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Regulation of non-classical immune parameters in immune thrombocytopenic purpura mice by a spleen-invigorating, qi-replenishing and blood-containing formula

Tiantian Li ^a, Hao He ^b, Li Hou ^a, Yue Xu ^b, Xiaoyong Wu ^c, Yanping Sun ^b, Lei Zheng ^b, Zhixiong Chen ^d, Xinyi Chen ^{a,*}, Bei Qin ^{b,*}

^a Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing 100700, China

^b School of Pharmacy, Xi'an Medical University, Shanxi 710021, China

^c No. 1 Hospital Affiliated to Guiyang University of TCM, GuiYang 550001, China

^d No. 1 Hospital Affiliated to Guangzhou University of Chinese Medicine, Guangzhou 510405, China

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KEYWORDS

Spleen-invigorating, qi-replenishing and blood-containing formula (SQBF); β -endorphin (β -EP); Vasoactive intestinal peptide (VIP); Salivary IgA (SIgA)

Abstract *Objective:* This study investigated the regulatory effect of non-classical immune parameters on immune thrombocytopenic purpura (ITP) mice by a spleen-invigorating, qi-replenishing and blood-containing formula (SQBF).

Method: A total of 80 BALB/c mice were randomly divided into four equal groups (20 mice each): control group, model group, prednisone group and spleen-invigorating, qi-replenishing and blood-containing (SQBF) group. Mice in the model group, prednisone group, and SQBF group were administered anti-platelet serum to induce ITP. The dynamic variations of platelet counts in ITP mice were measured with an automatic blood analyzer before modeling and 48 h, and 8, 12 and 15 days following APS injection. Levels of β -endorphin (β -EP), vasoactive intestinal peptide (VIP) and salivary IgA (SIgA) were detected by enzyme-linked immunosorbent assay (ELISA) on 15th day of experiment.

Results: SQBF enhanced peripheral blood platelet counts in ITP mice similar to that of prednisone, and both groups showed a statistically significant response compared with the model group ($P < .01$). The SQBF significantly decreased β -EP levels compared with the model and prednisone intervention groups ($P < .05$), significantly increased the levels of VIP and SIgA in

* Corresponding authors.

E-mail address: chenxinyisci@126.com (X. Chen).

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ITP mice compared with the model group ($P < .05$) and had significant protective effects on the thymus of ITP mice compared with the model group ($P < .01$).

Conclusions: The SQBF had a similar effect to prednisone with regards to enhancing peripheral blood platelet counts in ITP mice. Furthermore, it decreased β -EP levels and increased VIP and SIgA, and protected the thymus. This shows that, on base of the brain-gut axis functions, some non-classical immune vascular active factors or neurotransmitters are also involved in immune responses, and also have relationship with the onset of ITP and bleeding and/or hemostasis. It needs further study to determine whether a change in these active factors is related to immediate hemostasis.

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Introduction

The pathogenesis of immune thrombocytopenic purpura (ITP) is generally believed to be caused by abnormalities of immune cells and humoral immunity that lead to the generation of antibodies to platelets (PAIgG, PAIgM). These bind to platelet membrane glycoproteins, causing the destruction of platelets in the reticuloendothelial system. This causes a decrease in platelet numbers, a shortening of the platelet life span and change in platelet function.^{1–3} Currently, adrenocortical hormones such as prednisolone, prednisone, dexamethasone and hydrocortisone⁴ represent the first-line therapy for ITP. Shortly after receiving standardized treatment, the platelet count of some ITP patients increases significantly, with a significant improvement in bleeding tendency. However, the long-term and repeated use of adrenocortical hormones causes adverse drug reactions, including central obesity, hyperglycemia, edema, hypertension and bone necrosis. Furthermore, hormone dependence and ineffectiveness may also develop, reducing the clinical therapeutic effects of adrenocortical hormones.⁵ Therefore, we aimed to identify a single drug or combination of drugs from conventional Chinese herbal medicine that might provide clinical benefit to patients in the place of hormone therapy. A prospective central randomized controlled clinical trial (register ID: ChiCTR-TRC-13003602) reported that a spleen-invigorating, qi-replenishing and blood-containing formula (SQBF) might be an alternative treatment for ITP because it had efficacy in treating chronic ITP-afflicted patients with hormone dependence and ineffectiveness. The main therapeutic effects of SQBF were symptom relief, rapidly mitigating bleeding tendency and slowly increasing platelet counts. This study investigated whether the therapeutic effects of this formula were related to immune regulation.

Materials

Animals

Fifty guinea pigs weighing 250 g (25 males and 25 females) were purchased from the Tian Rui Experimental Animal Farm in Xing Ping, Shanxi Province, China [License number: SCXK (Shan Xi) 2012-001; common breeding environment].

Eighty BALB/c mice weighting 18–22 g (40 males and 40 females) were purchased from the Laboratory Animal Center of the Fourth Military Medical University [License number: SCXK (Shan Xi) 2014-002; specific pathogen free breeding].

Drugs

SQBF

SQBF consists of Radix Astragali Preparata (*Radix Astragali seu Hedysari Praeparatae*), Salviae miltiorrhizae (*Radix Codonopsis pilosulae*), Poria (*Poria*), Largehead Atractylodes Rhizome (*Rhizoma Atractylodis Macrocephalae*), Donkey-hide Gelatin (*Colla Corii Asini*) and Radix Rubiae (*Radix Rubiae*), prepared and provided by Xi'an Xinghua Drug Research Institute based on the pharmaceutical standards for new drugs. Drug preparation: the water extraction process was adopted, and the preparation process was as follows: Donkey-hide Gelatin (*Colla Corii Asini*) was ground into fine powder, and screened filtered through an 80-mesh sieve. Other drugs were soaked in water overnight, and decocted twice. First, eight cups (400 mL/cup) of cold water were added to the herbs in SQBF except Donkey-hide Gelatin (*Colla Corii Asini*) and boiled 2 h. The liquid was strained and kept as the decoction. Then six cups of water were added to the previously cooked herbs and they were boiled for 2 h. The two decoctions were subsequently blended, condensed into a thick paste without dregs, dried at 80°C to be powder which was screened through an 80-mesh sieve. The final medication was obtained by blending this powder and Donkey-hide Gelatin (*Colla Corii Asini*). Quality standards are according to the classification and reporting requirements for Chinese medicine, for natural medicines in the "drug registration management method". Radix Astragali Preparata (*Radix Astragali seu Hedysari Praeparatae*) was conducted using the thin-layer chromatography TLC identification method and High Performance Liquid Chromatography Evaporative Light Scattering Detector (HPLC-ELSD) methods, respectively. Results of thin-layer chromatography showed that the thin layer identification of Radix Astragali Preparata (*Radix Astragali seu Hedysari Praeparatae*) was consistent with the standards under the category of Radix Astragali Preparata (*Radix Astragali seu Hedysari Praeparatae*) in the *Chinese Pharmacopoeia 2010 edition*. Integral precision

test of astragaloside IV showed that relative standard deviation (RSD) of integral value of the peak area was 1.6%; the repeatability test showed that average content of that was 0.1224 mg/g, and the RSD was 3.14%; the sample recovery test showed that the average recovery rate of astragaloside IV was 94.90% and RSD was 2.19%. The results of three testing methods were found to be consistent with the content standards under the *Radix Astragali Preparata (Radix Astragali seu Hedysari Praeparatae)* in the *Chinese Pharmacopoeia 2010 edition*.

Prednisone

Prednisone was purchased from Shanghai Jingchun Scientific Co. Ltd. and dissolved in saline at a concentration of 1 mg/mL.

Reagents

Enzyme-linked immunosorbent assay (ELISA) kits for mouse SIgA, β -EP, and VIP were purchased from the Wuhan Gene Biotech Co. Ltd. Complete Freund's adjuvant and incomplete Freund's adjuvant were obtained from Sigma-Aldrich (St. Louis, Missouri, United States).

Apparatus

Electronic analytical balance (Satorius BSA224S, Germany); high-speed micro-centrifuge (Chang Sha Xiang Yi H1650-W, China); high-speed freezing centrifuge (Eppendorf 5804R, Germany); automatic animal blood analyzer (Perlong XFA6130, China); ultra-purified water system (Millipore Synergy UV, Germany); remote infrared thermometer (NC-9900, China); electronic balance; and timer.

Methods

Preparation of anti-platelet serum

Anti-coagulated whole blood from anesthetized BALB/c mice was subject to gradient centrifugation to obtain platelets, washed twice with phosphate buffered saline (PBS), resuspended in PBS, and counted. The concentration was then adjusted to 2.5×10^6 /mL. Platelets (the antigen) were evenly mixed with an equal amount of complete Freund's adjuvant or incomplete Freund's adjuvant. Complete Freund's adjuvant plus antigen was injected at week 0 into the paws of guinea pigs, back and subcutaneous areas (five areas), for a total injection volume of 1 mL. Incomplete Freund's adjuvant plus antigen was injected into the same areas and points at weeks 1, 2 and 4. Serum taken from guinea pig heart was centrifuged to obtain anti-platelet serum⁶ (GP-APS). GP-APS was then placed in a water bath at 56°C for 30 min to inactivate complement and allow erythrocyte adsorption. It was diluted with saline at a ratio of 1:4.

Measurement of GP-APS titer

Mouse platelets obtained by centrifugation were washed three times with PBS, and the concentration was adjusted

to 6×10^4 / μ L. Then, 50- μ L platelet suspension was added to microtiter wells and centrifuged for 13 min at $560 \times g$. Buffer (0.2% gelatin, 0.1% sodium azide, 0.05% polysorbate, 0.15 mol/L PBS) was added, incubated overnight at 4°C and washed. Next, 50- μ L test serum was added and the mixture was incubated for 1 h at 37°C and washed. Then, 100- μ L enzyme-linked A protein was added, incubated for 45 min at 22°C, and washed four times. 200 μ L PNPP ($C_6H_4NNa_2O_6P$) solution (PNPP dissolved in diethanolamine buffer, 1.5 mg/mL) was added and incubated for 30 min at 37°C. Finally, 50- μ L sodium hydroxide was added to terminate the reaction. The optical density (OD) was measured with a microplate reader at 405 nm wavelength.

Determination of experimental drug dose

Based on the Principle of Medical Experimental Animal Science, the dose (mg/kg) of drug used in mice = body surface area conversion factor \times human dose. The animal surface area was measured by the Meeh-Rubner equation: body surface area $m^2 = 9.1 \times$ body weight (g) $2 / (3 \times 1000)$. The body surface area conversion factor for mouse to human was 9.01. Referring to the human dosage of prednisone and the SQBF, the effective dosage of prednisone acetate was determined to be 1 mg/mL (20 times the clinical dose) concentration in this study. The effective dosage of the spleen-invigorating, qi-replenishing and blood-containing formula was calculated and determined by referring to the clinical dosage.

Modeling grouping

The number of platelets in blood taken from the tail vein of BALB/c mice was measured by an automatic animal blood analyzer. Eighty BALB/c mice were randomly divided into four equal groups (20 mice each): control group, model group, prednisone group and SQBF group. Mice in the blank control group were given an intraperitoneal injection of 100 μ L/20 g saline; mice in other groups were repeatedly given an intraperitoneal injection of 100 μ L/20 g GP-APS daily until the end of the experiment. On the eighth day following GP-APS injection, oral dose in mice was proceeded (0.2 mL per 10 g body weight). Mice in the blank control group were given an intraperitoneal injection of saline only.

Detection of peripheral blood platelets

The blood platelet count of blood from the tail vein of mice was measured by an automatic animal blood analyzer. The results were recorded and statistically processed. The time points for detection were before APS injection, 48 h after APS injection, and Days 8, 12 and 15 after starting the administration of APS injection.

Detection of immune parameters in mouse serum

After treatment, whole blood from anesthetized mice was centrifuged after standing. Serum was obtained. The levels of SIgA, β -EP, and VIP were measured by ELISA according to the manufacturer's instructions. A standard curve was

drawn and a regression equation was established according to the concentration of the standard and corresponding OD value provided in the kit. The corresponding sample concentration was calculated by the regression equation according to the OD values of samples. The results were recorded and statistically processed.

Detection of viscera indexes

Two days after the final administration of drugs, the body weight of mice was measured. Then spleen and thymus were removed and weighed. The viscera indexes were calculated by the following formula: viscera index = weight of viscera (mg)/body weight (g).

Statistical methods

The experimental results were expressed as the mean \pm standard deviation (SD) and comparisons between groups were conducted using ANOVA. Pairwise comparisons were conducted by t-test. The *P* value of 0.05 was considered statistically significant.

Results

Mouse anti-platelet serum titers

A titer assay was performed before the establishment of the ITP mouse model. The OD value in the APS serum group was twice that in the control group (Table 1).

Peripheral blood platelet counts

The peripheral blood platelet counts of mice in different groups are shown in Table 2.

Data from Table 2 indicates that before APS injection, there was no significant difference in platelet counts between the different groups (*P* > .05). And 48 h after APS injection, the platelet counts of mice in the model group, prednisone group and spleen-invigoration group were significantly decreased compared with the control group (*P* < .05). Between Day 8 and Day 12 after APS injection, the platelet counts in the model group, prednisone group and spleen-invigoration group were significantly decreased (the lowest point in the study) compared with the blank control group (*P* < .01). On Day 15 (the 8th Day after the administration of prednisone) following APS injection, platelet counts in the model group remained at a low level (*P* < .01) compared with the control group while the platelet count in the prednisone group and spleen-invigoration group increased significantly compared with the model group (*P* < .01).

Table 1 OD values of the APS serum titer assay.

Grouping	OD value (405 nm)
APS serum group	0.300 \pm 0.037*
Control group	0.163 \pm 0.012

Compared with the control group, **P* < .05.

Detection of immune parameters

The serum of mice in each group was collected after the experiment ended. Levels of SIgA, β -EP and VIP are shown in Table 3 and Fig. 1.

Table 3 and Fig. 1 show that β -EP was significantly increased in the model group compared with the control group (*P* < .05), but there was no difference in levels between the model control group and prednisone group (*P* > .05). However, β -EP levels were significantly decreased after intervention with SQBF. VIP levels were reduced compared with the blank control group (*P* < .05), and were significantly increased after prednisone or the SQBF (*P* < .05) compared with the model group. Compared with the blank control group, the levels of SIgA were significantly increased after prednisone intervention or SQBF (*P* < .05).

Thymus and spleen indexes

At the end of the experiment, mice were weighed and their spleen and thymus indexes calculated (Table 4, Figs. 2–4).

Table 4 and Figs. 2–4 indicate that the spleen and thymus weights in the model group were significantly enlarged by immune responses following APS injection compared with the control group (*P* < .05). After prednisone intervention, the spleen and thymus were significantly reduced in size compared with the model group (*P* < .01). After intervention with SQBF, there was a significant difference in thymus size (*P* < .01), but not in spleen size, compared with the model group (*P* > .05).

Discussion

Treatment and mechanism of ITP

ITP-treatment aims to increase the platelet count in the peripheral blood of patients to mitigate bleeding. To promptly control bleeding and reduce the risk of death, high-dose adrenocortical hormone, immunoglobulins and infusion of platelets are usually employed as the first-line therapy for patients suffering from severe bleeding or who are at risk of severe bleeding.⁷ For chronic ITP patients, maintenance treatment using adrenocortical hormones is one of the most effective therapies. A therapeutic effect can be achieved in some patients although complications related to treatment need to be addressed. For those ITP patients who develop resistance to or are dependent on first-line therapy, second-line therapy is usually recommended involving the use of the following medications: gonadotropin inhibitors (danazol), thrombopoietin, cyclosporin A, azathioprine, vincristine, vinblastine, and rituximab. These are effective but induce side effects including thrombosis, hematologic suppression, and organ lesions.^{8–10} Therefore, the auxiliary or combined use of Chinese herbal medicines might enhance the clinical therapeutic effect of the medications mentioned above, and help to relieve or reduce toxicity. In China, the treatment of ITP by integrated therapy of both traditional Chinese medicine and Western medicine has already become the best therapeutic protocol available. For patients that are

Table 2 Variations of platelet counts from peripheral blood of mice ($\times 10^9$).

APS injection time	Control group	Model group	Prednisone group	Spleen-invigoration group
Before treatment	401.17 \pm 162.54	345.50 \pm 36.46	297.00 \pm 80.95	335.17 \pm 39.15
48 h	408.00 \pm 110.39	279.33 \pm 70.69*	214.33 \pm 21.70*	156.50 \pm 95.23*
8th day	371.17 \pm 77.17	179.50 \pm 21.06 [▲]	169.83 \pm 37.99 [▲]	160.17 \pm 59.52 [▲]
12th day	436.50 \pm 60.13	183.33 \pm 10.44 [▲]	173.33 \pm 6.77 [▲]	187.00 \pm 22.08 [▲]
15th day	431.00 \pm 72.92	236.00 \pm 9.80 [▲]	360.83 \pm 93.54 ^Δ	342.00 \pm 28.09 ^Δ

Compared with the blank control group, * $P < .05$, [▲] $P < .01$; compared with the model group, ^Δ $P < .01$. Values are the mean \pm SD.

Table 3 Comparison of relevant immune parameters among different groups.

Grouping	Relevant immune parameters		
	β -EP (pg/mL)	VIP (pg/mL)	SIgA (μ g/mL)
Control group	5.97 \pm 0.32	8.63 \pm 2.45	1.64 \pm 0.11
Model group	7.91 \pm 1.53*	5.73 \pm 2.36*	1.47 \pm 0.44*
Prednisone group	7.15 \pm 1.39	8.64 \pm 1.93 ^Δ	1.96 \pm 0.31 ^Δ
Spleen-invigoration group	6.23 \pm 0.62 ^Δ	9.34 \pm 2.45 ^Δ	2.39 \pm 0.26 ^Δ

Compared with the blank control group, * $P < .05$; compared with the model group, ^Δ $P < .05$. Values are the mean \pm SD.

resistant to or dependent on adrenocortical hormones or show a poor response to second-line therapy, traditional Chinese medicine might be a replacement therapy.¹¹

Because the pathogenesis of ITP involves immune system disorder, the mechanism of ITP drug action is based on

classic T and B cells (total number of T and B cells, CD3, CD4, CD8, natural killer cells, CD4⁺ CD25⁺ T cell proportions, regulatory T cells, phagocytes, and immunoglobulins) and the regulation of related cytokines networks (IL-4, IL-10, interferon- γ).¹²⁻¹⁴ Closely related to the

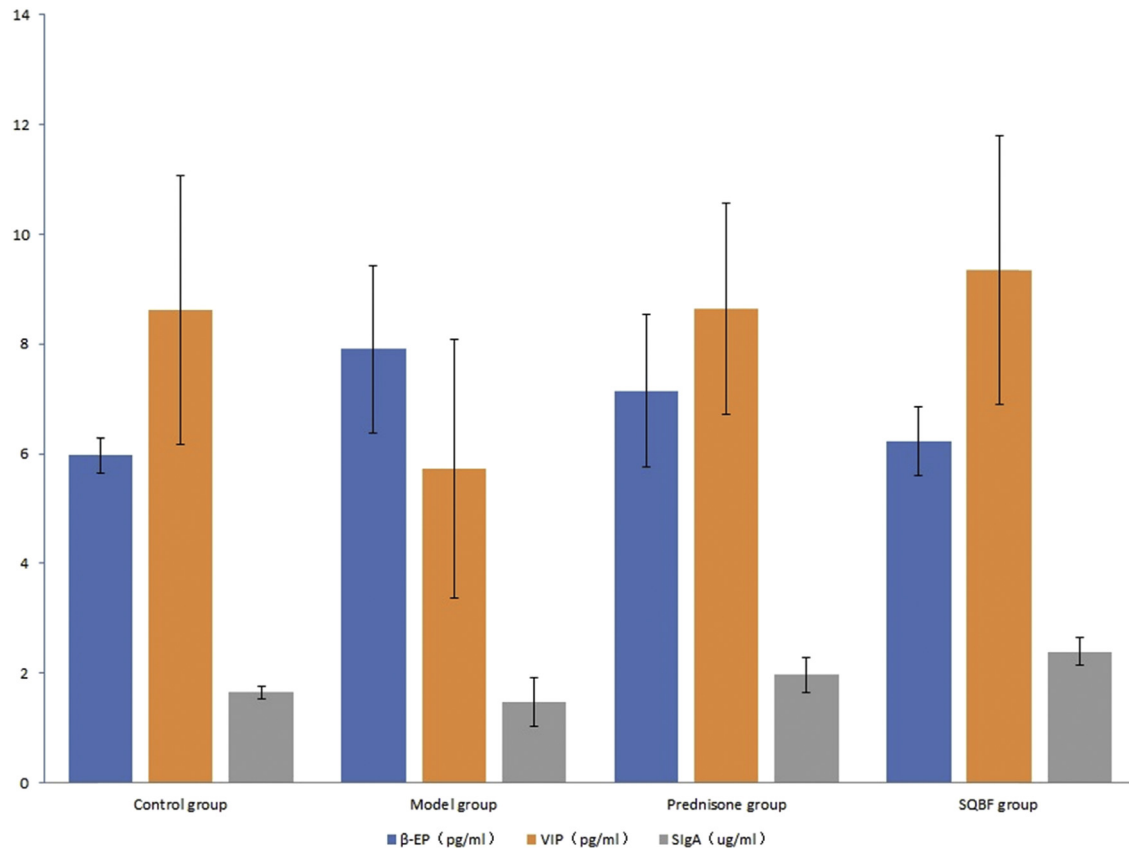
**Figure 1** Relevant immune parameters in each group.

Table 4 Viscera indexes of mice in each group (mg/g).

Viscera indexes	Control group	Model group	Prednisone group	Spleen-invigoration group
Spleen indexes	4.04 ± 0.52	13.89 ± 1.62*	9.15 ± 1.69 ^Δ	14.31 ± 1.69*
Thymus indexes	2.73 ± 0.49	3.62 ± 0.70*	2.48 ± 0.83 ^Δ	2.95 ± 0.59 [▲]

Compared with the blank control group, * $P < .05$; compared with the model group, [▲] $P < .05$, ^Δ $P < .01$. Values are the mean ± SD.

brain-gut axis functions, some non-classical immune vascular active factors or neurotransmitters such as SIgA, β -EP, VIP, and 5-HT are also involved in immune responses and the onset of ITP and bleeding and/or hemostasis. This remains an area for further study to determine whether a change in these active factors is related to immediate hemostasis.

Relationship between non-classical immune parameters and the onset of ITP and drug efficacy

SIgA was discovered in exocrine secretions in the early 1960s. It is mostly found in the exocrine secretions of breast milk, gastrointestinal fluid and respiratory secretions. Previous studies confirmed that SIgA is the first line of immune defense for the respiratory, gastrointestinal, and urogenital tracts as protection against pathogens and hazardous substances.¹⁵

β -EP is secreted by neurons from the pituitary, hypothalamic arcuate nucleus, hippocampus and amygdala. It is an important neuropeptide that has opiate-like effects and regulates respiratory, circulatory and digestive functions as well as functions of the thalamus, pituitary and adrenal axis. Additionally, many immune cells can synthesize and secrete β -EP as well as its receptor. β -EP also has important physiological functions in the immune system.¹⁶

VIP is related to the occurrence, development, outcome, treatment, and prognosis of many clinical diseases. Its main function is vasodilation, information transmission and immune regulation. VIP is important during immune cell injury and/or inflammation together with pituitary adenylate cyclase activating peptide. Delgado et al successfully treated models of multiple sclerosis (autoimmune encephalomyelitis) and increased the number of regulatory T cells with VIP. Pozo et al used VIP in an *in vitro* culture of human CD4⁺ CD25 T cells, which led to the successful induction of Foxp3⁺ CD4⁺ CD25^{high} T cells.

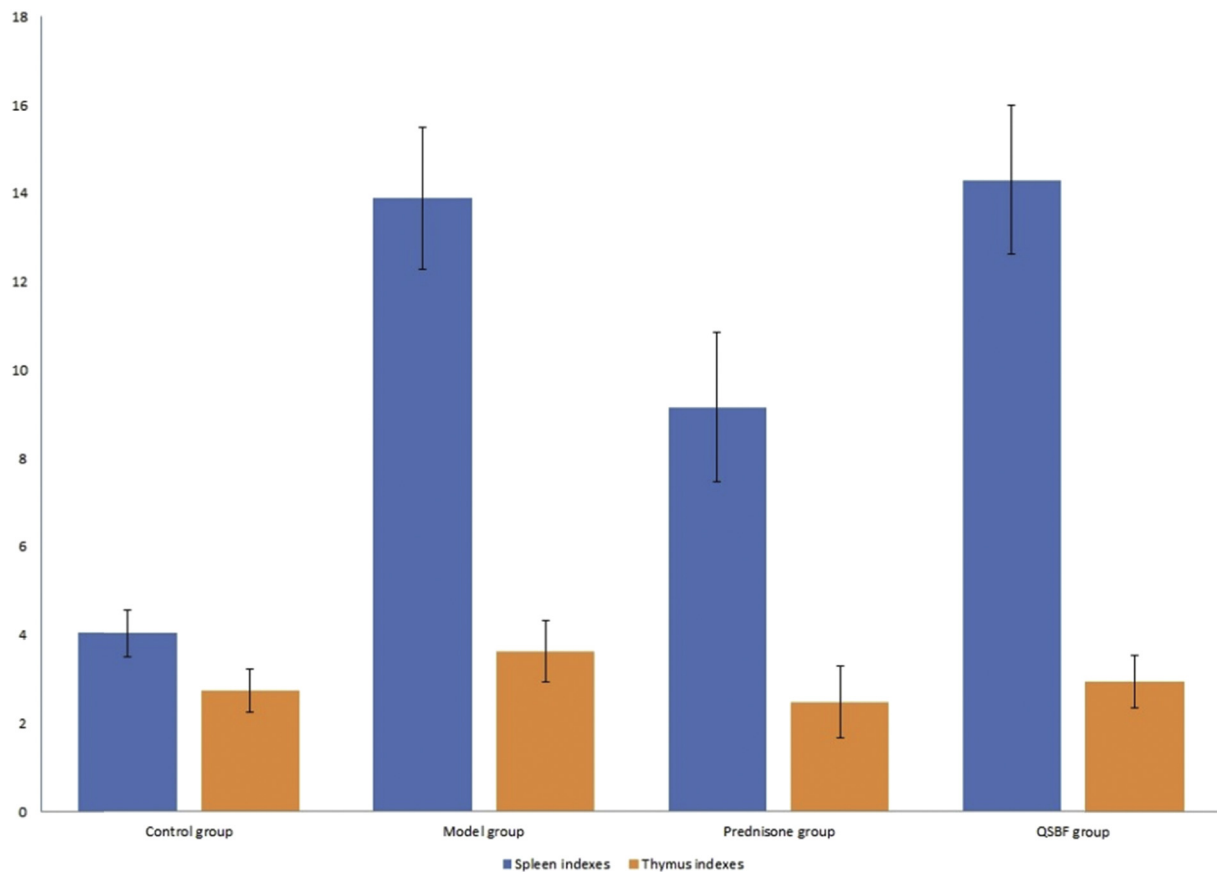


Figure 2 Spleen and thymus indexes in each group.

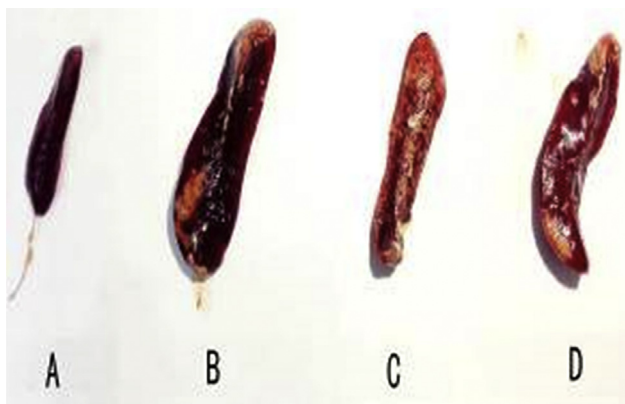


Figure 3 Comparison of spleen size in each group. A: control group, B: model group, C: prednisone group, D: spleen-invigoration group.

Foxp3 is the key regulatory molecule for Treg cell differentiation and development. VIP also plays an important role in immune regulation.¹⁷

The non-classical immune parameters mentioned above usually exert their immune effect in the progression of infectious diseases and chronic inflammatory diseases. However, to date, there has been little basic and pharmacodynamic research investigating whether the non-classical immune factors are important for the onset and treatment of ITP. In recent years, in our clinical practice of treating ITP with Chinese medicine, we have found that cipher prescription with the effect of invigorating spleen, replenishing qi and containing blood remarkably relieved the symptoms of ITP patients, mitigated bleeding tendency, and slowly improved peripheral blood platelet counts. In addition, SQBF as a primary or supplementary medication was useful for ITP patients with hormone dependence or ineffectiveness.¹⁸ Of note, its hemostatic effect is not directly proportionate to the increase in platelets, suggesting the hemostatic mechanism of SQBF may involve the regulation of non-classical immune parameters such as vasoactive substances and neurotransmitters.

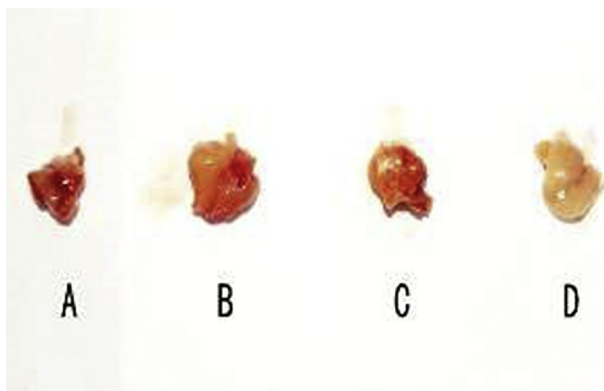


Figure 4 Comparison of the thymus size in each group. A: control group, B: model group, C: prednisone group, D: spleen-invigoration group.

Analysis of experimental results

Before APS injection, comparisons of platelet counts among different groups were not statistically significant ($P > .05$). Forty-eight hours after APS injection, the platelet count of mice in the model group, prednisone group and spleen-invigoration group were significantly reduced compared with the control group ($P < .05$).

In summary, SQBF enhanced peripheral blood platelet counts in ITP mice similar to that of prednisone, decreased β -EP and VIP levels and increased SIgA levels. It also maintained the relative stability of immune organs such as the spleen and thymus. Therefore, SQBF might be a replacement therapy for ITP patients with resistance to or a dependence on first-line and/or second-line therapy. The SQBF is mostly composed of herbs that are used as a pharmaceutical and edible resource, such as *Radix Astragali Preparata* (*Radix Astragali seu Hedysari Praeparatae*), *Salviae Miltiorrhizae* (*Radix Codonopsis pilosulae*), *Donkey-hide Gelatin* (*Colla Corii Asini*). Therefore, its long-term clinical use might be unlikely to lead to organ lesions or other adverse reactions.

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