반하 물 추출물의 ICR 마우스 골수세포를 이용한 소핵실험

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Micronucleus Test of Pinella Rhizoma Aqueous Extracts in Bone Marrow Cells of Male ICR Mice - In Vivo Genotoxicity

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In this research, the genotoxic effects of Pinelliae Rhizoma (PR) extracts, one of famous herbal agents in Korean medicine were evaluated using the mouse micronucleus test. PR extracts was administered once a day for 2 continuous days by oral gavage to male ICR mice at doses of 2000, 1000, and 500 mg/kg. Cyclophosphamide was used as a known genotoxic agent in a positive control. The appearance of a micronucleus is used as an index for genotoxic potential. No PR extracts treatment-related abnormal clinical signs, body weight changes and mortalities were detected. Significant ($p<0.01$) increases of the numbers of polychromatic erythrocytes contain micronucleus in prepared bone marrow cells were detected in CPA and PR extracts 2000 mg/kg treated groups as compared with intact control, respectively. The results of intraperitoneal dose mouse bone marrow cell micronucleus test of PR extracts were positive in the present study. It is considered that there were no problems from cytotoxicity of PR extracts tested in this study because the polychromatic erythrocyte ratio was detected as > 0.42 in all tested groups.

keywords : Pinelliae Rhizoma, *Pinellia ternata*, Micronucleus test, Mouse, Bone marrow cell

I. Introduction

A traditional Korean herbal medicine, Pinelliae Rhizoma (PR) is a dried tuber of *Pinellia ternata* (Thunberg) Breitenbach (Family: Araceae), and has been used as sedative and antiemesis agents for pregnant.[1-4] Until now, various pharmacological effects of PR have been revealed; anti-cancer effect,[5] anti-obesity effect,[6] emetic effect,[7] anti-emetic effect[4] and neuroprotective effect.[8] In addition, the therapeutic effects on hepatitis B infections,[9] neuroprotective effect,[10,11] anti-stress effect[12] and gastric profusion effect[13] of mixed herbal formula containing PR extracts also have been researched.

As increase of the concern in the functional food and well-being in life, the demands and consumption of functional food originated from natural sources are increased.[3] However, the toxicological aspects about these natural origin-functional foods have been neglected because of the reasons that they have been used as various purpose for long times. Therefore, it

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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. Although it has been regarded that PR is a representative toxic irritable herbs in Korean medicine,[14] the scientific toxicological studies about PR extracts have not conducted except for mouse single oral dose toxicity,[15] 4 weeks repeated oral dose toxicity test in rats,[3] cytotoxicity[16] and some poster presentational gentoxicity.[17]

The object of this study was to obtain preliminary *in vivo* genotoxicities, micro- nucleus test of PR extracts, lyophilized water extract of *Pinellia ternata* in male mice. The highest dosage used in the present study was selected as 2000 mg/kg/20ml, the limit highest dosages recommended by KFDA Guidelines,[18] and 1000 and 500 mg/kg were selected using common ratio 2 in the present study, respectively. In addition, intact control and positive control (cyclophosphamide, CPA; 70 mg/kg single intraperitoneally administered) groups were added. After 24 hrs end of second oral treatment of PR extract or single intraperitoneal treatment of CPA, all animals were sacrificed, and the changes on the number of polychromatic erythrocytes containing micronucleus (MNPCE) were evaluated on prepared Bone Marrow Cells. In addition, PCE/(PCE+ normochromatic erythrocytes (NCE)) ratio among 1000 erythrocytes were also evaluated for detecting possible cytotoxicity.

Ⅱ. Materials and Methods

1. Animals and husbandry

Total fifty male ICR mice (6-wk old upon receipt, SLC, JAPAN) were used after acclimatization for 7 days. Animals were allocated five per polycarbonate cage in a temperature (20-25℃) and humidity (40-45%) controlled room. Light : dark cycle was 12hr : 12hr and feed (Samyang, Korea) and water were supplied free to access. All animals were marked by picric acid.

2. Articles and formulation

Aqueous PR extracts (absorption rate 29.36%) were prepared by routine methods using rotary vacuum evaporator (BUCHI Rotavapor R-144, Switzerland) and programmable freeze dryer (IlShin Lab., Korea) from PR, which were purchased from local voucher (Cho-Heung Pharmaceutical Ind. Co. (Daegu, Korea)) after confirm the morphology under microscopy. Powders of PR extracts are light brown. PR extracts were stored in a refrigerator at -20°C to protect from light and degeneration. The appearance of PR extracts in vehicle is clear light brown solution in distilled water and it is well soluble upto 100 mg/ml concentration levels. CPA (Sigma, USA) was used as positive control drug according to KFDA guideline.[18]

3. Grouping and test article administration

PR extracts have been used as folk medicine and ingredients of medicinal food for long times and no mortalities were detected upto 2000 mg/kg in mouse single oral dose toxicity test.[15] The highest dosage level was selected as 2000 mg/kg according to the recommendation by KFDA Guidelines,[18] the limited dosages, and 1000 and 500 mg/kg were selected using common ratio 2 as middle and low dosages. In addition, intact control and positive control (CPA 70mg/kg single intraperitoneally administered) groups were added according to the recommendation of KFDA[18] and OECD[19] guidelines as <Table 1>. Three different dosages of PR extracts were orally administered at a volume of 20 ml/kg dissolved in distilled water, once a day for two days. CPA was single intraperitoneally administered at a volume of 20 ml/kg dissolved in distilled water (at second treatment of PR extracts). In intact control, only distilled water was intraperitoneally administered as same volume and treatment frequency as PR extracts.

<Table 1> Experimental test Groups used in this study

Group	Dose (mg/kg/day)
Control	Distilled water 20 ml/kg
Control	CPA (70 mg/kg), once
Active	PR extracts (2000 mg/kg), twice
Active	PR extracts (1000 mg/kg), twice
Active	PR extracts (500 mg/kg), twice

PR, Pinelliae Rhizoma PR extracts were orally administered, once a day for 2 days in a volume of 20 ml/kg of distilled water; CPA was once intraperitoneally dosed at second dose of PR extracts; Total 5 groups, 10 mice per group were used in the present study.

4. Observation of clinical signs

All abnormal clinical signs were recorded before and after dosing at least twice a day based on the functional observational battery test.[20)21)] If any abnormal clinical signs were detected, they were subdivided into 3 degrees according to the status of animals: 3+ Severe, 2+ moderate, 1+ slight.

5. Body weight measurements

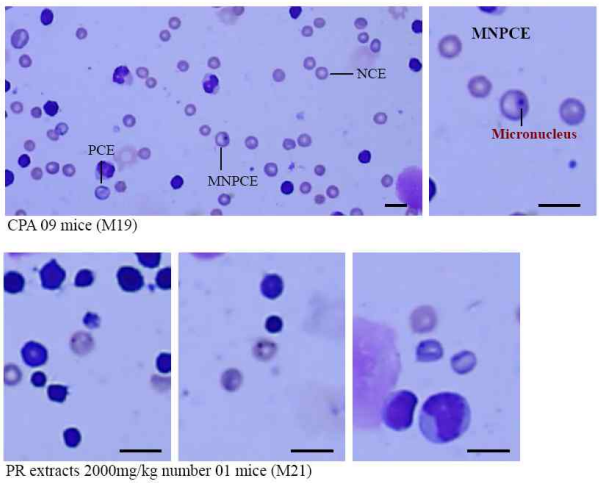
Body weights were measured at the 1 day before administration, immediately before treatments and sacrifice (24 hrs after end of 2nd treatment).

6. Bone marrow preparation and recoding of micronuclei

All survived animals were sacrificed at 24 hrs after end of 2nd treatment, and bilateral femurs were separated. Bone marrow preparations were made according to Schimid.[22)] In brief, bone marrow cells were collected from aforementioned femur in 3ml of inactivated fetal bovine serum (GIBCO BRL, USA), centrifuged, and smeared on slide. Preparations were dried, and fixed by submerging in absolute methanol (for 10~20min). Fixed slides were stained as follows;

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

Slides were randomly coded and examined under × 400 magnification by two different experts. Small round or oval shaped bodies, size of which ranging about 1/5 to 1/20 of diameter of PCE, were counted as micronuclei <Figure 1>. Attention was given to discriminate micronuclei from artifacts. Results were expressed as the number of MNPCEs in 2000 PCEs. Mean number of MNPCE ± S.D. was calculated for each treatment group. In addition, PCE/(PCE+NCE) ratio were also calculated by counting 1000 erythrocytes for detecting possibility of cytotoxicity.23)



<Figure 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 morphology. NCEs containing nucleus were not calculated. PR, Pinelliae Rhizoma Scale bars = 10μm

7. Statistical analyses

Multiple comparison tests for different dose groups were conducted. Variance homogen- eity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtained data were analyzed by one way ANOVA test followed by Sheffe test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogen- eity were observed at Levene test, a non-parametric comparison test, Kruskal- Wallis H test was conducted. When a sig-

nificant difference is observed in the Kruskal-Wallis H test, the Mann-Whitney test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 6.1.3., SPSS Inc., USA). 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/(PCE+NCE) ratio are greater than 0.20.[23)]

Ⅲ. Results

1. Mortality

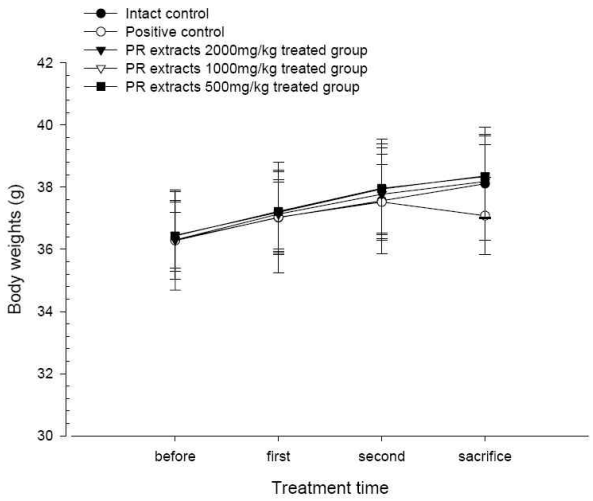
No unscheduled mortalities were detected in any groups in the present study, therefore, all of animals (10/10 100%) in CPA, three different dosages of PR extracts treated groups and intact control were subjected to bone marrow cell harvest.

2. Clinical signs

No meaningful clinical signs were detected in all tested groups in the present study.

3. Body weight changes

Except for slight non-significant decreases of body weights were detected in CPA treated mice as compared with intact control, no meaningful changes on the body weight were detected in all three different dosages of PR extracts treated groups as compared with intact control in the present study <Figure 2>.



<Figure 2> Changes on the body weights
Note that except for slight non-significant decreases of body weights were detected in CPA treated mice as compared with intact control, no meaningful changes on the body weight were detected in all three different dosages of PR extracts treated groups as compared with intact control in the present study. PR, Pinelliae Rhizoma Positive: cyclophosphamide 70mg/kg treated group; before means 1 day before first treatment.

4. Changes on the bone marrow MNPCEs

Significant ($p<0.01$) increases of the numbers of MNPCE among 2000 PCEs were detected in CPA or PR extracts 2000 mg/kg administered groups as compared with intact control, respectively. However, quite similar MNPCE numbers were detected in PR extracts 1000 and 500 mg/kg treated groups as compared with intact control, respectively <Table 2>.

<Table 2> Changes on the bone marrow cells: MNPCE numbers and PCE(PCE+NCE) ratio observed

Groups	MNPCEs/2000 PCEs	PCE(PCE+NCE) ratio/1000 erythrocytes
Control		
Intact	2.60 ± 2.46	0.46 ± 0.02
Positive	70.20 ± 14.99*	0.42 ± 0.01*
PR extracts treated		
2000 mg/kg	18.60 ± 9.65*	0.45 ± 0.05
1000 mg/kg	1.70 ± 2.75	0.46 ± 0.03
500 mg/kg	1.60 ± 1.17	0.46 ± 0.02

Values are expressed as mean ± SD of 10 mice; Positive: cyclophosphamide 70 mg/kg treated group; PR, Pinelliae Rhizoma MNPCE, PCE with one or more nuclei; PCE, Polychromatic erythrocyte; NCE, Normochromatic erythrocyte; * $p<0.01$ as compared with intact control.

Ⅳ. Discussion

In the present study, we investigated the preliminary *in vivo* genotoxicities, micronu- cleus test PR extracts, lyophilized water extract of *Pinellia ternata* in male mice. 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 by KFDA Guidelines,[18] the limited dosages, and 1000 and 500 mg/kg were selected using common ratio 2 as middle and low dosages. In addition, intact control and positive control (CPA; 70 mg/kg single intraperitoneally administered) groups were added according to the recommendation of KFDA and OECD.[18][19]

Bone marrow cytogenetics is a useful short-term technique for elucidating the mechanism as well as to identify the substances for their clastogenic and anticlastogenic activity.[24] Therefore, the genotoxicity of PR extracts is positive because significant ($p<0.01$) increases of MNPCE numbers in prepared bone marrow cells were detected in PR extracts 2000 mg/kg treated groups as compared with intact control. Traditionally, PR has been regarded as a representative toxic irritable herb in Korean medicine.[14] However, the prebrewed methods to reduce the toxicity of PR also have been developed.[16] Therefore, the micronucleus test along prebrewed methods seems to be need in future.

CPA is a widely used anti-neoplasic drug, employed alone or in combination with other products.[25] Used as an anticancer drug or in bone marrow transplantation conditioning regimes, treatment with CPA severely injures hematopoietic and lymphoid tissues, thereby leading to a profound leucopenia.[26] CPA is known to be biologically inactive by itself until after biotransformation by microsomal enzymes leading to the production of a number of active metabolites capable of alkylating nucleic acids[27] and damages chromosomes through generation of free-radicals and alkylating DNA thereby producing mutagenicity.[28] In addition, KFDA[18] recommended that CPA is suitable reference drug in mouse bone marrow cell micronucleus test. In the present study, CPA used as a positive control showed significant increase of MNPCE ratios, this indicates that the experiment protocol and results of the present study are acceptable.

In addition, the PCE/(PCE+NCE) was used as index of cytotoxicity and the study was accepted when all of the PCE/(PCE+NCE) ratio are greater than 0.20.[23] Although PCE numbers and PCE/(PCE+NCE) were significantly ($p<0.01$) decreased in CPA treated group as compared with intact control, they were showed at least > 0.42 in the present study. The frequency of MNPCE encounted and ranges of PCE/(PCE+NCE) in intact control and CPA 70 mg/kg treated groups were well corresponded to the results of previous reports.[29]

Ⅴ. Conclusion

The results of intraperitoneal dose mouse bone marrow cell micronucleus test of PR extracts were positive in the present study. In this study, CPA, a positive control, showed significant increase of MNPCE, and PCE(PCE+NCE) ratios in all test groups were detected > 0.42. These indicate that the experiment protocol and results of the present study are acceptable.

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