시호 물 추출물의 마우스 골수세포를 이용한 유전독성 평가

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Micronucleus Test of Bupleuri Radix Aqueous Extract in Bone Marrow Cells of Male ICR Mice

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In this research, the genotoxic effect of Bupleuri Radix (BR), the dried roots of *Bupleurum falcatum* Linne has been traditionally used as anti-inflammatory agent, was evaluated using the mouse micronucleus test. BR aqueous extract (yield = 16.52%) was administered once a day for 2 continuous days by oral gavage to male ICR mice at doses of 2,000, 1,000 and 500 mg/kg. Cyclophosphamide (CPA) 70 mg/kg was used as a known genotoxic agent in a positive control. The appearance of a micronucleus (MN) in polychromatic erythrocyte (PCE) is used as an index for genotoxic potential, and PCE ratio is used as an index of cytotoxicity. Although significant ($p<0.01$) increase of the number of PCE with one or more nuclei (MNPCE) was detected in CPA treated groups, no significant increases of MNPCE numbers were observed in all three different dosages of BR extracts treated mice with over 0.30 of the individual polychromatic erythrocyte ratio in all mice used in this study. The results obtained indicated that BR extract shows no genotoxicity effects up to 2,000 mg/kg dosing levels the limit dosage in rodents.

Key Words : Bupleuri Radix, micronucleus test, genotoxicity, mice

INTRODUCTION

Bone marrow cytogenetics, micronucleus test is a useful short-term technique for elucidating the mechanism as well as to identify the substances for their clastogenic and anticlastogenic activity[1)]. In Korea Food and Drug Administration (KFDA) guideline[2)], the genotoxicity should be tested prior to develop a new drug even though they have natural origin. Most of natural herbal agents, genotoxicity has been performed using in vivo like micronucleus test[1-4)].

Bupleuri Radix (BR) is a dried root of *Bupleurum falcatum* Linne (Umbelliferae), and it has been traditionally used as anti-inflammatory agent in Korea[5,6)]. BR has been showed anti-inflammatory[7,8)], immunomodulatory[9,10)], anti-ulcerative[11)], platelet activation inhibitory[12)], corticosterone secretory[13)], hepatoprotective[14)] and nephro-protective[8,15)] activities. However,

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there are no reports dealing the potential genotoxicity of BR extract upon our knowledge. The objective of the present study, therefore, was to obtain the genotoxic information about BR aqueous extracts, and further clarify their safety for clinical use.

MATERIALS AND METHODS

1. Animals

Thirty-five male ICR mice (6-wk old upon receipt, SLC, Japan) were used after acclimatization for 10 days. The body weights of animals at receipt are ranged in 28~32 g. Animals were allocated five per polycarbonate cage in a temperature (20-25℃) and humidity (30-35%) controlled room. Light : dark cycle was 12 h : 12 h and feed (Samyang, Korea) and water were supplied free to access. Animals were marked by picric acid. This study was carried out according to the guidelines of the Animal Ethical Committee, The University of Daegu Haany University (Gyeongsan, Korea).

2. Chemicals and agents preparation

Aqueous BR extracts (yield = 16.52%) were prepared by routine methods using rotary vacuum evaporator (Buchi Rotavapor R-144, Switzerland) and programmable freeze dryer (Freezone 1; Labconco Corp., MO, USA) from dried root of Bupleurum falcatum, which were purchased from Omniherb (Korea) after confirming the morphology under microscopy. In the present study, prepared herbs were boiled at 80℃, 3 hrs and then, evaporated and lysophilized. Powders of BR extracts are light brown color. BR extracts were stored in a refrigerator at -20℃ to protect from light and degeneration, and they are well soluble upto 200 mg/ml concentration levels in distilled water used as vehicle as clear light brown solution. The test article was orally administered at a dosage volume of 10 ml/kg, once a day for 2 days by oral gavage to mice; total 2,000, 1,000 and 500 mg/kg using distilled water as vehicle. Cyclophosphamide·H2O (CPA; Sigma, USA) was used as an identified genotoxic agents in a positive control group. CPA was dissolved in saline and once intraperitoneally administered at a volume of 10 ml/kg (70 mg/kg)

3. Agent treatments

The animals were allocated into five groups 7 mice each. The fixed highest dosage level of 2,000 mg/kg oral dosing was chosen in accordance to the KFDA guidelines[4], the limited highest dosage in rodent, and 1,000 and 500 mg/kg was selected using the common ratio 2. Control negative (taken vehicle) and control positive (CPA; 70 mg/kg-single treatment) were included by recommendation of KFDA and Organization for Economic Co-Operation and Development (OECD) guidelines[2,16].

4. Observation of clinical signs and body weights

All abnormal clinical signs were recorded before and after dosing at least twice a day based on the functional observational battery test[17,18], and body weights were measured once a day.

5. Bone marrow cells preparation

All animals were sacrificed 24 h post administration using carbon dioxide, and bilateral

femur was separated. Bone marrow preparations were made according to Schimid[19]. In brief, bone marrow cells were collected from aforementioned femur in 3 ml of inactivated fetal bovine serum (Gibco BRL, USA), centrifuged, and smeared on slides. Preparations were dried, and fixed by submerging in absolute methanol (for 10 ~ 20 min). Fixed slides were stained as follows;

May-Grunwald stain	3 min
May-Grunwald stain (1:1 diluted)	2 min
Giemsa stain (1:6 diluted)	10 min

6. Evaluation of genotoxicity

Slides were randomly coded and examined under 1000 magnification by two different experts. Small round or oval shaped bodies, size of which ranging from 1/5 to 1/20 diameter of polychromatic erythrocytes (PCE), were counted as micronuclei (MN). Attention was given to discriminate micronuclei from artifacts (Fig 1). Results were expressed as the number of MNPCEs in 2000 PCEs. Mean number of MNPCE standard deviation was calculated for each treatment group. In addition, PCE ratio (PCE/(PCE+ normochromatic erythrocytes (NCE)) ratio were also calculated by counting 1000 erythrocytes, for detecting the possibility of cytotoxicity[20].

7. Statistical analyses

Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test[21]. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by the Scheffe test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, the Mann-Whitney U (MW) test was conducted to determine the specific pairs of group comparison[22]. The result of statistical evaluation was regarded significantly when the P value was less than 0.05. In addition, the study was accepted when all of the PCE ratios are greater than 0.20[20]. Statistical analyses were carried out using SPSS for Windows (Release 14.0K, SPSS Inc., USA).

RESULTS

1. Mortalities

No test article-treatment related unscheduled mortalities were detected in all tested doses during the observation periods.

2. Clinical signs

During the observation period, no abnormal clinical signs were observed from BR extract treatment.

3. Body weight changes

No meaningful changes on body weights were detected in CPA and all tested doses of BR extract treated groups as compared to that of control negative group (taken vehicle only) (Table 1).

4. Changes on MNPCE numbers and PCE ratio

Significantly ($p<0.01$) increase of number of MNPCEs among 2000 PCEs was detected in CPA 70 mg/kg a positive control group. However, no

significant changes on MNPCE numbers were detected in all three different BR extract treated groups tested as compared with vehicle control. Although significant ($p<0.01$) decreases of the mean PCE numbers and ratio were detected in CPA treated mice as compared with negative control, the mean PCE ratio in total 1000 erythrocytes was detected above 0.35 (in individual mice, over 0.30) in all tested groups including negative and positive control (Fig 1, Table 2).

〈Table 1〉 Changes on the body weights

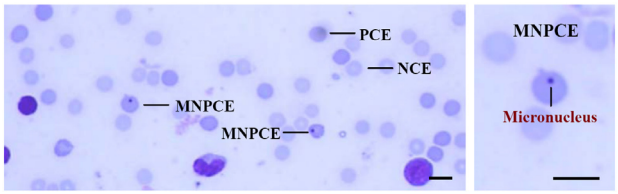
Groups	Day after dosing		
	Day 01)	Day 1	At a termination
Intact control	35.30 ± 2.04	39.27 ± 1.49	36.21 ± 1.68
CPA control	35.23 ± 1.75	39.29 ± 1.23	35.53 ± 1.94
BR extract			
2,000 mg/kg	35.06 ± 1.80	39.21 ± 2.04	36.01 ± 2.49
1,000 mg/kg	34.94 ± 1.66	39.39 ± 1.60	36.30 ± 1.74
500 mg/kg	35.41 ± 1.68	39.24 ± 1.06	35.99 ± 1.34

Values are expressed as mean SD, g of seven mice; [1)] Start day of test article administration; All animals were overnight fasted at Day 0 and a termination, respectively.

〈Table 2〉 Changes on MNPCE numbers and PCE/(PCE+NCE) ratio observed in mice

Group	MNPCEs/2000 PCEs	PCE/(PCE+NCE)	
		Ratio	Range
Intact control	1.14±0.69	0.43±0.03	0.40~0.48
CPA control	63.71±10.50**	0.35±0.04††	0.30~0.40
BR extract			
2,000 mg/kg	1.00±1.00	0.43±0.02	0.40~0.45
1,000 mg/kg	1.14±0.90	0.43±0.03	0.41~0.50
500 mg/kg	1.00±0.58	0.44±0.03	0.39~0.50

Values are expressed as mean SD of seven mice; PCE, polychromatic erythrocyte; MN, micronuclei; NCE, normochromatic erythrocyte; PCE+NCE = 1000 erythrocytes; ** $p<0.01$ as compared with intact control by MW test; †† $p<0.01$ as compared with intact control by Scheffe test



〈Fig. 1〉 Representative cytology of bone marrow cell smears. In prepared bone marrow cell smear, polychromatic erythrocyte (PCE), normochromatic erythrocyte (NCE), PCE with one or more nuclei (MNPCE) were counted based on the above morphology. NCE containing nucleus (MNNCE) was not calculated. Scale bars = 10㎛

DISCUSSION

In the present study, the genotoxic effects of BR extracts were evaluated using the mouse micronucleus test. As the results obtained in the present study, BR extract shows no genotoxicity effect up to 2,000 mg/kg dosing levels. The highest dosage used in the present study was selected as 2,000 mg/kg oral dosing was chosen in recommendation of the KFDA and OECD guidelines[2,16)], the limited highest dosage in rodent, and 1,000 and 500 mg/kg was selected using the common ratio 2.

Micronucleus assays were first introduced in the early 1970's for the examination of genotoxic activity of chemical agents[23,24)]. The procedure is based on the observation that mitotic cells with chromatid breaks or incomplete exchanges or with malfunction of the spindle apparatus suffer from disturbances in anaphase distribution of their chromatin. After telophase, a sizable portion of this displaced chromatin is not included in the nuclei of the daughter cells but forms single or multiple micronuclei in the cell cytoplasm. The frequency of the appearance of micronuclei depends both upon the rate of chromosome breakage or loss and the rate of cell division[20,25)]. Although micronuclei can occur in almost all

dividing cells, mouse bone marrow is usually the tissue used for the micronucleus test, and any agent which induces chromosomal aberrations can also produce micronuclei[20,26].

Because of its simplicity and efficacy, the micronucleus test has become a popular and useful in vivo procedure for the detection of chemically-induced chromosome damage. The number of reports from micronucleus testing has increased dramatically in the scientific literature during the past decade[27], and the value of this test for examining the mutagenicity and carcinogenicity of chemicals has been emphasized, particularly when it is used in combination with other cytogenetic assays[20].

The PCE ratio was used as index of cytotoxicity and the study was accepted when all of the PCE ratio are greater than 0.20[20]. Although significant ($p<0.01$) decreases of the mean PCE numbers and ratio were detected in CPA treated mice as compared with negative control, the mean PCE ratio was detected as over 0.35 in all tested groups including negative and positive control in the present study. That is no problem from cytotoxicity of the tested articles used in this work.

CPA is a widely used anti-neoplasic drug, employed either alone or in combination with other products[28]. The parent drug is biologically inactive, however after biotransformation by microsomal enzymes a number of active metabolites capable of alkylating nucleic acids[29], damage the chromosomes (through generation of free-radicals) and/or alkylating the DNA thereby producing mutagenicity were produced[30]. In the present study, CPA used as a positive control, and it showed a significant increases of MNPCE ratios. This indicates that the experiment protocol and the results of the present study are acceptable, and no meaningful increases of MNPCE were reported up to 2,000 mg/kg of BR extract.

Based on the results, it is concluded that BR extract shows no genotoxicity effects up to 2,000 mg/kg dosing levels. In addition, it is also considered that there were no problems from cytotoxicity of BR extract because the mean PCE ratio was estimated over 0.35 in all tested groups (in individual mice, over 0.30).

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