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The effects of Sa-Mul-Tang (Si-Wu-Tang), a Traditional Chinese Medicine, on Phenylhydrazine-induced Anemic Rats*

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Abstract – Sa-Mul-Tang (Si-Wu-Tang, SMT), a kind of Chinese medicine, has been used for the hemato-deficient disease for hundreds of years. In this work, investigations on the anti-anemic activity of an aqueous extract of SMT were undertaken in order to find the pharmacological basis for the ethnomedical use of the formulation. Three kinds of Angelicae species, such as *Angelica sinensis, Angelica acutiloba,* and *Angelica gigas,* were used for preparing the water extracts of SMT. Anemic model rats were induced by the treatment of phenylhydrazine (40 mg/kg/day, i.p.) for 4 days. After thc treatment of phenylhydrazine, rats were divided into several groups for their different treatment of three kinds of SMT. Red blood cell (RBC), hemoglobin (Hg), and hematocrit (Hct) were determined on the day 0, 3, 6, 10, 14 aftcr the treatment of SMTs and erythrocytes deformabilities were also determined at the end of experiments. Oral administration of SMT (1 g/kg/day) for 14 days did not ameliorate drug-induced anemic states evaluated by RBC counts, Hg contents, and Hct values. However, the erythrocyte deformabilities were improved in phenylhydrazine-treated group by the administration of SMTs ($p<0.05$). Especially, these effects were high in the *Angelica acutiloba* group. These results suggest that SMTs have an ameliorative effect on blood rheology related to the blood stasis syndrome in oriental diagnostics not on the blood deficient states related to the anemic syndrome.

Key words ☐ Sa-Mul-Tang, Anemia, Phenylhydrazine, Red blood cell, Deformability, Angelica species, Blood stasis

Sa-Mul-Tang (Si-Wu-Tang, SMT), a kind of Chinese medicine, is basic recipe for enriching the blood and regulating mensturation. It has been used for the blood disease, such as blood deficiency, abnormal blood coagulation, fibrinolysis, and atherosclerosis for hundreds of years. In the modern pharmacological studies, SMT possesses the anti-inflammatory and cognitive enhancing effects and also has the protective effects to the radiation-induced cell damage (Watanabe *et al.*, 1991; Sakuma *et al.*, 1998). SMT is consisted of four medicinal plants: Rehmannia root, Cnidium rhizome, Angelica root, and Peony root. Among these four constituents, Cnidium rhizome and Angelica root have been used to improve a state of insufficient blood circulation. Kobayashi *et al.*, (1992) reported that the anti-proliferative effects of SMT depend on the Angelica root and Cnidium rhizome. Yim *et al.,* (2000) suggested that *Angelica sinensis* and *Astragalus membranaceus* in the *Polygonum mutiflorum* extract have the myocardial protective effects. It is also reported that self-initiating regular consumption of the *Angelica sinensis* had beneficial effects on the hematopoietic systems (Bradly *et al.*, 1999). All these findings suggest that Angelica species play important roles in the blood circulation as well as other clinical effects. Until now, three kinds of Angelica species have been used for the preparation of SMT. *Angelica sinensis* is used for the preparation of SMT in China, *Angelica acutiloba* in Japan, and *Angelica gigas* in Korea, respectively. However, the relationship between Angelica species and some effect of SMT on the blood system remains unclear. The present study was undertaken to determine the relationship and to investigate using the anemic model whether any kind of Angelica species can be used for the preparation of SMT. Furthermore, we examined the effects of SMT on red blood cell deformabilities. As the anemic model, rats with hemolytic anemia induced by phenylhydrazine (PHZ), well known as a drug-related anemia, were used (Cynshi *et al.*, 1990).

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MATERIALS AND METHODS

Plant materials and water extract

Pharmacognostically specified dried parts of individual ingradients except the processed Rehmannia root were collected from the local market and authenticated by the one of authors (C. S. Yook). The processed *Rehmannia glutinosa* was purchased from the Dae-Yon Pharm. Inc., Inchean, Korea. The root of *Rehmannia glutinosa, Paeonia lactiflora,* and *Angelica sinensis* and rhizome of *Cnidium officinale* were added to 10 fold volume of double distilled water at the dry weight ratio of 1 : 1 : 1 : 1 and boiled until the volume was reduced to 5 fold volume (SMT-containing the *Angelica sinensis*, SMT-S). Then the extractive solution was filtered with Whatman No. 1 filter paper and concentrated on a water bath under reduced pressure. Then it was frozen and subjected to lyophilization (Elela, model FD-5N). SMT-containing the *Angelica acutiloba* (SMT-A) was prepared using the *Angelica acutiloba* instead of *Angelica sinensis.* SMT-containing the *Angelica gigas* (SMT-G) was prepared using *Angelica gigas* instead of *Angelica sinensis.*

Animals and treatments

Nine-week old male SD rats, weighing 280-290 g, were purchased from the Dae Han Laboratory Animal Research Center Co., LTD., Chungbuk, Korea, and used after 1 week of preliminary breeding under 12/12-h light/dark cycle at 24-26°C with food and tap water ad lib. For the PHZ-induced hemolytic anemia, rats received intraperitoneal injections of PHZ (Sigma Co. USA) by 40 mg/kg for 4 days. After PHZ injection, the rats were randomly assigned to control or treatment groups. Animals were selected for further studies into four groups, SMT-S treated group, SMT-A treated group, SMT-G treated group, and normal saline treated group as positive control. Animals received 14 daily injections of either SMT-S, SMT-A or SMT-G by 1 g/kg (p.o.).

Hematological examinations

Blood samples were obtained by cardiac puncture under the slightly anesthetic state on the day 0, 3, 6, 10, and 14 after the SMTs treatment in the anemic rats. Red blood cell (RBC), hemoglobin (Hg), and hematocrit (Hct) were determined using an automatic cell counter (Tilseac, model H5-M). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from the above data.

Assay for osmotic hemolysis

Blood was obtained from rats by cardiac puncture and collected in EDTA-treated tubes on day 14 after the last PHZ-injection. Erythrocyte were separated from plasma and buffy coat and washed three times with 6 volumes of the isotonic phosphate buffered saline (PBS). During the last wash, the erythrocytes were centrifuged at 2,500 rpm for 10 min to obtain a constantly packed cell preparation. The hemolysis experiments were carried out in erythrocyte suspension in PBS with hematocrit of 10%. Erythrocyte suspension (20 μl) was added to 1.8 ml of NaCl solution (0.1~1%) and left standing for 30 min at room temperature. And then the suspensions were centrifuged at 3,000 rpm for 10 min and hemolysis was estimated from the 540 nm of hemoglobin released into the supernatant (Nagano *et al.*, 1990). The results were expressed as percentage of total hemolysis by 0.1% NaCl solution.

Measurement of erythrocytes filterability

The measurement of erythrocytes filterability was carried out by recording the time passage of 1 ml of 10% erythrocytes hematocrit in a PBS solution through 3 μm pores of a polycarbonate filter (Corning Co., USA) at 20 cm negative water pressure (Reid *et al.*, 1976). The filtration was begun when the perfusion pressure was applied by opening the tap between the filtrate reservoir and the vacuum. Values for filterability time were expressed as the mean of duplicate determinations on each sample using two different filters.

Data analysis

The data were analyzed with one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method for multiple comparison. $P<0.05$ was considered to be significantly in all cases.

RESULTS

Effects of SMTs on hematological parameters in the blood

Hemolytic anemia was apparently induced by the treatment of PHZ as shown in Table I. In the anemic rats, RBC decreased about 60% compared with that in the control group. These phenomena were also observed in the other parameters such as Hg and Hct. The anemic conditions were gradually recovered both in the PHZ only treated group and the SMTs-treated group by ceasing the treatment of PHZ. Especially, Hg

Table 1. The effects of three kinds of SMTs, such as SMT-S, SMT-A and SMT-G on the RBC counts, Hg contents and Hct values in PHZ-induced anemic rats

	Day 0	Day 3	Day 6	Day 10	Day 14
Red blood cell counts (× 10^6 cells/ul)					
Control	8.09 ± 0.22	7.24 ± 0.33	7.91 ± 0.22	7.91 ± 0.20	7.65 ± 0.20
Phenylhydrazine	3.53 ± 0.21*	3.39 ± 0.07*	5.29 ± 0.10*	6.06 ± 0.27*	6.51 ± 0.15*
SMT-S	3.66 ± 0.17*	3.78 ± 0.29*	4.73 ± 0.44*	6.63 ± 0.16*	6.68 ± 0.26*
SMT-A	2.88 ± 0.33*	2.88 ± 0.33*	4.76 ± 0.35*	6.08 ± 0.23*	6.63 ± 0.14*
SMT-G	3.16 ± 0.14*	3.17 ± 0.18*	5.36 ± 0.19*	6.21 ± 0.28*	6.65 ± 0.09*
Hemoglobin (g/dl)					
Control	17.32 ± 1.10	14.62 ± 0.64	15.40 ± 0.91	15.65 ± 0.56	14.60 ± 0.23
Phenylhydrazine	19.35 ± 1.13	11.76 ± 0.18*	14.48 ± 0.25	16.02 ± 0.55	15.42 ± 0.22
SMT-S	21.98 ± 0.61*	11.30 ± 0.26*	12.90 ± 0.75	15.42 ± 0.09	14.82 ± 0.25
SMT-A	21.96 ± 1.03*	10.46 ± 0.55*	13.65 ± 0.19	14.36 ± 0.53	14.48 ± 0.43
SMT-G	17.44 ± 1.19	10.70 ± 0.22*	13.90 ± 0.26	15.04 ± 0.62	15.85 ± 0.72
Hematocrit (%)					
Control	51.33 ± 2.87	45.60 ± 2.05	47.98 ± 1.55	48.10 ± 1.92	45.38 ± 0.90
Phenylhydrazine	22.85 ± 1.17*	34.80 ± 0.54*	44.62 ± 0.36*	48.88 ± 3.10	48.12 ± 0.70
SMT-S	23.63 ± 0.93*	32.88 ± 0.37*	41.83 ± 0.65*	49.70 ± 1.19	46.20 ± 1.42
SMT-A	22.42 ± 1.25*	32.05 ± 0.31*	42.98 ± 0.72*	45.02 ± 1.60	48.68 ± 2.21
SMT-G	20.92 ± 0.70*	30.93 ± 1.10*	43.72 ± 1.14*	47.08 ± 2.21	51.95 ± 2.54

Time course of the effects of SMTs on the red blood cell counts, hemoglobin contents and hematocrit values in the phenylhydrazine (PHZ)-induced anemic rats. The drug-induced anemia was achieved by the treatments of PHZ (40 mg/kg for 4 days, i.p.). Following the treatments of PHZ, rats were administered either SMT-S, SMT-A, SMT-G or the same volume of normal saline for 14 days (1 g/kg/5 ml, p.o.). Day 0 means the subsequent day to the last treatment of PHZ. SMT-S, Sa-Mul-Tang containing *Angelica sinensis;* SMT-A, Sa-Mul-Tang containing *Angelica acutiloba;* SMT-G, Sa-Mul-Tang containing *Angelica gigas.* Each data represents the meanSEM of 5 or 6 rats. *$p<0.05$ as compared with control group.

contents and Hct values were reached to control level from 10 days. However, there were no significant changes on the hematological parameters by the treatment of any kinds of SMTs compared with those of PHZ-treated group.

Effects of SMTs on the osmotic hemolysis

In the osmotic hemolysis test performed on the day following the last day of administration of SMTs, the fragility of erythrocytes of PHZ-treated rats decreased compared with that of control rats at range from 0.4% to 0.55% of NaCl (Fig. 1). In the SMT-S and SMT-A groups, the trends of the fragility of erythrocytes were about the same as those of control group from 0.1% to 0.45% of NaCl concentration (Fig. 1A and 1B). And the fragility of erythrocytes was significantly decreased in the SMT-G-treated rats compared with that of PHZ-treated rats, especially at 0.45% of NaCl (Fig. 1C). However, in the all kinds of SMT groups, there were significantly decreased in the fragilities of erythrocytes at 0.5% of NaCl concentration compared with those of control group ($p<0.05$).

Effects of SMTs on the erythrocytes filterability

Fig. 2 shows the effects of SMTs on the filterability of rat erythrocytes. Intraperitoneal injection of PHZ decreased the filtration rate to about 3 times lower than that of the normal one. However, the PHZ-induced decrease of erythrocyte filterability recovered by the treatment of SMTs although the levels did not reached to the control levels. Among the three kinds of SMTs, filterability was much higher in SMT-A-treated rats than those in SMT-C and SMT-G-treated rats ($p < 0.05$).

DISCUSSION

In the present study, we investigated the effects of SMT on the anti-anemic activity and blood filtration rate. Furthermore, the role of Angelica root in the SMT was also investigated by varying the species in the preparation of SMT.

In the PHZ-induced anemic rats, there were significant decreased in the RBC counts and hemoglobin contents and gradually recovered from the anemic state either by the treat-

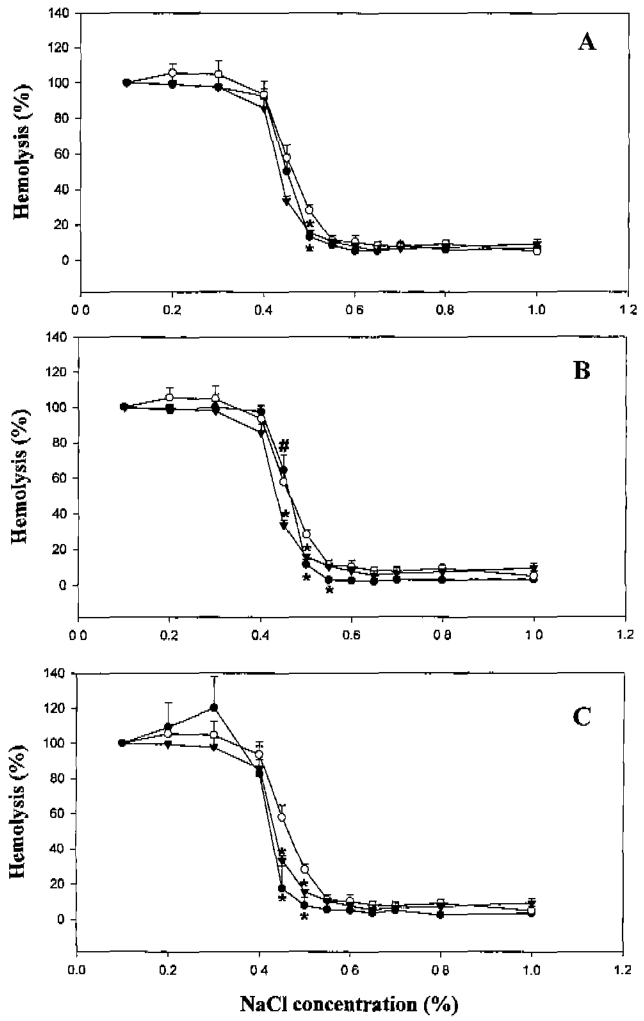

Fig. 1. Osmotic hemolysis of fresh erythrocytes to hypotonic pressure by the treatment of Sa-Mul-Tang containing *Angelica sinensis* (A), Sa-Mul-Tang containing *Angelica acutiloba* (B), and Sa-Mul-Tang containing *Angelica gigas* (C). (○), control group; (●), Sa-Mul-Tang-treated group; (▼), phenylhydrazine-treated group. Each points represents the mean±SEM of 5 or 6 rats. *$p<0.05$ as compared with the control group. #$p<0.05$ as compared with the phenylhydrazine-treated group.

ment of SMTs or by ceasing the treatment of PHZ. PHZ are well recognized as a hemolytic anemic inducer (Cynshi *et al.*, 1990). There were no significant changes in the RBC counts, Hg contents and Hct values by the treatment of SMTs, such as SMT-A, SMT-S, and SMT-G compared with those of PHZ-treated rats during the experiments (Table I). There were also no changes in the various parameters, such as MCV, MCH, and MCHC (data not shown). However, there are several reports that SMT plays a role in the anemic states (Kim, 1998. Ph.D. dissertation). Until now, it is unclear why there are different results derived between our results and others. Therefore, further

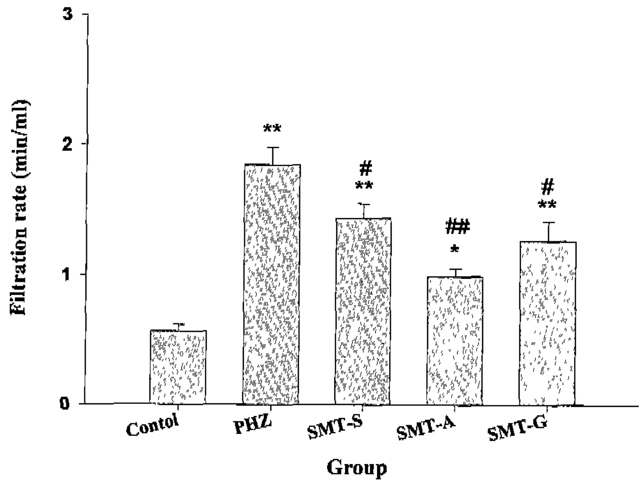

Fig. 2. Effects of Sa-Mul-Tangs on erythrocytes filterability in phenylhydrazine induced anemic rats. PHZ, phenylhydrazine; SMT-S, Sa-Mul-Tang containing *Angelica sinensis;* SMT-A, Sa-Mul-Tang containing *Angelica acutiloba;* SMT-G, Sa-Mul-Tang containing *Angelica gigas.* The bars are expressed as the meanSEM of 5 or 6 rats. *$p<0.05$, **$p<0.001$ as compared with control group. #$p<0.05$, ##$p<0.001$ as compared with phenylhydrazine-treated group.

study will be needed to examine these points in detail.

Several lines of evidence suggest that erythrocytes are changed to highly sticky and the membrane fluidity of erythrocytes is decreased by the treatment of PHZ (Rice-Evans and Hochstein, 1981). And the treatment of PHZ causes oxidative destruction of membrane of erythrocytes and resulted in loss of trans-membrane transport system of ions and water (Brugnara C. and de Franceschi L., 1993; Petty *et al.*, 1991). These evidences indicate that erythrocytes from the PHZ-treated rat may be more susceptible to osmotic injury. From our results in the fragility test, erythrocytes in the PHZ-treated rat are more susceptible to the osmotic pressure than that in the normal control rats. On the other hand, it was observed that erythrocytes in the SMT-S-treated rats were resistant to the osmotic pressure. These phenomena were also observed in the SMT-A-treated rats. However, in the SMT-G-treated rats, erythrocytes were less resistant to the osmotic pressure than others. These results indicate that resistance of erythrocytes to the osmotic pressure may vary from Angelica species, and that SMT-S and SMT-A have ameliorative effects on the changed resistance to osmotic pressure induced by the PHZ treatment.

Red blood cell deformability as measured by filterability was shown to be a major determinant of whole blood viscosity. The filterability of erythrocytes is changed by various disease states, such as diabetes, atherosclerosis, ischemia and so on (Schmid-Schobein H. and Volger E., 1976; Turchetti *et al.*, 1998). Such diseases may cause insufficient microcirculation. In the present study we observed that injection of PHZ decreased the filtration rate to about 3 times lower than that of the normal one (Fig. 2). These results are consistent with previous data from others (Shimizu *et al.*, 1999). Kubo *et al.*, (1996) reported that the processed Rehmanniae radix increased erythrocyte deformability and suggested that the quality of this herbal drug may be estimated by the improvable effects on the hemorheological parameters. And it was reported that Dangki-Jakyak-San (Tang-Kuei-Shao-Yao-San in Chinese) had strong inhibitory effects on the coagulation and platelet aggregation and might be related to blood stasis (Terasawa *et al.*, 1983). These hemorheological findings suggest that herbal medicine for the blood in the oriental diagnostics play ameliorative roles in the blood stasis. This suggestion is supported by our filtration results (Fig. 2) which show that filterability reduced by the PHZ was recovered by the treatment of SMTs. The filterability among the three kinds of SMTs was also varied by Angelica species like as the fragility test although the rate of recovery not reached to the control levels. SMT-A had much higher recovery effects than others. Unlike as the fragility test, SMT-S and SMT-G had about the same effects on the filterability. From the fragility and deformability tests, our results suggest that SMT has an ameliorative effect on blood rheology related to PHZ-induced blood stasis and SMT containing the *Angelica sinensis* (SMT-S) and *Angelica acutiloba* (SMT-A) are more useful for these syndrome than *Angelica gigas* (SMT-G). However, the reasons why the deformability and fragility of erythrocyte were changed by the varying with the Angelica species are not known but may be because of their constituents. The constituent compounds are phthalide derivatives in the *Angelica sinensis* and *Angelica acutiloba* but coumarin derivatives in the *Angelica gigas*.

The action mechanisms of SMTs on the blood circulations have been still unknown, but our results indicate that SMT affects the blood stasis through improving deformability and resistance to the osmotic pressures. In the traditional Chinese medicine, SMT has been used for the blood deficiency. From our results, it can be speculated that blood deficiency in the oriental diagnostics may be related not to the anemic sates but to the blood stasis syndromes.

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