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Single Oral Dose Toxicity Test of Water Extracts of Puerariae Radix in ICR Mice

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ABSTRACT. The object of this study was to obtain acute toxicity information (single oral dose toxicity) of lyophilized water extract of Puerariae Radix (PR) in both male and female mice. In order to investigate the 50% lethal dose (LD_{50}), approximate lethal dosage (ALD), test substances were once orally administered to female and male ICR mice at dose levels of 2000 and 0 (control) mg/kg (body wt.) according to the recommendation of KFDA Guidelines [2005-60, 2005]. The mortality and body weight changes, clinical signs and gross observation were monitored during 14 days after dosing. Organ weight and histopathology of 12 principal organs were measured. As the results, we could not find any mortality, clinical signs, body weight changes and gross findings except for PR extracts unrelated sporadic findings. In addition, no abnormal changes related PR extracts treatment on the organ weight and histopathology of principal organs were detected except for some sporadic findings including hyperplasia of lymphoid follicles in the popliteal lymph nodes and spleen as pharmacological effects of PR extracts. The results obtained in this study suggest that the PR extracts does not cause any toxicological signs except for pharmacological effects of enhancement of immune system. The LD_{50} and ALD of PR extracts in both female and male mice were considered as over 2000 mg/kg because no mortalities were detected up to 2000 mg/kg that was the highest dose recommended by KFDA and Organization for Economic Co-Operation and Development.

Keywords: Puerariae Radix, Water extracts, Single oral dose toxicity, Mice, Histopathology.

INTRODUCTION

Puerariae Radix (PR) is a dried root of arrowroot (*Pueraria thunbergiana* Benth) and has been used as a Korean traditional folk medicine for enhancing body resistance against various diseases (Huh *et al.*, 2006) or used as a medicinal ingredient of functional foods (Kim and Fung, 2004). PR is widely used as an antipyretic and analgesic drugs for treatment of the common cold (Kang *et al.*, 2005) and it is reported that saponins from the root of *Puerariae* showed preventive effects on in vitro immunological injury of rat primary hepatocyte cultures (Kim, 1996; Arao *et al.*, 1997). In addition, the protective effects of PR extracts on the oxidative stress (Lee, 2004; Kang *et al.*, 2005), and anti-osteoporotic (Wang *et al.*, 2004; Huh *et al.*, 2006), antidepressant (Yan *et al.*, 2004), antidipsotropic (Keung and Vallee, 1998a, b) and anti-bacterial (Kim and Fung, 2004) effects also has been evaluated.

As increase of the concern in the functional food and well-being in life, the demands and consumption of functional food originated form 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 has been used as various purpose for long times. Therefore, it is considered that more detail and systemic toxicological studies should be tested for control the abuse and potential toxicities even if they have been used as traditional folk medicine. The toxicological studies about PR also have been neglected and the reports dealing the toxicological aspects of PR extracts, even if the basic single dose toxicities in rodents are also seldom.

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

MATERIALS AND METHODS

Animals and husbandry

Each of ten female and male ICR mice (6-wk old upon receipt, SLC, Japan) was used after acclimatization for 8 days. Animals were allocated five per polycarbonate cage in a temperature (20~25°C) and humidity (40~45%) controlled room. Light : dark cycle was 12 hr : 12 hr and feed (Samyang, Korea) and water were supplied free to access. All animals were overnight fasted (about 18 hrs) before dosing and terminal necropsy. Animals were marked by picric acid.

Test substances and formulation

Aqueous Puerariae Radix (dried roots of *Pueraria thunbergiana* Benth: yield = 13~14%, extracted by Dong Wha Pharm. Ind. Co. Ltd., Korea) were prepared by routine methods using rotary vacuum evaporator (Lab. Camp, Korea) and programmable freeze dryer (IlShin Lab., Korea). PR were purchased from Bu-young Pharmaceutical Ind. Co. (Seoul, Korea) collected in China after confirm the morphology under microscopy. Powders of PR extracts are deep brown powder and containing 2.17% of Puerarin at high-performance lipid chromatographic analysis. PR extracts are well soluble up to 100 mg/ml concentration level and the appearance in distilled water is deep brownish solution. PR extracts was stored under -20°C of Deep freezer until using to protect from light and humidity. The test substance was single orally administered at a dosage volume of 20 ml/kg using distilled water as vehicle.

Groupings and dosing

The animals were distributed into 4 groups (5 mice per group) upon receipt. PR extracts have been used as folk medicine and ingredients of medicinal food for long times and no revealed toxicological data was available, the highest dosage level was selected as 2000 mg/kg according to the recommendation of by KFDA Guidelines (2005-60) [2005] and OECD Guidelines (#401, #423) [1987; 2001], the limited dosages. In addition, a vehicle control group was added as listed in Table 1. Animal was once orally dosed using a sonde attached to a syringe of 1 ml after overnight fasting (about 18 hr, water was not restricted). Feed and water were restricted further for about 3 hours after dosing.

Observation of clinical signs

All abnormal clinical signs were recorded before and after dosing at least twice a day.

Changes of body weights

Body weights were measured at the day of dosing (Day 0) immediately before treatment, 1, 2, 7, 13 and 14 days after dosing. In addition, to reduce the erratum originated from individual body weight differences of animals at initial dosing, body weight gains during Day 0~Day 7, Day 7~Day 13 and Day 0~Day 14 was also calculated based on measured body weight at each points.

Necropsy

All unscheduled death animals were grossly observed immediately after finding them and all survived animals were subjected to terminal necropsy. Animals were asphyxiated by diethyl ether (Duksan pure chemical Co., Ltd., Korea) and gross necropsy was performed in all animals at 14 days after overnight fasting (about 18hr, water was not restricted).

Specific organs grossly observed: Lung, Heart, Thymus, Kidney, Adrenal Glands, Spleen, Testis/Ovary, Liver, Pancreas, Brain, Epididymis/Uterus, Gastrointestinal tracts, Skins and Popliteal Lymph node.

Organ weight

The absolute organ weight was measured and then relative organ weight (% for body weight) was calculated for the following organs of all experimental animals when they were sacrificed.

Measured organs: Lung, Heart, Thymus, Left Kidney, Left Adrenal Gland, Spleen, Left Testis/Ovary, Liver,

Table 1. Experimental design used in this study

Group	Sex	No. of animals	Animal No.	Dosage (ml/kg)	Total dose (mg/kg)
G0M*	Male	5	G0M-01~G0M-05	20	0
GPRM	Male	5	GPRM-01~GPRM-05	20	2000 of PR extracts
G0F*	Female	5	G0F-01~G0F-05	20	0
GPRF	Female	5	GPRF-01~GPRF-05	20	2000 of PR extracts

* Vehicle control; distilled water 20 ml/kg as vehicle in this study; All test substances in vehicle were once orally dosed.

Splenic lobe of Pancreas, Brain, Left Epididymis/total Uterus and Left Popliteal Lymph node.

Histology

Principal organs listed below were sampled at terminal necropsy, and fixed in 10% NBF (neutral buffered formalin). After 18hrs of fixation, paraffin embedding was conducted and 3~4 μm sections were prepared by routine histological methods. Representative sections of each specified organs were stained with Hematoxylin & eosin for light microscopic examination.

Specific organs sampled: Lung, Heart, Thymus, Left Kidney, Left Adrenal Gland, Spleen, Left Testis/Ovary, Liver, Splenic lobe of Pancreas, Brain, Left Epididymis/ total Uterus and Left Popliteal Lymph node.

Statistical Analyses

Changes of body weights were analyzed by Mann-Whitney U-Wilcoxon Rank Sum W test (MW test) compared to those of vehicle control (G0F and/or G0M). LD_{50} was calculated by Probit method. Statistical analyses were conducted using SPSS for Windows (Release 6.1.3., SPSS Inc., USA). In addition, degree of clinical signs, gross and histopathological findings were subdivided into 3 degrees: 3+ Severe, 2+ moderate, 1+ slight.

RESULTS

Mortalities

No unscheduled or PR extracts-treatment related mortalities were detected in all dose levels tested in this study. At terminal day, all of animals (5/5; 100%) were survived in all dose levels tested including vehicle control.

Clinical Signs

In this study, no abnormal clinical signs related PR extract treatment were observed during observation periods regardless of male and female mice except for hair loss that was randomly detected from 5 days after dosing including vehicle control groups (Table 2).

Table 2. Clinical signs observed in male and female mice after single oral dose of PR extract[a]

Group ID		Clinical signs	
		Normal	Hair loss
Male	G0M	3/5 (60%)	2/5 (40%)
	GPRM	4/5 (80%)	1/5 (20%)
Female	G0F	2/5 (40%)	3/5 (60%)
	GPRF	4/5 (80%)	1/5 (20%)

[a]Observed animals/total animals (n = 5) (percentages).

Table 3. Body weight gains in male and female mice after single oral dose of PR extract[a]

Group ID		Interval		
		Day 0[b]~Day 7	Day 7~Day 13	Day 0~Day 14
Male	G0M	10.30 ± 3.68	3.68 ± 1.21	9.12 ± 1.63
	GPRM	8.46 ± 2.49	2.94 ± 1.20	7.38 ± 2.15
Female	G0F	5.42 ± 0.98	1.94 ± 1.13	4.10 ± 0.78
	GPRF	5.42 ± 1.42	3.54 ± 1.79	5.54 ± 2.13

[a]Values are expressed as mean ± S.D., g (n = 5).
[b]Day of dosing.

Changes of Body Weights and gains

No meaningful changes on body weight and gains were detected in all dosing groups tested compared to that of vehicle control in all dose levels tested (Table 3).

Changes of organ weights

No significant changes on the absolute and relative organ weight of 12 principal organs were observed in all

Table 4. Changes on the absolute organ weights observed in female and male mice after single oral dose of PR extract[a]

Group ID	Principal Organs											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis/ Ovary L	Liver	Pancreas S	Brain	Epididymis L/ Uterus	Lymph node L[b]
G0M	0.208 ± 0.014	0.176 ± 0.015	0.064 ± 0.016	0.355 ± 0.059	0.008 ± 0.004	0.110 ± 0.016	0.128 ± 0.025	1.739 ± 0.213	0.202 ± 0.030	0.488 ± 0.013	0.044 ± 0.002	0.023 ± 0.011
GPRM	0.205 ± 0.015	0.179 ± 0.012	0.068 ± 0.023	0.330 ± 0.055	0.010 ± 0.003	0.112 ± 0.032	0.113 ± 0.007	1.692 ± 0.204	0.215 ± 0.046	0.492 ± 0.035	0.047 ± 0.010	0.051 ± 0.008**
G0F	0.180 ± 0.011	0.145 ± 0.015	0.072 ± 0.016	0.203 ± 0.020	0.009 ± 0.003	0.124 ± 0.021	0.040 ± 0.013	1.268 ± 0.112	0.178 ± 0.016	0.468 ± 0.035	0.136 ± 0.032	0.022 ± 0.003
GPRF	0.191 ± 0.011	0.147 ± 0.007	0.072 ± 0.020	0.211 ± 0.014	0.008 ± 0.001	0.132 ± 0.026	0.044 ± 0.014	1.367 ± 0.202	0.195 ± 0.010*	0.485 ± 0.011	0.220 ± 0.085	0.051 ± 0.007*

[a]Values are expressed as mean ± S.D., g (n = 5); L, left sides; S, splenic lobes.
[b]Popliteal lymph node; * $p < 0.01$ and ** $p < 0.05$ compared to those of vehicle control (G0M or G0F, respectively) by MW test.

Table 5. Changes on the relative organ weights observed in female and male mice after single oral dose of PR extract[a]

Group ID	Principal Organs											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis/ Ovary L	Liver	Pancreas S	Brain	Epididymis L/ Uterus	Lymph node L[b]
G0M	0.533 ± 0.036	0.450 ± 0.030	0.161 ± 0.033	0.906 ± 0.106	0.021 ± 0.010	0.282 ± 0.028	0.331 ± 0.080	4.442 ± 0.309	0.516 ± 0.040	1.257 ± 0.116	0.113 ± 0.006	0.059 ± 0.028
GPRM	0.559 ± 0.050	0.481 ± 0.033	0.178 ± 0.053	0.892 ± 0.152	0.025 ± 0.007	0.296 ± 0.072	0.302 ± 0.026	4.557 ± 0.336	0.598 ± 0.109**	1.357 ± 0.141	0.131 ± 0.025	0.137 ± 0.027*
G0F	0.611 ± 0.034	0.491 ± 0.035	0.243 ± 0.065	0.688 ± 0.051	0.030 ± 0.009	0.421 ± 0.062	0.135 ± 0.048	4.302 ± 0.327	0.605 ± 0.043	1.588 ± 0.065	0.460 ± 0.101	0.075 ± 0.009
GPRF	0.626 ± 0.010	0.473 ± 0.021	0.248 ± 0.056	0.679 ± 0.025	0.027 ± 0.002	0.432 ± 0.066	0.157 ± 0.028	4.426 ± 0.523	0.638 ± 0.048	1.574 ± 0.070	0.641 ± 0.189	0.166 ± 0.018*

[a]Values are expressed as mean ± S.D., % (n = 5); L, left sides; S, splenic lobes.
[b]Popliteal lymph node; * $p < 0.01$ and ** $p < 0.05$ compared to those of vehicle control (G0M or G0F, respectively) by MW test.

dosing groups tested compared to that of vehicle control except for significant ($p < 0.01$ or $p < 0.05$) increases of relative weight of splenic pancreas weight in PR extracts-dosing male group and absolute weight of splenic pancreas in PR extracts-dosing female with increases of absolute and relative weight of popliteal lymph nodes in both female and male-dosing groups

Table 6. Necropsy findings observed in female and male mice after single oral dose of PR extract[a]

Necropsy findings	Male		Female	
	G0M	GPRM	G0F	GPRF
Lung				
Normal	3/5	4/5	2/5	4/5
Congestion	2/5	1/5	3/5	1/5
Heart				
Normal	5/5	5/5	5/5	5/5
Thymus				
Normal	3/5	4/5	4/5	4/5
Atrophy	2/5	1/5	1/5	1/5
Kidney				
Normal	4/5	4/5	3/5	5/5
Cyst	0/5	0/5	1/5	0/5
Discolorization	1/5	1/5	1/5	0/5
Adrenal gland				
Normal	5/5	5/5	5/5	5/5
Spleen				
Normal	3/5	4/5	4/5	4/5
Atrophy	2/5	1/5	1/5	1/5
Testis/Ovary				
Normal	5/5	5/5	5/5	5/5
Liver				
Normal	5/5	5/5	5/5	5/5
Pancreas				
Normal	5/5	5/5	5/5	5/5
Brain				
Normal	5/5	5/5	5/5	5/5
Epididymis/Uterus				
Normal	5/5	5/5	5/5	4/5
Pyometra	--	--	0/5	1/5
Politeal lymph node				
Normal	3/5	0/5	4/5	0/5
Hypertrophy	2/5	5/5	1/5	5/5
Others				
Normal	5/5	5/5	5/5	5/5

[a]Observed animals/total animals (n = 5).

Table 7. Histopathological findings observed in female and male mice after single oral dose of PR extract[a]

Histological findings	Male		Female	
	G0M	GPRM	G0F	GPRF
Lung				
Normal	2/5	3/5	3/5	5/5
Congestion	3/5	1/5	2/5	0/5
Hemorrhage	0/5	1/5	0/5	0/5
Heart				
Normal	5/5	5/5	5/5	5/5
Thymus				
Normal	5/5	5/5	5/5	5/5
Kidney				
Normal	5/5	5/5	5/5	5/5
Adrenal gland left				
Normal	5/5	5/5	5/5	5/5
Spleen				
Normal	5/5	1/5	5/5	0/5
Hyperplasia of lymphoid cells	0/5	4/5	0/5	5/5
Testis/Ovary left				
Normal	5/5	5/5	5/5	5/5
Liver				
Normal	2/5	4/5	4/5	4/5
Focal necrosis	1/5	0/5	0/5	1/5
Fatty changes	2/5	1/5	1/5	0/5
Pancreas splenic				
Normal	5/5	5/5	5/5	5/5
Brain				
Normal	5/5	5/5	5/5	5/5
Epididymis left/Uterus				
Normal	5/5	5/5	5/5	2/5
Uterus edema	--	--	0/5	2/5
Focal pyometra	--	--	0/5	1/5
Popliteal Lymph node				
Normal	5/5	0/5	4/5	1/5
Hyperplasia of lymphoid cells	0/5	5/5	1/5	4/5

[a]Observed animals/total animals (n = 5).

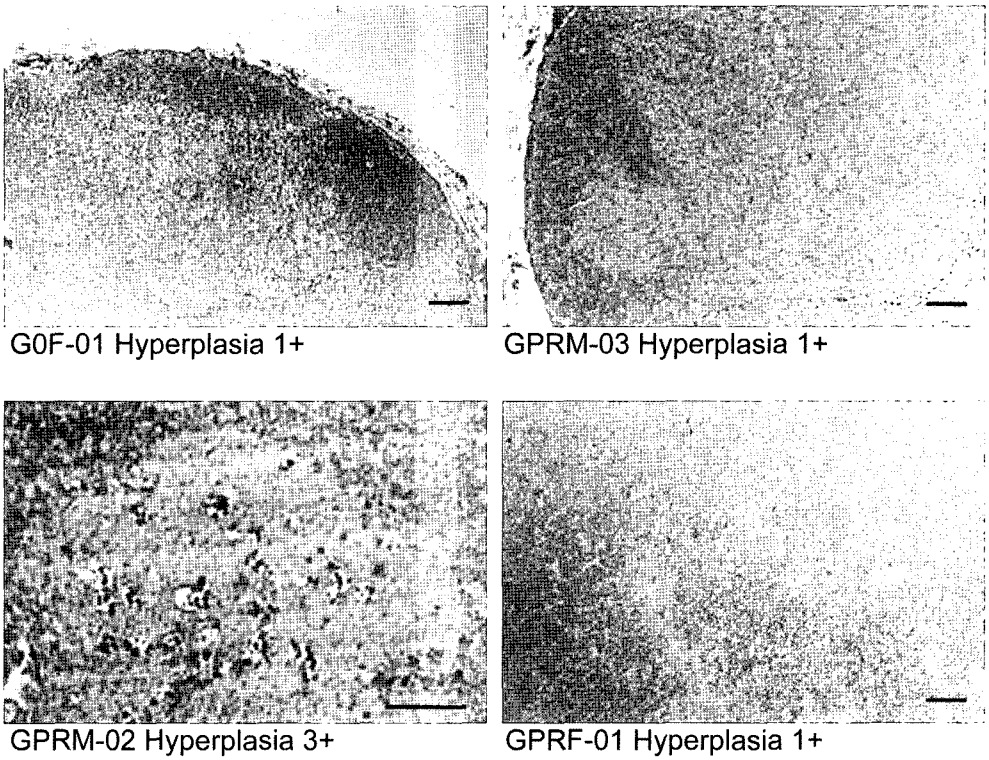

Fig. 1. Histopathological changes observed in the popliteal lymph node. Note that hyperplasia of lymphoid cells and follicles in the popliteal lymph node were detected in the present study. Although, these changes were also detected in some animals of vehicle control, the severities and frequencies encountered were increased in PR extracts-dosing female and male groups. All Hematoxylin & Eosin stain; Scale bars = 100 μm.

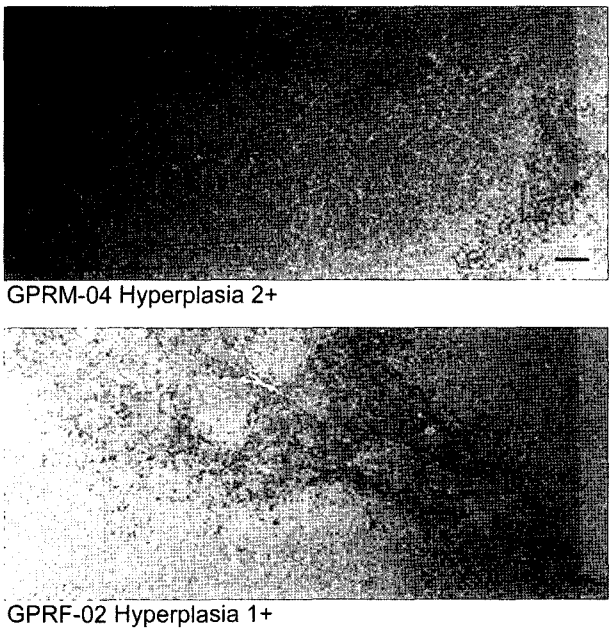

Fig. 2. Histopathological changes observed in the spleen. Note that hyperplasia of lymphoid cells and follicles in the spleen were detected and they were restricted to the PR extracts-dosing groups. All Hematoxylin & Eosin stain; Scale bars = 100 μm.

(Table 4, 5).

Necropsy findings

No PR extracts-treatment related gross findings of 12 principal organs were observed in all dosing groups tested compared to that of vehicle control except for increase of the incidence of hypertrophy of popliteal lymph nodes detected in PR extracts-dosing female and male groups, and some sporadic findings such as congestion of lung, atrophy of thymus, cyst and discoloration of kidney, spleen atrophy (Table 6).

Histopathological findings

Any PR extracts-treatment related histopathological findings of 12 principal organs were not observed in all dosing groups tested compared to that of vehicle control (Table 7) except for hyperplasia of lymphoid cells and follicles in popliteal lymph nodes (Fig. 1) and spleen (Fig. 2) detected in PR extracts-dosing groups, and some sporadic findings such as hypertrophy of lung alveolar wall and hemorrhage (Fig. 3), and focal pyometra and edematous changes in uterus (Fig. 4), and focal necrotic foci and fatty changes in liver (Fig. 5).

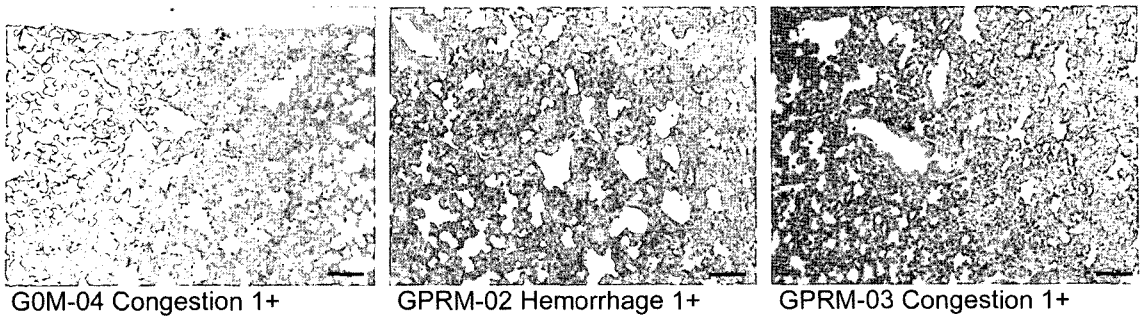

Fig. 3. Histopathological changes observed in the lung. Note that hypertrophy of alveolus and focal hemorrhages as congestion were randomly detected dispersed throughout the all tested groups including vehicle control. All Hematoxylin & Eosin stain; Scale bars = 100 μm.

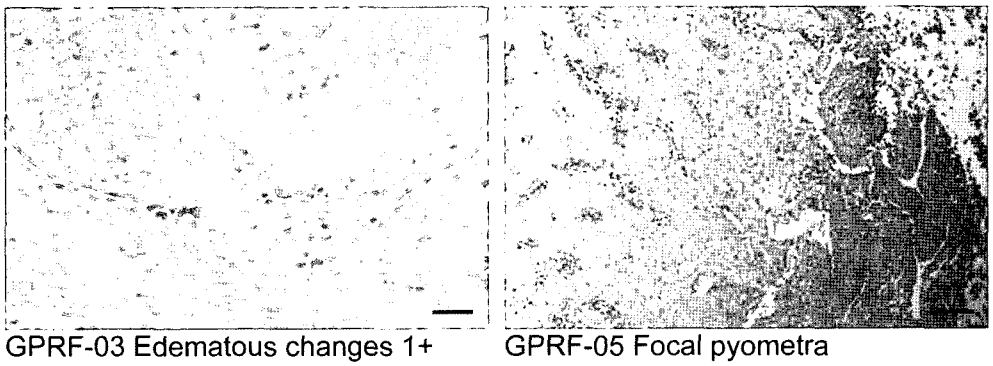

Fig. 4. Histopathological changes observed in the uterus. Note that edematous changes in uterus were restricted to two animals of PR extracts-dosing female group and are considered as a physiological signs related to the estrous cycle. In addition, one animal in PR extracts-dosing female group showed focal pyometra as one of accidental finding. All Hematoxylin & Eosin stain; Scale bars = 100 μm.

DISCUSSION

In the present study, we investigated the single oral dose toxicity with PR extracts, lyophilized water extract of *Pueraria thunbergiana* to mice as a part of the safety evaluation. In order to observe the LD_{50} and approximate lethal dose (ALD), test substances were administered orally to female and male ICR mice at dose levels of 2000mg/kg. As the results, we could not find any PR extracts treatment-related toxicological signs except for hair loss and hypertrophy of popliteal lymph nodes at gross observations and increase of the frequency of hyperplasia of lymphoid cells and follicles in spleen and popliteal lymph nodes, and some sporadic accidental findings at histopathological observations.

The increases in lymph node weight are secondary changes from the hypertrophy of the popliteal lymph nodes. Hypertrophy of lymph nodes detected in PR extracts-dosing groups was revealed as hyperplasia of lymphoid cells and follicles at histopathological observation, and these changes of immune system were also not considered as directly related to PR extracts-treatment toxicity but considered as the results of pharmacological effects by enhancing the immune system. In general, the hyperplasia of lymphoid cells and follicles were observed after treatment of the agents having the enhance effects on the immune systems (Lee *et al.*, 2005) and it is reported that saponins from the root of *Puerariae* showed preventive effects on *in vitro* immunological injury of rat primary hepatocyte cultures (Kim, 1996; Arao *et al.*, 1997). Generally, the animals enhanced immune system showed increase of the weights of spleen or lymphatic organs (Klein *et al.*, 1987; Iqbal *et al.*, 2001). Although, hyperplasia of lymphoid cells and follicles in popliteal lymph nodes were also detected in some animals of vehicle control, the severities and frequencies encountered were increased in PR extracts-dosing female and male groups. In addition, hyperplasia of lymphoid cells and follicles in spleen was observed in 4 or 5 animals restrict to PR extracts-dosing groups not in vehicle controls.

In KFDA Guidelines (2005-60) (2005) and OECD Guidelines (#401, #423) (1987, 2001), the recommended highest dose of test materials were 2000 mg/

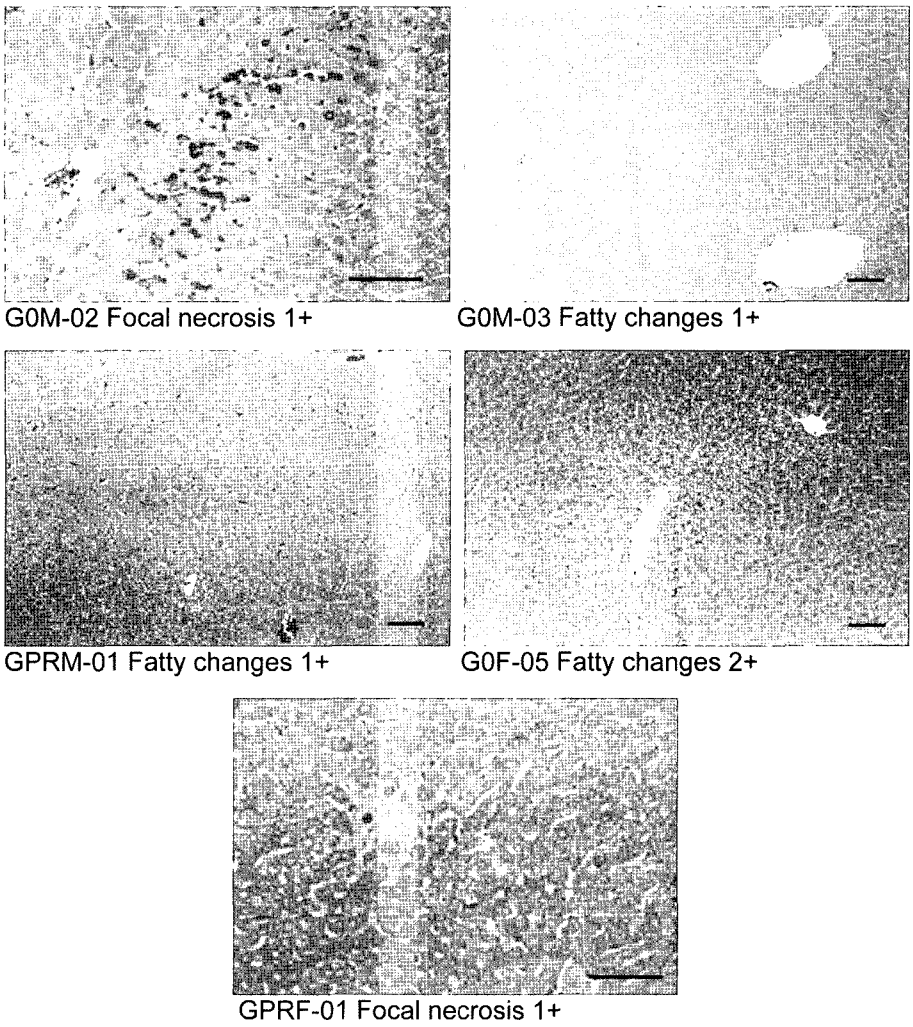

Fig. 5. Histopathological changes observed in the liver. Note that fatty changes and necrotic foci in the liver were randomly observed including vehicle controls. All Hematoxylin & Eosin stain; Scale bars = 100 μm.

kg, and they also recommended that in case of acute toxicity in mice, the dosage volume were below 20 ml/kg. In the present study, the highest dose of PR extracts was selected as 2000 mg/kg because PR extracts have been used as folk medicine and ingredients of medicinal food for long times and no revealed toxicological data was available, base on the recommendation of KFDA Guidelines (2005-60) (2005) and OECD Guidelines (#401, #423) (1987, 2001).

Hair loss sings detected in this study were not PR extracts-treatment related signs because they were also detected in the vehicle control even more frequencies encountered. The weight of pancreas sensitively changed at the weighing of sacrifice, and significant ($p < 0.05$) increases of the weight of pancreas detected in PR extracts-dosing groups was also considered as not PR extracts-treatment related changes because no changes on the histopathological profiles in the pancreas were observed, and the weight detected in this study well corresponded to the normal mice pancreas weight ranges as previous studies (Plata and Murphy, 1972; Yamaguchi *et al.*, 1983). Edematous changes in the uterus at histopathological observation detected in PR-extracts-dosing female group in the present study were also considered as results of differences of physiological estrus cycles (Banks, 1986; Pineda, 1989) not PR extracts-dosing relative changes.

Congestion of lung, atrophy of thymus, cyst and discolorization of kidney, spleen atrophy detected as gross findings, and hypertrophy of lung alveolar wall and hemorrhage, and focal pyometra and edematous changes in uterus, and focal necrotic foci and fatty changes in liver detected as histopathological findings were considered as accidental findings and they were

not considered as PR extracts-treatment related abnormal gross or histopathological findings because they were restricted in some individual animals and most of the case, they were also observed in vehicle control.

Although the Hodge and Sterner (1949) classify as non toxic materials those LD_{50} were 5000~15000 mg/kg and the materials those LD_{50} were 500~5000 mg/kg also classified as relatively low toxic (Class III) by US environmental Protection Agency [OPPTS 870.100; 1998], recently Notified Guidelines by Korean Food and Drug Administration [2005-60] and OECD Guidelines (#401, #423) (1987, 2001) recommended that the highest oral dose of test materials was 2000 mg/kg. The LD_{50} and ALD mice after single oral dose of PR extracts were considered as over 2000 mg/kg in both male and female in because no mortalities were detected upto 2000 mg/kg that was the highest dose recommended by KFDA and OECD. Therefore, oral gavage of PR extracts caused no serious toxic effect to the male and female mice up to 2000 mg/kg and is likely to be safe for clinical use.

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