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Original article

Single-dose Intravenous Toxicology Testing of Daebohwalryeok Pharmacopuncture in Sprague-Dawley Rats

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Key Words

aqua acupuncture, intravenous injection, pharmacopuncture, single-dose toxicity test

Abstract

Objectives: The aims of the study were to test the single-dose intravenous toxicity of Daebohwalryeok pharmacopuncture (DHRP) in Sprague-Dawley (SD) rats and to estimate the crude lethal dose.

Methods: The experiments were conducted at Biotoxtech Co., a Good Laboratory Practice (GLP) laboratory, according to the GLP regulation and were approved by the Institutional Animal Care and Use Committee of Biotoxtech Co. (Approval no: 110156). The rats were divided into three groups: DHRP was injected into the rats in the two test groups at doses of 10 mL/kg and 20 mL/kg, respectively, and normal saline solution was injected into the rats in the control group. Single doses of DHRP were injected intravenously into 6 week old SD rats (5 male and 5 female rats per group). General symptoms were observed and weights were measured during the 14 day observation period after the injection. After the observation period, necropsies were done. Then, histopathological tests were performed. Weight data were analyzed with a one-way analysis of variance (ANOVA) by using statistical analysis system (SAS, version 9.2).

Results: No deaths and no statistical significant weight changes were observed for either male or female SD rats in either the control or the test groups during the observation period. In addition, no treatment related general symptoms or necropsy abnormalities were observed. Histopathological results showed no DHRP related effects in the 20 mL/kg DHRP group for either male or female rats.

Conclusion: Under the conditions of this study, the results from single-dose intravenous injections of DHRP showed that estimated lethal doses for both male and female rats were above 20 mL/kg.

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1. Introduction

Pharmacopuncture is a new type of acupuncture treatment combining acupuncture based on the meridian theory and herbal medicine based on Qi and flavor theory [1]. Pharmacopuncture is categorized as

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meridian field pharmacopuncture (MFP), eight principle pharmacopuncture (EPP), animal based pharmacopuncture (ABP) and mountain ginseng pharmacopuncture (MGP). Pharmacopuncture is prepared by using various methods, such as alcohol immersion, distillation, low temperature extraction, pressing, and dilution [1].

Unlike EPP and MGP, which are made by using the distillation method, pharmacopuncture for Blood and Qi recuperation (BQRP) is made by using the low temperature extraction method. The low temperature extraction method is a novel method for producing pharmacopuncture: It separates the medicinal herb extract after decocting the herb compounds. This is followed by decompression and low temperature distillation. Therefore, BQRP can maximize the inherent effect of the medicinal herbs that were processed [1, 2]. A kind of BQRP is Chukyu (spine-healing) pharmacopuncture [3], Samgihwalryeok pharmacopuncture (SGHRP) [4], Biyeon pharmacopuncture, Cheonghyeol pharmacopuncture, Eunbisan pharmacopuncture, and so on [2].

Daebohwalryeok pharmacopuncture (DHRP) is a kind of BQRP that boosts energy, and it is used for general weakness and fatigue [2]. The indications of DHRP are similar to those of MGP and SGHRP [4-6]. SGHRP has been mainly applied in clinics by using intramuscular or subcutaneous methods. DHRP has a composition similar to that of SGHRP. DHRP, with the same dosage methods and development purposes of MGP, was developed for intravenous administration to increase the effect of SGHRP. Even though toxicity is critical for intravenous injections, studies on the intravenous injection of DHRP have no yet proven its safety whereas studies on the intravenous single-dose toxicity of MGP and on the intramuscular single-dose toxicity of SGHRP have reported those pharmacopunctures to be safe under those injection conditions [4, 6]. Thus, intravenous injection toxicity tests of DHRP are necessary prior to its application. The aims of the study were to test the single-dose intravenous toxicity of DHRP in Sprague-Dawley (SD) rats and to the estimate crude lethal dose.

2. Materials and Methods

For the preparation of DHRP, 400 g of DHRP were extracted with 90% ethyl alcohol (EtOH) for 72 hours. The DHRP extract was obtained after the separation of extracting EtOH, which was followed by a 40°C heat treatment to evaporate the volatile EtOH. Medicinal plant powder was mixed with water for injection (WFI) at an appropriate ratio in a low temperature low pressure extractor (Fine FA, Korea). After the extravasation, the pharmacopuncture was extracted at 0.1 Torr. The extract was balanced at a pH of 7.25 — 7.35 and a salinity of 0.9%. The extract was passed through a N_2 Gas filter (0.1 ㎛, Sartorius), was transferred to the filling tank/reservoir, and was fired in the vial for high pressure sterilization.

SD rats (Orientbio Inc., Korea) were used in this study as they are widely used for toxicity tests [3-4, 6]. Basic visual examinations were done for incoming animals, which were followed by weight measurements with an electronic scale (CP3202S, Sartorius, Germany). The general conditions were observed daily during the 7 day stabilization/conditioning period. After the general conditions had been observed, the animals were moved to an animal room/chamber for quarantine. The body weights, general basic conditions, and weight changes were measured on the last day of stabilization to confirm/ensure the health statuses of the SD rats. At the time of intravenous injection, the weight range of the 6 week old male rats was 178.4 — 195.8 g (n = 15) and that of the 6 week old female rats was 148.5 — 169.5 g (n = 15). Breeding conditions were as follows: a temperature of 19.0 — 23.2°C, a relative humidity of 33.0% — 59.5%, a ventilation rate of 10 — 15 times/hour, an illumination time of 12 hours/day (7:00 am — 7:00 pm), and an intensity of illumination of 150 — 300 Lux.

Telkad Certified Irradiated Global 18% Protein Rodent Diet 2918C (Harlan Laboratories, Inc., U.S.A.) was used to feed the animal Ad-libitum. This study was approved by the Institutional Animal Care and Use Committee of Biotoxtech Co. (Approval no: 110156). The tests were conducted according to the Good Laboratory Practice (GLP) regulation and the toxicity test guidelines of the Korea Ministry of Food and Drug Safety (MFDS). On the last day of stabilization (the grouping day), all animals whose weights were equal to the mean weight were randomly grouped into 3 groups, with 5 male rats and 5 female rats for each group. The groupings were as follows (Table 1).

DHRP were injected intravenously according to the schedule used in clinical settings. Dosages for the control and the high dose groups were 20 mL/kg each, and that for the low dose group was 10 mL/kg. For all groups, half of each dose was injected at a 2 hours interval. The initial dose for each animal was calculated based on the weight at the time of the injection. A 26G needle (3 mL) was used for all animals, and the injection speed was approximately 2 mL/minutes. The expected clinical dosage for DHRP was approximately 20 mL/human/day, which is approximately 0.33 mL/kg for an adult of 60 kg. In a pilot test (Biotoxtech Study no.: B10928P) no deaths were observed for single-dose intravenous injections of 20 mL/kg and 10 mL/kg in male and female rats. As a result, dosages for this study were set at 20 mL/kg, maximum available dose, for the high dose group, which is approximately 60 times the expected clinical dosage, and 10 mL/kg for low dose group. Normal saline (Choongwae Pharma Corp., Korea) was injected into the rats in the control group.

At the day of injection (day 0), general conditions (types of toxicity, onset time, recovery time) and deaths were observed 30 minutes, 1 hour, and 2 hours after the first injection, and at 30 minutes, 1, 2, 4, and 6 hours after the second

Table 1 Grouping of animals

Group	Dosage (mL/kg)	Number of animals	
		Male	Female
G1: Control group (Saline)	20	5	5
G2: Low-dose group (DHRP)	10	5	5
G3: High-dose group (DHRP)	20	5	5

DHRP, Daebohwalryeok pharmacopuncture.

injection. General conditions were noted daily for 14 day after injection. Body weights were measured on the day of the injection (before injection) and on the third, seventh, and fourteenth (necropsy day) days after injection. After the observation period, necropsy with CO_2 gas anesthesia, followed by abdominal aorta bloodletting, was conducted for all animals. Organs and tissues, including the brain, heart, liver, spleen, kidney, lung, and spinal nerve, were extracted from sacrificed animals and fixed with neutral buffered formalin solution. After the organs and tissues had been fixed, sections were produced with dehydrated paraffin. Histotomies were dyed with hematoxylin & eosin (H&E) solution. Residual organ, tissue and fixed organ, tissue were preserved with 10% neutral buffered formalin solution. Microscopic examinations were performed for all sections made for histotomy in both the control group and the high dose group.

Data were analyzed with statistical analysis system (SAS, version 9.2, SAS Institute Inc., U.S.A). A homoscedasticity check was done with Bartlett test (significance level of 0.05). When homoscedasticity was satisfied, a one-way analysis of variance (ANOVA) was done, followed by a Dunnett's *t*-test for a post-hoc analysis (significance levels of 0.05 and 0.01 were used for the two sided test).

3. Results

No deaths were observed for both male and female rats in the control and the test groups during the observation period (Table 2). In addition, no abnormal conditions were observed for both male and female rats in the control and the test groups during the observation period (Table 3). Moreover, no statistically significant weight changes were observed for both male and female SD rats in the test groups, compared with the SD rats in the control group, during the observation period (Figs. 1, 2).

As a result of necropsy, no macroscopic abnormalities were observed in the control and the test groups during the observation period (Table 4). No DHRP related effects were observed in the 20 mL/kg DHRP group for both male and female rats. Other findings that were observed in the kidneys and the livers in the control and the high dose groups, which were spontaneous and adventitious ones, were lesions commonly observed in age relevant SD rats and had no toxicological significance (Fig. 3, Table 5).

4. Discussion

The composition of DHRP is equal to that of SGHRP, which consists of *Panax ginseng radix, Cornu cervi parvum* (CC), *Angelicae gigantis radix* (AGR), *Ophiopogonis radix* (OR, *Liriope platyphylla*), and *Schisandrae fructus* (SF) [2, 4], but the dose rate of each herb medicine is different. The major organized herbal medicines of DHRP are as follows:

Panax ginseng radix is the dried root of *Panax ginseng* C. A. Mey. (family Aralicaceae). It has been used to tonify Qi, the spleen, and the lung, to tranquilize, to stimulate the secretion of body fluids to quench thirst, and to prevent shock and prostration. It has been demonstrated to excite and inhibit the central nervous system (CNS), to have cardiotonic, anti-fatigue, anti-shock, anti-aging, and anti-cancer effects, to strengthen anti-stress and immune functions, to stimulate hematogenesis, and to improve learning and memory [7-15].

CC is the hairy, young horn of a male deer or stag, *Cervus nippon* Temminck or *Cervus elaphus* L. (family Cervidae). It has been used to reinforce the vital function of the kidney's yang, to strengthen bones and muscles, and to treat the loss of spontaneous seminal emission and strength,

Table 2 Summary of mortalities

Sex	Group / Dose (mL/kg)	No. of animals	Days after dosing															Mortality (dead/total)
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Male	G1/ Saline (20)	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0% (0/5)
	G2/ DHRP (10)	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0% (0/5)
	G3/ DHRP (20)	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0% (0/5)
Female	G1/ Saline (20)	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0% (0/5)
	G2/ DHRP (10)	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0% (0/5)
	G3/ DHRP (20)	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0% (0/5)

DHRP, Daebohwalryeok pharmacopuncture.

Table 3 Summary of clinical signs

Sex	Group / Dose (mL/kg)	No. of animals	Clinical sign	Hours (Day 0) after dosing							
				1st dosing			2nd dosing				
				0.5	1	2	0.5	1	2	4	6
Male	G1/ Saline (20)	5	NOA	5	5	5	5	5	5	5	5
	G2/ DHRP (10)	5	NOA	5	5	5	5	5	5	5	5
	G3/ DHRP (20)	5	NOA	5	5	5	5	5	5	5	5
Female	G1/ Saline (20)	5	NOA	5	5	5	5	5	5	5	5
	G2/ DHRP (10)	5	NOA	5	5	5	5	5	5	5	5
	G3/ DHRP (20)	5	NOA	5	5	5	5	5	5	5	5

Sex	Group / Dose (mg/kg)	No. of animals	Clinical signs	Days after dosing														
				0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Male	G1/ Saline (20)	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	G2/ DHRP (10)	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	G3/ DHRP (20)	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Female	G1/ Saline (20)	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	G2/ DHRP (10)	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	G3/ DHRP (20)	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

DHRP, Daebohwalryeok pharmacopuncture; NOA, no observable abnormality.

impotence, and leukorrhea due to deficiencies of vital functions caused by chronic diseases [7, 8]. Also, CC has been reported to stimulate the synthesis of proteins and nucleic acids, hematogenesis, and the immune and sexual functions, to strengthen learning and memory ability, and to protect against fatigue and aging [7, 8, 16, 17].

AGR is the root of *Angelica gigas* NAKAI (family Umbelliferae) and has been known as the best medicine in gynecology as it nourishes the blood. It has been used to nourish the blood and to invigorate the blood circulation for the treatment of menstrual disorders and as an emollient and laxative for chronic constipation of the aged and debilitated. It tonifies an aversion to cold, a fever, and deficiencies induced by consumptive disease [7, 8, 18]. It has been known for its anti-thrombotic, anti-anemic, vasodilation, and hypotensive functions. Furthermore, AGR has been shown to have possible effects on hematogenesis stimulation, immune function improvement, and excitement or inhibition of bronchial and uterine smooth muscles [8, 19-24].

OR is the root of *Ophiopogon japonicas* Ker.-Gawl or *Liriope platphylla* WANG et TANG (family Liliaceae). It is used

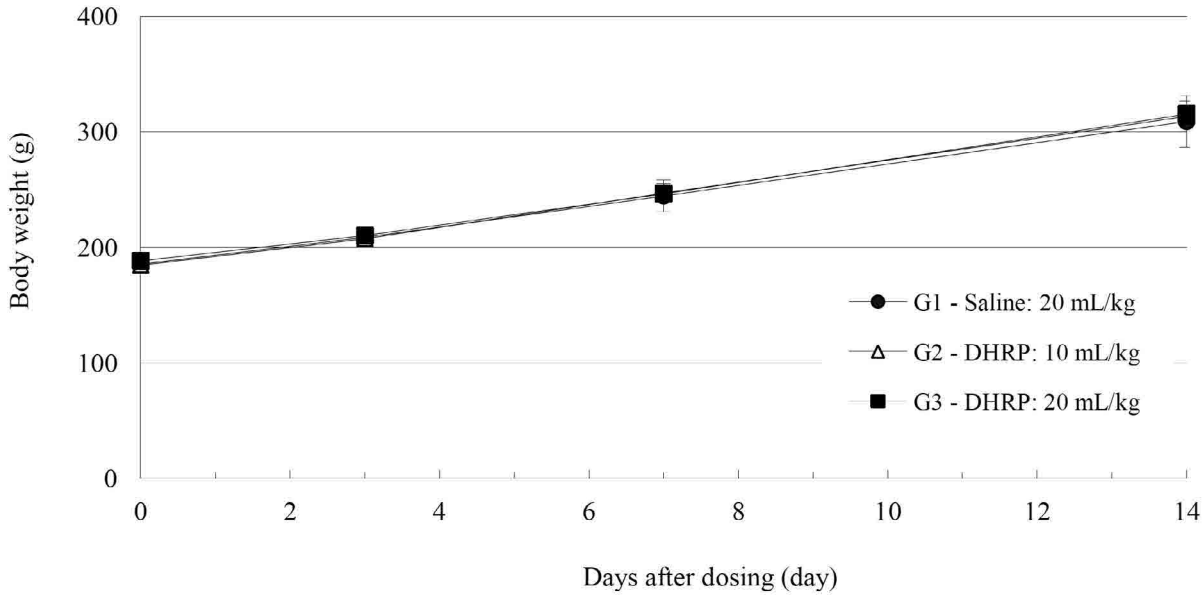

Figure 1 Body weights in male Sprague-Dawley rats. DHRP, Daebohwalryeok pharmacopuncture.

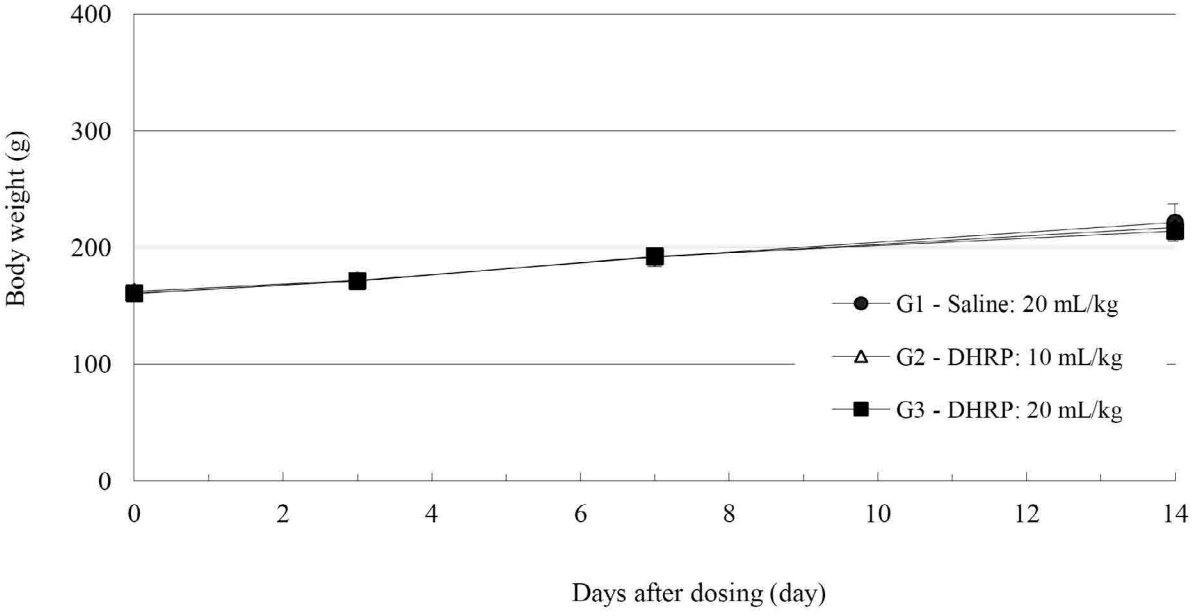

Figure 2 Body weights in female Sprague-Dawley rats. DHRP, Daebohwalryeok pharmacopuncture.

to replenish the vital essence, to promote the secretion of body fluids, to stop coughing by soothing the lung for the treatment of dipsosis, dry throat, dry cough and bloody sputum, and to nourish the heart for the treatment of palpitation and fearfulness [7, 8]. OR has been suggested to have a possible effect when used to treat atopic dermatitis, diabetes mellitus, and inflammation in autoimmune diabetes mellitus. It also has an immunomodulatory effect to protect against lung or liver injury and can be used to improve the lung's capacity, to improve the production and the secretion of respiratory mucus, to reduce apoptosis, and to improve neuro protection of neuron cells at the hippocampus [25-34].

SF is the dried fruit of *Schizandra chinensis* (Turez) Baillon (family Magnolicaceae) and causes contractions, arrests discharges, boosts Qi, engenderd fluid, tonifies the kidneys, and tranquilizes the heart [7, 8]. It has been used as an astringent for the treatment of dry cough, asthma, night sweating, liver hepatitis, seminal emission and chronic diarrhea; it has also been used as a tonic for neu-

rasthenia [7]. SF has been proposed to have hypotensive, anti-oxidative, anti-inflammation, and hypoglycemic effects, to suppress CNS, to protect liver function, and to inhibit the growth of prostate cancer [8, 35-40].

On the basis of the effect of the above herbs, DHRP can greatly tonify both the Qi and the blood system of the body. DHRP has an effect similar to that of SGHRP because DHRP has the same composition as the SGHRP herbs that tonify Qi, blood, fluid and humor. Thereby, we postulate that not only does DHRP produce healthy circulation that results in enhanced yang Qi, but also it can applied to treat diverse consumptive or intractable diseases due to Qi deficiency, blood deficiency, and Qi and blood deficiency, such as lassitude, gastric atony, chronic fatigue, stroke and cancer.

As MGP was developed for intravenous injection to enhance the effect of tonifying Qi and blood [6], DHRP was developed for intravenous administration to improve the effect of SGHRP [4]. Not only has MGP been widely used intravenously in clinics in Korea, but also the safety of MGP has been proven by using intravenous single-dose toxicity test [6].

On the basis of our single-dose toxicity study, we conclude that intravenous injection of DHRP can be safe because all SD rats showed tolerance to doses over 20 mL/kg. We secured the basic safety evidence that DHRP can be applied intravenously to patients with deficiency. However, further safety studies, such as 4-week recovery tests, 13-week, repeated intravenous dose toxicity tests and so on, and further efficacy studies will be needed in order to provide more conclusive results. In addition, we think DHRP can be injected into subcutaneous tissue or muscle to treat

Table 4 Summary of necropsy findings

Sex	Male			Female		
Group	G1	G2	G3	G1	G2	G3
Dose (mL/kg)	Saline	DHRP		Saline	DHRP	
	20	10	20	20	10	20
No. of animals	5	5	5	5	5	5
Unremarkable findings	5	5	5	5	5	5
No. of examined	5	5	5	5	5	5

External surfaces and all organs in the body cavities were unremarkable.
DHRP, Daebohwalryeok pharmacopuncture.

Table 5 Summary of histopathological findings

	Sex		Male		Female	
	Group		G1	G3	G1	G3
Organ / Findings	Dose (mL/kg)		Saline	DHRP	Saline	DHRP
			20	20	20	20
	No. of animals		5	5	5	5
Kidney	Basophilic tubules	±	0	0	0	1
	Mineralization, outer medulla	±	0	0	1	1
	No. of examined		5	5	5	5
Liver	Microgranuloma	±	0	1	1	0
	Vacuolation, hepatocyte, periportal	±	0	0	1	1
	No. of examined		5	5	5	5

There were unremarkable changes in the brain, heart, lung, spleen and spinal nerves of Groups 1 and 3.
DHRP, Daebohwalryeok pharmacopuncture; Grade- ±, minimal.

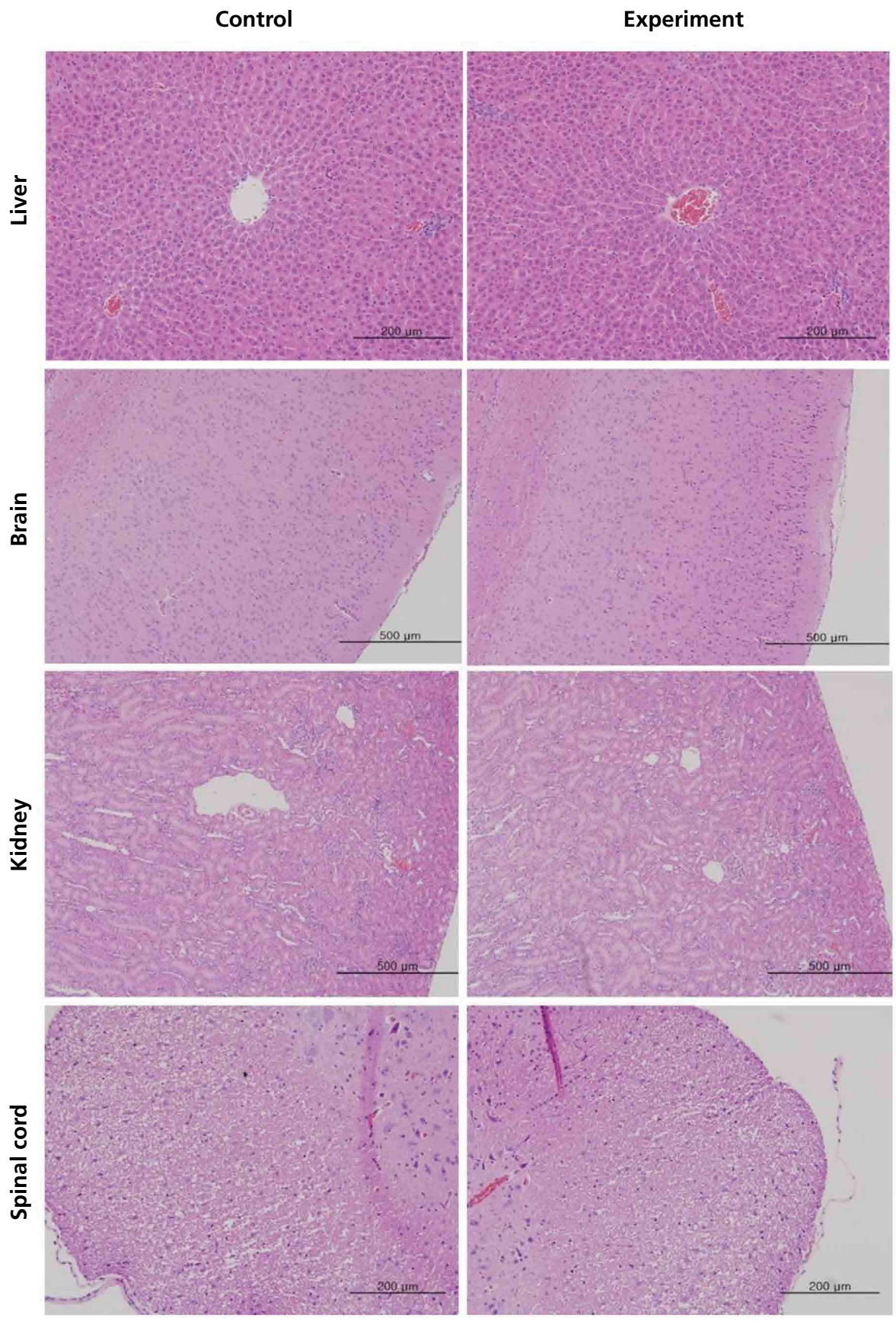

Figure 3 Histopathological observations (hematoxylin & eosin staining × 200).

diseases, just as SGHRP can [4], because DHRP consists of SGHRP and has the same applications in clinics. Thus, safety and efficacy studies for different dose methods will be needed.

5. Conclusions

Under the conditions of this study, single-dose intravenous injection of DHRP is safe because lethal doses were estimated to be over 20 mL/kg for both male and female rats.

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Conflict of interest

The authors declare that there are no conflict of interest.

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