Arch. Pharm. Res. Vol.21, No.4, pp. 391-397, 1998

Genotoxicity Study of Bojungchisup-tang, an Oriental Herbal Decoction-*In Vitro* Chromosome Aberration Assay in Chinese Hamster Lung Cells and *In Vivo* Supravital-Staining Micronucleus Assay with Mouse Peripheral Reticulocytes

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(Received March 13, 1998)

The toxicity evaluation of oriental herbal drugs is of great concern at present. Bojungchisup-tang (BCST, in Korean), a decocted medicine of oriental herbal mixture, is now well used in clinic at oriental hospitals for the treatment of edema of several diseases in practice. However, the toxicity of the oriental herbal decocted medicines such as genetic toxicity is not well defined until now. In this respect, to clarify the genetic toxicity of BCST, *in vitro* chromosome aberration assay with Chinese hamster lung (CHL) fibroblasts and *in vivo* supravital micronucleus assay with mouse peripheral reticulocytes were performed in this study. In the chromosome aberration assay, we used 5,000 μg/ml BCST as maximum concentration because no remarkable cytotoxicity in CHL cells was observed both in the presence and absence of S-9 metabolic activation system. No statistical significant differences of chromosome aberrations were observed in CHL cells treated with 5,000, 2,500 and 1,250 μg/ml BCST for 6 hour both in the presence and absence of S-9 metabolic activation. However, very weak positive result (6.5~8.0% aberration) of BCST was obtained in the absence of S-9 metabolic activation system at 5,000 μg/ml BCST when treated for 24 hour, i.e. 1.5 normal cell cycle time. And also, *in vivo* clastogenicity of BCST was studied by acridine orange-supravital staining micronucleus assay using mouse peripheral reticulocytes. We used 2,000 mg/kg as the highest oral dose in this micronucleus assay because no acute oral toxicity of BCST was observed in mice. The optimum induction time of micronucleated reticulocytes (MNRETs) was determined as 36 hours after oral administration of 2,000 mg/kg BCST. No significant differences of MNRETs between control and BCST treatment groups were observed *in vivo* micronucleus assay. From these results, BCST revealed very weak positive result in chromosome aberration assay *in vitro* with CHL cells and no clastogenicity in micronucleus assay *in vivo*.

Key words : Genotoxicity, Clastogenicity, Bojungchisup-tang, Herbal Decoction, Chromosome Aberration, Chinese hamster lung fibroblast, Micronucleus, Mouse peripheral reticulocyte

INTRODUCTION

There are many kinds of decocted oriental herbal mixture drugs used in practice traditionally and/or medically in Asian countries such as Korea, China and Japan. The establishment of toxicity of these oriental herbal medicines that may pose a genetic hazard in our health is one of subjects of great concern at present.

Several assay systems having rapidity and reliability have been introduced for genetic hazard, such as reversion test with bacterial gene mutation (Ames *et al.*, 1973, 1975; Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979) and micronucleus assay with rodents (Schmid, 1975; Hayashi *et al.*, 1982, 1990, 1992). These assay systems are now well used to evaluate the genotoxicity of some chemicals and drug candidates, and also frequently adopted as methods for an index of genotoxicity world-

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wide. Furthermore, it was well applied as a screening probe for the detection of possible carcinogenic substances for human health. Since the tens of thousands of man-made chemicals that have been introduced in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified. Our laboratory has been involved and experienced in the toxicity evaluation of some chemicals, especially in genetic toxicity (Ryu *et al.*, 1994a, 1994b, 1996a, 1996b). We also reported the clastogenicity of major trichothecene mycotoxins such as T-2, HT-2 toxin, nivalenol, deoxynivalenol (Ryu *et al.*, 1993a) using Chinese hamster lung (CHL) fibroblast cells *in vitro.*

Despite the many toxicological researches on synthetic chemicals and drug candidates, there are few reports on the genotoxicity of oriental herbal decocted drugs such as Kyoaesamul-tang (Woo *et al.*, 1997). So we aim to elucidate the clastogenicity of Bojungchisup-tang (BCST), a decocted oriental herbal medicine, with CHL cells *in vitro* and with peripheral reticulocytes of mouse *in vivo* in this study.

MATERIALS AND METHODS

Cell culture

A clonal sub-line of a Chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with Eagle's minimum essential medium (EMEM, Gibco, 410-1100EA) supplemented with 10% fetal bovine serum (FBS, Gibco, 26140-020). These cells were maintained at 37°C in 5% CO_2 humidified atmosphere.

Animals

Male ICR mice were purchased from Dae-Han Laboratory Animal Research Center Co., Ltd. (Eumsung, Korea). The animals were housed in an air-conditioned room having a 12 hr light/dark cycle and were acclimatized to the animal facility and environment for 1 week before use. Tap water and food (Samyang Co., Korea) were given *ad libitum.*

Reagents

Eagle's minimal essential medium, fetal bovine serum, antibiotic-antimycotic, trypsin-EDTA and colcemid were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, USA). Mitomycin C, cyclophosphamide and aciridine orange were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The test compounds were dissolved in dimethylsulfoxide (DMSO) for the chromosome aberration assay and in distilled water for the micronucleus assay. The preparation of rat liver S-9 fraction for the metabolic activation system was previously reported (Ames *et al.*, 1973; Maron and Ames, 1983). The S-9 fraction prepared was stored immediately at -80°C before use.

Preparation of BCST sample

All natural products for BCST sample preparation were purchased, and decocted by a routine method used for the preparation of oriental herbal drugs. The composition of one package of BCST was listed in Table I. To prepare the BCST sample, natural products of BCST corresponding to the amount of 40 packages were extracted with 6,000 ml of purified water by a conventional extractor for 2 hours. After extraction and filtration, the test sample was prepared by lyophilization for 24 hours.

In Vitro Chromosomal Aberration Assay in CHL Cells

The experiment was performed as described by OECD (1993) and Ishidate and Odashima (1977) with some minor modifications (Ryu *et al.*, 1993a, 1994b,

Table I. The composition of Bojungchisup-tang, an oriental herbal decoction

Herbal name	Scientific name	Gram per package
Ginseng Radix	*Panax ginseng*	3.75
Atractylodis macrocephalae Rhizoma	*Atractylodes marcrocephala*	3.75
Atractylodis Rhizoma	*Atractylodes japonica*	2.62
Citri pericarpium	*Citrus unshiu*	2.62
Poria	*Poria cocos*	2.62
Liriopis Tuber	*Liriope muscari*	2.62
Aucklandiae Radix	*Aucklandia lappa*	2.62
Angelicae gigantis Radix	*Glycyrrhiza uralensis*	2.62
Scutellariae Radix	*Scutellaria baicalensis*	1.87
Machili Cortex	*Machilus rimosa* *Machilus thunbergii*	1.12
Cimicifugae Rhizoma	*Cimicifuga heracleifolia*	1.12
Total		27.33

1996a, 1996b), which are briefly summarized as follows.

Determination of the 50% growth inhibition concentration: Test article dose levels were determined prior to the main study in a dose range-finding study performed in the presence and absence of a rat liver S-9 activation system. For the growth inhibition assay, CHL cells were seeded at the density of 5×10^4 cells/ml into 96 well plates. Twenty-four hours after seeding, several different doses of sample were separately added and incubated for 24 hours. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay (Mosmann, 1983).

Chromosome aberration assay: For the aberration assay, three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence of metabolic activation, cultures were treated for 24 hours with the test article, while in the presence of metabolic activation, cells were treated for 6 hours because of toxicity of S-9 and then maintained for 18 hours in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Treatment was followed by addition of medium containing colcemid at a concentration of 0.2 μg/ml. After 2 hr further incubation in the presence of colcemid, metaphase cells were harvested by centrifugation and trypsinization. The cells were swollen with hypotonic (0.075 M) KCl solution for 20 min at 37°C, and washed three times in ice-cold fixative (methanol:glacial acetic acid=3:1). After centrifugation, the fixative was removed, and cell pellet suspensions were prepared by pipetting gently. A few drop of cell pellet suspension were dropped onto precleaned glass microscope slides, and air dried. Slides were stained with 5% Giemsa buffered solution at pH 6.8 for scoring of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphases at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS (1988). Breaks less than the width of a chromatid were designated as gaps in our criteria, and not included as chromosomal aberration. The incidence of polyploid and endoreduplicated cells was also recorded when these events were observed. Solvent-treated cells served as controls in this experiment.

Evaluation and statistics: CHL cells usually have less than 3.0% cells with spontaneous chromosome aberrations. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fisher's exact test (Altman, 1993) with Dunnett's adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 200 metaphase cells were expressed as percentages, and then dose-dependent responses and the statistical significance in p- value will be considered as positive results in our judgement.

In Vivo Supravital Micronucleus Assay with Peripheral Blood Reticulocytes

The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange (AO)-supravital staining method was performed essentially as described by Hayashi *et al.* (1990) with minor modifications described as follows.

Animals and treatments: A single dose of mitomycin C (MMC) at 1 mg/kg was injected intraperitoneally as a positive control, and distilled water was administered orally with 10 ml/kg as a solvent control. The BCST sample was administered orally at doses of 2,000, 1,000 and 500 mg/kg body weight. Peripheral bloods were collected from mouse tail vein at 12 hr intervals from 36 to 60 hr after administration.

Preparation of AO-coated glass slides: The 10 μl of 1 mg/ml AO dissolved in distilled water was placed on a glass slide pre-heated at about 70°C, spread out, and dried at room temperature. These glass slides were stored in a dark and dry location at room temperature until used.

Peripheral blood cell preparations: Five μl of peripheral blood, collected by piercing the ventral tail, was placed directly without any anticoagulant on the center of an AO-coated glass slide. Glass slides were covered immediately with 24×40 mm coverslips, and allowed to be supravitally stained (Hayashi *et al.*, 1990).

Analysis and statistics: For the scoring and data analysis, two thousand reticulocytes (RETs) of type I, II, and III per animal were observed (Vander *et al.*, 1963; CSGMT, 1992; Hayashi and Sofuni, 1994) and micronucleated reticulocytes (MNRETs) were recorded under the fluorescent microscopy which had the combination of a blue excitation and a yellow to orange barrier filter. The data was analysed by the three-step method proposed by Hayashi *et al.* (1994a) for statistical differences between the control and specific treatment groups.

RESULTS AND DISCUSSION

It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1991). Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement (Zimmermann, 1971) which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of

cellular cancer genes (Radman *et al.*, 1982). Several short term methods with rapidity and reliability have been developed (Ames *et al.*, 1973; Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been applied to the evaluation of genotoxicity (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Hayashi *et al.*, 1982, 1990, 1992; Ryu *et al.*, 1993a, 1994b, 1996a, 1996b) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b; Heo *et al.*, 1997). Cytogenetic studies on mammalian cells *in vivo* (Schmid, 1975; Hayashi *et al.*, 1982, 1990, 1992; Heo *et al.*, 1997) as well as *in vitro* (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979) have also been widely used as a screening method for DNA-damaging substances.

There are many oriental herbal decocted mixture drugs used in practice. However, the toxicity of the oriental herbal decocted drugs especially genetic toxicity is not well defined until now. Recently, the mutagenicity and hepatotoxicity of Kyoaesamul-tang which is one of the well-known decocted mixtures used as oriental herbal medicines for the treatment of threatened abortion associated with pregnancy traditionally were studied by using the rec assay, the Ames assay and the SOS *umu* test (Woo *et al.*, 1997). The establishment of toxicity of these oriental herbal drugs that may pose a genetic hazard in our health is one of great concerns at present. In the present work, we evaluated the genotoxic potential of BCST in terms of its ability to induce chromosomal aberration in CHL cells and micronucleus induction in mice.

First of all, we used CHL cells because it was reported no differences of sensitivity between CHL and CHO (Chinese hamster ovary) cells in chromosome aberration study *in vitro* (Galloway *et al.*, 1997). Since no remarkable cytotoxicity of BCST in CHL cells was observed both in the presence and absence of S-9 metabolic activation system, 5,000 μg/ml of BCST was used as maximum concentration. The DMSO control revealed only 0.5~1.5 percent spontaneous chromosome aberration in 200 metaphase of CHL cells. However, the positive controls, cyclophosphamide (5 μg/ml) as an indirect mutagen that requires metabolic activation and mitomycin C (0.1 μg/ml) as a direct-acting mutagen, induced remarkable chromosome aberrations (20~44.5%) in CHL cells as shown in Table II. No statistically significant differences were observed in BCST treated groups without metabolic activation system for 6 hour treatment at 5,000, 2,500 and 1,250 μg/ml concentrations. Also, in the presence of S-9 metabolic activation system, no significant differences were observed in the 6 hour treatment group. However, high incidences (6.5~8.0%) of chromatid breakages and exchanges with statistical significance were observed at 5,000 μg/ml of BCST when treated for 24 hour, i.e. 1.5 normal cell cycle time, in the absence of S-9 metabolic activation system. This statistical significance of chromosome aberration of BCST was reproducible in an additional experiment as indicated

Table II. The frequency of chromosome aberrations induced by Bojungchisup-tang in Chinese hamster lung fibroblasts

Com.	Con. (μg/ml)	Treated hr.	Without (-) or with (+) S9 Mix	Chromosome aberrations/200 cells									
				Chromatid type		Chromosome type		Total aberration (%)	Extra aberration				
				Br	Ex	Br	Ex		ctg	csg	poly	endo	nor
DMSO	-	6	+	2	0	0	0	1.0	3	2	0	0	198
CP	5	6	+	7	65	2	0	29.5	5	3	1	0	140
BCST	5000	6	+	2	0	3	1	3.0	7	1	1	3	194
	2500	6	+	1	0	0	1	1.0	5	2	3	2	198
	1250	6	+	0	1	0	0	0.5	3	1	1	0	199
DMSO	-	6	-	2	0	1	0	1.5	4	0	2	0	197
MMC	0.1	6	-	5	39	1	1	20	2	0	2	0	160
BCST	5000	6	-	1	4	1	0	3.0	2	0	0	0	194
	2500	6	-	2	0	1	0	1.5	3	0	1	0	197
	1250	6	-	0	0	1	0	0.5	1	2	0	0	199
DMSO	-	24	-	0	1	0	0	0.5	3	0	0	0	199
MMC	0.1	24	-	14	110	4	0	44.5	0	0	0	0	111
BCST	5000	24	-	8	8	1	0	6.5*	8	1	2	0	186
	5000[a)]	24	-	9	7	2	0	8.0*	5	1	3	0	185
	2500	24	-	0	2	0	0	1.0	3	0	1	0	198
	1250	24	-	0	1	0	0	0.5	3	1	2	0	199

Com.: compound, Con.: concentration, Br: breakage, Ex: exchange, ctg: chromatid gap, chg: chromosome gap, poly: polyploid, endo: endoreduplicate, nor: normal, DMSO: dimethylsulfoxide, MMC: mitomycin C, CP: cyclophosphamide BCST: Bojungchisup-tang

*Statistical significance was observed ($p<0.05$).

[a)]This data was reproducible in an additional experiment.

Table III. Micronucleus induction in peripheral bloods of ICR mice by Bojungchisup-tang

Chemical	Route	Dose (mg/kg)	Number of mouse	Sampling time (hr)	%MNRETs[a] (Mean±S.D.)
Control	p.o.	·	5	·	0.26±0.10
MMC	i.p.	1	5	48	3.16±0.30
Bojungchisuptang	p.o.	2000	5	36	0.21±0.07
		2000	5	48	0.15±0.06
		2000	5	60	0.20±0.11
		1000	5	36	0.18±0.08
		500	5	36	0.26±0.19

[a]2000 Reticulocytes per mouse were counted
MNRETs: micronucleated reticulocytes, MMC: mitomycin C
i.p.: intraperitoneal, p.o.: *per os*

in Table II. Henderson *et al.* (1996) also reported that the extended harvest times are not necessary for the detection of *in vitro* clastogens in regulatory cytogenetic studies except it might help to resolve an equivocal result. Therefore, weak positive results of BCST were observed in chromosome aberration assay with CHL cells.

In general, the classical rodent micronucleus assay using bone marrow polychromatic erythrocytes as the target cells has been widely used to evaluate chemical genotoxicity *in vivo* (Schmid, 1975; Heddle *et al.*, 1983; Mavournin *et al.*, 1990). However, MacGregor *et al.* (1980) introduced the mouse peripheral blood instead of the bone marrow cells in the micronucleus assay. The peripheral blood has not been widely used until supravital staining with acridine orange was introduced to the micronucleus assay using the peripheral blood by Hayashi *et al.* (1990). In practical, the acridine orange-supravital staining micronucleus (AOSS-MN) assay with peripheral blood reticulocytes has several advantages compared with the classical MN assay (Schmid, 1975; Heddle *et al.*) with bone marrow cells. First, the cell population is more uniform and therefore easier to score. Second, a small amount of blood cells could repeatedly be taken from the tail of mice without killing, so it is possible to determine the optimal sampling time without preliminary experiment. It is also possible to take a very small amount of blood from other toxicity experiments without interferences (Hayashi and Sofuni, 1994), for example, transgenic animal study. On the basis of the AOSS-MN assay, we investigated *in vivo* clastogenicity of BCST using mouse peripheral bloods by the AOSS-MN assay. Since no acute lethal toxicity of BCST was observed in oral administration, it was assumed that the 50% lethal dose of BCST was above 5,000 mg/kg in a preliminary study. Therefore, we used 2,000 mg/kg of BCST as the highest oral dose in the AOSS-MN assay. The optimum induction time of micronucleated reticulocytes (MNRETs) was determined as 36 hours after administration as shown in Table III. Consequently, no significant differences of MNRETs induction between control and BCST treatment groups were observed in this micronucleus assay *in vivo*.

Recently, several new methods for the detection of genetic damages *in vitro* and *in vivo* were introduced according to the rapid progress in toxicology combined with cellular and molecular biology. Among these methods, the single cell gel electrophoresis (comet assay) which can detect DNA damages at a cellular level (Mckelvey-Martin *et al.*, 1993; Singh *et al.*, 1994; Ryu *et al.*, 1997), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Sawyer *et al.*, 1985), FISH (fluorescence *in situ* hybridization) (Hayashi *et al.*, 1994b), PRINS (primed *in situ* hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991; Ryu *et al.*, 1998) or *lac Z* (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on cellular and molecular toxicological approaches. Our laboratory is now under the progress of these assays to evaluate and to elucidate the mechanism of genetic toxicity and/or carcinogenesis, and will be presented in near future.

ACKNOWLEDGEMENTS

The authors thank to Professor Byung-Soo Kim, Department of Applied Statistics, College of Business and Economics, Yonsei University for his statistical analysis.

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