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Genotoxicity evaluation of *Hwanglyeonhaedok-tang*, an herbal formula

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ABSTRACT

Ethnopharmacological relevance: *Hwanglyeonhaedok-tang* (*Huanglianjiedu-tang*, *Orengedoku-to*), a traditional herbal formula, is used for the treatment of inflammatory, gastrointestinal and cardiovascular diseases.
Purpose: The purpose of this study was to evaluate the genotoxic potential of *Hwanglyeonhaedok-tang* water extract (HLHDT).
Methods: A genotoxicity test was conducted using a bacterial reverse mutation test (Ames test), an *in vitro* chromosome aberration test using Chinese hamster lung cells, and an *in vivo* micronucleus test using ICR mouse bone marrow.
Results: In the Ames test, which used different *Salmonella typhimurium* (*S. typhimurium*) and *Escherichia coli* (*E. coli*) strains, HLHDT did not increase the number of revertant colonies of *S. typhimurium* strains TA98, TA100 and TA1535 as well as *E. coli* strains with or without S9 mix. However, the number of revertant colonies with the *S. typhimurium* TA1537 strain and S9 mix increased in a dose-dependent manner. The chromosome aberration test showed that HLHDT did not increase the number of structural or numerical chromosome aberrations in a short-period test (6 h) with S9 mix. By contrast, HLHDT significantly increased the number of structural chromosome aberrations in a short-period (6 h) or continuous (22 h) test without S9 mix. In the micronucleus test, no significant increase was observed in micronucleated polychromatic erythrocytes, and no significant decrease was observed in polychromatic to total erythrocytes.
Conclusions: These results indicate that HLHDT might be genotoxic, based on both the Ames and chromosome aberration tests. Therefore, further *in vivo* studies will be needed to define the mechanism of this genotoxicity.

1. Introduction

Herbal medicine has been used for the prevention and treatment of diseases for a long time. Recently, the demand for and consumption of herbal formulas composed of various medicinal herbs have increased. However, the toxicological aspects of herbal formulas have been neglected, as natural treatments are generally considered to be safer than other medications (Lynch and Berry, 2007; Jordan et al., 2010). Many medicinal herbs may have toxic features that will result in damage to the human body, including the induction of genetic damage (Moreira et al., 2014). Therefore, toxicological studies of medicinal herbs, including herbal formulas, should be conducted to control both their abuse and potential toxicities.

According to Ministry of Food and Drug Safety (MFDS, 2015) guideline (No. 2015-82, 2015), a genotoxicity test should be conducted prior to developing a new drug, including natural origin. The basic requirement is to initially assess a drug's genotoxicity in a bacterial reverse mutation test, and then conduct tests in mammalian cells in both *in vitro* and *in vivo* models.

The traditional herbal formula *Hwanglyeonhaedok-tang* (*Huanglianjiedu-tang*, *Orengedoku-to*) is composed of four medicinal herbs, *Coptis chinensis* (Coptidis Rhizoma, China), *Scutellaria baicalensis* (Scutellariae Radix, Jeongseon, Korea), *Phellodendron chinensis* (Phellodendri Cortex, China), and *Gardenia jasminoides* (Gardeniae Fructus, Muju, Korea) (Hur, 2007). *Hwanglyeonhaedok-tang* has been used in clinical practice to treat inflammation, gastrointestinal dis-

Abbreviations: HLHDT, *Hwanglyeonhaedok-tang* water extract; *S. typhimurium*, *Salmonella typhimurium*; *E. coli*, *Escherichia coli*; KFDA, Korea Food and Drug Administration; CHL, Chinese hamster lung; 2-NF, 2-nitrofluorene; 2-AA, 2-aminoanthrancene; 9-AA, 9-aminoacridine; 4NQO, 4-nitroquinolone X-oxide; BP, benzo(a)pyrene; CPA, cyclophosphamide; EMS, ethyl methanesulfonate; ICR, CrljOri:CD1; IACUC, Institutional Animal Care and Use Committee; PP, polyploidy; ER, endoreduplication; MNPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte

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orders, hypertension, liver injury and cerebrovascular diseases (Lu et al., 2011). In addition, *Hwanglyeonhaedok-tang* has been reported to have preventive effects on both diabetes mellitus (Yue et al., 2013) and atherosclerosis *in vivo* (Sekiya et al., 2005). However, the potential genotoxicity of *Hwanglyeonhaedok-tang* has not yet been reported.

In the present study, we investigated the potential genotoxicity of *Hwanglyeonhaedok-tang* water extract (HLHDT) using standard tests, including an *in vitro* bacterial reverse mutation test (Ames test), an *in vitro* chromosome aberration test using Chinese hamster lung (CHL) cells and an *in vivo* micronucleus test recommended by the KFDA (2009).

2. Materials and methods

2.1. Preparation of HLHDT

Preparation of HLHDT was performed as previously reported (Seo et al., 2015). Voucher specimens (2008-KE-20-1~KE-20-4) have been deposited at K-herb Research Center, Korea Institute of Oriental Medicine. The high performance liquid chromatography (HPLC) profile of HLHDT has been previously reported (Seo et al., 2015). According to Seo et al. (2015), the correlation coefficients (r^2) of the marker compounds in HLHDT showed good linearity (≥0.9997). The contents of marker compounds in HLHDT were geniposide 36.54 ± 0.27 mg/g, baicalin 30.24 ± 0.72 mg/g, coptisine 0.97 ± 0.02 mg/g, palmatine 10.34 ± 0.47 mg/g and berberine 1.35 ± 0.02 mg/g.

2.2. Bacterial reverse mutation test (Ames test)

The bacterial reverse mutation test was conducted in accordance with OECD guideline No. 471 (1997) with previously described methods (Lee et al., 2015). The histidine-requiring *Salmonella typhimurium* (*S. typhimurium*) strains TA98 and TA1537 (for detecting frame-shift mutagens), TA100, and TA1535 as well as the tryptophan-requiring *Escherichia coli* (*E. coli*) strain WP2uvrA (for detecting base-pair substitution mutagens) were obtained from Molecular Toxicology Inc. (Boone, NC, USA), and were used as the tester strains. The positive control factors were 2-nitrofluorene (2-NF), 2-aminoanthrancene (2-AA), 9-aminoacridine (9-AA), 4-nitroquinolone X-oxide (4NQO), and benzo(a)pyrene (BP). A preliminary dose-range test was conducted to determine the highest concentration for the present study, which was used with all strains at doses of 8, 40, 200, 1000, 3000, and 5000 μg/plate with or without the S9 mix. The number of revertant colonies did not increase in the controls for any of the tester strains. However, there was an increased number of revertant colonies of TA1537 with the S9 mix. Based on these results, 5000 μg/plate was selected as the highest concentration for the mutagenicity study. The present study was tested using triplicates for each dose. Data were presented as the mean number of revertant colonies with standard deviations.

2.3. Chromosome aberration test

The chromosome aberration test was performed in CHL cells in accordance with OECD guideline No. 473 (2014a) with previously described methods (Lee et al., 2015). CHL cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in minimum essential medium (GIBCO, Invitrogen Corp., California, USA) supplemented with 10% fetal bovine serum (GIBCO), sodium bicarbonate (2.2 g/L), L-glutamine (2 mM), streptomycin sulfate (100 μg/mL), and penicillin G·Na (100 units/mL). Cells were incubated at 37 °C and 5% CO_2. To determine the maximum concentration for the present study, the preliminary dose-range study was conducted at a dose of 5000 μg/mL in the presence or absence of the S9 mix. In the dose-range finding study, over 50% cytotoxicity was observed with the 6 h and 22 h treatments in the absence of the S9 mix. Based on these results, the present study was designed by considering the solubility and cytotoxicity of HLHDT. Cyclophosphamide (CPA) was used as a positive control substance with metabolic activation and ethyl methanesulfonate (EMS) without metabolic activation.

2.4. Micronucleus test

The micronucleus test was performed in accordance with OECD guideline No. 474's "Mammalian Erythrocyte Micronucleus Test" (2014b); previously described methods were used with minor modifications (Lee et al., 2015). Specific pathogen-free male CrljOri:CD1 (ICR) mice (six weeks old, 27.8–30.9 g) were obtained from Orient Co., Ltd. (Seongnam, Korea). Mice were used in experiments after one week of quarantine and acclimatization. This study was reviewed and assessed by the Institutional Animal Care and Use Committee (IACUC) of the Korean Institute of Toxicology. All animals were cared for in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. A dose-finding test was conducted for the highest concentration to be used for the present study. HLHDT was administered once daily for two days by gavage to mice at doses of 500, 1000, or 2000 mg/kg. From these results, HLHDT treatment-related clinical signs were observed between male and female mice by 2000 mg/kg. Therefore, doses of 500, 1000, or 2000 mg/kg were selected in the micronucleus test. Cyclophosphamide monohydrate (CPA) in normal saline (10 mL/kg) was administered by intraperitoneal injection at 70 mg/kg as a positive control. Animals were sacrificed by CO_2 gas inhalation at 24 h after the last administration and bone marrow cells were prepared as described by Schmid (1975).

2.5. Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System (SAS) program (version 9.2, SAS Institute Inc., Cary, NC). No statistical analysis was conducted on the Ames test results. The statistical analyses for the *in vitro* chromosome aberration test were performed as described previously (Richardson, 1989; Lee et al., 2015). The number of aberrant metaphases and the number of [polyploidy (PP, ≥37 chromosomes)+endoreduplication (ER)] were analyzed. A χ^2 test and Fisher's exact test were conducted to compare the vehicle control and HLHDT-treated groups (Fisher, 1970). Fisher's exact test was used to compare the vehicle and positive control groups. $P < 0.05$ was considered to be statistically significant. *In vivo* micronucleus results were evaluated according the methods of Lovell et al. (1989), with minor modifications. *In vivo* micronucleus statistical analysis was performed as previously described (Lee et al., 2015). Differences were regarded as significant at $P < 0.05$.

3. Results

3.1. Bacterial reverse mutation test (Ames test)

Positive controls significantly increased the numbers of revertant colonies, confirming the sensitivity of the test system. No contaminant colonies were observed on the sterility plates for the highest concentration of HLHDT. As shown in Fig. 1, HLHDT did not increase the revertant colonies of any *S. typhimurium*, except for TA1537, or *E. coli* strains per plate at any dose, with or without S9 mix, in comparison with the spontaneous reversion rate in the vehicle control. However, in comparison with the vehicle control, the number of revertant colonies with the *S. typhimurium* TA1537 strain and S9 mix increased in a dose-dependent manner.

3.2. Chromosome aberration test

The results of the chromosome aberration test are presented in Table 1. HLHDT significantly increased the number of structural or numerical chromosome aberrations at 6 h or 22 h without S9 mix, in