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TOXICITY STUDY ON CHINESE HERBAL DRUGS USING THE MICRONUCLEUS ASSAY IN MURINE BONE MARROW ERYTHROCYTES

Ian C. Guest, Sang Ou Yoo, Nam Won Paik and Young Wook Lee
Ki Bong Oh*, Hyeong Cheol Yang*, Nan Joo Suh* and Il-Moo Chang*

*Graduate School of Public Health and Natural Products Research Institute**
Seoul National University, Seoul 110-460, Korea

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ABSTRACT: *A mouse whole animal bioassay was employed to screen for potential mutagenicity of ethanol/water extracts of 16 Chinese herbal drugs that are commonly prescribed in Korea. Specific cytogenetic toxicity was measured by recording evidence of clastogenesis via the mouse bone marrow micronucleus test. Male ICR mice administered ethanol extract of Pinelliae tuber (Pinellia eternata Breitenbach, ARACEAE, 半夏) and ddY female mice administered extract of Angelica Koreanae radix(Angelica Koreana Maximowicz, UMBELLIFERAE, 羌活) (both by oral administration, at a dose of 600 mg/kg), in a short-term dosing schedule, demonstrated significant increase in micronucleated polychromatophilic erythrocytes, indicating the increase of clastogenicity.*

Key words: *Toxicity of Chinese herbal drugs, Micronucleus assay, Clastogenicity/Mutagenicity of Pinelliae tuber(Pinellia ternata Breit.) and Angelicae koreanae radix(Angelica koreana Max.)*

INTRODUCTION

Although Western purified drugs have been subject to ever-increasingly strict safety regulations, herbal drugs (traditional Chinese medicines, derived from plants, that have appeared in Chinese pharmacopoeia for over 1,000 years) have until recently been subject to only limited toxicity testing, in Korea as well as in Japan and China. Some rather specialized studies (Chang *et al.*, 1982; Kalantari-Gotvandi *et al.*, 1986) and one comprehensive clinical study (Wang and Hu, in Chang *et al.*, 1984) have indicated that toxicity is not uncommon, sometimes being reported in up to 40% of herbal drug prescriptions (Chang, 1986).

Herbal samples that are used to make prescriptions are roots, stems, leaves and/or seeds of plants and so typically contain hundreds of different natural ingredients. With the realization that Chinese medicinal prescriptions are often polyprescriptions, con-

taining several different herbal drugs, the potential for toxicity becomes apparent. In regard to herbal drug extracts, representing complex mixtures of many unidentified ingredients, successful use of the Ames' mutatest, the SOS chromotest and the SOS umu test has been demonstrated (Chang *et al.*, 1987, 1989; Whong *et al.*, 1986).

The aim of this research was to determine if extracts of some commonly prescribed herbal drugs have any clastogenic effect in murine bone marrow. Twelve ethanol extracts and four water extracts of 16 different herbal drugs were administered to mice by oral intubation in a short-term dosing schedule. Then, incidence of micronuclei were recorded to examine potential clastogenicity.

MATERIALS AND METHODS

Animals

ICR male mice were obtained from the animal breeding laboratory, Seoul National University. ICR females were obtained from Yu Han Pharmaceutical Company, Seoul. In all cases mice were acclimatized for at least one week in our own animal laboratory at the Natural Products Research Institute. Mice were houses in plexiglass cages lined with wood shavings, maintained in a 12 hour light/dark cycle at 22°C ± 3° and 70% ± 10% humidity and given food (Laboratory Animal Pellets, Samyang Corporation, Seoul) and water *ad libitum*.

Chemicals

Cyclophosphamide (sodium salt, Lot Nos. LJ6BL, LJ7BU and LJ7BV) was obtained from JoongWeh Pharmaceutical Company, Seoul. Mitomycin C (Lot Nos. 384AGB and 305AFC) was from Kyowa Hakko Kogyo Co. Ltd., Tokyo. Harris' Haematoxylin (prepared solution) was purchased from Kukche Pharmaceuticals, Seoul. Both Giemsa and Eosin Y were obtained from Matheson, Coleman and Bell, Los Angeles, California. Hank's balanced buffered solution -HBBS (NaCl 8 g, KCl 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, Na_2HPO_4 0.048 g, $CaCl_2 \cdot 2H_2O$ 0.185 g, $NaHCO_3$ 0.35 g, KH_2PO_4 0.06 g, $MgCl_2 \cdot 6H_2O$ 0.1 g, glucose 1.0 g, distilled water 1 litre; adjusted to pH 7.2) was made fresh every week (Na_2HPO_4 and KH_2PO_4 were dried in an oven at 110°C for several hours before weighing). Sorensen's buffer used with the Giemsa stain was: KH_2PO_4 1.63 g, Na_2HPO_4 2.56 g, distilled water 1 litre; adjusted to pH 6.8. Canada balsam was from Merck.

Herbal Drugs

A list of the herbal drugs used in this research appears in Table 1. All were purchased from a local traditional pharmacy and taxonomically identified in the Natural Products Research Institute. Extracts were prepared according to the scheme outlined in Fig. 1. Extracts were dissolved in distilled water and except where noted were prepared to give a dosage of 600 mg/kg in a volume of 0.2 ml.

Animal Dosing and Treatment

Every mouse in each test group was administered a herbal drug extract once a day

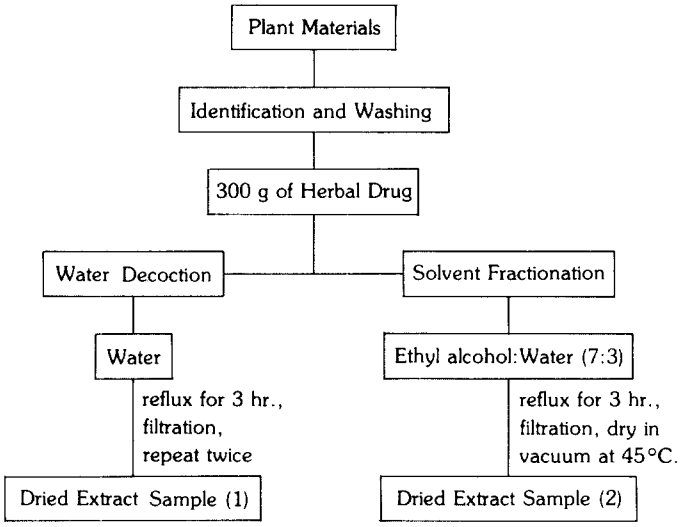

Fig. 1. Preparation of herbal drug extracts (8).

for 5 consecutive days. The injection was by oral intubation and always given between 7 and 8 a.m. Mice were housed 10 to a cage for the control groups and 11 per cage (10 test mice plus 1 positive control) for the treatment groups. They were killed by cervical dislocation 24 hours after the last dose.

Positive and Negative Controls

Negative control groups received distilled water p.o., 0.2 ml/mouse/day for 5 consecutive days. Positive control groups for baseline pilot studies received several dosages of two different carcinogens, as detailed in Table 2. Positive controls for the micronucleus assay involved one mouse housed with each treatment group, receiving identical dosing to the herbal drug treated mice, with cyclophosphamide replacing the herbal drug extract.

Micronucleus Assay

Mice were killed by cervical dislocation after being anaesthetized for the blood collection. The left rear femur and tibia, as one unit from the pelvis to the ankle, was removed, stripped of muscle and the femur was isolated. Smears were made according to the paintbrush technique (Albanese and Middleton, 1987) with slight modification. Briefly, the femur was split lengthwise and the tipe of a fine paintbrush, moistened in HBBS, was brushed lightly along the length of the exposed marrow, from the epiphyseal joint towards the centre of the femur. The paintbrush was then brushed along the length of a slide 4 times, to create 4 parallel bands. One slide was made per mouse. Slides were dried overnight at room temperature, fixed in 95% methanol for 10 minutes and dried. They were stained with haematoxylin and eosin, following the method of Pascoe and Gatehouse (Pascoe and Gatehouse, 1986). The slides were mounted with Canada balsam and examined at 400X and 1,000X.

For micronucleus scoring, 1,000 PCE's (polychromatophilic erythrocytes) were

counted per slide and the incidence of micronucleated PCE's was recorded. The ratio of PCE's to NCE's (normochromatophilic erythrocytes) was also recorded by noting how many PCE's were concurrently scored when 200 NCE's were tabulated.

Statistical analysis of the frequency of micronucleated PCE's was carried out by use of X^2 analysis.

RESULTS AND DISCUSSION

Table 1 show the herbal drugs used in the experiments. They were extracted with water or ethanol as shown in Fig. 1. Four herbs, Anglicae Koreanae radix, Ephedrae herba, Pinelliae tuber and Scutellariae radix were extracted with water, and others were extracted with ethanol.

As the data in Table 2 show, cyclophosphamide was used as a positive control and it showed highly increased micronucleus frequencies. Of the 16 herbal drugs tested, two gave positive, significant results in the micronucleus assay. Extracts of Pinelliae tuber and Angelicae koreanae radix caused micronucleated PCE frequencies of 5.1(negative control, 1.9) and 4.1(negative control, 1.6), $p < 0.05$.

Slight differences existed in the background micronucleus frequency among the four (ICR male, ICR female, ddY male and ddY female) negative control groups but all the values were within normally observed baseline frequencies (Mac Gregor, *et al.*, 1987). Small differences do exist between sexes and among strains, in response to particular compounds; male mice are sometimes more sensitive (and so recommended in the

Table 1. Herbal drugs used in the micronuleus test

Name of Herbal Drugs	Name in Chinese Script	Botanical name
Angelicae Koreanae Radix	羌活	Angelica koreana Maximowicz
Glycyrrhizae Radix	甘草	Glycyrrhiza glabra L. var. grandulifera
Ginseng Radix alba	人蔘	Panax ginseng C.A. Meyer
Pinelliae Tuber	半夏	Pinellia ternata Breit
Paeoniae Radix	白芍藥	Paeonia lactiflora Pall
Paeoniae Radix rubra	赤芍藥	Paeonia albiflora var. hortensis Makino
Scutellariae Radix	黃芩	Scutellaria baicalensis Geor.
Zingiberis Rhizoma	生薑	Zingiber officinale Rosco
Malvae Semen	冬葵子	Malva verticillata Linne
Ephedrae Herba	麻黃	Ephedra sinica Stapf
Scrophulariae Radix	玄蔘	Scrophularia buergeriana Miquel
Alismatics Rhizoma	澤瀉	Alisma orientale Juzepezuk
Gentianane scabrae Radix	龍膽	Gentiana scabra Bunge var. buergeri Max
Plantaginis Semen	車前子	Plantago asiatica
Zizyphi jujubae Fructus	大棗	Zizyhus jujuba Mill var. inermis
Anthrisci Radix	前胡	Anthriscus sylvestris Hoffmann

Table 2. The number of micronucleated PCE's among 1,000 PCE's and the PCE/NCE ratio in mice fed herbal extracts(i).

Treatment (ii)	Dose (mg/kg) body weight)	Mouse strain and sex	No. of mice	No. of PCE analyzed	No. of MNPCE/ 1,000 PCE (mean ± SE)	PCE/NCE ratio (mean)	Positive Control (iii)
Negative Control (iv)		ddY male	20	16,000	1.9 ± .33	.77	
Pinelliae Tuber	600	ddY male	10	10,000	3.8 ± .74	.66	10
Pinelliae Tuber (repeat)	600	ddY male	10	10,000	5.1 ± .67*	.74	15
Pinelliae Tuber (repeat)	300	ddY male	10	10,000	2.3 ± .40	.64	13
Gentianae scabrae Radix	600	ddY male	10	9,000	1.6 ± .41	.77	27*
Ginseng Radix alba	600	ddY male	10	10,000	1.7 ± .78	.65	13
Zingiberis Rhizoma	600	ddY male	10	10,000	2.9 ± .48	.84	10
Negative Control (iv)		ddY female	10	10,000	1.6 ± .51	.76	
Scrophulariae Radix	600	ddY female	10	10,000	3.3 ± .47	.76	15
Anthrisci Radix	600	ddY female	10	10,000	2.5 ± .31	.80	13
Angelicae Koreanae Radix	600	ddY female	10	10,000	4.1 ± .38*	.79	22
Plantaginis Semen	600	ddY female	10	10,000	2.1 ± .59	.72	35*
Scutellariae Radix	600	ddY female	10	10,000	1.7 ± .30	.72	42*
Negative Control (iv)		ICR male	20	15,000	1.73 ± .35	.82	
Paeoniae Radix	600	ICR male	10	9,000	1.5 ± .38	.71	7
Paeonia Radix rubra	600	ICR male	10	10,000	0.9 ± .23	.47	14
Alismatis Rhizoma	600	ICR male	10	10,000	1.6 ± .31	.91	20
Glycyrrhizae Radix	600	ICR male	10	10,000	1.7 ± .21	.47	11
Negative Control (iv)		ICR female	20	19,000	1.7 ± .25	.80	
Malvae Semen	600	ICR female	10	10,000	2.8 ± .48	.76	22
Ephedrae Herba	100	ICR female	10	9,000	3.9 ± .56	.88	11
Zingiberis rhizoma	600	ICR female	10	10,000	3.9 ± .69	.86	25
Zizyphi Fructus	600	ICR female	10	10,000	2.0 ± .37	.71	18

i. Dosing schedule: once a day for 5 consecutive days; sacrifice on day 6. All mice were 20 ± 2 grammes. Group size-10 mice

ii. For all treatments oral intubation was used, volume 0.2 ml.

iii. The number is that of MIPCE's in 1,000 PCE's in one mouse housed with the corresponding treatment group and fed cyclophosphamide at 25 mg/kg in the same dosing schedule as the treatment group. * indicates dose of cyclophosphamide was 50 mg/kg.

iv. Distilled water replaced herbal drug extract; same dosing schedule.

*Significant at $p < 0.05$

standard micronucleus test protocol) but any compound known to induce micronuclei in males also induces micronuclei in females (Aeschbacher, 1986; Collaborative study group, 1986).

The PCE/NCE ratio compares the turnover of immature erythrocytes (PCE's) to the more stable, mature erythrocyte (NCE) population and serves as an indicator of bone marrow depression by reflecting reduced maturing-cell populations (Albanese *et al.*, 1987; Amphlett *et al.*, 1984). With the exception of extracts of Paeonie radix rubra and Glycyrrhizae radix, both of which exhibited a PCE/NCE ratio of less than 0.5, all the herbal extracts did not affect this ratio in any significant way to deviate the values substantially from the control values (in the neighbourhood of 0.75). Extracts of Paeoniae radix rubra and Glycyrrhizae radix appear to be toxic to the bone marrow, as reflected by a reduced population of PCE's. Neither extract was positive in the micronucleus test.

Although oral intubation, when compared to intraperitoneal injection, gives a less decisive, less efficient dose to the intracellular space, it was chosen in order to duplicate the route of exposure of herbal drug extracts in humans. In fact, an interesting consequence of oral feeding might have appeared, as discussed below.

Most of the herbal extracts tested were ethanol preparations (12 extracts were ethanol concoctions, the remaining 4 were water decoctions, which is the common method of preparation for human prescriptions). Ethanol (alcohols in general) as a partition solvent preferentially dissolves glycosides (as does water) amongst other natural constituents when natural products are extracted in a scheme similar to that shown in Figure 1 (Chang, 1986).

Glycosides occur widely in the plant kingdom and in man's diet. Flavonoids and anthocyanins, the pigments of flowers and berries, are glycosides, as are many chemicals responsible for flavours and fragrance (Luckner, 1972; Torssell, 1983). Glycosides that have previously been negative in the Salmonella mutatest become mitagenic upon hydrolysis of the glycosidic linkage, which occurs by the action of enzymes present in the bacterial flora of man and animals (Batzinger *et al.*, 1978; Tamura *et al.*, 1980).

It is interesting to speculate that the clastogenic (and so probably mutagenic) potential (as determined by the increased frequency of micronucleated PCE's) of the extracts of Angelica koreanae radix and Pinelliae tuber might be due to such a hydrolysis of glycosides present in these extracts. Glycosides (as flavonoids) have been determined to be in both Angelica koreanae radix and Pinelliae tuber (Han, 1986; Kim, 1984).

All of the extracts tested in this research were negative in the Salmonella mutatest, the SOS chromotest and the SOS umu test (Chang *et al.*, 1987; Jhoun *et al.*, in press). It will be worthwhile in future research involving herbal drugs to attempt supplementation of glycosidases to the standard bacterial assays, in order to discover if previously negative results remain so.

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