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Repeated-Dose Toxicity Testing of Scolopendrid Pharmacopuncture in Sprague-Dawley Rats

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ABSTRACT

Background: The aim of this pilot study was to assess the safety and dosing of scolopendrid pharmacopuncture (SPP).
Methods: A total of 40 healthy Sprague-Dawley rats (males and 20 females 20) were selected following a 7-day inspection and acclimation period. SPP was administered via intramuscular injection, over a 2-week period using 3 doses including a high-dose [0.84 mg of scolopendrid per kg of body weight (BW)], a med-dose (0.42 mg/kg BW), and a low-dose (0.21 mg/kg BW). The control group was injected with sterile water into the muscles. Unusual changes caused by administration of the test substance were observed. Weight, feed intake, organ weight, and hematological examinations were compared among the groups. Using the SPSS statistical program, Levene's test was performed to evaluate the homogeneity of variances, and a one-way ANOVA test was subsequently performed to assess the significance between each test group.
Results: During the experiment no animals died. Weight change, food consumption, organ weight, hematological test, and blood biochemical tests showed no significant differences in the treatment groups compared to controls.
Conclusion: No toxicological changes related to the administration of test substances were observed. Therefore, the LD_{50} (lethal-dose that kills 50%) of scolopendrid pharmacoupuncture in rats was greater than 0.84 mg/kg.

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Introduction

Scolopendrid pharmacopuncture (SPP) is pharmacopuncture using an extract from dried centipedes (Scolopendra subspinipes mutilans) [1]. The centipede contains alanine, glycine, glutamic acid, and 16 amino acids, including proline, arginine, asparagine, and serine but it also contains substances such as histamine and hemolytic proteins which may be toxic. Therefore, the centipede's head, legs, and tail are removed due to their toxicity, before extracting scolopendrid [2,3].

The Scolopendra subspinipes mutilans has traditionally been used for back pain because its shape resembles the human spinal cord and nerve distribution. Scolopendrid was first mentioned in Sinnongbonchogyeong, and was used to treat infantile convulsion, erysipelas, kerion, scrofulosis, and "biting injuries" according to the text in Compendium of Material Medica [4]. Moreover, according to the Encyclopaedia of Chinese Medicine, scolopendrid has been used to treat vertigo, epilepsy, pediatric epilepsy, and

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facial palsy [5].

Pharmacopuncture, also known as herbal acupuncture, is a new acupuncture treatment combining acupuncture and the injection of herbal medicine to acupuncture points (acupoints). In pharmacopuncture treatment, the typical constitution, and condition of the individual patient are diagnosed, and specific amounts of herbal extracts are injected into meridians and acupoints, providing the effect of both acupuncture and herbal medicine. Furthermore, pharmacopuncture treatment can be combined with acupuncture where the patient has difficulty in taking oral medication. SPP has great potential for reducing pain, act as a sedative, anti-inflammatory or antipyretic, suggestive that it can be effective in musculoskeletal disease [6].

SPP is a complex therapy using the physical stimulation effects of acupuncture and the biochemical pharmacological effects of scolopendrid, by using injecting the extract processed from centipedes into disease-related areas and acupoints. It has been used especially for the treatment of joint pain diseases and nerve entrapment syndrome [7,8].

Currently, SPP is widely used in the clinical field and there has been some pharmacological studies of scolopendrid [9,10]. However, toxicity studies of SPP relating to its processing are still lacking. Son et al [11] conducted a single-dose toxicity test, but there has not been any published research where repeated-dose toxicity test has been used. This study was performed to assess repeated-dose toxicity and determine the approximate lethal-dose of SPP in rats. The aim of this study was to analyze the repeated-dose toxicity of SPP for 2 weeks in SD rats. In this study, all the experiments were conducted at the Korea Testing and Research Institute (KTR), an institution authorized to perform non-clinical studies, under the Good Laboratory Practice.

Materials and Methods

Scolopendrid for pharamacopuncture was produced by ethanol extraction in an extraction hood, in a laboratory at Wonkwang University Gwangju Medical Center (KTR code: TS-01695). Scolopendra subspinipes mutilans (CK corporation Boeun) was agitated for 3 hours at room temperature in a purified water/solvent mixture. Extraction was performed twice at 95°C for 3 hours, filtered, and concentrated at 38°C by reducing the pressure. Ethanol (90%) was added to the concentrate and agitated 3 times. The primary solvent was extracted by removing the fibers by filtration. The ethanol content was adjusted to 70% with purified water and the secondary solvent was extracted by agitating for 1 hour. Following dilution and sedimentation for 24 hours, floating material was removed using a 0.45μm filter and microorganisms were removed with 0.2 μm filter. To ensure stability of the product it was lyophilized then diluted with sterile water to a concentration of 3.0 mg/mL and stored at 2.0-8.0°C. Sterile water was used as a control in the repeated-dose intramuscular toxicity test.

The animals used in this study were 6-week-old Sprague-Dawley (SD) rats (typically used for toxicity testing allowing comparison between studies). The weights of the rats were 183.85 -204.16 g for males, and 152.71-172.10 g for females, at the time of injection. The temperature of the lab was 21.6-22.8°C, and the humidity was 56.9-61.6%. Sufficient food [Rodent Diet 20 5053 (Labdiet, USA)] and reverse osmosis water was provided freely. Following 7 days of acclimation all animals were visually inspected, monitoring of weight change, and general health condition. Healthy animals were randomly sampled so that the average weight and standard deviation between each group was uniform. In total, 20 male and 20 female rats were selected and distributed into 4 groups (5 mice

Table 1. Hematological Examination and Coagulation Test.

Items	Units	Analyzer
White blood cell count	10^3 cell/μL	ADVIA 2120i (Siemens, Germany)
Differential leucocyte count Neutrophils Lymphocytes Monocytes Eosinophils Basophils	10^3 cell/μL, %	
Red blood cell count	10^6 cell/μL	
Hemoglobin	g/dL	
Hematocrit	%	
Red blood cell indices Mean corpuscular volume Mean corpuscular hemoglobin Mean corpuscular hemoglobin concentration	 fL pg g/dL	
Reticulocyte	10^9 cell/L, %	
Platelet	10^3 cell/μL	
Prothrombin time	sec	ACL 7000 (Instrumentation Laboratory, USA)
Activated partial thromboplastin time	sec	

per group). Based on the clinical-dose of scolopendrid (1 mg/60 kg BW in humans), 0.84 mg/kg BW was selected in rats as the high-dose (G4, more than 50 times the exposure limit), 0.42 mg/kg BW was the mid-dose (G3), and 0.21 mg/kg BW was the low-dose (G2). The exposure limit capacity of 50 times was set by reference to guidelines for non-clinical drugs published by the Korea Food and Drug Administration. In the control group (G1), the same volume of sterile water as the experimental groups was injected into the rats. All animals were injected once a day for 2 weeks, into the leg muscle using a 1 mL syringe. This study was conducted with the approval of the Animal Ethics Committee in KTR (IAC2020-0240). Throughout the testing period, general symptoms were examined once a day. Weight was measured once a week at the time of introduction, grouping, and administration. The feed intake was measured once a week using the average intake (g/rat/day), calculated by dividing the difference between the day's supply and the next day's remaining amount. All animals were bled at the end of the experiment, they were euthanized with isoflurane, and a visual examination of the external surface and all orifices was conducted, (the cranial cavity, thoracic and abdominal cavities, and contents). At the necropsy, the wet weights of the following organs were recorded for all animals, and the relative organ weight ratio to fasting body weight was calculated. Of the following organs, the weight of organs with left and right counterparts (denoted *) was calculated by summing the left and right separately: the brain, pituitary gland, heart, lung, liver, spleen, kidneys*, adrenal glands*, testes*, epididymides*, ovaries*, uterus, thymus, prostate gland, seminal vesicle, thyroid gland, and submandibular gland.

Hematological examinations were conducted on the blood collected. There was 2 mL of blood collected in a CBC tube (EDTA 2K, BD vacutainer) processed by a blood analyzer (ADVIA 2120i, Siemens, Germany), 2 mL of blood collected in a vacutainer which was refrigerated, centrifuged, and the plasma processed to determine coagulation time using a blood coagulation analyzer (ACL 7000, Instrumentation Laboratory, Germany; Table 1). In addition, the remaining blood was coagulated at room temperature and centrifuged (3,000 rpm, 10 minutes) to collect the serum to determine blood biochemistry using a biochemical analyzer (TBA-120FR, TOSHIBA, Japan; Table 2).

Statistical analysis was performed using the SPSS statistical program (V. 19.0) for data on body weight, feed intake, hematological testing, blood biochemical testing, and organ weight. Levene's test was performed to evaluate the homogeneity of variances, and a one-way ANOVA test was subsequently performed to assess the significance between each test group. All statistical significance levels were verified at the 0.05 level. As a result of the one-way ANOVA test, significant tests were confirmed between the test groups. The post-hoc test (Scheffe multiple test for homogenous variances, Dunnett's T3 for heterogeneous variances) was conducted according to the homogeneity of variances.

Table 2. Blood Biochemical Test.

Items	Units
Total protein (TP)	g/dL
Albumin (ALB)	g/dL
A/G ratio (A/G)	-
Total bilirubin (T-BIL)	mg/dL
Total bile acid (TBA)	μmol/L
Alkaline phosphatase (ALP)	U/L
Aspartate aminotransferase (AST)	U/L
Alanine aminotransferase (ALT)	U/L
Gamma glutamyl-transferase (GGT)	IU/L
Creatinine (CREA)	mg/dL
Blood urea nitrogen (BUN)	mg/dL
Total Cholesterol (T-CHO)	mg/dL
Triglycerides (TG)	mg/dL
Glucose (GLU)	mg/dL
Calcium (CA)	mg/dL
Inorganic phosphorus (IP)	mg/dL
Creatine kinase (CK)	U/L
Cholinesterase (CHE)	U/L
Sodium (Na+)	mmol/L
Potassium (K+)	mmol/L
Chloride (Cl-)	mmol/L

Results

In this study, no deaths or abnormal symptoms occurred in any of the groups (Table 3) during the study. In 4 groups of male and female rats, the body weight was measured 3 times in 2 weeks, with no significant difference in weight change amongst each group. Food consumption was measured once a week, for 2 weeks by averaging daily intake and there was no significant difference amongst the groups (Table 4).

The animals were sacrificed and the necropsy was measured using the absolute weight and relative weight of organs including the brain, liver, lungs, and kidneys where no significant difference in organ weight amongst each group was observed (Table 5). The complete blood cell count and blood biochemical tests were performed including liver function test and serum electrolyte test was performed. There were no significant differences in the test indicators of each group (Tables 6 and 7).

The necropsy results showed no evidence of an effect from the test material. Histopathological examinations were not performed because none of the rats died or were thought to have developed toxicological changes during the study.

Discussion

Much experimental research and clinical trials using SPP have been conducted. Koh et al [12] reported that there was a significant decrease in the level of conscious pain in a clinical study where 10 patients diagnosed with lumbar disc herniation experiencing lower back pain, radiating lower extremity pain, or lower extremity weakness, were treated daily with SPP for 7 days. Lim et al [13] reported that in a group of 40 patients with carpal tunnel syndrome, acupuncture combined with SPP was more effective in treating clinical symptoms such as paresthesia, resting pain, and night pain compared with the control group where only

Table 3. Clinical Signs and Mortality.

Group (male)	Dose (mg/kg BW)	No. of animals	Mortality (dead/total)	Clinical signs	Day (s)													
					1		2		3		4		5		6		7	
					BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT
G1	0	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (male)	Dose (mg/kg BW)	No. of animals	Mortality (dead/total)	Clinical signs	Day (s)													
					8		9		10		11		12		13		14	
					BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT
G1	0	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (female)	Dose (mg/kg BW)	No. of animals	Mortality (dead/total)	Clinical signs	Day (s)													
					1		2		3		4		5		6		7	
					BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT
G1	0	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (female)	Dose (mg/kg BW)	No. of animals	Mortality (dead/total)	Clinical signs	Day (s)													
					8		9		10		11		12		13		14	
					BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT	BEF	AFT
G1	0	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	5	0% (0/5)	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5

BEF, before dosing; AFT, after dosing; BW, body weight.

Table 4. Body Weight and Food Consumption.

Group (male)	Dose (mg/kg BW)		Body weight (g) on week(s)			Food consumption (g) on week(s)	
			0*	1	2	1	2
G1	0	Mean	193.5	259.3	317.6	49.5	50.5
		SD	6.2	18.8	27.6	19.1	19.8
		n	15	15	15	3	3
G2	0.21	Mean	195.1	256.1	309.6	47.7	49.3
		SD	6.3	9.8	14.0	16.3	16.7
		n	10	10	10	3	3
G3	0.42	Mean	195.5	260.0	308.0	52.8	41.1
		SD	5.2	12.9	28.1	18.1	33.1
		n	10	10	10	3	3
G4	0.84	Mean	194.9	255.2	307.8	49.1	52.4
		SD	7.3	14.6	23.0	21.4	22.5
		n	15	15	15	3	3
Group (female)	Dose (mg/kg BW)		Body weight (g) on week(s)			Food consumption (g) on week(s)	
			0*	1	2	1	2
G1	0	Mean	160.6	188.6	215.1	39.8	41.1
		SD	5.2	8.8	8.7	10.0	13.8
		n	15	15	15	3	3
G2	0.21	Mean	160.5	184.5	215.5	39.4	39.7
		SD	5.5	13.0	14.7	10.9	11.6
		n	10	10	10	3	3
G3	0.42	Mean	163.9	194.1	215.1	36.1	40.0
		SD	6.6	7.5	7.4	13.5	16.3
		n	10	10	10	3	3
G4	0.84	Mean	160.8	195.7	221.8	33.3	41.7
		SD	5.3	8.5	11.5	10.4	9.6
		n	15	15	15	3	3

* body weight of group assignment.
Not significantly different from control group ($p > 0.05$). We performed Levene's test and one-way ANOVA test using the SPSS statistical program.
BW, body weight.

acupuncture treatment was performed. Park et al [14] reported subjective and objective pain levels of 2 patients with lateral epicondylitis where pain had decreased after an injection of SPP into the tender points.

Histamine and hemolytic proteins contained in Scolopendra subspinipes mutilans are toxic, which can affect the nervous system, cardiovascular system, circulatory system, urinary system, digestive system and hematopoietic system, or hypersensitive shock [15]. Bee venom, also used in pharmacopuncture, has been observed to cause itching, chills, fever, vomiting, diarrhea, fainting, dyspnea, and airway obstruction [16] and the determination dose-response [17] and repeated-dose toxicity is ongoing [18]. Aconitum ciliare Decaisne [19], and Drosera Rotundifolia [20] are more examples of repeated-dose toxicity testing. SPP has only been tested in single-dose toxicity tests to date.

SPP is often administered daily in the clinic due to its effectiveness in treating musculoskeletal pain and nerve entrapment syndrome therefore, in the course of a treatment multiple doses are delivered. In the case of bee venom, despite the presence of a negative skin test, anaphylactic shock is known to develop after further exposure to the antigen. Therefore, it is necessary to conduct repeated-dose toxicity, acute-chronic adverse effects and dose-response relationships in animal studies, prior to the development of protocols for pilot studies and clinical trials.

This study was conducted objectively and safely, and was evaluated using the systemic toxic responses in rats during 2 weeks of repeated administration. The LD50 of the SPP in SD rats was greater than 0.84 mg/kg, indicating that this dose was safe and did not cause histological problems. Animal studies in the future should extend the repeated-dose toxicity over a greater amount of time and with varying doses of scolopendrid. This study provided evidence for the safety of SPP, which is used widely in clinical practice at 1 mg/ 60 kg. There is a shortage of safety data compared to other medicines used in pharmacopuncture to treat various diseases, such as bee venom. The results of this animal study cannot be applied directly to humans but is a necessary safety step in the process of determining the safety of SPP in patients.

Conclusion

There were no abnormal symptoms or deaths during the 2-week repeated administration of SPP. Furthermore, there were no significant differences in body weight, food consumption, organ weight, hematological and blood chemical parameters among the groups. Consequently, the test results showed no toxicological changes related to the administration of the test substances up to 0.84 mg/kg, and no target organ toxicity was observed.

Table 5. Organ Weight.

(A) Absolute weight

Group (male)	Dose (mg/kg BW)		Liver (g)	Kidney (g)			Spleen (g)	Testis (g)			Brain (g)	Epididymis (g)			Heart (g)	Thymus (g)	Lung (g)	Seminal Vesicle (g)	Prostate (g)	SG (g)	Adrenal gland (g)			PG (g)	Thyroid gland (g)		
				Left	Right	Total		Left	Right	Total		Left	Right	Total							Left	Right	Total		Left	Right	Total
G1	0	Mean	9.97	1.49	1.48	2.98	0.77	1.54	1.58	3.12	2.16	0.39	0.42	0.81	1.18	0.62	1.56	1.43	0.51	0.72	0.033	0.030	0.063	0.012	0.015	0.014	0.029
		SD	1.16	0.20	0.23	0.42	0.09	0.19	0.20	0.39	0.11	0.04	0.12	0.15	0.15	0.10	0.21	0.15	0.10	0.09	0.003	0.002	0.004	0.001	0.003	0.003	0.005
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	9.51	1.44	1.45	2.89	0.60	1.51	1.51	3.03	2.08	0.38	0.40	0.78	1.26	0.70	1.56	1.21	0.54	0.68	0.031	0.028	0.059	0.011	0.016	0.017	0.033
		SD	0.76	0.10	0.16	0.25	0.08	0.08	0.09	0.18	0.04	0.05	0.03	0.06	0.02	0.11	0.10	0.06	0.10	0.03	0.006	0.006	0.012	0.000	0.002	0.004	0.004
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	Mean	9.62	1.47	1.50	2.97	0.70	1.52	1.52	3.05	2.08	0.37	0.35	0.72	1.20	0.64	1.44	1.28	0.54	0.69	0.034	0.033	0.066	0.012	0.018	0.016	0.034
		SD	1.12	0.29	0.29	0.58	0.08	0.13	0.11	0.23	0.13	0.04	0.03	0.07	0.22	0.25	0.18	0.10	0.11	0.07	0.006	0.005	0.011	0.002	0.004	0.004	0.007
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	9.15	1.59	1.55	3.13	0.74	1.47	1.50	2.97	2.15	0.38	0.41	0.79	1.24	0.68	1.48	1.19	0.47	0.73	0.036	0.032	0.068	0.011	0.016	0.016	0.032
		SD	0.95	0.32	0.34	0.66	0.20	0.24	0.17	0.41	0.08	0.05	0.05	0.07	0.11	0.22	0.18	0.21	0.16	0.14	0.003	0.005	0.007	0.003	0.005	0.002	0.006
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (female)	Dose (mg/kg BW)		Liver (g)	Kidney (g)			Spleen (g)	Brain (g)	Uterus (g)	Heart (g)	Thymus (g)	Lung (g)	SG (g)	Adrenal gland (g)			Ovary (g)			PG (g)	Thyroid gland (g)		
				Left	Right	Total								Left	Right	Total	Left	Right	Total		Left	Right	Total
G1	0	Mean	6.36	0.94	0.93	1.87	0.44	1.93	0.58	0.80	0.54	1.12	0.45	0.038	0.035	0.073	0.040	0.043	0.083	0.01	0.014	0.010	0.024
		SD	0.52	0.09	0.08	0.17	0.07	0.09	0.24	0.06	0.09	0.10	0.04	0.003	0.004	0.006	0.010	0.008	0.008	0.00	0.005	0.001	0.005
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	6.79	0.96	0.97	1.94	0.47	2.00	0.53	0.82	0.56	1.13	0.47	0.039	0.037	0.076	0.054	0.050	0.104	0.01	0.014	0.013	0.027
		SD	0.79	0.10	0.08	0.18	0.08	0.05	0.06	0.05	0.09	0.07	0.03	0.011	0.006	0.017	0.012	0.010	0.020	0.00	0.003	0.003	0.003
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	Mean	6.53	0.95	0.96	1.91	0.48	1.98	0.70	0.84	0.51	1.18	0.46	0.041	0.037	0.079	0.043	0.042	0.085	0.01	0.011	0.013	0.024
		SD	0.61	0.04	0.05	0.09	0.05	0.07	0.34	0.06	0.07	0.13	0.07	0.006	0.004	0.010	0.010	0.005	0.013	0.00	0.004	0.002	0.006
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	6.85	1.03	1.03	2.06	0.56	1.96	0.85	0.87	0.62	1.18	0.44	0.037	0.038	0.045	0.045	0.042	0.087	0.01	0.012	0.012	0.024
		SD	0.18	0.08	0.05	0.13	0.09	0.10	0.26	0.07	0.09	0.08	0.05	0.004	0.003	0.008	0.008	0.009	0.015	0.00	0.002	0.003	0.004
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Not significantly different from control group ($P > 0.05$). We performed Levene's test and one-way ANOVA test using the SPSS statistical program.
BW, body weight; SG, submandibular gland; PG, pituitary gland.

(B) Relative weight

Group (male)	Dose (mg/kg BW)		Body Weight (g)*	Liver (%)	Kidney (%)			Spleen (%)	Testis (%)			Brain (%)	Epididymis (%)			Heart (%)	Thymus (%)	Lung (%)	Seminal Vesicle (%)	Prostate (%)	SG (%)	Adrenal gland (%)			PG (%)	Thyroid gland (%)		
					Left	Right	Total		Left	Right	Total		Left	Right	Total							Left	Right	Total		Left	Right	Total
G1	0	Mean	292.5	3.40	0.51	0.51	1.01	0.27	0.53	0.54	1.06	0.74	0.13	0.14	0.28	0.40	0.21	0.53	0.49	0.18	0.25	0.011	0.010	0.022	0.004	0.005	0.005	0.010
		SD	26.0	0.16	0.03	0.03	0.06	0.04	0.03	0.04	0.07	0.06	0.01	0.03	0.04	0.04	0.03	0.07	0.03	0.03	0.04	0.001	0.001	0.002	0.000	0.001	0.001	0.001
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	286.5	3.32	0.50	0.51	1.01	0.21	0.53	0.53	1.06	0.73	0.13	0.14	0.27	0.44	0.24	0.54	0.42	0.19	0.24	0.011	0.010	0.021	0.004	0.006	0.006	0.011
		SD	17.3	0.15	0.04	0.06	0.09	0.02	0.02	0.03	0.05	0.04	0.02	0.01	0.02	0.03	0.03	0.02	0.04	0.04	0.02	0.002	0.002	0.003	0.000	0.001	0.002	0.002
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	Mean	287.6	3.34	0.51	0.52	1.03	0.24	0.53	0.53	1.06	0.73	0.13	0.12	0.25	0.41	0.22	0.50	0.45	0.19	0.24	0.012	0.011	0.023	0.004	0.006	0.006	0.012
		SD	21.0	0.20	0.07	0.07	0.13	0.01	0.06	0.06	0.12	0.02	0.01	0.02	0.03	0.05	0.07	0.04	0.03	0.02	0.03	0.002	0.002	0.004	0.000	0.001	0.001	0.003
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	278.7	3.28	0.57	0.55	1.12	0.26	0.53	0.54	1.07	0.77	0.14	0.15	0.28	0.44	0.25	0.53	0.43	0.17	0.26	0.013	0.012	0.025	0.004	0.006	0.006	0.011
		SD	18.3	0.24	0.10	0.11	0.20	0.06	0.08	0.06	0.14	0.04	0.02	0.01	0.02	0.03	0.08	0.04	0.06	0.05	0.05	0.001	0.002	0.003	0.001	0.001	0.001	0.001
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (female)	Dose (mg/kg BW)		Body Weight (g)*	Liver (%)	Kidney (%)			Spleen (%)	Brain (%)	Uterus (%)	Heart (%)	Thymus (%)	Lung (%)	SG (%)	Adrenal gland (%)			Ovary (%)			PG (%)	Thyroid gland (%)		
					Left	Right	Total								Left	Right	Total	Left	Right	Total		Left	Right	Total
G1	0	Mean	193.7	3.28	0.48	0.48	0.96	0.22	1.00	0.30	0.41	0.28	0.58	0.23	0.019	0.018	0.038	0.021	0.022	0.043	0.006	0.007	0.005	0.013
		SD	7.9	0.20	0.03	0.03	0.06	0.03	0.04	0.12	0.02	0.04	0.05	0.01	0.002	0.002	0.004	0.005	0.004	0.003	0.001	0.003	0.001	0.003
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	194.9	3.48	0.49	0.50	0.99	0.24	1.03	0.27	0.42	0.29	0.58	0.24	0.020	0.019	0.039	0.028	0.026	0.053	0.007	0.007	0.007	0.014
		SD	15.0	0.24	0.05	0.02	0.06	0.03	0.07	0.02	0.02	0.05	0.02	0.02	0.006	0.004	0.010	0.006	0.004	0.009	0.001	0.001	0.002	0.002
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	Mean	194.7	3.36	0.49	0.49	0.98	0.25	1.02	0.36	0.43	0.26	0.61	0.24	0.021	0.019	0.040	0.022	0.022	0.044	0.006	0.006	0.006	0.012
		SD	5.2	0.40	0.03	0.04	0.07	0.03	0.05	0.18	0.03	0.04	0.09	0.04	0.003	0.003	0.005	0.005	0.003	0.007	0.001	0.002	0.001	0.003
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	200.9	3.42	0.51	0.52	1.03	0.28	0.98	0.42	0.43	0.31	0.59	0.22	0.019	0.019	0.038	0.038	0.021	0.043	0.006	0.006	0.006	0.012
		SD	11.2	0.20	0.05	0.04	0.09	0.06	0.07	0.13	0.02	0.05	0.02	0.02	0.002	0.002	0.004	0.004	0.004	0.007	0.001	0.001	0.001	0.002
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

* fasting weight.
Not significantly different from control group ($p > 0.05$). We performed Levene's test and one-way ANOVA test using the SPSS statistical program.
BW, body weight; SG, submandibular gland; PG, pituitary gland.

Table 6. Hematological Parameters.

Group (male)	Dose (mg/kg BW)		WBC (103 cells/μL)	RBC (106 cells/μL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (109 cells/L)	Retic (%)	PLT (103 cells/μL)	PT (sec)	APTT (sec)	Differential leucocyte count (103 cells/μL)					Differential leucocyte count (%)				
															Neut	Lymph	Mono	Eos	Baso	Neut	Lymph	Mono	Eos	Baso
G1	0	Mean	7.89	6.77	14.8	42.5	62.7	21.8	34.8	298.7	4.43	1272	15.4	16.7	1.51.	6.13	0.16	0.04	0.01	19.1	77.7	2.1	0.5	0.1
		SD	1.60	0.29	0.6	1.6	1.9	0.5	0.7	21.5	0.47	148	0.7	0.9	0.50	1.26	0.05	0.01	0.00	4.2	4.7	0.6	0.2	0.0
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	6.95	6.79	14.5	42.6	62.8	21.4	34.1	296.9	4.38	1379	15.1	16.1	1.13	5.57	0.14	0.05	0.01	17.0	79.5	2.0	0.8	0.1
		SD	1.23	0.18	0.2	1.1	1.7	0.4	0.8	21.6	0.32	133	0.5	0.2	0.35	1.39	0.04	0.02	0.00	6.2	6.1	0.3	0.2	0.0
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	Mean	6.34	6.84	14.5	42.2	61.7	21.2	34.3	265.3	3.89	1272	15.9	16.4	0.98	5.15	0.14	0.04	0.00	15.3	81.4	2.1	0.6	0.1
		SD	1.23	0.19	0.6	1.2	0.8	0.5	0.5	52.9	0.82	78	0.6	0.9	0.32	0.93	0.04	0.01	0.01	2.9	3.4	0.4	0.2	0.0
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	7.51	6.96	14.7	42.4	60.9	21.1	34.7	291.0	4.21	1229	16.0	16.5	1.41	5.90	0.12	0.03	0.01	18.6	78.6	1.6	0.4	0.1
		SD	1.40	0.36	0.6	1.4	2.0	0.6	0.3	40.9	0.77	65	0.5	1.2	0.47	1.08	0.06	0.01	0.00	3.9	4.6	0.6	0.1	0.1
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (female)	Dose (mg/kg BW)		WBC (103 cells/μL)	RBC (106 cells/μL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (109 cells/L)	Retic (%)	PLT (103 cells/μL)	PT (sec)	APTT (sec)	Differential leucocyte count(103 cells/μL)					Differential leucocyte count (%)				
															Neut	Lymph	Mono	Eos	Baso	Neut	Lymph	Mono	Eos	Baso
G1	0	Mean	3.53	7.16	15.4	43.4	60.6	21.6	35.6	189.3	2.66	1460	15.8	15.8	0.44	2.99	0.06	0.04	0.00	12.6	84.2	1.7	1.2	0.1
		SD	1.05	0.47	0.9	2.2	1.4	0.5	0.5	15.7	0.37	135	1.0	1.2	0.13	0.94	0.02	0.01	0.01	2.8	3.0	0.4	0.3	0.1
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	4.65	6.89	14.5	41.4	60.1	21.2	35.2	203.4	2.96	1290	15.1	16.5	0.77	3.71	0.07	0.06	0.01	17.0	79.5	1.5	1.3	0.1
		SD	0.86	0.20	0.3	0.7	1.4	0.7	0.4	35.8	0.55	95	0.2	0.9	0.26	0.87	0.01	0.02	0.01	5.5	5.7	0.2	0.4	0.1
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.42	Mean	3.10	7.06	15.1	42.9	60.8	21.4	35.2	187.5	2.66	1165	15.8	15.5	0.51	2.48	0.04	0.06	0.00	16.3	80.0	1.4	1.7	0.1
		SD	0.61	0.21	0.4	0.9	1.1	0.3	0.2	33.6	0.52	374	1.7	3.1	0.15	0.43	0.01	0.06	0.00	2.7	3.7	0.3	1.3	0.1
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	5.12	7.00	14.9	42.4	60.7	21.2	35.0	227.4	3.26	1364	15.2	15.9	0.65	4.31	0.07	0.05	0.00	12.0	84.9	1.4	1.1	0.1
		SD	1.69	0.21	0.4	1.1	1.6	0.4	0.3	24.7	0.40	106	0.7	1.2	0.36	1.38	0.02	0.01	0.01	4.5	4.4	0.3	0.2	0.0
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Not significantly different from control group ($p > 0.05$). We performed Levene's test and one-way ANOVA test using the SPSS statistical program.
BW, body weight; WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Table 7. Blood Chemical Parameters.

Group (male)	Dose (mg/kg BW)		TP (g/dL)	ALB (g/dL)	A/G ratio	T-BIL (mg/dL)	ALP (U/L)	AST (U/L)	ALT (U/L)	CREA (mg/dL)	BUN (mg/dL)	T-CHO (mg/dL)	TG (mg/dL)	GLU (mg/dL)	CA (mg/dL)	IP (mg/dL)	GGT (IU/L)	CK (U/L)	TBA (μmol/L)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	CHE (U/L)
G1	0	Mean	5.4	3.6	2.1	0.00	740	97	30	0.37	11.3	62	31	136	9.8	9.1	0.95	471	13.5	143.2	4.72	103.4	123.9
		SD	0.3	0.1	0.2	0.01	75	7	5	0.02	2.0	6	7	8	0.5	0.4	0.42	115	5.3	1.1	0.23	1.4	7.7
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2	0.21	Mean	5.3	3.6	2.1	0.00	897	118	32	0.38	11.9	63	34	130	9.6	9.0	0.41	742	12.3	144.3	4.85	103.8	124.4
		SD	0.1	0.1	0.0	0.00	233	19	6	0.03	0.9	10	15	20	0.1	0.5	0.34	375	3.0	0.8	0.39	1.8	27.5
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3	0.48	Mean	5.3	3.6	2.1	0.01	691	96	31	0.38	11.3	54	34	135	9.8	8.5	0.14	507	11.9	143.1	4.63	103.8	130.7
		SD	0.2	0.1	0.1	0.01	214	17	3	0.01	1.7	10	17	23	0.1	0.7	0.24	232	4.9	1.9	0.25	1.1	20.7
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4	0.84	Mean	5.4	3.7	2.1	0.01	924	105	31	0.38	13.2	57	26	125	9.8	8.5	0.31	468	23.6	142.3	4.82	103.6	129.0
		SD	0.2	0.1	0.1	0.02	146	25	3	0.01	2.6	13	3	17	0.1	0.6	0.67	215	14.8	0.9	0.19	1.2	24.6
		n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Group (female)	Dose (mg/kg BW)		TP (g/dL)	ALB (g/dL)	A/G ratio	T-BIL (mg/dL)	ALP (U/L)	AST (U/L)	ALT (U/L)	CREA (mg/dL)	BUN (mg/dL)	T-CHO (mg/dL)	TG (mg/dL)	GLU (mg/dL)	CA (mg/dL)	IP (mg/dL)	GGT (IU/L)	CK (U/L)	TBA (μmol/L)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	CHE (U/L)
G1	0	Mean	5.7	3.9	2.2	0.00	442	79	20	0.42	14.8	60	14	136	9.8	7.9	0.30	280	18.2	141.9	4.13	104.8	594.4
		SD	0.2	0.1	0.1	0.00	92	10	6	0.06	2.4	13	3	8	0.2	0.9	0.42	127	17.2	1.3	0.20	2.1	151.7
		n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G2	0.21	Mean	5.7	3.9	2.2	0.00	605	84	23	0.43	12.8	49	13	136	9.8	7.5	0.17	294	12.3	141.3	4.06	103.5	733.5
		SD	0.1	0.1	0.0	0.00	126	12	3	0.06	3.8	10	4	10	0.3	0.7	0.26	144	5.3	1.1	0.31	2.3	271.5
		n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	0.48	Mean	5.6	3.8	2.1	0.00	421	86	22	0.40	14.6	63	15	131	9.8	7.7	0.55	282	15.8	141.8	4.17	103.3	649.1
		SD	0.3	0.2	0.1	0.01	61	10	3	0.02	2.4	15	5	16	0.2	0.4	0.96	104	6.8	1.1	0.30	1.5	128.6
		n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	0.84	Mean	5.5	3.7	2.1	0.00	572	80	23	0.38	12.3	54	15	129	9.7	8.0	0.79	219	14.2	141.3	4.24	104.2	611.9
		SD	0.3	0.1	0.2	0.00	91	4	3	0.03	2.6	7	5	13	0.2	0.6	0.38	68	6.6	1.2	0.34	1.2	233.2
		n	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Not significantly different from control group ($p > 0.05$). We performed Levene's test and one-way ANOVA test using the SPSS statistical program.
A/G, albumin/globulin; BW, body weight; TP, Total protein; ALB, albumin; T-BIL, total bilirubin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CREA, creatinine; BUN, blood urea nitogen; T-CHO, total cholesterol; TG, triglycerides ; GLU, glucose; CA, calcium; IP, inorganic phosphorus; GGT, gamma glutamyl-transferase; CK, creatine kinase; TBA, total bile acid; CHE, Cholinesterase.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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