

A 4-week Repeated dose Oral Toxicity Study of Mecasin in Sprague-Dawley Rats to Determine the Appropriate Doses for a 13-week, Repeated Toxicity Test

Eunhye Cha^{1,2}, Jongchul Lee^{1,2}, Seongjin Lee^{1,2}, Manyong Park^{1,2}, Inja Song³, Ilhong Son⁴, Bong-Keun Song⁵, Dongwoung Kim⁵, Jongdeok Lee⁶, Sungchul Kim^{1,2*}

¹ Department of Acupuncture & Moxibustion Medicine, Wonkwang University Gwangju Korean Medical Hospital, Gwangju, Korea

² Nervous & Muscular System Disease Clinical Research Center of Wonkwang University Gwangju Korean Medical Hospital, Gwangju, Korea

³ Department of Nursing, Gwang-Ju Womens University, Gwangju, Korea

⁴ Department of Neurology, Inam Neuroscience Research Center, Sanbon Medical Center, College of Medicine, Wonkwang University, Iksan, Korea

⁵ Department of Internal Medicine, Wonkwang University Gwangju Korean Medical Hospital, Gwangju, Korea

⁶ Department of Radiology, College of Korean Medicine, Wonkwang University, Iksan, Korea

Key Words

four week repeated toxicity test, gami jakyak gamcho buja decoction (mecasin), herbal medicine, sprague-dawley rat

Abstract

Objectives: In this study, we investigated the 4-week repeated-dose oral toxicity of gami-jakyak gamcho buja decoction (Mecasin) to develop safe treatments.

Methods: In order to investigate the 4-week oral toxicity of Mecasin, we administered Mecasin orally to rats. Sprague-Dawley (SD) rats were divided into four groups of five male and five female animals per group: group 1 being the control group and groups 2, 3, and 4 being the experimental groups. Doses of Mecasin of 500, 1,000, and 2,000 mg/kg of body weight were administered to the experimental groups, and a dose of normal saline solution of 10 mL/kg was administered to the control group. We examined the survival rate, weight, clinical signs, and gross findings for four weeks. This study was conducted under the approval of the Institutional Animal Ethics Committee.

Results: No deaths occurred in any of the four groups. No significant changes in weights or food consumption between the control group and the experimental groups were observed. Serum biochemistry revealed that some groups showed significant decrease in inorganic phosphorus (IP) ($P < 0.05$). During necropsy on the rats, one abnormal macroscopic feature, a slight loss of fur, was observed in the mid dosage (1,000 mg/kg) male group. No abnormalities were observed in any other rats. In histopathological findings, the tubular basophilia and cast of the kidney and extramedullary hematopoiesis of the spleen were found. However, those changes were minimal and had occurred naturally or sporadically. No other organ abnormalities were observed.

Conclusion: During this 4-week, repeated, oral toxicity test of Mecasin in SD rats, no toxicity changes due to Mecasin were observed in any of the male or the female rats in the high dosage group. Thus, we suggest that the doses in a 13-week, repeated test should be 0, 500, 1,000, and 2,000 mg/kg respectively.

1. Introduction

Gami-jakyak gamcho buja decoction (Mecasin) was developed for treating amyotrophic lateral sclerosis

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*Corresponding Author

Sungchul Kim, Department of Acupuncture & Moxibustion Medicine, Wonkwang Gwangju Oriental Medical Hospital, 543-8 Juweol 1-dong, Nam-gu, Gwangju 503-310, Korea.
Tel: +82-62-670-6441 Fax: +82-62-670-6767
E-mail: kscndll@hanmail.net

patients with pain, joint contracture and muscular weakness. gami-jakyak gamcho buja decoction, which is the major component of Mecasin, has been used in traditional medicine to relieve pain, muscle spasms and cold syndrome due to blood deficiency [1]. A recent study showed that jakyak gamcho decoction (JGT) and its constituents had a protective effect against tert-butyl hydroperoxide (t-BHP)-induced cytotoxicity in the hippocampal (HT22) cell line [2]. In addition, jkyak gamcho buja decoction, which consists of JGT and *Radix aconiti lateralis preparata*, is believed to be useful for suppressing the progress of osteoarthritis because of its anti-inflammatory effects and its ability to reduce pain with histopathological efficacy [3]. Because of these facts, Mecasin has been studied for treating patients with joint pain, muscle spasms, and arthralgia due to cold. The constituents of Mecasin are *Radix paeoniae ALBA*, *Radix glycyrrhizae*, *Radix aconiti lateralis preparata*, *Radix salviae miltiorrhizae*, *Rhizoma gastrodiae*, *Radix polygalae*, *Curcuma root*, *Fructus chaenomelis*, *Rhizoma atractylodis japonicae*.

The constituents of Mecasin are *Radix paeoniae ALBA*, *Radix glycyrrhizae*, *Radix aconiti lateralis preparata*, *Radix salviae miltiorrhizae*, *Rhizoma gastrodiae*, *Radix polygalae*, *Curcuma root*, *Fructus chaenomelis*, *Rhizoma atractylodis japonicae*, and *Polygala tenuifolia willd.* Several studies have been conducted about concerning the toxicity and the efficacy of these constituents. Also, objective oral toxicity testing of Mecasin, which is a complex combination of herbs, was conducted recently [4]. Nevertheless, 4-week repeated dose oral toxicity testing of Mecasin has not been conducted yet. Thus, we conducted a 4-week repeated oral dose, toxicity test of Mecasin in Sprague-Dawley (SD) rats as part of a safety evaluation of using Mecasin. The current research trend for oral toxicity testing of extracts is to study subacute toxicity through Good Laboratory Practice (GLP) regulations. All the experiments for this research were conducted at the Korea Testing & Research Institute (KTRI), an institution authorized to perform non-clinical studies, under the GLP regulations.

2. Materials and Methods

Mecasin was provided by the Nervous & Muscular System Disease Clinical Research Center of Wonkwang University Gwang-ju Korean Medical Hospital. It was mixed with pure water, without change of acidity, and the concentra-

tions were 50 mg/mL, 100 mg/mL, and 200 mg/mL. The completed extracts were stored at room temperature (15 — 25°C).

The animals used in this study were 6-week-old SD rats. The reason SD rats were chosen is that they have been widely used in safety tests in the field of medicine, so the results can be easily compared with many other databases. The mean weights of the rats were 167.6 — 185.7 g and 146.2 — 159.8 g, respectively, for the male and the female rats at the time of Mecasin administration. For all animals, a visual inspection was conducted; all animals were weighed at the beginning. During 7 days of acclimatization, the general symptoms of the rats were observed once a day. The weights of the rats were recorded on the last day of acclimatization. No abnormalities were found.

The temperature of the lab was 20.1 — 21.5°C, and the humidity was 44.3% — 56.8%. Enough food (Rodent Diet 20 5053) and reverse osmosis water were provided.

Groupings were done after 7 days of acclimatization. Animals were selected if their weights were close to the mean weight. In total, 20 male rats and 20 female rats were selected. The animals were randomly distributed into 4 groups (5 male and 5 female rats per group) as shown in Table 1.

In this study 2,000 mg/kg was set as a high dose, and 1,000 mg/kg and 500 mg/kg were set as mid and low doses, respectively. In the control group, 10 mL/kg body weight (BW) of normal saline solution was administered. Mecasin and normal saline were administered into the mouths of the rats in all groups by using syringes once a day for 4 weeks. This study was conducted under the approval of the Institutional Animal Ethics Committee of KTR Co., Ltd.

Throughout the treatment, the general symptoms (side effects, revealing time, recovery time, etc.) and mortality were examined once a day. The weights were measured immediately before treatment, at grouping, once a week during treatment, and at the time of necropsy. The amounts of food and water were measured once a week before they were supplied to each cage, and the leftover food and water were measured on the next day. The difference was calculated and regarded as the daily food and water consumption.

The animals were fasted for 18 hours prior to necropsy and blood collection. Blood samples were drawn from the abdominal aorta by using a syringe needle under isoflurane anesthesia. Blood samples were collected into tubes containing ethylene diamine tetraacetic acid (EDTA) and were analyzed by using a blood counting analyzer (AD-

Table 1 Groups of animals

Group	Mecasin administration (mg/kg BW)	Number of animals (serial number)	
		Male	Female
G1: Control group	0	5 (1101 — 1105)	5 (2101 — 2105)
G2: Low-dose group	500	5 (1201 — 1205)	5 (2201 — 2205)
G3: Mid-dose group	1,000	5 (1301 — 1305)	5 (2301 — 2305)
G4: High-dose group	2,000	5 (1401 — 1405)	5 (2401 — 2405)

Mecasin, gami-jakyak gamcho buja decoction; BW, body weight.

VIA 120, Siemens, U.S.A.) to determine the red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular cell hemoglobin concentration (MCHC), platelet count (PLT), white blood cell count (WBC), reticulocytes (Reti), prothrombin time (PT), and activated partial thromboplastin time (APTT).

The serum biochemistry analysis was performed using an auto-analyzer (TBA-120FR, TOSHIBA, Japan). Blood samples were centrifuged at 3,000 rpm for 10 minutes. Serum biochemistry parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (T-BIL), alanine aminotransferase (ALT), gamma-glutamyl-transferase (GGT), blood urea nitrogen (BUN), creatinine (Crea), total protein (TP), albumin (Alb), albumin/globulin ratio (A/G ratio), total cholesterol (T-Chol), triglycerides (TGs), glucose

(Glu), calcium (Ca), inorganic phosphorus (IP), creatine kinase (CK), sodium (Na⁺), potassium (K⁺), and chloride (Cl⁻) were examined.

The organs and tissues of all the animals were visually examined. Net weights were measured for the following organs: the brain, heart, liver, spleen, adrenal gland, testes, ovaries, uterus, thymus, lungs, prostate, pituitary gland, and kidneys. A relative weight ratio was calculated based on the fasting weight. The tissues from the following organs were obtained from all the animals and were fixed with 10% neutral buffered formalin solution: the brain, thymus, thyroid and parathyroid, heart, tongue, trachea, esophagus, lungs with bronchus, heart, liver, spleen, kidneys, adrenal gland, pituitary gland, stomach, small and large intestines, urinary bladder, pancreas, skin and mammary glands, salivary gland, seminal vesicle, prostate gland, ovaries, uterus, vagina, sternum, femur, spinal cord, mesenteric lymph node, skeletal muscles, and peripheral nerves. Testis and

Table 2 Body weights in grams

Group & Dose (mg/kg)	Sex	Mean S.D. N	Week (s)				
			0*	1	2	3	4
G1 (0)	Male	Mean	177.6	225.1	280.3	325.7	360.1
		S.D.	6.6	13.1	22.8	32.4	38.1
		N	5	5	5	5	5
	Female	Mean	154.0	176.5	201.4	224.3	234.2
		S.D.	4.1	4.8	8.2	8.4	1
		N	5	5	5	5	5
G2 (500)	Male	Mean	176.8	229.1	288.0	336.2	371.6
		S.D.	5.5	11.7	11.4	13.7	13.7
		N	5	5	5	5	5
	Female	Mean	152.4	170.8	195.6	211.6	227.6
		S.D.	3.5	3.5	10.1	14.6	14.4
		N	5	5	5	5	5
G3 (1,000)	Male	Mean	174.3	225.7	283.8	327.6	363.5
		S.D.	6.2	9.1	8.6	11.3	14.6
		N	5	5	5	5	5
	Female	Mean	151.7	178.2	199.2	219.3	231.7
		S.D.	3.9	6.6	14.5	17.9	17.4
		N	5	5	5	5	5
G4 (2,000)	Male	Mean	176.0	228.7	294.9	345.6	383.1
		S.D.	6.3	7.7	11.8	15.3	15.4
		N	5	5	5	5	5
	Female	Mean	153.6	173.9	197.7	219.7	227.7
		S.D.	4.3	5.7	11.4	15.2	20.3
		N	5	5	5	5	5

*Body weight of group assignment.

S.D., standard deviation; N, number of animals.

Table 3 Necropsy findings of male and female rats

Group	Dose (mg/kg)	Number of animals	Necropsy findings (male)		Necropsy findings (female)	
			External	Internal	External	Internal
G1	0	5	No gross findings (5)*	No gross findings (5)	No gross findings (5)*	No gross findings (5)
G2	500	5	No gross findings (5)	No gross findings (5)	No gross findings (5)	No gross findings (5)
G3	1,000	5	No gross findings (4), Loss of fur (back, slight) (1)	No gross findings (5)	No gross findings (5)	No gross findings (5)
G4	2,000	5	No gross findings (5)	No gross findings (5)	No gross findings (5)	No gross findings (5)

*Number of observed animals.

Table 4 Histopathological findings of rats

Organs	Number of animals	Histopathological findings	Male		Female	
			G1	G4	G1	G4
Liver	5	NAD	5	5	5	5
		NAD	4	5	4	5
Kidney	5	Tubular basophilia-minimal, multifocal	1	0	0	0
		Tubular casts-slight, multifocal	0	0	1	0
Heart	5	NAD	5	5	5	5
Lung (bronchus)	5	NAD	5	5	5	5
		NAD	3	2	5	5
Spleen	5	Extramedullary hematopoiesis-minimal	2	3	0	0

NAD, no abnormalities detected.

epididymis were fixed with Bouin solution. The eyeball was fixed with Davidson solution. Especially, liver, spleen, kidney, heart, and lung tissues were routinely processed, embedded in paraffin, and sectioned. The sections were stained with hematoxylin & eosin (H&E) dye for microscopic examination. All organs and tissues taken from all animals were examined microscopically.

Data on the weights, food consumption, hematology, serum biochemistry, and organ weights were tested using the SPSS program (SPSS 19.0). A Levene test was conducted to evaluate the homogeneity of the variance and the significance. The one-way analysis of variance (ANOVA) test was conducted when the homogeneity of the variance was recognized.

3. Results

No mortality was observed in any of the groups of SD rat during the 4-week observation period. No clinical signs occurred in the male and the female SD rats. However, one animal in the mid dosage (1,000 mg/kg) male group experienced slight loss of fur, but this symptom was assumed to be the result of an accidental change. No significant differ-

ences in weights were observed between the experimental groups and the control group during the 4 weeks (Table 2).

No significant differences in hematology and organ weights were observed between the experimental groups and the control group. Serum biochemistry revealed that the low (500 mg/kg) and the high (2,000 mg/kg) dosage male groups showed significant decreases in IP ($P < 0.05$). During necropsy on the rats, one animal in the mid dosage (1,000 mg/kg) male group showed an abnormal macroscopic feature, slight loss of (Table 3). However, this symptom was assumed to be the result of an accidental change. No abnormalities were observed in any other rats.

In the histopathological findings, a tubular basophilia of the kidney was found in one male rat in the control group, and a tubular cast of the kidney was found in one female rat in the control group. In addition, the two male rats in the control group showed extramedullary hematopoiesis of the spleen. In high dosage male group, an extramedullary hematopoiesis of the spleen was observed. However, those changes were minimal and had occurred naturally or sporadically. No other changes were observed in the livers, lungs, hearts of the control and the experimental groups (Table 4).

4. Discussion

Mecasin has been utilized in clinics to relieve pain, joint contracture and muscular weakness, especially for patients with amyotrophic lateral sclerosis [4]. A recent study showed that JGT and its constituents had a protective effect against t-BHP-induced cytotoxicity in the hippocampal HT22 cell line [2]. In addition, Jakyak Gamcho buja decoction, which consists of JGT and *Radix aconiti lateralis preparata*, is believed to be useful for suppressing the progress of osteoarthritis because of its anti-inflammatory effects and its ability to reduce pain with histopathological efficacy [3]. Because of these facts, Mecasin has been studied for treating patients with joint pain, muscle spasms, and arthralgia due to cold [1]. Several studies have been conducted to investigate the toxicities and efficacies of the constituents of Mecasin. Also, an objective oral toxicity test of Mecasin, which is complex combination of herbs, was conducted recently [4]. Nevertheless, 4-week repeated oral dose, toxicity testing of Mecasin has not been conducted yet. Thus, we conducted a 4-week, repeated oral dose, toxicity test of Mecasin in SD rats as part of a safety evaluation of using Mecasin.

Radix paeoniae ALBA is a herbal medicine that has been used for treating gastrointestinal disorders for hundreds of years [5]. *Radix paeoniae ALBA* extract has an anti-oxidant effect through Forkhead box O (FOXO) activation and thereby activations of FOXO target genes, Mn superoxide dismutase (MnSOD) and catalase [6]. *Radix glycyrrhizae* has been commonly used in the Orient for treating a variety of diseases and should be beneficial in the inhibition of allergic inflammatory response [7]. Park *et al* concluded that *Radix glycyrrhizae* had the potential to attenuate the behavioral and neurochemical impairments caused by stress [8].

Radix aconiti lateralis preparata has been widely used for the improvement of symptoms such as heart failure, inflammation, pain, and diarrhea for thousands of years in the Orient. However, the toxicity of *Radix aconiti lateralis preparata* has been the subject of controversy [9]. Some kinds of diester diterpenoid-type aconitum alkaloids, such as aconitine and mesaconitine, are major toxic ingredients. Some processing techniques such as pressure-steaming are known to be able to reduce the toxicity of aconitum alkaloids. Such methods use the facts that alkaloids can hydrolyze highly toxic diester-diterpene aconitum alkaloids to compounds of much lower toxicity such as aconine, benzoyleaconine and benzoylmesaconine [10]. Kim *et al* concluded that *Aconitum kusnezoffii reichb* pharmacopuncture was a relatively safe treatment [11]. *Radix aconiti lateralis preparata* aqua-acupuncture has analgesic and anti-inflammatory effects on the arthritis induced by Freund's complete adjuvant in rats. Jeong and Park measured the levels of plasma serotonin, WBC, RBC, haemoglobin (Hb), erythrocyte sedimentation rate (ESR) after injection of *Aconiti lateralis preparata* radix aqua-acupuncture. In that experiment, the levels of plasma serotonin, WBC, RBC, Hb, and ESR decreased, as did paw edema [12].

Radix salviae miltiorrhizae has been widely used for treating heart disease. It has been found to play a protective role against the blood-brain barrier impairment in the

intracranial hemorrhage (ICH) through suppression of inflammation in rat brain tissues [13]. Lee and Kim showed that *Radix salviae miltiorrhizae* reduced neuronal apoptosis [14].

Rhizoma gastrodiae extracts have depressor effect on blood pressure [15] and positive effects on memory improvement frontal lobe activation [16].

Radix polygalae has anti-inflammatory and gastroprotective effects [17]. Also, it inhibits pre-adipocyte differentiation and adipogenesis by blocking sterol regulatory element-binding protein-1c (SREBP-1c) gene expression in 3-day transfer, inoculum 3×10^5 cells (3T3-L1) cells. Therefore, *Polygala tenuifolia* can be used as an effective anti-obesity agent [18]. *Radix polygalae* extract can protect against N-methyl D-aspartate (NMDA) neurotoxicity and induced brain-derived neurotrophic factor expression, suggesting modulatory roles at glutamatergic synapses and possible anti-depressant action [19]. *Curcuma longa* extracts can have anti-cancer activities [20] and also have higher radical scavenging activities and anti-inflammatory effect [21]. *Chaenomeles fructus* suppress the exasperated intestinal motility by carbachol [22] and has an anti-inflammatory effect on controlling the over inflammatory reaction by RAW 264.7 cells [23].

Although Mecasin has been used in clinics, safety studies on Mecasin are insufficient. Toxicity tests provide important data and are essential for evaluating the safety of test substances in medications [24]. The current research trend for oral toxicity testing of extracts is to study subacute toxicity through GLP regulations. All the experiments for this research were conducted at the KTRI, an institution authorized to perform non-clinical studies, under the GLP regulations. Animal testing is the most fundamental and basic way to perform safety assessments [25]. The Korea Food & Drug Administration has testing protocol guidelines for the study of toxicity, and all experiments should be conducted following GLP regulations [26]. The present study was designed to evaluate a 4-week, repeated, oral dose, toxicity test of Mecasin in SD rats. Our study showed no significant changes in the weights, hematology, serum biochemistry, organ weights, necropsy findings, and histopathology of the rats. Also, no mortalities were observed. Although some changes were observed in several organs, those changes seemed to have occurred naturally or sporadically. During this 4-week, repeated, oral dose, toxicity test of Mecasin in SD rats, no toxicity changes due to Mecasin were observed in any of the male or female rats in the high dosage group. Thus, we suggest that the doses in a 13-week, repeated oral dose, toxicity test should be 0, 500, 1,000, and 2,000 mg/kg, respectively.

5. Conclusion

The results showed that administration of 500 — 2,000 mg/kg BW of Mecasin to SD male and female rats did not cause any changes in weight or in the results of necropsy examinations. It also did not result in any mortalities, which indicated that the lethal dose of Mecasin was higher than 2,000 mg/kg. During this 4-week, repeated, oral toxicity test of Mecasin in SD rats, no toxicity changes due to

Mecasin were observed in any of the male or the female rats in the high dosage group. Thus, we suggest that the doses in a 13-week, repeated test should be 0, 500, 1,000, and 2,000 mg/kg, respectively.

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Conflict of interest

The authors declare that there are no conflict of interest.

ORCID

Sungchul Kim. <http://orcid.org/0000-0003-3580-5290>.

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