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Original Article

Toxicity Assessment of a No-Pain Pharmacopuncture Extract Using a Standard Battery of *In Vitro* Chromosome Aberration Tests

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Objectives: Genotoxicity is evaluated through a chromosomal aberration test using cultured mammalian cells to determine the toxicity of no-pain pharmacopuncture (NPP), which has recently been used to treat musculoskeletal pain disorders in Korean medical clinical practice.
Methods: An initial test was performed to determine the dosage range of the NPP, followed by the main test. In this study, NPP doses of 10.0, 5.0, and 2.5%, and negative and positive controls were tested. An *in vitro* chromosome aberration test was performed using Chinese hamster lung cells under short-term treatment with or without metabolic activation and under continuous treatment without metabolic activation.
Results: Compared with the saline negative control group, NPP did not significantly increase the frequency of chromosomal abnormalities in Chinese hamster lung cells, regardless of the presence or absence of metabolic activation. Additionally, the number of cells with structural chromosomal abnormalities was significantly higher in the positive control group than that in the negative control group that received saline.
Conclusion: Based on the above results, the chromosomal abnormality-producing effect of NPP was determined to be negative under these test conditions.

Keywords: chromosome aberration test, genotoxicity test, mutong, no-pain pharmacopuncture, safety

INTRODUCTION

The human body is a complex system of diverse networks at the cellular, tissue, organ, and systemic levels. Western medicine is now incorporating multipharmacology to complement the traditional approach of using a single active ingredient to address a problem. Traditional herbal medicines are often recommended for their therapeutic benefits and reduced toxicity. Typically, a combination of herbal medicines with various effects is prescribed to achieve a synergistic effect, which has been scientifically validated [1]. However, in order to safely use these complex herbal formulas, in-depth verification studies on their toxicity and therapeutic effects are required [2].

A pharmacopuncture solution is a single or combined herbal medicine extract used for pharmacopuncture therapy, a modern acupuncture treatment method. In pharmacopuncture, herbal medicines are extracted, purified, and diluted before being injected into specific acupuncture points for treatment [3-5]. This therapy is typically used for musculoskeletal disorders [6, 7]. In Korea, pharmacopuncture is a widely utilized treatment method [8, 9], with various types of pharmacopuncture solutions being developed based on classical literature and clinical experience [10]. Previous research emphasized the importance of verifying the pharmacological effects and toxicity of herbal medicine solutions in a GLP (Good Laboratory Practice) facility to ensure their safety and effectiveness [7]. However, despite the active development and use of different pharmacopuncture solutions, there is limited verification of the toxicity of herbal medicines.

No-pain (Mutong) pharmacopuncture (NPP) is a pharma-

copuncture solution that has been clinically used since 2022 to relieve musculoskeletal pain. To date, there have been few studies on NPP, including one case report [11] and two toxicity assessments [12, 13]. The evaluation of genotoxicity is crucial in herbal medicines and traditional medicine practices as they have the potential to cause harmful mutations and damage genetic material, increasing the risk of diseases, such as malignant tumors [14]. This study aimed to evaluate the potential genotoxicity of NPP, an herbal complex extract, using an *in vitro* chromosomal aberration test in cultured mammalian cells.

MATERIALS AND METHODS

1. Preparation of test material and cell culture for NPP extract testing

NPP (6.3 mg/mL) consisting of Corydalis tuber (CT, 2 mg/mL), Chaenomelis Fructus (CF, 0.3 mg/mL), Paeoniae radix (PR, 2 mg/mL), and Glycyrrhizae Radix et Rhizoma (GR, 2 mg/mL) was prepared at Namsangcheon external herbal dispensary (Yongin, Korea) according to the extraction method described in a previous study [12, 13]. The positive control material, mitomycin C, was used for the "without metabolic activity" condition, while benzo(a)pyrene was used for the "with metabolic activity" condition. Physiological saline was used as the negative control. Chinese hamster lung (CHL/IU) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) on November 24, 2011. The cells were cultured in Eagle's minimum essential medium (Lonza Walkersville Inc., Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and were used within 26 passages. Satellite controls were included in both the dose-finding and main assays to confirm cytotoxicity.

2. Preliminary dose-range exploratory study

A preliminary dose-range exploratory study was conducted to determine high dose levels. A dose of 10% was established as a high dose and serially diluted by applying a geometric ratio of 2 to generate lower doses (5, 2.5, 1.25, 0.625, and 0.313%). At the conclusion of the pre-incubation period, the treatments were prepared and processed as indicated in Table 1, using one well per dose. After NPP treatment, the pH and osmolarity of both the negative control and the highest NPP dosage were assessed. It was determined that the pH and osmolarity of the highest NPP dosage did not exhibit a change exceeding 1.0 and 50 mOsm/kg, respectively, in comparison to the negative control. Consequently, the pH and osmolarity of the NPP groups,

Table 1. Treatment methods used in the dose setting test and main study

	Treatment method	S9 mix	Treatment group	Preparation amount (mL)			Dispense volume (mL/well)
				Culture medium	S9 mix	Negative or positive control, or NPP	
Dose setting test	Short time treatment	–	Negative control (saline)	2.7	-	0.3	2
			NPP	2.7		0.3	2
		+	Negative control (saline)	2.2	0.5	0.3	2
			NPP	2.2		0.3	2
	Continuous treatment	–	Negative control (saline)	2.7	-	0.3	2
			NPP	2.7		0.3	2
Main study	Short time treatment	–	Negative control (saline)	11.7	-	1.3	5
			NPP	11.7		1.3	5
			Positive control	12.87		0.13	5
		+	Negative control (saline)	9.53	2.17	1.3	5
			NPP	9.53		1.3	5
			Positive control	10.70		0.13	5
	Continuous treatment	–	Negative control (saline)	11.7	-	1.3	5
			NPP	11.7		1.3	5
			Positive control	12.87		0.13	2

NPP, no-pain pharmacopuncture (four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, Paeoniae Radix, and Glycyrrhizae Radix et rhizoma).

other than the highest dose group, were not subjected to measurement, and no alteration in the medium's color due to pH change was observed (Table 2).

3. *In vitro* chromosome aberration test

Based on the finding that there was no evidence of cytotoxicity or precipitation of NPP following short-term treatment with or without metabolic activation, as well as after continuous treatment without metabolic activation, the dose levels of NPP selected for the main study were 10%, 5%, and 2.5% in the absence (for short-term treatment and continuous treatment) and presence (for short-term treatment) of the S9 mixture (Table 1). After the test substances were metabolically activated for 6 h, the plate was rinsed with D-PBS, and a fresh culture solution was added for further incubation of 18 h. The test substance was continuously applied for 24 h in the absence of metabolic activation. The precipitation of the test substance at each dose was observed at the time of treatment with the test substance, end of the treatment, and end of the culture. The relative population doublings (RPDs) were calculated using the same methodology as in the dose-finding study.

4. Specimen slide observation

The specimen slides were examined sequentially, beginning with those subjected to short-term treatment and moving on to those subjected to continuous treatment. For each treatment method, the target dose for chromosome observation was set at three doses that could generate more than 300 metaphase cells per dose. Furthermore, 300 metaphase cells for each NPP dose were examined using a microscope with a 600x magnification (BX51, Olympus) and classified based on structural, numerical, or other chromosomal abnormalities. Images of chromosomal samples from the negative and positive controls were used as references (Fig. 1).

Table 2. Summary results of the dose setting study

Test substance	Dose (%)	S9 mix	Trt-rec time (h)	Relative population doubling (%)	PD
Negative control (saline)	0	–	6-18	100	1.53
NPP	0.313	–	6-18	98.5	-
	0.625	–	6-18	97.0	-
	1.25	–	6-18	95.5	-
	2.5	–	6-18	93.9	-
	5	–	6-18	91.8	-
	10	–	6-18	88.5	-
Negative control (saline)	0	+	6-18	100	1.53
NPP	0.313	+	6-18	98.5	-
	0.625	+	6-18	97.5	-
	1.25	+	6-18	95.9	-
	2.5	+	6-18	94.4	-
	5	+	6-18	90.0	-
	10	+	6-18	86.7	-
Negative control (saline)	0	–	24-0	100	1.54
NPP	0.313	–	24-0	98.5	-
	0.625	–	24-0	96.5	-
	1.25	–	24-0	94.5	-
	2.5	–	24-0	93.4	-
	5	–	24-0	90.2	-
	10	–	24-0	88.1	-

NPP, no-pain pharmacopuncture (A four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, Paeoniae Radix, and Glycyrrhizae Radix et Rhizoma; Trt-rec time, treatment-recovery times).
Relative population doubling (RPD) = (no. population doubling in treated cultures) (no. population doubling in control cultures) × 100.
Population doubling (PD) = [log (post-treatment cell number/initial cell number)]/log 2.

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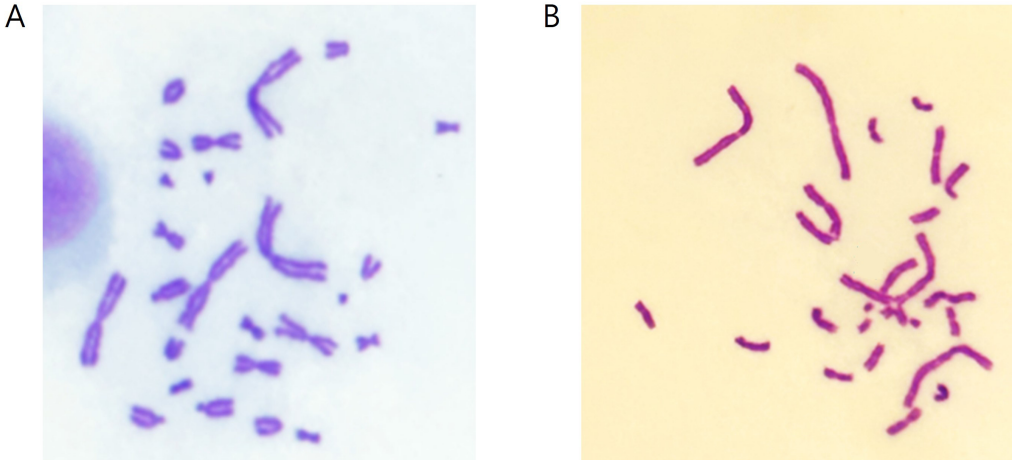

Figure 1. Negative and positive control reference images in the *in vitro* chromosomal aberration test. Images of cells without chromosomal abnormalities (negative control, A) and cells with chromosomal structural abnormalities (positive control, B) were used as reference data for the chromosomal abnormality test.

Table 3. Test suitability and determination of results

Test conditions (test established if all conditions are met).
1. The frequency of cells with chromosomal aberrations in the negative control group must be within the control range of the historical control data and within 95% of the historical control data.
2. The frequency of cells with chromosomal aberrations in the positive control must be within the control range of the historical control data and show a statistically significant increase compared to the negative control.
3. Cell proliferation criteria must be met in the negative control.
4. All three test conditions must be tested unless one of the three test conditions (absence and presence of metabolic activation in the short-term treatment, absence and presence of metabolic activation in the continuous treatment) is positive.
5. There should be at least three readable doses in the test substance group and at least 300 mitotic divisions per dose should be observed in the control and test substance groups.
6. Criteria for selection of the highest concentration should be appropriate.
Determination of results (The frequency of cells with chromosomal aberrations is considered positive if all of the following conditions are met; otherwise it is negative).
1. A statistically significant increase in the frequency of cells with chromosomal aberrations compared with the negative control at one or more doses.
2. The increase is dose dependent.
3. The frequency of cells with chromosomal aberrations increases beyond the control range of the negative control historical control data.

5. Test conditions and determination of results

This study was conducted, and its results were determined according to the test conditions presented in Table 3.

6. Statistical analyses

Statistical analyses were conducted using the SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). The frequency of cells exhibiting chromosomal abnormalities (excluding gaps) was compared between the saline negative control group and the NPP group, as well as between the saline negative control group and the positive control group, utilizing Fisher's exact test ($p < 0.05$, $p < 0.01$). Additionally, a Cochran-Armitage trend test was employed to assess the dose-dependence relationship among the NPP dose groups ($p < 0.05$, $p < 0.01$).

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Table 4. Results of the *in vitro* chromosome aberration test for the no-pain pharmacopuncture extract in CHL/IU cells with or without metabolic activation (S9 mixture)

Test substance	Dose (%)	RPD (%)	PD	S9 mix	Trt-rec time (h)	No. of cells analyzed	Number of cells with structural aberrations									Number of cells with numerical aberrations			Others[a]
							Ctb	Csb	Cte	Cse	Frg	Gap		Total (%)		End	Pol	Total (%)	
												Ctg	Csg	Gap-	Gap+				
Negative control (saline)	0	100	1.54	–	6-18	150	0	0	1	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		
NPP	2.5	95.1	-	–	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	0	0	0	0			0	0		
	5	92.0	-	–	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	0	0	0	0			0	0		
	10	89.9	-	–	6-18	150	0	0	0	0	0	1	0	0 (0.0)	1 (0.3)	0	0	0 (0.0)	0
							0	0	0	0	0	0	0			0	0		
MMC	0.1 mg/mL	51.4	-	–	6-18	150	12	0	22	0	0	0	0	63** (21.0)	63 (21.0)	0	0	1 (0.3)	0
							12	0	19	1	0	0	0			0	1		
Negative control (saline)	0	100	1.52	+	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	0	0	0	0			0	0		
NPP	2.5	98.5	-	+	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	0	0	0	0			0	0		
	5	91.7	-	+	6-18	150	0	0	0	0	0	0	0	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0
							0	0	1	0	0	0	0			0	0		
	10	87.3	-	+	6-18	150	0	0	0	0	0	1	0	1 (0.3)	1 (0.3)	0	0	0 (0.0)	0
							0	0	1	0	0	0	0			0	0		
B(a)P	20 mg/mL	55.4	-	+	6-18	150	13	0	24	0	0	1	0	64** (21.3)	64 (21.3)	0	0	0 (0.0)	0
							10	0	18	1	0	1	0			0	0		
Negative control (saline)	0	100	1.53	–	24-0	150	0	0	1	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		
NPP	2.5	96.0	-	–	24-0	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0
							0	0	0	0	0	0	0			0	0		
	5	93.9	-	–	24-0	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0
							0	0	0	0	0	0	0			0	0		
	10	92.4	-	–	24-0	150	0	0	0	0	0	1	0	0 (0.0)	1 (0.3)	0	0	0 (0.0)	0
							0	0	0	0	0	0	0			0	0		
MMC	0.1 mg/mL	52.7	-	–	24-0	150	13	0	23	0	0	1	0	66** (22.0)	66 (22.0)	0	0	0 (0.0)	0
							12	0	20	0	0	1	0			0	0		

B(a)P, benzo(a)pyrene; CHL/IU, Chinese hamster lung; csb, chromosome break; cse, chromosome exchange; csg, chromosome gap; ctb, chromatid break; cte, chromatid exchange; ctg, chromatid gap; end, endoreduplication; frg, fragmentation; gap-, total number of cells with structural aberrations excluding gap; gap+, total number of cells with structural aberrations including gap; MMC, mitomycin C; NPP, no-pain pharmacopuncture, a four-herb extract consisting of Corydalis tube, Chaenomelis Fructus, Paeoniae Radix, and Glycyrrhizae Radix et Rhizoma; pol, polyploidy; RPD, relative population doubling; Trt-rec time, treatment-recovery time.

[a]Others were excluded from the number of cells with chromosomal aberrations.

Significant difference from the negative control according to Fisher's exact test: **$p < 0.01$.

Relative population doubling (RPD) = (no. population doubling in treated cultures) (no. population doubling in control cultures) × 100.

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

RESULTS

1. RPD measurement results and test substance precipitation and chromosome aberration observation dose

The RPD was found to be higher than 89.9 at doses of 0, 2.5, 5, and 10% in the absence of metabolic inactivation when using the short-term method. It was higher than 87.3 at the same doses with metabolic inactivation, and greater than 92.4 at the same doses in the absence of metabolic inactivation when using the continuous method (Table 4).

Regarding test substance precipitation and chromosomal observations at the target dose, the RPD was 87.3 or higher at all NPP doses in both short-term treatments with and without metabolic inactivation, as well as in continuous treatment without metabolic inactivation. More than 300 cleavage metaphase cells were detected at the maximum dosage in each treatment series. Consequently, three doses (10%, 5%, and 2.5%), including the highest dose, were selected for observing chromosomal abnormalities.

2. Results of chromosome abnormality tests in cultured mammalian cells

As shown in Table 4, there was no significant variance in the occurrence of cells exhibiting chromosomal irregularities between the NPP and saline control groups during short-term treatment with and without metabolic activation, as well as continuous treatment without metabolic activation. Moreover, there was a notable increase in the number of cells displaying structural chromosomal abnormalities in the positive control group treated with mitomycin C compared to the negative control group ($p < 0.01$), confirming successful induction of a positive response.

3. Judgment of test establishment

In this study, cells displaying chromosomal abnormalities in the negative control group treated with saline were found to fall within the established range of historical control data (Table 5) and also within the 95% range of the historical control data. Similarly, the frequency of cells with chromosomal aberrations

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 ± SD)	Range (%)		95% control limit[c] (structural aberration cells/300 cells)	
					MIN	MAX		
Negative	–	6-18	46	0.304 ± 0.343	0	1.01*	0	< 3
	+	6-18	46	0.304 ± 0.384	0	1.09*	0	< 3
	–	24-0	44	0.280 ± 0.387	0	1.002*	0	< 3
Positive	–	6-18[a]	39	21.97 ± 6.264	8.60*	35.34*		
	+	6-18[b]	39	22.51 ± 5.418	11.03*	33.99*		
	–	24-0[a]	37	31.80 ± 9.077	13.26*	50.35*		
Historical control values of numerical aberrations								
Group	S9 mix	Trt-rec time (hr)	N	Numerical aberration cells excluding gap (%) (Mean ± SD)	Range (%)		95% control limit[c] (numerical aberration cells/300 cells)	
					MIN	MAX		
Negative	–	6-18	46	0.203 ± 0.285	0	0.95*	0	< 2
	+	6-18	46	0.145 ± 0.250	0	0.85*	0	< 2
	–	24-0	44	0.227 ± 0.247	0	1.01*	0	< 2

N, total number of chromosome aberration tests; Negative control, water for injection, dimethyl sulfoxide, acetone, etc.; SD, standard deviation; Trt-Rec time, treatment-recovery times.

[a]Mitomycin C (0.1 μg/mL).

[b]Benzo[a]pyrene (20 μg/mL).

[c]Poisson-based 95% control limits of the historical negative control data.

These historical control values were obtained from data pooled from May 6, 2015 to December 17, 2021.

*Range was calculated using the control limit of X derived from $\bar{X}$-$\bar{R}$-$\bar{R}$s value.

in the positive control group also fell within the control range of the historical control data, but a statistically significant increase was observed compared to the saline negative control group. Furthermore, both the control and NPP groups exhibited over 300 cell proliferation phases per dose, with at least three readable phases. The cell proliferation criterion was met in the saline-negative control group, as no positive results were observed with short-term treatment, with or without metabolic inactivation, under all three experimental conditions. In addition, a study was conducted to determine the appropriate dosage of NPP, which validated the criteria used to select the highest concentration and confirmed that the experiment was carried out under suitable conditions.

DISCUSSION

Herbal medicine has been utilized for thousands of years to treat human diseases and is still being used today due to its medicinal properties [15]. However, there is a growing concern regarding the safety of herbal medicines due to various factor such as increased awareness of food and drug safety, improved global living standards, rising environmental pollution, the importation of foreign medicinal materials, the use of plants of uncertain origin, and the indiscriminate use of health foods and traditional remedies containing medicinal herbs [16, 17]. The NPP extract is a combination of four medicinal herbs: CT, CF, PR, and GR, which have been used in traditional Asian medicine for centuries for pain relief, anti-inflammation, and muscle relaxation [12, 13, 18]. Previous studies on the safety of pharmacopuncture solutions containing these herbs have shown that CT pharmacopuncture mixed with PR, and CF, as well as CF pharmacopuncture mixed with Chelidonii herba and Clematidis Radix does not cause liver or kidney damage in experimental arthritis studies [19]. In addition, no toxicity was observed with single intramuscular and intravenous injections of Gamijakyak- gamchobuja-tang pharmacopuncture, which consists of nine medicinal ingredients including PR, GR, and CF [20]. In a safety study of NPP, no toxicity was detected in the single intramuscular administration toxicity test [12] and micronucleus test [13]. Furthermore, no special adverse reactions were indicated in a case report of plantar fasciitis [11]. Nevertheless, further research is needed to confirm the clinical safety of these ingredients.

Genotoxicity tests were used in the primary screening process to assess the potential carcinogenic effects of drugs. Several *in vitro* and *in vivo* genotoxicity tests are available to evaluate genetic mutations, chromosomal abnormalities, and DNA damage or repair potential [21, 22]. Due to the diverse range of toxicity mechanisms, multiple genotoxicity tests, such as battery assays, are needed to accurately evaluate the genotoxicity of test substances. The standard three-assay battery typically includes a bacterial reverse mutation test, an *in vitro* chromosome abnormality test, and an *in vivo* micronucleus test [23], conducted in accordance with guidelines established by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the Organization for Economic Co-operation and Development (OECD) [21, 22].

Chromosomal aberrations are frequently observed genotoxic responses associated with tumor initiation and progression. *In vitro* testing is conducted on cultured mammalian cells to identify factors that cause structural chromosomal aberrations [24]. These tests complement the Ames test [25, 26]. CHL cells are utilized for *in vitro* chromosomal aberration testing because of their ease of scoring and potential for repeatability, given their sensitivity to mutagens and the low number of chromosomes they possess [27]. In this particular study, an herbal acupuncture solution was utilized due to the special characteristics of the liquid sample. When the test substance solution was treated with the medium, the maximum dose was 10%, thus 10.0% NPP was set as the maximum dose. The capacities were set to 5.00, 2.50, 1.25, 0.625, and 0.313% at azeotropic ratios of 2. *In vitro* chromosomal aberration testing indicated no significant difference in the frequency of cells with chromosomal aberrations between the short-term treatment groups with and without metabolic activation and the continuous treatment groups without metabolic activation compared to the saline negative controls at all doses of NPP. These results indicated that NPP did not induce chromosomal abnormalities. It has been confirmed that this study was conducted under appropriate conditions based on the test set-up and result assessment conditions.

This study showed that NPP extract had no genotoxic effects on mammalian cells, suggesting that NPP did not cause mutations or chromosomal damage *in vitro*. However, as only one genotoxicity assay was performed in this study, additional toxicity assays should be performed in the future. Further research is required to assess the safety and efficacy of NPP in humans.

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CONCLUSION

It can be concluded that the test substance NPP did not cause chromosomal abnormalities within the parameters of this study. Additional research is required to establish the safety of NPP.

CONFLICTS OF INTEREST

The author declares no conflicts of interest in this work.

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