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Shenhong Buxue Granules Improve the Vascular Endothelial Injury and Hematopoietic Function in Rats with Aplastic Anemia

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ABSTRACT

Shenhong Buxue granule (SBG) is a traditional Chinese medicine compound recipe for treating blood deficiency syndrome. To investigate its effects on the hematopoietic function, aplastic anemia (AA) rat model was established by injection of cyclophosphamide (CTX) intraperitoneally. 60 SD rats were randomly divided into 6 groups, control, model, positive drug (compound E-jiao slurry), SBG high-, medium- and low-dose (SBG-H, SBG-M, SBG-L) groups. After 21 days of intervention, the changes of thymus and spleen indexs were compared, the levels of white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT) and platelet count (PLT) in peripheral blood were detected, and the changes of hemorheology were observed. Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), superoxide dismutase (SOD), malondialdehyde (MDA), lactate dehydrogenase (LDH) and reduced glutathione peroxidase (GSH-Px) were detected by ELISA. The expression level of TLR4/NF-κB, and HandE staining was used to observe the renal tissue. In the results, compared with model group, the spleen index and whole blood viscosity was significantly decreased, while the thymus index, peripheral blood WBC, RBC, Hb, HCT, PLT levels, and NO, SOD, GSH-Px levels in serum were significantly increased. The levels of TNF-α, IL-6, MDA, LDH in serum were significantly decreased. The protein expression levels of TLR4 and Nuclear factor-κB (NF-κB) p65 in kidney were significantly decreased. HandE staining on renal showed that SBG groups had a certain protective effect on renal injury, especially the high-dose group. In conclusion, SBG may improve hematopoietic function and improve vascular endothelial injury in AA model rats by regulating TLR4/NF-κB signaling pathway.

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Key words
Shenhong Buxue Granules (SBG), Aplastic anemia, Hematopoietic function, Vascular endothelial injury; TLR4/NF-κB pathway

INTRODUCTION

Aplastic anemia (AA) is a bone marrow hematopoietic failure caused by a variety of causes, the main reason is the loss or absence of bone marrow hematopoietic precursors caused by injury and resulting in peripheral pancytopenia (Shallis *et al*., 2018). Bone marrow injury occurs in many cases, including idiopathic (accounts for 65%), seronegative hepatitis, telomerase defects, eosinophilic fasciitis, and so on (Gadalla *et al*., 2018). The conventional treatment of AA in the world is immunosuppressive therapy (IST), which is generally treated with cyclosporine combined with androgen or hematopoietic stimulator, with strong toxic and side effects (Ding *et al*., 2018). Eltrombopag is approved for clinical use in severe patients in China, but it is usually associated with severe liver injury and visual impairment (Townsley *et al*., 2017). Hematopoietic stem cell transplantation (HSCT) can cure AA, but it is difficult to find a matching donor, with high cost and poor prognosis (Georges *et al*., 2018).

Traditional Chinese medicine (TCM) are often used to treat anemia and as supplements for ischemia and blood deficiency, showing sufficient curative effect with fewer side effects. Shenhong Buxue Granules (SBG) is a TCM compound recipe that used to treat blood deficiency syndrome, which is composed of 252.0 g *Ginseng Radix et Rhizoma Rubra*, 378.0 g *Rhodiola Crenulatae Radix et Rhizoma*, 252.0 g *Polygonati Rhizoma*, 252.0 g *Atractylodis Macrocephalae Rhizoma* and 252.0 g *Ophiopogonis Radix*. In which red ginseng mainly contains Ginsenosides Rb, which can significantly increase red blood cells (RBC) and hemoglobin (Hb) levels (Zheng *et al*., 2019). *Rhodiola rosa* is rich in salidroside (SAL), which can promote the proliferation of nucleated cells and hematopoietic progenitor cells in bone marrow of mice

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with bone marrow suppression anemia (Chen and Fang, 2019). Studies have shown that SBG can improve blood and immune functions, and maintain a stable of vascular endothelial by increasing the level of soluble vascular cell adhesion molecule-1 (VCAM-1). Moreover, it can improve the hematopoietic microenvironment and alleviate bone marrow suppression and hematopoietic incompetence (Li *et al*., 2015; Liu *et al*., 2017; Xu *et al*., 2018). However, its detailed mechanism remains unclear, which needs to be further investigated.

In this study, E-jiao (Colla corii asini) slurry, a compound TCM that commonly used to treat leucopenia and anemia, was selected as the positive drug. It is composed of *Asini Corii Colla, Ginseng Radix et Rhizoma, Rehmanniae Radix Praeparata, Codonopsis Radix* and *Crataegi Fructus* (Liu *et al*., 2014). Cyclophosphamide (CTX) was used to simulate the inhibition of bone marrow function to establish AA rat model. The protective effect of SBG on vascular endothelial injury and hematopoietic function was studied, and the regulation of TLR4/NF-κB pathway was observed. This study aims to provide a reference on further revealing the mechanism of SBG and provide ideas for clinical treatment of AA.

MATERIALS AND METHODS

Animals, materials and main reagents

Sprague Dawley (SD) rats (n=60, SPF grade, aged 4-6 weeks, body weight 200±20g) were purchased from Yisi Experimental Animal Technology Co., Ltd. Shenhong Buxue Granules (SBG) was prepared by the Preparation Center of the First Clinical Hospital Affiliated to Changchun University of Traditional Chinese Medicine, containing 1.39g of crude drug per gram. E-jiao slurry was from Dong-E E-jiao Co., Ltd. (Shandong, China).

Elisa Kits for lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione peroxidase (GSH-Px), nitric oxide (NO) were from Jiancheng BioEngineering Research Institute (Nanjing, China). Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) Elisa Kits were from Enzyme Industrial Co., Ltd, Jiangsu, China. HRP-labeled goat anti-rabbit IgG (H+L) (A0208), protein marker (P0068), SDS-PAGE (P0014D), electrophoresis buffer with Tris-Gly, 10X, western transfer buffer (P0021B), BeyoECL Plus (P0018S), SDS-PAGE sample loading buffer, 6X (P0015F) were from Beyotime Inc. (Shanghai, China). Total protein extraction kit (BC3710), BCA protein assay kit (PC0020), SDS-PAGE gel preparation kit (P1200) were from Solarbio, Beijing, China.

Groups and treatments

SD rats were randomly divided to 6 groups (10 rats per group): control, model (intraperitoneal injection cyclophosphamide, CTX), positive drug (6.25 mL/kg compound E-jiao slurry), SBG high-, medium- and low-dose (SBG-H, SBG-M, SBG-L; containing 4.5, 3.0, 1.5 g/kg of SBG, respectively) groups.

In drug groups, rats were given intragastric administration once a day for 21 days. In control and model groups, rats were given the same volume of distilled water. All the groups except control were intraperitoneally injected CTX normal saline solution 25 mg/kg/day from day 17 to 20. The control group was injected with normal saline of the same volume.

The thymus and spleen index

After the last administration, rats were weighted, and sacrificed under anesthesia. The thymus and spleen were extracted and wet weighed with residual blood dried with filter paper. The indexes were calculated: thymus index= thymus wet weight (mg)/mouse weight (g)×100% (Zhang *et al*., 2019).

Whole blood viscosity and complete blood count (CBC) test

Whole blood was collected and anticoagulated with heparin. The shear rates of low, medium and high whole blood viscosity (10, 60, 150 s^{-1}) were detected by semi-automatic hemorheometer analyzers (LBY-N6K, Prismlab, Shanghai, China) according to existing method (Akcaboy *et al*., 2018), which generally includes viscosity at high, medium and low shear rates. The CBC test including the levels of RBC, Hb, hematocrit (HCT), white blood cell (WBC) and platelet count (PLT), and were detected by automated hematology analyzers (BC-5300Vet, Mindray, Shenzhen, China).

ELISA test and H and E staining

The levels of TNF-α, IL-6, NO, SOD, LDH, MDA, and GSH-Px in serum were detected according to the instructions of the ELISA kits.

Rat kidneys were taken, the largest cross-section area was cut and trimmed into a cube of about 1/4 size, with the medulla and cortex fully preserved. After being fixed with 4% paraformaldehyde for 36 h, the tissues were dehydrated, transparent, waxed, embedded and sliced, and then were stained according to the instructions of the HandE staining kit (Solarbio, Beijing, China).

Western blot

The protein extractions of the kidney tissues were prepared as previously described (Leng *et al*., 2018). In brief, kidney tissues were lysed on ice with lysis buffer and

homogenized, then centrifuged and keep the supernatant. The protein concentrations of samples were determined using BCA Protein Assay Kit (Solarbio, Beijing, China). The loading buffer was added, then the sample was denatured at 100 °C for 4 min. The polyacrylamide gel was prepared and added to the sample (40 μg), electrophoresis at 90 V for 10 min, then adjust the conditions at 110 V for 100 min. After that, the gel was removed and transferred under the condition at 200 mA for 30 min on ice. The PVDF membrane was removed and blocked overnight with blocking solution at 4°C. Then discarded the blocking solution and the primary antibody was added for incubation at room temperature for 1 h. After the primary antibody was collected, the secondary antibody (Sanying Biotechnology, Wuhan, China) was added and incubated for 1 h.

Statistical analysis

SPSS version 21.0 (SPSS Inc. Chicago, IL, USA) was used to analyze the data. Data were expressed as mean antibody diluent ($\bar{x}\pm s$). One-way analysis of variance was used for comparison between arrays of multiple groups, and P <0.05 or P <0.01 was considered statistically significant.

RESULTS

Thymus and spleen indices in rats

As shown in Figure 1, in model group, the thymus index decreased and the spleen index increased, all significant compared with the control group (P < 0.01). In positive drug group, the spleen index decreased while thymus index increased, both significant compared with model group (P < 0.01). The thymus index in both SBG-H and SBG-M groups were significant increased (P <0.01) (Fig. 1A), in SBG-H group the spleen index was significantly decreased (P <0.05) (Fig. 1B), while in SBG-L group it had no significant effect. The results showed that there is a dose effect. A specific higher dose can improve the thymus atrophy and the compensatory enlargement of spleen in AA rats.

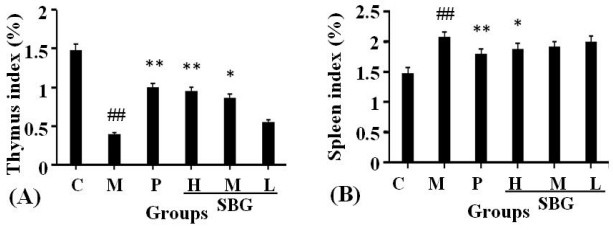

Fig. 1. Effect of SBG on thymus index (A) and spleen index (B) of rats.
Notes: C, control group; M, model group; P, positive drug group; SBG-H, SBG high-dose group; SBG-M, SBG medium-dose group; SBG-L, SBG low-dose group; n = 10, $\bar{x}\pm s$. Compared with control, ## P <0.01; Compared with model, * P <0.05, ** P <0.01.

Haematological parameters

As shown in Figure 2, the levels of RBC, HGB, HCT, WBC and PLT in peripheral blood of rats in model group decreased significantly (P < 0.01), indicating the AA model was established successfully. Those levels in positive drug group increased significantly (P < 0.01), while those levels in the SBG group increased in a dose-dependent manner (P < 0.05, P < 0.01) compared with model group, indicating that SBG can improve the hematopoietic function injury caused by cyclophosphamide.

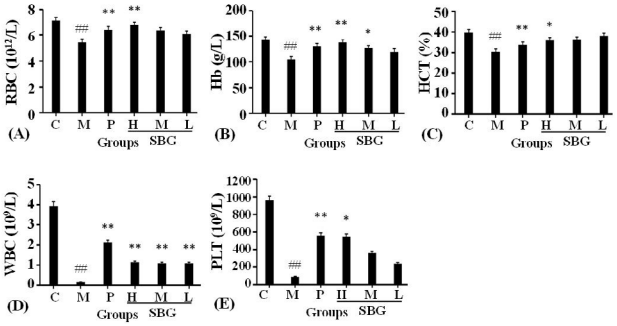

Fig. 2. Effect of SBG on haematological parameters of rats: A, RBC; B, Hb; C, HCT; D, WBC; E, PLT
RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; WBC, white blood cell; PLT, platelet count. For other abbreviations and statistical details, see Figure 1.

Whole blood viscosity

As shown in Figure 3, the low, medium and high shear rates of whole blood viscosity (10, 60 and 150 s^{-1}) in model group were significantly higher than those in control group (P <0.05, P <0.01). The positive drug and SBG-H groups had significant differences in the low, medium and high shear rates of whole blood viscosity compared with model group (p<0.05, P <0.01). SBG-M and SBG-L groups had significant difference (P <0.05) in the medium shear rates of whole blood viscosity (Fig. 3B); while SBG-M and SBG-L groups had no difference in the low and high shear rates of whole blood viscosity (Fig. 3A, C). It indicated that SBG has an effect in improving the whole blood viscosity on AA rats, there are certain differences in effects with different doses.

Serum levels of TNF-α, NO, IL-6, SOD, MDA, LDH and GSH-Px

As shown in Figure 4, compared with control group, the level of NO in serum of model group was significantly lower than that of control group (P <0.05), and the

levels of TNF-α and IL-6 in serum of model group were significantly higher than those of control group (P <0.01, P <0.05). Compared with model group, the level of NO in positive drug and SBG-H groups were significantly increased (p<0.05), and the levels of TNF-α and IL-6 were significantly decreased (p<0.05, P <0.01). The levels of TNF-α and IL-6 in SBG-M group were significantly decreased (p<0.05), level of IL-6 in SBG-L group was significantly decreased (p<0.05). It suggested that SBG had a good regulation effect on inflammatory factors and vasodilator factor NO in AA rats.

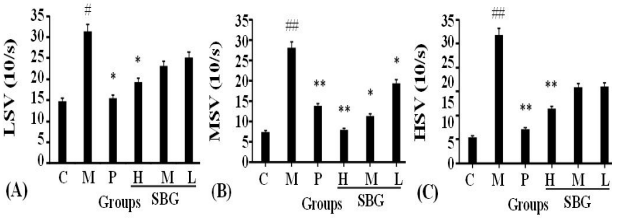

Fig. 3. Effect of SBG on shear rates of whole blood viscosity of rats: A, low shear viscosity (LSV); B, medium shear viscosity (MSV), and C, high shear viscosity (HSV). For other abbreviations and statistical details, see Figure 1.

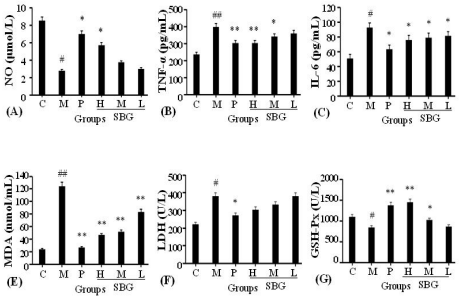

Fig. 4. Effect of SBG on NO (A), TNF-α (B), IL-6 (C), SOD (D), GSH-Px (E), MDA (F) and LDH (G) levels in serum of rats. NO, nitric oxide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; LDH, lactate dehydrogenase. For other abbreviations and statistical details, see Figure 1.

Compared with control group, the levels of SOD and GSH-Px in serum of model group were significantly decreased (P <0.01, P <0.05), while the levels of MDA and LDH were significantly increased (P <0.01, P <0.05). Compared with model group, SOD and GSH-Px levels in positive drug, SBG-H and SBG-M groups were significantly increased (P <0.05, P <0.01), and MDA levels in positive drug, SBG-H, SBG-M and SBG-L groups were significantly decreased (P <0.01), while LDH had no significant change, but showed a dose-dependent trend of decline. The results suggested that SBG had a certain regulating effect on oxidative damage in AA rats.

Pathological changes in kidney

In control group, no obvious lesions were observed in glomerulus and other structures, with compact and clear cell arrangement and no inflammatory cell infiltration or fibrosis observed in the interstitium (Fig. 5A, B). Compared with the control group, the cells in model groups were disorderly and loosely arranged, and the glomerular epithelial cells were vacuolar degeneration accompanied by hypertrophy and dilatation, some inflammatory infiltrates and hyalinosis were visible, and the renal vesicles were obviously shrinched (Fig. 5C, D).

The incidence of glomerular shrinkage, hypertrophy and dilation, and cavitation declined, and glomerular morphology tended to be normal in positive drug and SBG-H groups (Fig. 5E, H). In SBG-M and SBG-L groups, a certain improvement effect were observed, but there was still a small amount of hyalinosis (Fig. 5I, J). As can be seen (with 400x magnification) in each group that a certain renal damage occurred in AA rats. Compared with model group, the renal vessels in positive drug and SBG-H groups were clearer and fuller, suggesting that SBG promoted the formation of new renal vessels.

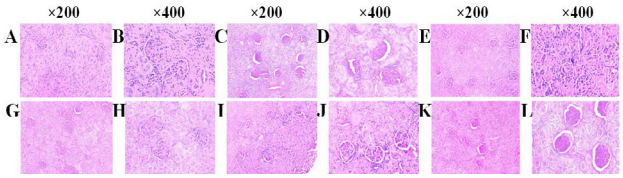

Fig. 5. Effect of SBG on histological structure of rats in (×200, ×400). **A, B**: control; **C, D**: Model group; **E, F**: Positive drug group; **G, H**: SBG-H dose group; **I, J**: SBG-M dose group; **K, L**: SBG-L dose group.
Stain: H & E. Magnification: A, C, E, G, I, K= 200X; B, D, F, H, J, L=400X.

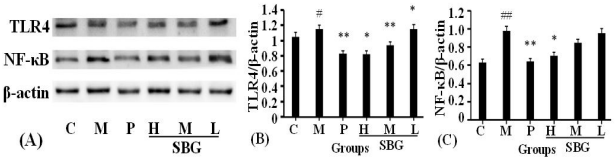

Fig. 6. Effect of SBG on western blot (**A**) and TLR4/β-actin (**B**), NF-κB p65/β-actin (**C**) protein expression levels kidney of rats. For other abbreviations and statistical details, see Figure 1.

TLR4/NF-κB related protein expressions in rat kidney

Compared with control group, the relative expression

of TLR4 and NF-κB p65 protein in kidney of model group was significantly increased (P <0.05, P <0.01). While the relative expression of TLR4 and NF-κB p65 protein in positive drug group was significantly increased (P <0.01), and those in SBG groups was significantly decreased (P <0.05, P <0.01), compared with model group (Fig. 6).

DISCUSSION

The spleen is the largest immune organ, and a storage site for blood as well as immune cell. The splenomegaly can cause hypersplenism, which leads to decreased blood cells and platelets in the blood and accelerates the occurrence of anemia (Föller *et al*., 2008). In this study, the reduction of AA rats thymocytes resulted in thymus structural destruction and thymus atrophy. This may be due to the fact that lymphocytes in the thymus are prone to apoptosis under stress or immunosuppression (Majumdar and Nandi, 2018). The results suggested that SBG could improve the hemogram, thereby regulating the immune organ dysfunction caused by anemia, and improving thymus atrophy and splenomegaly. The kidney can regulate blood pressure and bone marrow hematopoiesis. The results of H and E staining proved the hypothesis that AA is usually accompanied by renal injury. The kidney in model group showed a certain damage, and SBG can significantly improve the renal injury, which indirectly affected the hematopoietic function, and may promote the formation of new blood vessels in the kidney.

When the number of RBC, Hb, HCT, WBC and PLT cells in whole blood cell is reduced, the water in tissue is transferred to the blood vessels and the blood is diluted, thus leading to the decrease of whole blood viscosity (Caprari *et al*., 2019). The results of this study showed that SBG improved the blood viscosity and the CBC indicators, and it also suggested that SBG may play a certain role in angiogenesis.

Angiogenesis is a series of physiological processes that are strictly regulated (Hassan *et al*., 2016; Ma *et al*., 2021). Vascular endothelium has the functions of metabolism, immunity and participation in inflammatory response (Zhang *et al*., 2020). Inflammation and oxidative stress are the main causes of oxidative damage of vascular endothelial cells (Chao *et al*., 2020). Nitric oxide (NO) is known as endothelium-derived relaxing factor (EDRF), which can inhibit leukocyte adhesion and migration, platelet adhesion and aggregation. The occurrence of inflammation can reduce the synthesis of NO, leading to microcirculation disorder. At the same time, the damaged tissue releases two inflammatory mediators (Oshima *et al*., 2018), which interfere with the hematopoietic and immune functions of the body. When AA occurs, the metabolism is reduced, the ability of body to scavenge free radicals is reduced, causing oxidative damage exacerbated (Ritam and Sujata, 2018; Caprari *et al*., 2019). SOD and GSH-Px are antioxidant enzymes, which can antagonize the damage caused by oxidative stress. The results showed that SBG can increase the contents of NO, SOD and GSH-Px in blood, suggesting that SBG can improve the blood image and regulate the hematopoietic function of anemia rats.

Under the stimulation of CTX, macrophages produce inflammatory cytokines TNF-α and IL-6, which simultaneously leads to lipid peroxidation in serum, and the contents of intermetabolites MDA and LDH are increased. When the concentration of TNF-α was high, it could inhibit the formation of erythroid clone colony units, thus affecting the hematopoietic function of the body (Ma *et al*., 2016). When the concentration of IL-6 is increased, the expression of hepcidin is up-regulated, resulting in abnormal iron metabolism and anemia. MDA and LDH can cross-link proteins to denature biofilm, cause cell damage and aggravate anemia (Domingos *et al*., 2020). These results indicate that SBG can slow down the degree of oxidative damage and protect the vascular endothelial cell damage caused by oxidative stress.

As an important protein molecule involved in immune regulation, the activation of TLR4 can affect the survival, differentiation, proliferation, migration of bone marrow stromal cells and the secretion of proinflammatory cytokines. NF-κB is the downstream molecule signoal of TLR4, the activation of TLR4 can trigger the nuclear translation of NF-κB, which is the factor that necessary for the transcriptional activation of target genes such as NF-α, IL-1β, IL2 and IL-6, play an important role in promoting hematopoiesis and regulating immune function (Dejban, *et al*., 2020; Hasan *et al*., 2020). The activation of NF-κB pathway can reduce the expression of anti-apoptotic genes, apoptosis of hematopoietic stem cells, lead to hematopoietic disorders (Huijeong *et al*., 2019), and induce the expression of a variety of proinflammatory factors (Leng *et al*., 2018). The results showed that SBG could significantly reduce expression level of TLR4 and NF-κB p65, thus improve hematopoietic function and vascular endothelial injury in AA rats.

CONCLUSION

In conclusion, SBG has a protective effect on hematopoietic function and vascular endothelial injury in AA rats, can improve blood biochemical indexes and blood viscosity, protect kidney injury, improve the body's hematopoietic function, and promote angiogenesis by regulating angiogenesis related factors. SBG can inhibit

the inflammatory response and oxidative stress process, possibly by inhibiting the activation of TLR4/NF-κB signaling pathway.

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Ethical compliance

Research experiments conducted in this article with animals were approved by the Ethical Committee of Changchun University of Chinese Medicine (No.: 2020040) following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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