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Enhancement of immune response for Newcastle disease vaccine using a combined adjuvant solution of *Astragalus* polysaccharides, levamisole, and selenoprotein

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Abstract: The Newcastle disease vaccine strain I (ND I vaccine) was reconstituted using a 3-component combined adjuvant solution containing *Astragalus* polysaccharide (APS), levamisole (LMS), and selenoprotein (Se). A total of 200 Hy-Line brown chickens, 60 days of age, were randomly assigned into 10 groups. The first group was a negative control and was immunized using the phosphate buffered saline-reconstituted vaccine. The other 9 groups were immunized using the vaccine reconstituted with the combined adjuvant solution. The serum and blood samples were collected on days 7, 14, 21, 27, 35, and 42 after the vaccinations and were analyzed for IgG, IL-2, and IL-6 as well as T cell proliferation. The results showed that both the humoral and cellular immune responses were significantly enhanced compared to the negative control group. The IgG level was higher and prolonged. The IL-2 and IL-6 expressions as well as the T cell proliferation were enhanced in the experimental groups. The best combination was APS 9 mg/mL + Se 0.01 mg/mL + LMS 30 mg/mL. Our data confirmed the previous studies on immune enhancement of APS, Se, or LMS alone, but also indicated the synergy of APS + Se + LMS on the vaccine immunization.

Key words: *Astragalus* polysaccharides, selenium, levamisole, Newcastle disease I vaccine, reconstitution

1. Introduction

Frequent outbreaks of Newcastle disease (ND) and rapid dissemination of the ND virus have brought about enormous economic losses to the world poultry industry, especially in developing countries (1,2). The control of ND in poultry is through vaccination. The widely used method to prevent ND is mass vaccination with a mildly virulent virus among poultry (3). However, vaccine application alone has its own drawbacks, including weak immune response and negative side effects in the host (4). Therefore, it is important to use suitable adjuvants to increase the immune response of the ND vaccine (5). At present, various materials such as levamisole (LMS) (6,7), selenium (Se) (8), or aluminum (Al) (9) are incorporated in vaccine formulation.

LMS is a chemical immunostimulator. Since 1980 (10), numerous reports have confirmed its immunomodulatory effects (6,7,11–13). Due to producing cytokines, increasing IgG2a/IgG1 ratios, and enhancing antigen-specific T cell responses, LMS as an adjuvant has been successfully injected with vaccines against the foot mouth disease virus and porcine respiratory reproductive syndrome virus (7). Se supplementation has been demonstrated to modulate the immune system. Previous investigations showed that Se not only enhanced synthesis of IgM in precursors, but also improved the IgG absorption in newborn animals (14). The above 2 immunopotentiators are all applied as adjuvants in the prevention of infectious viruses. Notably, *Astragalus* polysaccharide (APS), the main bioactive component in the extract of *Radix astragali*, has been also confirmed to play an important role in immunoregulation, including T cell proliferation, cytokine (e.g., IL-2 and TNF) production, and complement activation (15–17). In addition, our laboratory recently reported that APS injected with infectious bursal disease virus enhanced the immune response of chickens (18). Recently, the application of combined adjuvants has been receiving increased attention; however, the effects of the combination of APS + Se + LMS on the vaccine remains largely unknown.

Commonly, freeze-dried ND I vaccine is usually diluted by 0.9% saline or phosphate buffered saline (PBS). In this study, we used various concentrations of LMS, Se, and APS to reconstitute the ND I vaccine and to study the immune response of chickens so as to find an alternative combined adjuvant for the ND I vaccine.

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2. Materials and methods

2.1. Reagents

APS, a white powder with the molecular weight of 1.1 × 10^4 Da and purity of 98.2%, was extracted from *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao by our laboratory. Its monosaccharide composition and molecular molar ratio were Rha:Glc:Gal:Ara = 1.19:72.01:5.85:20.95 (19). Selenoprotein with a concentration of Se 96% was provided by Wellroad Animal Health Co., Ltd. Levamisole (LMS) was purchased from Taiyuan Aofulai Animal Pharmacy Co., Ltd. Mildly virulent ND I vaccine (≥1.0 × 10^5 ELD_{50}) was obtained from Harbin Weike Biotechnology Development Co., Ltd., with lot number 20090925. RPMI 1640 medium (Hyclone) was added with 10% calf bovine serum. Concanavalin A (ConA) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma. IgG, IL-2, and IL-6 enzyme-linked immunosorbent assay (ELISA) test kits for ND were provided by CUSABIO (Wuhan, P. R. China).

2.2. Immunization and sample preparation

A total of 200 healthy Hy-Line brown chickens (60 days old, body weight 800 ± 50 g) were raised in an animal facility of our laboratory with commercial feed. After 1 week of adaption to the environment the chickens were randomly divided into 10 groups with 20 in each. The chickens in group 1 were injected intramuscularly with ND I vaccine reconstituted with PBS used as a control, and in groups 2–10 they were injected with ND I vaccine reconstituted with the solution of APS, Se, and LMS, as shown in the Table. On days 7, 14, 21, 27, 35, and 42 after vaccinations, blood samples for lymphocyte proliferation and serum preparation were collected from 3 chickens randomly selected from each group.

2.3. ELISAs for IgG, IL-2, and IL-6 in serum

After immunization, serum IgG, IL-2, and IL-6 levels were examined according to the ELISA manufacturer's protocol. Briefly, ELISA plates were coated with the standard preparation for IgG, IL-2, and IL-6, respectively. Each well was then added with 100 µL of serum samples, except for the control wells, in which distilled water was added. Subsequently, the plates were incubated with a 1:500 dilution of anti-IgG at 37 °C for 1 h, or anti-IL-2 (1:500), or anti-IL-6 (1:500). After 3 washes, 50 µL of substrate was added. Optical density (OD) at 450 nm was read with a plate reader after the stopping solution was added.

2.4. T cell proliferation

After 2-fold dilution by Hanks' buffer, the anticoagulated blood was gently layered on the surface of hydroxypropyl methyl cellulose and then centrifuged at 2000 rpm for 20 min. After discarding leukocytic cream, the rest of the sample was washed twice with RPMI 1640 and centrifuged at 1500 rpm for 15 min. The cell sample was then suspended in RPMI 1640 and seeded at 5 × 10^6 cells per well in a 96-well plate. Except for the cell control and the blank control (only 200 µL of RPMI 1640), ConA was added to each treatment well with the final concentration of 40 mg/L, followed by an inoculation at 39.5 °C with 5% CO_2 for 48 h. Then 15 µL of MTT was added to the cells and the plates were further cultured for 4 h. The supernatant was removed and 100 µL of DMSO was added to each well to dissolve the formazan crystals. After gently shaking the plates for 10 min, the OD value was read at 490 nm with a plate reader.

2.5. Statistical analysis

Data were analyzed with SPSS 18.0 with multifactor variance analysis. Duncan's test was applied for multiple comparisons. The data were expressed as mean ± SE. Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. Antibody response analysis

As shown in Figure 1, IgG levels were enhanced in all of the experimental groups and at almost all analyzed time

Table. Experimental design and animal treatment.

Group	n	APS (mg/mL)	Se (mg/mL)	LMS (mg/mL)	PBS (mL)
1	20	0.00	0.00	0.00	1.00
2	20	3.00	0.01	10.0	1.00
3	20	3.00	0.02	20.0	1.00
4	20	3.00	0.03	30.0	1.00
5	20	6.00	0.01	20.0	1.00
6	20	6.00	0.02	30.0	1.00
7	20	6.00	0.03	10.0	1.00
8	20	9.00	0.01	30.0	1.00
9	20	9.00	0.02	10.0	1.00
10	20	9.00	0.03	20.0	1.00

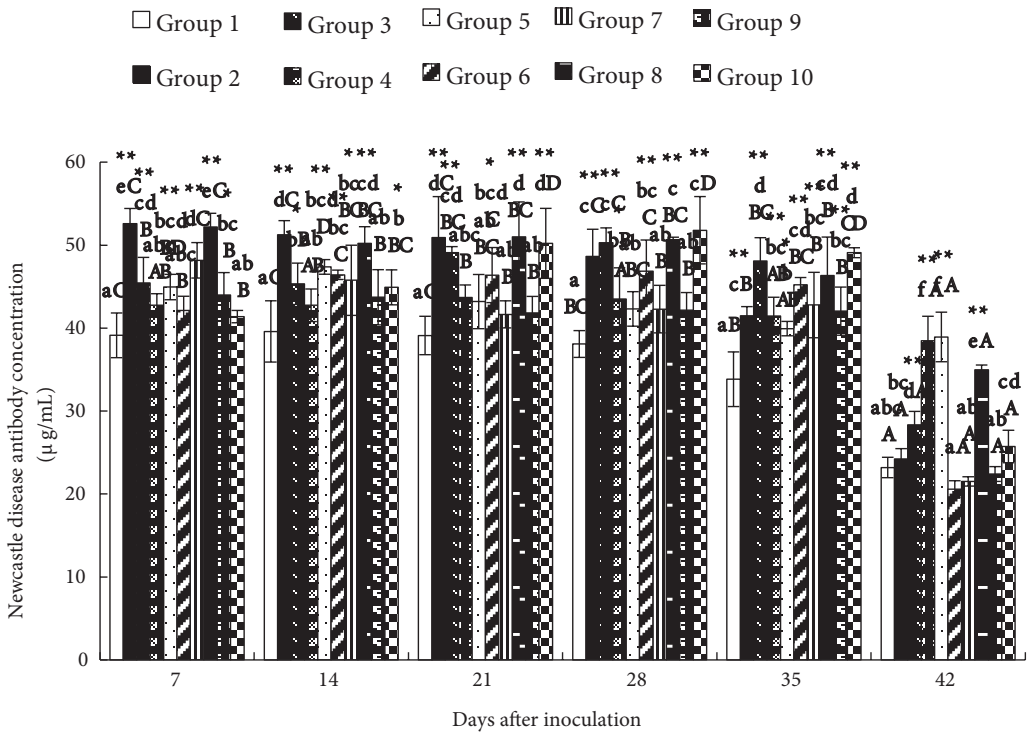

Figure 1. Levels of anti-ND vaccine IgG antibodies. Bars represent mean ± SE (n = 5). The asterisk indicates a statistically significant difference at *P < 0.05 and **P < 0.01. Statistical significance between treatment groups was marked with A, a, B, b, C, c according to the multifactor variance analysis.

points as compared to the negative control group. On days 7, 14, 21, 28, and 35 after the vaccinations, the IgG level in groups 2 and 8 were significantly enhanced, and especially at day 42, group 8 still remained higher. Furthermore, the IgG levels in groups 2 and 8 were remarkably higher than those in the rest of groups on days 7, 14, 21, and 28. Based on the multifactor variance analysis using SPSS 18.0, the P-values of APS and Se were less than 0.01, and the P-value of LMS was less than 0.05. The order was $F_{Se} > F_{APS} > F_{LMS}$. Thus, the main factor is Se, and then APS and LMS. Group 8 (APS 9 mg/mL + Se 0.01 mg/mL + LMS 30 mg/mL) was the best combination.

3.2. T cell proliferation analysis

T cell proliferation was analyzed using MTT assay (Figure 2). On days 7, 14, 28, 35, and 42 after the vaccination, the OD values of group 8 were elevated significantly compared to the control group. Enhanced T cell proliferation was also observed in other groups. The multifactor variance analysis using SPSS 18.0 indicated that the P-values of factors APS and Se were less than 0.01. The order was $F_{Se} > F_{LMS} > F_{APS}$. Se was the main factor, and then LMS and APS. Therefore, group 8 was the best match.

3.3. IL-2 analysis

As shown in Figure 3, the serum IL-2 was analyzed using ELISA. On days 35 and 42, IL-2 levels in all groups were significantly enhanced compared to the control group. The results of orthogonal design with regard to the 3 kinds of adjuvants showed that the P-values of factors APS, Se, and LMS were less than 0.01. The order was $F_{Se} > F_{LMS} > F_{APS}$. Se was the main factor, and then LMS and APS.

3.4. IL-6 analysis

The serum IL-6 level was also analyzed using ELISA, as shown in Figure 4. Compared to the control group the serum IL-6 level in almost all of the experimental groups was significantly enhanced from day 14 to day 35 after the vaccination. Analysis of the multifactor variance using SPSS 18.0 showed that the P-value of factors APS and Se were less than 0.01 and the P-value of factor LMS was less than 0.05. The order was $F_{APS} > F_{Se} > F_{LMS.}$ Therefore, the factor APS was the main factor, and then Se and LMS.

4. Discussion

In this study, for the first time we reconstituted the ND vaccine I with a combined solution of APS, Se, and LMS and examined whether the combined adjuvant could improve the immune efficacy of the ND I vaccine. The serum IgG in chickens was elevated and remained at a high level after vaccination. A strong T cell proliferation and an IL-2 level enhancement were also observed in the vaccinated groups with the combined adjuvant. The IL-6 level peaked on day 28 after vaccination with the ND vaccine reconstituted with APS + Se + LMS.

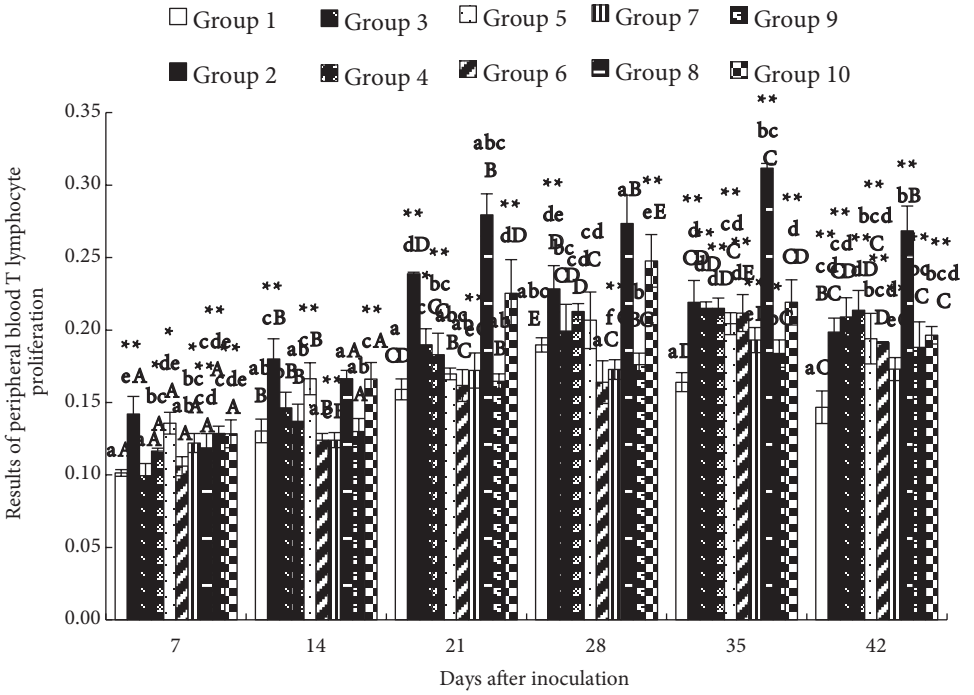

Figure 2. T cell proliferation in chickens inoculated with ND I vaccines reconstituted with a combined solution of APS, Se, and LMS on days 7, 14, 21, 27, 35, and 42 after vaccinations. Bars represent mean ± SE (n = 5). The asterisk indicates a statistically significant difference at *P < 0.05 and **P < 0.01. Statistical significance between treatment groups was marked with A, a, B, b, C, c according to the multifactor variance analysis.

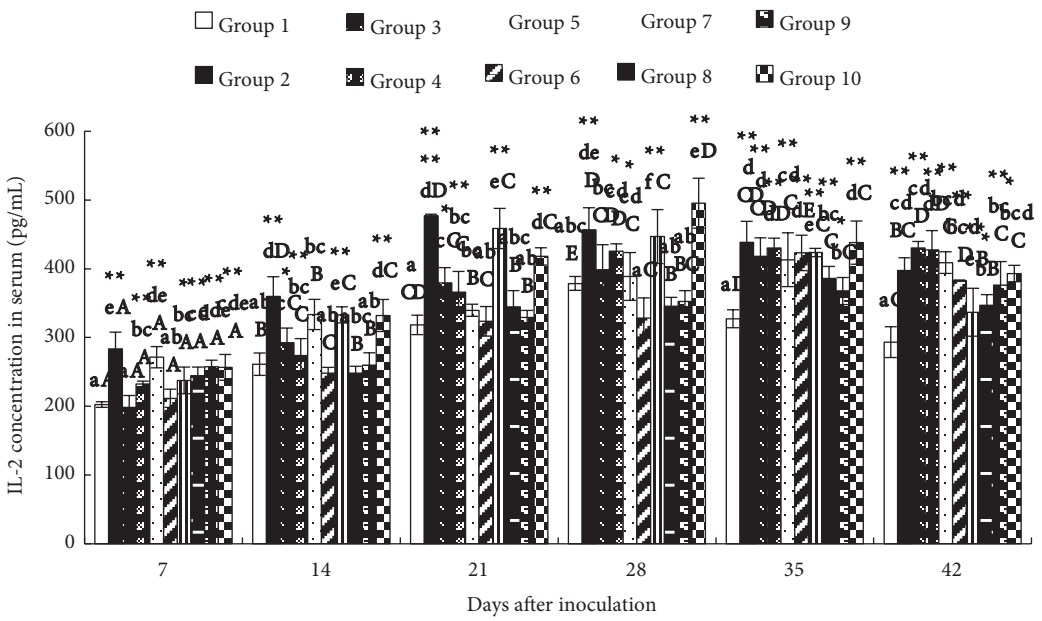

Figure 3. IL-2 levels in chickens inoculated with ND I vaccines reconstituted with a combined solution of APS, Se, and LMS on days 7, 14, 21, 27, 35, and 42 after vaccinations. Bars represent mean ± SE (n = 5). The asterisk indicates a statistically significant difference at *P < 0.05 and **P < 0.01. Statistical significance between groups was marked with A, a, B, b, C, c according to the multifactor variance analysis.

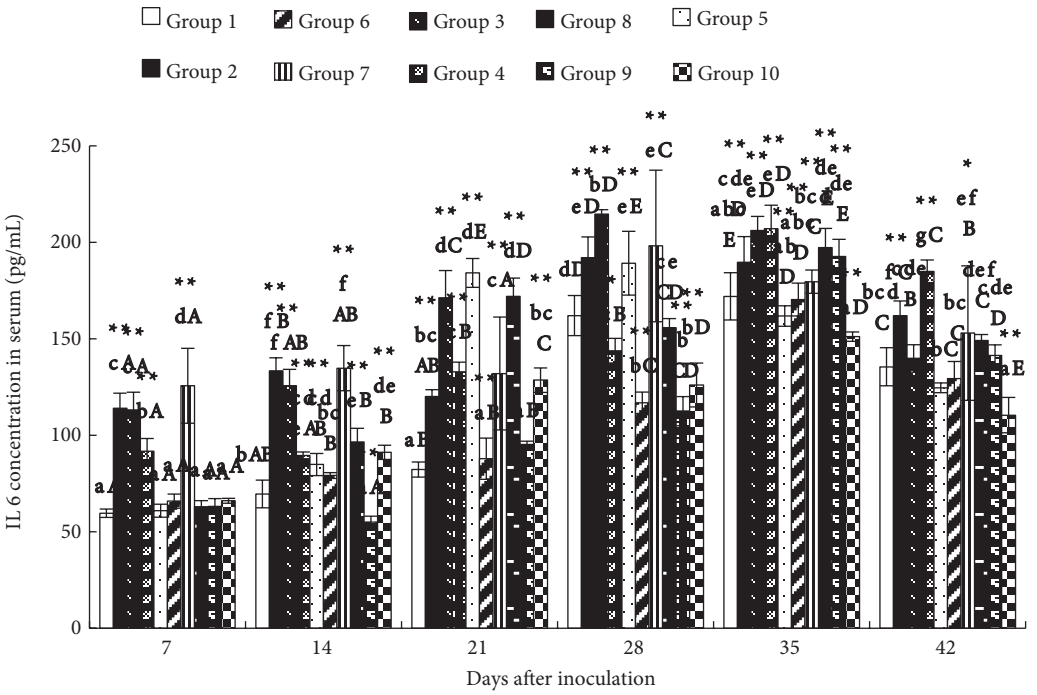

Figure 4. IL-6 levels in chickens immunized with ND I vaccines reconstituted with a combined solution of APS, Se, and LMS on days 7, 14, 21, 27, 35, and 42 after the vaccinations. Bars represent mean ± SE (n = 5). The asterisk indicates a statistically significant difference at *P < 0.05 and **P < 0.01. Statistical significance between groups was marked with A, a, B, b, C, c according to the multifactor variance analysis.

For improving the effectiveness of a vaccine, adjuvants have been studied extensively. Previous studies have demonstrated that reconstituted vaccines with ASP solution for infectious bursal disease and ND highly enhanced the antibody titer (9,18,20). Since Berenshtein (21) first reported the potential effects of Se on animal humoral immunity, numerous investigations have demonstrated that Se plus vaccines increased immune system activation in poultry (22,23). In addition, LMS as an adjuvant was also confirmed to increase the antibody titer in chickens (24). In this study, we found that at all of the analyzed time points, serum IgG levels in chickens injected with the vaccine reconstituted with the combined solution of APS + Se + LMS were significantly higher than those in the control group. The results not only confirmed the previous studies on immune enhancement of APS, Se, or LMS alone, but also indicated the synergy of the APS + Se + LMS on immunoregulation.

Among the 9 experimental groups, group 8 (APS 9 mg/mL + Se 0.01 mg/mL + LMS 30 mg/mL) had a high IgG response induced at every time point compared with the control group. Additionally, on days 7, 14, 21, and 28 after immunization, with the comparison to other experimental groups, IgG concentration in group 8 was also elevated obviously, remained at a high level within the first 35 days, and finally decreased at day 42. On the other hand, according to the statistical analysis, Se played the main role on high antibody titer. This is confirmed by the T cell proliferation and IL-2 analysis. However, data on IL-6 analysis revealed that APS was the primary factor, followed by Se and then LMS.

In the current investigation, data on T cell proliferation demonstrated that reconstitution solution containing APS, Se, and LMS significantly stimulated the T cell proliferation compared to the PBS-reconstituted group, which was consistent with earlier studies. Previously, it was reported Se supplementation can activate T cell-dependent immune response in humans (25), rats (26), and chickens (14). The elevated T cell proliferation was also observed in chickens inoculated with APS (9,18) or LMS (24) as the adjuvant. Therefore, the activation of T cells that can mediate the Th1 type responses in APS + Se + LMS groups may be the possible reason for the increased IgG level sustained for an extended period.

To further assess the immunoregulation of the mixed adjuvant, the level of IL-2 and IL-6 in the serum was detected. It is generally believed that IL-2, produced mainly by Th1 cells, NK cells, and cytotoxic T lymphocytes, can improve and maintain T cell proliferation and differentiation (27,28). Data on the IL-2 level showed that APS + Se + LMS stimulated IL-2 production and

indirectly supported the results of T cell proliferation in this study. On the other hand, the vaccination of the ND I vaccine with the mixed dilutions also enhanced the level of IL-6. The primary function of IL-6 was previously reported to be induction of B cell responses, including B cell proliferation and differentiation, and IL-6 is one of the key factors necessary for antibody production in B cells (29). The observed up-regulations of IL-2 and IL-6 in the present study agreed with earlier investigations on immune responses of APS (22), Se (20), and LMS (19) alone.

In summary, the inoculation of ND I vaccines reconstituted by APS, Se, and LMS combination produced broad immune responses involved in high and extended IgG levels and stimulation of T cell proliferation and cytokine expression. The findings may imply that reconstituted vaccine could be presented to antigen-presenting cells, resulting in cellular and humoral immunity. Notably, Se was shown to play a main role in immune regulation, and then APS and LMS, and the optimal formulation was APS 9 mg/mL + Se 0.01 mg/mL + LMS 30 mg/mL.

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References

1. Liang R, Cao DJ, Li JQ, Chen J, Guo X, Zhuang FF, Duan MX. Newcastle disease outbreaks in western China were caused by the genotypes VIIa and VIII. Vet Microbiol 2002; 87: 193–203.
2. Bogoyavlenskiy A, Berezin V, Prilipov A, Usachev E, Lyapina O, Korotetskiy I, Zaitceva I, Asanova S, Kydyrmanov A, Daulbaeva K et al. Newcastle disease outbreaks in Kazakhstan and Kyrgyzstan during 1998, 2000, 2001, 2003, 2004, and 2005 were caused by viruses of the genotypes VIIb and VIId. Virus Genes 2009; 39: 94–101.
3. Fan Y, Hu Y, Wang D, Guo Z, Zhao X, Guo L, Zhao B, Zhang J, Wang Y, Nguyen TL. Epimedium polysaccharide and propolis flavone can synergistically stimulate lymphocyte proliferation in vitro and enhance the immune responses to ND vaccine in chickens. Int J Bio Macromol 2010; 47: 87–92.
4. Bowersock TL, Martin S. Vaccine delivery to animals. Adv Drug Deliv Rev 1999; 38: 167–194.
5. Guo Z, Hu Y, Wang D, Ma X, Zhao X, Zhao B, Wang J, Liu P. Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. Vaccine 2009; 27: 660–665.
6. Jin H, Li Y, Ma Z, Zhang F, Xie Q, Gu D, Wang B. Effect of chemical adjuvants on DNA vaccination. Vaccine 2004; 22: 2925–2935.
7. Kang Y, Jin H, Zheng G, Xie Q, Yin J, Yu Y, Xiao C, Zhang X, Chen A, Wang B. The adjuvant effect of levamisole on killed viral vaccines. Vaccine 2005; 23: 5543–5550.
8. Goldson AJ, Fairweather-Tait SJ, Armah CN, Bao Y, Broadley MR, Dainty JR, Furniss C, Hart DJ, Teucher B, Hurst R. Effects of selenium supplementation on selenoprotein gene expression and response to influenza vaccine challenge: a randomised controlled trial. PLoS One 2011; 6: e14771.
9. Vecchi S, Bufali S, Skibinski DA, O'Hagan DT, Singh M. Aluminum adjuvant dose guidelines in vaccine formulation for preclinical evaluations. J Pharm Sci 2012; 101: 17–20.
10. Renoux G. The general immunopharmacology of levamisole. Drugs 1980; 19: 89–99.
11. Moertel CG, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Goodman PJ, Ungerleider JS, Emerson WA, Tormey DC, Glick JH et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 1990; 322: 352–358.
12. Monz BU, Konig HH, Leidl R, Staib L, Link KH. Cost effectiveness of adding folinic acid to fluorouracil plus levamisole as adjuvant chemotherapy in patients with colon cancer in Germany. Pharmacoeconomics 2003; 21: 709–719.
13. Tsavaris N, Gennatas K, Kosmas C, Skopelitis HM, Gouveris P, Dimitrakopoulos A, Zacharakis M, Kouraklis G, Vasiliou J, Felekouras E et al. Leucovorin and fluorouracil vs levamisole and fluorouracil as adjuvant chemotherapy in rectal cancer. Oncol Rep 2004; 12: 927–932.
14. Saad MB, Gertner LR, Bona TD, Santin E. Selenium influence in the poultry immune response--review. Recent Pat Food Nutr Agric 2009; 1: 243–247.
15. Chen Y, Wang D, Hu Y, Guo Z, Wang J, Zhao X, Fan Y, Guo L, Yang S, Sai F et al. Astragalus polysaccharide and oxymatrine can synergistically improve the immune efficacy of Newcastle disease vaccine in chicken. Int J Bio Macromol 2010; 46: 425–428.
16. Liu QY, Yao YM, Yu Y, Dong N, Sheng ZY. Astragalus polysaccharides attenuate postburn sepsis via inhibiting negative immunoregulation of CD4 + CD25 (high) T cells. PLoS One 2011; 6: e19811.
17. Li J, Zhong Y, Li H, Zhang N, Ma W, Cheng G, Liu F, Liu F, Xu J. Enhancement of *Astragalus* polysaccharide on the immune responses in pigs inoculated with foot-and-mouth disease virus vaccine. Int J Bio Macromol 2011; 49: 362–368.
18. Li HQ, Lloyd RJ, Wang JD. Effect of *Astragalus* polysaccharides on erythrocyte immune adherence of chickens inoculated with infectious bursal disease virus. Agr Sci China 2007; 6: 1402–1408 (article in Chinese with an abstract in English).
19. Li HQ, Qiu JD, Yang LH. Regulation of *Astragalus* heteropolysaccharides on synoviocytes apoptosis and proinflammatory cytokine secretion of rats with adjuvant arthritis. Acta Pharmaceut Sin 2009; 44: 731–736 (article in Chinese with an abstract in English).

20. Jiang J, Wu C, Gao H, Song J, Li H. Effects of *Astragalus* polysaccharides on immunologic function of erythrocyte in chickens infected with infectious bursa disease virus. Vaccine 2010; 28: 5614–5616.

21. Berenshtein GF. Effect of selenium on the sensitivity of the organism to hormones regulating carbohydrate metabolism. Nauchnye Doki Vyss Shkoly Biol Nauki 1973; 115: 42–44.

22. Baowei W, Guoqing H, Qiaoli W, Bin Y. Effects of yeast selenium supplementation on the growth performance, meat quality, immunity, and antioxidant capacity of goose. J Anim Physiol Anim Nutr (Berl) 2011; 95: 440–448.

23. Brennan KM, Crowdus CA, Cantor AH, Pescatore AJ, Barger JL, Horgan K, Xiao R, Power RF, Dawson KA. Effects of organic and inorganic dietary selenium supplementation on gene expression profiles in oviduct tissue from broiler-breeder hens. Anim Reprod Sci 2011; 125: 180–188.

24. El-Kholy H, Kemppainen B, Ravis W, Hoerr F. Pharmacokinetics of levamisole in broiler breeder chickens. J Vet Pharmacol Ther 2006; 29: 49–53.

25. Walpole D. Effects of selenium supplementation on New Zealanders' health. N Z Med J 2007; 120: U2482.

26. Behne D, Alber D, Kyriakopoulos A. Effects of long-term selenium yeast supplementation on selenium status studied in the rat. J Trace Elem Med Biol 2009; 23: 258–264.

27. Castro I, Yu A, Dee MJ, Malek TR. The basis of distinctive IL-2-and IL-15-dependent signaling: weak CD122-dependent signaling favors CD8 + T central-memory cell survival but not T effector-memory cell development. J Immunol 2011; 187: 5170–5182.

28. Liao W, Lin JX, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Curr Opin Immunol 2011; 23: 598–604.

29. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, Yusuf I, Crotty S. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. PLoS One 2011; 6: e17739.