Original Article

Assessment of genotoxicity of *Ssanghwa-tang*, an herbal formula, by using bacterial reverse mutation, chromosome aberration, and *in vivo* micronucleus tests

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Objectives: *Ssanghwa-tang* (SHT) is a traditional herbal formula comprising nine medicinal herbs, and it is used for reducing fatigue in Korea. SHT exerts various effects such as anti-inflammatory, antioxidant, and anti-aging activities, and protection against acute hepatotoxicity. However, the genotoxicity of SHT has not yet been established.

Methods: Ten components were identified in SHT water extract by using high-performance liquid chromatography analysis. We assessed the genotoxicity of SHT by using bacterial reverse mutation (Ames test), chromosome aberration, and *in vivo* micronucleus tests.

Results: The contents of paeoniflorin, glycyrrhizin, and liquiritin apioside in SHT were 15.57, 6.94, and 3.48 mg/g extract, respectively. SHT did not increase the revertant colonies of *Salmonella typhimurium* and *Escherichia coli* strains in the presence or absence of metabolic activity. Although SHT did not induce structurally abnormal chromosomes in Chinese hamster lung (CHL) cells in the presence of metabolic activity, the number of structurally aberrated chromosomes increased dose-dependently in the absence of metabolic activity. In the *in vivo* micronucleus test, SHT did not affect the formation of micronuclei compared with the vehicle control.

Conclusions: Genotoxicity of SHT was not observed in the Ames test and *in vivo* micronucleus test. However, based on the results of chromosome aberration test, it can be presumed that SHT has the potential to induce genotoxicity because it induced structurally abnormal chromosomes in the absence of metabolic activity.

Key Words : *Ssanghwa-tang*, Genotoxicity, Bacterial reverse mutation test, Chromosome aberration test, *In vivo* micronucleus test

Introduction

Herbal medicines have been traditionally used for a long time and are now widely used to replace pharmaceutical drugs. The use of herbal medicines for the prevention and treatment of

several diseases is expanding rapidly worldwide¹⁾. Herbal medicines have long been considered safe. However, no experimental basis has been established to determine the toxicity of traditional herbal medicines²⁾. Recently, there have been increasing concerns on various toxicities, such as

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hepatotoxicity and nephrotoxicity, caused by traditional herbal medicines. Therefore, it is necessary to perform experiments to establish the basic safety of various herbal medicines³⁻⁷.

Ssanghwa-tang (SHT), a traditional herbal formula, has long been used in Korea for the reduction of fatigue caused by colds and for the enhancement of physical strength. Korean pharmaceutical companies produced 39 million bottles of SHT in 2016, and it was in the top 100 over-the-counter drugs⁸. In recent studies, SHT has been reported to possess many functions, such as protection against acute hepatotoxicity, anti-melanogenic activity, and inhibitory effects on bone loss⁹⁻¹¹. It has also been reported to exert analgesic, anti-convulsant, anti-oxidative, anti-aging and anti-inflammatory effects¹²⁻¹⁴. A recent study showed that SHT exerted anti-inflammatory effects on cigarette smoke-induced airway inflammation by modulation pro-inflammatory cytokines through the matrix metalloproteinase 9 (MMP-9) and extracellular signal-regulated kinase (ERK) signaling pathways¹⁵. Although various effects of SHT have been identified, studies on its toxicity have not been performed sufficiently. The acute and repeated oral dose toxicity studies of SHT have been archived in previous reports^{16,17}, but the genotoxicity has not yet been studied.

Genotoxicity test is performed to confirm that there is no possibility of carcinogens or mutagens. Therefore, in this study, we aimed to test the genotoxicity of SHT by using bacterial reverse mutation (Ames test), chromosome aberration, and *in vivo* micronucleus tests. These tests were performed according to the Organization for Economic Cooperation and Development (OECD)

guidelines¹⁸⁻²⁰.

Materials & Methods

1. Chemicals and reagents

Albiflorin (ALB, 99.8%), paeoniflorin (PAE, 98.8%), ferulic acid (FA, 98.0%), liquiritin (LIQ, 99.6%), and glycyrrhizin (GLY, 99.0%) were purchased from Wako Pure Chemical (Osaka, Japan). Cinnamic acid (CA, 99.0%), coumarin (COU, 99.0%), and 5-(hydroxy-methyl)furfural (5-HMF, 99.0%) were acquired from Merck KGaA (Darmstadt, Germany). Liquiritin apioside (LIQA, 98.0%) and nodakenin (NOD, 99.5%) were obtained from Shanghai Sunny Biotech (Shanghai, China) and ChemFaces Biochemical (Wuhan, China), respectively. High-performance liquid chromatography (HPLC)-grade solvents (methanol, acetonitrile and water) were purchased from J.T. Baker (Phillipsburg, NJ). Anhydrous acetic acid (glacial) for HPLC was ACS reagent grade and purchased from Merck KGaA (Darmstadt, Germany).

2. Plant materials

The nine raw materials (Table 1) of SHT decoction were obtained from Kwangmyungdang Medicinal Herbs (Ulsan, Korea) in October 2016, and identified by Dr. Goya Choi of the Korea Institute of Oriental Medicine (KIOM). The voucher specimens (2016-KE21-1- KE21-9) have been stored at KIOM.

3. Preparation of *Ssanghwa-tang* water extract

To obtain the SHT decoction extract, the nine raw materials described in Table 1 were mixed

Table 1. Composition of *Ssanghwa-tang*.

Herbal name	Scientific name	Family	Original region	Amount per Chup (g)	Amount for extraction (kg)
Paeoniae Radix	<i>Paeonia lactiflora</i> Pallas	Paeoniaceae	Hwasun, Korea	9.38	37.50
Rehmanniae Radix Preparata	<i>Rehmannia glutinosa</i> Liboschitz ex Steudel	Scrophulariaceae	Jangheung, Korea	3.75	15.00
Astragali Radix	<i>Astragalus membranaceus</i> Bunge	Leguminosae	Jeongseon, Korea	3.75	15.00
Angelicae Gigantis Radix	<i>Angelica gigas</i> Nakai	Umbelliferae	Yeongcheon, Korea	3.75	15.00
Cnidii Rhizoma	<i>Cnidium officinale</i> Makino	Umbelliferae	Yeongcheon, Korea	3.75	15.00
Cinnamomi Cortex	<i>Cinnamomum cassia</i> Presl	Lauraceae	Vietnam	2.81	11.25
Glycyrrhizae Radix et Rhizoma	<i>Glycyrrhiza uralensis</i> Fischer	Leguminosae	China	2.81	11.25
Zingiberis Rhizoma Recens	<i>Zingiber officinale</i> Roscoe	Zingiberaceae	Seosan, Korea	3.75	15.00
Zizyphi Fructus	<i>Zizyphus jujube</i> Miller var. <i>inermis</i> Rehder	Rhamnaceae	Yeongcheon, Korea	3.75	15.00
Total				28.12	150.00

Fig. 1. Chemical structures of the ten marker components in *Ssanghwa-tang*.

and extracted with distilled water (DW, 1,500 L) at 80°C for 2 h using the reflux method in Sungil Bioex Co., Ltd. (Hwaseong, Korea). The liquid extract was spray-dried to obtained powdered extract of 39.0 kg (extraction yield: 26.0%).

4. HPLC analysis of *Ssanghwa-tang* decoction

Analysis of the 10 marker components (Fig. 1),

ALB, PAE, FA, LIQ, GLY, CA, COU, 5-HMF, NOD, and LIQA, in *Ssanghwa-tang* was conducted using the Shimadzu Prominence LC-20A series HPLC system (Kyoto, Japan) equipped with a photodiode array (PDA) detector and LCsolution (Version 1.24, SP1, Kyoto, Japan) for measurement and processing of chromatographic data. The 10 components were separated on a Phenomenex Gemini C₁₈ column (250 × 4.6 mm, 5 μm particle

size, Torrance, CA, USA) at 40°C. The mobile phases were 1.0% (v/v) acetic acid in DW (A) and 1.0% acetic acid in acetonitrile (B). The gradient flow conditions were as follows: 5–60% B for 0–40 min, 60–100% B for 40–45 min, 100% B for 45–50 min, and 100–5% B for 50–55 min. The analysis was performed at a flow rate of 1.0 mL/min and an injection volume of 10 μ L.

5. Preparation of S9 mixture

Many materials may induce mutagenicity after metabolism, even if they are not mutagenic before metabolism in the body. To confirm metabolism-induced mutagenicity, *in vitro* genotoxicity tests were performed by adding metabolic activation system extracted from the liver of rat. The S9 mixture was prepared by mixing the exogenous metabolizing system (S9 fraction) and cofactor. The S9 fraction was obtained from Molecular Toxicology, Inc. (Boone, NC, USA). The final concentration of cofactors was 8 μ M MgCl₂, 33 μ M KCl, 5 μ M glucose-6-phosphate, 4 μ M nicotinamide adenine dinucleotide phosphate (NADPH), 4 μ M nicotinamide adenine dinucleotide (NADH), and 100 μ M sodium phosphate buffer.

6. Bacterial reverse mutation test (Ames test)

The Ames test was carried out according to OECD guideline¹⁸⁾. The experiment was performed by the method of Maron and Ames²¹⁾. Histidine-requiring *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, and the tryptophan-requiring *Escherichia coli* strain WP2uvrA are known to be sensitive to mutagens and are

commonly used in bacterial mutagenicity studies. All strains were obtained from Molecular Toxicology, Inc. (Boone, NC, USA). SHT was dissolved in DW and the maximum treated concentration was 5000 μ g/plate, as determined by OECD guidelines. Each strain was treated at concentrations of 0, 1250, 2500, and 5000 μ g/plate in the presence or absence of S9 mix. Sodium azide (SA), 2-nitrofluorene (2-NF), 9-aminoacridine (9-AA), 4-nitroquinoline N-oxide (4NQO), 2-aminoanthracene (2-AA), and benzo(a)pyrene (BP) were respectively used as positive controls. The results are presented as the mean of the number of revertant colonies that occurred at each concentration three times on each plate with the standard deviation (mean $\pm$ SD).

7. Chromosome aberration test

The chromosome aberration test was conducted in accordance with OECD guideline¹⁹⁾, using the method described previously, with minor modifications^{22–24)}. Chinese hamster lung (CHL) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The number of chromosomes in CHL cells was 25 and the doubling time was approximately 15 h. The cells were cultured at 37°C in a 5% CO₂ atmosphere in minimum essential medium containing 10% fetal bovine serum, 2.2 g/L of sodium bicarbonate, 2 mM of L-glutamine, 100 μ g/mL of streptomycin sulfate and 100 units/mL of penicillin G·Na. The concentration of SHT was determined based on solubility and cytotoxicity in a preliminary range-finding study. SHT 5000 μ g/mL was used as the highest concentration and serially diluted twofold to a final concentration of

19.53 $\mu\text{g/mL}$. The cells were treated with SHT in the presence (+S) and absence (-S) of S-9 mix. The relative population doubling (RPD) was calculated and used as an index of cell proliferation. In the presence of S9 mixture, the concentrations of SHT were 2600, 4000, and 5000 $\mu\text{g/mL}$ for 6 h. In the absence of S9 mixture, the concentration of SHT was 1000, 2600, 3800, and 4000 $\mu\text{g/mL}$ and 1000, 2000, 2600, and 2800 $\mu\text{g/mL}$ for 6 and 22 h, respectively. After completion of treatment with SHT, the cells were washed with Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline (CMF D-PBS) and fresh medium was added. SHT, positive control, and vehicle control (DW) were treated for 6 h with or without S9 mixture, and 22 h without S9 mixture. Ethyl methanesulfonate (EMS) was used as a positive control in the absence of S9 mixture, and cyclophosphamide monohydrate (CPA) was used as a positive control in the presence of S9 mixture. After incubation, the cells were fixed in fixation solution (methanol:glacial acetic acid = 3:1, v/v) for 20 min at 4°C and replaced with fresh fixation solution after centrifugation at 1000 rpm for 5 min. The fixed cells were dropped on slides and stained with 3% (v/v) Giemsa solution prepared with Gurr buffer. The chromosomal aberrations were differentiated and counted according to the 'Atlas of chromosome aberration by chemicals (JEMS-MMS, 1988)',²⁵⁾.

8. *In vivo* micronucleus test

The *in vivo* micronucleus test was performed according to OECD guideline²⁰⁾. Six-week-old male ICR specific-pathogen-free mice were

obtained from Orient Co., Ltd. (Seongnam, Korea). The mice were housed 3 per cage and maintained at a temperature of 22±3°C, relative humidity of 40–70%, 12 h light/dark cycle, light intensity of 150–300 Lux, and air ventilation frequency of 10–20 times/h. The highest SHT concentration was set at 2000 mg/kg, and SHT was orally administered for 2 days at concentrations of 500, 1000, and 2000 mg/kg, respectively. CPA (Sigma-Aldrich, USA) was used as a positive control. It was intraperitoneally administered at 70 mg/kg. After 23 h from the final administration, the mice were sacrificed by CO₂ asphyxiation, and bone marrow cells were separated and stained with 5% (v/v) Giemsa solution²⁶⁾. Circular and oval bodies showing the same staining pattern as the nuclei of surrounding nucleated cells and with size ranging from 1/5–1/20th of the diameter of the cell were counted as micronucleus (MN). The number of micronucleated polychromatic erythrocytes (MNPCEs) among 2000 polychromatic erythrocytes (PCEs) per individual was measured and expressed as the induced frequency of micronucleus. The ratio of PCEs/[PCEs + normochromatic erythrocytes (NCEs)] was measured by counting 500 erythrocytes regardless of micronucleus.

The *in vivo* micronucleus test protocol was approved by the Institutional Animal Care and Use Committee of Korea institute of Toxicology (approval number: 1706-0244).

Results

1. Marker components of SHT

In the present study, an optimized HPLC-PDA

Fig. 2. Representative HPLC chromatogram of the standard mixture (A) and *Ssanghwa-tang* decoction (B) at 254 nm. 5-HMF (1), ALB (2), PAE (3), LIQA (4), LIQ (5), FA (6), NOD (7), CA (8), COU (9), and GLY (10).

method was successfully applied for the quantitative analysis of the 10 main components in SHT. All the components were separated within 45 min. Representative, HPLC chromatograms of the standard mixtures and SHT sample are shown in Fig. 2. For the quantitative analysis of each compound, ultraviolet absorbance was monitored at the following wavelengths: 230 nm for ALB and PAE; 254 nm for GLY; 275 nm for LIQA,

LIQ, COU, and CA; 280 nm for 5-HMF; 320 nm for FA; and 335 nm for NOD, respectively. The quantity of the 10 marker components in SHT sample was in the range of 0.09–15.57 mg/g of the extract (Table 2).

2. Ames test

In the *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain

Table 2. Amounts of the Ten Marker Components in *Ssanghwa-tang* Determined by HPLC (n=3).

Compound	Mean (mg/extract g)	SD ($\times 10^{-1}$)	RSD (%)	Source
5-HMF	0.94	0.03	0.34	<i>R. glutinosa</i>
ALB	1.65	0.18	1.12	<i>P. lactiflora</i>
PAE	15.57	1.15	0.74	<i>P. lactiflora</i>
LIQA	3.48	0.35	1.02	<i>G. uralensis</i>
LIQ	0.87	0.19	2.14	<i>G. uralensis</i>
FA	0.50	0.09	1.85	<i>C. officinale</i>
NOD	1.85	0.17	0.92	<i>A. gigas</i>
COU	0.15	0.02	1.20	<i>C. cassia</i>
CA	0.09	0.01	0.81	<i>C. cassia</i>
GLY	6.94	0.046	0.66	<i>G. uralensis</i>

WP2uvrA, no reverse mutation was observed in any concentration (1250, 2500, and 5000 $\mu\text{g/mL}$) groups treated with SHT in the presence or absence of metabolic activation (Table 3). The positive controls significantly increased the number of revertant colonies, indicating that the

test was valid. This result showed that SHT has no mutagenic or carcinogenic potential.

3. Chromosomal aberration test

In the preliminary dose range-finding study, no cytotoxicity was observed when CHL cells were

Table 3. Results of Bacterial Reverse Mutation Test of *Ssanghwa-tang* in the Presence or Absence Metabolic Activity.

Tester Strain	Chemical Treated	Concentration ($\mu\text{g}/\text{plate}$)	Without Activation System		With Activation system ^a	
			Revertant Colonies/plate (Mean $\pm$ SD) [Fold-increases] ^b		Revertant Colonies/plate (Mean $\pm$ SD) [Fold-increases]	
TA100	Vehicle Test Item	0	129 $\pm$ 11		139 $\pm$ 4	
		1250	133 $\pm$ 4	[1.0]	151 $\pm$ 18	[1.1]
		2500	136 $\pm$ 5	[1.1]	145 $\pm$ 7	[1.0]
		5000	177 $\pm$ 21	[1.4]	164 $\pm$ 3	[1.2]
TA1535	Vehicle Test Item	0	14 $\pm$ 2		12 $\pm$ 3	
		1250	14 $\pm$ 3	[1.0]	11 $\pm$ 1	[1.0]
		2500	12 $\pm$ 3	[0.9]	11 $\pm$ 2	[1.0]
		5000	15 $\pm$ 5	[1.1]	13 $\pm$ 1	[1.1]
TA98	Vehicle Test Item	0	25 $\pm$ 2		38 $\pm$ 1	
		1250	27 $\pm$ 7	[1.1]	31 $\pm$ 6	[0.8]
		2500	25 $\pm$ 4	[1.0]	36 $\pm$ 7	[0.9]
		5000	24 $\pm$ 3	[1.0]	33 $\pm$ 7	[0.9]
TA1537	Vehicle Test Item	0	8 $\pm$ 2		13 $\pm$ 3	
		1250	10 $\pm$ 4	[1.2]	11 $\pm$ 2	[0.9]
		2500	8 $\pm$ 2	[1.0]	13 $\pm$ 3	[1.0]
		5000	8 $\pm$ 2	[1.0]	13 $\pm$ 1	[1.0]
WP2uvrA	Vehicle Test Item	0	19 $\pm$ 2		23 $\pm$ 2	
		1250	20 $\pm$ 2	[1.1]	21 $\pm$ 1	[0.9]
		2500	20 $\pm$ 3	[1.1]	23 $\pm$ 3	[1.0]
		5000	18 $\pm$ 2	[1.0]	20 $\pm$ 2	[0.9]
Positive Controls						
TA100	SA	0.5	301 $\pm$ 8	[2.3]	-	-
	BP	2	-	-	660 $\pm$ 59	[4.7]
TA1535	SA	0.5	199 $\pm$ 17	[14.5]	-	-
	2-AA	2	12 $\pm$ 2	[0.9]	216 $\pm$ 18	[18.5]
TA98	2-NF	2	236 $\pm$ 11	[9.6]	-	-
	BP	2	21 $\pm$ 4	[0.9]	195 $\pm$ 17	[5.1]
TA1537	9-AA	50	194 $\pm$ 39	[24.2]	-	-
	BP	2	-	-	77 $\pm$ 4	[5.9]
WP2uvrA	4NQO	1	179 $\pm$ 14	[9.6]	-	-
	2-AA	4	-	-	64 $\pm$ 11	[2.8]

^a Metabolic activation system containing 5% v/v of S9 fraction

^b No. of revertant colonies of treated plate/No. of revertant colonies of vehicle control plate

treated with SHT for 6 h in the presence of metabolic activity. In the absence of metabolic activity, treatment with SHT at a concentration of 3800 $\mu\text{g/mL}$ for 6 h and 2600 $\mu\text{g/mL}$ for 22 h resulted in approximately 50% cytotoxicity (6-S, RPD 51%; 22-S, RPD 50%, Supplementary Table 1 and 2). Turbidity was observed at a concentration greater than 1250 $\mu\text{g/mL}$ in all conditions. In the presence of metabolic activity (+S), SHT showed no significant increases in the numerical aberrant metaphase cells and frequency of metaphases

with structural aberration compared with the vehicle control. In the absence of metabolic activity, there were significant increases in the frequency of metaphases with structural aberration depending on the concentration of SHT and duration (6 or 22 h) compared with the vehicle control (Table 4). There was no observable increase the number of aberrant metaphase cells. The positive controls significantly increased the frequency of metaphases with structural aberration, suggesting that the test was valid.

Table 4. Results of Chromosomal Aberration Test and Relative Population Doubling of *Ssanghwa-tang* in the Presence or Absence of Metabolic Activation System.

Concentration ($\mu\text{g/mL}$)	Times (hours) ^a	Mean Aberrant Metaphases ^b	Mean Total Aberrations	Mean of PP+ER	RPD (%)
6 h treatment (+S9)					
0	6-18	1.0 / 0.0	1.5 / 1.0	1.0+0.0	100
2600 ^{#&}	6-18	0.0 / 0.0	1.5 / 1.5	0.0+0.0	115
4000 ^{#&}	6-18	2.0 / 0.5	0.5 / -0.5	0.5+0.0	110
5000 ^{#&}	6-18	0.5 / 0.0	0.5 / 0.5	0.0+0.0	98
CPA (6)	6-18	22.5 / 20.0 ^{**}	31.0 / 26.5	0.0+0.0	60
6 h treatment (-S9)					
0	6-18	1.5 / 0.0	1.5 / 0.0	0.0+0.0	100
1000	6-18	0.0 / 0.0	0.0 / 0.0	0.0+0.0	105
2600 ^{#&}	6-18	1.5 / 1.5	1.5 / 1.5	0.0+0.0	71
3800 ^{#&}	6-18	15.0 / 13.5 ^{***§}	26.5 / 24.0	0.0+0.0	47
4000 ^{#&}	6-18		Not counted		39
EMS (800)		18.0 / 18.0 ^{**}	19.5 / 19.5	0.5+0.0	79
22 h treatment (-S9)					
0	22-2	1.0 / 0.0	1.0 / 0.0	0.5+0.0	100
1000	22-2	1.0 / 0.0	1.0 / 0.0	0.0+0.0	100
2000 ^{#&}	22-2	5.5 / 4.0 [*]	8.5 / 6.5	0.5+0.5	72
2600 ^{#&}	22-2	8.0 / 6.5 ^{***§}	14.0 / 11.0	0.0+0.0	47
2800 ^{#&}	22-2		Not counted		34
EMS (600)	22-2	20.5 / 19.5 ^{**}	22.5 / 21.5	0.0+0.0	67

Abbreviations: PP, polyploid; ER, endoreduplication; +S9, treated S9 mixture; -S9, treated without S9 mixture; RPD, relative population doubling; CPA, cyclophosphamide monohydrate; EMS, ethyl methanesulfonate

[#] Visible turbidity of test item was observed at the beginning of the treatment.

[&] Visible turbidity of test item was observed at the end of the treatment.

^{*} ** Significantly different from the control at P(0.05 or P(0.01.

[§] Significantly from Cochran-Armitage trend test at P(0.01.

^a Treatment time-recovery time.

^b Gaps included/excluded, means of duplicate cultures; 150 metaphases were examined per culture.

4. Micronucleus test

Body weight showed no significant changes in mice administered SHT compared with that in mice administered vehicle control (Supplementary Fig. 1). Moreover, there were no deaths and adverse clinical signs associated with SHT in mice but also no significant increase in the number of MNPCs and ratio of PCEs/(PCEs + NCEs) at any dose of SHT (Table 5). This test was appropriated because the number of MNPCs and ratio of PCEs/(PCEs+NCEs) increased in the CPA-treated group, which was a positive control.

Discussion

In traditional medicine, medicinal herbs have been used for centuries. In recent years, its use has been increasing as an alternative or supplement to counter the limitations of modern medicines. Consumers expect herbal medicines to be more user-friendly and safer than conventional drugs such as synthetic chemicals because these originate from nature and have many clinical applications. However, some herbal medicines

adverse events. We investigated such medicines to establish safety data for widely used herbal formulas in Korea. In previous studies, we have reported the acute and sub-acute toxicity of SHT in rats. There was no death or any adverse clinical signs at the highest dose of SHT (2000 mg/kg) in both male and female rats. In the 4-week repeated oral administration toxicity study of SHT, no adverse signs were observed at the dose 2000 mg/kg, while minor changes were observed at the highest dose of 5000 mg/kg. In the present study, the genotoxicity of SHT was estimated by bacterial reverse mutation, *in vitro* chromosomal aberration and *in vivo* micronucleus formation tests. The maximum dose of SHT for the micronucleus formation test in mice was 2000 mg/kg, which converts to a human equivalent dose of 9,756 mg/60 kg adult²⁷⁾. In humans, the dose of 1 Chup of SHT is 28.12 g as a herbal mixture before extraction (Table 1), and when the extraction yield (26.0%) is reflected, it corresponds to 7,311 mg as an extract powder.

Genotoxicity includes mutagenicity and

Table 5. Incidence of *in vivo* Micronucleated Polychromatic Erythrocytes in the Bone Marrow of Mice Administered with *Ssanghwa-tang*.

Treatment (mg/kg)	No. of animals	MNPCEs ^a		PCEs/(PCEs+NCEs) ^b	
		(mean ± SD)			
0	3	4.00 ± 1.73		0.52 ± 0.06	
500	3	2.67 ± 0.58		0.49 ± 0.06	
1000	3	1.67 ± 0.58		0.48 ± 0.02	
2000	3	4.00 ± 1.73		0.55 ± 0.03	
CPA (70)	3	105.33 ± 21.83		0.52 ± 0.05	

Abbreviation: MNPCs, micronucleated polychromatic erythrocytes; PCEs, polychromatic erythrocytes; NCEs, normochromatic erythrocytes; CPA, cyclophosphamide monohydrate.

^a 2000 polychromatic erythrocytes were examined per animal.

^b 500 erythrocytes were examined per animal.

contain toxic ingredients that cause serious

carcinogenicity according to DNA damage. The

Ames test is a bacterial reverse mutation assay that can be performed in a short period to confirm whether a substance has the potential to cause gene mutations through genetic damage²⁸⁾. The *in vitro* mammalian chromosome aberration test was conducted to detect the structural chromosomal aberrations²⁹⁾. The *in vivo* micronucleus test is a method to confirm genotoxicity by detecting chemical substance-induced micronucleus. Micronuclei are generated by chromosome aberrations, defects of the cell repair system, and accumulation of DNA damage. The formation of micronucleus induced by substances with genotoxicity can lead to cell death or cancer³⁰⁾.

In the present study, SHT did not cause reverse mutagenesis in all five bacterial strains, nor did it induce micronucleus formation in mice. In contrast, in CHL cells, chromosomal abnormalities were induced by more than 1000 $\mu\text{g/mL}$ of SHT. In a previous study, *Paeoniae Radix*, *Rehmanniae Radix Preparata*, *Angelica Gigantis Radix*, *Cnidii Rhizoma*, and *Cinnamomi Cortex* showed negative results for the chromosomal aberration test in the absence of metabolic activation system in CHL cells at 100 $\mu\text{g/mL}$ extract concentration. On the other hand, *Paeoniae Radix*, *Angelica Gigantis Radix*, and *Cnidii Rhizoma* in the presence of the metabolic activation system induced chromosomal abnormalities in 9%, 7%, and 6% of the observed cells, respectively³¹⁾. The conditions that induced chromosomal abnormalities in CHL cells by SHT were the absence of metabolic activation system and more than 1000 $\mu\text{g/mL}$ of SHT. It means that SHT itself, not metabolites of SHT, causes chromosomal abnormalities. Also, when SHT taken at normal dose, a concentration of 1000 μ

g/mL is considered too high to reach in the body. Chromosomal aberration can be caused by a mechanism related to cytotoxicity even by drugs that do not attack DNA, and it is known that the *in vitro* chromosomal aberration test has a high possibility of showing 'false positive' by inducing cytotoxicity of the treated drug³²⁾. In addition, there is also a study that confirmed the plasma levels of chemicals which showed negative results in the Ames test and *in vivo* micronucleus test, but showed positive results in the *in vitro* chromosomal aberration/micronucleus test. According to the study, when the maximum concentration in plasma (C_{max}) of the chemical in the *in vivo* test was lower than the lowest positive concentration (LPC) of the chemical in the *in vitro* test, negative results were obtained in the *in vivo* test³³⁾. There is a limitation to this study. It is difficult to confirm that this result alone is indicative of genotoxicity.

Conclusions

SHT did not cause genotoxicity in the Ames test and *in vivo* micronucleus test, but it caused chromosomal abnormalities in CHL cells. We suggest that although SHT showed a positive result in the *in vitro* chromosomal aberration test, 1) because the concentration of SHT inducing positive chromosomal aberration showed about 50% cytotoxicity in CHL cells, it may be a 'false positive' and 2) because SHT did not induce micronucleation in mice, the C_{max} of SHT in mice may be lower than the LPC of SHT in CHL cells. Additional tests such as comet assay should be performed to ensure genotoxicity.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Fig. 1. Body weight changes after single administration of Ssanghwa-tang extract at dose level of 0, 500, 1000, and 2000 mg/kg in male mice. Cyclophosphamide monohydrate (CPA, 70 mg/kg) was used as a positive control. Values are expressed as means $\pm$ SD ($n = 3$ per group).

Supplementary Table 1. Individual relative population doubling (RPD) data from the first study for dose range-finding of Ssanghwa-tang (SHT) in CHL cells

Nominal Conc. of SHT ($\mu\text{g/mL}$)	Metabolic Activation System	Times ^a (hours)	Cell Counts ^b		Mean	PD ^c	RPD (%) ^d
			One Flask/Concentration				
6-hour treatment (6+S)							
0	+	6-18	6905	6944	6925	1.163	100
19.53	+	6-18	6567	6425	6496	1.071	92
39.06	+	6-18	6745	6801	6773	1.131	97
78.13	+	6-18	6920	6923	6922	1.162	100
156.25	+	6-18	7049	7000	7025	1.184	102
312.5	+	6-18	7174	7191	7183	1.216	105
625	+	6-18	7128	7084	7106	1.200	103
1250 #&	+	6-18	6897	6881	6889	1.156	99
2500 #&	+	6-18	6565	6436	6501	1.072	92
5000 #&	+	6-18	5762	5711	5737	0.891	77
6-hour treatment (6-S)							
0	–	6-18	10014	10071	10043	1.699	100
19.53	–	6-18	10551	10607	10579	1.774	104
39.06	–	6-18	10796	10682	10739	1.796	106
78.13	–	6-18	10678	10733	10706	1.792	105
156.25	–	6-18	10899	10820	10860	1.812	107
312.5	–	6-18	11017	10954	10986	1.829	108
625	–	6-18	11378	11276	11327	1.873	110
1250 #&	–	6-18	10428	10550	10489	1.762	104
2500 #&	–	6-18	8463	8466	8465	1.453	85
5000 #&	–	6-18	4434	4430	4432	0.519	31
22-hour treatment (22-S)							
0	–	22-2	8461	8361	8411	1.444	100
19.53	–	22-2	8415	8283	8349	1.433	99
39.06	–	22-2	8235	8222	8229	1.412	98
78.13	–	22-2	8633	8624	8629	1.480	103
156.25	–	22-2	8563	8562	8563	1.469	102
312.5	–	22-2	8409	8476	8443	1.449	100
625	–	22-2	8833	8884	8859	1.518	105
1250 #&	–	22-2	8191	8277	8234	1.413	98
2500 #&	–	22-2	5757	5696	5727	0.889	62
5000 #&	–	22-2	3761	3630	3696	0.257	18
Cell number at the treatment			3104	3081	3093		

Visible turbidity of SHT was observed at the beginning of the treatment.

& Visible turbidity of SHT was observed at the end of the treatment.

^a Treatment time-recovery time

^b Each culture was trypsinized and suspended with 1.0 mL of 0.05% trypsin-EDTA and 9.0 mL of culture medium. The cell suspensions of 0.8 mL/culture were diluted 25 times with 19.2 mL of Isoton solution. The cells in 0.5 mL Isoton solution were counted twice/culture using Coulter Counter[®] model ZM. Actual number of cells per culture = Mean Count $\times$ 500.

^c Population Doubling = $[\log (\text{Post-treatment cell number} \div \text{Initial cell number})] \div \log 2$

^d RPD = $\frac{\text{No. of Population doubling in treated cultures}}{\text{No. of Population doubling in control cultures}} \times 100 (\%)$

Abbreviations:

RPD, Relative Population Doubling; +, Presence of metabolic activation system; -, Absence of metabolic activation system

Supplementary Table 2. Individual relative population doubling (RPD) data from the second study for dose range-finding of Ssanghwa-tang (SHT) in CHL cells

Study No. P117008								
Nominal Conc. of SHT ($\mu\text{g/mL}$)		Metabolic Activation System	Times ^a (hours)	Cell Counts ^b		Mean	PD ^c	RPD (%) ^d
				One Flask/Concentration				
6-hour treatment (6-S)								
0		–	6-18	10130	9999	10065	1.618	100
2600	#&	–	6-18	7814	7981	7898	1.268	78
2900	#&	–	6-18	6741	6697	6719	1.035	64
3200	#&	–	6-18	6305	6405	6355	0.954	59
3500	#&	–	6-18	5859	5762	5811	0.825	51
3800	#&	–	6-18	5806	5776	5791	0.820	51
4100	#&	–	6-18	5429	5307	5368	0.711	44
4400	#&	–	6-18	5148	5032	5090	0.634	39
4700	#&	–	6-18	4751	4753	4752	0.535	33
5000	#&	–	6-18	3604	3647	3626	0.144	9
22-hour treatment (22-S)								
0		–	22-2	9473	9532	9503	1.535	100
2000	#&	–	22-2	7109	6998	7054	1.105	72
2600	#&	–	22-2	5562	5589	5576	0.765	50
2900	#&	–	22-2	5070	5060	5065	0.627	41
3200	#&	–	22-2	4635	4505	4570	0.478	31
3500	#&	–	22-2	4336	4280	4308	0.393	26
3800	#&	–	22-2	3806	3711	3759	0.196	13
4100	#&	–	22-2	3745	3607	3676	0.164	11
4400	#&	–	22-2	3558	3466	3512	0.099	6
4700	#&	–	22-2	3092	3120	3106	-0.079	-5
Cell number at the treatment				3248	3312	3280		

Visible turbidity of SHT was observed at the beginning of the treatment.

& Visible turbidity of SHT was observed at the end of the treatment.

^a Treatment time–recovery time

^b Each culture was trypsinized and suspended with 1.0 mL of 0.05% trypsin–EDTA and 9.0 mL of culture medium. The cell suspensions of 0.8 mL/culture were diluted 25 times with 19.2 mL of Isoton solution. The cells in 0.5 mL Isoton solution were counted twice/culture using Coulter Counter® model ZM. Actual number of cells per culture = Mean Count $\times$ 500.

^c Population Doubling = $[\log (\text{Post-treatment cell number} \div \text{Initial cell number})] \div \log 2$

^d RPD = $\frac{\text{No. of Population doubling in treated cultures}}{\text{No. of Population doubling in control cultures}} \times 100 (\%)$

Abbreviations:

RPD, Relative Population Doubling; +, Presence of metabolic activation system; –, Absence of metabolic activation system