

# Evaluation of Genotoxicity of SU-Eohyeol Pharmacopuncture Using an In Vitro Chromosome Aberration Test in Chinese Hamster Lung Cell

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**Objectives:** This study was conducted to evaluate the safety of SU-Eohyeol pharmacopuncture (SUEP) by assessing its potential to cause chromosomal abnormalities in Chinese hamster lung cells (CHL/IC).

**Methods:** A dose-curve was conducted to determine the highest dose of SUEP. Doses of 10, 5, 2.5, 1.25, 0.625, and 0.313% were used, and no cytotoxicity or SUEP precipitation was observed. SUEP doses of 10, 5, and 2.5%, with positive and negative controls, were used in a chromosome aberration test.

**Results:** In this study, the frequency of abnormal chromosomal cells in the SUEP group did not show a statistically significant difference from that of the negative control group in short-term treatments with and without metabolic activation and the continuous treatment without metabolic activation. Compared with the negative control group, the positive control group had a significantly higher frequency of cells with structural chromosomal abnormalities. This test's results satisfied all conditions for determining the results.

**Conclusion:** SUEP did not induce chromosomal aberrations under the conditions of this study. Other toxicity evaluations, safety studies in humans, and various clinical trials are required to evaluate the safety and efficacy of SUEP.

**Keywords:** safety, in vitro chromosome aberration test, jungsongouhyul pharmacopuncture, cervi parvum cornu, su-eohyeol, korean medicine

## INTRODUCTION

Determining the safety of pharmaceuticals and botanical products for human use requires systematic toxicity studies using various experimental models. These studies aim to predict the toxicity and to establish criteria for selecting doses that are safe for humans. Genotoxicity tests have been mainly used to predict the carcinogenicity of chemicals since these tests can identify potential carcinogenic and mutagenic compounds. Despite the frequent use of medicinal plants in traditional medicines, few studies have been conducted to determine the safety of these traditional herbs [1-4]. Pharmacopuncture therapy using acupuncture agents prepared in single or mixed forms of herbal medicines is a commonly used treatment method in Ko-

rean medicine (KM). Pharmacopuncture combines the use of meridians and herbal factors and improve the delivery of herbal medicines to the target site [5-8]. It is important to note that the herbs used in pharmacopuncture are natural substances, and the manufacturing process may vary depending on the level of active ingredients present in the formulation [8, 9]. Pharmacopuncture treatments are currently treated as non-reimbursable items in Korea since any standard fees have been agreed upon, and the safety and effectiveness of these KM herbal medicines have not been verified scientifically [10]. In a previous survey, KM doctors agreed that the expansion of health insurance coverage and verified safety and effectiveness were most important for the further development and use of pharmacopuncture [10]. Therefore, the verification of pharmacological mechanism of

action and potential toxicity was selected as an important goal for good laboratory practice (GLP)-level institutions [10]. Although a wide variety of pharmacopuncture agents are used in KM clinical trials, the verification of the toxicity of pharmacopuncture agents in GLP-level institutions is insufficient.

Recently, SU-Eohyeol pharmacopuncture (SUEP) was developed by combining the blood flow stimulation, stagnant elimination, anti-inflammatory, and analgesic effects of Jung-songouhyul pharmacopuncture (JOP) [11] with the hydration and nutritional replenishment effects of Cervi Parvum Cornu (CPC) pharmacopuncture on the musculoskeletal system, tendon, and tissue [5, 6]. There have been no clinical reports yet, and the potential toxicity of SUEP is still being evaluated. However, many mechanisms can lead to toxicity. Therefore, it is necessary to perform a battery of genotoxicity tests to accurately measure the genotoxicity of a substance. Currently, the most widely performed three-battery assays include the bacterial reverse mutation test, in vitro chromosomal aberration assay, and in vivo micronucleus assay [12]. The results for the genotoxicity of SUEP in two of the three battery assays have already been published [13, 14], yet no studies using mammalian cells for chromosomal abnormalities have been published.

Therefore, we aimed to further evaluate the potential genotoxicity of SUEP. Biototech (Cheongwon, Korea), a non-clinical GLP testing institution, assessed the potential for SUEP to cause chromosomal abnormalities in a Chinese hamster lung cell line (CHL/IU).

## MATERIALS AND METHODS

### 1. Preparation of SUEP extract and control materials

SUEP is a pharmacopuncture agent with a concentration of 305 mg/mL mixed with nine herbs (Table 1). SUEP was manufactured by mixing and filtering after each extraction process using centrifugal partition chromatography (CPC) and other medicinal herbs at an external herbal dispensary (EHD), meeting Korean Good Manufacturing Practice standards (Namsangcheon EHD, Yongin, Korea) [13]. SUEP was provided in a transparent vial containing a pale-yellow liquid and was refrigerated at 4.2–5.4°C. SUEP was dissolved in saline for treatment. For the highest dose, SUEP was used undiluted and was diluted stepwise with saline for lower doses.

**Table 1.** Prescription of SU-Eohyeol pharmacopuncture agent

Botanical name	Scientific name	Ratio (mg/mL)
Cervi Parvum Cornu	<i>Cervus elaphus</i> Linné	50
Gardeniae Fructus	<i>Gardenia jasminoides</i> Ellis	75
Olibanum	<i>Boswellia carterii</i> Birdwood	30
Myrrha	<i>Commiphora myrrha</i> Engler	30
Corydalis Tuber	<i>Corydalis ternata</i> Nakai	30
Persicae Semen	<i>Prunus persica</i> Batsch	22.5
Salviae Miltiorrhizae Radix	<i>Salvia miltiorrhiza</i> Bunge	22.5
Paeoniae Radix	<i>Paeonia lactiflora</i> Pallas	22.5
Sappan Lignum	<i>Caesalpinia sappan</i> Linné	22.5

### 2. Negative control and positive control

Saline solution was used as a negative control (Lot No./Batch No. 21039, 21081; JW Pharmaceutical Co., Ltd., Republic of Korea). Mitomycin C (lot no. MKCN1342) and benzo[a]pyrene (Lot No. SLCD4874) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as positive controls. Dimethyl sulfoxide (DMSO, Lot No.: K51637131, Merck, Germany) was added as an excipient, dissolved, subdivided, and dispensed into a tube in a cryogenic freezer (−80 to −60°C, OPR-DFU-657CEV, Operon, Korea). It was then stored frozen and thawed on the day of treatment.

### 3. Preparation of metabolic activation system S9 mix

S9 (Lot No.:21080605) and Cofactor C (Lot No.: C21080405) were purchased from ORIENTAL YEAST Co., LTD. (Japan), stored in a cryogenic freezer (−80 to −60°C), and used before the expiration date. They were thawed and mixed at a ratio of 2:4.7. Based on the total liquid volume of 1 mL, the composition of the S9 mixture was 0.3 mL Cofactor C, 0.1 mL (5 μmol) 50M MgCl<sub>2</sub>, 0.1 mL (33 μmol) 330M KCl, 0.1 mL (5 μmol) 50M glucose-6-phosphate, 0.1 mL (4 μmol) 40M NADP, 0.2 mL (4 μmol) 20M HEPES buffer (pH 7.2), and 0.1 mL purified water. The S9 mixture was prepared at the time of use in the required amount.

### 4. Cell line and medium

The CHL/IU cell line was selected due to its high detection sensitivity and frequent use in chromosomal aberration tests [15]. CHL/IU cells were purchased from American Type

Culture Collection (USA) on November 24, 2011, placed in a 75 cm<sup>2</sup> flask (Nunc, Denmark) containing Eagle's Minimum Essential medium (EMEM, Lonza Walkersville Inc., USA) and 10% fetal bovine serum (FBS, Gibco, USA), and cultured in a 37°C incubator (MCO-20AIC, SANYO, Japan) supplemented with 5% CO<sub>2</sub>. CHL/IU cells were checked for mycoplasma contamination using the Hoechst Stain Kit (MPBIOMEDICALS, Japan), and 0.25% Trypsin-EDTA solution was added to the culture flask to separate the cells from the bottom of the flask. After centrifugation of the cell suspension for five minutes at 1,000 rpm, the supernatant was discarded. FBS was added to a concentration of 1 × 10<sup>6</sup> cells/mL, DMSO was added to a final concentration of 10%, and the cells were aliquoted into a tube for freezer storage.

## 5. Cell culture method

For cultivation, the frozen cells were thawed, and the cell

morphology was observed when the cells grew to more than 70-80% of the bottom area of the culture flask. The cells were then detached using 0.25% trypsin-EDTA. After centrifugation of the cell suspension at 1,000 rpm for five minutes and removal of the supernatant, the cells were suspended in EMEM containing 10% FBS. The cell suspension was transferred to a 75 cm<sup>2</sup> flask and incubated. Cells that were passaged two or more times were collected into a cell suspension of 5 × 10<sup>4</sup> cells/mL. A dose-curve was conducted in a six-well plate (2 mL/well, Nunc, Denmark), and the chromosome aberration test was dispensed in a 60 mm plate (5 mL/plate, BD, U.S.A.) and six-well plate (2 mL/well) and incubated for one day at 37°C in a 5% CO<sub>2</sub> incubator. In addition, for cytotoxicity confirmation, a satellite control group was prepared in separate wells.

## 6. Dose-curve analysis

A dose-curve was performed to determine the highest SUEP

**Table 2.** Results of the dose range-finding study

Test substance	Dose (%)	S9 mix	Trt-Rec time (h)	Relative Population Doubling (%)	PD
Normal saline injection	0	-	6-18	100	1.56
SU-Eohyeol pharmacopuncture	0.313	-	6-18	97.6	-
	0.625	-	6-18	97.6	-
	1.25	-	6-18	94.0	-
	2.5	-	6-18	90.9	-
	5	-	6-18	88.2	-
	10	-	6-18	84.9	-
Normal saline injection	0	+	6-18	100	1.56
SU-Eohyeol pharmacopuncture	0.313	+	6-18	98.5	-
	0.625	+	6-18	96.0	-
	1.25	+	6-18	90.3	-
	2.5	+	6-18	88.1	-
	5	+	6-18	87.5	-
	10	+	6-18	84.1	-
Normal saline injection	0	-	24-0	100	1.58
SU-Eohyeol pharmacopuncture	0.313	-	24-0	98.1	-
	0.625	-	24-0	94.7	-
	1.25	-	24-0	94.7	-
	2.5	-	24-0	94.7	-
	5	-	24-0	88.6	-
	10	-	24-0	83.7	-

Trt-Rec time, Treatment-Recovery times.

Population doubling (PD) = [log (post-treatment cell number/initial cell number)]/log 2.

starting dose in the chromosome aberration test. Considering the specificity of SUEP, a liquid sample, the highest dose used in the dose-curve analysis was the stock solution (305 mg/mL). Six doses (10, 5, 2.5, 1.25, 0.625, and 0.313%) were tested.

The dose-curve analysis was performed as shown in **Table 2**. After SUEP treatment, the pH and osmolality of the negative control and highest SUEP doses were measured. The pH and osmotic pressure of the highest SUEP dose did not change by more than 1.0 and 50 mOsm/kg, respectively, compared to the negative control group. Therefore, the pH and osmotic pressure at the other SUEP doses were not measured. No pH-induced color change of the medium was observed. The dose-curve was conducted in the same manner as the chromosome aberration test, and specimen preparation was not performed.

Relative population doubling (RPD) was calculated by counting the number of cells using a hemocytometer in the satellite control group at the time of SUEP treatment and after the end of SUEP treatment.

$$\text{RPD (\%)} = \frac{(\text{No. of population doublings in treated cultures})}{(\text{No. of population doublings in control cultures})} \times 100$$

Population doubling =

$$[\log (\text{post-treatment cell number}/\text{initial cell number})]/\log 2$$

As a result of the dose-curve, cytotoxicity and precipitation of SUEP were not evident in the short-term treatments with and without metabolic activation and the continuous treatments without metabolic activation. Therefore, 10% SUEP was set as the highest dose for the chromosome aberration test, and azeotrope 2 was added. In addition, negative and positive control groups were established (**Table 3**).

## 7. In vitro chromosome aberration test

### 1) Treatment method

The chromosome aberration test was performed, as shown in **Table 3**. Two plates were used at each dose. For the calculation of RPD, the negative control, SUEP-treated, and positive control groups were treated with 2 mL each in six wells. After a short-term treatment (six hours) with SUEP in the absence and presence of metabolic activation, the inside of the plate was washed with D-PBS, and a fresh culture solution was added, followed by further incubation for 18 hours. SUEP treatment was continued for 24 hours without metabolic activation. Both

the short-term treatment and continuous treatment methods were conducted in a 37°C incubator supplied with 5% CO<sub>2</sub>. Precipitation of SUEP was observed for each dose at the time of treatment, the end of the treatment, and the end of the culture (before colcemid treatment). This study was conducted at Biototech (Cheongwon, Korea) in accordance with the Good Laboratory Practice Regulations of the Ministry of Food and Drug Safety, the Organization for Economic Co-operation and Development (OECD) [16, 17].

### 2) Calculation of RPD

The colcemid solution (Gibco, U.S.A.) was added to a final concentration of 0.2 µg/mL to stop cell division in the metaphase two hours before the end of the culture. After completion of the culture, cells were removed from the plate bottom with 0.25% trypsin-EDTA (Gibco, U.S.A.) and centrifuged at 1,000 rpm for five minutes (FLETA 5, Hanil Science Industrial Co., Ltd., Republic of Korea). After removal of the supernatant, 5 mL of a 0.075 mol/L KCl aqueous solution maintained at 37°C was added and incubated at 37°C for 20 minutes. After adding 1 mL of cold fixative and centrifuging at 1,000 rpm for five minutes to remove the supernatant, 5 mL of cold fixative was added and centrifuged at 2,000 rpm for an additional five minutes. This fixing operation was repeated. Cells were suspended in a small amount of cold fixative, and a specimen slide was prepared by dropping one drop of cells on two glass slides and dried. After staining with 3% Giemsa staining solution, cells were washed with ultrapure water, dried, and sealed with an encapsulant (Entellan® new, Merck, Germany).

### 3) Observation of sample slides

The observation of specimen slides was performed in order of short-term treatment method to continuous treatment method. To assess the target dose for chromosome observation, three doses were set in which more than 300 chromosomes fixed during metaphase were observed per dose for each treatment method. Using a microscope (600X magnification, BX51, Olympus, Japan), 300 fragmented metaphase chromosomal abnormalities were observed per dose and classified into structural abnormalities, numerical abnormalities, and others. Chromatid breaks, chromatid exchanges, chromosome breaks, chromosome exchanges, chromatid gaps, chromosome gaps, and fragmentation were observed. In metaphase, multiple gaps and cuts were recorded as fragmentations. A gap was defined as a non-stained region narrower than the chromatid width.

**Table 3.** Treatment method in dose range-finding study and main study

Dose range finding study	S9 mix	Treatment group	Preparation amount (mL)			Amount to be dispensed (mL/well)
			Culture medium	S9 mix	Negative control or test substance	
Short time treatment	-	Negative control	2.7	-	0.3	2
		Test substance (SU-Eohyeol pharmacopuncture)	2.7	-	0.3	2
	+	Negative control	2.2	0.5	0.3	2
		Test substance (SU-Eohyeol pharmacopuncture)	2.2	-	0.3	2
Continuous treatment	-	Negative control	2.7	-	0.3	2
		Test substance (SU-Eohyeol pharmacopuncture)	2.7	-	0.3	2
Main study	S9 mix	Treatment group	Preparation amount (mL)			Amount to be dispensed (mL/well)
			Culture medium	S9 mix	Negative control, positive control or test substance	
Short time treatment	-	Negative control (Normal saline)	11.7	-	1.3	5
		Test substance (SU-Eohyeol pharmacopuncture)	11.7	-	1.3	5
		Positive control	12.87	-	0.13	5
	+	Negative control (Normal saline)	9.53	2.17	1.3	5
		Test substance (SU-Eohyeol pharmacopuncture)	9.53	-	1.3	5
		Positive control	10.70	-	0.13	5
Continuous treatment	-	Negative control (Normal saline)	11.7	-	1.3	5
		Test substance (SU-Eohyeol pharmacopuncture)	11.7	-	1.3	5
		Positive control	12.87	-	0.13	2

Polyploidy and endoreduplication were also observed as hydrodynamic abnormalities. Cells with one or more of these abnormalities were counted as one abnormal cell, and the types were recorded. Abnormal cells with or without gaps were recorded separately. For others, the type and number were recorded as those not included in the structural and numerical abnormalities.

## 8. Experimental conditions and analysis

The test was completed when the following conditions were satisfied. First, the frequency of cells with chromosomal structural abnormalities in the negative control group was expected. Second, the frequency of cells with chromosomal structural

abnormalities in the positive control group was expected, and there was a statistically significant increase compared with the negative control group. Third, the cell proliferation criteria in the negative control group were met. All three conditions were tested until any one of them (absence and presence or absence of metabolic activation of the continuous treatment method) was positive. When all three conditions were met, the criteria for selecting the highest concentration was appropriate.

The results were judged as positive if the following three conditions were satisfied. The frequency of cells with chromosomal aberrations (without gaps) and those with chromosomal abnormalities showed a statistically significant increase compared to the negative control group at one or more doses. Second, the results showed a dose-dependent increase. Third,

**Table 4.** Summary results of the main study

Test substance	Dose (%)	RRPD (%)	PD	S9 mix	Trt-Rec time (h)	No. of cell analyzed	Number of cells with structural aberrations										Number of cells with numerical aberrations						
							ctb	csb	cte	cse	frg	gap	csg	gap-	gap+	end	pol	Total (%)	Others <sup>a)</sup>				
Normal saline injection	0	100	1.53	-	6-18	150	0	0	1	0	0	0	0	0	0	0	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0	
SU-Eohyeol pharmacopuncture	2.5	90.7	-	-	6-18	150	0	0	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0	
	5	88.5	-	-	6-18	150	0	0	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0	
	10	83.9	-	-	6-18	150	0	0	0	0	1	0	0	0	0	0	0 (0.0)	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0
MMC	0.1 µg/mL	49.8	-	-	6-18	150	5	0	17	0	0	0	0	0	0	0	45** (15.0)	45 (15.0)	0	0	1 (0.3)	0	
							6	0	17	1	0	0	0	0	0	0			0	0	1		
Normal saline injection	0	100	1.53	+	6-18	150	0	0	1	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0	
SU-Eohyeol pharmacopuncture	2.5	88.9	-	+	6-18	150	0	0	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0	
	5	87.2	-	+	6-18	150	0	0	0	0	0	0	0	0	0	0	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0	
	10	84.3	-	+	6-18	150	0	0	1	0	0	1	0	0	0	0	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0	
B[a]P	20 µg/mL	48.3	-	+	6-18	150	7	0	17	0	1	0	0	0	0	0	47** (15.7)	49 (16.3)	0	0	0 (0.0)	0	
Normal saline injection	0	100	1.53	-	24-0	150	0	0	1	0	0	0	0	0	0	0	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0	
							0	0	0	0	0	0	0	0	0	0			0	0	0	0	

**Table 4. Continued**

Test substance	Dose (%)	RRPD (%)	PD	S9 mix	Trt-Rec time (h)	No. of cell analyzed	Number of cells with structural aberrations										Number of cells with numerical aberrations										
							ctb	csb	cte	cse	frg	ctg	csg	gap	gap-	gap+	end	pol	Total (%)	Others <sup>a)</sup>							
SU-Eohyeol pharmacopuncture	2.5	94.9	-	-	24-0	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	5	89.5	-	-	24-0	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	10	83.7	-	-	24-0	150	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
MMC	0.1 µg/mL	48.3	-	-	24-0	150	7	0	18	0	0	1	0	47**	(15.7)	49	(16.3)	0	0	0	0	0	0	0	0	0	0
							6	0	16	0	0	1	0														

Abbreviations: ctg, chromatid gap; csg, chromosome gap; ctb, chromatid break; cte, chromatid exchange; cse, chromosome exchange; frg, fragmentation; end, endoreduplication; pol, polyploidy; MMC, Mitomycin C; B[a]P, Benzo[a]pyrene; RPD, Relative Population Doubling; Trt-Rec time, Treatment-Recovery times; gap-, total number of cells with structural aberrations excluding gap+; gap+, total number of cells with structural aberrations including gap.

<sup>a)</sup>Others were excluded from the number of cells with chromosomal aberrations.

Significant difference from negative control by Fisher's exact test: \*\*p < 0.01.

Population doubling (PD) = [log (post-treatment cell number/initial cell number)]/log 2.

the frequency of cells with chromosomal aberrations showed an unexpected increase for the negative controls.

### 9. Statistical analysis

SAS (version 9.4, SAS Institute Inc., USA) was used for statistical analysis of the frequency of cells with chromosomal abnormalities. A Fisher's exact test was conducted on the frequency of cells with chromosomal abnormalities (gaps not included) to test the significance between the negative control group and the SUEP-treated group, negative control group, and positive control group (p < 0.05, p < 0.01). The dose-dependence of the SUEP-treated group was tested for significance between the SUEP doses by conducting a Cochran-Armitage trend test (p < 0.05, p < 0.01).

## RESULTS

### 1. RPD measurement

RPD was more than 83.9% with SUEP doses of 0, 2.5, 5, and 10% in the absence of metabolic activation in the short-term treatment method, and 84.3% with doses of 0, 2.5, 5, and 10% in the presence of metabolic activation in the short-term treatment method. In the absence of metabolic activation with continuous treatment with SUEP, RPD was greater than 83.7% at doses of 0, 2.5, 5, and 10% (Table 4).

### 2. Precipitation of test substance and chromosomal abnormality observation

#### 1) Capacity

At all doses, in the absence and presence of metabolic activation in the short-term treatment method and the absence of metabolic activation in the continuous treatment method, RPD was observed to be 83.7% or more, and no precipitation of SUEP was observed (Table 4). More than 300 fission metaphases were observed at the highest dose in each treatment series. Therefore, the doses used for chromosomal abnormalities were set at 10, 5, and 2.5%.

### 3. Frequency of chromosomal abnormalities

In the SUEP-treated group, no significant difference was observed in the frequency of cells with chromosomal abnor-

malities in the absence and presence of metabolic activation in the short-term treatment method and the absence of metabolic activation in the continuous treatment method compared with the negative control group. In the positive control group for each treatment, a statistically significant increase was observed in the frequency of cells with structural abnormalities compared to the negative control group ( $p < 0.01$ ) (Table 2).

#### 4. Establishment of the test

The frequency of cells with chromosomal structural abnormalities in the negative control group was expected as it was within the control range of historical control data (Table 5) and within 95% of historical control data. The frequency of cells with chromosomal structure abnormalities in the positive control group was also expected as it was within the control range of the historical control data and showed a statistically significant increase compared to the negative control group. Additionally, more than 300 chromosomes fixed during meta-

phase were observed per dose in the control and SUEP-treated groups, and three or more readable doses were secured in the SUEP-treated group. The cell proliferation criteria in the negative control group were met, and all three conditions were tested because none of the three experimental conditions (in the absence and presence of metabolic activation in the short-term treatment method and the absence of metabolic activation in the continuous treatment method) were positive. In addition, since the highest concentration of SUEP was set by performing a dose-curve, the criteria for selecting the highest concentration were also suitable, and it was confirmed that the test was conducted under appropriate test conditions. Therefore, in the short-term and continuous treatment methods, the observed doses of SUEP were set at 10, 5, and 2.5%.

## DISCUSSION

Pharmacopuncture therapy is also called herbal acupuncture, aqua acupuncture therapy, or acupoint injection therapy.

**Table 5. Historical control data**

Historical control values of structural aberrations								
Group	S9 mix	Trt-Rec time (hr)	N	Structural aberration cells excluding gap (%) (Mean $\pm$ S.D.)	Range (%)		95% control limit <sup>(c)</sup> [structural aberration cells/300 cells]	
					MIN	MAX		
Negative	-	6-18	41	0.317 $\pm$ 0.357	0	1.067*	0	< 3
	+	6-18	41	0.333 $\pm$ 0.394	0	1.17*	0	< 3
	-	24-0	39	0.282 $\pm$ 0.370	0	0.98*	0	< 3
Positive	-	6-18 <sup>a)</sup>	31	22.57 $\pm$ 6.346	8.68*	36.46*		
	+	6-18 <sup>b)</sup>	31	23.21 $\pm$ 5.377	11.54*	34.87*		
	-	24-0 <sup>a)</sup>	29	33.52 $\pm$ 8.503	13.27*	53.77*		

  

Historical control values of numerical aberrations								
Group	S9 mix	Trt-Rec time (hr)	N	Numerical aberration cells excluding gap (%) (Mean $\pm$ S.D.)	Range (%)		95% control limit <sup>(c)</sup> [numerical aberration cells/300 cells]	
					MIN	MAX		
Negative	-	6-18	41	0.220 $\pm$ 0.294	0	1.02*	0	< 2
	+	6-18	41	0.154 $\pm$ 0.259	0	0.90*	0	< 2
	-	24-0	39	0.239 $\pm$ 0.253	0	1.01*	0	< 2

Negative control: Water for injection, Dimethyl sulfoxide, Acetone, etc.

Trt-Rec time, Treatment-Recovery times; S.D., standard deviation.

<sup>a)</sup>Mitomycin C (0.1  $\mu$ g/mL).

<sup>b)</sup>Benzo[a]pyrene (20  $\mu$ g/mL).

<sup>c)</sup>Poisson-based 95% control limits of the historical negative control data.

N: The total number of chromosome aberration test.

The above historical control values were obtained from the data pooled from May. 6, 2015 to Aug. 9, 2021.

\*The range was calculated by the control limit of X derived from X-R-Rs value.



Pharmacopuncture is a new form of acupuncture that combines acupuncture and herbal medicine, the most common treatments in traditional Asian medicine [5, 6], and is widely used for musculoskeletal disorders in Korea [7]. In KM, JOP has been used to treat acute and traumatic musculoskeletal pain, peripheral nerve palsy, pain, and edema caused by impaired blood flow [12]. CPC pharmacopuncture has been reported to have anti-inflammatory effects on arthritis and alleviate musculoskeletal pain in most clinical studies [18]. SU is an English transcription of the Korean pronunciation of the Chinese character 秀, which means excellent. SUEP is developed in the form of a qi formula (water type) by adding highly concentrated CPC to JOP to optimize the analgesic effect and the effect of essence supplementation. SUEP can manage pain from acute injury to chronic muscle pain, dystonia, arthralgia, joint deformity, ligament inflammation, and neurogenic pain.

Herbal medicine has been developed based on clinical experiences and KM's unique theoretical system. Since the 1990s, the importance of evidence-based medicine (EBM) has grown to provide the best care for patients by integrating the experience of doctors with relevant scientific evidence. It is necessary to verify the therapeutic effect of KM through well-designed clinical trials to clarify the efficacy and safety according to EBM [19, 20]. High-quality clinical trials are used to develop new drugs, evaluate the efficacy and safety of existing drugs, and enhance insurance coverage [20]. Evaluating the toxicity of SUEP is needed to justify clinical trials. According to the guidelines for non-clinical testing of herbal medicines by the Korea Food and Drug Administration, toxicity tests of herbal medicines are classified as single administration, repeated administration, genotoxicity tests, reproductive toxicity tests, and carcinogenicity tests [21].

The genotoxicity test is the primary screening method for predicting carcinogenicity and should be used with other toxicity tests. There are several *in vitro* and *in vivo* test methods that use genetic mutations, chromosomal abnormalities, DNA damage, or their reparability as indicators of carcinogenicity [22]. The mechanisms that induce toxicity are diverse, and accurate measurements of the genotoxicity of a substance require several tests using a battery rather than a single genotoxicity test. Currently, the most widely used three-battery assay includes the bacterial reverse mutation test, *in vitro* chromosomal aberration assay, and *in vivo* micronucleus assay [11]. These tests are conducted according to the International Conference on Harmonization of Technical Requirements for Registration of

Pharmaceutical for Human Use (ICH) and OECD guidelines [22].

Chromosomal abnormalities are classical genotoxic responses to tumor initiation and progression [23]. *In vitro* chromosomal aberration testing has been performed to identify the factors causing structural chromosomal abnormalities in cultured mammalian cells [24, 25]. CHL cells are sensitive to mutagens and have a small number of chromosomes, which makes them easy to score and can be used for repeated measurements. Therefore, these cells were selected for *in vitro* chromosomal aberration testing [14]. In the dose-curve analysis, 10% SUEP was selected as the high dose, and cytotoxicity and precipitation were not observed in doses of 10, 5, 2.5, 1.25, 0.625, and 0.313%. Therefore, the chromosome aberration test was conducted using 10, 5, and 2.5% SUEP in the absence and presence of metabolic activation in the short-term treatment method and the absence of metabolic activation in the continuous treatment method. The results showed that the frequency of cells with chromosomal abnormalities was not significantly increased with any SUEP dose in the short-term treatments with and without metabolic activation and the continuous treatment without metabolic activation. In contrast, the positive control group showed a statistically significant increase in the frequency of cells with structural chromosomal abnormalities compared with the negative control group. Since the experimental results satisfied all the test conditions, it was confirmed that this test was conducted under appropriate conditions.

## CONCLUSION

We determined that SUEP did not induce chromosomal aberration. These results indicate that SUEP does not induce genotoxicity and is safe according to the three-battery assay; however, more testing should be done to ensure SUEP is safe for clinical use. This study was conducted at a GLP-level institution and further provides evidence that pharmacopuncture is safe and enhances health insurance coverage of pharmacopuncture. Other toxicity assays, human safety, and various clinical trials are needed to evaluate the safety and efficacy of SUEP.

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

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