JTCM 中医杂志 Journal of Traditional Chinese Medicine

Online Submissions: http://www.journaltcm.com
info@journaltcm.com

J Tradit Chin Med 2017 October 15; 37(5): 643-649
ISSN 0255-2922

RESEARCH ARTICLE

Flavone from Zhongjiefeng (*Herba Sarcandrae Glabrae*) inhibits platelet apoptosis in immune-induced bone marrow failure through mitochondrial pathway

Jiang Yiling, Zheng Qin, Zhang Aiping, Cui Lele, Xia Lemin, Luo Meihong

Jiang Yiling, Zheng Qin, Zhang Aiping, Cui Lele, Xia Lemin, Luo Meihong, Department of Hematology, Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine; Department of Hematology, Baoshan Branch of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine), Shanghai 201999, China

Supported by Research Project of Shanghai Municipal Health and Family Planning Commission: Flavone from Zhongjiefeng (*Herba Sarcandrae Glabrae*) Inhibits Platelet Apoptosis in Immune-induced Bone Marrow Failure Through Mitochondrial Pathway (No. 201640144); Baoshan Health Systems of Young Medical Talent Training Project (No. bswsyq-2016-A11); National Nature Science Foundation Project of Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine: Research on Xijiao Dihuang Decoction Suppressing Platelet Apoptosis in Bone Marrow Failure Secondary to Abnormal Immune Response Based on Mitochondria-mediated Pathway (No. GZRPY-JJ-201601)

Correspondence to: Dr. Xia Lemin, Department of Hematology, Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai 201999, China. roby_0_0_2000@163.com; **Prof. Luo Meihong,** Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai 201999, China. lmh021009@163.com

Telephone: +86-21-56601100-410

Accepted: December 16, 2016

Abstract

OBjECTIVE: To investigate the effect of Flavone from Zhongjiefeng (*Herba Sarcandrae Glabrae*) on the platelet number in immune-induced bone marrow failure (BMF) and its mechanism of mitochondrial apoptotic pathway.

METHODS: Immune-induced BMF model, established in mice, was randomly divided into four groups: normal control group without BMF, BMF control group, cyclosporine (CSA) group and flavone group (n = 10 in each group). Mice were given 0.027 g/kg cyclosporine or 0.2 g/kg flavone lavage daily in either the cyclosporine or flavone group respectively. Platelet count, mitochondrial transmembrane potential (ΔΨm), cytochrome C (Cyt C), phosphatidylserine (PS), changes of calcium ion (Ca^{2+}), and protein expression of mitochondrial apoptotic pathway including B-cell lymphoma-2 (bcl-2) Homologous Antagonist-Killer Protein (Bak), bcl-2-associated X protein (Bax), caspase-3, caspase-8, and caspase-9 were examined and compared.

RESULTS: Compared with the normal control group, the BMF group had significantly lower levels of platelet count, ΔΨm, and expressions of caspase family proteins as well as higher levels of Cyt C, PS, Ca^{2+}, and expressions of Bak and Bax (all $P < 0.05$). Compared with the BMF group, the CSA and flavone groups had significantly higher ΔΨm and expressions of caspase family proteins (all $P < 0.05$) whereas the levels of Cyt C, PS, Ca^{2+}, and expressions of Bak and Bax were reduced (all $P < 0.05$). More importantly, the flavone group had higher levels of Cyt C, Ca^{2+} and expressions of Bak and Bax compared with the CSA group (all $P < 0.05$), while the levels of PS and caspase family proteins were reduced (all $P < 0.05$).

CONCLUSION: Flavone from Zhongjiefeng (*Herba Sarcandrae Glabrae*) significantly increases the platelet number and prevents its apoptosis through mitochondrial pathway.

Keywords: Flavone; Herba Sarcandrae; Mitochondria; Apoptosis regulatory proteins

INTRODUCTION

Bone marrow failure (BMF) is a type of diseases characterized by reduced blood cell regeneration capability,[1] especially the platelet regeneration, which threatens life. Excessive platelet apoptosis is one of the pathogenic causes for immune-induced BMF. Mitochondrial apoptotic pathway is one of the important pathways modulating platelet apoptosis. The mechanisms include early manifestation of reduction in mitochondrial transmembrane potential ($\Delta\Psi m$) and formation of mitochondrial permeability transition pore (MPTP) followed by multi-transportation of mitochondrial proteins to cytoplasm to exert a pro-apoptotic effect. Pro-apoptotic proteins translocate to mitochondria and cytochrome C releases from mitochondrial intermembrane space to cytoplasm. In platelet, cysteinyl aspartate specific proteinase (caspase)-8, caspase-9 and caspase-3 are activated subsequently, hence splitting cytoskeleton. The membrane phosphatidylserine (PS) is then exposed extracellularly and the platelet then undergoes shrinking, dropping of microparticles and apoptosis.[2] The commonly-used medication for thrombocytopenia is cyclosporine (CSA), which exerts its effect through inhibiting apoptosis by mitochondrial pathway.[3] As found out by our previous pilot study, mitochondrial pathway inducing platelet apoptosis could lead to abnormal blood coagulation and hemorrhage.[4]

Zhongjiefeng (*Herba Sarcandrae Glabrae*) is the dried whole-plant of Chloranthaceae Caoshanhu and a type of commonly-used Traditional Chinese Medicine (TCM) with its effect on "promoting blood circulation, eliminating mass and relieving swelling, and cooling blood and hemostasis". Clinically, it has been prescribed for hemorrhagic disease caused by thrombocytopenia. Current study has found out the major chemical composition of Zhongjiefeng (*Herba Sarcandrae Glabrae*) includes sesquiterpenes (atractylenolide-II, III, IV, and chloranthalactone A, B, E, F and G), flavone (isoliquiritigenin, isoliquiritin and 7-O-methyl naringenin), coumarins (isofraxidin, fraxidin and fraxin), organic acids (fumarate, succinic acid and chlorogenic acid) and volatile oils.[5] Total flavonoids from Zhongjiefeng (*Herba Sarcandrae Glabrae*) is the effective component. Previous mouse research has demonstrated its effect on promoting proliferation of megakaryocytic series,[6] improving the post-chemo therapy thrombocytopenia,[7] increasing white blood cells and platelet count.[8] However, its mechanism has not been elucidated. It still remains unknown whether its effect on increasing platelet count is through inhibiting mitochondrial apoptotic pathway. Therefore, we aim to investigate the effect of flavone from Zhongjiefeng (*Herba Sarcandrae Glabrae*) on the platelet number in mice with immune-induced bone marrow failure and elucidate whether its effect is through mitochondrial apoptotic pathway.

MATERIALS AND METHODS

Animal

Forty C57BL/6 mice (20 male and 20 female mice) aged from 8 to 12 weeks, weighing (20 ± 2) g were purchased from Shanghai Slac Laboratory Animal Corporation (Shanghai, China). Mice were housed in the animal center of the company with free access to water and food. The animal approval number was SCXK 2012-0002. The study was approved by the experimental animal ethics commitee of Shanghai Baoshan hospital of Integrated Traditional Chinese and Western Medicine.

Flavone and cyclosporine preparation

Flavone from Zhongjiefeng (*Herba Sarcandrae Glabrae*) is isolated and purified by using HPD400 macroporous adsorptive resin.[9] Its characteristic figure by high performance liquid chromatography (HPLC) is presented in Figure 1. Cyclosporine (S0408, 25 mg × 50 tablets, Novartis Pharma, Freiburg Area, Germany) is made into 4 mg/mL solution with sterile saline. Lavage solution is then diluted to 0.1 mL per 10 grams of mouse weight.

Bone marrow failure animal model

The animal model of bone marrow failure is established by following method from Liu *et al.*[10] Briefly, thymus glands were dissected from DBA/2 mice sacrificed by cervical dislocation, filtered through Nylon filters and passed through the size-4 syringe needle to form single-cell suspension. Trypan Blue staining was used to assess the cell viability, hence determining the cell number. A total of 1×10^6 cells were then administered through the tail vein of a C57BL/6 mouse for 4 h after exposure to ^{60}Co-γ radiation [5.5Gy (1.1 Gy/min × 5 min)]. Three days after modeling, peripheral blood was drawn from the mouse-tail vein and detected by the automatic blood cell analyzer. When pancytopenia was shown, it suggested that the model was successful.

Study design

Forty C57BL/6 mice were randomly assigned to four groups with 10 mice in each group: normal control group, BMF control group, CSA group and flavone group. Mice of the normal control group were healthy C57BL/6 mice without BMF modeling. Mice of the BMF control group were exposed to radiation and cell transfusion and had no treatment with either CSA or flavone. Mice of the CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA whereas the

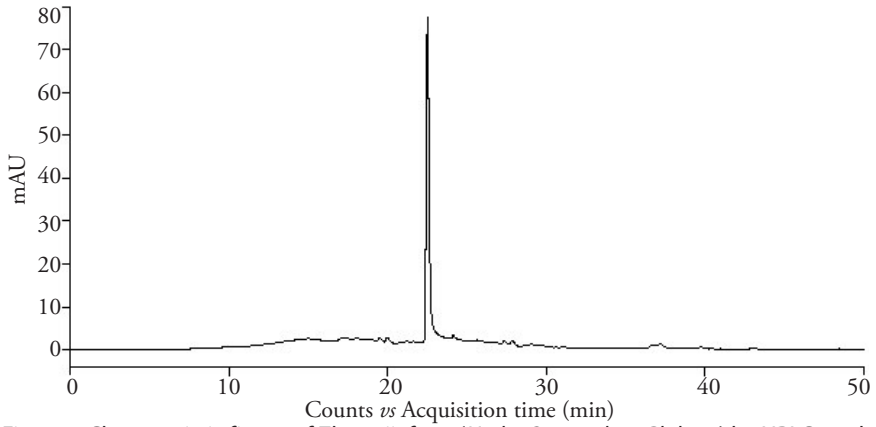

Figure 1 Characteristic figure of Zhongjiefeng (*Herba Sarcandrae Glabrae*) by HPLC method
HPLC: high performance liquid chromatography. The retention time of hydrolysis products was 21 min.

mice of the flavone group received daily lavage with 0.2 g/kg of flavone. Mice of the normal control group and BMF control group received daily lavage with the same volume of saline. All lavage interventions continued for 3 consecutive days.

Platelet count and preparation for washed platelet

After 3 days of lavage, 10% of chloral hydrate was intra-abdominally administered with a concentration of 0.005 mL/g for anesthesia. Blood was then drawn from the tail vein for complete blood count by using the auto-analyzer (Sysmex XE-2100, Sysmex Co., Ltd., Kobe, Japan). Platelet rich plasma (PRP) was obtained by combining 1 mL of whole blood with 7ml of ACD (2.5% sodium citrate, 2.0% glucose, and 1.5% citric acid) and centrifuged at 1300 rpm for 20 min (FRESCO 17 centrifuge, Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA). Modified Tyrode's buffer (pH 7.4; 2.5 mM Hepes, 150 mM sodium chloride, 2.5 mM potassium chloride, 1mM calcium chloride, 1 mM magnesium chloride, 12 mM sodium bicarbonate and 5.5 mM glucose) were used for re-suspension of PRP. Washed platelet suspension was then obtained and the cell counter chamber was used for platelet counting. The washed platelet suspension was then diluted to a concentration of 3×10^8/mL and rested at the room temperature for 60 min.

Flowcytometry assay

Platelet $\Delta\Psi$m, Cyt C, PS and Ca^{2+} were examined by flowcytometry (CYTOMICS FC 500, Beckman Coulter Co., Ltd, Brea, CA 92821, USA).

Western blotting

Protein expression of Bak, Bax, caspase-3, caspase-8, and caspase-9 were examined by western blotting by using β-actin as internal control. Protein electrophoresis was performed by using Mini Protein 3 Cell (Bio-Rad Co., Ltd., Hercules, California, USA). BandScan software was used for analyzing the signal intensity of each protein band and calculating the level of expression.

Statistical analysis

All data are presented as mean ± standard deviation ($\bar{x} \pm s$). Data analysis was made by SPSS software (SPSS Inc., Chicago, IL, USA). Quantitative data was compared by using the test whereas categorical data were compared by χ^2 test. Multi-group comparison was performed by one-way analysis of variance. Less than 0.05 was considered as statistically significant.

RESULTS

Platelet count number

Compared with mice of the normal control group, the mice of the BMF group were marked by significantly less platelet count ($P < 0.05$). On the contrary, mice of the CSA and flavone lavage groups had significantly higher platelet count than mice of the BMF group ($P < 0.05$). Therefore, treatment of both CSA and flavone served to restore the platelet count in mice with bone marrow failure (Figure 2).

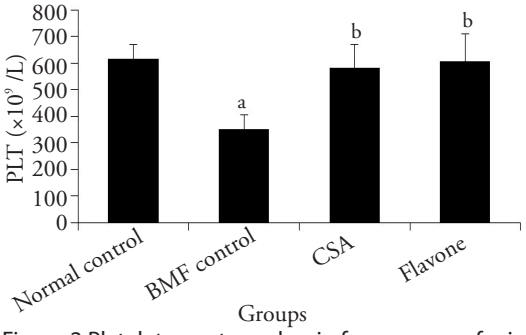

Figure 2 Platelet count number in four groups of mice
Normal control group and BMF control group received daily lavage with 0.1 mL/10 g saline solution. The CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA. The flavone group received daily lavage with 0.2 g/kg of flavone. All lavage interventions continued for 3 consecutive days. BMF: bone marrow failure; CSA: cyclosporine. $^aP < 0.05$, *vs* normal control, $^bP < 0.05$, *vs* BMF control.

Changes in mitochondrial transmembrane potential and biochemics

Mice of the BMF group significantly reduced $\Delta\Psi$m as compared with mice of the normal control ($P < 0.05$) whereas CSA and flavone treatments increase $\Delta\Psi$m. However, it was still significantly lower than the nor-

mal control ($P < 0.05$) (Figure 3). Levels of Cyt C, PS and Ca^{2+} were significantly higher in the BMF group *vs* control but they were all remarkably reduced in the CSA and flavone groups. More specifically, compared with the CSA group, the flavone group also had a higher level of Cyt C, a lower level of PS and a higher level of Ca^{2+} (Figures 4-6) (both $P < 0.05$).

Pro-apoptotic protein expressions

Compared with normal control, BMF mice had significantly higher levels of Bak, Bax, and lower levels of caspase-3, caspase-8 as well as caspase-9 expression (all $P < 0.05$) (Figure 7). Treatment of both CSA and flavone reduced Bak and Bax levels significantly compared with BMF mice. However, flavone treatment still showed higher expression than CSA ($P < 0.05$). BMF mice also had significantly lower expression of caspase proteins than normal control whereas treatment of both CSA and flavone increased these levels. Flavone shows less potent effect than CSA in increasing these protein expressions (all $P < 0.05$).

DISCUSSION

This study demonstrates a platelet protective effect by flavone subtracted from Zhongjiefeng (*Herba Sarcandrae Glabrae*) through inhibiting mitochondrial apoptotic pathway in mouse immune-induced bone marrow failure. Flavone significantly restores the platelet number, mitochondrial transmembrane potential and modulate pro-apoptotic protein expressions.

This finding is of great significance as it indicates a new drug candidate. Thrombocytopenia in BMF is associated with an increased risk of hemorrhage, and is a thorny problem in the clinic.[11] Current treatments of

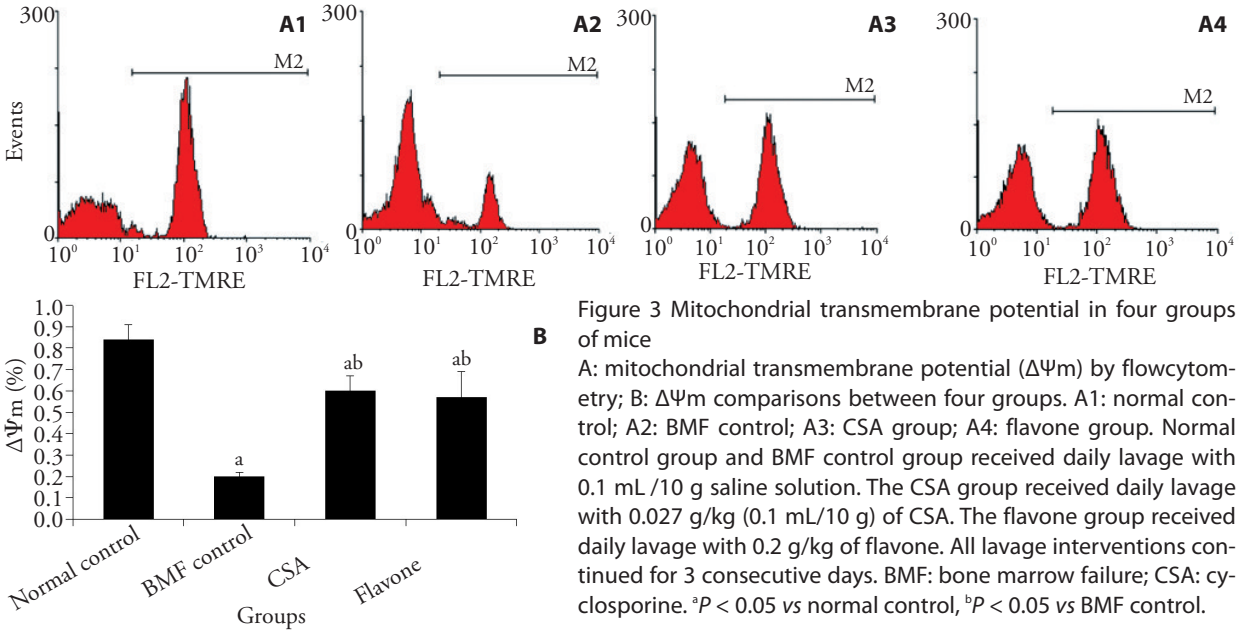

Figure 3 Mitochondrial transmembrane potential in four groups of mice

A: mitochondrial transmembrane potential (ΔΨm) by flowcytometry; B: ΔΨm comparisons between four groups. A1: normal control; A2: BMF control; A3: CSA group; A4: flavone group. Normal control group and BMF control group received daily lavage with 0.1 mL /10 g saline solution. The CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA. The flavone group received daily lavage with 0.2 g/kg of flavone. All lavage interventions continued for 3 consecutive days. BMF: bone marrow failure; CSA: cyclosporine. [a]$P < 0.05$ *vs* normal control, [b]$P < 0.05$ *vs* BMF control.

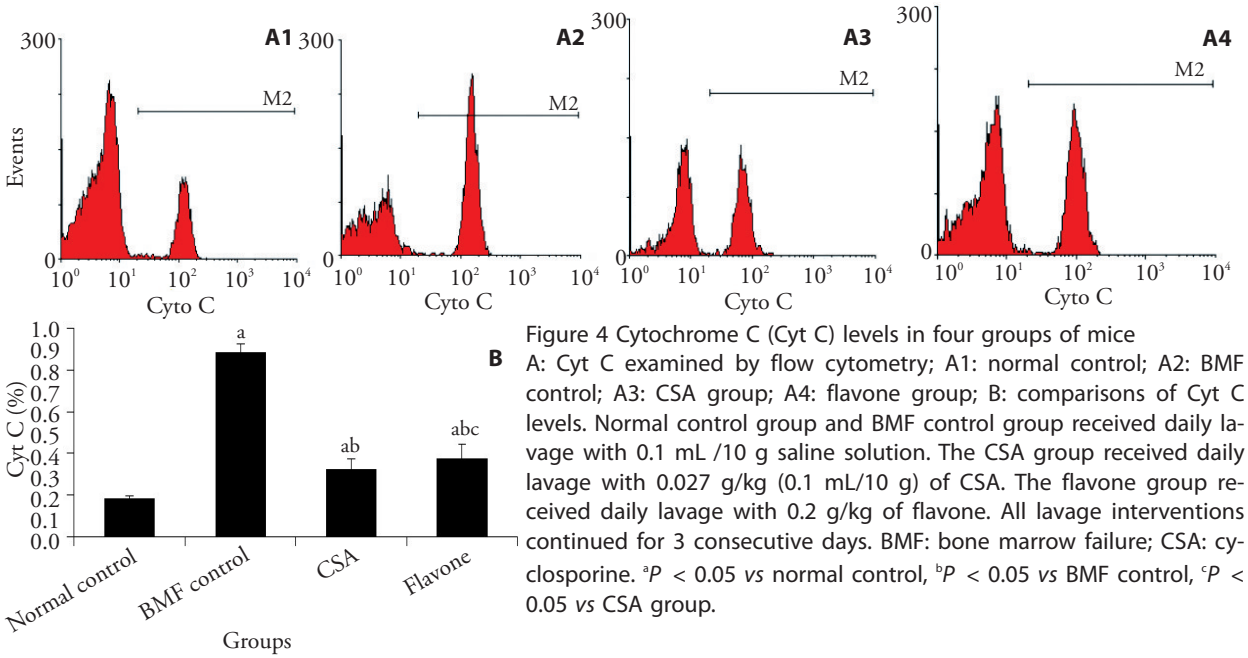

Figure 4 Cytochrome C (Cyt C) levels in four groups of mice

A: Cyt C examined by flow cytometry; A1: normal control; A2: BMF control; A3: CSA group; A4: flavone group; B: comparisons of Cyt C levels. Normal control group and BMF control group received daily lavage with 0.1 mL /10 g saline solution. The CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA. The flavone group received daily lavage with 0.2 g/kg of flavone. All lavage interventions continued for 3 consecutive days. BMF: bone marrow failure; CSA: cyclosporine. [a]$P < 0.05$ *vs* normal control, [b]$P < 0.05$ *vs* BMF control, [c]$P < 0.05$ *vs* CSA group.

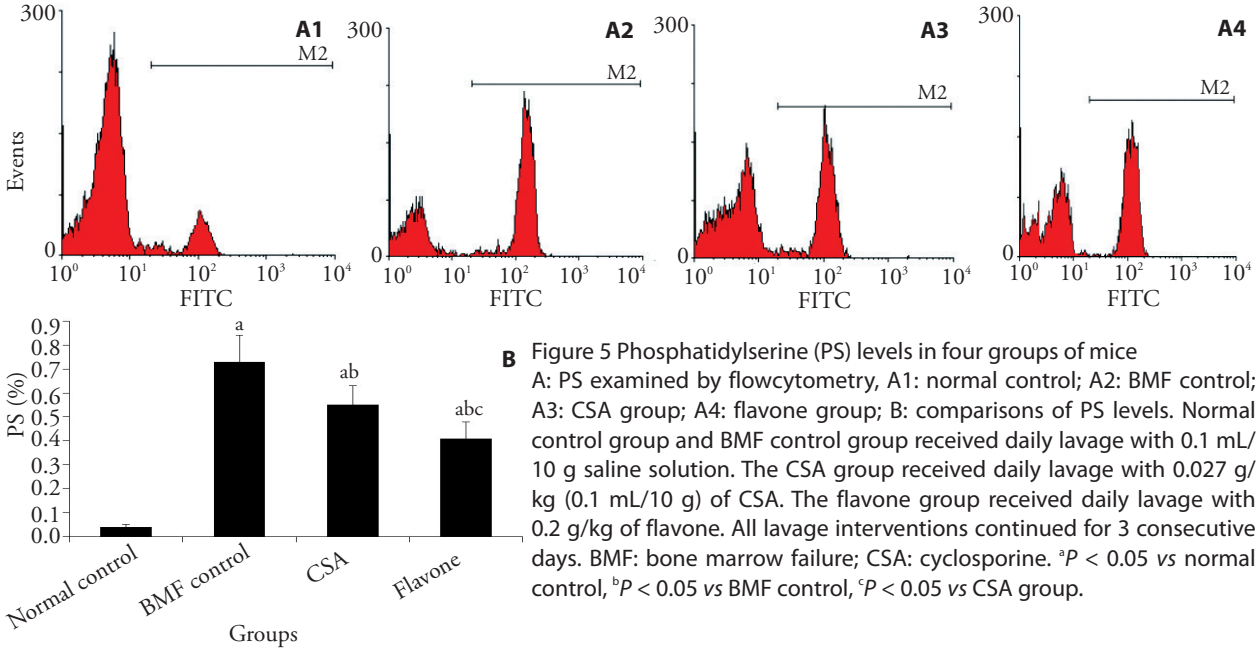

Figure 5 Phosphatidylserine (PS) levels in four groups of mice
A: PS examined by flowcytometry, A1: normal control; A2: BMF control; A3: CSA group; A4: flavone group; B: comparisons of PS levels. Normal control group and BMF control group received daily lavage with 0.1 mL/10 g saline solution. The CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA. The flavone group received daily lavage with 0.2 g/kg of flavone. All lavage interventions continued for 3 consecutive days. BMF: bone marrow failure; CSA: cyclosporine. $^{a}P < 0.05$ *vs* normal control, $^{b}P < 0.05$ *vs* BMF control, $^{c}P < 0.05$ *vs* CSA group.

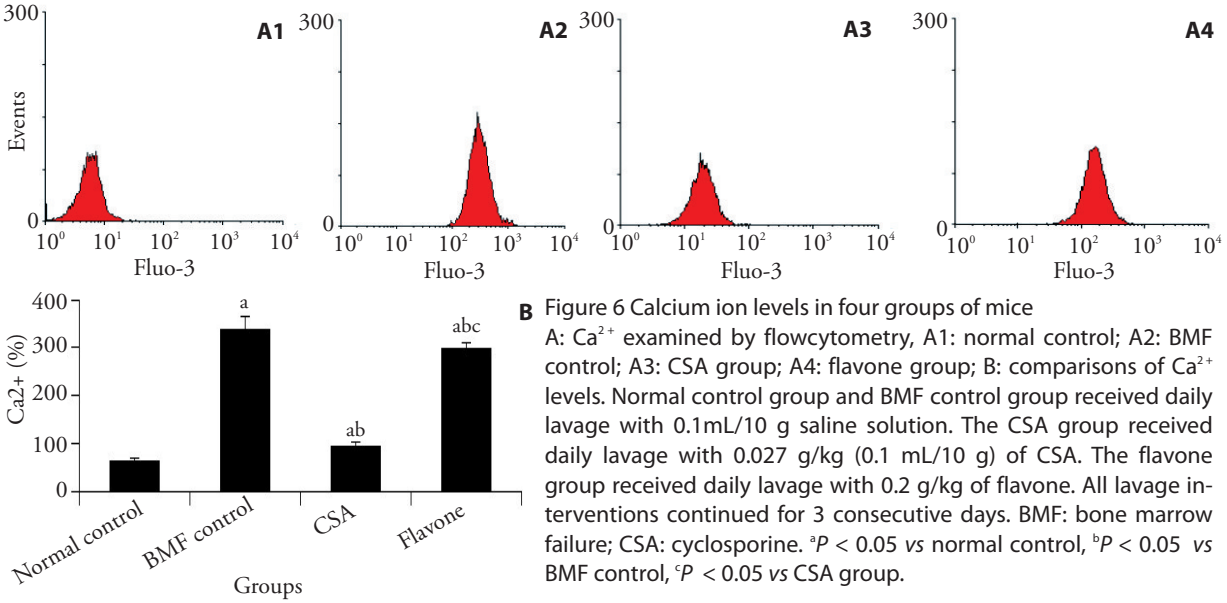

Figure 6 Calcium ion levels in four groups of mice
A: Ca^{2+} examined by flowcytometry, A1: normal control; A2: BMF control; A3: CSA group; A4: flavone group; B: comparisons of Ca^{2+} levels. Normal control group and BMF control group received daily lavage with 0.1mL/10 g saline solution. The CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA. The flavone group received daily lavage with 0.2 g/kg of flavone. All lavage interventions continued for 3 consecutive days. BMF: bone marrow failure; CSA: cyclosporine. $^{a}P < 0.05$ *vs* normal control, $^{b}P < 0.05$ *vs* BMF control, $^{c}P < 0.05$ *vs* CSA group.

thrombocytopenia include the transfusion platelet and γ-globin and glucocorticoid therapy. However, blood transfusion, commonly used in patients with severe thrombocytopenia, is limited to blood supply, and more importantly, it can induce platelet antibodies which hamper the treatment efficacy. γ-globin transfusion is expensive and glucocorticoid therapy has multiple side-effects. Therefore, searching for new therapy approach is of great importance. Abnormality in the platelet number and function is the leading cause for platelet-associated diseases.[12] Recent research has shown that platelet apoptosis is regulated mainly through the endogenous pathway.[13] As also shown by previous studies, mice with immune-induced bone marrow failure had peripheral cytopenias including thrombocytopenia, accompanied with reduction in apoptotic protein expressions such as caspase-8 and caspase-3, suggesting the etiology of thrombocytopenia in BMF is related to apoptosis modulation.[14,15] Current research has drawn much attention targeting at platelet apoptosis pathway in hope of new drug invention,[16] in which Traditional Chinese Medicine could be a novel source. In TCM, platelet-associated diseases are treated by "clearing heat, cooling blood and hemostasis". Zhongjiefeng (*Herba Sarcandrae Glabrae*), as this type of TCM, is widely used in treating hemorrhage diseases including macules due to blood heat-rash, rheumatic arthralgia, and traumatic injury. Our study demonstrates a platelet protective effect of flavone, which is the effective component from Zhongjiefeng (*Herba Sarcandrae Glabrae*).

We have also demonstrated that the effect of flavone is through inhibiting platelet apoptosis via mitochondrial pathway. It restores $\Delta\Psi m$ level, reduces the translocation of pro-apoptotic proteins, hampers the release of Cyt C from mitochondrial intramembrane space to cytoplasm and the lysis of cytoskeleton, and finally, inhibit platelet apoptosis. The effect of flavone is close to

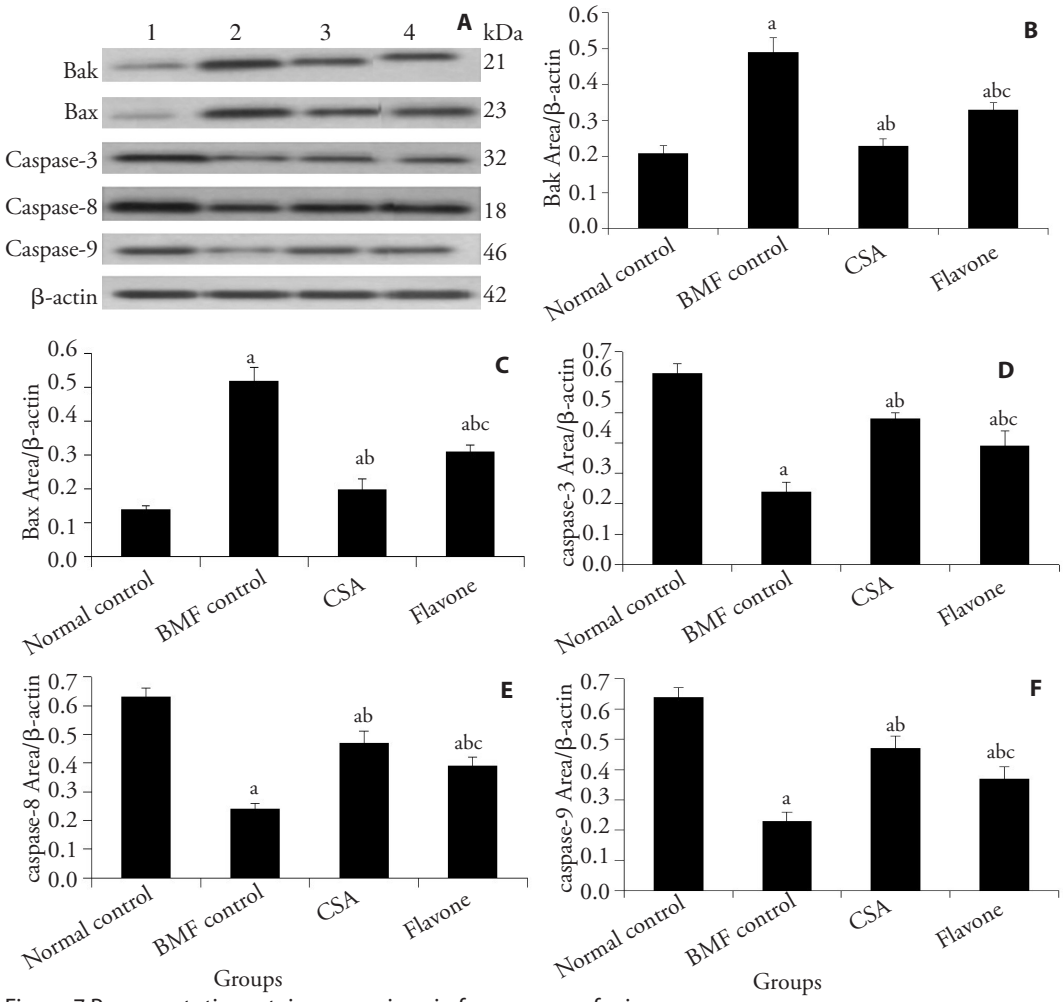

Figure 7 Pro-apoptotic protein expressions in four groups of mice

A: blots of pro-apoptotic protein expressions in each group; B-F: bar graph showing the expressions of Bak, Bax, caspase-3, caspase-8, caspase-9, respectively. 1: normal control; 2: BMF control; 3: CSA group; 4: flavone group. Normal control group and BMF control group received daily lavage with 0.1 mL/10 g saline solution. The CSA group received daily lavage with 0.027 g/kg (0.1 mL/10 g) of CSA. The flavone group received daily lavage with 0.2 g/kg of flavone. All lavage interventions continued for 3 consecutive days.Bak: B-cell lymphoma-2 (bcl-2) homologous antagonist-killer protein; Bax: bcl-2-associated X protein; BMF: bone marrow failure; CSA: cyclosporine. $^{a}P < 0.05$ *vs* normal control, $^{b}P < 0.05$ *vs* BMF control, $^{c}P < 0.05$ *vs* CSA group.

that of CSA, indicating that they may share similar mechanism to exert their therapeutic effect on treating immune-induced BMF.

In conclusion, flavone increases the platelet number and prevents its apoptosis in immune-induced bone marrow failure *via* the mitochondrial pathway. Future study with other animal models or randomized control trials in patients with bone marrow failure could further elucidate the usefulness and weakness of flavone application in clinical setting.

REFERENCES

1 **Nakao S**. Diagnostic problems in acquired bone marrow failure syndromes. Int J Hematol 2016; 104(2): 151-152.

2 **Valery L**. Apoptosis in the anucleate platelet. Blood Rev 2012; 26(2): 51-63.

3 **Leytin V**, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. Lab Invest 2009; 89(4): 374-384.

4 **Wang ZC**, Cai F, Chen XY, Luo MH, Hu LL, Lu Y. The role of mitochondria-derived reactive oxygen species in hyperthermia-induced platelet apoptosis. PLoS One 2013; 8 (9): e75044.

5 **Li X**, Zhang YF, Zeng Xing, Yang L, Deng Y. Chemical profiling of bioactive constituents in Sarcandra glabra and its preparations using ultra-high-pressure liquid chromatography coupled with LTQ Orbitrap mass spectrometry. Rapid Commun Mass Spectrom 2011; 25 (17): 2439-2447.

6 **Tang XL**, Huang LX, Zeng ZJ, Zhang QY, Shan YM, Xu GL. Effects of flavonoids of sarcand glabrain on the expansion of mature megakaryocytes and colony forming unit-megakaryocyte *in vitro*. Zhong Guo Shi Yan Fang Ji Xue Za Zhi 2010; 16(1): 79-82.

7 **Zhong L**, Liu T, Chen Y, et al. The study on effect of Sarcandra glabra on prevention and treatment of thrombocytopenia by chemotherapy. Zhong Yao Cai 2005; 28(1): 35-38.

8 **Zhang WQ**, Su M, Chen Q. Synergistic effects and decreasing toxicity on the total flavonoids of Herba Sarcandrae for S180 mouse sarcoma treated by Cyclophosphamide. Zhong Guo Yi Yao Dao Bao 2011; 8(31): 17-18.

9 **Xu GL**, Xiao BH, Zou HB, Chen Q. Separation of total flavone in Sarcandra glabra by macroporous adsorption resins. Zhong Cao Yao 2006; 37(7): 1014-1016.
10 **Liu HT**, Zhao JM, Chu JX. Experimental study of low dose irradiation for treatment of immuno-mediated aplastic anemia in mice. Zhong Guo Shi Yan Xue Ye Xue Za Zhi 2007; 15(3): 510-514.
11 **Yamazaki H**. Acquired aplastic anemia. Rinsho Ketsueki 2016; 57(2): 91-97.
12 **Wang B**, Zheng JS. Platelet generation *in vivo* and *in vitro*. Springerplus 2016; 5(1): 787.
13 **Winkler J**, Rand ML, Schmugge M, Speer O. Omi/HtrA2 and XIAP are components of platelet apoptosis signalling. Thromb Haemost 2013; 109(3): 532-539.
14 **Satyamitra M**, Ney P, Graves JI, Mullaney C, Srinivasan V. Mechanism of radioprotection by δ-tocotrienol: pharmacokinetics, pharmacodynamics and modulation of signalling pathways. Br J Radiol 2012; 85(1019): e1093-e1103.
15 **Chen J**, Desierto MJ, Feng X, Biancotto A, Young NS. Immune-mediated bone marrow failure in C57BL/6 mice. Exp Hematol 2015; 43(4): 256-267.
16 **Jagadish S**, Rajeev N, NaveenKumar SK, et al. Platelet protective efficacy of 3, 4, 5 trisubstituted isoxazole analogue by inhibiting ROS-mediated apoptosis and platelet aggregation. Mol Cell Biochem 2016; 414(1-2): 137-151.