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Original article

Assessment of the Toxicity and the Stability of Saeng Mak San by Using Repeated Intravenous Injections in Sprague-Dawley Rats

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Key Words
intravenous injection, pharmacopuncture, Saeng Maek San, toxicity test

Abstract

Objectives: This study used repeated intravenous injections of Saeng Maek San (SMS) injection in Sprague-Dawley (SD) rats to assess the toxicity and the stability of SMS.

Methods: Six-week-old male and female SD rats reared by Orient bio Inc were chosen for this pilot study. They were randomly split into four groups: Group 1 (G1), the control group (0.3 mL of normal saline solution/day/animal), and Groups 2, 3 and 4 (G2, G3 and G4), the experimental groups (0.1, 0.2 and 0.3 mL/day/animal of SMS), respectively. Each animal received an intravenous injection of SMS once a day for four weeks. Clinical signs, body weight changes, and food consumption were monitored during the observation period, and urinalysis and hematology were conducted after four weeks of SMS or saline administration.

Results: No deaths occurred in any of the four groups during the observation period. Compared to the control group, male and female rats in groups 3 and 4 (0.2 and 0.3 mL/animal/day) showed hemoglobinuria, but the low-dosage group (G2, 0.1 mL/animal/day) showed no significant changes in the clinical signs test. No significant changes due to SMS were observed in the experimental groups regarding body weight changes, food consumption urinalysis, or hematology.

Conclusion: During this study, no mortalities were observed in any of the experimental groups and no hemoglobinuria was observed in the low dosage group (0.1 mL/animal/day) while it was intermittently observed in groups 3 and 4 (0.2 and 0.3 mL/animal/day). Thus, we suggest that the no-observed adverse-effect level (NOAEL) is 0.1 mL/animal/day in male and female SD rats.

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1. Introduction

Pharmacopuncture is a new form of acupuncture treatment in traditional Korean medicine [1]. Pharmacopuncture does not pass through the digestive system, so it works faster and is more effective compared to medicines that are administered orally [2]. For these reasons, pharmacopuncture is widely used.

The constituents of the Saeng Maek San (SMS) are three herbs, *Panax ginseng*, *Ophiopogon japonicas*, and *Schisandra chinensis* [3]. In traditional Chinese medicine (TCM), SMS is used as a remedy or clinical prescription to treat symptoms related to cardiovascular diseases [4]. In previous studies, SMS was found to inhibit inflammatory cytokines, such as tumor necrosis factor-α and interleukin-8, and to reduce the systemic inflammatory reaction. Protective effects against oxidative damage in mitochondria, cells, and tissues,

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as well as amyloid-β-induced cytotoxicity in PC12 cells, were also verified [5-8]. Additionally, SMS is known to enhance humoral immunity and to inhibit cellular immunity after a cardiopulmonary bypass [9].

In a previous single-volume toxicity study (Biotoxtech study No: B12877), 0.1, 0.5 and 1.0 mL of SMS were administered to the experimental groups and 1.0 mL of saline to the control group. In all four groups, the administration of 1.0 mL/animal of SMS did not cause any significant changes or any incidence of mortality. Therefore, SMS administration up to this volume was determined to be a safe option for treatment. However, signs of hematuria were noted in the animals that received SMS doses of 0.5 and 1.0 mL/animal. Therefore, in this study, 0.3 mL/animal was set as the high dosage and 0.2 and 0.1 mL/animal as the medium and the low dosages, respectively.

2. Materials and Methods

All experiments were performed at Biotoxtech (Chungwon, Korea), an institute certified to perform non-clinical studies under the regulations of Good Laboratory Practice (GLP). The SMS consisted of *Panax ginseng*, *Ophiopogon japonicas*, and *Schisandra chinensis* in the ratio 2 : 1 : 1, respectively (total: 5,500 g). The SMS pharmacopuncture was prepared in a sterile room at the Korean Pharmacopuncture Institute (K-GMP). SMS was extracted by decocting the dried herbs in distilled water for 2 hours (total extracts: 12 L), and the pH was controlled to between 7.0 and 7.5 by adding NaOH to make a 0.9% isotonic solution. The final solution was stored at 4°C.

In this study, 5-week-old male and female Sprague-Dawley (SD) rats with weights in the ranges of 119.3 — 137.0 g and 101.9 — 118.2 g, respectively, were provided by Orient bio Inc. (Gyeonggi, Korea). SD rats have been widely used in drug-safety tests, so the use of SD rats in this study allowed the data obtained to be easily compared to similar data available in numerous existing databases. The animals were housed in stainless-steel wire-mesh cages (260 mm (W) × 350 mm (D) × 210 mm (H)) at a constant temperature of 21.8 — 23.5℃ under a relative humidity of 48.7% — 68.1% with 10 — 15 air changes per hour. The room was provided with artificial lighting (150 — 300 Lux) from 07:00 to 19:00. The animals were allowed free access to tap water and commercial rodent chow (Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C, Harlan Laboratories, Inc., USA). This study was conducted with the approval of the Institutional Animal Ethics Committee (No. 130387).

Forty male and forty female SD rats were used as the subjects of this test after a week of adaptation. Rats of each gender were randomly distributed, based on average weights, into four groups, with 10 rats per group (Table 1). At the first injection, the 6-week-old male and female SD rats had weights in the ranges of 189.6 — 212.5 g and 149.0 — 175.1 g, respectively.

According to a previous single-volume toxicity study (Biotoxtech study No: B12877), 0.1, 0.5 and 1.0 mL of SMS were administered to the experimental groups and 1.0 mL of saline to the control group. In all four groups, no deaths occurred, but hematuria was noted in the animals that received SMS in doses of 0.5 and 1.0 mL/animal. Therefore, in this study, 0.3 mL/animal was set as the high dosage and 0.2 and 0.1 mL/animal as the medium and the low dosages, respectively.

All animals were observed daily for clinical signs for 4 weeks from the first injection day. The body weight and food consumption of each rat were measured at the initiation of treatment and once a week during the treatment period. The amounts of food and water intake were averaged every week during the treatment period.

Ophthalmological examinations and urinalyses of five rats in each group were carried out at the end of the recovery period. In the ophthalmological examinations, after the use of a mydriatic (Lot No.: 12K21B, isopto atropine eye drops 1%, Alcon, Korea), and the anterior segment, lenses, vitreous body and fundus were examined by using an opthalmoscope (ALL PUPIL Ⅱ, Keeler, U.K.). Urinalyses were conducted on fresh urine to assess specific volume, protein, bilirubin, and occult blood; a Combur^{10}Test®M stick (Roche, Germany) system (MIDITRON®) Junior II, Roche, Germany) was used.

Hematological analyses were performed before autopsy; all animals were anesthetized by using isoflurane after fasting for more than 18 hours, and blood was collected from the abdominal aorta. The blood samples, about 1 mL, were collected into tubes with ethylene diamine tetraacetic acid (EDTA) and were analyzed using a blood counting analyzer (ADVIA 2120i, Siemens, Germany). For the blood coagulation analyses, about 2 mL of blood were collected into tubes with 3.2% sodium citrate, centrifuged at 3,000 rpm for 10 minute, after which measurements were taken using an Automated Coagulation Analyzer (Coapresta 2000, Sekisui, Japan). The serum biochemistry analyses were performed using an auto-analyzer (7180, Hitachi, Tokyo, Japan). Serum samples were acquired and then centrifuged at 3,000 rpm for 10 minutes.

Biochemical tests were performed by using an Automatic Analyzer (7180, Hitachi, Japan) and an Electrolyte Analyzer (ILyte, Instrumentation Laboratory, USA).

Weight, food intake, hematology and blood biochemistry data were analyzed using the statistical analysis system (SAS) software (versions 9.3, SAS Institute Inc., USA). The Bartlett test ($P < 0.05$) was conducted to evaluate the homogeneity of the variance and the significance. If the test had equal variance, the data were analyzed by using the one-way analysis of variance (ANOVA) ($P < 0.05$) and multiple range tests for Dunnett's *t*-test for a post-hoc analysis ($P < 0.05$, $P < 0.01$). If the test did not have equal variance, the data were analyzed by using the Kruskal-Wallis test ($P < 0.05$) and multiple range tests for the Steel test for a post-hoc analysis ($P < 0.05$, $P < 0.01$).

3. Results

During the observation period, no mortality occurred in any of the four groups. Compared to the control group, the male and the female rats in groups 3 and 4 (0.2 and 0.3 mL/animal/day) showed hemoglobinuria, but the low-dosage group (G2, 0.1 mL/animal/day) showed no significant

changes in the clinical signs test (Table 2). No changes in body weight were observed (Table 3). In addition, no significant differences in food consumption were observed (Table 4). In the ophthalmological tests, no abnormalities were detected in any group (Table 5). In the urinalysis, occult blood of male rats in groups 3 and 4 (0.2 and 0.3 mL/animal/day) increased. The female rats in groups 3 and 4 (0.2 and 0.3 mL/animal/day) exhibited significantly increased protein. In addition, groups 2, 3 and 4 (0.1, 0.2 and 0.3 mL/animal/day) showed increased bilirubin, and occult blood (Table 6). No remarkable changes were observed in the hematology (Table 7). Finally, no changes were observed in the blood chemistry, the necropsy, or the histopathology.

4. Discussion

SMS, a traditional medicine, is a mixture of *Panax ginseng*, *Ophiopogon japonicas,* and *Schisandra chinensis*. In a recent study, SMS was used to treat symptoms of cardiovascular diseases, such as heart failure and stroke, as well as neuronal damage [10-12]. Even though SMS is widely used in clinics, further research is needed to assess the safety of the medication by using tox-icity tests. Toxicity tests are mostly used to examine the toxicity of a specific sample and to calculate the No-Observed Adverse-Effect Level (NOAEL) volume.

In this study, the toxicity test was performed at Biotoxtech (Chungwon, Korea), an institute certified to perform non-clinical studies under the regulations of GLP. During the observation period, no mortality occurred in any of the four groups. The medium- and the high-dosage groups (G3

Table 1 Grouping of the animals

Group	SMS injection (mL/animal)	Number of animals (serial numbers)	
		Male	Female
G1 (Control group)	0	10 (1101 — 1110)	10 (2101 — 2110)
G2 (Low-volume group)	0.1	10 (1201 — 1210)	10 (2201 — 2210)
G3 (Mid-volume group)	0.2	10 (1301 — 1310)	10 (2301 — 2310)
G4 (High-volume group)	0.3	10 (1401 — 1410)	10 (2401 — 2410)

SMS, Saeng Maek San.

Table 2 Summary of clinical signs

Group volume (mL/animal)	Sex	Number of animals	Clinical sign	Number of animals affected
G1 (0)	Male	10	Hemoglobinuria Hemoglobinuria (green)	NOA NOA
	Female	10	Hemoglobinuria Hemoglobinuria (green)	NOA NOA
G2 (0.1)	Male	10	Hemoglobinuria Hemoglobinuria (green)	NOA NOA
	Female	10	Hemoglobinuria Hemoglobinuria (green)	NOA NOA
G3 (0.2)	Male	10	Hemoglobinuria Hemoglobinuria (green)	8 NOA
	Female	10	Hemoglobinuria Hemoglobinuria (green)	10 2
G4 (0.3)	Male	10	Hemoglobinuria Hemoglobinuria (green)	10 NOA
	Female	10	Hemoglobinuria Hemoglobinuria (green)	10 4

NOA, no observable abnormality.

Table 3 Mean body weights (g)

Group volume (mL/animal)	Sex	Number of animals	Week 0	1	2	3	4
G1 (0)	Male	10	201.4 ± 7.5	259.6 ± 17.0	310.5 ± 30.0	351.0 ± 40.3	377.1 ± 47.1
	Female	10	160.8 ± 8.4	180.6 ± 8.8	203.3 ± 15.4	220.7 ± 18.5	234.2 ± 18.9
G2 (0.1)	Male	10	201.6 ± 5.4	260.0 ± 12.5	310.1 ± 20.6	350.3 ± 26.8	377.6 ± 29.7
	Female	10	160.8 ± 7.9	184.5 ± 11.3	213.1 ± 14.0	231.6 ± 18.4	244.5 ± 19.5
G3 (0.2)	Male	10	201.8 ± 5.9	259.4 ± 10.9	307.5 ± 20.0	342.5 ± 22.3	366.9 ± 25.6
	Female	10	161.0 ± 8.4	183.2 ± 14.3	204.9 ± 15.9	226.5 ± 18.1	239.2 ± 21.6
G4 (0.3)	Male	10	201.5 ± 6.4	261.3 ± 14.2	311.6 ± 18.0	356.2 ± 24.9	384.1 ± 31.7
	Female	10	161.1 ± 6.9	182.5 ± 9.3	207.5 ± 15.7	225.5 ± 14.5	237.5 ± 14.4

Table 4 Mean food intake (g)

Group volume (mL/animal)	Sex	Number of animals	Week 0	1	2	3	4
G1 (0)	Male	10	27.6 ± 2.7	29.4 ± 3.1	31.9 ± 4.6	32.4 ± 4.9	32.3 ± 4.6
	Female	10	20.8 ± 3.8	20.8 ± 2.2	21.6 ± 2.5	22.5 ± 2.3	23.2 ± 2.8
G2 (0.1)	Male	10	27.2 ± 1.4	28.7 ± 1.7	31.2 ± 2.7	31.1 ± 2.6	31.6 ± 2.5
	Female	10	19.9 ± 3.7	21.9 ± 1.8	23.0 ± 2.4	23.9 ± 2.8	24.7 ± 3.0
G3 (0.2)	Male	10	26.3 ± 1.8	28.2 ± 2.2	30.7 ± 3.0	29.8 ± 2.6	29.9 ± 2.8
	Female	10	20.2 ± 3.7	21.3 ± 2.4	22.1 ± 2.5	23.2 ± 2.6	23.1 ± 3.2
G4 (0.3)	Male	10	27.1 ± 1.9	28.2 ± 3.0	30.6 ± 2.9	31.5 ± 3.2	31.9 ± 4.1
	Female	10	19.6 ± 2.9	20.3 ± 1.5	21.4 ± 2.3	22.2 ± 1.9	22.6 ± 1.4

and G4, 0.2 and 0.3 mL/animal/day, respectively) showed signs of hemoglobinuria while the low-dosage group (G2, 0.1 mL/animal/day) showed no significant signs of hemoglobinuria. In the medium-dosage group (0.2 mL/animal/day), from the 4th day, hemoglobinuria was observed in 8 male rats, and from the 9th day, it was observed in 10 female rats. In the high-dosage group (0.3 mL/animal/day), hemoglobinuria was observed in all animals from the 1st day.

No significant differences in body weight and food consumption were observed.

Additionally, no abnormalities were detected in the ophthalmological tests. Compared to the control group, male rats in groups 3 and 4 (0.2 and 0.3 mL/animal/day) exhibited increased occult blood, and female rats in groups 3 and 4 (0.2 and 0.3 mL/animal/day) exhibited significantly increased protein. Also, groups 2, 3 and 4 (0.1, 0.2 and 0.3 mL/animal/day) showed increased bilirubin and occult blood. In the medium-dosage group (0.2 mL/animal/day) and the high-dosage group (0.3 mL/animal/day), one and two cases, respectively, of amber-colored urine were observed in female rats. However, corpuscular, creatinine and histopathological findings showed no significant changes. No remarkable changes were observed in the hematological examination. Finally, no changes were observed in the blood chemistry, necropsy, or histopathological examinations.

Table 5 Summary of ophthalmological examination

Sex		Male and Female			
Group		G1	G2	G3	G4
Volume (mL/animal/day)		0	0.1	0.2	0.3
Number of animals		5	5	5	5
Findings		Normal	Normal	Normal	Normal
Right eye	Pupil light reflex	5	5	5	5
	Anterior segment	5	5	5	5
	Transparent media	5	5	5	5
	Fundus	5	5	5	5
Left eye	Pupil light reflex	5	5	5	5
	Anterior segment	5	5	5	5
	Transparent media	5	5	5	5
	Fundus	5	5	5	5

Table 6 Summary of urinalysis results

Sex		Male			
Group		G1	G2	G3	G4
volume (mL/animal/day)		0	0.1	0.2	0.3
Number of animals		5	5	5	5
Volume (mL)	Mean	9.8 ± 3.2	10.5 ± 3.8	10.4 ± 6.1	8.0 ± 5.0
Color	Pale yellow	—	2	3	—
	Yellow	5	3	2	5
	Amber	—	—	—	—
Protein (mg/dL)	—	—	1	3	—
	25	4	4	1	4
	75	1	—	1	—
	150	—	—	—	1
	500	—	—	—	—
Bilirubin (mg/dL)	—	5	4	5	4
	1	—	1	—	1
	3	—	—	—	—
	6	—	—	—	—

(Continued)

	—	3	4	1	—
	10	1	1	2	—
Occult blood (Ery/μL)	25	1	—	—	—
	50	—	—	1	2
	150	—	—	—	1
	250	—	—	1	2

Sex		Female			
Group		G1	G2	G3	G4
Volume (mL/animal/day)		0	0.1	0.2	0.3
Number of animals		5	5	5	5
Volume (mL)	Mean	3.8 ± 1.6	4.9 ± 1.4	3.9 ± 1.0	8.9* ± 3.6
	Pale yellow	—	—	—	1
Color	Yellow	5	5	4	2
	Amber	—	—	1	2
	—	—	—	—	1
	25	5	5	1	1
Protein (mg/dL)	75	—	—	3	1
	150	—	—	1	2
	500	—	—	—	—
	—	4	3	—	3
Bilirubin (mg/dL)	1	1	2	5	2
	3	—	—	—	—
	6	—	—	—	—
	—	4	1	—	1
	10	1	—	—	—
Occult blood (Ery/μL)	25	—	1	—	—
	50	—	—	—	—
	150	—	—	—	—
	250	—	3	5	4

Significantly different from control by Dunnett's *t*-test: *$P < 0.01$.

Table 7 Mean hematological parameters

Sex	Male			
Group	G1	G2	G3	G4
Volume (mL/animal/day)	0	0.1	0.2	0.3
Number of animals	10	10	10	10
RBC ($\times 10^6$ cells/μL)	7.86 ± 0.48	7.78 ± 0.28	7.62 ± 0.38	7.60 ± 0.47
HGB (g/dL)	15.5 ± 0.7	15.5 ± 0.5	15.1 ± 0.6	15.0 ± 0.6

(Continued)

HCT (%)		43.7 ± 1.9	43.4 ± 1.3	42.6 ± 1.8	42.3 ± 1.8
RBC Indices	MCV (fL)	55.7 ± 2.0	55.8 ± 1.1	55.9 ± 2.0	55.7 ± 1.7
	MCH (pg)	19.8 ± 0.7	19.9 ± 0.4	19.8 ± 0.7	19.7 ± 0.7
	MCHC (g/dL)	35.5 ± 0.5	35.7 ± 0.5	35.5 ± 0.4	35.4 ± 0.2
PLT ($\times 10^3$ cells/μL)		940 ± 71	997 ± 81	968 ± 88	983 ± 148
Reti (%)		2.59 ± 0.49	2.75 ± 0.48	2.62 ± 0.32	3.24 ± 0.74*
WBC ($\times 10^3$ cells/μL)		8.18 ± 2.36	9.13 ± 2.37	8.66 ± 2.94	6.93 ± 1.28
WBC Differential Counting (%)	NEU	16.2 ± 6.0	14.5 ± 6.2	18.8 ± 4.8	15.6 ± 4.9
	LYM	80.8 ± 6.0	82.4 ± 6.0	78.0 ± 5.0	81.2 ± 4.7
	MONO	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.5	1.5 ± 0.5
	EOS	0.8 ± 0.4	0.8 ± 0.3	0.9 ± 0.2	0.9 ± 0.4
	BASO	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
PT (sec)		16.7 ± 0.7	17.0 ± 0.5	17.0 ± 0.8	17.4 ± 0.6
APTT (sec)		15.2 ± 1.9	14.6 ± 1.4	14.9 ± 1.7	15.2 ± 1.2

Sex		Female			
Group		G1	G2	G3	G4
Volume (mL/animal/day)		0	0.1	0.2	0.3
Number of animals		10	10	10	10
RBC ($\times 10^6$ cells/μL)		7.71 ± 0.16	7.48 ± 0.25	7.53 ± 0.32	7.55 ± 0.22
HGB (g/dL)		15.1 ± 0.3	14.8 ± 0.3	14.8 ± 0.5	14.9 ± 0.3
HCT (%)		41.4 ± 0.9	40.9 ± 1.0	40.5 ± 1.5	40.8 ± 0.9
RBC Indices	MCV (fL)	53.7 ± 1.2	54.7 ± 1.2	53.8 ± 1.3	54.1 ± 1.5
	MCH (pg)	19.6 ± 0.4	19.9 ± 0.5	19.6 ± 0.6	19.8 ± 0.6
	MCHC (g/dL)	36.5 ± 0.5	36.3 ± 0.5	36.5 ± 0.7	36.6 ± 0.6
PLT ($\times 10^3$ cells/μL)		1056 ± 120	1075 ± 96	1050 ± 65	1087 ± 123
Reti (%)		2.37 ± 0.40	2.57 ± 0.56	2.52 ± 0.38	2.82 ± 0.38
WBC ($\times 10^3$ cells/μL)		5.41 ± 1.85	6.32 ± 2.04	5.32 ± 1.27	6.00 ± 2.06
WBC Differential Counting (%)	NEU	15.8 ± 5.1	14.6 ±6.9	13.5 ± 2.2	11.9 ± 4.9
	LYM	81.4 ± 4.8	81.8 ± 6.8	83.0 ± 2.6	85.2 ± 4.8
	MONO	1.3 ± 0.3	1.8† ± 0.3	1.6 ± 0.4	1.2 ± 0.4
	EOS	0.9 ± 0.3	0.9 ± 0.2	1.1 ± 0.3	0.8 ± 0.3
	BASO	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
PT (sec)		18.3 ± 0.8	17.8 ± 0.4	17.8 ± 0.5	17.9 ± 0.6
APTT (sec)		14.5 ± 1.3	14.0 ± 0.7	13.6 ± 2.1	14.2 ± 0.9

Significantly different from control by Dunnett's *t*-test: *$P < 0.05$, †$P < 0.01$.

RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular cell hemoglobin concentration; PLT, platelet; Reti, reticulocytes; WBC, white blood cell; NEU, neutrophils; LYM, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils; PT, prothrombin time; APTT, active partial thromboplastin time.

5. Conclusion

In conclusion, the present study corroborated that administration of 0.1 mL/animal/day of SMS did not cause any significant changes in body weight, food consumptions or the results of hematological, blood biochemistry, and necropsy examinations. Also, no mortality was observed in any group, which indicates that SMS pharmacopuncture can be used as a safe treatment.

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Conflict of interest

The authors declare that there are no conflict of interest.

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