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ORIGINAL PAPER

Toxicological evaluation of the isoflavone puerarin and its glycosides

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Abstract Puerarin, an isoflavone derived from kudzu roots, has strong biological activities. However, its bioavailability in vivo is often limited by its insolubility. A novel transglycosylase increases the solubility of puerarin >100-fold, by converting it to puerarin glycosides. Since over-consumption of an isoflavone might have toxic effects, therefore, we investigated the potential antimutagenic activity, bone marrow micronucleus test, and a 28-day oral repeated administration test with puerarin and its glycosides. In Ames tests, neither puerarin nor its glycosides exhibited mutagenic effects up to 200 μg/plate. Puerarin and its glycoside, glucosyl-α-(1,6)-puerarin, significantly reduced the mutagenic effect of 4-nitroquinoline-1-oxide by up to 41%. In bone marrow micronucleus tests using ICR mice, neither puerarin nor glucosyl-α-(1,6)-puerarin interfered with erythrocyte production in the bone marrow. Both compounds decreased the prevalence of polychromatic erythrocytes. Sprague–Dawley rats were orally dosed with puerarin and its glycosides daily for 28 days. Neither puerarin nor its glycosides caused significant alterations in histology, and biochemical and hematologic parameters. These results suggest that puerarin and its glycosides do not have significant toxic effects, at least in rodents, either in vitro or in vivo at doses of up to 250 mg/kg per day.

Keywords Puerarin · Glycosylation · Genotoxicity · Twenty-eight-day oral repeated administration test

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Introduction

Puerariae radix, the root of the familiar kudzu vine (*Pueraria lobata*) [1, 2], has been used in traditional Eastern Asian medicine to prevent and treat various symptoms including muscle and joint stiffness, neck and eye problems, and febrile diseases [3], and puerarin (daidzein 8-C-glucoside) is one of the major isoflavones in *Puerariae radix* [4]. Isoflavones are phytoestrogens that have various biological activities relevant to animal health [5]; genistin, for example, competes with estrogen for binding to the estrogen receptor and may prevent breast and prostate cancers in humans [6], regulate bone metabolism and prevent osteoporosis [7].

Previously, we showed that puerarin and its glycosides have antioxidative and hypocholesterolemic effects in vitro and in vivo [8], consistent with the report of Shen and Xie [9]. Puerarin also improves alcohol metabolism in the liver [10], regulates retinal functions [11] and may prevent Parkinson disease [12]. In spite of their potential beneficial effects, however, the biological activities of dietary isoflavones also raise issues of safety. For example, intake of isoflavone-containing soy products has been shown to raise plasma homocysteine levels in postmenopausal women [13] and may increase menopausal symptoms [14] and risk of breast cancer.

Isoflavone toxicity has been the focus of several studies. Genistein showed no mutagenic or clastogenic effects in a *Salmonella typhimurium* assay and in micronucleus tests, but in a mouse lymphoma assay appeared to act as a clastogen, markedly increasing the prevalence of small colonies and causing breaks in chromosomes [15]. In the in vivo toxicity tests, daily oral dosing of genistein at 1,000 mg/kg for 28 days appeared to have endocrine-disrupting effects and increased triglyceride levels, total protein levels and liver weights. As a result, 120 mg/kg per day of genistein was selected as the level at which no adverse effects were observed [16]. On the other hand, Wistar rats fed genistein at 50 mg/kg per day in dietary mixtures showed no adverse effects, whereas rats fed genistein at 500 mg/kg per day showed acute, subchronic and chronic toxicity [17].

The safety of puerarin in rats was examined in a toxicity study of the Chinese herbal preparation NPI-28 [18], which contained puerarin as a major component. Dosages of NPI-28 as high as 2 g/kg per day did not lead to specific harmful changes, and the authors concluded that a daily intake of 2 g/kg NPI-28 was safe for long-term use.

Other than the above-described study, few data are available for evaluating puerarin toxicity; therefore, we investigated the toxicity of puerarin and its water-soluble glycosides. Puerarin glycosides were enzymatically synthesized using the novel transglycosylase, *Bacillus stearothermophilus* maltogenic amylase (BSMA), to increase water solubility according to the previously described method [19]. Improving water solubility of isoflavones, such as puerarin, is advantageous in product development as foods and cosmetics might help increasing the bioavailability as well. To test genotoxicity, we performed the Ames test using *S. typhimurium* [20] and the micronucleus test using ICR mice [21]. We also performed a 4-week repeated oral administration test in Sprague–Dawley rats to determine the short-term toxicity of puerarin and its glycosides in vivo.

Our results suggest that puerarin and its glycosides are not mutagenic in vitro or in vivo. In fact, they may confer some protection against drug-induced mutagenicity. At dosages of up to 250 mg/kg per day, neither puerarin nor its glycosides induced the formation of abnormal erythrocytes in bone marrow, as assessed by the micronucleation of polychromatic erythrocytes in vitro, or affected the biochemical or hematological parameters of blood in rats in vivo.

Materials and methods

Puerarin and its glycosides

To increase its water solubility, puerarin was enzymatically altered using *Bacillus stearothermophilus* maltogenic amylase (BSMA) [19], resulting in the formation of puerarin glycosides with greatly increased water solubility, which were up to 168 times more than that of puerarin. In the synthesis, purified cornstarch (donor) and puerarin (>99%, acceptor) were incubated with the enzyme called BSMA. Then, BSMA cleaved glucose and maltose from the non-reducing end of cornstarch and simultaneously transferred to puerarin molecule to produce puerarin glycosides. In the in vitro mutagenesis and anti-mutagenesis tests, as well as in the micronucleus formation test, purified glucosyl-α-(1,6)-puerarin was used as the puerarin glycoside. In repeated oral administration tests for 28 days, rats were fed with puerarin or with a puerarin glycosides mixture containing glucosyl-α-(1,6)-puerarin [32% (w/w)], maltosyl-α-(1,6)-puerarin [39.8% (w/w)], glucosyl-α-(1,3)-puerarin [9.5% (w/w)] and residual carbohydrates [17.5% (w/w)]. This sample was partially purified with column chromatography after enzymatic reaction of BSMA. The residual carbohydrates were glucose, maltose, isomaltose and maltodextrins derived from cornstarch. Test formulations of puerarin and its glycosides were prepared by dissolving the compounds in deionized water. Deionized water was used as a vehicle control. The solutions were stored at 4 °C until use.

Salmonella mutagenicity test (Ames test)

The Ames test was performed according to a previously described method using *S. typhimurium* strains, TA100 and TA98 [20, 22], which were gifts from Professor S. S. Ham of Kangwon National University, Korea. *S. typhimurium* TA98, TA100 as well as TA102, TA1535 and TA1537 have been used for mutagenicity tests; however, we used TA98 and TA100 model strains for frame-shift mutation and base pair substitution, respectively. These two are the most commonly used strains in mutagenicity tests as well. Aliquots (50-μL) of puerarin or glucosyl-α-(1,6)-puerarin solution containing 50–200 μg of the test compound were dissolved in test tubes containing 100 μL of *S. typhimurium* culture media, and phosphate-buffered saline (PBS) was added to bring the final volume in each test tube to 700 μL. These mixtures were incubated with shaking at 120 rpm for 20 min at 37 °C. Then, 4 mL of top agar (45 °C) containing histidine/biotin was added to each test tube, and the contents of each test tube were poured onto minimal glucose agar plates. The plates were incubated for 48 h at 37 °C, and the mutagenicity of puerarin and glucosyl-α-(1,6)-puerarin was determined by counting His^+-revertant colonies.

Salmonella anti-mutagenicity test (Ames test)

To investigate anti-mutagenic effects of puerarin and its glycosides, the mutagen 4-nitroquinoline-1-oxide (4NQO;

Sigma, St. Louis, MO, USA) was used to induce mutagenesis [23]. The experimental procedure was the same as that described above for the *Salmonella* mutagenicity test, except that 50 μL of 4NQO solution was added to each test tube containing puerarin or glucosyl-α-(1,6)-puerarin and 100 μL of *S. typhimurium* culture media before the final volume was brought to 700 μL with PBS. Each plate contained 50–200 μg puerarin or glucosyl-α-(1,6)-puerarin and 0.15 μg 4NQO. As in the mutagenicity test, His^+-revertant colonies were counted to determine the anti-mutagenic effects of the test compounds. These effects were represented as the inhibition rates (%) of the compounds against the mutagen, calculated as shown below:

$$\text{Inhibition rate } (\%) = \frac{M - S_1}{M - S_0} \times 100$$

where M the number of His^+-revertant colonies on the positive control plates containing the mutagen, S_1 the number of His^+-revertant colonies on the sample plates containing the mutagen, and S_0 the number of His^+-revertant colonies (spontaneous revertants) on the negative control plates.

Micronucleus test with ICR mice

Animal care and handling were performed according to protocols approved by the Animal Experimentation and Ethics Committee of Korea University. Male ICR mice (4–5 weeks old; Samtako, Korea) were randomly assigned to groups and acclimatized for 1 week. Then, 0, 500, 1,000, 2,000 or 5,000 mg/kg of puerarin or glucosyl-α-(1,6)-puerarin was orally administered. Mitomycin C treatment (2 mg/kg) was used as a positive control [24]. At 36 h (normally 18–72 h), all mice were killed, their femurs extracted, and the femur bone marrow cells were flushed with syringes into tubes containing 0.5 mL fetal bovine serum (Wellgene Co, Seoul, Korea). The tubes were centrifuged at 1,000 rpm for 5 min, and the resuspended cell pellets were dropped onto clean, dry slides. Cells were fixed with absolute methanol and stained with 5% Giemsa stain in Sorensen's phosphate buffer (pH 6.8). To determine the percentage of erythrocytes that were polychromatic, 500 erythrocytes per animal were examined. The polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were identified, and the percentage of PCE/(PCE + NCE) was calculated [25]. The percentage of PCEs that were micronucleated (MNPCEs%) was determined from an analysis of 1,000 PCEs per animal.

Repeated (28-day) oral administration test

Animals

Male and female Sprague–Dawley rats (4 weeks old; Samtako, Korea) were randomly assigned to groups, and their health was observed while they were acclimatized for 1 week. Rats were housed separately in 22.5 × 20 × 19 cm^3 ($w \times l \times h$) stainless steel cages in an air-conditioned room maintained at 20 ± 3 °C with a relative humidity of 50 ± 10% on a 12-h/12-h light/dark cycle. The rats were allowed free access to water and a commercial solid diet (NIH31, Samtako, Seoul, Korea) except when fasted prior to killing [26]. All experimental protocols followed the guidelines suggested by the National Institute of Toxicological Research, Korea, and experiments were performed according to the animal protocol approved by the Animal Experimentation Committee of Korea University.

Oral administration of puerarin and its glycosides

During the 4-week toxicity test, puerarin and its glycosides were orally administered to 10–12 rats (5–6 of each sex) by gavage once daily at 10 a.m. The test dosages were 0 (vehicle control), 50, 250, 500 and 2,000 mg/kg and were determined based on previously published genistein experiments [15]. The rats were observed for clinical symptoms, visible health conditions and mortality twice daily (before and after dosing) following the method of Okazaki et al. [16]. Body weight, and food and water consumption were recorded once per week. At the end of the experiment, rats were fasted for 18 h and then killed using avertin (2,2,2-tribromoethanol; Sigma) as an anesthetic.

Biochemical and hematological parameters

Rats were fasted for 18 h before blood collection. Blood was collected in MiniCollect EDTA tubes (Greiner Bio-One, Austria) for biochemical analysis and in Vacuette K3EDTA tubes (Greiner Bio-One, Austria) for hematological analysis. Blood samples were centrifuged at 15,000 rpm for 10 min at 4 °C to collect plasma, and the plasma was biochemically analyzed for levels of total protein, cholesterol, glutamate pyruvate transaminase (ALTL), glutamate oxaloacetate transaminase (ASTL), glucose, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) using an automated clinical chemistry analyzer (Cobas C111; Roche, Madison, WI, USA). The hematological parameters, including hemoglobin concentration (Hb), hematocrit (Hct),

mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell count (WBC), red blood cell count (RBC) and platelet count were measured at the blood chemistry lab at the Korea University Anam Hospital (Seoul, Korea).

Necropsy

After the rats were killed, their brains, hearts, thyroid glands, livers, lungs, kidneys, adrenal glands, spleens, testes and ovaries were extracted and weighed. For investigation of potential liver damages with oral intakes of puerarin and its glycosides, pathologic examination of liver sections was performed with the aid of a pathologist at the Korea University Anam Hospital (Seoul, Korea). The liver was sectioned normally, fixed with 10% formaldehyde and stained with hematoxylin and eosin according to standard procedures [17].

Statistical analysis

All data were analyzed using the Student's *t* test for group comparisons. Results shown represent means ± SEM unless otherwise specified. Differences were considered statistically significant at $P < 0.05$.

Results

Salmonella mutagenicity and anti-mutagenicity tests

Treatment with puerarin or glucosyl-α-(1,6)-puerarin had no significant dose-dependent effect on the number of His^+-revertant colonies formed by TA98 or TA100 *S. typhimurium* (Table 1). The numbers of spontaneously His^+-revertant colonies in the negative control samples were within normal range (TA98: 15–30; TA100: 120–250). Both puerarin and glucosyl-α-(1,6)-puerarin dose-dependently inhibited 4NQO-induced mutagenesis in both the TA98 and TA100 strains, with a maximum inhibition rate of 41% (Fig. 1).

Micronucleus test with ICR mice

No signs of toxicity were observed in ICR mice during the 36-h micronucleus test period, and no mice died. Neither puerarin nor glucosyl-α-(1,6)-puerarin caused significant changes in the percentage of MNPCE at any dose compared with negative controls. However, the percentage of PCE/(PCE + NCE) was significantly decreased in groups treated with puerarin or glucosyl-α-(1,6)-puerarin at dosages higher than 500 mg/kg per day. The mitomycin C-dosed group (positive control) exhibited significant increases in the percentage of MNPCE and decreases in the percentage of PCE/(PCE + NCE), as expected (Table 2).

Four-week repeated oral administration test

Clinical symptoms and mortality

One female rat in the group treated with 2,000 mg/kg per day of puerarin died during the repeated oral administration test. However, its necropsy revealed no noticeable differences from those of the rats that lived to the end of the experimental period, also the organ weights and pathologic characteristics were not distinguishable from those of the other rats. However, both the male and female rats in the groups dosed with 2,000 mg/kg per day puerarin were less active than the rats in other groups. No specific clinical signs were observed in males or females of any of the other groups. None of the rats treated with puerarin glycosides

Table 1 Mutagenicity of puerarin and its glycosides in *Samonella typhimurium* TA 98 and 100

Dose (μl/plate)	Puerarin				Puerarin glycosides			
	His+ revertants/plate TA 98 (S9)		His+ revertants/plate TA100 (S9)		His+ revertants/plate TA 98 (S9)		His+ revertants/plate TA 100 (S9)	
	(−)[a]	(+)[b]	(−)[a]	(+)[c]	(−)[a]	(+)[b]	(−)[a]	(+)[c]
0	18 ± 5	14 ± 1	166 ± 5	112 ± 6	24 ± 3	14 ± 1	171 ± 2	213 ± 17
50	20 ± 1	14 ± 1	130 ± 13	100 ± 3	24 ± 4	22 ± 2	204 ± 4	203 ± 20
100	16 ± 2	14 ± 3	117 ± 11	125 ± 6	26 ± 0	17 ± 4	71 ± 39	224 ± 18
150	18 ± 1	22 ± 3	136 ± 10	120 ± 40	26 ± 4	13 ± 2	233 ± 32	210 ± 5
200	14 ± 1	16 ± 4	134 ± 25	118 ± 4	19 ± 4	15 ± 1	238 ± 30	189 ± 15
Positive controls	205 ± 7	151 ± 35	691 ± 25	460 ± 38	205 ± 7	151 ± 35	691 ± 25	542 ± 18

Each value represents the mean ± SE of three plates

Positive controls [a]2-aminoanthracene; [b]2-nitrofluorene; [c]sodium azide

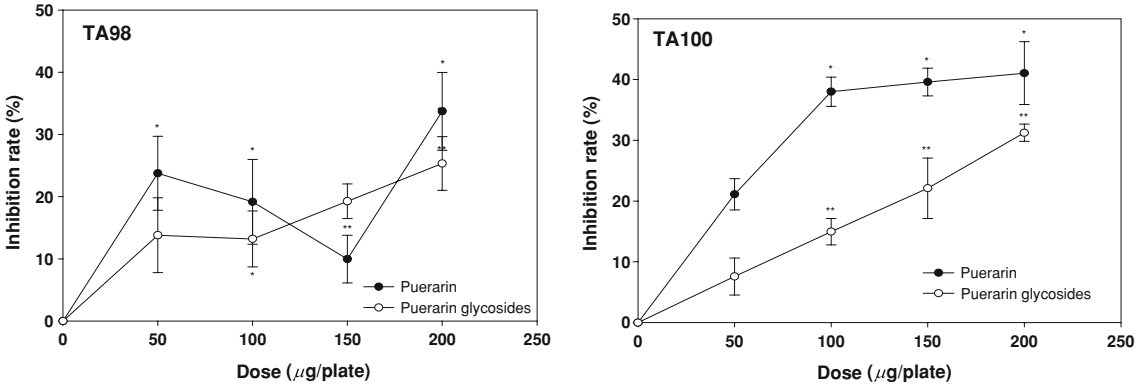

Fig. 1 Anti-mutagenic effects of puerarin and glucosyl-α-(1,6)-puerarin on mutagenesis induced by 4NQO (0.15 μg/plate) in *S. typhimurium* strains TA98 and TA100. Each value shown represents the mean ± SE of three replicates. *$P < 0.05$ compared to 4NQO-treated controls

Table 2 The bone marrow micronucleus test in male ICR mice administered puerarin and its glycosides

Compound	Dose (mg/kg)	No. of animals	Exposure time (h)	Mean ± SE	
				MNPCE[a] (%)	(%PCE[b])[c]/(PCE + NCE[d])
Puerarin	5,000	5	36	0.16 ± 0.05	9.36 ± 2.42*
Puerarin	2,000	5	36	0.18 ± 0.04	18.64 ± 5.09*
Puerarin	1,000	5	36	0.16 ± 0.04	13.60 ± 1.56*
Puerarin	500	5	36	0.12 ± 0.02	26.16 ± 4.63*
Puerarin-G	5,000	5	36	0.12 ± 0.02	23.92 ± 3.87*
Puerarin-G	2,000	5	36	0.14 ± 0.02	20.88 ± 2.19*
Puerarin-G	1,000	5	36	0.14 ± 0.04	22.64 ± 1.78*
Puerarin-G	500	5	36	0.12 ± 0.04	25.52 ± 2.84*
Negative Control	0	5	36	0.14 ± 0.02	39.68 ± 2.57
Mitomycin C	2	5	36	5.48 ± 0.78*	16.00 ± 2.32*

* $P < 0.05$ compared to negative control; Puerarin-G, puerarin glycosides containing 100% glucosly-α-(1,6)-puerarin

[a] %MNPCE (Micronucleated polychromatic erythrocytes/polychromatic erythrocytes) × 100

[b] PCE, polychromatic erythrocytes

[c] %PCE (PCE + NCE), PCE = [PCE/(PCE + NCE)] × 100

[d] NCE, normochromatic erythrocytes

exhibited any abnormal clinical symptoms or mortality during the 4-week oral administration period.

Body weight and food and water intake

Body weight and food and water consumption were recorded weekly for all rats. No changes in body weight or water or food intake were observed among the puerarin-treated groups. In the groups treated with puerarin glycosides at 500 or 2,000 mg/kg per day, body weights were decreased relative to those of the other groups. This result was statistically significant in both male and female rats after 2 weeks of treatment. However, water and food intake did not vary among the five groups (data not shown).

Hematological parameters

Most hematological parameters, such as Hb, Hct, RBC count, MCV, MCH, MCHC, RDW and WBC count were unaffected by puerarin treatment at any dose. In female rats, no notable differences in hematological parameters were observed. However, platelet counts were significantly lower in male rats dosed at more than 250 mg/kg per day of puerarin than in the negative controls (Table 3).

In rats treated with puerarin glycosides, the hematological parameters did not differ significantly among male rats dosed at less than 250 mg/kg per day. In male rats dosed at more than 500 mg/kg per day, the Hb, Hct and RBC count were higher than those of the controls. In male rats dosed at 2,000 mg/kg per day, the WBC and platelet

Table 3 Hematological parameters in male and female rats after 4 weeks of administration of puerarin or puerarin glycosides

Dose (mg/kg per day)	Male Puerarin/puerarin glycosides					Female Puerarin/puerarin glycosides				
	0	50	250	500	2,000	0	50	250	500	2,000
Parameters										
Hb[a] (g/dL)	15.4 ± 0.3[b]/ 15.5 ± 0.2	16.3 ± 0.3/ 15.3 ± 0.4	14.5 ± 0.3/ 15.7 ± 0.2	15.4 ± 0.4/ 16.3 ± 0.2*	14.4 ± 1.0/ 17.5 ± 0.3*	15.5 ± 0.4/ 16.3 ± 0.3	15.5 ± 0.3/ 16.0 ± 0.2	15.9 ± 0.2/ 15.7 ± 0.3	16.3 ± 0.2/ 16.2 ± 0.2	15.9 ± 0.2/ 16.2 ± 0.2
Hct[c] (%)	44.8 ± 0.3/ 46.3 ± 0.6	46.3 ± 1.0/ 45.9 ± 1.0	42.7 ± 2.0/ 47.4 ± 0.4	46.9 ± 1.8/ 48.7 ± 0.5*	42.0 ± 3.5/ 52.8 ± 1.4*	42.3 ± 1.9/ 48.1 ± 0.8	42.0 ± 1.4/ 48.1 ± 0.5	44.5 ± 0.4/ 46.7 ± 0.9	45.8 ± 0.5/ 48.3 ± 1.2	44.3 ± 0.5/ 48.6 ± 0.8
RBC Count[d] ($\times 10^3/\mu L$)	7.0 ± 0.5/ 7.9 ± 0.1	7.6 ± 0.1/ 7.8 ± 0.2	6.9 ± 0.2/ 8.2 ± 0.1	7.5 ± 0.2/ 8.5 ± 0.0*	6.9 ± 0.5/ 8.9 ± 0.2*	7.3 ± 0.4/ 8.5 ± 0.2	7.1 ± 0.2/ 8.5 ± 0.1	8.0 ± 0.1/ 8.2 ± 0.2	8.0 ± 0.1/ 8.5 ± 0.2	7.8 ± 0.0/ 8.6 ± 0.2
MCV[e] (fL)	59.7 ± 0.5/ 58.3 ± 0.6	60.7 ± 0.5/ 59.2 ± 1.4	61.4 ± 1.1/ 58.0 ± 0.5	62.4 ± 1.3/ 57.6 ± 0.5	60.3 ± 1.5/ 59.6 ± 0.3	58.0 ± 0.4/ 56.4 ± 0.8	59.1 ± 0.4/ 56.6 ± 0.7	56.4 ± 1.1/ 57.1 ± 0.3	57.0 ± 0.7/ 57.1 ± 0.6	57.0 ± 0.5/ 56.6 ± 0.6
MCH[f] (pg)	20.9 ± 0.2/ 19.5 ± 0.3	21.3 ± 0.2/ 19.7 ± 0.3	20.9 ± 0.4/ 19.2 ± 0.2	20.6 ± 0.5/ 19.2 ± 0.1	20.8 ± 0.4/ 19.8 ± 0.2	21.4 ± 0.8/ 19.1 ± 0.2	21.8 ± 0.3/ 18.9 ± 0.2	19.8 ± 0.2/ 19.1 ± 0.2	20.3 ± 0.3/ 19.2 ± 0.1	20.5 ± 0.2/ 18.8 ± 0.3
MCHC[g] (g/dL)	35.1 ± 0.1/ 33.4 ± 0.2	35.1 ± 0.2/ 33.2 ± 0.3	34.1 ± 1.2/ 33.2 ± 0.2	33.0 ± 1.3/ 33.4 ± 0.2	34.6 ± 1.3/ 33.1 ± 0.5	36.8 ± 1.1/ 34.0 ± 0.3	36.9 ± 0.7/ 33.3 ± 0.1	35.6 ± 0.0/ 33.6 ± 0.2	35.7 ± 0.2/ 33.7 ± 0.4	35.9 ± 0.1/ 33.3 ± 0.3
RDW[h] (%)	13.0 ± 0.1/ 13.3 ± 0.3	13.0 ± 0.3/ 13.4 ± 0.5	13.3 ± 0.2/ 13.4 ± 0.2	13.0 ± 0.2/ 13.5 ± 0.3	12.9 ± 0.3/ 14.7 ± 1.0	11.8 ± 0.3/ 12.5 ± 0.2	11.9 ± 0.2/ 13.0 ± 0.7	12.5 ± 0.2/ 12.8 ± 0.5	11.9 ± 0.3/ 13.0 ± 0.5	12.6 ± 0.2/ 13.3 ± 0.6

Hb hemoglobin concentration, *Hct* hematocrit, *RBC* red blood cell count, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red cell distribution width

counts were also significantly increased, but no hematological changes were found (Table 3).

Biochemical parameters

In groups dosed with puerarin at less than 500 mg/kg per day, the major biochemical parameters were unchanged. At high dosages, however, puerarin did cause some changes; for example, in the male rats dosed at 2,000 mg/kg per day, ASTLs were significantly higher than in the control groups, and dosages of puerarin of more than 500 mg/kg per day caused a dose-dependent decrease in total cholesterol. HDL and LDL levels were also dose-dependently decreased in the male rats. On the other hand, the female rats did not exhibit any significant changes in biochemical parameters (Table 4).

Treatment with puerarin glycosides at dosages of less than 250 mg/kg per day did not affect major biochemical parameters, but some changes were found in the high-dose groups. In male rats, dosing at more than 500 mg/kg per day caused a dose-dependent increase in total protein. At dosages higher than 250 mg/kg per day, total cholesterol, HDL and LDL levels were dose-dependently decreased. ALTL was significantly increased in the male group dosed at 2,000 mg/kg per day. In female rats, treatment with puerarin glycosides at any dose significantly decreased the total cholesterol level, and HDL and LDL levels trended similarly. The glucose and triglyceride levels were significantly increased in female rats dosed at more than 500 mg/kg per day, and the ASTL was significantly decreased in the 2,000 mg/kg per day group (Table 4).

Necropsy

Absolute weight of all organs exhibited no differences among groups. Uterus in female rats showed no noticeable alteration in appearance and absolute weights. Neither puerarin nor its glycosides affected absolute organ weights in any male or female group. However, some changes in relative organ weights were observed, particularly in the high-dose groups. For example, in groups dosed at more than 500 mg/kg per day puerarin, the relative lung, adrenal gland, thyroid gland and spleen weights were increased in both sexes, and in groups dosed with puerarin glycosides at more than 250 mg/kg per day, changes were observed in the relative brain, adrenal gland and kidney organ weights in both sexes (data not shown)

Liver histology

After killing, livers were sectioned and examined histologically. Some abnormalities, such as swollen hepatocytes with nuclear pleomorphism, prominent nucleoli, occasional

Table 4 Biochemical parameters in male and female rats after 4 weeks of administration of puerarin or puerarin glycosides

Dose (mg/kg/day)	Male Puerarin/puerarin glycosides					Female Puerarin/puerarin glycosides				
	0	50	250	500	2,000	0	50	250	500	2,000
Parameters										
Total protein (g/dL)	5.5 ± 0.1[a]/ 5.5 ± 0.0	5.7 ± 0.1/ 5.4 ± 0.1	5.5 ± 0.1/ 5.7 ± 0.1	5.8 ± 0.2/ 5.8 ± 0.1*	5.9 ± 0.2/ 6.2 ± 0.2*	5.7 ± 0.1/ 5.7 ± 0.1	6.0 ± 0.2/ 5.6 ± 0.1	6.0 ± 0.1/ 5.7 ± 0.1	5.9 ± 0.1/ 6.0 ± 0.1	6.0 ± 0.1/ 5.9 ± 0.1
Cholesterol (mg/dL)	88.2 ± 8.8/ 71.5 ± 1.7	87.0 ± 4.0/ 76.2 ± 4.1	67.1 ± 6.6/ 54.9 ± 4.8*	47.5 ± 11.6*/ 51.4 ± 2.0*	41.7 ± 3.0*/ 41.9 ± 4.8*	64.5 ± 7.7/ 83.7 ± 6.8	76.0 ± 10.3/ 61.6 ± 2.4*	57.6 ± 17.3/ 44.5 ± 7.1*	53.8 ± 12.6/ 35.2 ± 4.4*	59.6 ± 12.4/ 43.4 ± 6.8*
ALTL (U/L)	33.6 ± 1.7/ 33.0 ± 2.2	38.1 ± 2.4/ 55.5 ± 17.7	33.9 ± 1.4/ 36.0 ± 0.7	34.8 ± 2.4/ 39.3 ± 4.4	37.9 ± 4.0/ 41.9 ± 4.8	26.1 ± 0.9/ 29.6 ± 1.1	25.4 ± 2.0/ 25.8 ± 1.4	25.3 ± 2.2/ 53.0 ± 28.3	29.0 ± 1.5/ 26.8 ± 3.1	26.7 ± 3.5/ 25.0 ± 2.5
ASTL (U/L)	85.7 ± 7.1/ 84.4 ± 4.6	90.8 ± 7.31/ 46.4 ± 42.0	86.7 ± 4.0/ 85.6 ± 3.7	86.7 ± 3.9/ 105.3 ± 11.9	113.5 ± 6.6*/ 111.4 ± 15.3	104.7 ± 6.8/ 92.2 ± 4.2	97.6 ± 9.6/ 81.3 ± 2.6*	95.4 ± 6.1/ 162.7 ± 77.3	112.2 ± 8.7/ 149.9 ± 51.9	83.2 ± 5.8/ 73.1 ± 4.2*
Glucose (mg/dL)	74.9 ± 17.0/ 168.1 ± 7.5	91.9 ± 9.3/ 171.4 ± 12.0	85.7 ± 4.0/ 139.4 ± 4.4*	95.3 ± 7.1/ 143.7 ± 7.8*	81.3 ± 15.0/ 126.3 ± 11.9*	80.0 ± 8.5/ 118.8 ± 16.2	89.8 ± 4.1/ 158.6 ± 7.1	88.8 ± 4.8/ 159.0 ± 4.8	74.8 ± 8.6/ 193.1 ± 26.2*	79.9 ± 13.7/ 169.7 ± 10.8*
Triglyceride (mg/dL)	30.9 ± 6.0/ 17.6 ± 1.7	43.3 ± 4.8/ 21.7 ± 1.4	27.7 ± 3.8/ 20.0 ± 2.1	34.2 ± 4.0/ 26.4 ± 2.6*	42.7 ± 6.5/ 34.7 ± 4.0*	23.6 ± 2.4/ 17.2 ± 1.3	21.9 ± 3.6/ 21.4 ± 4.5	25.3 ± 1.9/ 20.6 ± 1.6	27.8 ± 3.6/ 35.1 ± 7.6*	25.7 ± 4.0/ 18.3 ± 1.5
HDL (mg/dL)	71.3 ± 6.3/ 59.2 ± 1.4	70.9 ± 4.8/ 62.0 ± 3.4	58.1 ± 5.5/ 46.0 ± 4.2*	40.1 ± 11.0*/ 43.9 ± 2.0*	35.1 ± 3.1*/ 41.0 ± 3.0*	55.9 ± 6.2/ 66.0 ± 4.3	68.1 ± 8.9/ 54.2 ± 2.2*	51.2 ± 16.1/ 37.7 ± 6.1*	45.6 ± 12.5/ 28.6 ± 5.0*	53.6 ± 12.1/ 37.3 ± 6.7*

Hb hemoglobin concentration, *Hct* hematocrit, *RBC* red blood cell count, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red cell distribution width, *ALTL* alanine transaminase, *ASTL* aspartate transaminase, *HDL* high-density-lipoproteins

appearance of double nuclei, and clear, vacuolated cytoplasm, were observed in some of the rats in groups dosed with puerarin or puerarin glycosides at 2,000 mg/kg per day. Other groups did not exhibit any specific histological differences from the control groups (Fig. 2).

Discussion

Puerarin is a major isoflavone found in the roots of the kudzu plant, *Pueraria lobata*. As in other isoflavones, puerarin has several properties beneficial to health, such as the ability to reduce cholesterol and regulate glucose metabolism [8]. However, some concerns regarding the safety of isoflavones have been reported. For example, genistein can cause reproductive and development toxicity if exposure occurs during early, critical periods of development, especially puberty, and at high concentration (Lamartiniere et al. 1998) [5]. Moreover, both genistein and daidzein are toxic to rat primary neuronal culture at high concentration [6, 7, 27, 28]. Genistein could potentially suppress male fertility via inhibited sperm capacitation and suppression of acrosome reaction at higher doses, whereas it can stimulate male fertility by promoting acrosome reaction at lower doses (Kumi-Dika and Townsend 2003) [8]. However, limited information is available on the potential toxicity of puerarin and thus we chose to investigate it.

We examined the genotoxic effects of puerarin and its glycosides at various concentrations in vitro using the Ames test with *S. typhimurium* strains, TA98 and TA100. The data revealed no dose-dependent induction of His^+-reversion by puerarin or glucosyl-α-(1,6)-puerarin and, furthermore, these compounds exhibited some activity against mutagenesis. The number of colonies exhibiting 4NQO-induced His^+-reversion was decreased by up to 41%. Summing up, plates containing less than 200 μg of puerarin or glucosyl-α-(1,6)-puerarin were not mutagenic in vitro and, in fact, displayed anti-mutagenic effects against the mutagen 4NQO.

In our next experiments, we investigated the potential genotoxicity of puerarin and glucosyl-α-(1,6)-puerarin in vivo using ICR mice. As a positive control, we used mitomycin C, which can alter the structure or number of chromosomes, thus decreasing the percentage of erythrocytes that are PCEs [%PCE/(PCE + NCE)] while increasing the percentage of PCEs that are MNPCEs (%MNPCE). However, feeding of puerarin or glucosyl-α-(1,6)-puerarin at more than 500 mg/kg per day did not significantly influence the percentage of MNPCE values, suggesting that these compounds do not disrupt normal cell division by damaging chromosomal structure. However, we found that extremely high doses of puerarin or glucosyl-α-(1,6)-puerarin (e.g., 500 mg/kg) caused erythrocyte numbers to change over

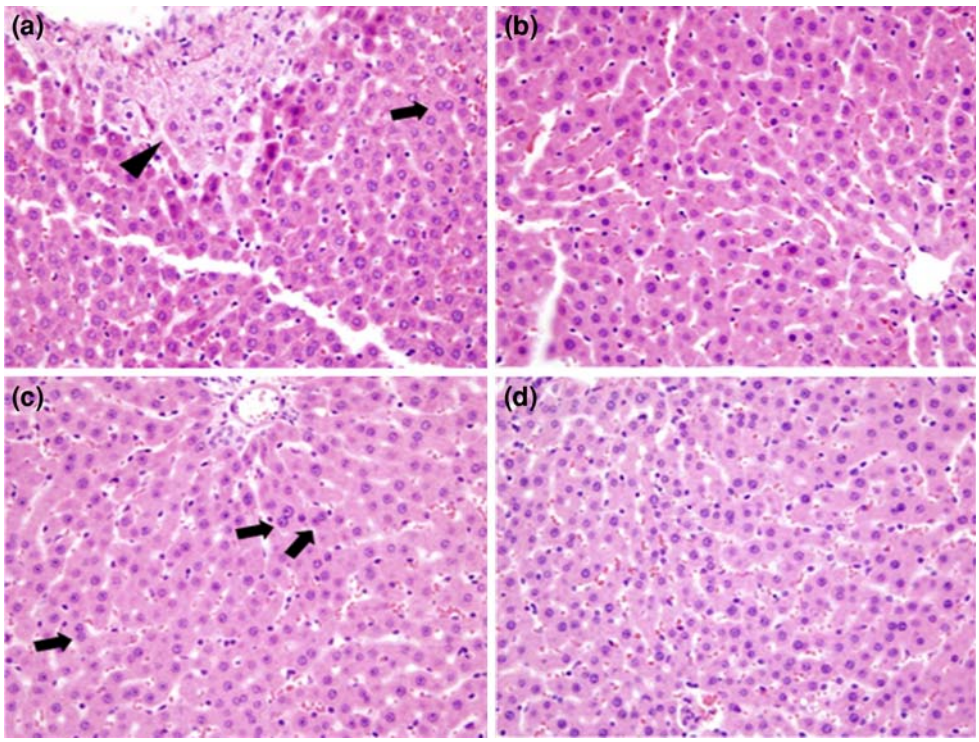

Fig. 2 Microscopic images of hematoxylin- and eosin-stained rat livers after 4 weeks of repeated administration of puerarin or puerarin glycosides. **a** Puerarin, 2,000 mg/kg per day (male rats); **b** control rats (male); **c** puerarin glycosides, 2,000 mg/kg per day (female rats); **d** control rats (female). All images are at ×400. *Arrows* indicate double nuclei, and *arrowheads* indicate swollen hepatocytes with clear, vacuolated cytoplasm

36 h. In groups dosed at more than 500 mg/kg, the percentage of PCE/(PCE + NCE) value was dramatically decreased, to a level similar to that of the positive controls, within 36 h. We concluded that puerarin and glucosyl-α-(1,6)-puerarin at dosages greater than 500 mg/kg might adversely affect erythrocyte formation in the bone marrow within 36 h. Therefore, we conclude that a safe dose of puerarin or glucosyl-α-(1,6)-puerarin would be 500 mg or less of puerarin or glucosyl-α-(1,6)-puerarin per kg.

To assess the potential toxicity of puerarin and its glycosides in short-term administration in rats, a 28-day repeated oral administration test was performed. During this test, one female rat dosed at 2,000 mg puerarin/kg per day died, and some others dosed at the same level exhibited decreased physical activity, although necropsy results did not suggest any specific abnormalities in these rats. These findings suggest that puerarin is harmful at this dosage level, and the median lethal dose (LD_{50}) of oral puerarin might be slightly greater than 2,000 mg/kg per day. Body weights and food and water intake amounts did not vary significantly among treated rats, as expected. However, the body weights of the rats in the high-dose groups (>250 mg/kg per day) were reduced in a dose-dependent manner after 3–4 weeks of treatment, suggesting that puerarin and its glycosides might be harmful at this dosage.

In male and female rats dosed at less than 250 mg/kg per day, neither puerarin nor its glycosides influenced hematological parameters, including Hb, Hct, MCV, MCHC, RDW, and WBC, RBC, and platelet counts. However, in male rats dosed at more than 500 mg/kg per day, Hb, Hct, and WBC, RBC, and platelet counts were altered. In female rats, treatment with puerarin or its glycosides was not associated with any major changes or specific trends in hematological parameters. These compounds did not affect ALTL or ASTL, which are markers for liver damage, in either sex in groups dosed at less than 500 mg/kg per day, although plasma lipid and glucose levels fluctuated slightly.

Reductions in total cholesterol, and HDL and LDL levels were found only in the high-dose groups, in contrast to our previous results obtained with mice [8]. Puerarin appeared to effectively regulate cholesterol levels in rats, and puerarin glycoside dosages of greater than 250 mg/kg per day decreased glucose levels in male rats. This result is consistent with the observed hypoglycemic effect of puerarin in C57BL/6J-ob/ob mice [29], although the dosages used in the present study were higher than those used by Meezan et al. Since ASTL and ALTL were increased in rats dosed at 2,000 mg/kg per day, dosages of puerarin and its glycosides of less than 500 mg/kg per day may not be hepatotoxic.

Necropsy results revealed no toxicologically relevant observations in any of the rat groups. Neither puerarin nor its glycosides affected the absolute organ weights in any group, but relative organ weights did fluctuate in the high-dose groups. Histopathologic and morphologic changes suggesting liver damage were found in groups dosed with puerarin or its glycosides at 2,000 mg/kg per day, but not in other groups, suggesting that at excessively high doses

(>2,000 mg/kg per day) these compounds may cause liver damage, as suggested by the biochemical changes in ALTL and ASTL.

In conclusion, our investigation of the potential toxicity of puerarin and its glycosides revealed no mutagenicity in vitro and a protective effect against drug-induced mutagenicity (up to a 41% decrease). Furthermore, neither puerarin nor its glycosides induced the formation of abnormal erythrocytes in bone marrow in vivo, as assessed by the percentage of MNPCE. At dosages of less than 250 mg/kg per day, they did not affect liver enzyme levels, hematological parameters or liver histology. Therefore, we conclude that moderate consumption of puerarin and its glycosides is largely safe at dosages of less than 250 mg/kg per day, at least in rodents, for short-term administration. Further studies are required to ensure the safety of puerarin and its glycosides in humans.

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