

Original Article

Keishibukuryogan is not carcinogenic in Sprague-Dawley rats

Masanao Kanitani^{1*}, Nobuo Nishimura², Hiroshi Edamoto², and Yoshio Kase¹

¹Tsumura Research Laboratories, Kampo Scientific Strategies Division, 3586 Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki 300-1192, Japan

²Gotemba Laboratory, Bozo Research Center Inc., 1284 Kamado, Gotemba-shi, Shizuoka 412-0039, Japan

Abstract: Keishibukuryogan is a traditional Japanese medicine widely administered to patients with menopausal symptoms. Because humans use it on a long-term basis, we believed that a carcinogenicity study was warranted. We orally administered keishibukuryogan (TJ-25) extract powder to 6-week-old Sprague-Dawley rats [CrI:CD(SD)], which were divided into four dosage groups-0 (water for injection), 100, 500 and 2,500 mg/kg/day for 24 months. We found that TJ-25 did not affect the survival rate of either sex. Furthermore, it did not affect the clinical condition of the rats, number of superficial tumors found by palpation, body weight, food consumption, hematology, or gross pathological findings. The severity of degeneration of muscle fiber in the femoral skeletal muscle increased slightly in males and females in the 2,500 mg/kg/day group, but TJ-25 did not increase the number of tumors found on histopathological examination. In our study, oral administration of TJ-25 extract powder in rats for 24 months was not associated with an increased incidence of tumors. (DOI: 10.1293/tox.2015-0017; J Toxicol Pathol 2016; 29: 103–110)

Key words: TJ-25, keishibukuryogan, oral administration, carcinogenicity, rat

Introduction

Keishibukuryogan is a pharmaceutical-grade traditional Japanese medicine known as “Kampo medicine” in Japan. Keishibukuryogan consists of five crude drugs: Cinnamon cortex, Radix paeoniae, Cortex Moutan, Poria, and Semen persicae. It is routinely used in modern medicine to treat uterine and adnexal inflammation, endometritis, menoxenia, dysmenorrhea, leukorrhea, menopausal disorders (headache, dizziness, hot flash, stiff shoulder), sensitivity to cold, peritonitis, contusion, hemorrhoids disease and orchitis. Many pharmacologic studies have reported that it has hormonal effects^{1,2}, changes the heteromorphic properties of red blood cells^{3,4} and acts as an anti-inflammatory agent⁵.

In a previous study on rats in which the toxicity of a single oral dose of keishibukuryogan (TJ-25) extract powder was examined, researchers estimated the lethal dose to be greater than 10,000 mg/kg⁶. In the same study, repeated doses of TJ-25 over 13 weeks (with a 4-week recovery period between doses) did not cause any deaths in rats of either

sex, and the maximum tolerated dose of TJ-25 was estimated to be 500 mg/kg/day.

In another study conducted in rats⁷, TJ-25’s potential for reproductive toxicity was assessed. TJ-25 did not affect the reproductive ability of parents, and no external, visceral or skeletal malformations/variability in fetuses were found. Genotoxicity tests, including gene mutation tests using *Salmonella typhimurium* and Chinese hamster lung (CHL/IU) cells, chromosomal aberration tests using CHL/IU cells and in vivo micronucleus tests using bone marrow cells of mice orally treated with TJ-25⁸, have not revealed any mutagenic potential for TJ-25. Because of this, TJ-25 is not expected to have carcinogenic effects. However, TJ-25 is used on a long-term basis in humans. Considering its hormonal effects, a carcinogenicity study was warranted.

Our study is the first carcinogenicity study of a Kampo medication conducted in compliance with carcinogenicity study guidelines. We orally administered TJ-25 extract powder to rats for 24 months. Drug concentrations in plasma were determined to evaluate systemic exposure, and we observed the rats for the presence or absence of carcinogenic activity.

Materials and Methods

This study was conducted by Bozo Research Center Inc. in compliance with good laboratory practice (GLP)⁹ standards and in accordance with the guidelines for toxicity studies¹⁰ and animal welfare.

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*Corresponding author: M Kanitani

(e-mail: kanitani_masanao@mail.tsumura.co.jp)

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Test and control materials

Keishibukuryogan (TJ-25) extract powder, composed of equal parts Cinnamomi cortex, Paeoniae radix, Moutan Cortex, Poria and Persicae semen, was obtained from Tsunmura Co. Ltd. (Tokyo, Japan) in the form of a dried powder extract. The test article was stable for approximately 38 months, which included the animal experimental period. Water for injection (D.W., Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was used as the vehicle and negative control. Test suspensions were prepared at the time of use and not stored. For each dosage, the test suspension was analyzed for concentration and homogeneity of paeoniflorin, a representative ingredient. We found the nominal value for concentration and coefficient of variation to be within acceptable ranges.

Animals and animal husbandry

Male and female Sprague-Dawley rats [294 each; CrI:CD(SD), Atsugi Breeding Center, Charles River Laboratories Japan, Inc.] were obtained at 4 weeks of age and quarantined/acclimated at the test facility (14 days for males and 15 days for females). At 6 weeks of age, rats that showed regular body weight gain without clinical abnormalities were selected and used in the study (255 males and 255 females).

Animals were housed individually in bracket-type, metallic wire-mesh cages. Rooms were maintained at 20 to 26°C, with a relative humidity of 35 to 84%, air ventilation set at 12 to 17 air changes per hour and 12 hours of illumination per day. Animals were allowed free access to a pellet diet (radiation-sterilized CR-LPF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water (Gotemba City water, via an automatic water supply system). All animals were treated humanely according to institutional guidelines, and the experimental procedure was approved by our institutional ethics committee.

Group assignment and dose levels

The rats were divided into two groups: the main group for toxicological evaluation (220 males and 220 females) and the satellite group for toxicokinetic (TK) analysis (35 males and 35 females). Both the main group and satellite group were subdivided into four dosage groups: 100, 500 and 2,500 mg/kg/day of TJ-25 and a control group. Each dosage group within the main group consisted of 55 males and 55 females. Within the satellite group (TK analysis), there were 10 animals of each sex per TJ-25 dosage group and 5 animals of each sex in the control group.

In accordance with the toxicity study guidelines, the route of administration was oral (the expected clinical route of administration), and the administration period was 24 months. The dose volume was set at 10 mL/kg body weight. Animals in the control group received the vehicle (water for injection) in the same manner.

Clinical observations and pathological examinations

All animals were observed for clinical signs such as external appearance, nutritional condition, posture, behavior and abnormality of feces. They were palpated for masses once a week. All animals were weighed before dosing on the measurement days. Body weight gain was calculated. Food consumption was measured for all animals before dosing on the day of measurement. Animals that were moribund or for which euthanasia was judged to be appropriate from the standpoint of animal welfare were euthanized and quickly subjected to pathological examination after blood samples were collected for hematological examinations. Animals that died were subjected to pathological examinations as soon as they were found dead.

For survivors, necropsy was performed the day following the end of the administration period. All animals (unfasted) were subjected to laparotomy under ether anesthesia. Blood samples were collected via the abdominal aorta into blood collection tubes containing EDTA-2K (SB-41, Sysmex Corporation, Kobe, Japan) and examined. Red blood cell count, white blood cell count and platelet count were counted electronically using a Coulter Counter T890 (Beckman Coulter K.K., Tokyo, Japan). After blood sampling, animals were sacrificed by exsanguination via the abdominal aorta and observed for any abnormality in external appearance. All organs and tissues, including those in the cephalic, thoracic, and abdominal cavities, were carefully examined and recorded.

Systemic organs of all animals were examined. The eyeballs were fixed in phosphate buffered 3% (w/v) glutaraldehyde/2.5% (v/v) formalin and then preserved in phosphate buffered 10% (v/v) formalin. Other organs/tissues were fixed in phosphate buffered 10% (v/v) formalin. All organs/tissues were embedded in paraffin and sectioned, after which specimens stained with hematoxylin/eosin (H&E) were prepared and examined microscopically. Since TJ-25-related changes were observed in femoral skeletal muscles, representative examples of the femoral skeletal muscles were photographed. After completion of the histopathological examination, a histopathological peer review was performed by an outside pathologist.

Analysis for plasma concentration of test article

Blood samples were taken from the satellite group and processed for plasma. For each blood draw, approximately 0.3 mL of blood was collected via the cervical vein under light ether anesthesia. Samples were drawn just before dosing and 0.5, 1, 2, 4, 8 and 24 hours after dosing (just prior to the next dose) on day 1 of administration and at weeks 26 and 52 (day 176 and 358). For the control group, blood was collected only at 0.5 hours after dosing. The concentration of paeoniflorin, the main ingredient of Paeoniae radix or Moutan Cortex, was determined using liquid chromatography-tandem mass spectrometry (LC/MS/MS; LC, 2690 Separations Module, Nihon Waters K.K., Tokyo, Japan; MS/MS, API 3000, Applied Biosystems/MDS SCIEX, Tokyo, Japan). For each dose group, the maximum drug concentra-

tion (C_{\max}) and the time to reach the maximum drug concentration (T_{\max}) were calculated from the means of measured values for each time point. The area under the concentration vs. time curve (AUC_{0-24h}) was calculated by the trapezoid method using the means of the measured values.

Statistical analysis

The survival function was estimated for each dose level by the Kaplan-Meier method (product limit estimator)¹¹ and graphed (Kaplan-Meier curve). The Tarone test¹² was performed to evaluate survival functions for dose-related positive tendencies, and then paired comparisons between the control group and each dose group were performed with the log-rank test¹³ (level of significance: 0.05, unilateral). Body weight, food consumption and hematological examination data were analyzed using one-way analysis of variance and Bartlett's test for homogeneity of variance^{14, 15}. The plasma drug concentration (mean \pm standard deviation) was calculated for each point of measurement.

The tumors generated were divided into two categories: common tumors (incidence of greater than 1% in the background data) and rare tumors (incidence of 1% or less in the background data). For evaluation of positive dose-related trends, survival was adjusted. Analysis for all groups was performed using a method by Peto *et al.*¹⁶, and the level of significance was set at 0.005 (unilateral) for general tumors and 0.025 (unilateral) for rare tumors. For all tumors, paired comparison tests were run between the control group and each dose group by Fisher's exact test. The level of significance was set at 0.01 (unilateral) for general tumors and 0.05 (unilateral) for rare tumors. For the tumors with a 5% or greater incidence in any of the groups including the control group, paired comparison tests between the control group and each dose group were performed using the method described by Peto *et al.*¹⁶. The level of significance was set at 0.01 (unilateral) for general tumors and 0.05 (unilateral) for rare tumors.

The histological findings of skeletal muscle degeneration were analyzed by a Wilcoxon rank sum test. Statistical significance was set at the two-sided 5% level of probability level. The analysis was performed with the integrated statistical package SAS Release 9.1.3 (SAS Institute Inc.).

Results

Survival, general condition, body weight and food consumption

The number of animals that died and survival rate at the end of the administration period are summarized and shown in Table 1. There were no significant differences in survival rate between the TJ-25-treated groups of either sex and their respective controls.

The causes of deaths (including moribund sacrifices), which were estimated from clinical observation and histopathological examination, are summarized in Table 2. Of the 104 males that died or were sacrificed moribund, the number of deaths/moribund sacrifices caused by tumor was 74, and thus 71% of the male animals died from tumors. The percentage of deaths by tumor for males in the 0, 100, 500 and 2,500 mg/kg/day groups were 78%, 66%, 74% and 68%, respectively. Of the 142 females that died or were sacrificed moribund, the number of deaths/moribund sacrifices caused by tumors was 138, and thus 97% of the female animals died of tumors. The percentages of deaths by tumor for females in the 0, 100, 500 and 2,500 mg/kg/day groups were 97%, 97%, 100% and 95%, respectively. Animals died or were sacrificed moribund for non-tumor lesions, but no specific lesions appeared to be related to the administration of TJ-25 in either sex. Furthermore, TJ-25 was not associated with an increased number of tumor-related deaths, nor was it associated with an increased incidence of death caused any specific tumors in either sex.

For males, significantly low values for food consumption were sporadically observed in the 2,500 mg/kg/day group from week 92 of administration onward, but this reduction was not believed to be TJ-25-related since it was not continuous. For females, food consumption in the 100 and 500 mg/kg/day groups was comparable to that of the control group. Females in the 2,500 mg/kg/day group sporadically experienced significantly low values of food consumption compared with that of the control group from week 17 to week 68 of administration, but they were judged not to be TJ-25 related since there were no significant differences thereafter. Regardless of food consumption, the body weights of males and females in each dose group were comparable to those of the control group throughout the study period (Fig. 1).

Table 1. Summary of the Mortality and Survival Rates at the End of the Administration Period

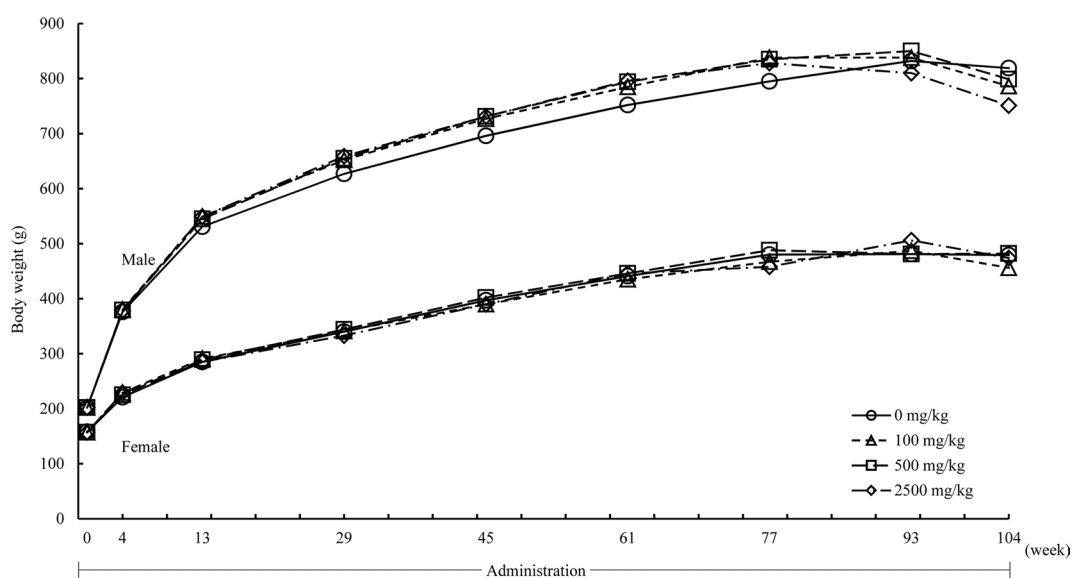
Sex	Male				Female			
	0	100	500	2500	0	100	500	2500
Dose level (mg/kg/day)								
Number of animals used	55	55	55	55	55	55	55	55
Week 26 ^a	1	0	0	1	0	1	0	0
Week 52 ^a	2	3	2	1	0	1	1	4
Week 78 ^a	5	12	6	2	13	12	14	14
Week 104 ^a	23	29	27	25	35	34	33	40
Number of survivors at necropsy	32	26	28	30	20	21	22	15
Survival rate (%)	58.2	47.3	50.9	54.5	36.4	38.2	40.0	27.3

^aCumulative number of deaths/moribund sacrifices.

Table 2. Estimated Causes of Deaths (Including More Abundant Sacrifices)

Sex	Males				Females			
	0	100	500	2500	0	100	500	2500
Dose level (mg/kg/day)								
Number of animals died	23	29	27	25	35	34	33	40
Neoplastic lesions	18	19	20	17	34	33	33	38
Adrenal cortex tumor	0	0	0	0	1	0	0	0
Adrenal medulla tumor	0	0	1	0	0	0	0	0
Body cavity tumor	0	1	0	0	0	1	0	0
Histiocytic tumor	0	0	2	0	0	0	1	0
Intestinal tract tumor	0	1	0	0	0	0	0	0
Large granular lymphocytic leukemia	0	0	0	0	0	0	0	1
Granular leukemia	1	0	0	0	0	0	0	0
Mammary gland tumor	0	0	0	0	1	2	5	7
Malignant lymphoma	1	1	0	0	0	0	0	0
Mesothelioma	0	0	0	0	0	0	0	1
Nerve tumor	1	0	0	2	0	0	0	0
Ovary tumor	–	–	–	–	0	1	0	0
Pituitary tumor	13	14	13	14	30	28	25	23
Kidney tumor	0	0	0	1	0	0	0	0
Skin tumor	0	1	1	0	0	0	1	1
Subcutaneous tumor	2	1	2	0	2	1	0	2
Thymus tumor	0	0	1	0	0	0	0	2
Uterus tumor	–	–	–	–	0	0	1	1
Nonneoplastic lesions	1	1	2	1	0	1	0	0
Anorexia	0	0	0	1	0	0	0	0
Cardiovascular lesions	0	0	1	0	0	0	0	0
Thoracic cavity lesions	0	0	0	0	0	1	0	0
Foot lesions	1	0	0	0	0	0	0	0
Gastrointestinal lesions	0	0	1	0	0	0	0	0
Kidney lesions	0	1	0	0	0	0	0	0
Incidental deaths	0	0	0	0	1	0	0	0
Unknown	4	9	5	7	0	0	0	2

–: Not applicable

**Fig. 1.** The mean body weights of the male and female rats in each TJ-25 dose group over the two-year period*Clinical conditions, palpable masses and hematology*

Clinical conditions that were observed at relatively high incidences (observed in at least 5 males or females in a group) are summarized in Table 3. There were no TJ-

25-related clinical manifestations in any survivors, nor were there TJ-25-related clinical manifestations in animals that died or were sacrificed moribund. No clear differences in the incidence of palpable masses between the males and fe-

Table 3. Main Clinical Signs and Incidence of Occurrence

Sex	Males				Females			
	0	100	500	2500	0	100	500	2500
Dose level (mg/kg/day)								
Number of animals	55 (23)	55 (29)	55 (27)	55 (25)	55 (35)	55 (34)	55 (33)	55 (40)
Decrease,spontaneous movement	9 (9)	14 (13)	11 (9)	9 (8)	21 (19)	14 (13)	24 (24)	24 (23)
Prone/lateral position	8 (8)	6 (6)	6 (6)	8 (8)	18 (18)	11 (11)	9 (9)	7 (7)
Torticollis	1 (1)	0 (0)	0 (0)	1 (1)	9 (7)	6 (4)	10 (9)	9 (9)
Bradypnea	12 (12)	14 (14)	11 (11)	13 (12)	29 (29)	24 (22)	30 (30)	27 (27)
Hypothermia	3 (3)	8 (8)	4 (4)	5 (5)	6 (6)	6 (6)	8 (8)	14 (14)
Unkempt fur	3 (3)	4 (3)	3 (3)	2 (2)	9 (9)	4 (4)	12 (12)	6 (5)
Pale skin	0 (0)	0 (0)	0 (0)	0 (0)	2 (2)	3 (3)	6 (6)	10 (9)
Emaciation	2 (2)	2 (2)	3 (3)	2 (2)	5 (5)	6 (3)	8 (8)	5 (5)
Opacity, eyeball	2 (0)	3 (2)	2 (2)	5 (1)	0 (0)	1 (0)	2 (0)	7 (5)
Swelling, hind limb	6 (2)	8 (3)	5 (3)	4 (0)	1 (1)	0 (0)	2 (0)	0 (0)

The numbers in parentheses indicate the numbers of deaths/moribund sacrifices.

Table 4. Numbers of Tumors and Tumor-bearing Animals

Sex	Males				Females			
	0	100	500	2,500	0	100	500	2,500
Dose level (mg/kg/day)								
Number of animals	55	55	55	55	55	55	55	55
Incidence of benign tumors	86	77	70	102	91	97	113	108
Incidence of malignant tumors	14	14	20	16	50	33	44	63
Total incidence of tumors	100	91	90	118	141	130	157	171
Number of benign tumor-bearing animals	43	39	36	47	47	46	46	45
Number of malignant tumor-bearing animals	13	13	17	14	27	26	27	36
Number of multiple tumor-bearing animals	28	25	25	34	34	37	35	40
Number of tumor-bearing animals	48	41	43	49	54	54	54	54

males in the control group and the males and females of any dose group were observed.

There were no significant differences in hematological findings between the control group and any dose group in males. In females, the erythrocyte count was significantly lower in the 2,500 mg/kg/day group than that of the control group. Otherwise, there were no significant differences between the control group and any dose group.

Pathology

The macroscopic findings that were observed at relatively high incidence (defined as five or more males or females in any dose group) are summarized in Table 3. The macroscopic findings observed in the male dose groups showed no clear differences from those of the male control group. For females in the 2,500 mg/kg/day group, macroscopic findings for pale skin, opacity and enlargement of the spleen were slightly higher than those of the control group. Otherwise, there were no clear differences between the control group and any dose group. For instance, when results for the females in the control group were compared with those of the females in the 100, 500 and 2,500 mg/kg/day TJ-25 groups, pale skin was observed in 1/55, 5/55, 3/55 and 8/55 rats; ocular opacity was observed in 0/55, 0/55, 1/55 and 7/55 rats; and enlargement of the spleen was observed in 5/55, 7/55, 5/55 and 13/55 rats, respectively.

The number of tumors and the number of tumor-bearing animals are shown in Table 4. The incidence of benign

tumors, malignant tumors and total incidence of tumors did not differ between the TJ-25 groups and control group. The number of benign tumor-bearing animals, malignant tumor-bearing animals, multiple tumor-bearing animals and total number of tumor-bearing animals in each TJ-25 dose group were also comparable to those of the control group for both sexes. There were no TJ-25-related effects, and earlier onset of tumors was not noted.

Neoplastic lesions that were observed at relatively high frequency (benign and malignant tumors of the same tissue origin that were observed in 5 males or 5 females or more in a group) are shown in Table 5. In males, there were no TJ-25-related increases in the incidence of tumors in any dose group. There were no tumors that showed a tendency toward increase compared with the control group. In females from the 2,500 mg/kg/day group, a significantly high value (Peto's trend test) was observed for bronchioloalveolar epithelium adenoma. However, it was judged to be spontaneous for the following reasons: only two rats in the 2,500 mg/kg/day group were affected; it was also observed in males in the control group, and it is a tumor observed in untreated animals.

Histopathological changes in femoral smooth skeletal muscle fibers are listed in Tables 6. Minimal to moderate degeneration was observed in all groups. The numbers of animals in the control group and 100, 500 and 2,500 mg/kg/day groups that showed mild or moderate changes were as follows: 21, 25, 26 and 33 males and 9, 10, 7 and 24 females,

Table 5. Main Neoplastic Lesions

Sex	Male				Female			
	0	100	500	2500	0	100	500	2500
Dose level (mg/kg/day)								
Number of animals	55	55	55	55	55	55	55	55
Adrenal								
Pheochromocytoma	9	8	12	7	3	0	2	1
Mixed type pheochromocytoma	0	0	0	1	0	0	0	0
Malignant pheochromocytoma	1	2	2	2	2	0	0	0
Liver								
Hepatocellular adenoma	2	6	5	3	1	1	6	2
Hepatocellular carcinoma	0	0	0	2	0	0	0	0
Lung								
Bronchioloalveolar adenoma	1	0	0	1	0	0	0	2 ^s
Mammary gland								
Adenoma	1	0	0	0	7	2	4	4
Fibroadenoma	0	2	0	0	19	20	19	25
Adenocarcinoma	0	0	0	1	18	13	16	24
Mixed tumor, malignant	0	0	0	0	0	2	1	1
Pancreas								
Acinar cell adenoma	4	5	0	1	0	0	2	0
Islet cell adenoma	18	12	11	24	3	6	4	1
Adenoma/acinar-islet cell	1	0	0	0	0	1	0	0
Acinar cell carcinoma	0	1	0	0	0	0	0	0
Islet cell carcinoma	4	2	2	1	1	0	0	0
Pituitary								
Anterior lobe adenoma	29	24	21	28	32	34	35	33
Anterior lobe carcinoma	1	1	6	2	12	7	8	8

The numbers in the table indicate the numbers of affected animals. ^sp<0.05 (Trend test, significantly different from the control group, fatal + incidental Peto test)

Table 6. Histopathological Changes in the Femoral Skeletal Muscle

Sex	Male				Female			
	0	100	500	2500	0	100	500	2500
Dose level (mg/kg/day)								
Number of animals	55	55	55	55	55	55	55	55
Femoral skeletal muscle								
Degeneration of muscle fiber	53	53	55	54**	44	38	41	51**
Minimal	32	28	29	21	35	28	34	27
Mild	18	22	23	15	9	10	7	20
Moderate	3	3	3	18	0	0	0	4

The numbers in the table indicate the numbers of affected animals. **Significantly different from the control group (p>0.01).

Table 7. TK Parameters

	Sex	Males			Females			
		Dose level (mg/kg)	100	500	2500	100	500	2500
T _{max} (h)	Day 1		0.5	0.5	1.0	0.5	0.5	0.5
	Week 26		1.0	1.0	1.0	0.5	0.5	0.5
	Week 52		1.0	1.0	1.0	0.5	1.0	1.0
C _{max} (ng/mL)	Day 1		25.9	73.7	224.6	18.8	59.2	167.9
	Week 26		26.5	58.7	154.3	30.9	76.6	132.0
	Week 52		24.0	72.9	197.2	29.8	75.9	234.1
AUC _{0-24h} (ng·h/mL)	Day 1		50.5	203.1	741.6	27.9	134.1	442.5
	Week 26		55.0	160.5	637.3	30.8	109.6	418.5
	Week 52		56.7	207.3	867.2	55.4	161.1	794.6

The values in the table indicate the mean values.

respectively. The changes were more severe for males and females in the 2,500 mg/kg/day group, significantly higher than those of the control group (p<0.01) and believed to be

TJ-25 related. Other nonneoplastic changes did not show clear differences between the control group and TJ-25 group, and so they were thought to be incidental.

TK parameters in plasma

The TK parameters of paeoniflorin are summarized in Table 7. On day 1 and in weeks 26 and 52 of administration, the plasma concentration of paeoniflorin increased quickly after dosing in all TJ-25 groups, and the T_{\max} for all TJ-25 groups was 0.5 or 1.0 hours. Both C_{\max} and AUC_{0-24h} increased as the dose level increased. We also noted that these TK parameters did not change with repeated administration of TJ-25.

Discussion

In our study, administration of TJ-25 extract powder did not result in any negative clinical effects. TJ-25 did not affect survival rate, body weight or food consumption, and the deaths observed were deemed to have been caused by spontaneous lesions or tumors not associated with administration of the TJ-25 extract powder.

The dose levels for this study were selected by considering the results of a preliminary carcinogenicity study. In that study, rats were divided into three dosage groups (100, 500 and 2,500 mg/kg/day TJ-25), each consisting of 14 males and 14 females. They were orally administered TJ-25 for 26 weeks. The results showed mild anemia, mildly increased hematopoiesis and diffuse acinar hyperplasia in the mammary glands of rats in the 2,500 mg/kg/day TJ-25 group. The maximum applicable dose level of 2,500 mg/kg/day was calculated from the maximum applicable dose volume for long-term repeated administration (10 mL/kg) and the maximum preparable concentration of 250 mg/mL. Therefore, for our study, we selected the maximum applicable dose level of 2,500 mg/kg/day as the high dose and 500 and 100 mg/kg/day as the middle and low dose levels, respectively.

We did note a low erythrocyte count in females from the 2,500 mg/kg/day TJ-25 group. However, we believe the low erythrocyte count was caused by hemorrhage from subcutaneous masses and thus not TJ-25 related. Pale skin at necropsy may also have been caused by hemorrhage from the subcutaneous masses, and enlargement of the spleen may be a reactive change to hemorrhage. Our results suggest that the decreased red blood cell count was caused by the presence of subcutaneous masses. Since histopathological examination revealed that the tumors observed in this study were all spontaneous, not TJ-25 related, we believe administration of TJ-25 did not cause the decline in red blood cell count.

Degeneration of muscle fiber in the femoral skeletal muscle was observed in all groups including the control group. Although this degeneration was spontaneous, the slightly higher incidence in the 2,500 mg/kg/day TJ-25 group suggests that administration of TJ-25 might have exacerbated it. However, because histological examination revealed that TJ-25 did not worsen radiculoneuropathy and sciatic nerve degeneration, which are associated with degeneration of skeletal muscles, we were unable to discern the mechanism of muscle degeneration from our results.

It is well known that glycyrrhizin, the main component of *Glycyrrhizae radix*, induces adverse effects such as rhabdomyolysis in humans by causing pseudoaldosteronism¹⁷. However, since TJ-25 does not contain any glycyrrhizin, the mechanism of degeneration of femoral muscle fibers is unknown. In a three-month repeated dose study in dogs, no similar findings were observed in the muscles (unpublished observations). Hence, this effect can be considered to be specific to rats.

Under macroscopic examination, we found that the incidence of ocular opacity tended to be higher for females in the 2,500 mg/kg/day TJ-25 group (0/55 females in the control group and 7/55 females in the 2,500 mg/kg/day group, five of which died or were sacrificed moribund). However, we do not believe that this was related to administration of the TJ-25, because histopathological examination indicated that the ocular opacity was caused by keratitis or calcification and thus spontaneous. In addition, ocular opacity was observed in many animals that died or were sacrificed moribund but not observed frequently in the animals that were necropsied on schedule after a longer administration period.

In our TK analysis, the plasma concentration of paeoniflorin increased quickly after dosing in males and females in all dose groups, and T_{\max} was approximately 0.50 or 1.0 hours. The C_{\max} and AUC_{0-24h} values increased as the dose level increased. Repeated administration did not change these TK parameters much.

The recommended clinical daily dose of TJ-25 granules for humans is 7.5 g, which contains 1.75 g of dried TJ-25 extract. Given that the average body weight is 60 kg, the daily dose of dried TJ-25 extract ingested by humans is 30 mg/kg. The maximum dose level for rats in this study was 2,500 mg/kg, which is 80 times higher than the recommended clinical dose for humans.

In conclusion, oral administration of TJ-25 extract powder to rats for 24 months was not associated with an increase incidence of tumors.

Disclosure of Potential Conflicts of Interest: We certify that there is no actual or potential conflict of interest in relation to this article.

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