Single Oral Dose Toxicity Test of Kong-Jin-Dan, a Polyherbal Formula in ICR Mice

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Abstract – The object of this study was to evaluate the single dose toxicity of Kong-Jin-Dan (KJD), a polyherbal formula in male and female mice. KJD was administered to female and male ICR mice as an oral dose of 2000, 1000 and 500 mg/kg (body wt.) according to the recommendation of KFDA Guidelines. Animals were monitored for the mortality and changes in body weight, clinical signs and gross observation during 14 days after dosing, upon necropsy, organ weight and histopathology of 12 principle organs were examined. As results, we could not find any mortality, clinical signs, and changes in the body and organ weight except for increases of lymphoid organ weights in KJD-dosing groups. These increases of lymphoid organ weights considered that related to the immune modulate effect of KJD not toxicological signs. In addition, no KJD-treatment related abnormal gross findings and changes in histopathology of principle organs were detected except for some sporadic accidental findings. The results obtained in this study suggest that the KJD does not cause any toxicological signs. The LD₅₀ and approximate LD of KJD extracts in both female and male mice were considered as over 2000 mg/kg.

Key words □ Kong-Jin-Dan, single oral dose toxicity, mice, histopathology

INTRODUCTION

A polyherbal formula, Kong-Jin-Dan (KJD) is one of the most famous tonic agents, in Korean traditional folk medicine, and consisted of 4 herbs – Angelicae gigas radix, Ginseng steamed red, Corni fructus and Rehmanniae radix preparata, and 2 animal resources – antler and musk. These 6 agents were plastered using honey, and coated by gold plates. The hypolipemic and immune stimulatory effects of KJD are relatively well documented (Kim and Bae, 2001; Kim *et al.*, 1999).

As increase of the concern in the functional food and well being in life, the demands and consumption of functional food originated from natural sources are increased (Lee *et al.*, 2003). However, the toxicological aspects about these natural origin-functional foods has been neglected because of the reasons that they have been used as various purpose for long times. Therefore, it is considered that more detailed and systemic toxicological studies should be performed to control the abuse and potential toxicities even if they have been used as traditional

folk medicine. The toxicological studies about KJD also have been neglected and the reports dealing the toxicological aspects of KJD, even if the basic single dose toxicities in rodents are also seldom.

The objective of the present study, therefore, was to obtain the primary safety information about KJD, and to further clarify their safety for clinical use.

MATERIALS AND METHODS

Animals and husbandry

Each of twenty female and male ICR mice (6-wk old upon receipt, Charles River, Japan) was used after acclimatization for 14 days. Animals were allocated five per polycarbonate cage in a temperature (20-25°C) and humidity (45-50 %) controlled room. Light : dark cycle was 12hrs : 12hrs, and feed (Samyang, Korea) and water were supplied free to access. All animals were overnight fasted before dosing and terminal necropsy. Animals were marked by picric acid. The experimental protocols were conducted in accordance with internationally accepted principles for laboratory animal use and care as found in the Korea Food and Drug Administration (KFDA) guidelines.

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Preparation and Administration of KJD

KJD used in this study was purchased from Daegu Oriental Hospital of Daegu Hanny University (Daegu, Korea) as listed in Table I. Deep brown gold-coated plasters, KJD was stored in a refrigerator at -20°C to protect from light and degeneration. KJD was well suspended upto 200 mg/ml concentration levels and appeared to be a deep brown homogenous suspension. The animals were grouped into 8 with 5 mice per group upon receipt. KJD have been used as folk medicine for long times, and no revealed toxicological data was available, the highest dosage level was selected as 2000 mg/kg according to the recommended by KFDA Guidelines (2005-60, 2005), the limited dosages and 1000 and 500 mg/kg was selected using common ratio 2. In addition, a vehicle control group was added as listed in Table II. Animals were given an oral dose of KJD prepared in water using a zonde attached to a syringe of 1ml after overnight fasting (about 18hr, water was not restricted). Food and water were restricted further for about 3 hrs after dosing.

Abnormal behavior, clinical sign and body weight

All abnormal clinical signs and behaviors were recorded before and after dosing at least twice a day based on the functional observational battery test (Irwin, 1968; Dourish, 1987). Body weights were measured on the day of dosing (Day 0) prior to treatment, 1, 2, 7, 13 and 14 days after dosing. In addition, to reduce the differences from individual body weight differences of animals at treatment, body weight gains during Day 0 ~ Day 7, Day 7 ~ Day 13 and Day 0 ~ Day 13 was also calculated based on measured body weight at each point.

Necropsy

Necropsy was done on the animals found dead during the experiment and all animals were subjected to terminal necropsy at the end of experiment at Day 14 after overnight fasting

Table II. Experimental design used in this study

Group	Sex	No. of animals	Total Dose (mg/kg) of Kong-Jin-Dan
G0M*	Male	5	0
G1M	Male	5	2000
G2M	Male	5	1000
G3M	Male	5	500
G0F*	Female	5	0
G1F	Female	5	2000
G2F	Female	5	1000
G3F	Female	5	500

*Vehicle control; distilled water 10 ml/kg as vehicle in this study; All test articles in vehicle were once orally dosed.

(about 18hr, water was not restricted). Animals were euthanized by carbon dioxide and gross necropsy was performed.

Organ weight measurements and sampling

The absolute organ weight was measured and then relative organ weight (% for body weight) was calculated. The following organs were collected for histopathological observation.

Measured and sampled organs: lung, heart, thymus, left kidney, left adrenal gland, spleen, left testis or ovary, liver, splenic lobe of pancreas, brain, left epididymis or total uterus and left popliteal lymph node.

Histopathology

Samples were fixed in 10 % neutral buffered formalin. After 18 hrs of fixation, paraffin embedding was conducted and 4 µm sections were prepared by routine histological methods. Representative sections of each specified organs were stained with hematoxylin-eosin for light microscopical examination.

Statistical analyses

Multiple comparison tests for different dose groups were

Table I. Composition of Kong-Jin-Dan Used in This Study

Herbs	Scientific Names	Amounts (g/1 pill)
Antler (<i>Cornus cervi parvum</i>)	<i>Cervus elaphus</i> Linne	0.683
Angelicae gigantis radix	<i>Angelica gigas</i> Nakai	0.683
Ginseng steamed red	<i>Panax ginseng</i> CA Mey.	0.683
Corni fructus	<i>Cornus officinalis</i> Sieb. Et Zucc	0.683
Rehmanniae radix preparata	<i>Rehmannia glutinosa</i> (Gaertner) Liboschitz	0.683
Musk	<i>Moschus moschiferus</i> Linne	0.122
Honey	<i>Apis indica</i> Radoszkowski	2.506
Gold plate		0.006
Total	8 types	6.050

Kong-Jin-Dan used in this study was purchased from Daegu Oriental Hospital of Daegu Hanny University (Daegu, Korea)

conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by Tukey HSD test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, the Mann-Whitney U-Wilcoxon Rank Sum W test was conducted to determine the specific pairs of group comparison, which are significantly different. LD₅₀ and 95% confidence limits were calculated by Probit method. Statistical analyses were conducted using SPSS for Windows (Release 6.1.3., SPSS Inc., USA) and a *p*-value of less than 0.05 was considered to be a significant difference. In addition, degree of clinical signs, gross and histopathological findings were subdivided into 3 degrees: 3+ Severe, 2+ moderate, 1+ slight.

RESULTS

Mortality rate

No sudden mortality was detected in all dose levels tested. All animals (5/5; 100%) survived in all dose levels tested including vehicle control.

Abnormal clinical signs detected

In this study, no KJD treatment related abnormal clinical signs were observed during observation periods regardless of male and female mice.

Changes of body weights

No significant changes in body weight were detected compared to that of vehicle control in all dose levels tested (Table III).

Table III. Body weight gains in female and male mice after once orally dose of Kong-Jin-Dan

Group		Interval		
		Day 0 ^a - Day 7	Day 7 - Day 13	Day 0 - Day 13
MALE	G0M	5.64±1.62	1.78±1.80	7.42±1.74
	G1M	5.72±0.99	1.80±0.80	7.52±1.36
	G2M	5.32±0.94	1.30±1.75	6.69±1.14
	G3M	5.32±1.17	1.38±1.33	6.70±1.50
FEMALE	G0F	4.34±1.72	1.60±2.45	5.94±1.06
	G1F	5.30±1.54	1.64±2.19	6.94±1.05
	G2F	3.00±1.60	1.14±0.63	4.14±1.44
	G3F	4.60±1.75	1.16±0.93	5.76±2.11

Values are expressed as mean ± S. D., g (n=5); ^aDay of dosing.

Changes of organ weights

No significant changes in the absolute and relative weight of 12 principle organs were observed in all dose groups compared to that of vehicle control except for lymphatic organ weights. In KJD 2000 and 1000 mg/kg-treated male and female mice showed significantly (*p*<0.01 or *p*<0.05) increases of absolute and relative spleen and popliteal lymph node weights as compared with vehicle control, respectively. In addition, 2000 mg/kg of KJD-treated female mice also showed significantly (*p*<0.05) increase of absolute thymus weight, and significant (*p*<0.01) increase of relative heart weight was detected in KJD 1000 mg/kg-treated male mice as compared with vehicle control. In 500 mg/kg of KJD-treated females, significantly (*p*<0.05) increases of absolute and relative popliteal lymph node weights and relative thymus weights were detected as compared with vehicle control, respectively (Table IV, V).

Necropsy findings

No significant changes in the gross findings of 12 principle organs were observed in all dosing groups compared to that of vehicle control except for increased incidences of the hypertrophy of spleen encountered in KJD treated male and female groups. Some animals showed sporadic accidental findings such as congestion spots of lung, atrophy of thymus, cyst of kidney, spleen atrophy, liver atypical white foci and edematous changes of uterus (Table VI).

Histopathological findings

No significant histopathological findings were observed in 12 principle organs of all dose groups tested compared to that of vehicle control except for hypertrophy of lung alveolus wall with hemorrhage (Fig. 1), focal necrotic foci and inflammatory cell infiltration in liver (Fig. 2), cyst in kidney (Fig. 3), depletion of lymphoid cells in red pulps of spleen (Fig. 4), and desquamation of mucosa in uterus (Fig. 5) (Table VII).

DISCUSSION

In the present study, we investigated the single oral dose toxicity of KJD, a polyherbal formula to mice as a part of the safety test. In order to observe 50% lethal dose (LD₅₀) and approximate lethal dosage (LD), test substances were administered orally to female and male ICR mice at dose levels of 2000, 1000 and 500 mg/kg. However, we could not find any mortality, clinical signs, and changes in the body and organ weight except for increases of lymphoid organ weights in KJD-

Table IV. Changes in the absolute organ weights observed in female and male mice after a oral dose of Kong-Jin-Dan

Group	Principal Organs										
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis/Ovary L	Liver	Pancreas S	Brain	Epididymis L/Uterus Lymph node L ^a
G0M	0.195±0.011	0.159±0.013	0.046±0.009	0.303±0.045	0.009±0.003	0.112±0.011	0.130±0.009	1.608±0.134	0.230±0.023	0.475±0.010	0.051±0.004
G1M	0.191±0.011	0.166±0.011	0.058±0.010	0.286±0.043	0.008±0.001	0.134±0.007*	0.126±0.015	1.577±0.157	0.211±0.028	0.469±0.016	0.056±0.005
G2M	0.189±0.010	0.166±0.009	0.055±0.013	0.305±0.030	0.006±0.001	0.128±0.008**	0.117±0.013	1.629±0.118	0.223±0.035	0.479±0.019	0.048±0.007
G3M	0.194±0.019	0.167±0.011	0.056±0.015	0.295±0.034	0.008±0.001	0.135±0.031	0.126±0.007	1.593±0.115	0.219±0.015	0.473±0.018	0.048±0.006
G0F	0.183±0.011	0.148±0.008	0.057±0.008	0.204±0.012	0.008±0.002	0.128±0.023	0.026±0.007	1.436±0.138	0.190±0.026	0.486±0.035	0.196±0.054
G1F	0.185±0.005	0.145±0.007	0.074±0.013**	0.225±0.030	0.008±0.002	0.165±0.023**	0.027±0.016	1.371±0.108	0.189±0.025	0.493±0.016	0.177±0.068
G2F	0.190±0.016	0.150±0.008	0.063±0.019	0.205±0.021	0.008±0.002	0.165±0.020**	0.030±0.002	1.365±0.077	0.183±0.014	0.492±0.018	0.160±0.056
G3F	0.188±0.016	0.146±0.002	0.073±0.017	0.205±0.013	0.007±0.002	0.132±0.022	0.033±0.011	1.308±0.073	0.183±0.010	0.489±0.028	0.204±0.089

Values are expressed as mean±S. D., g (n=5); L, left sides; S, splenic lobes; ^aPopliteal lymph node; *p<0.01 and **p<0.05 as compared with vehicle control (G0M or G0F).

Table V. Changes in the relative organ weights observed in female and male mice after a oral dose of Kong-Jin-Dan

Group	Principal Organs										
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis/Ovary L	Liver	Pancreas S	Brain	Epididymis L/Uterus Lymph node L ^a
G0M	0.505±0.013	0.412±0.022	0.120±0.028	0.782±0.083	0.022±0.007	0.290±0.035	0.336±0.010	4.161±0.183	0.594±0.030	1.234±0.070	0.132±0.012
G1M	0.493±0.025	0.430±0.022	0.152±0.030	0.738±0.082	0.021±0.003	0.346±0.018*	0.326±0.030	4.080±0.424	0.548±0.086	1.215±0.096	0.144±0.012
G2M	0.504±0.023	0.441±0.014*	0.148±0.035	0.812±0.070	0.015±0.002	0.342±0.023*	0.312±0.046	4.339±0.332	0.590±0.065	1.278±0.082	0.129±0.022
G3M	0.509±0.041	0.439±0.021	0.149±0.042	0.773±0.071	0.020±0.002	0.355±0.086	0.332±0.016	4.185±0.251	0.576±0.033	1.245±0.075	0.127±0.018
G0F	0.564±0.034	0.457±0.035	0.177±0.027	0.630±0.064	0.023±0.005	0.395±0.065	0.080±0.022	4.418±0.273	0.583±0.053	1.504±0.169	0.608±0.186
G1F	0.567±0.055	0.443±0.030	0.226±0.046	0.682±0.066	0.024±0.006	0.499±0.035*	0.083±0.054	4.168±0.195	0.573±0.044	1.504±0.088	0.531±0.169
G2F	0.609±0.048	0.483±0.031	0.202±0.054	0.660±0.071	0.024±0.006	0.532±0.089*	0.095±0.008	4.381±0.220	0.587±0.027	1.580±0.055	0.512±0.177
G3F	0.607±0.049	0.470±0.036	0.233±0.039*	0.664±0.086	0.022±0.006	0.428±0.086	0.107±0.045	4.213±0.125	0.590±0.054	1.578±0.141	0.665±0.303

Values are expressed as mean±S. D., % vs body weights (n=5); L, left sides; S, splenic lobes; ^aPopliteal lymph node; *p<0.05 as compared with vehicle control (G0M or G0F)

Table VI. Necropsy findings in female and male mice after once orally dose of Kong-Jin-Dan

Group	MALE				FEMALE			
	G0M	G1M	G2M	G3M	G0F	G1F	G2F	G3F
Lung								
Congestion	2/5	1/5	1/5	2/5	1/5	1/5	1/5	0/5
Thymus								
Atrophy	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Kidney								
Cyst	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen								
Atrophy	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Hypertrophy	0/5	1/5	2/5	2/5	0/5	2/5	2/5	1/5
Liver								
Atypical foci	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Uterus								
Edematous changes					2/5	2/5	1/5	2/5

Values are expressed as observed animals/total observed animals (n = 5)

Table VII. Histopathological findings in female and male mice after once orally dose of Kong-Jin-Dan

Group	MALE				FEMALE			
	G0M	G1M	G2M	G3M	G0F	G1F	G2F	G3F
Lung								
Congestion	2/5	1/5	1/5	2/5	2/5	1/5	1/5	0/5
Kidney								
Cyst	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen								
DL ¹⁾	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Liver								
IF-FN ²⁾	2/5	0/5	1/5	1/5	1/5	1/5	1/5	2/5
Uterus								
DM ³⁾					3/5	2/5	0/5	2/5

Values are expressed as observed animals/total observed animals (n = 5); ¹⁾DL, depletion of lymphoid cells in red pulps; ²⁾IF-FN, focal necrosis with inflammatory cell infiltration; ³⁾DM, desquamation of mucosa.

dosing groups. In addition, no KJD-treatment related abnormal gross findings and changes in histopathology of principle organs were detected except for some sporadic accidental findings

No KJD extracts related histopathological changes were detected in all three lymphatic organs; lymph nodes, spleen and thymus observed in the present study, although the hypertrophy of spleen in gross findings and increases of lymphatic organ weights. The increases in the weights of lymphatic organs were frequently observed after treatment of immune enhancers with (Klein *et al.*, 1987; Iqbal *et al.*, 2001) with hypertrophy and hyperplasia of lymphoid cells (Lee *et al.*, 2005). These changes detected in the present study, therefore considered that related to the immune modulate effect of KJD not toxicological signs.

Fig. 1. Histopathological changes detected in the lung. Note that hypertrophy of alveolus as congestion with hemorrhage (CG) were randomly detected and dispersed throughout the all tested groups including vehicle control. All Hematoxylin & Eosin stain; Scale bars = 8 μ m

The immune enhancer effects of KJD were already reported (Kim *et al.*, 1999). Significant increase of relative heart weight restricted to KJD 1000 mg/kg-treated male group was considered as accidental findings, and they were not considered as KJD-treatment related abnormal sign because they did not showed any dose-dependency and no meaningful changes on the heart at histopathological and gross observations in the present study.

In KFDD Guidelines (2005-60, 2005), the recommended highest dose of test materials is 2000 mg/kg or the maximum solubility in a volume below 20 ml/kg for studies of single toxicity in mice. In the present study, the highest dose of KJD was selected as 2000 mg/kg because KJD have been used as traditional medicine for long times and no revealed toxicological data was available.

Congestion spots of lung, atrophy of thymus, cyst of kidney,

Fig. 2. Histopathological changes detected in the liver. Note that focal necrotic foci with inflammatory cell infiltration (IF-FN) were randomly observed including vehicle controls as accidental findings. All Hematoxylin & Eosin stain; Scale bars = 40 μ m.

spleen atrophy, liver atypical white foci and edematous changes of uterus detected as gross findings, and hypertrophy of lung alveolus wall with hemorrhage, focal necrotic foci and inflammatory cell infiltration in liver, cyst in kidney, depletion of lymphoid cells in red pulps of spleen, and desquamation of mucosa

Fig. 4. Histopathological changes detected in the spleen. Note that depletion of lymphoid cells (DL) in red pulps was detected only one animal of female vehicle control as accidental findings. All Hematoxylin & Eosin stain; Scale bars = 80 μ m.

Fig. 5. Histopathological changes detected in the uterus. Note that desquamation of mucosa (DM) in uterus were randomly detected and dispersed throughout the all tested groups including vehicle control. All Hematoxylin & Eosin stain; Scale bars = 80 μ m.

in uterus detected as histopathological findings were also considered as accidental findings, and they were not considered as

Fig. 3. Histopathological changes detected in the kidney. Note that cyst was detected only one animal of KJD 1000 mg/kg-treated male as one of accidental findings. All Hematoxylin & Eosin stain; Scale bars = 40 μ m.

KJD-treatment related abnormal gross or histopathological findings because they were restricted in some individual animals and most of the case, also observed in vehicle control. In addition, most of them were rarely observed in normal mice (Lee *et al.*, 2005; Lee *et al.*, 2006). Liver atypical white foci detected at gross observations in the present study were revealed as relatively broaden focal necrotic foci with inflammatory cell infiltration in liver at histopathological observations.

Hodge and Sterner (1949) classify as non toxic materials for those with LD₅₀ of 5000~15000 mg/kg and those with LD₅₀ of 500~5000 mg/kg as relatively low toxic (Class III) in US Environmental Protection Agency (OPPTS 870.100; 1998). However, recent guideline by Korean Food and Drug Administration (2005-60, 2005) recommended that the highest oral dose of test materials should be 2000 mg/kg. The LD₅₀ and approximate LD in mice after single oral dose of KJD were detected over 2000 mg/kg in both male and female in the present study. Therefore, oral administration of KJD caused no serious toxic effect to the male and female mice upto 2000 mg/kg – the highest dosage tested in this study and is likely to be safe in humans.

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