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Effects of Astragalus Polysaccharides on Associated Immune Cells and Cytokines in Immunosuppressive Dogs

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Abstract

The aim of this study was to determine the effects of Astragalus Polysaccharide (APS) on associated Immunity Cells and Cytokines in the immunosuppressive dogs and its dose-effect correlation. One hundred two-month-old male Chinese Countryside Dogs were randomly assigned to five groups: Control group (CG), immunosuppressive group (IG), APS low dose group (50 mg/kg, LDG), APS median dose group (100 mg/kg, MDG), and APS high dose group (200 mg/kg, HDG), each group with twenty animals. After successfully established the dexamethasone-induced immunosuppressive models, with intravenous administer the CG and IG groups were daily dosed with saline, and the other three groups were daily dosed with APS for 7 days. On day 4 and 11 venous blood samples were collected and analyzed to determine the percentages of peripheral blood ANAE$^+$ T lymphocytes, CD4$^+$, CD8$^+$ cells and CD4$^+$/CD8$^+$ ratio; the phagocytic index and percentage of the peritoneal macrophages; and the contents of INF-γ and IL-2. After 7 days administration, the measured parameters as described above in three treated groups increased significantly ($P<0.05$). Our findings show that the dosage of 200 mg/kg APS can significantly enhance the cellular immune level of the immunosuppressive dogs. This study has provided evidence and basis for Astragalus polysaccharides development as companion animal health products as well as for its clinical application.

Key words: Astragalus Polysaccharides; dog; immunosuppression; immune cell; Cytokine

1. Introduction

With the growing of companion dog population, canine infectious diseases occurrence has become increasingly serious, among these diseases, virus diseases are a big threat against the dog's health (13). It has been reported that the virus diseases account for 45.95% of the dog diseases in Beijing (31). Canine distemper, canine parvovirus disease, infectious canine hepatitis etc. result in the canine multisystem syndrome with the severe immunosuppression, and virus-induced non-inflammatory lymphocyte apoptosis, invade and destroy the immune system (18, 21). In recent years, the researches on immune modulation showed that Astragalus polysaccharides (APS), extracted from Astragalus membranaceus, have an extensive effect on the specific immunity and non specific immunity, such as on enhancing the immune organs index, the transformation of T lymphocytes (3, 22), the activities of the peritoneal macrophages cells, B lymphocytes, DC cells and NK cell (19), and the percentage of peripheral CD4$^+$ lymphocytes; regulating the ratio of CD4$^+$/CD8$^+$ lymphocytes (20); activating the reticuloendothelial system and the complement system (9); promoting the

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cytokine expression of IL-2, IL-3, IL-4, and INF-γ (4, 25, 26, 29); improving the immune defensive function; and resisting the invasion of the external pathogens. Astragalus polysaccharides can produce not only the direct effects on immune cells, but also the mutual regulatory effects among the nerve-endocrine-immune system network to enhance the intracellular signal transduction (11, 12), correct immune imbalance and make the immune function recover to normal level.

In this study the immunosuppressive model was established with the experimental dogs by intraperitoneal injection of dexamethasone (DEX). Three different doses from low to high levels of the Astragalus polysaccharide were intravenously injected to the dogs in comparison with the control groups to observe the influence of APS on the immune cells and the cytokines of immunosuppressive dogs.

2. Materials and Methods

2.1. Animals and Treatment

One hundred and twenty two-month-old male Chinese Countryside Dogs (body weights 2.0 ± 0.15kg) were obtained from Tongxian Dog Farm (Beijing, China). Based on the results of general examination and laboratory testing (blood), and one hundred health dogs were recruited into the study. All recruited dogs were not vaccinated and randomly assigned to 5 groups: control group (CG), immunosuppressive group (IG), APS low dose group (50 mg/kg, LDG), APS median dose group (100 mg/kg, MDG), and APS high dose group (200 mg/kg, HDG), twenty animals each group. The study was approved by the China Agricultural University (CAU) Institutional Animal Care and Use Committee. All dogs were housed in a controlled environment, with the same condition and diet regulation. Basal diet was formulated to meet the nutrient requirements contained 25.00% crude protein, 10% crude fat, 1.8% Calcium, 0.8% Total Phosphorus, 1.8% NaCl, 5000 IU/kg Vitamin E, and 50 IU/kg Vitamin A.

2.2. Agents and Drugs

Rat anti canine CD8: alexa fluor 647 Ab, the product from Ab-direct, UK, No. 0304; Rat anti canine CD4: PRE Ab, the product from Ab-direct, UK, No. 1105; Dog IFN-γ Elisa Kit, the product from Rapid Bio-Rad Co., USA, No. 07240601; Dog IL-2 Elisa Kit, the product from Rapid Bio-Rad Co., USA, No.09110602.

Dexamethasone sodium phosphate injection (DEX), 5mg/ml, was purchased from Tianjin Pharmaceutical Ltd. Co., CHINA, No. 07042211. Astragalus polysaccharide (MC018) powder injection, 100mg/g, was provided by Beijing Medicass Biotechnologies Co. Ltd. Beijing, China, No. HD006001. The content of its main components or polysaccharides was measured and determined by a standard HPLC-RID method of Chinese State Drug Administration [WS-330 (Z-029)-2001]: Analysis was performed using a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), consisting of a quaternary pump solvent management system, an on-line degasser and an autosampler. The raw data were detected by HPLC-RID method, acquired, and processed with Empower™ Software. The analytical column used is a Shodex chromatographic column (Asahipak GS series), GS-320 7E (7.6 mmID×250 mmL, particle size 9 μm, exclusion MW (Pullulan) 4×10^4) and GS-620 7G (7.6 mmID×500 mmL, particle size 9 μm, exclusion MW (Pullulan) 200×10^4) (Showa Denko, JAPAN), cascade connection. Pre-Column is GS-2G 7B (Guard Column, 7.6 mmID×50 mmL). Apparatus and chromatographic conditions: mobile phase is composed of 0.7% sodium sulfate solution; column temperature at 20°C; the flow rate of mobile phase 1.0 mL/min and detected with differential refractometer detector; adequate ASP sample were weighed precisely and dissolved in mobile phase. The sample injection volume for HPLC analysis was 100 μL, retention time 40 min.

2.3. Empirical procedures

During the whole experimental period of 11 days, the animals were examined and recorded daily for clinical symptoms. The immunosuppressive models were successfully established according to the document reported methods (2, 8, 17). Except the CG was given normal saline, the other four groups were given by intraperitoneal injection of DEX 10 mg/kg body weight, one time daily for the first continual 3 days. Then CG and IG were given normal saline and other three APS treatments were respectively given by intravenous injection of APS 50 mg/kg, 100 mg/kg, and 200 mg/kg according to their weight, one time each day for the following 7 days. At day 4 (before APS injection) and day 11, venous blood was sampled from each animal of all groups to detect the percentages of peripheral blood $ANAE^+$ T lymphocytes, $CD4^+$, $CD8^+$ cells and $CD4^+/CD8^+$ ratio, the phagocytic index and percentage of the peritoneal macrophages, and the contents of INF-γand IL-2. The experiment was terminated on day 11.

2.4. Assay of Ratio and function in Immune cells

2.4.1. ANAE$^+$ Peripheral blood lymphocytes (PBL)

Previously reported method (32) was used to count 100 PBL under BH2 biological microscope (Olympus, Japan), the percentage of ANAE$^+$ PBL = ANAE$^+$ PBL numbers / 100 PBL numbers×100%.

2.4.2. Peripheral blood CD4$^+$, CD8$^+$ lymphocytes

According to the procedures of instruction, 100 μL anticoagulated blood sample and the antibodies were added to an Eppendoff Tube, which had been treated with appropriate amount of 10% EDTA-Na_2 solution, to detect the percentage of peripheral blood CD4$^+$, CD8$^+$ lymphocytes with FACM Calibur Flow Cytometry (BD, Bioscience, USA).

2.4.3. The cytophagic index and percentage of peritoneal macrophages

The cytophagic index and percentage of peritoneal macrophages were detected with the methods described previously (1, 7, 23). Cytophagic index = the number of macrophages phagocytizing chicken erythrocytes / 200 macrophages×100%; Cytophagic percentage = the total number of phagocytized chicken erythrocytes / 200 macrophages×100%.

2.5. Assay of Serum INF-γ and IL-2 Contents

The Serum INF-γ and IL-2 contents were assayed using a commercially available Dog ELISA kit according to kit introductions. Optical densities of the INF-γ and IL-2 were read on a 680 micro plate reader (Bio-Rad Laboratories, USA) at 450 nm.

2.6. Statistical Analysis

Data obtained was performed using Statistical Package of the SPSS 12.0 software, Analysis of One-Way ANOVA procedure was used to assess the association between the different experimental groups.

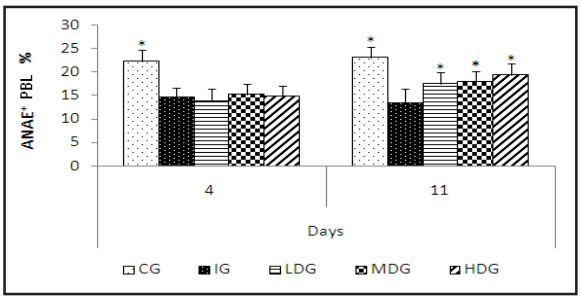
3. Results

3.1. Clinical Symptoms

After 3 days of injecting DEX, all animals with the immunosuppression showed signs of depression, lethargy, with rough clothing hair, lack of appetite, diarrhea and even leading to secondary respiratory and gastrointestinal diseases. Compared with the IG, all animals of three APS treatments had alleviation of these clinical symptoms after injection for seven days.

3.2. Percentage of peripheral blood ANAE$^+$ lymphocyte

On the 4th day, after the immunosuppressive models were successfully established, the percentage of peripheral blood ANAE$^+$ lymphocyte in CG was significantly higher ($P<0.05$) than that in other four groups. After APS injection for 7 days, on the 11th day, the values of the three APS treatments increased significantly ($P<0.05$) higher than that of IG, and close to that of CG without obvious difference (Figure 1).



Results are expressed as mean ± S.D. (n = 6). *P < 0.05 vs. IG.

Figure 1. Percentage of ANAE$^+$ PBL.

3.3. ANAE stain of peripheral blood lymphocytes.

The result of ANAE stain on 11th day (Figure 2) showed that: There were less $ANAE^+$ cells in the campus visualis of IG compared with CG, and the red blood cells deposited sparse. With the increasing dose of APS, the quantity of $ANAE^+$ cells in the campus visualis became larger, density of red blood cells increasing.

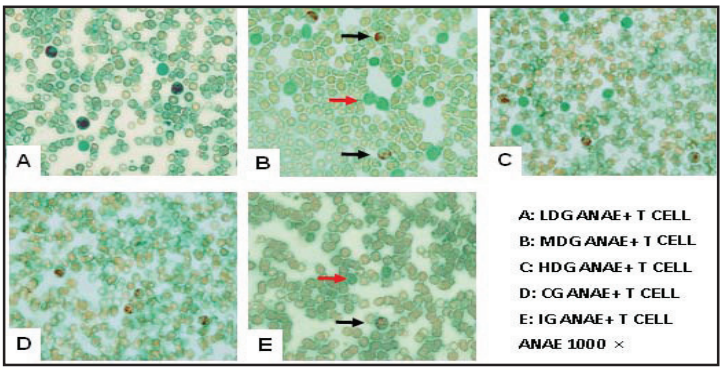


Figure 2. ANAE stain of peripheral blood lymphocytes.
The result of ANAE stain on 11th day, 1000×, under oil-Lens the nucleus of lymphocytes were stained into dark green. The dark green cells which had brown granulation were $ANAE^+$ cells, the other dark green cells were $ANAE^-$ cells, and the light green cells were red blood cells.

3.4. Percentages of peripheral blood $CD4^+$, $CD8^+$ lymphocytes

Percentage of peripheral blood $CD4^+$ lymphocytes in CG was much higher ($P<0.05$) than that in IG on the 4th day (Figure 3), After APS injection for seven days, on the 11th day, the values of the three APS treatments increased significantly ($P<0.05$) higher than that of IG. On the 4th day, percentage of peripheral blood $CD8^+$ lymphocytes in CG was much smaller than ($P<0.05$) that in IG, After APS injection for seven days, on the 11th day, the value of the three APS treatments decreased significantly ($P<0.05$) lower than that of IG, the dogs administered much higher dose of APS got a lower percentage of peripheral blood $CD8^+$ lymphocytes.

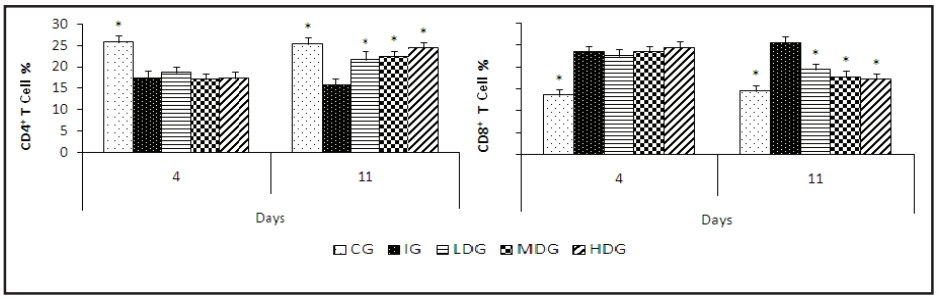


Figure 3. Percentages of peripheral blood $CD4^+$, $CD8^+$ lymphocytes.
Results are expressed as mean ± S.D. (n = 6). *P < 0.05 vs. IG.

3.5. Proportionality of peripheral blood $CD4^+/CD8^+$ cell

After APS injection for seven days, on the 11th day, the proportionality of peripheral blood $CD4^+/CD8^+$ cell below 1 in IG (Figure 4), the proportionality in three APS treatments were much closer to the CG than that in IG, the proportionality in the HDG has nearly recovered to normal level.

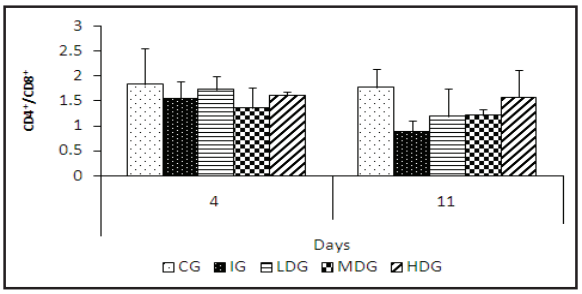


Figure 4. Proportionality of peripheral blood $CD4^+/CD8^+$ cell.
Results are expressed as mean ± S.D. (n = 6). *P < 0.05 vs. IG.

3.6. Cytophagic index and percentage of peritoneal macrophages

Cytophagic index and percentage of peritoneal macrophages in CG was much higher (P<0.05) than that in IG on the 4th day (Figure 5), there was no significant difference between other groups. After APS injection for 7 days, on the 11th day, the value of the APS treatments markedly (P<0.05) increased than that of IG, the cytophagic percentage was much closer to normal level than the cytophagic index.

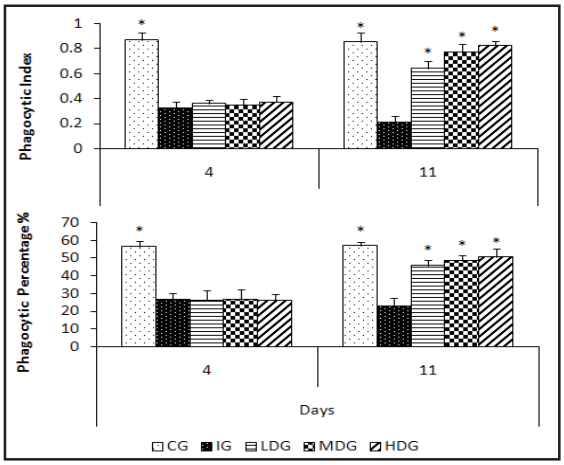


Figure 5. Effect of APS on the Phagocytic Index and Percentage of the Peritoneal Macrophage.
Results are expressed as mean ± S.D. (n = 6). *P < 0.05 vs. IG.

3.7. Contents of IL-2 and INF-γ in serum

In serum, the content of IL-2 and INF-γ in CG was significantly different (P<0.05) compared with that in IG on the 4th day (Figure 6), there were no significant difference between IG and APS groups. On 11th day, contents of IL-2 and INF-γ in three APS treatments were markedly higher (P<0.05) than that in IG, the effect of APS was dose dependent.

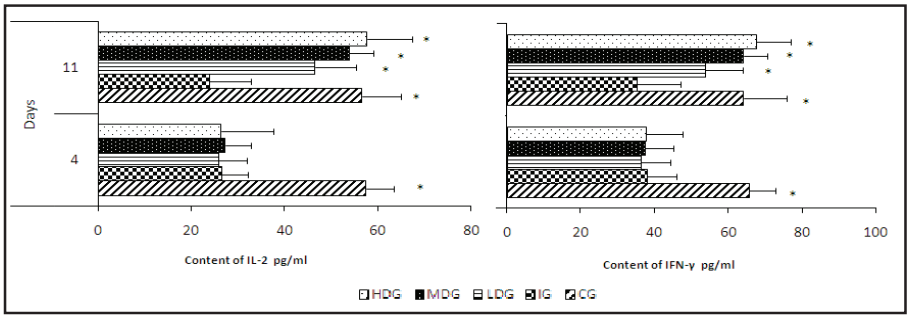


Figure 6. Contents of IL-2 and INF-γ in serum.
Results are expressed as mean ± S.D. (n = 6). *P < 0.05 vs. IG.

4. Discussion

The viruses of canine viral infectious disease invade the body and often destroy the immune system, resulting in the multiple organ dysfunction syndromes with serious immunosuppression (6, 18, 21). Dexamethasone (DEX) is an inflammatory reaction inhibitor, as an anti-inflammatory and immunosuppressive drug, it has being extensively used in autoimmune and allergic diseases (8, 15). It can inhibit the proliferation of mouse spleen cells in vitro□induce cell apoptosis□and decrease the secretion of IL-2, IL-4, INF-γ in spleen cells (17, 24). The preliminary test indicated that the dose of 10 mg/kg DEX was a reasonable dose to establish the canine immunosuppressive models. The dogs were given DEX by intraperitoneal injection 1 time daily for 3 days, the main clinical signs were observed and the immune parameters such as immune cells and cytokines were analyzed. The immunosuppressive models were successful when each immune parameter changed significantly ($P<0.05$) compared with the CG on the 4th day. And then the immune-modulating effects of different dose APS were explored on the basis of these models.

Lots of previous researches have reported that Astragalus polysaccharides can significantly promote the lymphocyte transformation and proliferation in chicken and mice in vivo and in vitro (3, 14, 16). But up to now, there is no research about the effects of Astragalus polysaccharides on the peripheral lymphocyte transformation in dogs. After the Astragalus polysaccharide administration, the percentages of peripheral $ANAE^+$ T lymphocytes in three different treatments increased significantly ($P<0.05$), and the dosage of 200 mg/kg showed the best effect, but the value from IG decreased significantly. This suggests that APS has obvious antagonistic action against DEX-induced inhibitory effect on the canine peripheral lymphocytes with a dose-dependent manner.

$CD4^+$ cells can identify the MHC class II antigens, participate in delayed hypersensitivity, assist and enlarge the effect of antibody formation and the activation of macrophages, NK and CTL cells. While $CD8^+$ cells can identify the MHC class I antigenic short peptides, specifically and directly kill the target cells, and have the cytotoxic and immunosuppressive effects (5, 10). Under normal condition, the $CD4^+/CD8^+$ ratio is about 2/1 in animal's peripheral blood, the ratio can quietly embody the cellular immune level in the clinic (27). The results showed that the $CD4^+/CD8^+$ ratio in IG and three APS groups was below 1.0 after DEX administration, that indicated TS cell subgroup hold a great proportion, and the body was in a state of immunosuppression. APS made the percentage of $CD4^+$ T lymphocytes markedly improved, but that of $CD8^+$ T lymphocytes has not obviously changed. On the 11th day, the $CD4^+/CD8^+$ ratio increased to 1.2-1.3, it may due to the increasing of TH1 cell subgroups. We supposed that APS regulated the $CD4^+/CD8^+$ ratio by increasing the number of TH1 cell subgroups and depressing the percentage of TS/TC cells, and finally certificated the immune imbalance caused by the DEX-induced immunosuppression in dogs.

The phagocytic index and percentage of the peritoneal macrophage can well reflect the animal's nonspecific immune function as well as the effect of immunoenhancer. Previous report (12, 28) found that Astragalus polysaccharides acted on mouse macrophages and strongly stimulated the gene expression of transcription factor NF-κB / Rel-dependent receptor. In our study after APS administration, the phagocytic index and percentage of the peritoneal macrophage significantly increased ($P<0.05$) in immunosuppressive dogs, and the function of macrophages has been recovered to normal level. We found that the effect of APS on peritoneal macrophage is obviously dose-dependent.

IL-2 and IFN-γ are the main cytokines that improve cell immunity. IL-2, the main proliferation factor of B and NK cells, acts on most of the T cells, and the most important function of IFN-γ is to activate macrophages and NK cells, and promote the differentiation from TH0 cells to TH1 cells (30). Results in our study showed that the contents of IL-2 and IFN-γ in serum increased significantly in APS administration groups, but decreased significantly without APS. We believe that APS can effectively promote the secretion of IL-2 and IFN-γ in serum, and antagonize the immunosuppressive effects of DEX.

5. Conclusion

In this study with the DEX-induced immunosuppressive dogs, the Astragalus polysaccharide has showed positive effects on improving the clinical signs and regulating the immune cells, cytokines and other immune-related parameters. The dosage of 200 mg/kg APS can significantly enhance the cellular immune level of the immunosuppressive dogs. These results have a great significance on research and development of companion animal health products. Astragalus Polysaccharide could be used as an immune adjuvant to regulate and to improve the immune system of the immune-suppressed dogs for different cause, keeping the dogs healthy.

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