Pakistan J. Zool., vol. 51(5), pp 1927-1935, 2019. DOI: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1927.1935

Recovery of Chemotherapy-induced Anemia by Angelica Polysaccharides in C57BL/6N Mice and Elucidation of Underlying Mechanism

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ABSTRACT

Angelica polysaccharides (APs) has been used for many years in clinical practice and in blood invigoration. In this article, we want to elucidate the molecular mechanism underlying the effects of APs in the recovery of anemia. Red blood cell count and hemoglobin level in peripheral blood were measured using a fully automatic blood cell counter. Flow cytometry was performed to detect the changes in red blood cell counts and number of T cells in peripheral blood and the spleen. Western blotting was performed to detect Stat5, Bcl-2, and Cyclin D1 protein expression in peripheral blood. The red blood cell count and hemoglobin levels decreased in 5-Fu group compared with the control group ($P < 0.05$). 5-Fu+APs group showed a marked increase in the number of erythrocytes and a significant increase in hemoglobin content ($P < 0.05$). Compared with the control group, 5-Fu group showed a significant decreased in the number of CD71/Ter119-labeled erythrocytes ($P < 0.001$) and CD4/CD8-labeled T cells ($P < 0.001$) in peripheral blood and the spleen of mice, which was restored significantly in the number of CD71/Ter119-labeled erythrocytes and CD4/CD8-labeled T cells in 5-Fu+APs group ($P < 0.001$). Compared with the control group, 5-Fu group showed significantly downregulated Cyclin D1 ($P < 0.001$), Stat5 ($P < 0.01$), and Bcl-2 ($P < 0.05$) expression in peripheral blood cells, and it was restored in 5-Fu+APs group ($P < 0.05$). APs promoted erythrocyte proliferation and increased hemoglobin content. The possible mechanism is by increasing Cyclin D1, Stat5 and Bcl-2 protein expression.

Article Information
Received 18 March 2019
Revised 12 May 2019
Accepted 03 June 2019
Available online 26 July 2019

Authors' Contribution
MD designed the study and supervised the study. TZ, ZL, BY and PH performed the experiments. QG, HZ and YY helped in the experimental work.

Key words
APs, Hemoglobin content, Flow cytometry, Red blood cells, Western blotting.

INTRODUCTION

Chemotherapy refers to the use of cytotoxic drugs such as cyclophosphamide, 5-fluorouracil, mitomycin, and other anti-tumor drugs to treat malignant tumors. These drugs have poor selectivity and act on normal cells as well, especially bone marrow hematopoietic cells, resulting in inhibition and killing of these cells, and consequently bone marrow suppression. The other side effects of chemotherapy include decreased red blood cell, white blood cell, and platelet counts, nausea, vomiting, loss of appetite, and anemia.

The incidence of anemia in patients with malignant tumor is as high as 50%. Anemia can cause injury to organs and tissues of the whole body and aggravate the original disease. These problems are predominant in advanced tumors and patients undergoing chemoradiotherapy.

Previous studies have shown that chemotherapy and the cause of disease itself can induce and aggravate the occurrence of cancer anemia during cancer development (Groopman and Itri,1999; Verbeke *et al*., 2012; Steinmetz *et al*., 2011). Cancer anemia can be an independent factor affecting the quality of life and survival of cancer patients (Zhang *et al*., 2017).

In traditional Chinese medicine, anemia belongs to the category of "deficiency," and often includes blood deficiency, Qi deficiency, Yin deficiency, and Yang deficiency. Qi and blood are interdependent, and a balance between these two should be maintained. Therefore, the primary treatment for anemia is to improve blood levels.

Angelica sinensis is a traditional medicine that has been used for more than 2000 years in China (Sawadogo *et al*., 2012). *Angelica* polysaccharides (APs), a bioactive constituent of *Angelica*, have been shown to be responsible for maintaining blood levels (Igor *et al*., 2013; Shi *et al*., 2005). Li *et al*. (2016) showed that *A. sinensis* (Oliv.) *Diels* polysaccharide can promote the proliferation of hematopoietic cells by stimulating the expression of cell

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Abbreviations used: Con, control; 5-Fu, Fluorouracil; APs, *Angelica* polysaccharide; EPO, Erythropoietin; PBS, phosphate-buffered saline

cycle protein D2. Cao and Huang (2013) showed that *A. sinensis* (Oliv.) *Diels* polysaccharide plays a role in many important biological activities, such as hematopoietic, immunoregulatory, anti-tumor, antioxidation, radiation protection and hypoglycemic effects.

To date, no study has investigated the mechanism by which APs increased erythrocyte count and enhanced immune. In this study, the effects of APs on the number of red blood cells, hemoglobin content, cell surface markers, molecular signaling pathways, and cell proliferation were determined in C57BL/6N mice. The effects of APs in the treatment of cancer provide experimental basis for its role in the recovery of anemia.

MATERIALS AND METHODS

EPO was selected as the positive control to investigate the prevention and treatment effect of APs on cancer anemia caused by 5-Fu. Before 5-Fu was given, C57BL/6N mice in the drug group were given EPO and APs for 7 days, and then 5-Fu was given at the same time for 15 days.

Drugs

Angelica polysaccharide (APs) was purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). 5-Fluorouracil (5-FU; Shanghai Xudong Haipu Pharmaceutical Co., Ltd.) was purchased from the First Hospital of Jilin University. Dilute APs with PBS solution to a concentration of 480mg/mL.

Animals

Thirty-two, male, 11-week-old, C57BL/6N mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Beijing, China. The experiment was performed at room temperature (20 + 2) °C with 12:12 h light-dark cycle. The mice were provided sterilized drinking water and quantitative fixed-formula granular feed, and the experiments were performed in an ultra-clean platform to achieve a sterile environment. The mice were bred in a cage system with external bottle (Nexgen Company; size: 194 x 181 x 398 mm) for one week, and then randomly divided into four groups (control, 5-Fu, 5-Fu+EPO and 5-Fu+APs groups) of eight mice each.

Study design

No drug was administered to the control group (n=8). The 5-Fu group (n=8) was given an intravenous injection 0.2 mL of 5-Fu (150 mg/kg) for 15 days, once daily.

The 5-Fu+EPO group (n=8) was given EPO for 7 days (155IU/day,subcutaneous), then given EPO and 0.2 mL of 5-Fu (150 mg/kg) for 15 days at the same time once a day.

The 5-Fu+APs group (n=8) was given APs (480 mg/mL) was orally gavage administered every day (0.5 mL) for 7 days, then given APs and 0.2 mL of 5-Fu (150 mg/kg) for 15 days at the same time once a day.

Blood was collected from the caudal vein on days 3, 7, 9, 12, and 15, added to a test tube containing anticoagulant to determine the cell count (Fig. 1A).

The mice were weighed after administration. After 15 days, the mice were euthanized by cervical dislocation. Then, the body was dissected and the spleen was excised for analysis.

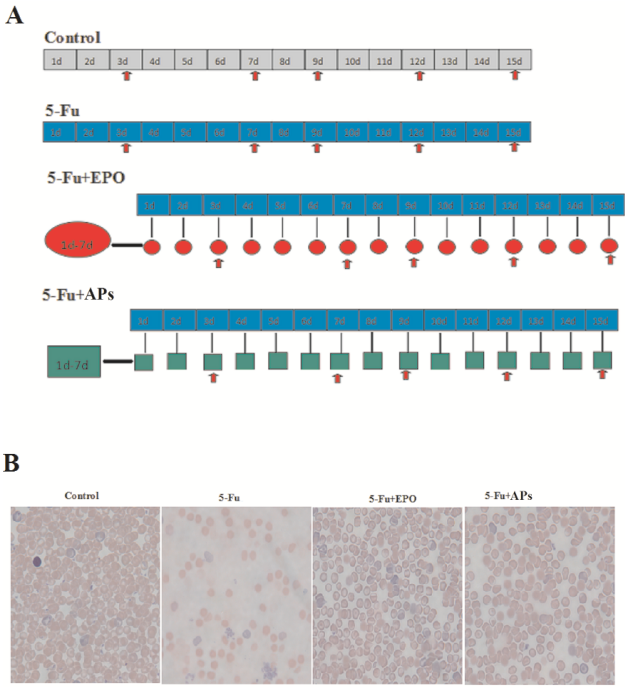

Fig. 1. Study design of this research (A) and blood smear with microscopic observation on the 15th day (B).

Erythrocyte count and hemoglobin content

One drop of peripheral blood was placed on Fisherbrand premium superfrost microscope slides (Catalog No. 12-544-7) on the 15th day and the 22th day, and a blood smear was made, stained. The cell form performance was examined under Fisherbrand AX-1100 series advanced research microscope [Catalog No. 11-350-116; (40X)].

The peripheral blood (60 μL) was collected and heparin was added to the test tube. After mixing completely, the heparin-blood sample was used to measure red blood cell count and hemoglobin content in peripheral blood by using automatic multi-species small animal blood analyzer (HEMAVE950FS, US) (Borsook *et al.*, 1962; Guo *et al.*, 2015).

Flow cytometric analysis of erythrocytes

Peripheral blood was added to the test tube containing anticoagulant. The red blood cells were cleaved using red blood cell lysis solution. The spleen was used to make a single cell suspension. The red blood cell lysis solution was added to cleave the cells.

The lysis reaction was stopped by adding phosphate-buffered saline (PBS), followed by centrifugation at 350 ×*g* (2–8°C) to discard the supernatant to obtain a clear solution. The solution was washed twice with PBS, centrifuged at 350 ×*g*, and the supernatant was discarded to obtain a clear solution. Then, the cells were collected.

Ter119 and CD71 expression levels on the surface of erythrocytes increase during erythropoiesis and maturation. Therefore, Ter119 and CD71 are commonly used as markers on the surface of red blood cells. FITC-Ter119 and PE-CD71 antibodies were added according to the manufacturer's protocol (Krutzik *et al*., 2005; Miroslav *et al*., 2011). The cells were incubated at room temperature for 20 min, followed by centrifugation at 350 ×*g* for 5 min. The supernatant was removed and the remaining solution was washed two times with 2 mL PBS. PBS (250 μL) was added to the upper part of the flow cytometer for detection of erythrocytes.

Flow cytometry analysis of T cells

Peripheral blood was added to the test tube containing anticoagulant. The red blood cells were cleaved using red blood cell lysis solution. The spleen was used to make a single cell suspension. The red blood cell lysis solution was added to cleave the cells.

The lysis reaction was stopped by adding PBS, followed by centrifugation at 300–400 ×*g* (2–8°C) to discard the supernatant to obtain a clear solution. Then, the solution was washed twice with PBS and centrifuged. The supernatant was discarded to obtain a clear solution, and the cells were collected.

CD4 and CD8 are commonly used as markers on the surface of T cells. FITC-CD4 and PE-CD8 antibodies were added according to the manufacturer's protocol (Aleksandr *et al*., 2017). The cells were incubated at room temperature for 20 min, followed by centrifugation at 1000 ×*g* for 5 min. The supernatant was removed and the remaining solution was washed two times with 2 mL PBS.

PBS (250 μL) was added to the upper machine detection for flow cytometry analysis. For histopathology analyses, the femurs were harvested, fixed in formaldehyde, decalcified, and paraffin embedded. The spleens were treated in the same way, except for the decalcification step. Then, 4.5-μm thick sections were cut and mounted on slides and stained with H&E.

Western blotting

According to the requirements of BCA protein determination kit (total protein extraction kit), the standard curve was plotted and the sample protein concentration was determined. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4% concentrated glue and 12% separation glue and transferred onto polyvinylidene difluoride (PVDF) membrane. Stat5, Bcl-2, and CylinD1 antibodies (Bioss, Beijing, China) (Chen, 2009) were incubated at 4 °C overnight. Then, they were placed on a shaking table at room temperature and incubated for 2 h, followed by rinsing with rinse buffer three times. Next, horseradish peroxidase (HRP)-labeled antibody was added and incubated for 2 h at room temperature, followed by rinsing three times. An electrochemiluminescence (ECL) kit was used to detect protein expression. The expression of proteins was measured using the relative gray values in the band (relative to β-actin as internal standard). Stat5 expression level, Bcl-2/β-actin as Bcl-2 expression level, and cylinD1/β-actin as CylinD1 expression level were measured.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 software, and the numerical value was expressed by $\bar{x}$ +s. ANOVA was used for comparison between groups, and the difference between $P<0.05$ and $P<0.01$ was statistically significant.

RESULTS

Before the experiment, there was no statistically significant difference in body weight between the groups.After the experiment,the weight in four groups (control, 5-Fu, 5-Fu+EPO and 5-Fu+APs groups) is 23.82g ± 1.21,20.95g ± 1.05,21.54g ± 1.02, 21.42g ± 1.14 respectively. There was no statistically significant difference in body weight between the groups.

Erythrocyte count and hemoglobin content

The experimental results showed that the number of red blood cells decreased and shrinking in the 5-Fu group compared with the control group. The number of red blood cells in the 5-Fu+APs group increased significantly compared with the 5-Fu group,and reduced compared with the 5-Fu+EPO group.The number of red blood cells in peripheral blood is shown in Figure 1B.

The experimental results showed that the number of red blood cells and the hemoglobin content were decreased significantly in the 5-Fu group on the 3th, 7th, 9th, 12th and 15th day when compared with the control group. The

number of red blood cells and the hemoglobin content were increased significantly on the 3th, 7th, 12th and 15th day of administration in the 5-Fu+EPO group compared with the 5-Fu group. The number of red blood cells and the hemoglobin content were increased significantly on the 7th, 9th, 12th and 15th day of administration in the 5-Fu+APs group compared with the 5-Fu group (Table 1 and Table II).

Table I.- Red blood cell statistics in peripheral blood (106/μl, $\bar{x}$ ±SD).

Group	n	Con	5-Fu	5-Fu +EPO	5-Fu +APs
d0	8	70.23±1.06	69.58±1.04	71.14±2.13	70.86±1.12
d7	8	68.25 ±1.15	48.32±1.03***	69.21±2.05	61.23±1.04###
d9	8	69.13±1.08	34.15±0.93***	68.09±2.13	58.49±1.25###
d12	8	71.28 ±1.06	30.86±0.88***	67.23±2.07###	55.09±1.12###
d15	8	72.34±1.24	26.58±0.86***	66.17±1.59###	52.0±2.36###

Note: * 5-Fu group compare with control group. *P<0.05, **P<0.01, ***P<0.001. # 5-Fu+EPO and 5-Fu+APs group compare with 5-Fu group. # P<0.05, # # P<0.01, # # # P<0.001.

Table II.- Hemoglobin content statistics in peripheral blood (g/L, $\bar{x}$ ±SD).

Group	n	Con	5-Fu	5-Fu +EPO	5-Fu +APs
d0	8	156.2±18.3	157.3 ±20.3	157.18±18.25	155.9±20.6
d3	8	153.6±19.5	148.6 ±19.6***	156.78±13.56###	154.76 ±19.8
d7	8	153.8 ±18.6	139.5± 17.8***	156.34±14.58##	152.62 ± 18.5
d9	8	155.2 ±19.3	131.8±16.7***	155.69±16.89	151.47 ±17.9###
d12	8	156.3 ±18.9	126.5±15.9***	154.10±17.56#	150.28 ± 18.3###
d15	8	157.9 ±20.2	120.3±15.3***	153.25±18.46###	149.2±17.8###

For statistcal details, see Table I.

Flow cytometry analysis of erythrocytes and T cells

Flow cytometry was used to assess the number of red blood cells labeled with CD71/Ter119 and T cells labeled with CD4/CD8 in the peripheral blood and spleen cells. Compared with 5-Fu group, the 5-Fu+EPO group and the 5-Fu+APs group showed a significant increase in the number of CD71/Ter119-labeled red blood cells in the peripheral blood and spleen (Fig. 2A and 2B; $P < 0.001$); The 5-Fu+EPO group and the 5-Fu+APs group showed a significant increase in the number of CD4/CD8-labeled T cells in the peripheral blood and spleen (Fig. 3A and 3B; $P < 0.001$). Histology examination of H&E stained the spleen sections of different group mice. Compare to the control group, the number of cell in 5-Fu group decreased and the cells are shriveled in shape. The number of cell in the 5-Fu+EPO group and the 5-Fu+APs group increased and the cells are circular in shaped (Fig. 3C). APs can prevent and restore cancer anemia through effects on the Cyclin D1 signaling pathway

To elucidate whether APs can prevent and restore cancer anemia through Cyclin D1 signaling (Li *et al.*, 2016) pathway, levels of Cyclin D1-Stat5 and Bcl-2 in different treatment groups were determined (Socolovsky, 2001). Compared with that in the 5-Fu group, Cyclin D1-Stat5 and Bcl-2 levels in 5-Fu+EPO group and 5-Fu+APs group decreased significantly. Overall, APs can prevent and restore cancer anemia through effects on the Cyclin D1 signaling pathway (Fig. 4).

DISCUSSION

The diverse biological effects of traditional Chinese medicines have gained much attention in modern disease research. In previous studies, APs has been found to have therapeutic effects in a variety of disease states (Shi *et al.*, 2005). Continuing this work in the present study, we revealed an additional potential use for APs in the recovery of anemia. Anemia is a common complication in cancer patients and it can lead to other diseases.

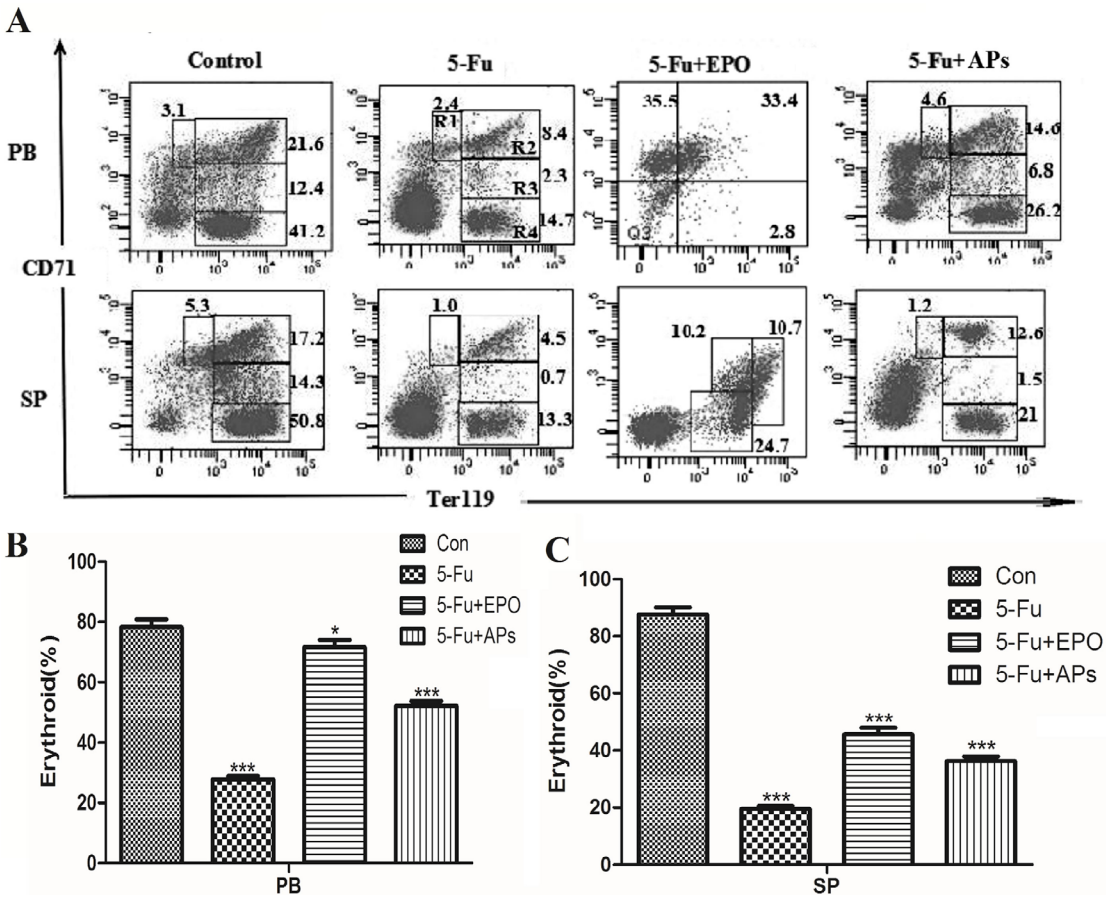

Fig. 2. CD71/Ter119-labeled red blood cells in control, 5-Fluorouracil (5-Fu), 5-Fu+EPO and 5-Fu+APs groups. Flow cytometric analysis of red blood cells in the peripheral blood and spleen, CD71 and Ter119 are the red blood cell surface marker protein (A); statistical analysis of CD71/Ter119-labeled red blood cells in peripheral blood (B); statistical analysis of CD71/Ter119-labeled red blood cells in spleen (C). Data are presented as the mean ± standard deviation; ***$P < 0.001$ versus the control group. PB, peripheral blood; SP, spleen.

C57BL/6N mice has been widely used to evaluate the cancer anemia (Chen *et al*., 2008). 5-Fu has been widely used to make the cancer anemia (Cao and Huang, 2013). For these reasons, we used C57BL/6N mice and 5-Fu for the therapeutic evaluation of APs. Similar to previous studies, we devided the mice into four groups, given an intravenous injection 0.2 mL of 5-Fu (150 mg/kg) for 15 days. This experiment revealed that APs can protect cancer anemia.

Red blood cells are the most common type of cells in the human body, and its main physiological function is to exchange oxygen and carbon dioxide through hemoglobin present in the cells. Erythrocyte count and hemoglobin content are widely used to evaluate the anemia (Cui and Fan, 2011). Anemia is a condition in which the amount of blood that circulates throughout the body is below normal levels. In China, the concentration of Hemoglobin in the peripheral blood of adult males is less than 120 g/L, in the peripheral blood of adult females is less than 110 g/L, and in the peripheral blood of pregnant women is less than 100 g/L, which is defined as anemia. The results showed that APs can increase the number of red blood cell and hemoglobin content.

There are several immune-related molecules on the cell membrane surface. CD71/Ter119 are the red blood cell surface marker and CD4/CD8 are T cell surface markers (Krutzik *et al*., 2005; Miroslav *et al*., 2011; Aleksandr *et al*., 2017). The results showed that APs can increase the number of red blood cell and improve body immunity.

Stat5 regulates the antiapoptotic protein Bcl-2, while the promoter regions of the cell cycle regulation factor Cyclin D1 can activate the hematopoietic growth factor-related genes, which result in the proliferation and differentiation of red blood cells (Qian *et al*., 2013; Li *et al*., 2014; Shila *et al*., 2015).

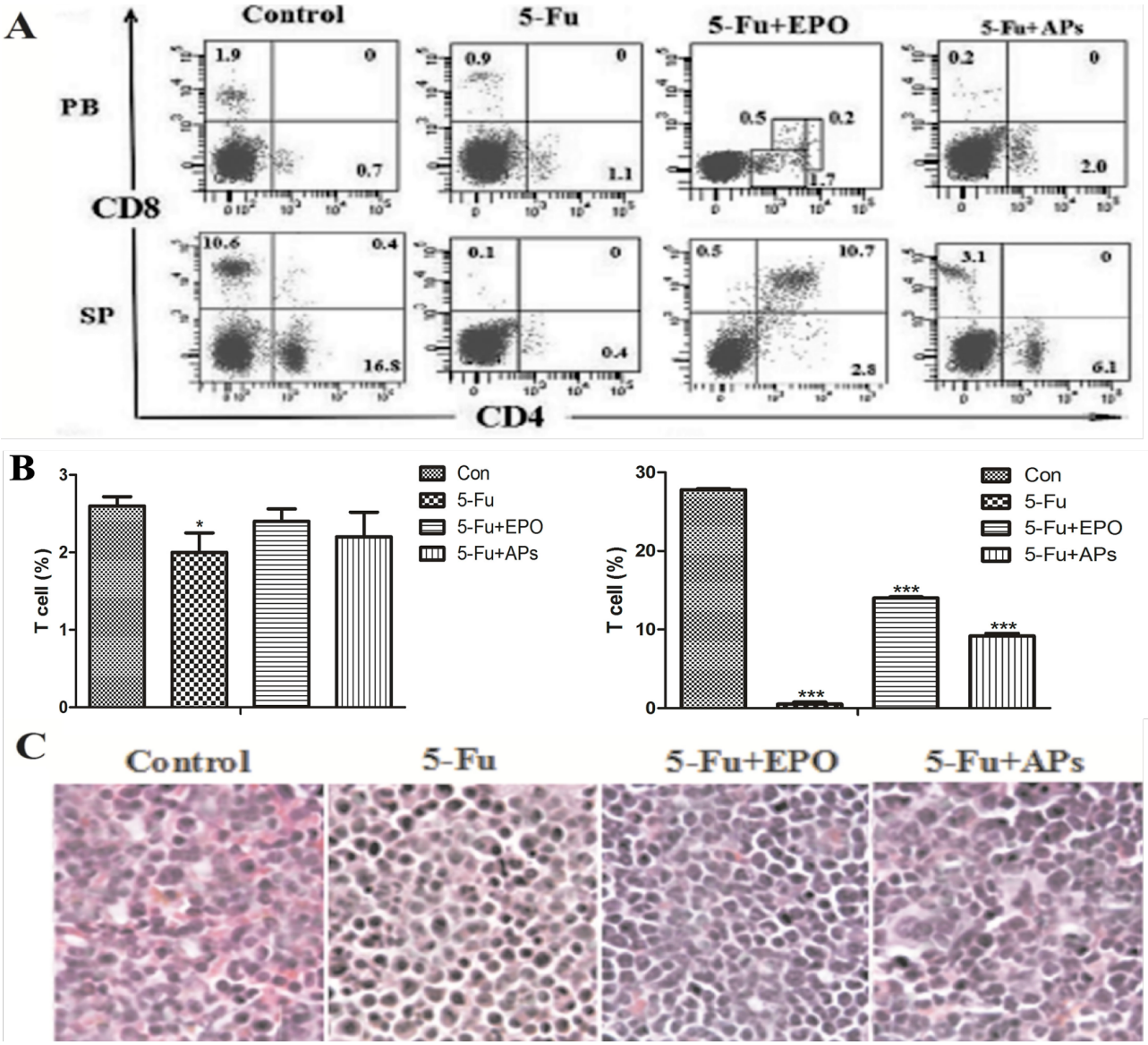

Fig. 3. CD4/CD8-labeled T cells in control, 5-Fluorouracil (5-Fu), 5-Fu+EPO and 5-Fu+APs groups. (A) Flow cytometric analysis of T cells in the peripheral blood and spleen. CD4 and CD8 are the T cell surface marker protein; (B) Left: statistical analysis of CD4/CD8-labeled T cells in peripheral blood; Right: statistical analysis of CD4/CD8-labeled T cells in spleen. (C) H&E staining of paraffin-embedded sections of spleen for control group, 5-Fu treatment group, 5-Fu +EPO group,and 5-Fu +APs group. Data are presented as the mean ± standard deviation; ***$P < 0.001$ versus the control group. PB, peripheral blood; SP, spleen.

The expression of Cyclin D1, Stat5, and Bcl-2 in peripheral blood cells of 5-Fu group was significantly downregulated compared with the control group. The expression levels of Cyclin D1, Stat5, and Bcl-2 in peripheral blood cells in 5-Fu+APs group significantly increased compared with the 5-Fu group. 5-Fu+APs group exhibited a good regulatory effect on the number of erythrocytes and on immune function in mice with chemotherapy-induced anemia. However, the mechanism needs to be investigated further. In future research, we plan to use Hela cells as a model to elucidate the mechanism of APs for treatment of anemia in cancer patients.

CONCLUSION

In conclusion, our research shows that APs can promote erythrocyte proliferation and increase hemoglobin content. APs can increased the number of red blood cells

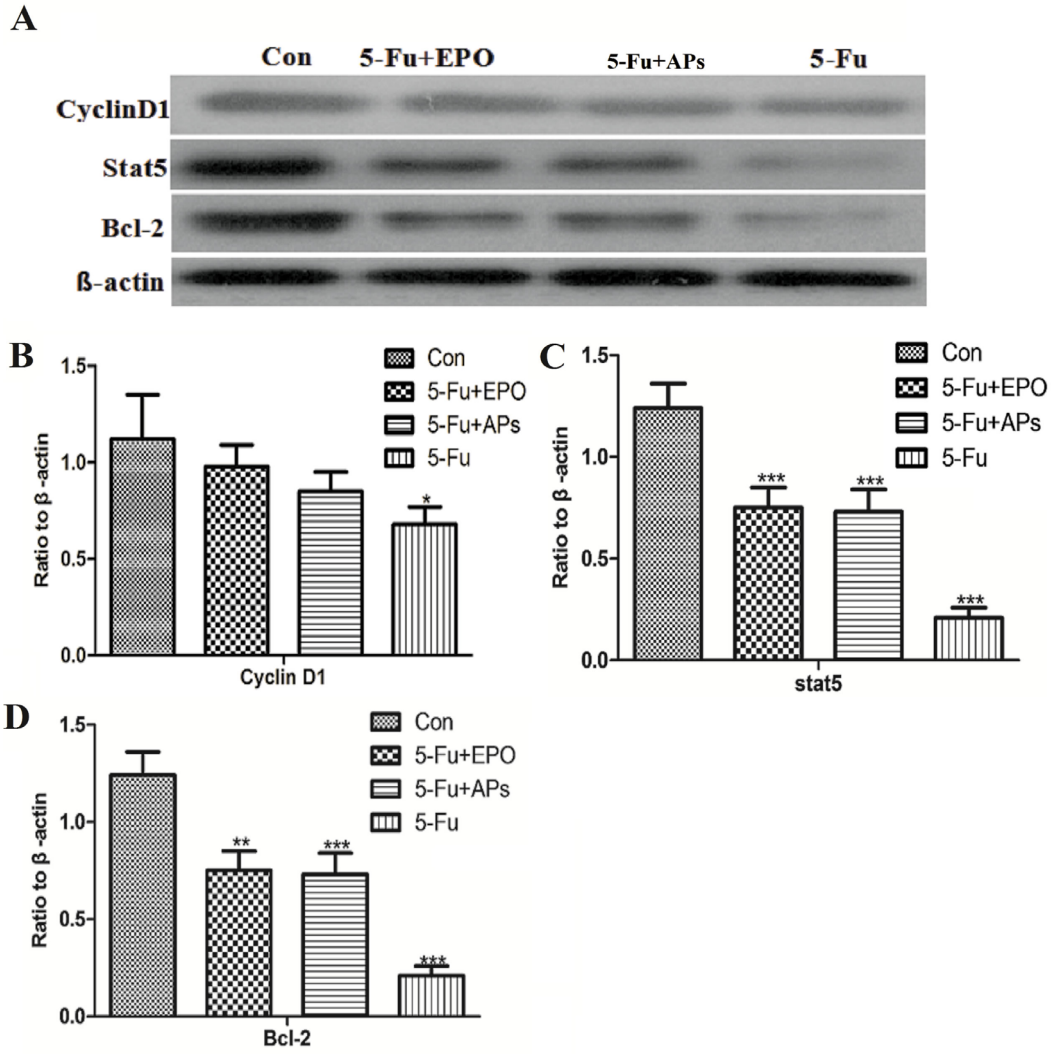

Fig. 4. Expression levels of cyclin D1, Stat5, and Bcl-2 in peripheral blood cells. (A) Western blot analysis of the expression of cyclinD1, Stat5, and Bcl-2 in control, 5-Fluorouracil (5-Fu), 5-Fu+EPO and 5-Fu+APs groups. (B-D) β-actin was used as the internal control for grayscale analysis. Data are presented as the mean ± standard deviation; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the control group.

and T cells. These changes are mediated by upregulation of the expression of Cyclin D1, Stat5, and Bcl-2 proteins. These results demonstrate potentially therapeutic roles for APs in preventing and restoring cancer anemia, and necessitate future studies investigating the utility of APs in cancer anemia.

ACKNOWLEDGEMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Statement of conflict of interest

The authors declare that there is no conflict of interests.

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