Effects and mechanisms of Bazhen decoction, Siwu decoction, and Sijunzi decoction on 5-fluorouracil-induced anemia in mice

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Abstract

OBJECTIVE: To investigate the effects of Bazhen decoction (BZD), Siwu decoction (SWD) and Sijunzi decoction (SJZD) in mice with anemia induced by 5-fluorouracil (5-FU) and discussed the possible pharmacological hematopoietic mechanism to provide experimental evidence for the clinical use of the three classical prescriptions in the treatment of anemia.

METHODS: Anemia was induced by intravenous injection of 5-FU and 80 female Kunming mice were randomly assigned to oral administration of SWD, SJZD, or BZD daily for 10 days. Peripheral blood cells count and bone marrow cell cycle were monitored to evaluate anti-anemia effects. Serum cytokines, interferon-γ (IFN-γ), interleukin-3 (IL-3), erythropoietin (EPO), granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) were assayed. EPO mRNA expression was assayed in kidney and liver tissue homogenates.

RESULTS: BZD and SWD significantly increased the number of red blood cells, hemoglobin concentration, and hematocrit, promoted bone marrow cells to enter the cell cycle, proliferate and differentiate, significantly increased IL-3 secretion, and significantly inhibited IFN-γ secretion. BZD stimulated transcription of EPO mRNA in the kidney and liver and enhanced serum EPO expression. A therapeutic effect of SJZD was not observed.

CONCLUSION: BZD and SWD treatment specifically enhanced hematopoietic function and mediated myelopoiesis by altering serum cytokines levels and accelerating entry of bone marrow cells into the cell cycle. Better curative effects were achieved via nourishing both Qi and blood (BZD) than by enriching the blood (SWD) or invigorating Qi alone.

Key words: Anemia; Blood-deficiency; Fluorouracil; Bazhen decoction; Siwu decoction; Sijunzi decoction

INTRODUCTION

In Traditional Chinese Medicine (TCM), blood deficiency is a syndrome with pathological changes such as pale/sallow complexion, brittle nails, dizziness, palpitation, numbness of the hands and feet, scanty and light-colored menses, pale tongue and weak pulse.
TCM theory, blood deficiency is related to the intricate balance of blood and Qi. In TCM, blood is the red fluid circulating through the blood vessels and nourishing the body tissues, a concept that corresponds in part to that of modern Western Medicine. Qi is the invisible basic element or energy that makes up the human body and supports vital activities of the human body. In brief, insufficient blood generates blood deficiency, subsequently resulting in Qi deficiency, which in turn aggravates blood deficiency, ultimately forming a vicious circle.

In recent decades, many practitioners of TCM have come to regard blood deficiency as similar anemia as defined in modern medicine. According to WHO criteria, anemia is defined as a hemoglobin (Hb) concentration < 13 g/dL in men and < 12 g/dL in women. The pathogenesis of anemia is multifactorial. For example, hematopoietic damage caused by 5-FU can be improved by prescription of blood tonics or drugs. TCM researchers develop animal models of blood deficiency by methods including radiation exposure, chemical agents, and immune modulation. Recent studies of blood deficiency syndrome have utilized a mouse model in which anemia is induced by 5-fluorouracil (5-FU). 5-FU is a antineoplastic, cell-cycle specific drug that is widely used to treat a variety of tumors including colorectal, breast and liver carcinomas. Following administration, 5-FU is converted to fluorouracil deoxyxynucleotide, which binds thymidine synthase, leading to disruption of RNA, DNA, and protein biosynthesis. However, 5-FU usually suppresses the bone marrow, and causes adverse gastrointestinal reactions because nucleic acid metabolism in tumors is similar to that in normal tissues. 5-FU administration at a dose of 150 mg/kg is known to result in long-term lesions of erythropoiesis and erythropoietin (EPO) production in which mature erythroid cells (reticulocytes, erythrocytes) and erythropoietic precursors (erythroid colony-forming units, erythroid burst-forming units) in the bone marrow were severely reduced.

TCM is an excellent example of alternative and complementary medicine with a long history, unique theory system, and a variety of herbal remedies. In TCM, enriching blood and balancing Qi and blood are the basic therapies for blood deficiency. Siwu decoction (SWD), Sijunzi decoction (SJZD), and Bazhen decoction (BZD), have been used in TCM clinical practice to treat blood deficiency, Qi deficiency and blood- and-Qi deficiency, respectively. It is reported that BZD promotes the proliferation of bone marrow cells of anemic mice, and that addition of BZD to the media in cultures of spleen cells, macrophages, lung and skeletal muscle resulted in strong stimulation of hematopoietic cells. Previous studies of SWD have shown that it affected the expression of apoptosis proteins, proliferation and differentiation of hematopoietic progenitor stem cells, ameliorated bone marrow damage after radiation. It has been proposed as an effective, nontoxic, orally administered agent for cancer chemoprevention via activation of the nuclear factor-erythroid 2-related factor-2 (Nrf2) pathway. Combined chemotherapy with SJZD plus low-dose mitomycin C significantly inhibited tumor growth in mice with bladder carcinoma, while attenuating toxicity attenuation and increasing efficacy. A comparison of the radioprotection conferred by SWD and SJZD found that SJZD and its ingredients were not as effective as SWD. The three decoctions are closely linked by TCM pharmacology, but comparative studies of the three formulae have not been conducted.

The aim of this study was to compare the anti-anemia effects of BZD, SWD and SJZD in mice with 5-FU-induced anemia and to investigate the hematopoietic mechanisms of these three TCM preparations. It is hoped that this preliminary laboratory data will support the scientific rationale for further clinical applications, reflect the modern Qi and blood concept in Chinese medicine.

Table 2: Effects of the three decoctions on hematologic parameters determined on day 15 (± ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>RBC (×10^12/L)</th>
<th>HGB (g/L)</th>
<th>WBC (×10^9/L)</th>
<th>HCT (%)</th>
<th>RC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.597±0.324</td>
<td>131.83±5.981</td>
<td>6.698±1.014</td>
<td>44.66±1.954</td>
<td>0.67±0.055</td>
</tr>
<tr>
<td>Model</td>
<td>5.438±0.244</td>
<td>83.5±9.731</td>
<td>2.91±0.438</td>
<td>28.68±3.544</td>
<td>0.09±0.029</td>
</tr>
<tr>
<td>BZD</td>
<td>6.986±0.728</td>
<td>106.33±8.981</td>
<td>4.03±0.560</td>
<td>35.33±3.075</td>
<td>0.27±0.042</td>
</tr>
<tr>
<td>SWD</td>
<td>6.897±0.623</td>
<td>109.6±8.620</td>
<td>3.9±0.328</td>
<td>33.6±4.665</td>
<td>0.14±0.034</td>
</tr>
<tr>
<td>SJZD</td>
<td>6.02±0.422</td>
<td>90.85±7.081</td>
<td>3.6±0.556</td>
<td>28.08±2.803</td>
<td>0.126±0.035</td>
</tr>
</tbody>
</table>

Notes: mice in the SWD, SJZD and BZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. Mice were sacrificed on day 15 and femur was aseptically removed for cell cycle analysis. After administrated with three decoctions for 10 days (0.2 mL/10 g body weight), about 100 μL of retro-orbital sinus blood was collected using EDTA-coated capillary tubes. RBC, HGB, WBC, HCT and RC were measured. BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction; RBC: red blood cell; HGB: hemoglobin; WBC: white blood cell; HCT: hematocrit; RC: reticulocyte. *P < 0.001, †P < 0.01, ‡P < 0.05 vs Model group; †P < 0.001 vs Normal group.
**MATERIALS AND METHODS**

**Materials**
The Traditional Chinese Medicines were supplied by the Traditional Chinese Medicine hospital in Beibei, Chongqing, China. Their Chinese names, Latin names, Plant scientific name and dose (g) used in three decoctions were presented in Table 1. BZD, SWD, and SJZD were crushed into small pieces, suspended in 1 L distilled water and washed twice for 30 min. The mixtures were filtered, and the filtrates were combined and concentrated to 2.0 g/mL using a routine method. The decoctions were stored at 4 °C until used.

5-Fluorouracil (250 mg/10 mL) was obtained from Jindecoctions were stored at 4 °C. The reagents were of analytical grade. All other reagents were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Taq polymerase and DNaseI (RNase free) were purchased from Promega (Beijing, China). M-Mulv Reverse Transcriptase Kits were supplied by Roche Pharmaceuticals Ltd. (Shanghai, China). TRIpure reagent was obtained from Dingguo Changsheng Biotech Co., Ltd. (Tianjin, China). All enzymes were of analytical grade.

**Animals and experimental protocols**
A total of 80 female Kunming mice, weighing (27 ± 2) g and 12 weeks of age, were supplied by the Laboratory Animal Center of Chongqing Medical University, Chongqing, China (Certificate of Conformity: 2002A 040). All animal studies were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals (1985) developed by the Council for International Organizations of Medical Sciences and relevant institutional guidelines. Mice were maintained in standard polypropylene transparent cages in a 12 h light/dark cycle and had free access to water and food. The study was approved by the experimental animal ethics committee of Southwest University of Traditional Chinese Medicine. After a 1 week acclimatization period, the 80 mice were randomly divided into five groups of 16 mice each using a table of random numbers. These were a normal group (Normal), anemia model group (Model), and anemia groups treated with Bzhen decoction (BZD), Siwu decoction (SWD) or SJunzi decoction (SJZD). The observation period was from day 0 to day 15. There was no significant between-group differences in the peripheral hemogram on day 0.5-FU at a dose of 150 mg/kg was intravenously administered to the four groups of anemic mice on day 5. Mice in the SWD, SJZD and BZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. Blood cell counts were obtained in all five groups on days 5, 10, and 15. All mice were sacrificed on day 15, and blood samples were collected for cytokine analysis. Bone marrow cells were harvested from both femurs for cell morphology and bone marrow cell-cycle assays. Kidney and liver tissues were stored in liquid nitrogen for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) assay.

**Hematology analysis**
Peripheral blood was drawn from the retro-orbital venous plexus on days 5, 10, 15 and collected in tubes containing EDTA. The red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), white blood cells (WBCs), and reticulocytes (RCs) in each sample were determined using an automatic analyzer.

**Bone marrow cell morphology and cell cycle analysis**
Mice were sacrificed on day 15 and both femurs were aseptically removed for assay of bone marrow cellular morphology and cell cycle analysis. Bone marrow obtained from the femurs was stained by Wright-Giemsa method for the morphological analysis, and the number of nucleated bone marrow cells was determined at high magnification (’400). For cell cycle analysis, bone marrow was carefully pipetted up and down in phosphate-buffered saline (PBS) until the cells had dispersed, forming a single cell suspension. The supernatant was discarded following centrifugation for 5 min at 1000 rpm at 4 °C. The precipitates were washed twice with PBS, fixed with 70% ethanol at 4 °C, washed twice with PBS, incubated with 10 mg/L RNase and 1 mL 50 mg/L propidium iodide at 4 °C for 30 min in the dark. The cell cycle assay was by flow cytometry, and the proliferation index (PI) was calculated as (S+G2/M)/(G0/G1+S+G2/M) ’100%.

**Cytokine assay**
Sera obtained from blood samples collected from the retro-orbital venous plexus on day 15 were in the cytokine assays. The concentrations of interferon-γ (IFN-γ), interleukin-3 (IL-3), erythropoietin (EPO), granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) in the serum were measured by enzyme-linked immunosorbent assay (ELISA).

**qRT-PCR assay of EPO transcription in kidney and liver tissue**
On day 15, the kidneys and liver were removed in an RNase-free environment and stored in liquid nitrogen until assayed for the expression of EPO mRNA by qRT-PCR technology. The sequences of the EPO primers were: upstream, 5′-GAGGCAAGAAAAAGTCACGATG-3′; downstream: 5′-CTTCCCACCTCATTCTTTTCCC-3′, and the PCR product size was 112 bp. The β-actin primers were: upstream, 5′-ATGGGATTAGGATATCGCT-3′; downstream 5′-ATGAGGTAATGCTGTCAGGT-3′, and the PCR product size was 569 bp.
Total RNA was extracted from tissues using TRIpure reagent following the manufacturer’s protocol, and 5 μL of each total RNA sample was reverse transcribed into cDNA using 1 μL of M-Mulv reverse transcriptase. PCR was performed using 2 μL of template cDNA, 1 μL forward primer and 1 μL reverse primer, 0.5 μL Taq polymerase, and 1 μL of dNTP mix. PCR amplification was carried out using a protocol with an initial denaturing step at 94 °C for 4 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. After amplification, 8 μL of each PCR product was fractionated on a 1% agarose gel and visualized by ethidium bromide staining. β-actin was used as internal control. The band intensity of ethidium bromide fluorescence was measured using Quantity One v4.62 gel analysis software from BioRad (Hercules, CA, USA). The expression of target genes was presented as the ratio of target to the β-actin control fluorescence intensity.

**Statistical analysis**

The results were expressed as means ± standard deviation (x ± s) and analyzed by SPSS v.20.0 statistical software (IBM, Chicago, IL, USA). Statistical significance was determined by one-way analysis of variance followed by the least significant difference test. P values < 0.05 and < 0.01 were considered significant and extremely significant, respectively.

**RESULTS**

**Effects of the three prescriptions on the peripheral hemogram**

As shown in Table 2, RBC, HGB, HCT, WBC and RC values were significantly lower in the Model group than in the Normal group. BZD and SWD treatment significantly increased RBC count, HGB concentration (P < 0.001), WBC count (P < 0.001 and P < 0.01 respectively), HCT (P < 0.01 and P < 0.05 respectively), and RC count (P < 0.001 and P < 0.01) compared with the Model group. The RC count on day 15 suggested that BZD-treated mice might have a hematopoiesis activity similar to that of normal mice, but the RC counts of BZD-treated mice did not reach a normal level. The RBC counts and HGB concentrations in each group (Figure 1) indicate that both BZD and SWD had a significant therapeutic effect in anemic mice, and that BZD achieved better therapeutic effects than SWD. However, SJZD increased only the levels of WBC (P < 0.05), and was not shown to significantly promote hematopoiesis in the anemic mice.

**Table 1 Compositions and dose of three decoctions**

<table>
<thead>
<tr>
<th>Chinese name</th>
<th>Latin name</th>
<th>Plant scientific name</th>
<th>BZD dose (g)</th>
<th>SWD dose (g)</th>
<th>SJZD dose (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danggui</td>
<td>Radix Angelicae Sinensis</td>
<td>Angelica sinensis (Oliv.) Diels</td>
<td>10</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Shudi</td>
<td>Radix Rehmannia Libouch</td>
<td>Rehmannia glutinosa (Gaertn.) DC.</td>
<td>15</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Baishao</td>
<td>Radix Paeoniae Alba</td>
<td>Paeonia sterniana H.R.Fletcher</td>
<td>8</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Chuanxiong</td>
<td>Rhizoma Chuanxiong</td>
<td>Ligusticum striatum DC.</td>
<td>5</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Fuling</td>
<td>Poria Cocos</td>
<td>Poria cocos wolf</td>
<td>8</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Gancao</td>
<td>Radix Glycyrrhiza</td>
<td>Glycyrrhiza uralensis Fisch.</td>
<td>5</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Baizhu</td>
<td>Rhizoma Astracylodis Macrocephala</td>
<td>Atractylodes macrocephala Koidz</td>
<td>10</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Dangshen</td>
<td>Radix Codonopsis</td>
<td>Codonopsis pilosula (Franch.) Nannf.</td>
<td>3</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 1 Effects of the three decoctions on RBC and HGB in each group on days 0, 5, 10, 15

A: RBC; B: HGB. Mice in the SWD, SJZD and BZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. Mice were sacrificed on day 15 and femur was aseptically removed for cell cycle analysis. BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction; RBC: red blood cell; HGB: hemoglobin. aP < 0.001, bP < 0.01 vs Model group.

**Effects of the three prescriptions on the proliferation of bone marrow cells**

The numbers of nucleated bone marrow cells in each
smear were counted at high magnification (× 400). As shown in Figure 2, myeloproliferative capacity was greatly suppressed in the Model group (P < 0.001 vs Normal), while the numbers of nucleated cells in the BZD and SWD groups indicated active bone marrow hyperplasia (both P < 0.001 vs Model). The results showed that BZD and SWD treatment promoted the recovery of myeloproliferative ability in anemic mice, however, SJZD did not have a significant effect on bone marrow hyperplasia (P < 0.001 vs Normal).

Effects of the three prescriptions on bone marrow cell cycle
Flow cytometry was used to assay bone marrow samples harvested on day 15. The effects on the bone marrow cell cycle in each experimental group are shown in Figure 3 and Table 2. The proportion of bone marrow cells in G0/G1 (the Model group, both BZD and SWD decreased the bone marrow hyperplasia (both P < 0.001 vs Model)), while the numbers of nucleated cells in the G2/M phase (P < 0.001 vs Normal), showed that BZD and SWD treatment promoted the G1-S phase and SG2-/M phase transitions.

Effects of the three prescriptions on transcription of EPO in kidney and liver tissue

Based on the finding that BZD affected the EPO concentration in serum, we assumed that it was able to promote the transcription of EPO. To confirm this, we assayed the expression of EPO mRNAs in kidney and liver tissue by qRT-PCR. Ten days after the injection of 5-FU, EPO mRNA expression had significantly decreased in liver (P < 0.05) and kidney (P < 0.001) tissue of mice in the Model group. As shown in Figures 5 and 6, BZD significantly increased the expression of EPO mRNA in the kidney and liver of the mice (both P < 0.01); SWD only up-regulated the expression of EPO mRNA only in kidney tissue (P < 0.01). However, SJZD did not increase EPO mRNA expression of the transcripts.

DISCUSSION

Based on the TCM theory of ‘Qi and blood’, SWD, SJZD and BZD, have been used to treat blood deficiency, Qi deficiency, and Qi-and-blood deficiency for more than 500 years even though their mechanisms of action have not been determined. Herein, we established a mouse model of blood deficiency induced by 5-FU, following methods used to induce anemia,24 to investigate possible mechanisms using a modern biomedical approach. The pharmacological action of 5-FU includes the impairment of DNA synthesis through the inhibition of thymidylate synthetase activity. Therefore, 5-FU is particularly toxic to actively proliferation tissues such as bone marrow, which is the “factory” where all blood cells are produced.25 The intravenous administration of 5-FU led to a dramatic decrease in RBC, WBC, and RC counts, HGB concentration, and HCT in the mice during the 10 day observation period. The changes in these routine blood indexes demonstrated that the hemopoiesis was inhibited or impaired, resulting in severe cytopenia. Consistent with the peripheral blood
Figure 3 Effects of the three decoctions on the bone marrow cell cycle on day 15
A: normal group; B: model group; C: BZD group; D: SWD group; E: SJZD group. Mice in the SWD, SJZD and BZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. Mice were sacrificed on day 15 and femur was aseptically removed for cell cycle analysis. Bone marrow cells were stained with propidium iodide and analyzed by flow cytometry. BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction.

Table 3 Percentage of bone marrow cells in different cell cycle phases (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>54.4 ± 8.8</td>
<td>30.6 ± 5.0</td>
<td>15.0 ± 4.3</td>
<td>45.6 ± 9.2</td>
</tr>
<tr>
<td>Model</td>
<td>69.3 ± 9.1</td>
<td>27.2 ± 3.2</td>
<td>3.5 ± 1.2</td>
<td>30.7 ± 5.9</td>
</tr>
<tr>
<td>BZD</td>
<td>51.9 ± 6.6</td>
<td>32.4 ± 5.6</td>
<td>15.7 ± 2.5</td>
<td>48.1 ± 6.6</td>
</tr>
<tr>
<td>SWD</td>
<td>52.5 ± 8.3</td>
<td>38.6 ± 6.5</td>
<td>8.9 ± 2.6</td>
<td>47.5 ± 8.3</td>
</tr>
<tr>
<td>SJZD</td>
<td>58.7 ± 9.9</td>
<td>29.3 ± 3.1</td>
<td>12.0 ± 3.6</td>
<td>41.3 ± 7.7</td>
</tr>
</tbody>
</table>

Notes: mice in the SWD, SJZD and BZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. Mice were sacrificed on day 15 and femur was aseptically removed for cell cycle analysis. Bone marrow cells in five groups were removed on day 15 after 10 days of treatment. Data are expressed as x ± s (n = 8). BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction; PI: proliferation-index. *P < 0.05, **P < 0.01, ***P < 0.001 vs model group; †P < 0.05, ††P < 0.001, †††P < 0.01 vs normal group.
Figure 4 Effects of the three decoctions on erythropoietin transcripts in liver and kidney
A: liver; B: kidney. 1: Normal group; 2: Model group; 3: BZD group; 4: SWD group; 5: SJZD group. Mice in the BZD, SWD, and SJZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. On day 15, the kidneys and liver were removed in an RNAse-free environment and assayed for the expression of EPO mRNA by qRT-PCR technology. The intensities of bands were normalized against β-actin intensity. BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction. EPO: erythropoietin. aP < 0.001, bP < 0.01, cP < 0.05 vs Model group.

Figure 5 Effects of the three decoctions on erythropoietin transcripts in liver and kidney
A: liver; B: kidney. Mice in the BZD, SWD, and SJZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction. On day 15, the kidneys and liver were removed in an RNAse-free environment and assayed for the expression of EPO mRNA by qRT-PCR technology. The intensities of bands were normalized against β-actin intensity.

Figure 6 Effects of the three prescriptions on erythropoietin transcripts in liver and kidney
A: liver; B: kidney. Mice in the BZD, SWD, and SJZD groups were given the decoctions by intragastric administration at a daily dose of 0.2 mL/10 g body weight for 10 days. Mice in the Normal and Model groups were given an equal volume of normal saline. On day 15, the kidneys and liver were removed in an RNAse-free environment and assayed for the expression of EPO mRNA by qRT-PCR technology. The intensities of bands were normalized against β-actin intensity. BZD: Bazhen decoction; SWD: Siwu decoction; SJZD: Sijunzi decoction. aP < 0.05, bP < 0.01, cP < 0.001 vs Model group.
data, injection of 5-FU reduced the cellular components of bone marrow and greatly reduced the myeloproliferative capacity of the mice. Evaluation of bone marrow smears confirmed that the number of nucleated cells in the Model group mice was significantly lower than that in Normal mice, which suggests depressed bone marrow proliferation. These observations confirmed that we successfully established a mouse anemia model. Oral administration of BZD and SWD was followed by remarkably improvement of RBC, WBC, and RC counts, HGB concentration, and HCT. At the same time, increase in the number of nucleated cells in the bone marrow of mice given BZD and SWD demonstrated active bone marrow hyperplasia. BZD was the most effective of the three decoctions in promoting the recovery or the enhancement of hemopoiesis.

The cell cycle distribution of bone marrow cells is an important indicator for evaluating hematopoietic system function and reflects the status of bone marrow cell proliferation. Most hematopoietic stem cells (HSCs), which originate in the bone marrow, are in G0 and temporarily or permanently out of the cell cycle. A minority are in the proliferative state.26 Hemopoiesis is sensitive to chemotherapeutic agents such as 5-FU, which usually damage the bone marrow and affect normal hematopoiesis, changing the proportions of cells in different phases of cell cycle. The DNA damage caused by chemotherapeutics prevents entry into the cell cycle and causes cell cycle arrest.27 Releasing bone marrow cells from G0 phase (first gap phase) arrest, promoting S-phase (DNA synthesis phase) entry, and progression to the G2 (second gap phase)/M (mitosis phase) phase are the important mechanisms to overcome the myelosuppression caused by chemotherapeutic drugs. The flow cytometry assay revealed that nearly 70% of the mouse bone marrow cells were arrested in G0 phase and had not entered into division and proliferation after treatment of 5-FU. It was noted that all three prescriptions promoted recovery from myelosuppression by accelerating the G0/G1-S phase and S-G2/M phase transitions and increased the PI of bone marrow cells. The findings indicated that BZD, SWD, and SJZD all stimulated bone marrow nucleated cells to enter the cell cycle and promoted the proliferation and differentiation of hematopoietic progenitor cells. However, SJZD did not promote the recovery of hematopoiesis in the bone marrow even though it promoted entry to the cell cycle, possibly owing to being unable to increase cytokine production. We conclude that BZD and SWD played vital roles in enhancing the proliferation of myeloid progenitors, thus having an anti-anemia effect via hematopoietic lineage development. Hematopoietic progenitor cells (HPCs), especially early HPCs with high proliferation potential, may not all be cycling (i.e., actively proliferating). HSCs and HPCs in G0 phase partly depend on the regulation of hematopoietic cytokines to enter the cell cycle. Thus, we assayed the effects of the three prescriptions on some cytokines that are thought to either suppress or stimulate immature erythroid cells.28 EPO, IL-3, and IFN-γ, have been reported to influence erythropoiesis.29-31 IL-3, a multipotent hematopoietic growth factor, plays a significant role in the regulation of hematopoietic function and immunology in vivo.2 EPO is an essential factor for the viability and proliferation of erythroid progenitors32 and for the production of red blood cells.22 IFN-γ, an erythroid cell suppressing cytokine in the bone marrow, can cause the early death of erythroid progenitor cells, thereby antagonizing the anti-apoptotic action of EPO.33-34 5-FU injection resulted in a significant decrease in EPO and IL-3 concentrations and an increase in IFN-γ concentration. SJZD did not improve abnormal cytokine secretion, but BZD and SWD contributed to improvement. BZD and SWD promoted erythropoiesis, possibly by inhibiting the secretion of IFN-γ and promoting the release of EPO and IL-3, which would be expected to improve the proliferation and differentiation of immature erythroid cells. These results suggest that BZD and SWD enhanced the production of hematopoietic factors and the proliferation of hematopoietic progenitor cells. The recovery of mice from 5-FU-induced anemia, indicates that BZD and SWD enhanced cytokine production by lymphocytes.

EPO is a glycoprotein hormone that regulates the production of red blood cells. It is primarily synthesized in the kidney and secreted by renal cortical interstitial cells in response to tissue hypoxia. A small amount of extra-renal EPO is produced by the liver. Recombinant human EPO has been widely used to treat anemia caused by chronic renal failure.28 In our experimental model, BZD greatly upregulated EPO mRNA expression in the liver and kidneys of myelosuppressed mice. BZD also promoted the synthesis and secretion of EPO and the formation of RBCs. Even though SWD significantly increased the expression of EPO mRNA in the kidney, the concentration of EPO in the serum of SWD-treated mice was lower than that observed in the Normal group. We thus infer that BZD could affect more factors related to the secretion and synthesis of EPO than SWD.

Our experimental results demonstrate establishment of a blood deficiency model that was useful in studying "Qi and blood theory", an important component of TCM.35 Qi is the Yang, whereas blood constitutes the Yin aspects of the body.3 Qi is the commander of blood, and blood is the mother of Qi. When humans or animals are in a state of blood deficiency, the lack of blood would lead loss of Qi and subsequent worsening of blood deficiency. With insufficient Qi, the organs cannot produce, move, or control the blood, ultimately resulting in Qi and blood deficiency. In brief, it is believed that Qi is the commander of blood, and blood is the mother of Qi. When humans or animals are in a state of blood deficiency, the lack of blood would lead loss of Qi and subsequent worsening of blood deficiency. With insufficient Qi, the organs cannot produce, move, or control the blood.
a representative prescription of a blood-supplementing formula, and BZD, which enriches both blood and Qi, had significant therapeutic effects on 5-FU-induced anemia in our mouse model. Associated with “Qi and blood theory”, promoting Qi could contribute blood production and relieve blood stagnation. Moreover, enriching blood could bring adequate Qi to the organs. SJZD could strengthen only the spleen and replenish Qi, which was used for Qi deficiency and not blood deficiency. SJZD could theoretically promote hematopoiesis because it enhanced Qi, while lack of blood in the mice led to the loss of carrier (blood is the mother of Qi), which implies that there was no significant difference between SJZD group and Model group in our study. The study results are consistent with the “Qi and blood theory” of TCM and provide preliminary laboratory data for eventual clinical applications.

In conclusion, both the Bazhen and Siwu decoctions had therapeutic effects on blood deficiency as well as anemia. The assay results indicated that BZD and SWD ameliorated the bone marrow toxicity induced by 5-FU, and improved hematopoiesis by improving cytokine expression. It is noteworthy that BZD and SWD inhibited the secretion of IFN-γ and promoted the release of IL-3, and activated the expression of EPO mRNA, especially in the kidney. Our observations indicate that BZD and SWD are potential therapeutic drugs for anemia induced by chemotherapeutic agents like 5-FU. The results support further research on the effectiveness of pro-hematopoietic monomers in these TCM prescriptions and on their biological targets to provide additional evidences for the international use of TCM.

REFERENCES


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