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Original Article
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Single-Dose Intramuscular Toxicity Study of SU-Eohyeol Pharmacopuncture in Rats

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Objectives: This toxicological study was performed to assess for potential toxicity and to determine the approximate lethal dose of SU-Eohyeol pharmacopuncture (SUEP) following a single intramuscular injection of SUEP into male and female Sprague-Dawley (SD) rats.
Methods: The groups in our experiment consisted of an experimental group treated with SUEP at a dose of 1.0 mL/animal and a control group injected with a normal saline solution, and five male and female rats were placed in each group. Each animal was administered a single intramuscular injection. We monitored all rats for clinical signs and body weight changes for 14 days after administration. At the end of the observation period, the rats were euthanized and autopsied, and localized tolerance examinations were conducted at the site of administration of the test substance.
Results: There were no deaths in either sex in the SUEP-treated group. There was no significant difference between the SUEP-treated group and the control group in the clinical signs and weight changes among the rats. In addition, no significant SUEP-related changes were observed on autopsy findings or local tolerance examinations at the injection site by histopathological examination.
Conclusion: Our results suggest that the approximate lethal dose of a single intramuscular administration of SUEP in female and male rats under the conditions of this study is greater than 1.0 mL/animal. To determine the safety of the use of SUEP in Korean medical clinical practice, additional toxicity studies will be needed.

Keywords: safety, acupuncture, single intramuscular toxicity, jungsongouhyul pharmacopuncture, cervi parvum cornu, su-eohyeol

INTRODUCTION

Pharmacopuncture (acupoint injection, herbal acupuncture, aqua-acupuncture) used in traditional Chinese and Korean medicine (TCM and TKM, respectively) is a new form of acupuncture involving the injection of various herbal medicines based on both the meridian and Qi flavor theory. Unlike oral administration through the digestive system, pharmacopuncture can deliver herbal medicine directly to the target site; therefore, it is known to have a larger and faster effect than oral administration [1, 2]. Currently, in Korea, many types of pharmacopuncture agents are being used as staples in clinical practice [3, 4], and new pharmacopuncture agents are actively being developed based on literature and clinical experience.

Jungsong-ouhyul pharmacopuncture (JOP), which consists of eight medicinal herbs such as Gardeniae Fructus, Olibanum, Myrrha, Corydalis Tuber, Persicae Semen, Salviae Miltiorrhizae Radix, Paeoniae Radix, and Sappan Lignum, is known to have effects such as such as promoting blood circulation, relieving pain, and reducing inflammation [5, 6]. Cervi Parvum Cornu (CPC), a predominant nontoxic agent in TCM and TKM has also been reported to have the effect of replenishing Blood and Essence, tonifying Kidney Yang, and strengthening bones and muscles [7, 8]. In addition, CPC has been pharmacologically

demonstrated to improve immunity and possess antifatigue, antioxidant, stress relief, anti-inflammatory, anti-cancer, and analgesic effects [7, 9]. Thus, CPC pharmacopuncture is a type of immune (meridian, meridian-field) pharmacopuncture that can be used to treat a wide range of diseases by compensating for deficiencies in Qi, Blood, Yin and Yang. However, early reports have suggested that when CPC is prepared by alcohol immersion, it becomes fibrous when exposed to air and causes burning pain when injected into patients [1, 5].

SU-Eohyeol pharmacopuncture (SUEP) was recently developed by adding CPC to JOP to supplement essential body fluids in human tissues. SU is the English transcription of the Korean pronunciation of the Chinese character 秀, which means excellent. This was developed by changing the existing preparation method to include CPC to improve the durability of the treatment effect. By reducing the size of particles through nano-extraction and microfiltration and increasing the concentration of the immersion solution under reduced pressure concentration, SUEP can also be applied more efficiently to weak patients.

However, newly developed pharmacopuncture agents require toxicity evaluation data to be recognized as safe pharmacopuncture agents. Although there have been many studies on the effectiveness of JOP as a widely used agent in clinical practice [4, 10], no toxicological evaluation studies have been conducted. In terms of SUEP, a clinical study was reported in which a mixed pharmacopuncture of Jungsong-ouhyul and CPC was used to treat lower back pain [11]; however, no other clinical or experimental studies have been performed. In this study, to determine the scientific basis for the safety of the newly developed SUEP, the safety of SUEP was evaluated through a single-dose intramuscular toxicity test in male and female Sprague-Dawley (SD) rats.

MATERIALS AND METHODS

1. Preparation of SUEP and control materials

SUEP is a pharmacopuncture agent with nine herbs. SUEP used in this study was manufactured at an external herbal dispensary (EHD) facility (Namsangcheon EHD, Yongin, Korea) that meets the Korean Good Manufacturing Practice standards. The amount of dry raw herbs used for extraction based on 1 L water was as follows: CPC (50 g), Gardeniae Fructus (75 g), Olibanum (30 g), Myrrha (30 g), Corydalis Tuber (30 g), Persicae Semen (22.5 g), Salviae Miltiorrhizae Radix (22.5 g), Paeoniae Radix (22.5 g), and Sappan Lignum (22.5 g). Two extraction methods were used in this study: 1) In the case of CPC, the tips of Russian deer velvet antlers were powdered, water was added to the distillation extractor for injection, and the extract was recovered by circulation extraction. After adding alcohol, stirring, paper filtering, and removing the alcohol from the filtrate using a reduced pressure concentrator, the extract was subjected to ultrafiltration with a molecular weight fraction of 10,000 Da. 2) In the case of other herbal medicines, water for injection was added to the distillation extractor, and the distillate was recovered after circulation. 3) CPC concentrates and other herbal distillates that had undergone ultrafiltration were mixed and filtered, and 0.9% NaCl was added, dissolved by stirring, and then titrated to pH 7.4. The filtrate was then filtered a final time through a 0.45-0.2-μm filter and stored in a sealed container.

In this single-dose intramuscular toxicity test, 2 mL of pale-yellow liquid SUEP sealed in a transparent vial was used, and normal saline was set as the control.

2. Experimental animals

SD rats are widely used in drug safety testing [12] and were selected because of the abundance of basic data available for comparison. We purchased SD rats (5-week-old males and females, n = 6 per group) from Orient Bio Inc. (Seongnam, Korea) for the single-dose intramuscular toxicity testing. On the first arrival of the animals, visual inspection was performed; then, their weights were measured and recorded. The body weight was in the range of 120.9-138.4 g and 110.1-118.7 g for male and female rats, respectively. During the quarantine and acclimatization periods, their general symptoms such as appearance, posture, attitude, behavior, nervous system, respiration, body temperature, and excretion were observed once a day. At the end of these periods, all SD rats were weighed again, and general symptoms and lack of changes in weight confirmed that there were no abnormalities in any of the animals. At the end of the acclimatization period, 10 males and females each of an average weight (186.1-204.9 g and 150.6-168.5 g for male and female rats, respectively) were selected and grouped into two groups (5 males and 5 females per group). The selected animals were randomly placed such that the average body weight of each group was equal. SD rats were housed in controlled environmental conditions of 20.3-24.4℃ ambient temperature, 44.9-60.9% relative humidity, 12-h/12-h light/dark cycle, 10-

15 ventilation times/hour, illuminance of 150-300 Lux, and free access to food (Envigo RMS Inc., Huntingdon, UK) and water.

The animals were cared for and treated in accordance with the guidelines of the Korean National Institute of Health (KNIH) and the Korean Academy of Medical Sciences (KAMS). This study was conducted at Biotoxtech (Cheongwon, Korea) (experiment number: B211217). Biotoxtech obtained full certification from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in 2010.

Our toxicity evaluation study was conducted in accordance with the following Good Laboratory Practice (GLP) Regulations: "GLP Regulation for Nonclinical Laboratory Studies" (Notification No. 2018-93, Ministry of Food and Drug Safety [MFDS] [November 21, 2018]) [13], and "OECD Principles of GLP" (OECD, ENV/MC/CHEM(98)17 [as revised in 1997]) [14].

This test was approved by the Animal Ethics Experimentation Committee of Biotoxtech Co., Ltd. based on the Animal Protection Act (Act No. 4379 of May 31, 1991, partially revised Act No. 16977 of February 11, 2020) (Approval No.: 210819).

3. Single-dose intramuscular toxicity test in SD rats

1) Experimental group designation and administration method

The expected clinical application route of SUEP is through the muscle; therefore, in this study, the muscle route was selected. The expected clinical application dose of SUEP is 0.1-1.0 mL/human (0.1 mL/time). Thus, in this experiment, a dose of 1.0 mL/animal and 0.5 mL/site was administered once each into the left and right thigh muscles of the rats using a disposable syringe (Table 1).

As a preliminary test for this study (Biotoxtech Study No.: B211217P1), one male and female rat underwent a single intramuscular administration of 1.0 mL/animal. Since no deaths were observed, the doses of SUEP and control saline were set to 1.0 mL/animal, and a total amount of 1.0 mL/animal was intramuscularly injected into the left and right thigh muscles.

2) Methods of observation and examination of animals

On the day of administration, the general state of the rats (type of toxic signs, time of onset, recovery period, etc.) and any potential deaths were noted at 30 min and 1, 2, 4, and 6 h after administration. Thereafter, general symptoms were observed in SD rats once a day for 14 days (from the 2nd day to the 15th day of administration). The body weight of rats was measured on the day of administration (before administration) and on days 4, 8, and 15 (necropsy day).

3) Histopathology

All SD rats used in the main experiment were euthanized by inhalation of CO_2 gas on the day of autopsy, exsanguinated from the abdominal aorta, and then autopsied. For all animals subjected to autopsy, the extracted tissues from the injection site were fixed in 10% neutral-buffered formalin (NBF). Organs and tissues subjected to histopathological examination were prepared according to the standard operating procedure for histopathological specimen preparation, and the remaining organs and tissues were preserved in 10% NBF. For histopathological examination, the organ and tissue samples were examined through a localized tolerance examination of the injection site of all animals.

4. Statistical analysis

The body weights obtained in the experiment were statistically analyzed using SAS (version 9.4, SAS Institute Inc., Cary, NC, USA), and the measured values were tested for equality of variance with the Folded-F test ($p < 0.05$). Equal variance was recognized, and significance was confirmed by Student's t-test ($p < 0.05$, $p < 0.01$).

Table 1. Group designation of a single-dose intramuscular toxicity test for SU-Eohyeol pharmacopuncture in Sprague-Dawley (SD) rats

Group		Dose of SUEP (mL/animal)	Injection dose amount (mL/animal)	Number of animals (object number)	
				Male	Female
G1	Control (normal saline)	0	1.0	5 (1101-1105)	5 (2101-2105)
G2	Test substance (SUOP)	1.0	1.0	5 (1201-1205)	5 (2201-2205)

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RESULTS

1. Observations of general health and weight changes

During the 14-day observation period, no general health-related abnormalities or deaths were observed in the control or SUEP-treated group in all male and female SD rats (Table 2).

For the change in body weight during our experiment, the body weight of male rats in the SUEP-treated and control groups changed from 200.3 ± 3.7 g (G1) and 196.9 ± 6.9 g (G2), respectively, to 330.4 ± 17.5 g (G1) and 327.1 ± 16.1 g (G2), respectively, whereas that of female rats changed from 161.5 ± 4.6 g (G1) and 156.3 ± 4.2 g (G2), respectively, to 220.9 ± 7.7 g (G1) and 212.8 ± 15.1 g (G2), respectively. No significant difference was observed in the body weight changes in male and female rats treated with SUEP 1.0 mL/animal compared with those in the control group (Fig. 1, Table 3).

2. Macroscopic examination following necropsy

There were no abnormalities in the male and female SD rats in the control group or SUEP-treated group on macroscopic examination following necropsy.

3. Histopathological examination

The results of the local tolerance test of SUEP were observed by microscopic examination of the tissues obtained from the administration sites of all male and female SD rats. As a result of microscopic histopathological examination, no SUEP-related changes were observed at the injection site. Treatment with SUEP was considered to be tolerated by all animal groups (Fig. 2).

Table 2. SU-Eohyeol pharmacopuncture-related effects on clinical sign changes in a single-dose intramuscular toxicity study in Sprague-Dawley (SD) rats

Sex	Group		Day 1 (hour)					Day													
	Dose (mL/animal)	Animal ID	0.5	1	2	4	6	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Male	G1 (n = 5) 0	1101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1103	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1105	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G2 (n = 5) 1.0	1201	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1202	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1203	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1204	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1205	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Female	G1 (n = 5) 0	2101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2103	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2105	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G2 (n = 5) 1.0	2201	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2202	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2203	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2204	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2205	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No observable abnormalities in clinical signs.
G1: Control group administered with normal saline (1.0 mL/animal).
G2: SU-Eohyeol pharmacopuncture administration group (1.0 mL/animal).

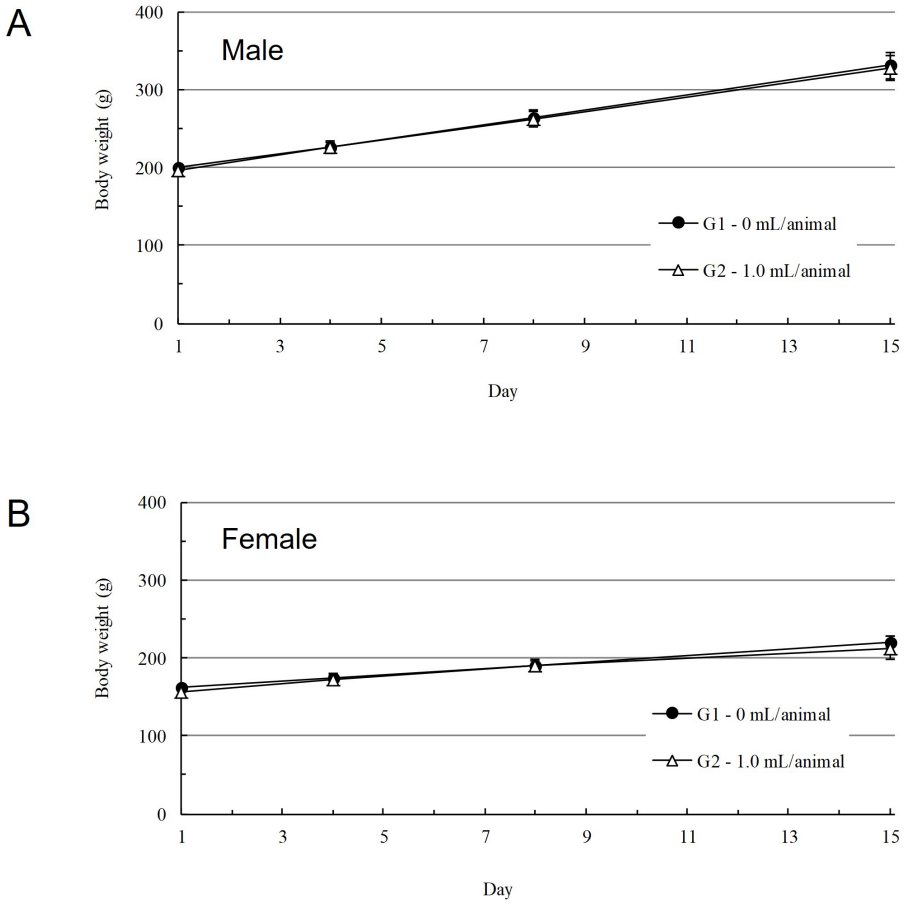

Figure 1. Effects of SU-Eohyeol Pharmacopuncture on the body weight of male (A) and female (B) Sprague-Dawley (SD) rats.

Table 3. Effects of SU-Eohyeol pharmacopuncture on changes in mean body weights in a single-dose intramuscular toxicity test in Sprague-Dawley (SD) rats

Sex	Group/Dose (mL/animal)		Day 1	Day 4	Day 8	Day 15	Day 1-15 Gain (g)
Male	G1 0	Mean	200.3	226.7	264.1	330.4	130.1
		S.D.	3.7	5.3	9.7	17.5	15.9
		N (g)	5	5	5	5	5
	G2 1.0	Mean	196.9	226.2	261.9	327.1	130.2
		S.D.	6.9	8.5	10.1	16.1	10.8
		N (g)	5	5	5	5	5
Female	G1 0	Mean	161.5	174.2	190.2	220.9	59.4
		S.D.	4.6	3.3	5.3	7.7	7.6
		N (g)	5	5	5	5	5
	G2 0	Mean	156.3	173.0	189.9	212.8	56.6
		S.D.	4.2	7.1	8.8	15.1	11.8
		N (g)	5	5	5	5	5

Significantly different from control by Student's test.
S.D., standard deviation; N, number of animals.

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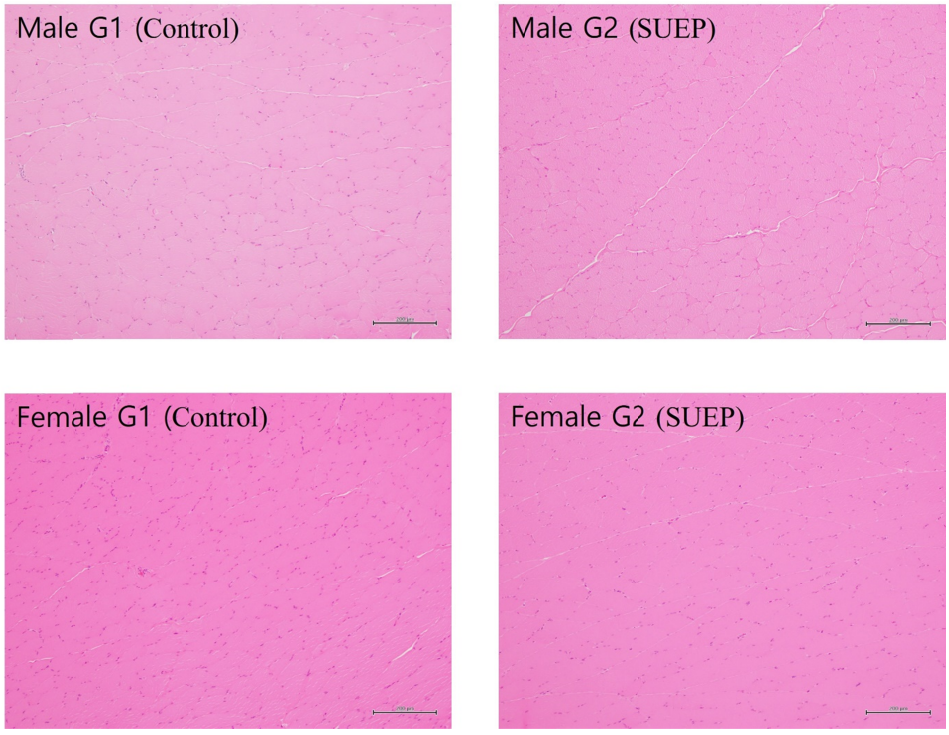

Figure 2. Histopathological results of a single-dose intramuscular toxicity test for SU-Eohyeol Pharmacopuncture (SUEP) in male and female Sprague-Dawley (SD) rats Histopathological examination images of injection sites in Sprague-Dawley (SD) rats were captured at ×200 (scale bar = 200 μM).

DISCUSSION

After Nam Sang-cheon officially disclosed pharmacopuncture to the TKM community in 1967, pharmacopuncture has continued to develop and has subsequently become one of the most used TKM treatment methods in Korea [1-5]. In a survey study on the usage status and satisfaction of pharmacopuncture conducted by TKM doctors in Korea, 88% of respondents had experience using pharmacopuncture in the previous year, pharmacopuncture was mainly used for the treatment of musculoskeletal, nervous system, and gastrointestinal diseases, and the two main types used in TKM clinical practice were bee venom and ouhyul pharmacopuncture [4].

While medicinal products are usually administered by the MFDS, pharmacopuncture is implemented in Korea according to prescriptions issued to EHDs by TKM and TCM institutions [15, 16]. An EHD is a type of pharmacy that offers various types and formulations of herbal medicines to TKM institutions according to TKM doctors' prescriptions based on each patient's condition and was legalized by the Ministry of Health and Welfare (MoHW) in Korea in 2008. In September 2018, the MoHW announced that the Accreditation System of EHDs would be applied in TKM to evaluate and certify herbal and pharmacopuncture preparations [15-17].

With the MoHW certification mark, EHDs are said to provide safely formulated pharmacopuncture preparations; however, in order for these preparations to be scientifically recognized, the efficacy and safety of newly developed pharmacopuncture drugs needs to be evaluated both non-clinically and clinically.

Conversely, the MFDS requires non-clinical data on safety, such as genotoxicity, single-dose toxicity, repeated dose toxicity, and efficacy, to approve herbal medicines for clinical trials [18-22]. JOP, one of the most widely used pharmacopuncture techniques in TKM, has many reports of its clinical efficacy [4, 10], but no toxicity evaluation reports have been conducted to date. Therefore, SUEP, which was developed by adding CPC to JOP, should be scientifically verified for safety through toxicity evaluation. It is expected that public trust will be gained through these efforts.

Single-dose toxicity studies are conducted to describe the qualitative and quantitative data of time-related toxic phenomena and occurrences after administration of a single dose of a substance or a combination of substances [23]. There are species and phylogenetic differences in biological responses to test substances. In the selection of an animal species, an animal species with a similar metabolic profile to that of a human is preferred. However, during the stage of single-dose toxicity test-

ing, it may be difficult to select an animal species or lineage that satisfies the above criterion; therefore, an animal that is easy to handle, is qualitatively uniform, and has abundant background data is usually selected.

The most commonly preferred rodent is the rat, and the most preferred animal for non-rodent experiments is the dog. In addition, depending on the drug, there may be sex-specific differences in the appearance of toxicity. Thus, toxicity should be evaluated in at least one male and female animal [21, 24].

We performed a SUEP single-dose toxicity study in female and male SD rats. Our toxicological study was performed to assess for potential toxicity and to determine the approximate lethal dose of SUEP after a single intramuscular injection of SUEP into the thigh of 6-week-old male and female SD rats. Our findings showed that there were no deaths in either sex in the SUEP-treated group. There was no significant difference between the SUEP-treated group and the control group in the general condition, weight change, or clinical signs of male or female SD rats. In addition, no significant SUEP-related changes were observed in autopsy findings or local tolerance examinations at the injection site by histopathological examination. According to our toxicity study results, the lethal dose of SUEP is approximately greater than 1.0 mL/animal (approximately 1,525 mg/kg) in both male and female SD rats following a singular intramuscular injection of SUEP.

The expected clinical application dose of SUEP is 1.0 mL at a time based on an adult weight (60 kg), and the dose of 1.0 mL/animal used in this experiment is approximately 500 times higher than the actual clinical dose of SUEP used (1.0 mg/kg). The appropriateness and safety of the SUEP dose used in clinical practice has thus been determined. Therefore, the findings of this study allow us to infer that acupuncture using SUEP agents can be safely administered to humans at a dose of 1.0 mL or less.

However, since our study only investigated the toxic effects of a single dose of SUEP only for an observation period of 14 days, in the future, additional intramuscular toxicity tests, such as multiple-dose and long-term toxicity observations, and various toxicological studies will be needed to establish the effectiveness and safety of SUEP.

Fortunately, pharmacopuncture-related adverse reactions have not been described in clinical reports using a combination of JOP and CPC pharmacopuncture [11]; however, no official clinical study has been conducted on the side effects of SUEP to date. Therefore, many experimental and clinical studies will need to provide scientific evidence regarding the efficacy and safety of SUEP.

CONCLUSION

According to the results of our toxicity study, the approximate lethal dose of SUEP was greater than 1.0 mL/animal (approximately 1,525 mg/kg) in both male and female SD rats following a single intramuscular injection under the conditions of this study. This finding suggests that, in TKM clinical trials, SUEP can be considered safe at doses below 1.0 mL. To determine the safety of the use of SUEP in TKM clinical practice, additional toxicity studies will be needed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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