Humoral immune response against FMD infection has been well documented[20,21] and the contribution of antibodies to the major immune defense against FMD is obvious[21–23]. Studies in animal models have shown that specific antibodies play an important role in the immune defense against FMD[6,24,25]. Figure 1 shows that oral administration of GSLS significantly increased antibody response in comparison with

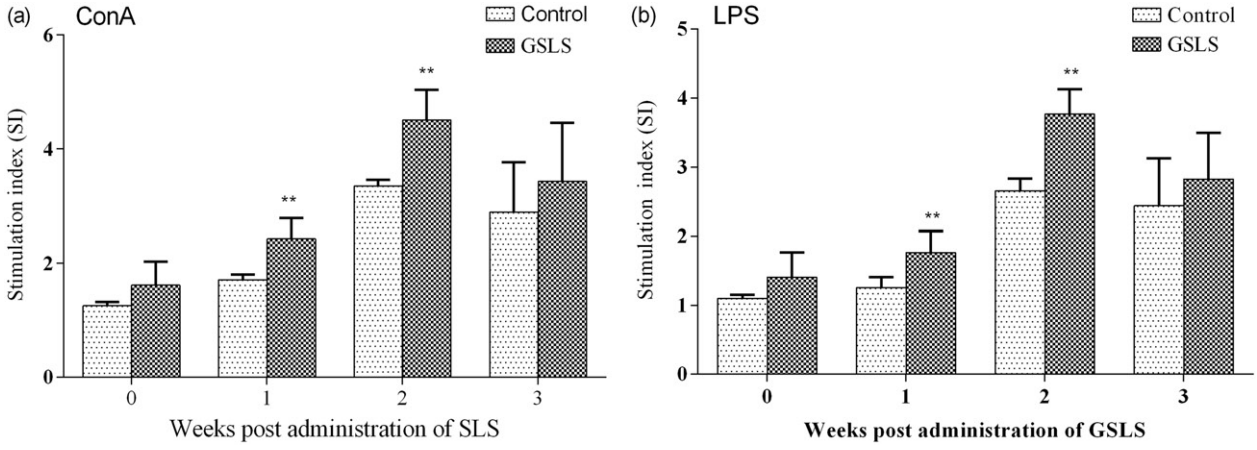

Figure 3. Splenocyte proliferative responses. Mice ($n = 20$/group) were orally administrated GSLS (0.5 mg) or saline solution. After that, five mice were sacrificed from each group at one day, and one, two and three weeks after GSLS administration. Splenocytes were prepared and MTT method was used to determine the proliferative responses to Con A (a) and LPS (b). Data are presented as mean SI ± SD. Values with ** are considered significantly different when compared with the control group ($p < 0.05$).

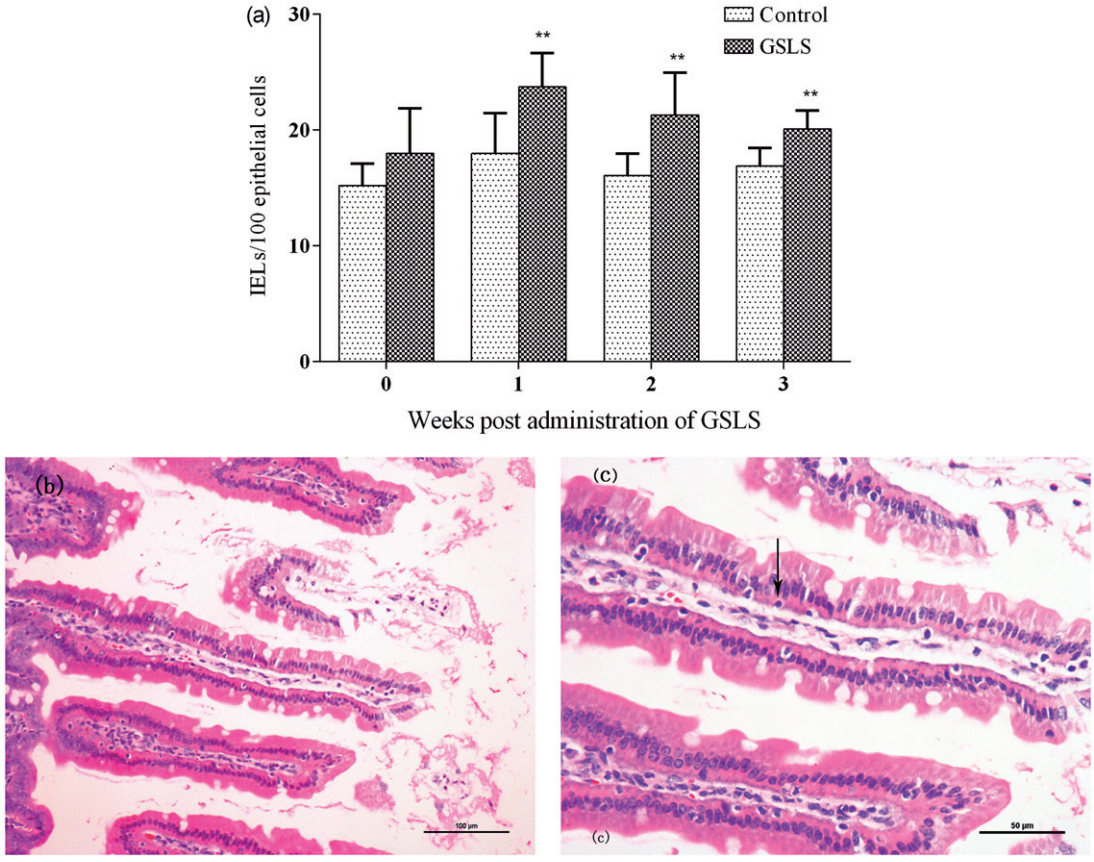

Figure 4. IELs in the duodenum. Mice ($n = 20$/group) were orally administrated GSLS (0.5 mg) or saline solution. After that, five mice were sacrificed from each group at one day, and one, two and three weeks after GSLS administration. Duodenum samples were collected for analysis of IELs which was indicated with the arrow. The number of IELs per 100 epithelial cells in five different microscope fields of intestinal villi were counted using Nikon NIS element BR 2.30 software (Nikon, Shizuoka, Japan). Data are represented mean ± SD. Values with ** are considered significantly different when compared with the control group. (b) 200×; (c) 400×.

the control. The enhancement of IgG depended on the dose of GSLS administered. The dose-dependent IgG response was also found in other studies when saponins were used as adjuvant in vaccines[18,25–27]. There are different subclasses of IgG immunoglobulins such as IgG1, IgG2a, IgG2b and IgG3 that provide the bulk of immunity to most infectious agents. In mice, IgG1 isotype is indicated for Th2 type immune response stimulated by IL-4 and IL-10; IgG2a and IgG3 for Th1 type responses stimulated by IFN-γ. Figure 2 shows that oral administration of GSLS significantly increased all types of IgG subclasses, suggesting that both Th1 and Th2 were enhanced.

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses[28,29]. Different mitogens stimulate different lymphocyte subtypes. T lymphocytes are responsive to Con A; B lymphocytes are responsive to LPS[30–32]. In this study, proliferative responses to either Con A or LPS of the lymphocytes were significantly increased after administration of GSLS when compared to the control (Figure 3), indicating that both T and B lymphocytes were activated.

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, existing in the intestinal lamina propria, are of notable dominance in the intestinal mucosal immunity as approximate 80% of all IgA + cells situate in the gut mucosa[17,33,34]. The secretory IgA (sIgA) dominates humoral mucosal immunity, similar to IgG dominates systemic immune system, is early evidence for the distinct nature of mucosal immunology[35]. SIgA serves as the first line of defense in protection of the intestinal epithelium from enteric toxins and pathogens by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities[33,36]. Despite of the unique role of sIgA in mucosal immunity, to directly evaluate it is difficult, whereas IgA + cells can be counted as a parameter of intestinal mucosal immunity since they are closely correlated with sIgA and the location is relatively fixed[37,38]. IELs, on the other hand, locate at the basolateral side of the epithelial layer, are the first cells in the immune system to encounter pathogens that have invaded through the gastrointestinal tract[39]. The majority of IELs 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-17, hence establishing an immune surveillance and guarding the safety of mucosa integrity[40–43]. Therefore, measuring the number of IELs can be used as an important marker for intestinal mucosal immunity. Although it has been long accepted as a conventional method for quantifying mucosal immune response to enumerate IELs based on the morphology in histology sections[44,45], a more accurate method to

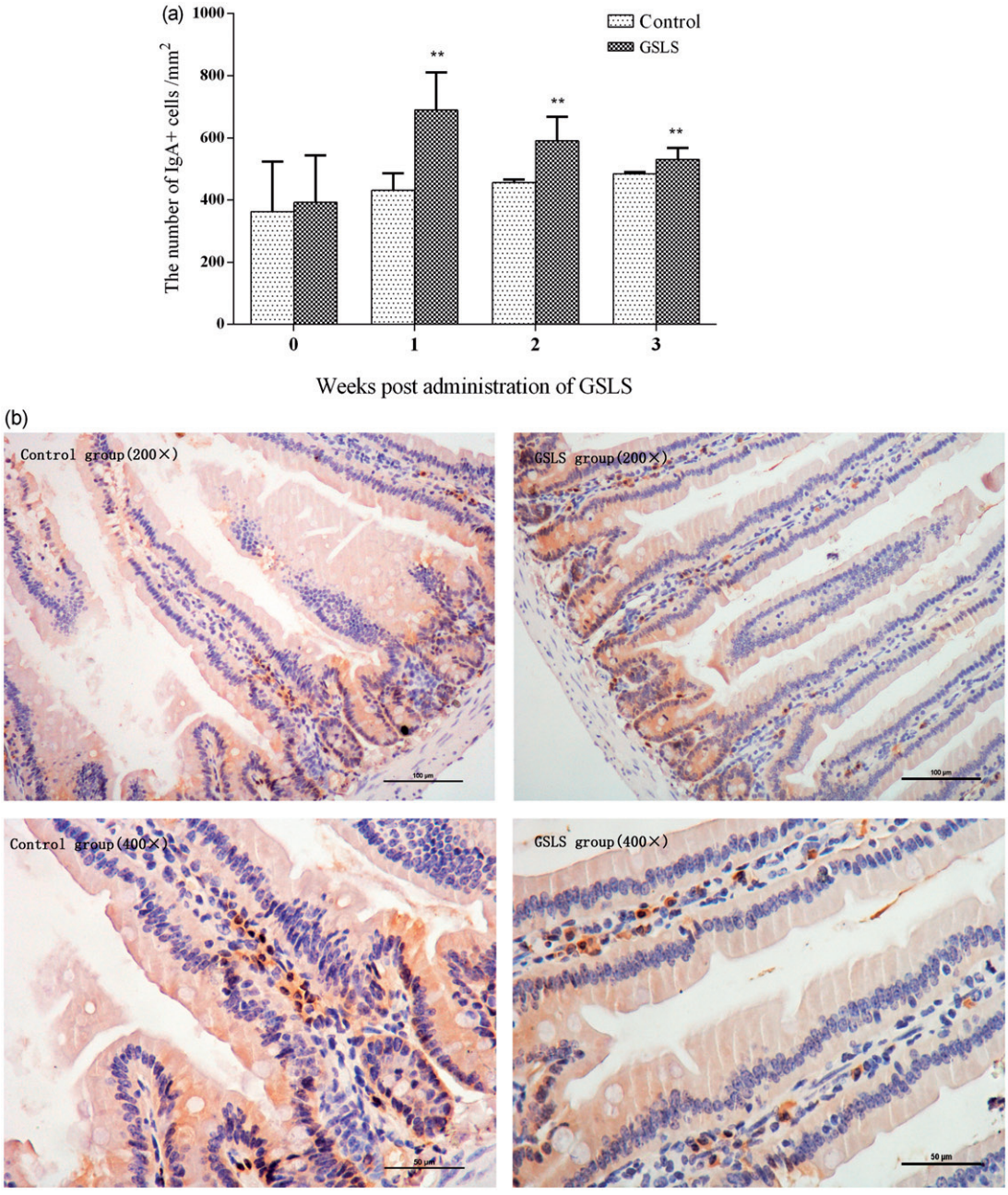

Figure 5. IgA + cells in the duodenum. Mice ($n = 20$/group) were orally administrated GSLS (0.5 mg) or saline solution. After that, five mice were sacrificed from each group on one day, and one, two and three weeks after GSLS administration for analysis of IgA + plasma cells which were identified with the immunohistochemical staining method (a) and were indicated with the arrow (b). IgA + cells per square milliliter in five different microscope fields of intestinal villi were counted using Nikon NIS element BR 2.30 software (Nikon, Shizuoka, Japan). Data are represented mean ± SD. Values with ** are considered significantly different with the control ($p < 0.05$).

identify IELs by immunohistochemicaly staining the cells using CD45 specific antibodies should be used in our next investigation. Because our previous studies found that oral administration of GSLS promoted both the numbers of IgA + cells and IELs in the duodenum, jejunum and ileum, of which the most apparent changes were in the duodenum, thus we counted IgA + plasma cells and IELs in the duodenum to evaluate the effect of GSLS on the mucosal immunity. In this study, we observed that administration of GSLS significantly increased the numbers of IgA + cells and IELs, indicating the intestinal mucosal immune responses were enhanced by the herbal extract (Figrues 4 and 5).

Taken together, this study demonstrated that oral administration of GSLS enhanced the immune responses to FMD vaccine antigen in mice. When GSLS was orally administered before immunization of FMD vaccine antigen, FMDV-specific IgG and the isotypes responses were significantly enhanced. Oral administration of GSLS also significantly increased splenocyte proliferation induced by ConA and LPS, as well as the numbers of IgA + cells and IELs. Therefore, GSLS should be further studied as a potential agent to improve vaccination against FMD in susceptible animals.

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Disclosure statement

The authors declare no conflicts of interests.

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