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Immunity Against Avian Influenza Viruspanax Ginseng Polysaccharide (GPS) Can Improve Immunity Against H9N2 Avian Influenza Virus in Chickens

Research Article

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ABSTRACT

The humoral immunization potential of panax ginseng polysaccharide (GPS) against H9N2 avian influenza virus (H9N2 AIV) in chickens was investigated. The effects of GPS treatment before and during H9N2 AIV infection were determined in chicken embryo fibroblast (CEF) by MTT (3(4, 5-dimethylthiazol-2-yl)-2, 3-diphenyl tetrazolium bromide) assays and quantitative RT-PCR analysis of MHC and cytokine expression. The percentages of CD3+, CD4+ and CD8+ T cells in peripheral blood lymphocytes and serum antibody titers were examined *in vivo*. High expressions of MHCII and the cytokines IL-2, IL-4, and IL-10 were observed in CEF treated with GPS before and during H9N2 infection. These results indicated that the anti-viral activity of GPS was enhanced by pretreatment of CEF and that GPS promotes early humoral immune responses in young chickens

KEY WORDS antibodies, cytokines, H9N2 avian influenza virus, immunity, Panax ginseng polysaccharide.

INTRODUCTION

Influenza is a severe respiratory disease causing recurrent outbreaks which significantly affect human health, livestock and the global economy (Yoo *et al.* 2012). Illness and death caused by this disease have economic and social costs. According to FAO, in 2010, the infection was spread by migratory birds, inert substrates such as contaminated cages and clothing, and internationally-traded live poultry. Previously WHO in 2010 emphasized that the disease can be transmitted to humans through exposure to infected birds or through the handling infected carcasses (Obayelu, 2007).

Outbreaks of avian influenza caused by H9N2 viruses with low pathogenicity have occurred in poultry, resulting in serious economic losses in Asia and the Middle East. It has been difficult to eradicate H9N2 because of its low pathogenicity frequently causing in apparent infection. Another important problem facing eradication efforts is that influenza virus has a wildlife reservoir.

In humans, current vaccines are only effective if they are well-matched with influenza strains that are predicted to circulate during the next season. In addition, a number of influenza variants have evolved to develop resistance to antiviral drugs (Moscona, 2005). A preventive measure that would have protective effects against influenza virus regardless of strain is highly desirable.

Panax ginseng is an acidic polysaccharide which contains ginsenosides, essential oil, peptidoglycans, polysaccharides, nitrogen-containing compounds, fatty acids and phenolic compounds (Zhang *et al.* 2001; Dou, 2001). Among these, the polysaccharides have been shown to have effects on immunologic defense functions (Yoo *et al.* 2012). Panax

ginseng is a well-known traditional Chinese herbal medicine.

Panax ginseng polysaccharide (GPS) has multiple functions such as promoting the production of cytotoxic T-cells against tumors and stimulating macrophages to produce Th1 and Th2 cytokines (Kim, 1998 and Yuan, 2010). GPS also modulates antioxidants such as superoxide dismutase and glutathione peroxidase, probably via the induction of regulatory cytokines (Han, 2005). An anti-inflammatory response at an early phase resulted in enhanced antimicrobial activities and protection of mice from *Staphylococcus aureus*-induced sepsis (Ahn *et al.* 2006a and Ahn *et al.* 2006b). However, the effect of GPS on viral infection remains unknown and its research application in poultry is rarer than its use in mice.

Most researchers have concentrated on the small molecular weight of components such as GPS. In mice, acidic GPS can stimulate lymphoid cells to produce regulatory cytokines such as IL-1 and IL-2, while reducing the production of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IFN-γ, IL-12 and IL-18 at the early phase of sepsis (Hwang, *et al.* 2011). GPS has been described as an immunologic response modifier.

We evaluated the effect of GPS applied in different concentrations and its antiviral action on the propagation of H9N2 AIV in chick embryo fibroblasts (CEF). The mechanism of the antiviral effect was investigated by analysis of IL-2, IL-4 and IL-10 mRNA expression. Changes in peripheral blood lymphocyte and humoral immune responses were also analyzed to estimate the immunological enhancement of GPS in chickens.

MATERIALS AND METHODS

Ethics

Animal experiments and husbandry complied with the guidelines of the South China agricultural university animal care and use committee, which operates under the animal welfare law and regulations of the department of health and human services. The South China agricultural university animal care and use committee approved all protocols.

Extraction and purification of GPS

GPS was extracted and purified as described (Kallon *et al.* 2013). Briefly, GPS ground powder obtained from South China agricultural university (Guangzhou, China) was boiled in distilled water for four hours at 100 °C. After filtration to remove debris, the filtrate was concentrated in a rotary evaporator. Protein was removed using the Savage method (Zhang 1999). Crude polysaccharide fractions were obtained by precipitation with three volumes of ethanol and desiccation *in vacuo*. The precipitate was re-dissolved in distilled water and loaded on a D101 macro-porous resin column (2.6cm×60cm) to remove pigment. The effluent was collected and the polysaccharide fractions were quantitatively determined using a phenol-sulfuric acid assay (Dubois *et al.* 1951) with glucose as the reference standard.

Purification of H9N2 AIV

Embryonating specific pathogen free (SPF) chicken eggs (10 days old from Guangdong Dahuanong Animal Health Products Co. ltd Guangzhou China) were inoculated with H9N2 virus (0.2mL/egg). Infected allantoic fluids were harvested after 48 hours and concentrated with a 100K tangential flow filtration capsule (Pall Life Sciences) by centrifugation at 40,000 rpm for one hour. The suspension was loaded onto a 30- to 60% (wt/wt) sucrose gradient and subjected to centrifugation at 26000 rpm at 4 °C with SW-40 Ti rotor (Beckman Instruments, Palo Alto, CA) for 3 hours using the slowest acceleration and braking rates. The viral pellets were washed and centrifuged at 40000 rpm at 4 °C for one hour. Subsequently, pellets were re-dissolved in 1ml of PBS, filtered through 0.22 Millipore paper, and stored at -70 °C (Suomalainen *et al.* 2011). CEF cultures were prepared from 10-day-old chicken embryos according to standard procedures. Briefly, Dulbecco's Modified Eagle Medium (Gibco-Invitrogen) was used. Embryo tissue was cut into pieces and diluted to 1×10^6 cells/mL. After filtration, cells were cultivated in a 5 % (v/v) CO_2 incubator at 37°C for 48 hours (Yang *et al.* 2010).

Determination of CEF safety concentration

The CEF safety concentration was determined as described (Xiaoyan *et al.* 2008). Briefly, CEF monolayers in 96-well plates were exposed to serial dilution of four wells per concentration. After culture for 72 hours at 38.5 °C in a humid atmosphere of 5% CO_2, the supernatant was removed and 100µL of DMSO was added. The plates were shaken for five minutes t7o dissolve the crystals completely. The absorbance at 570nm (A_{570}) of each well was measured in a microliter enzyme-linked immunosorbent assay reader (Model DG-3022, East China Vacuum Tube Manufacturer). A_{570} is correlated to the number of live cells.

Determination of appropriate concentration of GPS in CEF Sextuplicate confluent monolayers of CEF in 96-well culture plates were overlaid with media containing GPS at ten doubling dilutions from 4.833 to 2500µg/mL. Plates were cultured for 8 hours at 37 °C in a humid thermostat with 5% CO_2. The effects on cell metabolism were determined by using the MTT (3(4, 5-dimethylthiazol-2-yl)-2, 3-diphenyl tetrazolium bromide) assay. Twenty microliters of MTT (Amresco, solon, OH, 5µg/mL) were added into each well at a final concentration of 0.5µg/ml and cells were incubated at 37 °C in a humid atmosphere with 5% (v/v)

CO_2 for four hours. At the end of this incubation, 10µL of dimethyl sulphoxide (DMSO) (Sigma, Kent, UK) was added and plates were incubated at 37 °C for five minutes to dissolve Formazan crystals. Absorbance was measured at A_{570} using a microtiter ELISA reader (Molecular Devices Ema x, CA, USA). A_{570} in GPS-treated plates, compared to the control, was recorded to assess the cytotoxicity of GPS in CEF.

Antiviral assays

Antiviral activity was detected as described (Kallon *et al.* 2013). In our previous work we used APS but in the present work we used GPS. Antiviral detection procedures were slightly changed. Three stages were involved: before GPS addition, after GPS addition and simultaneous addition of GPS and H9N2 virus.

However, detection of antiviral in present study detected only first stage. Briefly, ten different GPS concentrations (100µL/well; five wells/concentration) were added to CEF prior to the addition of virus incubated at 38.5 °C at 5% CO_2 for two hours. Supernatant were removed and cells washed twice with PBS. 100µL of virus was added and plates were incubated for 72 hours. When a complete cytopathic effect (CPE) was observed for the H9N2 control group (~72 hours), CEF viability was measured by the MTT assay. The mean cellular A_{570} indicated antiviral activity (Kallon *et al.* 2013).

Animal experiment

One hundred and fifty, 5-day-old SPF White Leghorn broilers (Guangdong Dahuanong Animal Health Products Co. ltd, Guangzhou China) were randomly assigned into three sample groups and two control groups (vaccinated and unvaccinated). Each group comprised 30 birds. All groups were maintained in five positive pressure isolators. Seven days post hatching (dph), birds in the three sample groups were hypodermically injected with GPS at 5 mg/kg, 10 mg/kg and 20mg/kg, daily for five successive days. The control groups were treated with PBS. At 12 dph, 6 birds per group were killed by cervical dislocation and blood was collected. Peripheral blood lymphocytes separated from the blood samples (5mL per bird) were used for flow cytometry and real-time PCR. The immune organs were weighed and stored at -70 °C. Simultaneously, 9 birds per group were challenged with $10^{7.5}$ EID_{50}/0.2 mL of H9N2 AIV by intramuscular injection. The remaining 15 birds from each group except the control group were immunized subcutaneously with an inactivated oil-emulsion vaccine of H9N2 AIV (Guangdong Dahuanong animal health products Co. ltd Guangzhou China) at 13 and 20 dph. The control group was kept PBS-treated. Antibody titers were measured at 7 and 14 days post immunization. At 26 dph, experimental procedures were performed as described at 12 dph (sacrifice and sampling) for all remaining birds.

Three color flow cytometry Blood samples were collected with sodium heparin and mixed with an equal volume of Hanks' balanced salt solution (HBSS, pH7.2~7.4). 5 mL of this mixture was layered over 5 mL lymphocyte separation medium (LSM).

The tubes were centrifuged at 2000 rpm for 20 minutes in a swinging-bucket rotor. The peripheral blood lymphocyte was recovered from the LSM-HBSS interface, then washed three times with PBS and re-suspended in RPMI 1640 medium containing 10% (v/v) bovine serum. PBMC (2×10^6) were incubated with 20µl anti-chicken CD3-PE (Southernbiotech Cat. 8200-09), CD4-FITC (Southernbiotech Cat. 8200-02) or CD8-CY5 (Southernbiotech Cat. 8200-15). The blending was washed twice with PBS and centrifuged at 1200 rpm for 10 minutes after subsequent incubation in the dark for 20 minutes. The supernatant was discarded and cells were re-suspended in 500µL PBS (Fair *et al.* 2008). Cells were then analyzed by flow cytometry (version 6.1, BD FACSAria).

Measurement of antibody titer was measured by the hemagglutination inhibition (HI) test. The HI test was a standard beta test, using 4 hemagglutinating units of antigen in 96-well plates, where the test serum had been diluted twofold. HI endpoint titers were determined as the reciprocal of the highest serum dilution that produced complete inhibition of hemagglutination. Blood samples (1.0mL/bird) obtained from the main brachial vein were drawn into Eppendorf tubes and allowed to clot at 37 °C for two hours. Serum was separated by centrifugation and stored at −20 °C for HI antibody determination.

Briefly, two-fold serial dilutions (1:2 to 1:2,048) of heat inactivated (56 °C for 30 min), serum were added to a 96-well, V-bottomed microtiter plate containing 50 µL/well cm F-PBS, and 50 µL/well H9N2 antigen (four hemagglutination units), except the last row which was used for controls: positive serum, negative serum, erythrocytes and antigens. The antigen-serum mixture was incubated for 10 minutes at 37 °C, 50 µL of 1% rooster erythrocyte suspension added to each well and the plates were re-incubated for 30 minutes. The highest dilution of serum causing complete inhibition of erythrocyte agglutination was considered the end point. The geometric mean titer was expressed as reciprocal log2 of the highest dilution that displayed anti-H9N2 HI.

Real-time PCR

Total CEF RNA was extracted using Trizol (Takara Biotechnology, Dalian, China) to detect the expressions of cytokines or H9N2 AIV. RNA concentrations were measured by spectrophotometry (260/280). The isolated RNA was digested and purified with recombinant DNase (Takara

Biotechnology, Dalian, China) at 37 °C for 30 minutes. 1µg of total RNA was used for reverse transcriptase with Rever Tra Ace QPCR RT kit (Toyobo Osaka, Japan). After reversing the RNA to cDNA, real-time PCR was performed in a total volume of 20µl containing 150ng cDNA template, 2×SYBR green Real-time PCR master mix (Roch Mortlake Australia), and 200nM of each primer (Table 1). After initial denaturation for 10 minutes, the amplification was carried out through 40 cycles, each consisting of denaturation at 95 °C for 10 seconds and primer annealing at 58 °C for 30 seconds.

Melting curves were obtained, and quantitative analysis of the data was performed by the 2–□□Ct method, with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as endogenous reference gene used to normalize the expression level of target genes.

Statistical analysis

Analysis was performed using SPSS 17.0 (SPSS, Chicago, IL, USA). All data were expressed as mean ±SEM. Comparisons between two groups were performed using unpaired Student's T tests, and among multiple groups by post-hoc ANOVA and Tukey's multiple comparison tests at 5% significance levels. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

Optimum GPS concentration and antiviral assay

The optimum concentration of GPS and results of antiviral assays are shown in Table 2. The optimum concentrations of GPS for CEF cells proliferation detected were 321.25µg/mL and 2500µg/mL. The A_{570} values for both GPS and the pre-addition (321.25µg/mL and 2500µg/mL) were significantly higher than observed for corresponding controls. These results revealed that CAF proliferation was improved, with the greatest proliferation at a GPS concentration of 321.25µg/mL and 2500µg/mL. Also, both were significantly higher than for the corresponding virus control, indicating that virus activity was inhibited at all GPS and pre-addition concentrations. However, for both GPS and the pre-addition a number of the A_{570} values of 156.6 µg/mL - 4.833µg/mL were closely similar or lower than cell controls suggesting that at such concentrations, viral activity was not inhibited (Table 2).

Relative expression of H9N2 AIV in CEF

The relative expression of H9N2 AIV for pre-addition of GPS at various GPS concentrations, particularly 321.25 µg/mL and 2500 µg/mL, were significantly low. The proliferation of H9N2 AIV was approximately 11% lower than for the pre-addition of GPS at 321.25 µg/mL and 2500 µg/mL, when compared with 4.833 µg/mL and 0µg/Ml respectively (Figure 1).

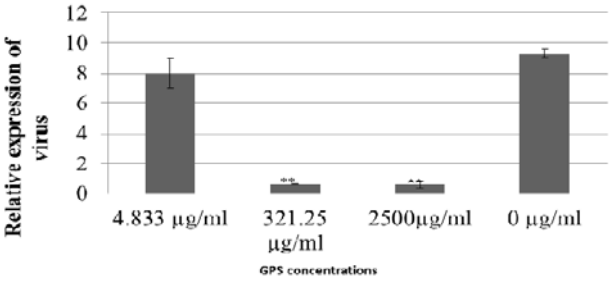

Figure 1 The relative expression of H9N2 AIV in CEF treated at different concentrations of GPS for groups treated by pre-adding GPS. The inhibition efficiency of GPS was significantly higher (P<0.01) at 321.23 µg/mL and 2500 µg/mL concentration than at 4.833 µg/mL and 0µg/ml.

**Difference is highly significant (P<0.01) in comparison with control

Relative expression of MHC and cytokines in CEF

The relative expressions of MHC and cytokines in CEF for the different groups are shown in Figures 2 and 3. MHC 1 and MHC 11 expressions were low following GPS treatment; but increased after H9N2 AIV infection. The expressions of IFN-α, IFN-β, IFN-γ, IL-6 and LITAF were down regulated in all groups. However the relative expressions of IL-2, IL-4 and IL-10 in GPS-treated groups were higher than control groups before and after H9N2 AIV infection (Figure 2 and 3).

Differential induction of CD3+, CD4+, and CD8+ T-cells

The proportion of lymphocytes of CD3+, CD4+, CD8+ and T-cell surface markers are presented in Figures 4,5 and 6.

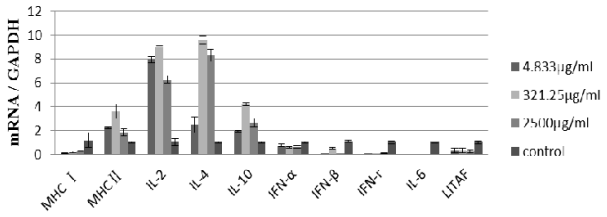

Figure 2 Real-time quantitative PCR analysis of MHC class Ⅰ, MHC class Ⅱ, IL-2, IL-4, IL-10,IL-6, IFN-γ, IFN-β, IFN-α and LITAF in GPS-treated CEF

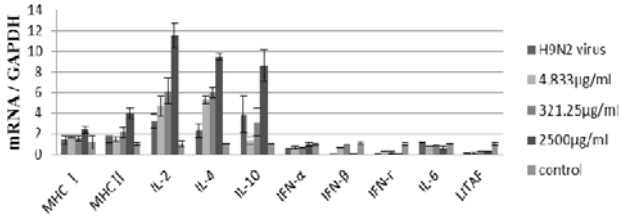

Figure 3 Real-time quantitative PCR analysis of MHC class Ⅰ, MHC class Ⅱ, IL-2, IL-4, IL-10, and IFN-α, IFN-β, IFN-γ and LITAF in GPS-treated CEF infected with H9N2 AIV injection

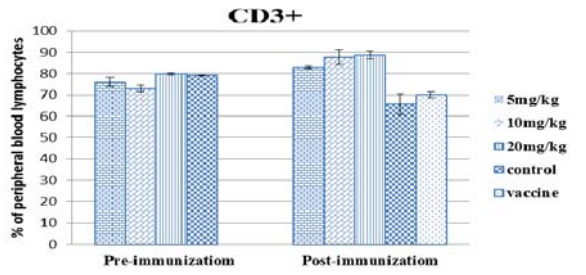

Figure 4 Percentages of CD8+ T-cells *in vivo*
* Difference is significant (P<0.05) * *Difference is highly significant (P<0.01) in comparison with control

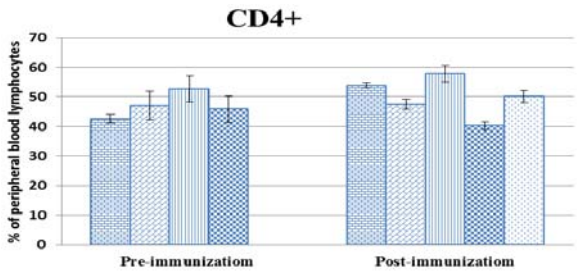

Figure 5 Percentages of CD4+T cells *in vivo* test

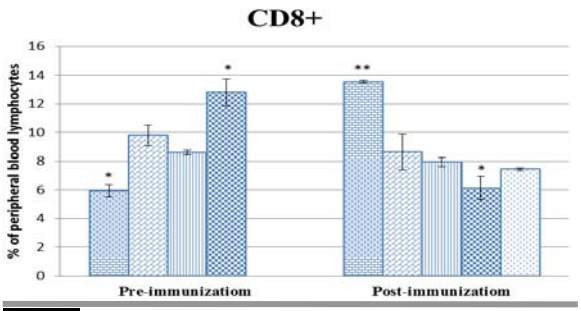

Figure 6 Percentages of CD8+ T-cells *in vivo*
* Difference is significant (P<0.05) * *Difference is highly significant (P<0.01) in comparison with control

The percentage composition of lymphocytes expressing CD3+, CD4+, and CD8+ were detected. No significant difference in the percentages of CD3+ or CD4+ lymphocytes in peripheral blood was detected following GPS administration or H9N2 AIV infection following GPS pre-addition treatment compared with the control. Furthermore, lower levels of CD8+ cytotoxic T-cells were observed following with GPS compared with the control. However, CD8+ ratios were significantly higher than the control after GPS treatment. In particular, CD8+ ratios in the 5 mg/kg group was significantly lower (P<0.05) than the control group before vaccination, but expanded after vaccination to become higher (P<0.01) than the control group (Figure 6).

Antibodies

Antibody titers in GPS-treated groups increased significantly more quickly than the control groups in the first week after H9N2 AIV challenge, the antibody titers of the 5 mg/kg and 10 mg/kg groups were higher than vaccine and blank control groups (Figure 9). After immunization, the highest antibody titers were observed in the group treated with 5 mg/kg of PSP following administration of inactivated vaccine (Figure 9). No significant difference in antibody titer was detected between the GPS-treated groups and the control group at 14 days post immunization (Figure 10). The 5mg/k g APS group attained the highest antibody titer at 7 and 14 days post immunization with inactive vaccine (Figures 9 and 10)

Ginsan is currently available as an over- the-counter preparation and is used as a natural, proactive alternative to strengthen immunity. The cellular immune response is critical to the host defense system against infection by accelerating the clearance of pathogens and secreting many cytokines for the regulation of the immune response (Zhang *et al.* 2010). The A_{570} value is an index reflecting the number of living cells, cell growth, and polysaccharide inhibition of virus infection (Wang *et al.* 2010). The experimental results revealed that higher CEF proliferation was observed in the presence of 2500 µg/mL and 321.25µg/mL of GPS compared with control groups. Based on these observations and the practicability of GPS administration all subsequent *in vitro* experiment s were carried out using 2500µ g/mL - 4.833 µ g/mL GPS. The optimum concentration for GPS treatment of CEF was established as 321.25 µ g/mL and 2500µg/mL. Cell proliferation was enhanced in the presence of 321.25 µg/mL and 2500µg/mL GPS (compared with the control group) and the cell viability of H9N2 AIV-infected cells was greater at this concentration of GPS than for the other concentrations investigated. When GPS was pre-added to cultures of CEF, A_{570} for groups within the range 4.833 to 2500µ g/mL GPS were significantly higher than for the corresponding virus control group, indicating that APS could prevent H9N2 AIV infection at these concentrations (Table 2).

MHC molecules can identify and present antigen to T-lymphocytes, then activate CD4+ and CD8+ T-cells and induce specific immunity (Wang, 2001). MHC molecules are responsible for binding to degraded peptides of invading pathogens, their subsequent presentation on the surface of the infected cell for T-cell (Th and cytotoxic T-cell) recognition, and ultimately for the ensuing destruction of the infected cell. MHC II molecules interact predominantly with CD4+ helper T-cells (Th), while MHC I molecules interact with CD8+ cytotoxic (killer) T-cells. Helper T-cells help to trigger an appropriate adaptive immune response. The T-cell response is described by the total T-cell count (CD3+) and T-cell subsets (CD 4+ helper and CD8+ cytotoxic cells; Guleria *et al.* 1993). In this study, the expression of MHC 1 was low after GPS treatment and H9N2 AIV challenge. However MHC expression increases after

Table 1 Primers used in real-time PCR

RNA target	Primer sequences		Size of PCR product (bp)
	Sense（5’ 3’）	Antisense（5’ 3’）	
MHC class I	AAGAAGGGGAAGGGCTACAA	AAGCAGTGCAGGCAAAGAAT	222
MHC class II	CTCGAGGTCATGATCAGCAA	TGTAAACGTCTCCCCTTTGG	312
Interferon γ	TGAGCCAGATTGTTTCGA	ACGCCATCAGGAAGGTTG	118
IL-2*	CGGGATCCATGATGTGCAAAGTACTG	CGGTCGACTTATTTTTGCAGATATCT	80
IL-4	GAGAGGTTTCCTGCGTCAAG	TGACGCATGTTGAGGAAGAG	76
LITAF	TTCTATGACCGCCCAGTT	CAGAGCATCAACGAAAA	165
IL-6	ATTAAATCCCGATGAAAGTGG	CTCACGGTCTTCTCCATAAA	146
IL-10	CAATCCAGGGACGATGAAC	GCAGGTGAAGAAGCGGTGA	94
H9N2	ATGCGGTGGAAGATGGG	AGGCGACAGTCGAATAAATG	198
GAPDH*	CCTCTCTGGCAAAGTCCAAG	CATCTGCCCATTTGATGTTG	200

MHC: major histocompactability complex.
GAPDH: glyceraldehydes 3-phosphate dehydrogenase

Table 2 A_{570} of the optimal concentration and antiviral assays at 2500 μg/ML-4.833μ/mL

Groups	GPS concentration									
	2500μg/mL	250μg/mL	625μg/mL	321.25μg/mL	156.6 μg/mL	78.215μg/mL	39.063μg/mL	19.513μg/mL	9.766μg/mL	4.833μg/mL
GPS	0.325±0.012^{a}*	0.302±0.051^{b}*	0.302±0.008^{b}*	0.327±0.016^{a}*	0.247±0.006^{c}*	0.241±0.008^{c}*	0.251±0.009^{c}*	0.251±0.007^{c}*	0.222±0.007^{d}*	0.212±0.007^{d}*
Pre-added GPS	0.270±0.010^{a}*	0.238±,010^{b}*	0.229±0.018^{c}*	0.274±0.010^{a}*	0.244±0.014^{b}*	0.249±0.014^{b}*	0.248±,014^{b}*	0.216±0.009^{b}*	0.225±0.010^{c}*	0.207±0.008^{d}*
Virus control	0.160±0.005									
Cell control	0.244±0.007*									

$^{a-d}$ data within row without the same superscripts differ significantly(P<0.05).
∗ data differed significantly (P<0.05) when compared with virus control.

H9N2 AIV infection and the relative expressions of IL-2, IL-4 and IL-10 in GPS-treated groups were higher than control groups before and after H9N2 AIV infection. In addition, the expressions of IL-2 and IL-4 were highly expressed in the 321.25μg/mL group and attained higher increases than any other group after GPS treatment (Figure 2), while the 2500μg/mL had the highest IL-2 IL-4 and IL-10 expressions after H9N2 AIV challenge (Figure 3). IL-2 and IFN-γ are secreted by Th1 cells and promote cell-mediated immunity (Guleria *et al.* 1993). These cytokines are involved in the differentiation of naïve T-cells into Th1 cells. IL-2is known as a T cell-stimulating factor, which can stimulate the growth and function of T-cells (Hsieh *et al.* 1993).

It stimulates the production of IFN-γ and TNF-α from T and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ. In this study the production of IFN-γ was decreased but the expression of IL-2 was increased in response to H9N2 AIV challenge. The expressions of IL-4 and IL-10 are important in the generation of mammalian Th2 type responses, and inhibit Th1 cells (Hilton *et al.* 2002; Delves and Roitt, 2000). These cytokines were evidently higher in GPS-treated groups than in control groups.

The expressions of IL-2, IL-4 and IL-10 in GPS-treated groups were higher than in control groups, suggesting that GPS probably stimulated cytokine production through B lymphocyte and macrophage activation. Individual cytokines often have multiple effects, the results of which are dependent on their target cell. CD3+ and CD4+ are important T lymphocyte markers. CD + T lymphocytes are primarily responsible for mediating cytotoxic effects , and the main function of CD4+ T lymphocytes is the secretion of cell factors which induce and enhance the immune response (Sun *et al.* 2002). In our study, CD3+ and CD4+ levels did not differ between experimental and control groups before vaccination (Figure 4 and 5). However, after H9N2 AIV infection the proportion of CD3+ and CD4+ in the GPS treated-groups were numerically higher than in vaccinated and control groups (Figures 7 and 8). Moreover, the CD8+ level of the 5mg/kg group was than that of the control group (P<0.05) before immunization, but after immunization it was higher (P<0.01) (Figure 9). Recent studies revealed that Ginseng extract in aluminum hydroxide adjuvant vaccines improves the antibody response in pigs against porcine parvovirus and the bacterium *Erysipelothrix rhusiopathiae* (Rivera *et al.* 2003). In addition, standardized ginseng extract G115 has been used for potentiating vaccination against the human common cold and / or influenza to increase antibody titers and induce a prolonged response with accompany memory (Scaglione *et al.* 1996).

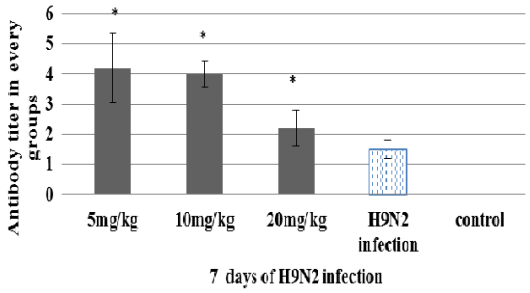

Figure 7 Antibody titer of each group after H9N2 AIV infection was carried out by HI test 7 days after infection by H9N2 AIV

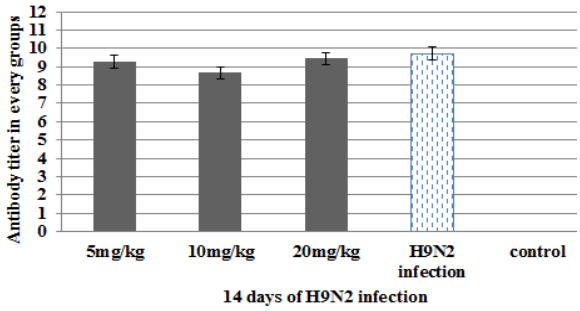

Figure 8 Antibody titer of each group after H9N2 AIV infection was carried out by HI test 14 days after infection by H9N2 AIV

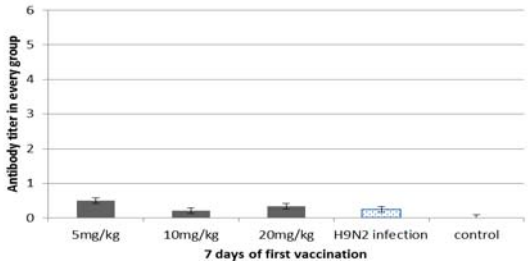

Figure 9 Antibody titer of each group after inactive vaccine immunization, measured by HI test

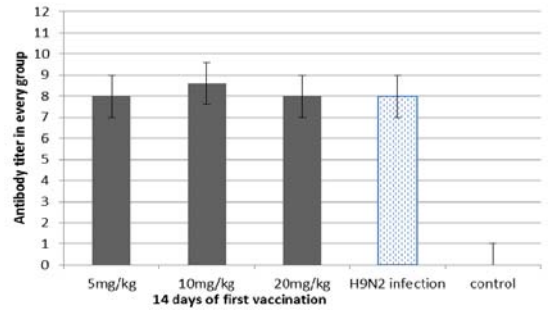

Figure 10 Antibody titer of each group after inactive vaccine immunization, measured by HI test

In agreement with these findings, our study showed that the antibody titer of GPS-treated groups significantly surpassed that of control groups after one week of H9N2 AIV challenge. This could be due to B cells having been activated before H9N2 AIV infection in GPS-treated groups. Seven days after H9N2 infection or immunization by inactivated vaccine, the 5 mg/kg GPS=treated group reached the highest antibody titer when compared with corresponding H9N2 AIV infection and control groups (Figures 7). There was no significant difference between GPS-treated groups and control groups two weeks post H9N2 infection (Figures 8). Collectively, the results obtained from this study indicate that the administration of the appropriate dose of APS could enhance antibody production and improve humoral immunity, and that GPS had the properties of an antiviral agent. These observations agreed with those reported by Kong *et al.* (2006).

The antibody titers of experimental groups were higher than controls following both H9N2 AIV challenge and inactive vaccine immunization, suggesting that GPS could enhance humoral immunity in broiler chickens. Indeed on day 7 after vaccination the anti-H9N2 AIV antibody titers of GPS-treated groups (5 mg/kg, 10 mg/kg and 20 mg/kg) were higher than those in vaccinated and control groups, indicating a more rapid response by the GPS-treated group (Figures 9 and 10).

CONCLUSION

This present study demonstrated that GPS at optimum concentration could promote cell growth and protect CEF against H9N2 AIV. We found an immunological regulatory effect of GPS, when virus was inhibited in the most effective way, and when MHC class Ⅱ and cytokines such as IL-2, IL-4 and IL-10 were at the highest levels in 321.25 μg/mL and 2500 μg/mL GPS-treated CEF.

1: at appropriate concentrations (321.25 μg/mL and 2500 μg/mL) GPS can inhibit the propagation of H9N2 AIV.

2. GPS enhanced the proliferation of CEF cells above 9.766 μg/mL concentration.

3. GPS stimulated the expressions of MHC class Ⅱ and cytokines such as IL-2, IL-4 and IL-10 after H9N2 infection.

4. GPS improved the antibody titers of GPS-treated groups (5 mg/kg, 10 mg/kg and 20 mg/kg) 7 and 14 days post H9N2 AIV infection and inactive vaccine immunization.

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