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In Vivo Genotoxicity Evaluation of a No-Pain Pharmacopuncture Extract Using the Micronucleus Test

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Objectives: We aimed to evaluate the genotoxicity of a recently developed no-pain pharmacopuncture (NPP) targeting muscle relaxation and analgesia using the micronucleus test.
Methods: To evaluate the potential of NPP extracts to induce micronuclei in rat bone marrow cells, a micronucleus test was performed using male Sprague-Dawley rats. The test substance NPP was administered intramuscularly at concentrations of 0.25, 0.5, and 1 mL/animal. Saline was used as the negative control and cyclophosphamide as the positive control.
Results: No NPP treatment-related deaths or abnormal changes in general appearance were observed at any dose level during the experimental period. No statistically significant differences in body weight were observed in any of the NPP dose groups compared to the saline negative control group. NPP did not cause a significant increase in the incidence of micronucleated polychromatic erythrocytes (PCEs) and PCEs or in the ratio of PCE-to-total erythrocytes.
Conclusion: The NPP extract did not exhibit genotoxic in Sprague–Dawley rat bone marrow cells under the conditions of this study. Further toxicity studies of the NPP extract are required.

Keywords: safety, genotoxicity test, in vivo micronucleus test, toxicity, Mutong pharmacopuncture, no-pain pharmacopuncture

INTRODUCTION

To use natural products and herbal medicines as safe therapeutic agents, in-depth toxicity studies and verification of their therapeutic effects are required [1]. Despite their long-term use, only 15% of studies evaluating herbal medicines have provided information on safety or side effects [2]. Moreover, some herbal medicines and their components have been suspected of being carcinogenic [3]. Compared to chemical-based medicines, natural medicines have multi-component and multi-target properties with many substances and sites of action, which make it difficult to confirm their mechanism of action, side effects, and quality. In addition to the toxicity of natural products themselves, there are concerns about the mixing of pesticides or heavy metals during the cultivation process, which requires strict management and monitoring [4].

In the case of Korean pharmacopuncture, as pharmacopuncture extracts are prepared using standardized herbal medicines in external herbal dispensaries (EHDs) certified by the Ministry of Health and Welfare (MOHW) in accordance with Practice for Pharmaceutical products in Korea, the adoption of quality control criteria in the preparation of these extracts is evident to some extent. The use of various pharmacopuncture preparations continues to increase, and Korean medicine doctors recognize the importance of pharmacopuncture toxicity verification according to the Good Laboratory Practice regulations [5-

7]. However, studies on pharmacopuncture toxicity are limited.

No-pain (Mutong) pharmacopuncture (NPP), a complex extract of Corydalis tuber (CT), Paeoniae radix (PR), Glycyrrhizae Radix et Rhizoma (GR), and Chaenomelis Fructus (CF), has been clinically used since 2022 to achieve simultaneous pain relief and muscle relaxation. It is made with qi formula (water type) to reduce pain during acupuncture and promote absorption [8]. However, while NPP continues to be used clinically, to the best of our knowledge, no studies have reported its efficacy and safety, other than a case report of one case of plantar fasciitis [9] and a single-dose muscle toxicity study [8].

Genotoxicity assessment is important for folk remedies containing medicinal herbs because of their potential to damage genetic material, which can lead to lethal mutations and increase the risk of diseases such as cancer [10]. Thus, in this study, in compliance with OECD guidelines [11, 12], we aimed to evaluate the potential genotoxicity of NPP and determine its safety profile for clinical use by performing a micronucleus test using Sprague–Dawley rats.

MATERIALS AND METHODS

1. Preparation of the NPP extract

The NPP extract used was manufactured at Namsangcheon EHD (Yongin, Korea), an EHD certified by the MOHW. Dried CT (4 g), CF (0.6 g), PR (4 g), and GR (4 g) were dissolved in 1 L of water (Table 1). After washing the herbal medicines and drying them using a non-woven fabric, they were mixed with water in a distillation extractor. The extract was diluted with water, filtered using filter paper, dissolved in 0.9% NaCl, and titrated to pH 7.4. Finally, it was filtered using a 0.45-0.2-μm filter, sealed in a sterilized container, and stored at room temperature until required for further analysis.

2. Experimental animals

Seven-week-old male specific pathogen-free Crl:CD Sprague–Dawley rats were purchased from Orient Bio Inc. (Seongnam, Korea) and housed in a room maintained at a temperature of 22 ± 3℃, relative humidity of 55% ± 15%, and illumination of 150-300 lux, with 10-15 air changes per hour. Animals had ad libitum access to a solid diet and ultraviolet-sterilized tap water. This toxicity study was approved by the Animal Experimentation Ethics Committee of Biotoxtech Co., Ltd. (Cheongju, Korea) with complete certification from the International Association for Assessment and Accreditation of Laboratory Animal Care in 2010, based on the Animal Protection Act in Korea (No.: 220415).

After a 6-day quarantine and acclimatization period, the rats were randomly assigned to one of five groups (n = 5 per group). We used the muscle route of injection given that the muscle route is the clinical application route for NPP. Using a disposable syringe, 0.25 mL of low dose, 0.5 mL of medium dose, 1 mL of high dose of NPP, and 1 mL of saline negative control were administered twice into the thigh/animal muscle at approximately 24-h intervals. The test substance/control was divided into doses so as not to exceed 0.5 mL per site. During the second administration of NPP, cyclophosphamide was administered orally once using a disposable syringe, as a positive control (Table 2).

3. *In vivo* micronucleus test of NPP in Sprague–Dawley rats

In the "Single Intramuscular Dose Toxicity Preliminary Study of NPP in Sprague–Dawley Rats" (Biotoxtech Study No.: B22465P1), 1 mL of NPP/animal did not cause mortality in the rats; hence, a dose of 1 mL NPP/animal was used as the high dose in the current study. A geometric ratio of 2 was applied to generate two additional low-dose levels (0.5 and 0.25 mL/animal), and both positive and negative controls were estab-

Table 1. Crude components of the no-pain pharmacopuncture extract

Herbal name	Latin name	Scientific name	Amount (mg/mL)
Corydalis	Corydalis tube	*Corydalis ternata Nakai*	2
Red peony	Paeoniae Radix	*Paeonia lactiflora Pallas*	2
Licorice root	Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis Fischer*	2
Quince	Chaenomelis Fructus	*Chaenomeles sinensis*	0.3

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

Table 2. Group settings in the micronucleus test in Sprague–Dawley rats

Group		Dose (mL/animal)	Dose amount (mL/animal)	Frequency of administration	Number of animals
G1	Negative control (normal saline)	0	1	2	5
G2	NPP (low-dose)	0.25	0.05	2	5
G3	NPP (mid-dose)	0.5	0.1	2	5
G4	NPP (high-dose)	1	0.2	2	5
G5	Positive control (cyclophosphamide)	20 mg/kg	10 mL/kg	1	5

NPP, no-pain pharmacopuncture.

lished. General symptoms related to appearance, behavior, and excretion were observed twice daily during the administration period (immediately before and after administration) and once daily during the non-administration period. Rats were weighed on the day of initiation of administration and on the day of bone marrow collection.

Animals were euthanized under CO_2 gas anesthesia 24 h after the second administration of NPP. Rat femurs were removed, and bone marrow cells were collected. Briefly, 0.5 mL of 10% neutral formalin was added to the bone marrow cell suspension for 5 min for fixation, followed by centrifugation (4℃, 1,000 rpm) for 5 min to remove the supernatant. Thereafter, 0.3 mL of 10% neutral formalin was added to the precipitated bone marrow cells to suspend them, before filtering the cells filtered through a cell strainer and transferring to a storage tube. After dropping the fixed bone marrow cell suspension on a coverslip, the slide for observation was prepared by placing the coverslip on a glass slide coated with 20 μL of 0.05% acridine orange.

The slides were observed under a microscope (600 times magnification; BX51, Olympus, Japan). A total of 4,000 polychromatic erythrocytes (PCEs) were observed per individual, and the appearance rate of micronucleated PCEs (MNPCEs) was calculated per individual. The observation results confirmed that the ratio of PCE to the total number of erythrocytes, also called red blood cells (RBCs), in the NPP group was ≥ 20% compared to that in the saline negative control group. As an indicator of suppression of bone marrow cell proliferation, 500 total erythrocytes per individual were observed, and the ratio of PCE-to-total erythrocytes was obtained.

4. Statistical analyses

All statistical analyses in this study were conducted using version 9.4 SAS software (SAS Institute, Inc., Cary, NC, USA). The Kruskal–Wallis test was performed to confirm the significance of the differences in the frequencies of MNPCEs, and the Mann–Whitney U test was performed when significance was confirmed after comparison with the saline negative control group ($p < 0.05$, $p < 0.01$). For the NPP test substance group, the Cochran–Armitage trend test was conducted to test the significant difference in dose dependance between the test groups ($p < 0.05$ and $p < 0.01$). The Bartlett test was used to determine the frequency and weight of PCEs relative to total erythrocytes and the equal variance between the negative control and NPP groups ($p < 0.05$). After confirmation of equal variance, significance was confirmed using one-way analysis of variance ($p < 0.05$), and Dunnett's t-test was performed following confirmation of significance. The Kruskal–Wallis test was not performed if the equal variance was not rejected. To compare the negative and positive controls, an equal variance test was performed using the Folded-F test. If equal variance was recognized, significance was confirmed using Student's t-test ($p < 0.05$, $p < 0.01$), and if the equal variance was not rejected, the Aspin–Welch t-test was not performed.

RESULTS

1. Effect of NPP on the overall health and body weight of male Sprague–Dawley rats exposed to no-pain pharmacopuncture in the in vivo micronucleus test

During the experimental period, no NPP treatment-related deaths or abnormal changes in general appearance were observed in any of the NPP dose groups (0.25, 0.5, and 1.0 mL/animal). Moreover, compared to the saline negative control group, no statistically significant differences were observed in the body weights of rats in the NPP group at any dose (Table 3).

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Table 3. Changes in body weight of male Sprague–Dawley rats exposed to no-pain pharmacopuncture in the in vivo micronucleus test

Group		Dose (mL/animal)	Route	Days after dosing (g)	
				Before the first dosing	1 day after the second dosing
Negative control	Normal saline	0	IM	260.9 ± 10.32	276.8 ± 7.91
Test substance	NPP	0.25	IM	261.0 ± 6.87	274.5 ± 9.68
		0.5	IM	262.1 ± 9.45	281.0 ± 12.21
		1.0	IM	262.8 ± 8.06	279.7 ± 7.78
Positive control*	CPA	20 mg/kg	PO	263.5 ± 8.62	273.8 ± 11.08

CPA, cyclophosphamide; IM, intramuscular; NPP, no-pain pharmacopuncture; PO, per os (by mouth).
*The positive control substance was administered once 24 h before the sampling time.
Data are presented as mean ± standard deviation.

Table 4. Ratio of PCEs-to-total erythrocytes in male Sprague–Dawley rats in the in vivo micronucleus test

Group		Dose (mL/animal)	Route	Hours after dosing (h)		PCE (PCE + NCE)	MNPCE/PCE
Negative control	Normal saline	0	IM	24	Total	806/2,500	8/20,000
					% (Mean ± SD)	32.2 ± 0.77	0.040 ± 0.022
Test substance	NPP	0.25	IM	24	Total	800/2,500	6/20,000
					% (Mean ± SD)	32.0 ± 0.73	0.030 ± 0.021
		0.5	IM	24	Total	802/2,500	9/20,000
					% (Mean ± SD)	32.1 ± 1.15	0.045 ± 0.021
		1.0	IM	24	Total	805/2,500	8/20,000
					% (Mean ± SD)	32.2 ± 1.21	0.040 ± 0.029
Positive control	CPA	20 mg/kg	PO	24	Total	800/2,500	883††/20,000
					% (Mean ± SD)	32.0 ± 0.71	4.415 ± 0.080

CPA, cyclophosphamide; IM, intramuscular; MMC, mitomycin C; MNPCEs, micronucleated polychromatic erythrocytes; NCE, normochromatic erythrocytes; NPP, no-pain pharmacopuncture; PO, per os (by mouth); PCEs, polychromatic erythrocytes; SD, standard deviation.
$^{††}p < 0.01$, a significant difference from the negative control according to the Mann–Whitney U test.

2. Incidence of MNPCE in PCE and PCE-to-total erythrocytes in the micronucleus test for NPP

Compared to the saline negative control group, no statistically significant increase in the incidence of MNPCE among PCEs was observed in any of the NPP dose groups. Additionally, there was no statistically significant difference in the ratio of PCEs to total erythrocytes between the NPP and saline negative control groups (Table 4). The incidence rates of MNPCEs and PCEs in the positive control (cyclophosphamide) group were significantly higher than those in the negative control group, while no significant difference was observed in the ratio of PCEs-to-total erythrocytes between the positive and negative control groups, indicating that this experiment was conducted under established test conditions.

The frequency of MNPCE in the saline-negative and cyclophosphamide-positive control groups was within the control range of historical control data. The frequency of MNPCE was significantly increased in the cyclophosphamide-positive control group compared to the saline-negative control group (Table 5). The number of dosing groups and cells to be observed was appropriate and the highest dose level was set according to the dose setting standards; thus, the toxicity test was confirmed to be conducted under appropriate experimental conditions.

DISCUSSION

The NPP extract consists of four medicinal herbs that have been traditionally used in Asian medicine, including CT, CF, PR, and GR. CT has been reported to have significant analgesic,

Table 5. Historical control data

Historical control values of micro nucleated polychromatic erythrocytes (MNPCE)							
Group	Hours after dosing (hr)	Dose (mL/kg)	N	MNPCE/PCE(%) (Mean ± S.D.)	Range [MNPCE/PCE] (%)		95% control limit[a] [MNPCE/PCE]
					MIN	MAX	
Negative control	24	0	20	0.03 ± 0.02	0	0.065*	< 12
Positive control	24	20	19	4.32 ± 0.61	3.913*	4.717*	-
Historical control values of ratio of polychromatic erythrocytes (PCE) to total erythrocytes							
Group	Hours after dosing (hr)	Dose (mL/kg)	N	PCE/(NCE + PCE) (%) (Mean ± S.D.)	Range [PCE/(NCE + PCE)] (%)		
					MIN	MAX	
Negative control	24	0	20	32.4 ± 2.56	30.3*	34.5*	
Positive control	24	20	18	31.3 ± 2.08	29.1*	33.5*	

N, The total number of micronucleus test; Negative control, water for injection, phosphate buffered saline, 0.5% methyl cellulose 1,500 centipoise solution, *etc.*; NPP, no-pain pharmacopuncture; Positive control, cyclophosphamide (20 mg/kg, P.O., single dosing).
The above historical control values were obtained from the data pooled from Feb. 26, 2016 to Dec. 3, 2021.
*The range was calculated by the control limit of X derived from X-R value.
[a]Poisson-based 95% control limits of the historical negative control data.

sedative, and hypnotic effects and has been shown by modern medical research to have clinical treatment effects for various conditions, including gastric ulcer, arrhythmia, and coronary heart disease [13, 14]. CF has been traditionally used to treat muscle and joint pain, inflammation, and weak muscles and bones [15, 16]. The Jakyak–Gamcho decoction (PR and GR) is effective in relaxing gastrointestinal smooth and skeletal muscles and in relieving muscle spasms [17, 18]. However, to the best of our knowledge, the efficacy or toxicity of NPP extract has not been previously evaluated, with the exception of one study that conducted a single-dose toxicity assessment [8], and a clinical report of efficacy in a case of plantar fasciitis [9].

Genotoxicity refers to the property of a substance to cause damage to cellular DNA or chromosomes [19]. Genotoxic carcinogens initiate cancer development by damaging DNA, thereby causing mutations in genes [20]. Genotoxicity testing is used for primary screening of anti-cancer drugs and needs to be supplemented with other toxicity test results; however, it provides an important clue for identifying the substances that need to be tested for carcinogenicity. In this study, the in vivo micronucleus test, one of the most widely used genotoxicity tests, was performed to test the potential genotoxicity of NPP extract.

The micronucleus test is a representative in vivo assay used to determine the mutagenicity of a test substance, given that the induction of micronuclei is closely related to the induction of chromosomal aberrations [21]. In this study, the potential of NPP to induce micronuclei in rat bone marrow cells was investigated by administering NPP intramuscularly to 8-week-old male Sprague–Dawley rats at doses of 0.25, 0.5, and 1.0 mL/animal twice at 24-h intervals. The Sprague–Dawley rat was chosen because it is the most widely used rodent for various toxicity evaluations, including micronucleus testing, has accumulated a large amount of baseline data for comparison, and is a guideline-recommended species [22].

In this study, intramuscular administration, which is the clinical route for NPP, was selected as the route of administration in the toxicity testing [22]. Considering that the general clinical application dose of NPP is 1 mL, the maximum dose for animal testing was set at 1 mL/animal. In a preliminary toxicity study of NPP by single intramuscular administration in Sprague–Dawley rats, no deaths were observed in the male and female groups administered 1 mL/animal. Accordingly, the highest dose used in this study was 1 mL/animal. Moreover, no NPP treatment-related deaths or abnormal changes in appearance were observed in rats at any concentration during the experiment, and no statistically significant change in body weight was observed compared to the saline negative control group on the day of treatment initiation and bone marrow harvest.

In interpreting the result, the test is considered positive if there is a statistically significant dose-related increase in the number of MNPCEs or if a positive response is reproducible at one or more dose levels. Statistical processing is used to evaluate whether a significant increase or dose–response relationship is observed compared to the natural frequency of micronuclei occurrence in the negative control group [22]. In this study, the

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results of the micronucleus test showed no significant difference in the incidence of MNPCEs and PCEs between the NPP and negative control groups. Additionally, the ratio of PCE to total erythrocytes in the NPP administration groups was not significantly different from that in the saline negative control group. Meanwhile, in the CPA-treated positive control group, a statistically significant increase in the frequency of MNPCE among PCEs was confirmed compared to that in the saline-treated negative control group. Additionally, there was no statistically significant difference in the ratio of PCEs to total erythrocytes compared to the negative control group.

As we confirmed that the study was conducted under appropriate conditions, the frequency of MNPCE in the negative control group was within the control range of the historical control data and within 95% of the historical control data. The frequency of micronucleated polycythemia in the positive control group was within the control range of the historical control data and exhibited a statistically significant increase compared to the negative control group. The number of treatment groups and cells to be monitored was appropriate, and the highest dose was set according to the standards for dose setting. Therefore, it was concluded that this study was conducted under appropriate experimental conditions. Therefore, it was concluded that the NPP extract had no potential to induce micronucleus formation in murine bone marrow cells. If the in vitro test system shows relatively high activity but the micronucleus test is negative, it is possible that the test substance or its metabolites are not reaching the bone marrow. In this case, it is advisable to add the necessary tests and submit the results [22]. It is also important to confirm the activity of NPP in an in vitro test system by conducting additional testing.

NPP is commonly used clinically at 1 mL per adult (60 kg), which is 0.105 mg/kg, while the 1 mL/animal dose used in this study was 21.79 mg/kg. The confirmation of safety at a dose of NPP that was approximately 207 times greater than the actual clinical dose indicates that its use is safe in actual clinical practice. Although the NPP extract passed the single dose toxicity and micronucleus tests, there remain limitations in confirming the safety of NPP. In particular, as this study was conducted with a short observation period of 24 h, delayed genotoxic effects cannot be captured. Therefore, additional evaluation of delayed persistence toxicity is required. Additional genotoxicity assessments, other forms of toxicity assessments, and human safety studies are needed to demonstrate the safety and efficacy of NPP.

CONCLUSION

In conclusion, in this toxicity study, we demonstrated that the NPP extract showed no potential for inducing micronucleus formation in murine bone marrow cells. However, additional experimental and clinical studies are necessary to ensure the safety of NPP when used clinically.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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