Effects of Four Si-Wu-Tang’s Constituents and Their Combination on Irradiated Mice

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Effects of four Si-Wu-Tang (SWT)’s constituents, fructose (Fru), paeoniflorin (Pae), ferulic acid (FA), tetramethyl pyrazine (TP), and their combination on irradiated mice as model of anaemia were investigated, with the purpose of further understanding the relationship between SWT’s constituents and activities. Similarly to SWT, oral administration of Fru, Pae, FA, TP and their combination, to some extent, all showed effects of increasing the number of peripheral leukocyte and increasing four types of progenitor cells in bone marrow, including colony-forming unit-granulocyte-macrophage (CFU-GM), colony-forming unit-mature erythroid (CFU-E), colony-forming unit-immature erythroid (BFU-E) and colony-forming unit-multipotential (CFU-mix). Pae and FA showed significant body weight reducing effect, which were largely abolished when they were combined with Fru and TP. The SWT, Fru and combination significantly increased the thymus index while Pae significantly decreased it. Both SWT and TP significantly increased the spleen index but the combination did not. The results suggested that multiple constituents contribute to the promoting effect of SWT on hematopoiesis. Although being a very common compound in plants, the Fru has a special contribution to SWT’s effect, which cannot be neglected. It may be an important active constituent that is responsible for SWT’s promoting effect on hematopoiesis and immunity. Another suggestion is that when being combined, some effect of one constituent, sometimes is unexpected side effect, may be abolished by other. This may reflect the advantage of multiple constituent characteristics possessed by most TCMs.

Key words  Si-Wu-Tang; fructose; paeoniflorin; ferulic acid; tetramethyl pyrazine; irradiation

Si-Wu-Tang (SWT), a traditional Chinese formula consisting of Rehmanniae Radix, Angelica Radix, Chuanxiong Rhi zona and Paeoniae Radix, has traditionally been used in China for about one thousand years.1) Dai et al. reported that SWT has been used for the treatment of gynecologic diseases (e.g., dysmenorrhea, menoxenia, metrorrhagia, abortion), cutaneous diseases (e.g., pruritus, urticaria, eczema, dermatitis), and chronic inflammation (e.g., chronic nephritis, pelvic inflammation).2) It has been reported to possess sedative, anti-coagulant and antibiotic activities and to exhibit vasodilatation, hematopoiesis, enhancement of cellular immunity and radio-protection.3)4) However, the knowledge on what constitutes are responsible for SWT’s activities is still very limited. Paeoniflorin (Pae), a major constituent of Paeon niae Radix, was reported to be an active constituent of Paeo niae Radix that mainly contributes to SWT’s cognitive enhancing effect.5) The antiproliferative effect of SWT seemed to depend on its Chuanxiong-derived phthalides.6) Some phenolic compounds, including ferulic acid (FA) that is contained in both Angelica Radix and Chuanxiong Rhizoma, strongly inhibited platelet aggregation.6) Our interest has been focused on SWT’s hematopoiesis-related activities. Using 3.5Gy 60Co γ-rays irradiated mice as a model of anaemia, we found that SWT increase the number of peripheral leukocyte and four types of progenitor cells in bone marrow, CFU-GM, CFU-E, BFU-E and CFU-mix.7) This to some extent is consistent with the previous studies conducted by Hsu et al.8) and Lee et al.,9) which showed the protective effects of SWT in irradiated mice, such as increasing the radiodisensitivity of bone marrow stem cells or the formation of endogenous spleen colony. In the present study, we investigated some effects of SWT and four of its major constituents, fructose (Fru), Pae, FA, tetramethyl pyrazine (TP), as well as the combination of the four constituents on irradiated mice, with the purpose of further understanding the contributions of different constituents to SWT’s activities. The effects investigated included changes of peripheral leukocyte count, colony-forming unit count of hematopoietic progenitor cells in bone marrow, body weight, thymus index and spleen index.

MATERIALS AND METHODS

Animals  C57/BL/6J female mice (6—8 weeks old, weighing 18—22 g) were purchased from the Institute of Experimental Animal, Chinese Academy of Medical Sciences. They were housed 10—11 in each group for the study of peripheral leukocyte count, colony-forming unit count of hematopoietic progenitor cell colony forming assay, at 23 ± 5°C and 55 ± 15% relative humidity with free access to standard animal chow and tap water, and were allowed at least three days for acclimatization before an experiment. Each mouse was used once and treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Drugs  An extract of SWT was prepared by decocting the dried prescription of herbs with boiling water. After the first decoction the duration of which was about 30 min, the suspension was filtered and water was added for the second decoction the duration of which was about 20 min. The filtered and mixed suspension from two decoction was condensed to dry gel. The dried prescription of herbs with boiling water. After the first decoction the duration of which was about 30 min, the suspension was filtered and water was added for the second decoction the duration of which was about 20 min. The filtered and mixed suspension from two decoction was condensed to dry gel and then stored at 5°C and 55% relative humidity with free access to standard animal chow and tap water, and were allowed at least three days for acclimatization before an experiment. Each mouse was used once and treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

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Angelica sinensis (OLIV.) (Umbelliferae) DIELS, PALL (Paeoniaceae), respectively. These plant materials were administered normal saline. Blood samples were collected from the tail end and cell counts were done at the day before irradiation. The animals, after irradiation, were administered stored drug solutions daily intragastrically at a dose of 0.2 ml/20 g body weight for 7 consecutive days. The concentrations of drug solutions were 1 g dried herb weight/ml for SWT, 3.34 mg/ml for Fru, 2.2 mg/ml for Pae, 0.2 mg/ml for FA and 1.1 mg/ml for TP. Solution of constituent combination contained 33.4 mg/ml of Fru, 2.2 mg/ml of Pae, 0.2 mg/ml of FA and 1.1 mg/ml of TP. Control mice were administered normal saline.

Peripheral Leukocyte Count

Table 1 shows the peripheral leukocyte count of each group before and after irradiation. After irradiation the number of peripheral leukocyte promptly decreased and then recovered very slowly. The groups administrated with SWT, Fru, Pae, FA and TP combination all showed enhancement of number of peripheral leukocyte at one or more time point, as compared to the irradiation control group. *p<0.05, **p<0.01, ***p<0.001 as compared to irradiation control group. a) bw: body weight.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before irradiation</th>
<th>Day 3 after irradiation</th>
<th>Day 5 after irradiation</th>
<th>Day 7 after irradiation</th>
<th>Day 10 after irradiation</th>
<th>Day 13 after irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.0±2.4</td>
<td>0.8±0.2</td>
<td>1.1±0.2</td>
<td>1.8±0.6</td>
<td>1.9±0.5</td>
<td>1.8±0.8</td>
</tr>
<tr>
<td>SWT</td>
<td>13.2±5.0</td>
<td>0.9±0.2</td>
<td>1.9±0.9**</td>
<td>2.6±0.8*</td>
<td>3.5±1.2**</td>
<td>2.9±1.1*</td>
</tr>
<tr>
<td>Fru 6.68 mg/20 g bw</td>
<td>10.7±2.1</td>
<td>0.9±0.2</td>
<td>1.7±0.3***</td>
<td>3.2±0.7***</td>
<td>2.8±0.3***</td>
<td>2.6±0.9</td>
</tr>
<tr>
<td>Pae 0.44 mg/20 g bw</td>
<td>11.2±1.7</td>
<td>1.2±0.4**</td>
<td>1.4±0.3</td>
<td>3.2±1.0**</td>
<td>2.4±0.6</td>
<td>2.1±0.7</td>
</tr>
<tr>
<td>FA 0.04 mg/20 g bw</td>
<td>10.6±3.7</td>
<td>1.1±0.3*</td>
<td>1.3±0.2*</td>
<td>3.0±0.8***</td>
<td>2.1±0.4</td>
<td>1.9±0.6</td>
</tr>
<tr>
<td>TP 0.22 mg/20 g bw</td>
<td>11.3±3.5</td>
<td>0.9±0.2</td>
<td>1.8±0.8*</td>
<td>2.3±1.6</td>
<td>2.8±0.7*</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td>Combination</td>
<td>12.0±4.0</td>
<td>1.1±0.4</td>
<td>1.6±0.4**</td>
<td>1.8±0.6</td>
<td>1.9±0.8</td>
<td>2.4±0.6</td>
</tr>
</tbody>
</table>

Irradiation and Administrations

The animals received total body irradiation of 3.5 Gy Co\textsuperscript{60}  \gamma-rays at a dose-rate of 1.60 Gy per minute. After irradiation, stored drug solutions were administered daily intragastrically at a dose of 0.2 ml/20 g body weight for 7 consecutive days. Control mice were administered normal saline.

Peripheral Leukocyte Count, Body Weight, Thymus Index and Spleen Index

Blood samples were collected from the tail end and cell counts were done at the day before irradiation and the third, fifth, seventh, tenth and thirteenth day after irradiation using a microcell counter (Sysmex F-820, Japan). At the thirteenth day, all mice were weighted and sacrificed, and the thymus gland and spleen from each mouse were excised and weighted to calculate the thymus index and spleen index. Both index were calculated as (organ weight/body weight) x 1000.

Hematopoietic Progenitor Cell Colony Forming Assay

All mice in each group were sacrificed at the seventh day after irradiation and bone marrow from femur were collected and mixed. Bone marrow cells were cultured directly in McCoy’s 5A medium (GIBCO). For CFU-GM, the culture medium contained the following reagents in final concentration: 0.3% agar, 26% horse serum, and 20% cultured supernatant from mouse fetal liver adherent cell line\textsuperscript{9} (kindly offered by professor Shifu Zhao, Beijing Institute of Radiation Medicine). For BFU-E, CFU-E and CFU-mix the culture medium contained 1% methylcellulose, 25% horse serum, 2 U/ml erythropoietin (Huaxin pharmaceutical and bioengineering Co., Nanjing, China), 10% cultured supernatant from IL-3 providing cell line (WEHI-3, kindly offered by Manchester Institute), 0.01 mM 2-mercaptoethanol, and 0.045% glutamine. Bone marrow cells (2 x 10\textsuperscript{5}/ml for CFU-GM, 5 x 10\textsuperscript{5}/ml for BFU-E, CFU-E and CFU-mix) were incubated in a humidified incubator at 37°C in 5% CO\textsubscript{2} with sample replicate number of four. The number of colonies was counted at day 3 for CFU-E, day 6 for BFU-E, CFU-mix, and day 7 for CFU-GM using an inverted microscope.

Statistical Analysis

Indices were expressed as mean±standard deviation. The significance of difference between two groups was analyzed statistically by Student’s t-test.

RESULTS

Peripheral Leukocyte Count

The effect of SWT, constituents and combination of constituents on bone marrow progenitor cells in irradiated mice were summarized in Table 2. All samples, compared to the irradiation control group, significantly increased the four types of progenitor cells, CFU-GM, CFU-E, BFU-E and CFU-mix, except that the effect of FA on CFU-GM was not significant (p>0.05). As compared to the SWT group, all constituents and the combination showed significantly decreased colony counts in one or more type of cell. The difference between Fru and SWT was significant only in CFU-E (p<0.05), while the difference between FA and SWT was significant in all types of cells, CFU-GM (p<0.001), CFU-E (p<0.001), BFU-E (p<0.01) and CFU-mix (p<0.01). Similar pattern was found when compared to the Fru group. That is, Pae, FA, TP and combination all showed significantly
lower colony count in two or more types of cells, and no significant difference in the other. The difference between FA and Fru was also significant in all types of cells, CFU-GM (p<0.001), CFU-E (p<0.001), BFU-E (p<0.001) and CFU-mix (p<0.001).

**Body Weight**  Figure 1 shows the changes of body weight of each group of mice at the thirteenth day after irradiation. Oral administration of Pae or FA significantly decreased the body weight (p<0.001) compared to the irradiation control group, but this effect was largely abolished when the mice were administered with the combination of the four constituents (p>0.05).

**Thymus Index**  Figure 2 shows the changes of the thymus index in each group at the thirteenth day after irradiation. Oral administration of SWT (p<0.05), Fru (p<0.001) or the combination (p<0.01) significantly increased the thymus index while administration of Pae decreased it (p<0.05), compared to the irradiation control group.

**Spleen Index**  Figure 3 shows the changes of the spleen index in each group at the thirteenth day after irradiation. Oral administration of SWT (p<0.05) or TP (p<0.001) significantly increased the spleen index compared to the irradiation control group.

**DISCUSSION**

SWT has been reported to possess sedative, anti-coagulant and antibacterial activities and to exhibit vasodilatation, hematopoiesis, enhancement of cellular immunity and radio-protection, but the knowledge concerning active constituents that is responsible for these activities is still very limited. Our interest has been focused on SWT’s hematopoiesis-related
activities and to find out some relationships between constituents and activities. Using 3.5 Gy $^{60}$Co γ-rays irradiated mice as a model of anaemia, we found that SWT increase the number of peripheral leukocyte and four types of progenitor cells in bone marrow, CFU-GM, CFU-E, BFU-E and CFU-mix, and that the n-butanol fraction of SWT showed the similar effects on peripheral leukocyte and bone marrow progenitor cells. The major constituents in the fraction were Pae, monosaccharide and disaccharide, and Fr and was one of the major components in SWT's monosaccharides. Our further experiment showed that Pae and Fr increased the number of peripheral leukocyte and the four types of progenitor cells in bone marrow. A significant weight reducing effect of Pae was also observed. In the present study, we investigated the activities of SWT's four major constituents, Pae, Fr, FA, and TP as well as the combination of the four constituents, with the purpose of further understanding the relationships between SWT's constituents and activities.

The selection of the four constituents was based on either that they were major constituents in SWT, or that they may have potential contribution to SWT's activity on hematopoiesis according to previous report or our preliminary experiment. Pae, Fr and FA were found as major constituents in SWT in our previous reports. Pae is from Paeoniae Radix while Rehmanniae Radix seems to be an important source for Fr, and both Angelica Radix and Chuanxiong Rhizoma contain FA. Moreover as mentioned above Pae and Fr have showed enhancing activities on hematopoiesis in our preliminary study. TP was reported to be one of the major constituents in Chuanxiong Rhizoma and its improving or protecting activity on hematopoiesis has been reported. The selected doses for Pae, Fr and FA were based on high-performance liquid chromatography (HPLC) analysis in our preliminary experiment, which showed that the natural content of Pae, Fr and FA in SWT decoction were about 0.22%, 3.34% and 0.02% respectively. Therefore the concentrations for Pae, Fr and FA were 2.2, 33.4, and 0.2 mg/ml respectively. Unfortunately our HPLC failed to detect TP in SWT decoction, however a capillary electrophoresis study had showed that the content of TP in Chuanxiong Rhizoma was about 6 mg/g. So with the assumption that all TP from 6 g of Chuanxiong Rhizoma in 41 g of SWT could be completely extracted when preparing the SWT decoction, we selected the concentration of 1.1 mg/ml for TP. Peripheral leukocyte count and hematopoietic progenitor cell colony forming assay were performed to indicate the effect on hematopoiesis. We also investigated the body weight change because a weight reducing effect had been observed for Pae in our preliminary experiment as mentioned above. Considering that SWT also have a promoting effect on immunity and the close relationship between immunity and hematopoiesis, the thymus index and spleen index were also investigated.

As indicated in our previous report, SWT showed the effect of increasing the number of peripheral leukocyte and increasing four types of progenitor cells in bone marrow, CFU-GM, CFU-E, BFU-E and CFU-mix. These once again confirmed the enhancing effect of SWT on hematopoiesis. To some extent, the four constituents and their combination all showed this effect. These suggested that Pae, Fr, FA and TP all have some contributions to SWT's effect on hematopoiesis. However it could not be determined of which the effect was the strongest according to the data of peripheral leukocyte count, because the changes of peripheral leukocyte count were all very small. But as showed by the data of progenitor cells, the effect of SWT and Fr were stronger than other samples including the constituent combination, and the effect of SWT was even stronger than Fr concerning CFU-E ($p<0.05$). FA showed very poor effect because in all types of progenitor cells the colony counts of FA group were significantly lower than SWT or Fr. Therefore Fr seemed to be an important constituent that contributed to SWT's enhancing effect on hematopoiesis.

As for body weight change, Pae and FA decreased mice's weight significantly ($p<0.001$), which once again confirmed our findings about Pae in preliminary experiment. However, the effects almost completely disappeared when they were combined with Fr and TP. These indicated that Fr or TP or the combination of Fr and TP very efficiently antagonized the weight reducing effect of Pae or FA, which was probably an unexpected side effect as to the SWT's therapeutic effect.

Similar phenomenon was observed in the thymus index. Fr showed a significant decreasing effect on thymus index, but when combined with the other three constituents the parameter was increased. Fr was most probably responsible for this, because among the four constituents tested only Fr increased thymus index significantly ($p<0.001$). As expected, SWT also increased the thymus index. These once again indicated that the contribution of Fr to SWT was important. TP had a significant increasing effect on the spleen index and SWT also increased it as expected. However all other constituents and even the combination showed no significant effect on spleen index. These reflected that the relationship between SWT's constituents and its effect on spleen index was complicated and needed further investigations.

It was very amazing that Fr showed significant promoting effect on hematopoiesis and to our knowledge there is no previous report of such an effect. Besides, the effect of Fr on thymus index was also impressive. All these suggest that although being a very common compound in plants, Fr has a special contribution to SWT's effect, which cannot be neglected. It may be an important active constituent that is responsible for SWT's promoting effect on hematopoiesis and immunity. According to Riby et al., Fr is found mostly in natural food products as a constituent of the disaccharide sucrose while free Fr, in the monosaccharide form, is found in notable quantities only in honey and a few fruits (date, figs, apples, grapes and most berries). Interestingly honey, which is also a widely used TCM and ranks at the top among naturally occurring mixtures contains free Fr (37—47.5%) in excess of glucose, was reported to increase humoral immunity and to stimulate antibody production during primary and secondary immune responses in mice. Notable quantities of Fr was also found in Rehmanniae Radix, a major ingredient in SWT (15 g in 41 g), which showed positive effects in mice on both hematopoiesis and immune function. In the present study, the effects of Fr were similar to that of SWT in most observed parameters, except that Fr was not as efficient as SWT in colony count of CFU-E and spleen index. However it does not mean that Fr is fully responsible for the therapeutic effect of SWT on hematopoiesis and immunity because our experiments studied just some
certain aspects. It seems that there is not a ready explanation for why and how Fru showed the effects on hematopoiesis and immunity, but after all Fru does have some special and positive activities. According to Craig,\textsuperscript{27} Fru feeding before or during exercise can enhance physical performance under certain conditions and the addition of fructose to the diet during ultraendurance events can improve performance by 126\%. Having an exceptional chelating ability among the common sugars, Fru forms stable complex with iron and promotes its absorption and also that of zinc, both of which are essential and important nutrients.\textsuperscript{28} Fru also showed cell-protection against several types of injury, the mechanism of which is not clearly understood.\textsuperscript{29—31} Moreover there are reports of Fru’s effects of enhancing platelet function and disrupting the thromboxane/prostacyclin ratio,\textsuperscript{32} enhancing mitogenicity of diploid human cells\textsuperscript{33} and, at certain doses, improving memory in rats.\textsuperscript{34} Further studies are needed to understand the exact role of Fru in SWT as well as the mechanism of Fru’s promoting effect on hematopoiesis and immunity. Another impressive result was that the weight reducing mechanism of Fru’s promoting effect on hematopoiesis and immunity, but after all Fru does have some special and positive activities. According to Craig,\textsuperscript{27} Fru feeding before or during exercise can enhance physical performance under certain conditions and the addition of fructose to the diet during ultraendurance events can improve performance by 126\%. Having an exceptional chelating ability among the common sugars, Fru forms stable complex with iron and promotes its absorption and also that of zinc, both of which are essential and important nutrients.\textsuperscript{28} Fru also showed cell-protection against several types of injury, the mechanism of which is not clearly understood.\textsuperscript{29—31} Moreover there are reports of Fru’s effects of enhancing platelet function and disrupting the thromboxane/prostacyclin ratio,\textsuperscript{32} enhancing mitogenicity of diploid human cells\textsuperscript{33} and, at certain doses, improving memory in rats.\textsuperscript{34} Further studies are needed to understand the exact role of Fru in SWT as well as the mechanism of Fru’s promoting effect on hematopoiesis and immunity. Another impressive result was that the weight reducing effect of Pae or FA almost completely disappeared when they were combined with Fru and TP. This may reflect an advantage of multiple constituent characteristics possessed by most TCMs and by many other natural medicines. That is when multiple constituents are acting together, some effect of one constituent, sometimes is unexpected side effect, may be abolished by other.

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