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ORIGINAL ARTICLE

Polysaccharides from Radix Astragali Exert Immunostimulatory Effects to Attenuate the Dampness Stagnancy Due to Spleen Deficiency Syndrome

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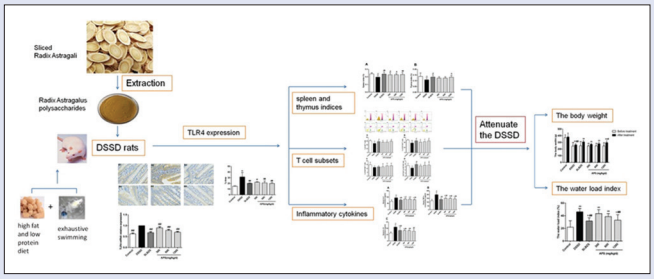
ABSTRACT

Background: Radix Astragali is an herb with tonifying qi or adaptogenic effects and commonly used for spleen-qi deficiency in traditional Chinese medicine. Astragalus polysaccharides (APS) is one of the major bioactive ingredients, and little is known on APS in attenuating the dampness stagnancy due to spleen deficiency (DSSD). **Objective:** The objective of the study is to investigate the potential mechanism of action of APS underlying attenuating water and fluid retention of rats with the DSSD syndrome in order to provide novel insights into the application of APS on modulating the DSSD syndrome. **Materials and Methods:** Four-week-old Wistar rats were fed with one high-fat and low-protein diet and subjected to exhaustive swimming to induce the DSSD model for 8 weeks. APS (300, 600, and 1200 mg/kg) and Shen Ling Bai Zhu San (SLBZS) (2.5 g/kg) were administrated starting at 9 weeks for 2 weeks. **Results:** APS increased the body weight, decreased the water load index, and attenuated symptoms of the DSSD syndrome. APS also increased spleen and thymus indices, the percentage of $CD3^+$, the percentage of $CD3^+$ $CD4^+$, and $CD3^+$ $CD4^+$ to $CD3^+$ $CD8^+$ ratio and decreased levels of serum interleukin (IL)-6, IL-10, and tumor necrosis factor-α in DSSD rats. Significant effects were seen for toll-like receptor 4 (TLR4) at both protein and gene expression levels with amelioration of expression levels in APS groups. **Conclusion:** These findings suggest that APS may be used to attenuate the fluid retention of the DSSD syndrome through the immunoregulatory effect, which is driven in part by the modulation of TLR4.

Key words: Astragalus polysaccharides, cytokine secretion, dampness stagnancy due to spleen deficiency, immunostimulatory effects, toll-like receptor 4

SUMMARY

- Radix Astragali is well known as an herbal remedy because of its tonic, diuretic, and immunostimulatory property. Epidemiological studies show many chronic diseases even the general populations are usually accompanied by the dampness stagnancy due to spleen deficiency (DSSD) syndrome, which characterized by dizziness, heavy limbs, laziness, and frequently edema. The impaired spleen-qi due to unhealthy lifestyle implicated in the pathogenesis of the DSSD. It involves in changes of T-cell subsets, inflammation cytokines levels, and toll-like receptor 4 (TLR4) expression.
- In the present study, Astragalus polysaccharides (APS), an important bioactive ingredient, extracted from Radix Astragali, was evaluated for its modulatory effect on the body weight and water load index of rats with the DSSD syndrome. APS exhibited a significant effect, including regulated T-cell subsets; decreased serum interleukin (IL)-6, IL-10, and tumor necrosis factor-α levels, and TLR4 in the tissue of the rats with the DSSD syndrome. This study revealed the potential mechanism of APS in invigorating spleen and removing dampness.



Abbreviations used: APS: Astragalus polysaccharides; DSSD: Dampness stagnancy due to spleen deficiency; SLBZS: Shen Ling Bai Zhu San; IL: Interleukin; TNF-α: Tumor necrosis factor-α; TLR4: Toll-like receptor 4; TCM: Traditional Chinese medicine; ELISA: Enzyme-linked immunosorbent assay; SD: Standard deviation; ANOVA: One-way analysis of variance.

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INTRODUCTION

Radix Astragali dried root referred to as "Huangqi" in China is an herb with tonic, diuretic, and hepatoprotective property. It affects spleen meridians and is commonly used for strengthening the host defense system. Radix Astragali is indicated for qi deficiency symptoms such as fatigue, anemia, and edema.[1-3] Radix Astragali contains multiple complicated components, including polysaccharides, flavonoids, and astragalosides.[4] Astragalus polysaccharides (APS) is identified as one of the major bioactive components, which has effects of immunomodulation, antioxidant, anti-inflammation, and antidiabetes.[5-8] In our previous study, we found that APS ameliorates conditions of the dampness stagnancy due to spleen deficiency (DSSD) syndrome.[9] However, the mechanism of APS in attenuating the symptoms of the DSSD syndrome remains unclear.

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The spleen is the hub of qi transportation and water metabolism in traditional Chinese medicine (TCM) theory. Water and food essences are extracted and distributed to the whole of the body under the action of spleen-qi.[10] Spleen-qi deficiency will lead to water and fluid retention, which called DSSD. In modern life, the DSSD syndrome is very common and characterized by gastrointestinal dysfunction, dizziness, heavy limbs, laziness, and frequently edema and is usually accompanied by chronic disease including diabetes, obesity, and Alzheimer's disease.[11-13] The DSSD syndrome is hard to be ameliorated, making disease recurrence probable.[14] However, there is no notion on the DSSD syndrome in modern medicine and no good treatment. In addition, the present majority studies focus on the food digestions and absorption dysfunction rather than water and fluid retention. It is very necessary to investigate the mechanism and treatment of the DSSD syndrome.

As recorded in Miraculous Pivot (Lingshu), defensing is one of the major functions of spleen which closely associated with its immune function in Western medicine. "A healthy spleen can keep one from evils of the four seasons" said by Zhongjing Zhang in the golden chamber synopsis. He has explicitly pointed out the significance of the spleen in the defense of the immune system. Spleen-qi deficiency could lead to the immune system disorder. For example, studies show that spleen-qi deficiency involves in low-grade inflammation characterized by increased levels of cytokines and the DSSD syndrome is closely related to inflammatory mediators including cytokines.[15,16] Therefore, one of the important etiologies of the DSSD syndrome appears to be involved in the immune dysfunction, and one kind of spleen-invigorating, qi-tonifying, and water-eliminating herb used to improve immune response process is the important way for TCM to treat the DSSD syndrome.

In this study, we extracted APS from Radix Astragali and examined its potential mechanisms of action on rats with DSSD through detecting the T-cell subsets, inflammation cytokine levels, and toll-like receptor 4 (TLR4) expression. The purpose of this study is to explore the hypothesis that APS improves immune function through TLR4 signal pathway and regulates the cytokine secretion and T-cell subsets to ameliorate the DSSD syndrome, with particular focus on water and fluid retention.

MATERIALS AND METHODS

Experimental drugs

Sliced Radix Astragali (*Astragalus membranaceus* Bunge, No. 130107) was purchased from TCM pharmacy in Jinan, China, and identified as the root of *A. membranaceus* by the Department of TCM identification, Shandong University of TCM, China. Shen Ling Bai Zhu San (SLBZS), a traditional Chinese patent medicine (China SFDA: Z14020346, product lot No. 20140704), was purchased from Shanxi Huakang Pharmaceutical Co., Ltd. in Yuncheng, China.

Main reagents

Mouse anti-rat antibodies such as CD3 FITC (No. 4233838), CD4 APC (No. B7075558), and CD8a PerCP (No. 5254871) were all purchased from BD Biosciences (San Diego, USA); erythrocyte lysis buffer was purchased from Beijing Solarbio Science and Technology Company (No. 20161220); interleukin (IL)-6 (No. CK-E30646R), IL-10 (No. CK-E30651R), and tumor necrosis factor-α (TNF-α) (No. CK-E30635R) enzyme-linked immunosorbent assay (ELISA) kits were all obtained from Shanghai Chuangxiang Biological Technology Co., Ltd (Shanghai, China); TLR4 rabbit polyclonal antibody (No. GB13187) was purchased from Wuhan Servicebio Technology Co., Ltd (Wuhan, China).

Preparation and quantitative analysis of Astragalus polysaccharides

About 2.49-kg sliced Radix Astragali was extracted in 10 volumes of boiling water for 1 h. This was done twice. Then, aqueous extracts were mixed and concentrated to 2 L. The mixed decoction was precipitated by adding 95% alcohol to a concentration of 80%. The residue was then suspended in 1 L of distilled water and alcohol precipitation again. APS (266.83 g) was finally obtained after lyophilization. The content of the APS was 56.29% which was determined by the sulfuric acid-anthrone method.

Animal and animal model

Specific pathogen-free, 4-week-old Wistar rats of both genders weighing 100–120 g were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd. in Beijing, China (License No. SCXK (Jing) 2012-0001). All procedures were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals. Rats were kept in individual ventilation cages with controlled temperature of 23°C ± 2°C and humidity of 50%–70% and allowed free access to food and water. Rats were induced the DSSD syndrome according to the method previously reported.[9] Rats were fed with a high-fat and low-protein diet. Meanwhile, rats were subjected to exhaustive swimming with a load until their noses were under water for 10 s once a day in the afternoon. Rats in normal control group were fed with AIN-76A diet. The time of model building lasted for 8 weeks.

Experimental protocol

Model rats were randomly divided into the DSSD group, SLBZS group (2.5 g/kg), and APS (300 mg/kg, 600 mg/kg, and 1200 mg/kg) group, 8 rats in each group. SLBZS and APS were administered to the respective groups once a day for 2 weeks by gavage, 1 ml/100 g. The low dose of APS and the dose of SLBZS were equivalent to six times of the human adult dose to adjust for the 5–10-fold increased metabolism that exists in rodents compared to humans. Equal volume of normal saline was fed to rats in both the DSSD and control groups. At the end of intragastric administration, 500-μl blood sample was collected into BD Vacutainer tube containing EDTA-K2 which is used for flow cytometry, and the rest of blood was centrifuged to separate serum samples. Rats were euthanized, and the duodenum was immediately dissected, frozen in liquid nitrogen, and stored in −80°C for quantitative real-time polymerase chain reaction (qRT-PCR) assay.

Water load index

The water load index was measured based on the method of the previous reference.[9] The body weight was weighed after fasting for 12 h, as a control value. Then, normal saline in the volume equal to 10% of the body weight was administered into rats by intraperitoneal injection. Rats were put into the metabolic cage, and the body weight was measured again after 0, 1, 2, 4, and 6 h. During this time, food and water were banned. Ratio of the body weight loss (%) = (the body weight after water load − body weight before water load)/body weight before water load × 100. Water load chart between ratio of the body weight loss and time was drawn; water load index was area under curve of 6 h water load.

Thymus and spleen indices

The body weights of rats were evaluated before being sacrificed. The thymus and spleen were immediately removed, dried, and weighed. Thymus and spleen indices were calculated as follows: Thymus/spleen index (%) = (Thymus/spleen weight in grams/body weight in grams) ×100.

Flow cytometry

$CD4^+$ and $CD8^+$ T-lymphocytes in peripheral blood were determined by flow cytometry, following manufacturer's instructions. About 100 μl of EDTA-K2 anticoagulative blood samples were immunostained with mouse anti-rat CD3-FITC, CD4-APC, and CD8a-PerCP in the dark for 15 min at room temperature. Then, erythrocyte lysis buffer was added to lytic red cell and centrifuged for 5 min at 1200 g. Supernatants were decanted, cells were collected and resuspended in 500 μl phosphate-buffered saline (PBS) buffer, and then detected by the flow cytometer (Beckman coulter, CA, USA). Data were analyzed using CytExpert software (Beckman Coulter, USA).

Enzyme-linked immunosorbent assay

The serum samples were obtained after centrifugation for ELISA analysis following the instructions, and levels of IL-6, IL-10, and TNF-α were detected. The optical density values from the samples were measured with a microplate reader at the 450-nm wavelength.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from frozen duodenum tissues using the TRIzol reagent (Invitrogen, USA). cDNA was synthesized by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The primers used in this study are as follows: TLR4: forward 5'-TGGCATCATCTTCATTGTCC-3' and reverse 5'-CAGAGCATTGTCCTCCC-3'; GAPDH: forward 5'-TTCCTACCCCCAATGTATCCG-3' and reverse 5'-CATGAGGTCCACCACCCTGTT-3'. GAPDH was used as the internal control. Real-time PCR was performed on 7500 FAST Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and SYBR Green Master Mix for 40 cycles. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry staining

Duodenum tissues were fixed in 4% (w/v) paraformaldehyde (pH 7.4) and embedded in paraffin. The 3-μm tissue sections were dewaxed and placed into water. According to the kit instructions, TLR4 antibody (1:200) was added and incubated at 4°C overnight. Subsequently, the horseradish peroxidase-coupled secondary antibody was added and incubated for 50 min at room temperature. The sections were washed with PBS, developed with diaminobenzidine, and finally, counterstained with hematoxylin and dehydration and mounted. Semi-quantitative analysis of per stained area was performed using Image J software. All results were repeated at least three times, and the representative images were presented.

Statistical analysis

Data are presented as mean ± standard deviation. Mean comparison in groups was conducted with one-way analysis of variance test, and the pairwise comparison was performed with least significant difference (LSD) method using SPSS version 21.0 (IBM Corp, Armonk, NY, USA). All statistical tests were two-sided, with $P < 0.05$ considered as statistically significant.

RESULTS

Effects of Astragalus polysaccharides on the body weight and the water load index

As shown in Figure 1a, the body weight was significant reduced in the DSSD rats compared with normal rats ($P < 0.01$). Compared with the DSSD group, APS and SLBZS increased the body weight of the DSSD rats ($P < 0.01$ in SLBZS and $P < 0.05$ in 1200 mg/kg APS). The water load index in the DSSD group was significant increased compared with the control group ($P < 0.01$) and APS and SLBZS decreased the water load index when compared with the DSSD group ($P < 0.01$ in 1200-mg/kg APS and SLBZS) [Figure 1b].

Effects of Astragalus polysaccharides on spleen and thymus indices

Spleen and thymus indices were significantly decreased in the DSSD group compared with the control group ($P < 0.01$). APS increased the spleen index ($P < 0.01$ in 1200 mg/kg and $P < 0.05$ both in 300 and 600 mg/kg) and thymus index ($P < 0.05$ in 1200 mg/kg) [Figure 2]. SLBZS had similar effect on rats with DSSD syndrome.

Effects of Astragalus polysaccharides on T-cell subsets in the peripheral blood

T-cell sets changes were shown in Figure 3. The results in Figure 3a, b, and d showed that the percentage of $CD3^+$, the percentage of $CD3^+$ $CD4^+$ T-cells, and $CD3^+$ $CD4^+$ to $CD3^+$ $CD8^+$ ratio were decreased in the DSSD group when compared with the control group ($P < 0.01$ and $P < 0.05$); APS was found to increase the percentage of $CD3^+$, the percentage of $CD3^+$ $CD4^+$ T-cells ($P < 0.05$ in 1200 mg/kg), and $CD3^+$ $CD4^+$ to $CD3^+$ $CD8^+$ ratio ($P < 0.05$ both in 300 and 600 mg/kg).

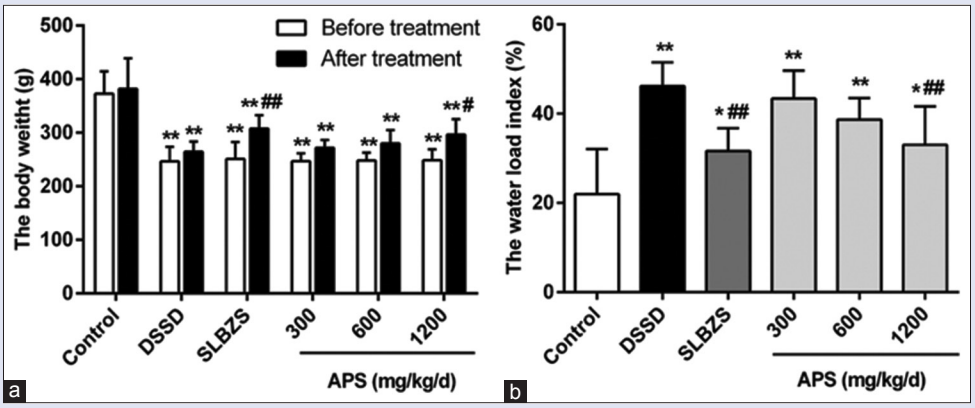

Figure 1: Effects of Astragalus polysaccharides on the body weight and the water load index in dampness stagnancy due to spleen deficiency rats. The dampness stagnancy due to spleen deficiency rats was treated with Astragalus polysaccharides for 14 days, and the body weight and the water load index were tested (a and b, respectively). Data are expressed as mean ± standard deviation for eight animals in each group, $^*P < 0.05$, $^{**}P < 0.01$ between the control group and other groups, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between dampness stagnancy due to spleen deficiency group and drug-treated groups

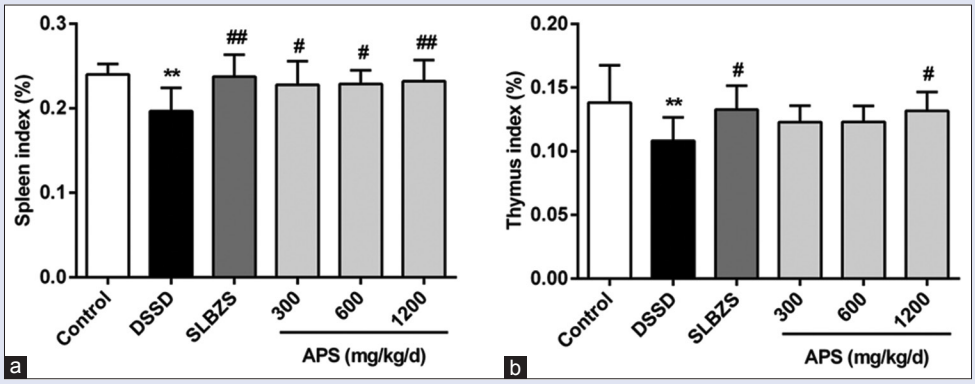

Figure 2: Effects of Astragalus polysaccharides on the organ index in dampness stagnancy due to spleen deficiency rats. The dampness stagnancy due to spleen deficiency rats was treated with Astragalus polysaccharides for 14 days, and the spleen index and the thymus index were tested (a and b respectively). Statistical information is the same as Figure 1

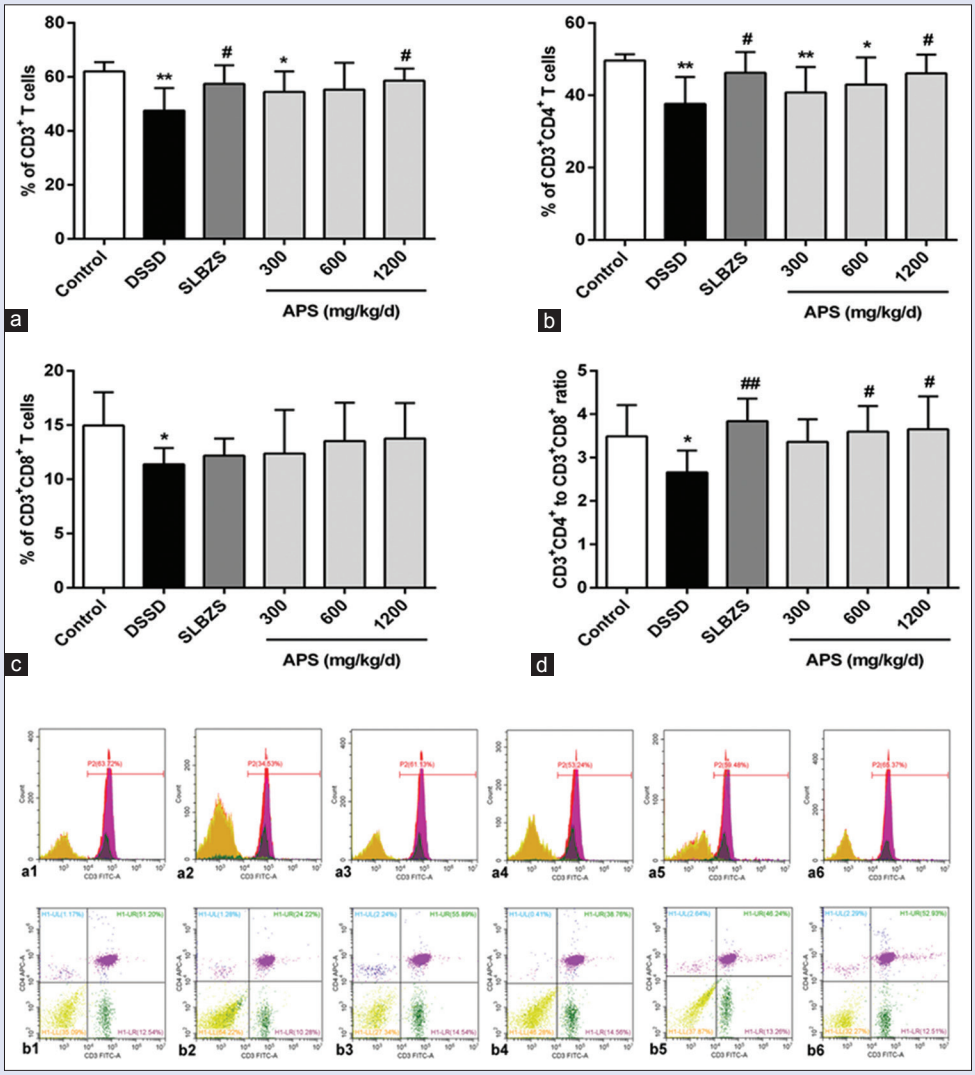

Figure 3: Effects of Astragalus polysaccharides on the T-cell subsets. Dampness stagnancy due to spleen deficiency rats was treated with Astragalus polysaccharides for 14 days. The percentage of $CD4^+$ T-cells, $CD3^+$ $CD4^+$ T-cells, and $CD3^+$ $CD8^+$ T-cells and $CD3^+$ $CD4^+$ to $CD3^+$ $CD8^+$ ratio were determined by flow cytometry (a, b, c, and d, respectively). Images are shown in the control group (a1 and b1), dampness stagnancy due to spleen deficiency group (a2 and b2), Shen Ling Bai Zhu San group (a3 and b3), and Astragalus polysaccharides group (a4-a6 and b4-b6). Data are expressed as mean ± standard deviation for six animals in each group, $^*P < 0.05$, $^{**}P < 0.01$ between the control group and other groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between the dampness stagnancy due to spleen deficiency group and drug-treated group

The percentage of $CD3^+$ $CD8^+$ was also reduced in the DSSD group when compared with the control group ($P < 0.05$) [Figure 3c], and APS increased the percentage of $CD3^+$ $CD8^+$ ($P > 0.05$). SLBZS also showed modulation effect of these alterations.

Effects of Astragalus polysaccharides on levels of serum inflammatory cytokines

The results in Figure 4 showed that higher levels of IL-6, IL-10, and TNF-α were observed in the DSSD group, compared with the control group ($P < 0.01$). Compared with the DSSD group, APS obviously decreased the levels of IL-6 ($P < 0.05$ in 300 and 600 mg/kg groups and $P < 0.01$ in 1200 mg/kg group), TNF-α ($P < 0.01$ in 300, 600, and 1200 mg/kg groups), and IL-10 ($P < 0.01$ in 300, 600, and 1200 mg/kg groups). SLBZS also resulted in significant attenuation of these alterations.

Effects of Astragalus polysaccharides on the toll-like receptor 4 expression

Figure 5a showed that TLR4 in DSSD rats was activated by the increased messenger RNA expression ($P < 0.01$) and APS reduced its expression ($P < 0.01$ in 300, 600, and 1200 mg/kg groups). On immunohistochemical examination, TLR4 proteins were found to be expressed in epithelial cells and inflammatory cells in the small intestine; compared with the DSSD group, APS decreased the percentage of the positive stain area ($P < 0.05$ in 300 mg/kg group and $P < 0.01$ in 600, 1200 mg/kg groups) [Figure 5b]. SLBZS also showed similar effects on rats with the DSSD syndrome.

DISCUSSION

The DSSD syndrome is a common yet poorly understood syndrome. Spleen-qi deficiency and spleen failing to transform and transport induced by overfatigue, poor diet, and emotional disorders are the most important reasons of the DSSD syndrome based on the TCM theory.[17] SLBZS, a famous classical formula originally described in "Tai Ping Hui Min Heji Ju Fang," was widely used to treat the DSSD syndrome in clinical practice because of its significant functions in tonifying spleen-qi.[18,19] However, there is still no optimal single herb or component indicated for the DSSD syndrome. In previous studies, results showed that Radix Astragali can protect against the DSSD syndrome and APS as one of significant components produced better effects than others,[9,20,21] but the mechanism of action of APS treatment for the DSSD syndrome is not fully understood yet.

In this study, we found that APS could attenuate the symptoms of the DSSD syndrome such as weakness, slow movement, weight loss, and fluid retention. Meanwhile, APS increased the spleen and thymus indices and corrected $CD4^+$ and $CD8^+$ T-lymphocyte imbalance. The effects were positively related to the dosage of application, and SLBZS also had a similar effect on rats with DSSD syndrome. A growing body of research shows that APS possesses the excellent immunopotentiatory property, including regulating T-cell immunity, promoting dendritic cells (DC) maturation, and inhibiting LPS and palmitate-induced inflammation.[8,22-24] Previous studies also showed that similar related results. For example, the percentage of $CD4^+$ and $CD4^+/CD8^+$ decreased in rats with spleen-qi deficiency, and APS promotes the humoral immunity by increasing C3 and IgG levels in rats with the DSSD syndrome.[25,26] Therefore, our results indicated that enhancing immune system is one of the important ways for APS to alleviate symptoms. This also potentially indicated that APS has the efficacy of Radix Astragali in invigorating spleen for diuresis.

We also examined the inflammatory cytokine levels in serum. The results showed that APS decreased levels of serum IL-6, TNF-α, and IL-10 in rats with the DSSD syndrome. The high dosage of APS worked better.

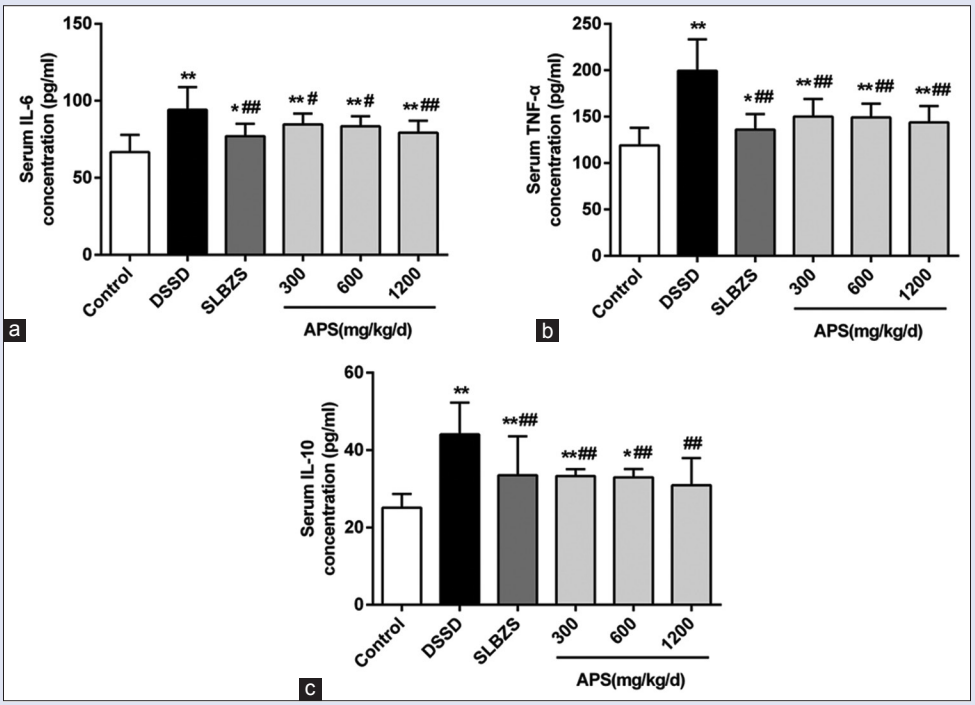

Figure 4: Effects of Astragalus polysaccharides on serum cytokine levels in dampness stagnancy due to spleen deficiency rats. Rats were treated with Astragalus polysaccharides for 14 days, and the serum IL-6, tumor necrosis factor-α, and IL-10 were measured (a, b, and c, respectively). Data are expressed as mean ± standard deviation for eight animals in each group. $^*P < 0.05$, $^{**}P < 0.01$ between the control group and other groups, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between the dampness stagnancy due to spleen deficiency group and drug-treated group

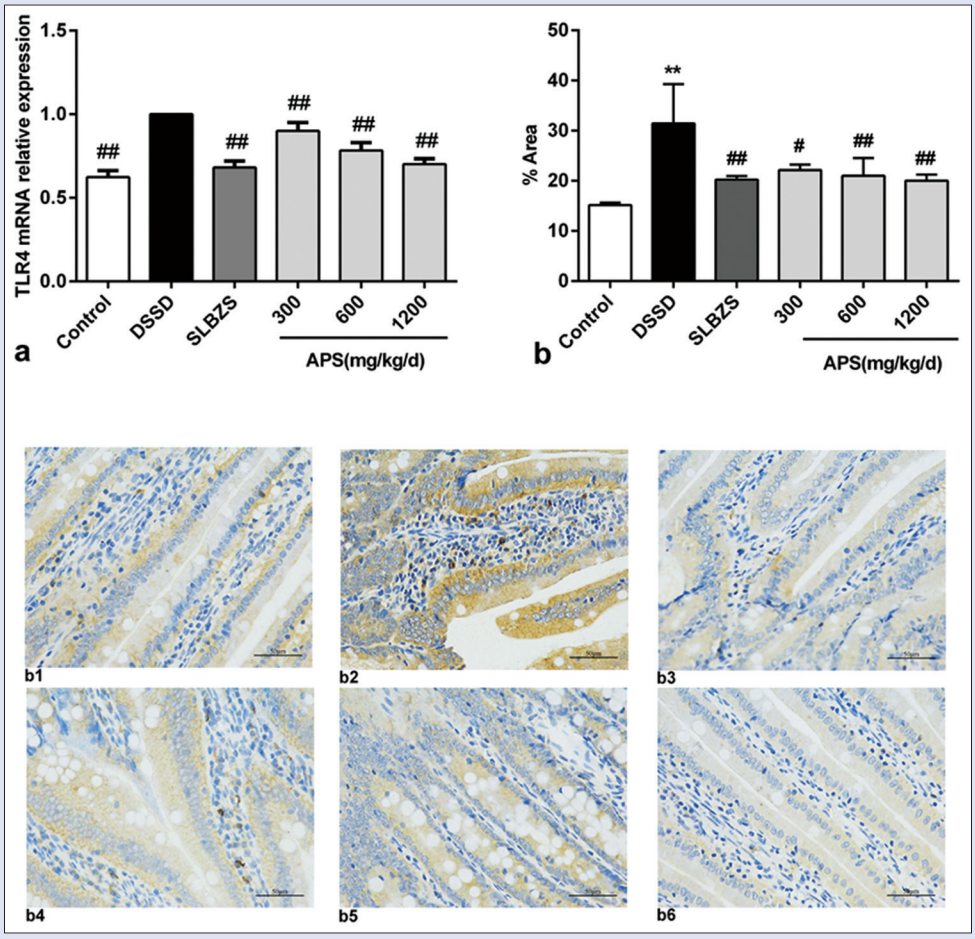

Figure 5: Effects of Astragalus polysaccharides on the toll-like receptor 4 expression. Dampness stagnancy due to spleen deficiency rats was treated with Astragalus polysaccharides for 14 days, and toll-like receptor 4 expression was determined by quantitative real-time polymerase chain reaction and immunohistochemistry (a and b, respectively). The representative images are showed in the control group (b1), dampness stagnancy due to spleen deficiency group (b2), Shen Ling Bai Zhu San group (b3), and Astragalus polysaccharides groups (b4-b6). Data are expressed as mean ± standard deviation for three animals in each group, $^{\#\#}P < 0.01$ the control group and Astragalus polysaccharides-treated group compared with the dampness stagnancy due to spleen deficiency group (a); $^{*}P < 0.05$, $^{**}P < 0.01$ between the control and the dampness stagnancy due to spleen deficiency group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between the dampness stagnancy due to spleen deficiency group and drug-treated group (b)

The essential roles of IL-6, TNF-α, and IL-10 in inflammation reaction have been widely recognized. IL-6 and TNF-α act as pro-inflammatory cytokines can evaluate the inflammation.[27-29] IL-10 exerts immunosuppression through inhibiting secretion of pro-inflammatory cytokine from immune cells and decreasing the function of APC.[30,31] Our results suggested that spleen deficiency led to inflammatory factor level imbalance. In turn, the cytokines disorder is one characteristic of the DSSD syndrome. A great number of studies report that spleen deficiency exists together with inflammatory cytokine disorder.[10,32,33] Therefore, we speculate that APS ameliorated the DSSD symptom through regulating inflammatory cytokine disorder rather than damage or loss of function of the infiltrated tissue.

We further detected TLR4 expression using qRT-PCR and immunohistochemistry staining methods. The results found TLR4 expression increased in DSSD rats and APS decreased its expression. TLR4, a key component of the innate immune response, is particularly implicated in immune dysfunction.[34] Bacterial LPS and food productions such as short-chain fatty acids trigger intestinal TLR4 response and convey signals to produce inflammatory cytokines, such as IL-6, TNF-α, help DC mature, and modulate the activation of T-cells.[35,36] Our results indicated that immune disorder including T-cell sets and cytokine imbalance was involved in activation of TLR4 signaling under the condition of the DSSD syndrome. In addition, TLR4 expression was coincident with the production of IL-6, TNF-α, and IL-10. These results are similar with the reports that the DSSD syndrome is associated with intestinal environment disorder such as gut flora imbalance and food digestive dysfunction, which trigger TLR4 pathway.[37] Therefore, APS may regulate T-cell-mediated immunity system through TLR4 pathway, enhancing spleen-qi and eliminating fluid retention.

CONCLUSION

Taken together, our results provide insight into a molecular mechanism for the efficacy of APS in ameliorating the DSSD syndrome, enhancing immunity function through regulating inflammatory cytokine, and interfering with TLR4-dependent signaling, which can be effective against the DSSD syndrome. Further study is needed for the detail of the TLR4 regulation mechanism.

Acknowledgements

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Nil.

Conflicts of interest

There are no conflicts of interest.

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