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Original Article
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Single-Dose Intramuscular Toxicity Test Using No-Pain Pharmacopuncture in Sprague–Dawley Rats

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Objectives: This study aimed to evaluate the potential toxicity of a recently developed and clinically used No-Pain pharmacopuncture (NPP) solution. We also assessed the lethal dose of the NPP agent following a single intramuscular injection in Sprague-Dawley (SD) rats.
Methods: Animals were divided into two groups: the NPP test material group and the normal saline control group. A single intramuscular injection of the NPP agent (1.0 mL/animal) was administered to rats of the NPP test material group. The control group rats received the same volume of normal saline. Both female and male rats were included in each group. All rats were monitored for clinical signs and body weight changes for 14 days after administration of the test substance or saline. At the end of the observation period, a gross necropsy was conducted and localized tolerance at the injection site was analyzed.
Results: No mortality was observed in the NPP test material and control groups. Moreover, no test substance-related effects were observed on clinical signs, body weight, necropsy findings, and localized tolerance at the injection site.
Conclusion: The approximate lethal dose of the NPP agent is greater than 1.0 mL/animal under the conditions used in this study. Additional toxicity evaluations and clinical studies are needed to confirm the safety of NPP use in clinical practice.

Keywords: safety, single intramuscular toxicity, No-Pain (Mutong) pharmacopuncture, corydalis tube, jakyak gamcho decoction, shakuyaku-kanzo-to

INTRODUCTION

It is believed that herbal medicines are safe and effective since herbal remedies generally have no side effects [1]. The safety and therapeutic efficacy of herbs used in Korean medicine (KM) and traditional Chinese medicine (TCM) have been verified clinically [2]. The Korean Ministry of Food and Drug Safety has excluded safety and efficacy reviews for herbal medicines (crude drugs) that correspond to prescriptions listed in the Korean Medical Classics [3]. However, some side effects of herbal medicines, including hepatotoxicity and genotoxicity, have been reported in previous studies [4-6]. Results of the non-clinical toxicity tests can provide evidence to support the safety of medicinal plants and herbal medicines [7-9]. Therefore, the evaluation of the toxicity of herbal medicines is of prime importance.

Pharmacopuncture is a novel type of acupuncture therapy that improves body functions and pathological conditions by delivering herbal medicine to acupuncture points via injection [10, 11]. In KM, pharmacopuncture is frequently used for musculoskeletal disorders [12].

Moreover, in KM and TCM, several pharmacopuncture agents are used [13, 14]. Furthermore, in KM, based on the literature and clinical experience, the development of new herbal pharmacopuncture agents is being actively pursued [15]. To promote the development and utilization of pharmacopuncture, the most important concern is to expand the scope of health insurance coverage and to verify its safety and therapeutic efficacy. Therefore, elucidation of the pharmacological mechanism and potential toxicity is important and should be performed at

the good laboratory practice (GLP) level [16]. However, toxicity verification of the pharmacopuncture agents has not been extensively performed at GLP-compliant institutions.

A recently developed No-Pain (Mutong) pharmacopuncture (NPP) agent is composed of four medicinal herbs: Corydalis tubers (CT), also known as morphine in traditional medicine, and possess strong analgesic properties [17, 18], Jakyak Gamcho (Shakuyaku-kanzo-to, Shao-Yao-Gan-Cao) decoction, which is composed of two herbs, *Paeonia lactiflora* Pallas (PLP) and *Glycyrrhizae Radix* et Rhizoma (GRR) [19], and Chaenomelis Fructus (CF) [20], which is effective for muscle relaxation. The NPP agent was developed as an aqueous solution to reduce pain during injection and facilitate absorption, with the goal of providing both pain relief and muscle relaxation. Since several studies have analyzed the effects of individual herbal medicines and performed pharmacological analysis [17-20], the efficacy and mechanism of the NPP agent can be predicted to some extent. Although the NPP agent, a complex pharmacopuncture solution composed of four herbs, is already used in KM, the related safety studies are insufficient. Herein, to analyze the safety of the newly developed NPP agent, we evaluated its toxicity using the single-administration muscle toxicity test.

MATERIALS AND METHODS

1. Preparation of the test substance

The NPP test substance used in this study contained four herbs and was manufactured at an external herbal dispensary (EHD) facility certified by the Ministry of Health and Welfare, Namsangcheon EHD (Yongin, Korea). The facility is compliant with the Korean Good Manufacturing Practice standards. Table 1 shows the certificate of analysis based on the test results of the Namsangcheon EHD quality control team. To prepare 1 L of the NPP agent, 4 g CT, 0.6 g CF, 4 g PLP, and 4 g GTT were used. All herbs were weighed, washed, placed in a non-woven fabric, and then circulated in a distillation extractor with sterile water. The distillate was collected, diluted with sterile water, filtered, dissolved in 0.9% NaCl, and titrated to a pH of 7.4. After filtration through a 0.45-0.2 μm filter, the distillate was stored in a sterilized and sealed container. Normal saline (JW Pharmaceutical Co., Ltd., Seoul, Korea) was used as the control.

2. Experimental animals

Sprague-Dawley (SD) rats (5-week-old males and females, n = 12) were purchased from Orient Bio Co., Ltd. (Seongnam, Korea). Visual inspection was performed on the day of arrival and body weight was recorded. At the time of acquisition, the body weight was 117.6-131.3 g for males and 107.7-120.5 g for females. During acclimatization, systemic symptoms such as appearance, posture, behavior, attitude, body temperature, respiration, nervous system function, and excretion were observed once every day. During this period, the animals exhibited no abnormalities and no change in general symptoms and body weight. After acclimatization, the animals were divided into two groups of ten rats each (five males and five females) with similar body weights using the stratified sampling method so that the average weight of each group was approximately the same (Table 2). Animals were housed in controlled environmental

Table 1. Certificate of analysis of No-Pain pharmacopuncture

Test items	Specifications	Results
Appearance	Transparent vial pharmacopuncture agent of clear colorless liquid	Conform
pH	4.0-9.0	7.86
Endotoxin	< 150.15 EU/mL	Conform
Insoluble matter	No foreign objects visible to the naked eye	Conform
Insoluble particulates	10 μm or more ≤ 6,000 pieces 25 μm or more ≤ 600 pieces	Conform
Actual capacity	2.2 ± 0.1 mL	Conform
Sterile	Microorganisms not detected	ND
Confidentiality	No traces or leaks of methylene blue solution	Conform

No-Pain pharmacopuncture: A four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma.
ND, Not detected.

Table 2. Group designation of a single-dose intramuscular toxicity test for No-Pain pharmacopuncture in Sprague–Dawley rats

Group			Injection		Number of animals (object number)	
			Dose amount (mL/animal)	Dose of NPP (mL/animal)	Male	Female
G1	Control group	Normal saline	1.0	0	5 (1101–1105)	5 (2101–2105)
G2	Test substance group	NPP	1.0	1.0	5 (1201–1205)	5 (2201–2205)

NPP, No-Pain pharmacopuncture (a four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma).

conditions with a temperature of 19.7-22.3℃, relative humidity of 53.1-61.6%, and free access to food and water. All animal experiments were approved by the Animal Ethics Experimentation Committee of Biotoxtech Co., Ltd. based on Korea's Animal Protection Act (Approval No.: 220426).

3. NPP single-dose intramuscular toxicity test in SD rats

1) Animal groups and dosing

In clinical practice, the NPP agent is administered via the muscles, therefore, we chose the intramuscular route for this study. In humans, the clinically applicable dose of the NPP agent is 0.1-1.0 mL/individual (0.1 mL/point) (Biotoxtech Study No.: B22465P1). We selected a dose of 1.0 mL, which is the maximum clinically applicable dose, for our preliminary tests. No mortality was observed in male and female rats following a single intramuscular injection of the NPP agent (1.0 mL/animal). Based on this result, 1.0 mL of the NPP agent or saline was intramuscularly injected into the left and right thighs of rats.

2) Observation and examination of animals

The general condition of the rats (signs of toxicity, onset time, recovery period, etc.) and mortality were assessed at 30 min and 1, 2, 4, and 6 h after the administration and then once every day for 14 days. The body weight was measured before administration and on the 4th, 8th, and 14th day of drug administration.

3) Histopathology

All animals were euthanized on the day of performing necropsy by CO_2 inhalation followed by exsanguination from the abdominal aorta. Tissues were removed from the injection sites and fixed in 10% neutral-buffered formalin (NBF). Histopathology samples of organs and tissues were prepared according to standard operating procedures, and the remaining organs and tissues were preserved in 10% NBF. A histopathological examination was performed to evaluate local tolerance at the injection site.

4. Statistical analysis

Values are expressed as the mean ± standard deviation (SD). All statistical analyses were performed using Provantis™. F-test was used to assess sample variance. Groups with equal variance were compared using the Student's t-test.

RESULTS

1. Mortality and other clinical signs

During the observation period, no mortality was observed in the control and NPP groups (Table 3). Moreover, no abnormal clinical signs and no general health-related unusual findings were observed in animals of both groups on the day of drug administration, at 30 min and 1, 2, 4, and 6 h after administration, and 14 days after administration (Table 4).

2. Changes in body weight

The body weight of the male SD rats before drug administration, on the 4th and 8th day of drug administration, and on the day of necropsy was 197.76 ± 4.53 g, 226.42 ± 4.21 g, 266.65 ± 6.27 g, and 336.92 ± 9.20 g, respectively, in the control group and 196.71 ± 2.50 g, 227.45 ± 3.52 g, 267.55 ± 3.10 g, and 332.92 ± 9.95 g, respectively, in the NPP group. The body weight of female SD rats before drug administration, on the 4th and 8th day of drug administration, and on the day of necropsy was 157.97 ± 4.22 g, 169.57 ± 8.22 g, 185.59 ± 8.15 g, and 210.49 ± 12.55 g, respectively, in the control group and 159.90 ± 2.69 g, 171.86 ± 6.91 g, 186.41 ± 6.57 g, and 204.49 ± 7.51 g, respectively, in the NPP group. Compared to the control group, no significant change in body weight was observed in male and female SD rats of the NPP group (Fig. 1).

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Table 3. Mortality in a single-dose intramuscular toxicity test for No-Pain pharmacopuncture in Sprague–Dawley rats

Sex	Group		Day(s) relative to start date													
			1	1→2	1→3	1→4	1→5	1→6	1→7	1→8	1→9	1→10	1→11	1→12	1→13	1→14
Male	G1	Count positives	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Proportion ratio	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Proportion alpha	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	G2	Count positives	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Proportion ratio	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Proportion alpha	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Female	G1	Count positives	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Proportion ratio	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Proportion alpha	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	G2	Count positives	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Proportion ratio	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Proportion alpha	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

No-Pain pharmacopuncture: A four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma.

Table 4. No-Pain pharmacopuncture-related effects on clinical sign changes in a single-dose intramuscular toxicity study in Sprague–Dawley rats

Sex	Group	NPP dose (mL/animal)	Observation type : all types	Day(s) relative to start date																	
				Day 1 (hour)					Day												
				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	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	G2 (n = 5)	1	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Female	G1 (n = 5)	0	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	G2 (n = 5)	1	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Values represent the specific number of animals affected.
G1: Control group administered with normal saline (1.0 mL/animal).
G2: No-Pain pharmacopuncture administration group (1.0 mL/animal).
NPP, No-Pain pharmacopuncture (a four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma).

3. Macroscopic examination following necropsy

Autopsy results showed no gross abnormal changes in males and females of the control group and NPP group (Table 5).

4. Histopathological examination

For the local tolerance test, a histopathological examination of the administration site was performed. Results showed no significant differences between the control and NPP groups. Thus, NPP treatment can be considered acceptable in animals (Table 6).

DISCUSSION

CT, a herbal medicine that improves circulation, moves qi,

and offers pain relief [21], reportedly possesses considerable analgesic, sedative, and hypnotic properties. Besides, CT is clinically effective in various conditions such as arrhythmia, gastric ulcers, and coronary heart disease [17, 21-23]. In traditional medicine, CT is called "morphine" among herbal medicines, and several studies from China showed that CT alleviates pain in patients by acting on gamma-aminobutyric acid or opioid receptors [22]. Moreover, the analgesic, anticonvulsant, antiulcer, antitumor, anti-inflammatory, and anti-arthritic effects of the CT pharmacopuncture solution have been evaluated in experimental models [24-28]. A combined pharmacopuncture solution containing CT and *Erycibae Caulis* has been used for the treatment of rheumatoid arthritis [29]. Moreover, CT is one of the herbal ingredients of the widely used Eohyeol pharmacopuncture [30]. Analysis of the serum components in experimental studies related to arthritis revealed that CT pharmacopuncture solution mixed with PLP and CF does not induce liver and kidney damage [28].

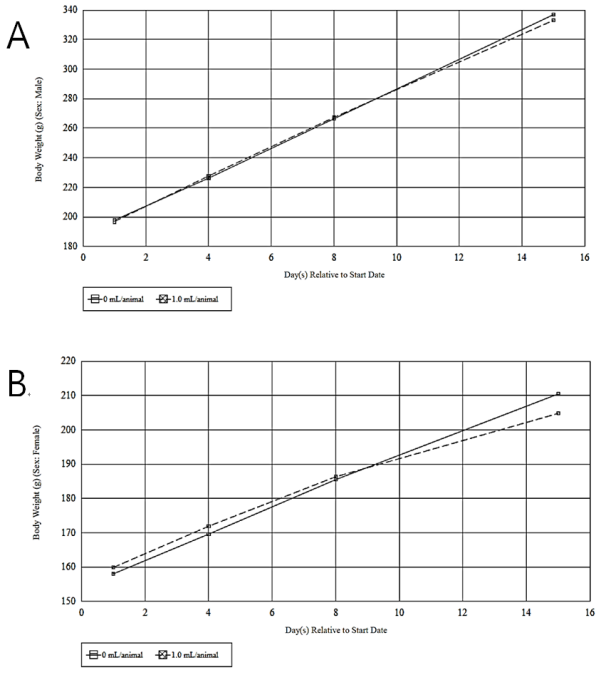

Figure 1. Effects of No-Pain pharmacopuncture on the body weight of male (A) and female (B) Sprague–Dawley rats. The mean weight and standard deviation of each group were calculated, and equal variance was recognized in the F-test and tested using Student's t-test. No-Pain pharmacopuncture: A four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma.

CF, an herb that harmonizes the stomach and resolves dampness [21], has been used in traditional medicine for the treatment of patients with weak muscles and bones, muscle pain, and arthritis [20, 31]. Recently, it has been reported that CF is effective in the treatment of arthritis due to its anti-inflammatory properties [31]. The therapeutic effects of CF pharmacopuncture have been studied in patients with arthritis, and its effects on leukocytes and c-reactive protein have been compared with those of acupuncture. Moreover, oral administration of CF has also been reported [32]. Analysis of serum components in experimental studies related to arthritis showed that CF pharmacopuncture solution mixed with *Chelidonii herba* and *Clematidis Radix* does not induce liver and kidney damage [28].

Jakyak Gamcho decoction is known to exert therapeutic effects on the gastrointestinal smooth muscles and skeletal

Table 5. Necropsy findings in a single-dose intramuscular toxicity test for No-Pain pharmacopuncture in Sprague–Dawley rats

Group		Number of animals			
		Male		Female	
		Group 1	Group 2	Group 1	Group 2
Dose of NPP (mL/animal)		0	1.0	0	1.0
All tissue findings	Examined	5	5	5	5
	Normal	5	5	5	5
Animal excluded		None			
Observation type		Gross			
Measurement		Pathology observation			

Pathology of all organs on the external surface and inside the body cavity was observed.
G1: Control group administered with normal saline (1.0 mL/animal).
G2: No-Pain pharmacopuncture administration group (1.0 mL/animal).
NPP, No-Pain pharmacopuncture (a four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma).

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Table 6. Histopathological findings in a single-dose intramuscular toxicity test for No-Pain pharmacopuncture in Sprague–Dawley rats

Group		Number of animals			
		Male		Female	
		Group 1	Group 2	Group 1	Group 2
Dose of NPP (mL/animal)		0	1.0	0	1.0
Injection site findings	Examined	5	5	5	5
	Normal	5	5	5	5
Animal excluded		None			
Observation type		Histo-non-neoplastic only			
Measurement		Pathology observation			

G1: Control group administered with normal saline (1.0 mL/animal).
G2: No-Pain pharmacopuncture administration group (1.0 mL/animal).
NPP, No-Pain pharmacopuncture (a four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, *Paeonia lactiflora* Pallas, and *Glycyrrhizae Radix* et Rhizoma).

muscles and relieve muscle cramps [19, 33]. It has been reported that Jakyak Gamcho decoction effectively relieves painful muscle cramps in patients with lumbar spinal stenosis [19, 33, 34]. Regarding pharmacopuncture, there have been reports on the analgesic and anticonvulsive effects of peony licorice-tang herbal acupuncture. A single-dose toxicity evaluation of intramuscular and intravenous injections of Gami-Jakyak Gamcho buja decoction pharmacopuncture solution containing PLP, GRR, and CF has been performed in previous studies. However, there are no reports on a complex pharmacopuncture solution containing CR, PLP, GRR, and CF.

Toxicity studies using various experimental models should be conducted systematically to determine the safety of pharmaceuticals and herbal products. Toxicity can be single-dose toxicity, repeated-dose toxicity, reproduction/developmental toxicity, genotoxicity, antigenicity, immunotoxicity, carcinogenicity, local toxicity, local tolerance, single-dose inhalation toxicity, and repeated-dose inhalation toxicity [7, 8]. For combination drugs, oral dosage forms, injections and infusions, single-dose toxicity, repeated-dose toxicity for 1 month, and repeated-dose toxicity for 3 months or longer should be evaluated. Through single-dose toxicity studies, qualitative and quantitative data on time-related toxic events and their occurrence following a single dose of a substance or combination of substances can be described. Due to species level and phylogenetic differences in biological responses to test substances, animal species with efficacy or metabolic profiles similar to that of humans should be selected for experiments. In single-dose toxicity studies, it is common to select animals that are easy to handle, qualitatively uniform, and have abundant background data. The most preferred animals are rats for rodent studies and dogs for non-rodent studies. Moreover, because there might be sex-dependent differences in the toxicity response, toxicity should be evaluated in at least one male and one female animal [8]. This study aimed to evaluate the potential toxicity of the NPP solution and determine its approximate lethal dose using a single intramuscular injection in female and male SD rats.

In our study, no mortality was observed in either sex following NPP solution administration, and no NPP-related effect was observed on clinical signs and body weights. Additionally, no macroscopic abnormalities related to NPP were observed at autopsy, and no significant changes related to NPP were observed in the local tolerance test results at the administration site through histopathological examination.

Based on our toxicity evaluation, the approximate lethal dose of the NPP solution under the conditions of this study was found to be greater than 1.0 mL/animal in both male and female SD rats. The actual clinically applied dose of the NPP agent when performing intramuscular administration is 1 mL once per adult (60 kg body weight). The 1 mL/animal dose used in this experiment is approximately 300 times higher than that clinically used in humans. Therefore, it can be inferred that the administration of NPP formulations at doses of 1.0 mL or less in clinical practice is safe for humans. However, since this study evaluated toxicity for only a short period of 14 days with a single administration of the NPP agent, multiple doses, long-term toxicity observations, route of administration, and toxicity evaluation of the liver and kidneys should be performed in the

future. In addition, evidence on the safety and effectiveness of NPP should be clarified through clinical studies.

CONCLUSION

In humans, NPP generally uses a dose of approximately 1.0 mL, therefore, we considered this as the lethal dose in our study. Results of our toxicity test suggest that the approximate lethal dose of the NPP agent following a single intramuscular injection in female and male SD rats is greater than 1.0 mL/animal under the conditions of this study. Thus, administration of up to 1.0 mL of NPP solution to humans can be considered safe. However, other toxicity studies on NPP are needed to further determine the precise lethal dose. Animal experiments and clinical studies should be conducted in the future to verify the efficacy of NPP.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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