Original Article

Rehmanniae Radix provides most of the free fructose and glucose in Si-Wu-Tang decoction

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ABSTRACT: Our previous study showed that free fructose is an important active constituent responsible for Si-Wu-Tang's (SWT) effect promoting hematopoiesis and immunity. However, the contribution from SWT's four ingredient drugs to the free fructose content in the SWT decoction was not clear. To answer this question, in this study, the fructose, glucose and sucrose content in the SWT decoction, in the decoctions of each single ingredient drug, and in the decoctions of the four formulae lacking each single ingredient drug were determined by HPLC-ELSD. The results showed that the fructose and glucose content in the decoction of single *Rehmanniae Radix* were almost the same as those in the SWT decoction. In the single *Rehmanniae Radix* decoction concentrations were: 4.25 ± 0.53 mg/mL for fructose, and 3.43 ± 0.60 mg/mL for glucose; in the SWT decoction concentrations were: 4.10 ± 0.43 mg/mL for fructose, and 3.42 ± 0.32 mg/mL for glucose, while the content of fructose and glucose in the decoctions of single *Angelica Radix*, single *Paeoniae Radix*, single *Chuanxiong Rhizoma* and the formula lacking *Rehmanniae Radix* were either very small or undetectable. On the other hand, the fructose and glucose content in the decoctions of the formulae lacking *Angelica Radix*, lacking *Paeoniae Radix* and lacking *Chuanxiong Rhizoma* also were approximately the same as those in the SWT decoction. This indicated that *Rehmanniae Radix* provides most of the free fructose and glucose in the SWT decoction, and therefore plays an important role in SWT's effect promoting hematopoiesis and immunity. As for sucrose in the SWT decoction, *Angelica Radix* was shown to be a major donor.

Keywords: Si-Wu-Tang, fructose, glucose, *Rehmanniae Radix*

1. Introduction

Si-Wu-Tang (SWT), a traditional Chinese formula consisting of *Rehmanniae Radix*, *Angelica Radix*, *Paeoniae Radix*, and *Chuanxiong Rhizoma*, 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 antibacterial activities, and has been reported to exhibit effects on vasodilatation, hematopoiesis, enhancement of cellular immunity and radio-protection (*3*,*4*). Our interest has been focused on SWT's hematopoiesis-related activities. Using 3.5 Gy ^{60}Co γ-ray irradiated mice as a model of anemia, we found that SWT increases the number of peripheral leukocytes and four types of progenitor cells in bone marrow, 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) cells (*5*). In our latest report, free fructose (*i.e.*, dissociative form of fructose) was shown to be an important active constituent that is responsible for SWT's effect promoting hematopoiesis and immunity after oral administration of the SWT decoction (*6*). Oral administration of pure fructose, at a dose equal to the natural content of free fructose in the SWT decoction, showed positive effects on peripheral leukocytes, on bone marrow progenitor cells and on thymus index, which were similar to the SWT decoction. Therefore, comprehensive studies on the nature of fructose in the SWT decoction have been required. This may help us to further understand the mechanism of

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SWT's bioactivity and the reasons for the composition of the SWT formula. One of the fundamental questions is which ingredient drug is the donor of free fructose in the SWT decoction. However, to our knowledge, there is no research concerning this question. It had been reported that *Rehmanniae Radix* contains a significant amount of free fructose (*7*,*8*), but whether or not other ingredient drugs of SWT also contain free fructose, or how much does each ingredient drug contribute to the total amount of free fructose in the SWT decoction is unknown. To answer these questions, in this study, we determined the content of free fructose in the SWT decoction, in the decoctions of each single ingredient drug of SWT, and in the decoctions of the four formulae each lacking an ingredient drug, by high performance liquid chromatography (HPLC) with evaporated light scattering detection (ELSD). Since this method is capable of simultaneous determination of fructose, glucose and sucrose, the glucose and sucrose content in the above-mentioned decoctions were also determined.

2. Materials and Methods

2.1. *Drugs and reagents*

Rehmanniae Radix, *Angelica Radix*, *Paeoniae Radix*, and *Chuanxiong Rhizoma*, corresponding to *Rehmannia glutinosa* LIBOSCH. (Scrophulariaceae), *Angelica sinensis* (OLIV.) (Umbelliferae) DIELS, *Paeonia lactiflora* PALL (Paeoniaceae), and *Ligusticum chuanxiong* HORT. (Umbelliferae), respectively, were purchased from Tongrentang Ltd. (Beijing, China) and identified by Dr. Baiping Ma in our laboratory. Fructose was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glucose and sucrose were purchased from Beijing Chemical Reagent Company (Beijing, China). Acetonitrile was of HPLC grade (Fisher Scientific, Fair Lawn, New Jersey, USA). Deionized water was prepared using a Millipore water purification system.

2.2. *Chromatographic apparatus and conditions*

A Waters Acquity UPLC system consisting of a binary solvent manager, a sample manager and an evaporative light scattering detector (ELSD), and an analytical workstation with Waters Masslynx (v4.1) software was used. Separations were carried out with a SHISEIDO carbohydrate column (5 μm, 150 × 2.0 mm). The mobile phase was isocratic acetontrile and water (80:20). The temperature of the column was kept at 24°C and the flow rate was 0.6 mL/min. The drift tube temperature, gas pressure and gain of ELSD were 40°C, 40 psi and 25, respectively. The sample injection volume was 2 μL. The compounds were identified by comparing their retention time values with standards.

2.3. *Calibration curve, Sample preparations and measurement*

Each standard compound, fructose, glucose, and sucrose, was accurately weighed and dissolved in 50% acetonitrile to give serial concentrations within the range of 0.8-10 mg/mL. Log/log calibration curves were obtained from the log values of the peak areas over the log values of the concentrations of the standard solutions. Thirty-six samples were divided into nine groups (4 repetitive samples in each group). The formula composition of each group is listed in Table 1. Drugs of each sample were accurately weighed, dropped into 650 mL water for 60 min at room temperature and boiled for 30 min. The suspension was filtered and 410 mL water was added for the second decoction of 20 min duration. The filtered and mixed suspension from the two decoctions was adjusted to a volume of 820 mL. 20 mL of the decoction was precipitated by adding 80 mL alcohol and filtered. The filtrates were evaporated to less than 1 mL at about 50°C *in vacuo*. The evaporated residue was dissolved with 50% acetonitrile into a volumetric flask. The final volume of the sample solution was set to 25 mL. The solutions of standards and samples were filtered through a 0.45 μm membrane before injection into the UPLC system. The injection volume was 2 μL.

3. Results

3.1. *Typical chromatograms*

Typical chromatogram of the standard compounds and

Table 1. Formula composition of each group

Groups	Composition
1	*Rehmanniae Radix* (30 g), *Angelica Radix* (20 g), *Paeoniae Radix* (20 g), *Chuanxiong Rhizoma* (12 g)
2	*Rehmanniae Radix* (30 g)
3	*Angelica Radix* (20 g)
4	*Paeoniae Radix* (20 g)
5	*Chuanxiong Rhizoma* (12 g)
6	*Angelica Radix* (20 g), *Paeoniae Radix* (20 g), *Chuanxiong Rhizoma* (12 g)
7	*Rehmanniae Radix* (30 g), *Paeoniae Radix* (20 g), *Chuanxiong Rhizoma* (12 g)
8	*Rehmanniae Radix* (30 g), *Angelica Radix* (20 g), *Chuanxiong Rhizoma* (12 g)
9	*Rehmanniae Radix* (30 g), *Angelica Radix* (20 g), *Paeoniae Radix* (20 g)

typical chromatograms of the nine groups of decoctions are given in Figures 1 and 2, respectively. Fructose, glucose, and sucrose were perfectly separated. Among the four groups of single ingredient drug (group 2 through group 5), obvious peaks of fructose and glucose could be seen only in the *Rehmanniae Radix* group (group 2). On the other hand, among the four groups of the formulae lacking each ingredient drug (group 6 through group 9), only the exclusion of *Rehmanniae Radix* (group 6) showed a significant decrease of fructose and glucose. As for sucrose, both *Angelica Radix* (group 3) and *Chuanxiong Rhizoma* (group 5) showed obvious peaks, and the exclusion of *Angelica Radix* (group 7) showed a significant decrease of peak intensity.

3.2. *Calibration curve, contents of fructose, glucose and sucrose in each decoction group*

Log/log calibration curves showed a good linear relation between the log values of the peak areas and the log values of the concentrations of the standard solutions (Table 2) for fructose, glucose, and sucrose. The contents of fructose, glucose, and sucrose in each decoction group are summarized in Table 3. The contents of fructose and glucose in the decoction of *Rehmanniae Radix* alone (group 2) were almost the same as those of the SWT decoction (group 1) (in the *Rehmanniae Radix* alone decoction: 4.25 ± 0.53 mg/mL was seen for fructose, and 3.43 ± 0.60 mg/mL for glucose; in the SWT decoction: 4.10 ± 0.43

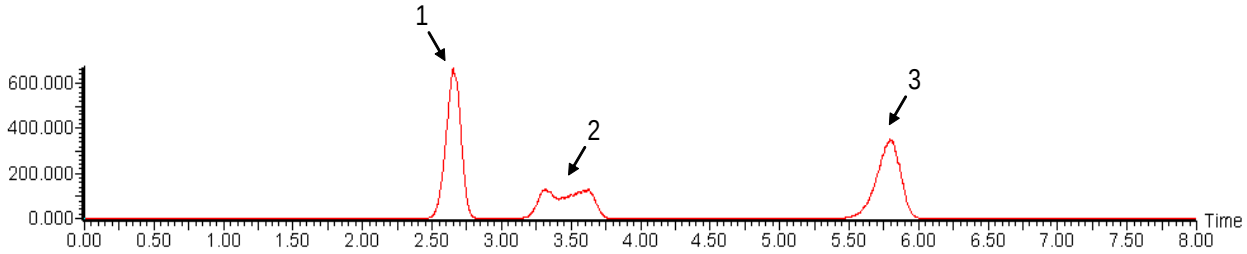

Figure 1. Typical chromatogram of the standard compounds. Peaks 1-3 denote fructose, glucose, and sucrose, respectively.

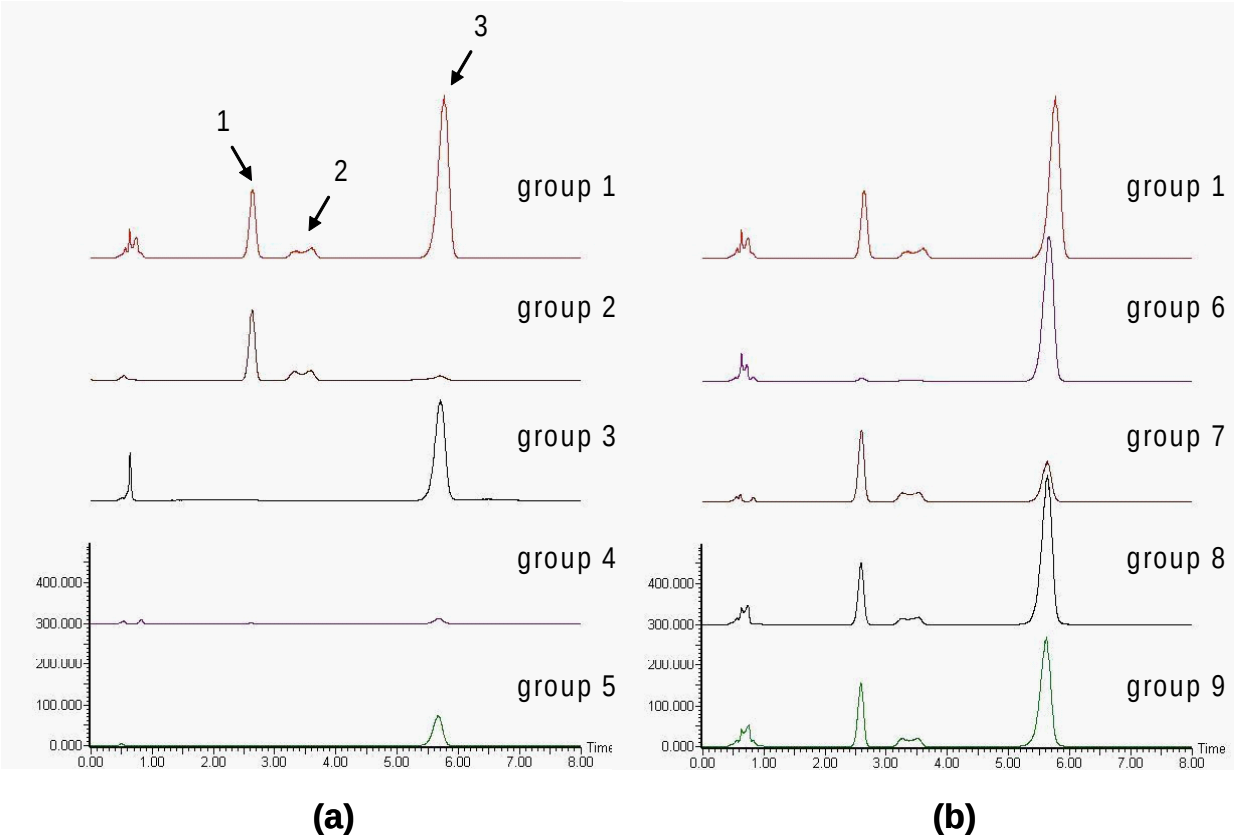

Figure 2. Typical chromatogram of groups 1-5 (a) and groups 1 and 6-9 (b). Peak intensity of all samples was normalized to a uniform value. Peaks 1-3 denote fructose, glucose, and sucrose, respectively.

Table 2. Linear relation between the log values of the peak areas and the log values of the concentrations of the standard solutions

Compounds	Regression equation	*r*	Linear range (mg/mL)
Fructose	y = – 2.44885 + 0.70281x	0.99874	0.8-10
Glucose	y = – 2.01709 + 0.64336x	0.99964	0.8-10
Sucrose	y = – 2.24556 + 0.67214x	0.99914	0.8-10

x: log value of peak area; y: log value of concentration.

Table 3. Content of fructose, glucose, and sucrose in each group of decoctions

Groups[a]	Concentration[b] (mg/mL)		
	Fructose	Glucose	Sucrose
1	4.10 ± 0.43	3.42 ± 0.32	12.33 ± 1.25
2	4.25 ± 0.53[§]	3.43 ± 0.60[§]	0.76 ± 0.50[**]
3	0.47 ± 0.48[**]	n.d.	9.90 ± 0.30[*]
4	0.18 ± 0.01[**]	n.d.	1.12 ± 0.13[**]
5	n.d.	n.d.	2.85 ± 1.30[**]
6	0.43 ± 0.11[**]	0.52 ± 0.35[**]	11.69 ± 1.93[§]
7	4.33 ± 0.32[§]	3.73 ± 0.53[§]	4.33 ± 0.72[**]
8	3.81 ± 0.35[§]	3.34 ± 0.33[§]	11.67 ± 1.40[§]
9	4.29 ± 0.17[§]	3.82 ± 0.29[§]	10.78 ± 0.64[§]

[a] See Table 1; [b] Data expressed mean ± S.D., $n = 4$; n.d.: not detected.
[*] $p < 0.05$, [**] $p < 0.001$, [§] $p > 0.1$ as compared to group 1.

mg/mL was seen for fructose, and 3.42 ± 0.32 mg/mL for glucose), while the contents of fructose and glucose in decoctions of *Angelica Radix* alone (group 3), *Paeoniae Radix* alone (group 4), *Chuanxiong Rhizoma* alone (group 5) and the formula lacking *Rehmanniae Radix* (group 6) were either very small or undetectable. On the other hand, the contents of fructose and glucose in the decoctions of the formulae lacking *Angelica Radix* (group 7), lacking *Paeoniae Radix* (group 8) and lacking *Chuanxiong Rhizoma* (group 9) were also approximately the same as those in the SWT decoction. The content of sucrose in the decoction of *Angelica Radix* alone (group 3) was 9.90 ± 0.30 mg/mL, about 4/5 of that seen in the SWT decoction (12.33 ± 1.25 mg/mL, group 1). The sucrose content when *Angelica Radix* was excluded (group 7, 4.33 ± 0.72 mg/mL) was about 1/3 of the amount in the SWT decoction.

4. Discussion

This is the first report concerning the contributions among SWT's four ingredient drugs to the content of the SWT decoction's free fructose, which was shown in our previous study as an important active constituent responsible for the effect promoting hematopoiesis and immunity from the use of SWT (*6*). In this study, in order to assess the ability to provide free fructose from each ingredient drug of SWT when decocted, we determined the free fructose content not only in the decoctions prepared from each single ingredient drug, but also in decoctions prepared from the four formulae lacking each ingredient drug. They were all compared with the free fructose content in the SWT decoction. The adoption of such experimental design was with the consideration of possible interactions between different ingredient drugs when decocted together, which may affect the release of fructose from each ingredient drug. All groups of samples were prepared with the identical method to ensure their comparability. The determination method was established and reported by ourselves in 2004 (*9*). This method is capable of simultaneous determination of fructose, glucose and sucrose in the SWT decoction.

The results clearly indicated that *Rehmanniae Radix* provides most of the free fructose and glucose in the SWT decoction, because the contents of fructose and glucose in the decoctions were approximately equal to those in the SWT decoction if only the formula contained *Rehmanniae Radix*. The fructose and glucose content was either very low or undetectable if the formula did not contain *Rehmanniae Radix*. Although it had been reported that *Rehmanniae Radix* contains a significant amount of free fructose (*7*,*8*) and we had anticipated that the contribution of *Rehmanniae Radix* to the free fructose content in the SWT decoction should be significant, we had not expected that the contribution was so exclusive. We had not expected that the contribution of *Rehmanniae Radix* to the free glucose content in the SWT decoction was also exclusive. Since free fructose was shown to be an important active constituent responsible for SWT's effect promoting hematopoiesis and immunity after oral administration of SWT (*6*), and *Rehmanniae Radix* provides most of the free fructose in the SWT decoction, it is inferred that *Rehmanniae Radix* plays an important role in SWT's effect promoting hematopoiesis and immunity. In fact, according to the conventional theory of traditional Chinese medicine (TCM) which uses "Jun" ("emperor", the most important ingredient or plays a central role in a formula), "Chen" ("minister"), "Zuo" ("assistor"), and "Shi" ("emissary") to identify the importance and roles of different ingredient drugs in a formula, *Rehmanniae Radix* is the "Jun" in the SWT formula (*10*). Our deduction based on modern studies is consistent with judgment based on traditional TCM theory. In addition, it is necessary to point out that the *Rehmanniae Radix* used in SWT is not raw but has been processed by braising, and our recent study, the data of which will be published elsewhere, has revealed that the major amount of fructose and glucose contained in *Rehmanniae Radix* used in SWT is produced during the braising process. Perhaps such an intentional choice of drug variety, *i.e.,* the choice of processed *Rehmanniae Radix*, which contains significantly more free fructose and glucose than the raw drug, indicates again the importance of these monosaccharides for SWT's

therapeutic effects. The results of this study showed that *Angelica Radix* is a major donor of sucrose in the SWT decoction, and next to *Angelica Radix* is *Chuanxiong Rhizoma*. Interestingly they both belong to the Umbelliferae family.

The mechanism of the bioactivity of free fructose in SWT on hematopoiesis and immunity is still to be studied. Current knowledge of fructose's metabolism has shown that the liver is the primary metabolic site of fructose disposal, where fructose is metabolized by fructokinase to fructose-1-phosphate that is cleaved by aldolase B to form dihydroxyacetone phosphate and glyceraldehyde, both of which can be further metabolized in the glycolytic pathway (*11*). It was reported that low-dose fructose (infused into the duodenum) could increase hepatic glucose uptake and glycogen storage, which is possibly due to the activation of glucokinase by trace amounts of fructose acting on the glucokinase regulatory protein (*12*). The stimulating effects on insulin-stimulated hepatic glycogen synthesis of low-dose fructose were also reported (*13*). Therefore it is necessary to consider the possibility that fructose may help the body's hematopoiesis and immunity by participating in and/or regulating glucose metabolism. In our former studies on SWT and fructose's bioactivities (*5*,*6*), the model of anemia was induced by γ-ray radiation. Interestingly, according to Fang Yunzhong (*14*), a higher energy intake is required for irradiated experimental animals because radiation may: (*i*) inhibit the oxidation phosphorylation process and lead to a low P/O ratio; (*ii*) affect the Krebs cycle and decrease production of NADH and FADH which are materials for the oxidation phosphorylation process; and (*iii*) increase the basal metabolic rate. Lack of energy or nutrition increases the body's sensitivity to radiation, and experiments showed that enhanced energy supplies prevents dogs from loss of body weight after irradiation. And among four different sugars (sucrose, dextrin, cornstarch and glucose), glucose showed the most significant therapeutic effect against radiation injury, but when comparing the therapeutic effect of glucose with fructose, fructose was even better. According to Fang, radiation decreases the activity of hexose kinase and thereby interrupts the transformation from glucose to glucose-6-phosphate, which is the first step of the glycolytic pathway. The transformation from fructose to fructose-1-phosphate is unaffected because the activity of fructokinase is not changed by radiation. The current study has affirmed the exclusive role of *Rehmanniae Radix* in providing free fructose and glucose in SWT and to some extent revealed the meaning of *Rehmanniae Radix* in the composition of the SWT formula.

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