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Safety assessment of *Oryeong-san*, a traditional herbal formula: Study of subacute toxicity and influence of cytochrome P450s and UDP-glucuronosyltransferases

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ABSTRACT

Oryeong-san is a traditional herbal formula that is used for the treatment of common genitourinary diseases in Korea and other Asian countries. However, little is known about its safety and influence on drug metabolism. In the present study, we investigated the subacute toxicity of an *Oryeong-san* water extract (OSWE) in rats and its effects on activities of drug-metabolizing enzymes. Subacute toxicity was modeled in animals exposed to treatment with the extract at multiple doses. Rats were given OSWE by oral gavage at 0, 1000, 2000 and 5000 mg/kg/day for 4 weeks. We checked general observations and investigated any changes of body/organ weight, food consumption, hematology, serum biochemistry, and urinalysis *in vivo*; and the activities of human microsomal cytochrome P450s (CYP450s) and UDP-glucuronosyltransferase (UGT) isozymes *in vitro*. We found that OSWE caused no significant toxicological changes at the doses tested. Therefore, the no observed adverse effect level of OSWE was more than 5000 mg/kg/day for male and female rats. OSWE inhibited the activities of CYP2C19 (IC_{50}: 737.69 μg/mL) and CYP2E1 (IC_{50}: 177.77 μg/mL). These results indicate that OSWE may be safe with no drug-related toxicity for up to 4 weeks and provide useful information concerning its potential to interact with conventional drugs or other herbal medicines.

1. Introduction

Safety assessment of drugs is recognized as essential in their development. The dosage of a drug is a main determinant of its safety and efficacy, and establishing a safe dose of a drug is an important task in drug development (Atuah et al., 2004). Toxicity studies are performed as a fundamental assessment of the safety or hazards of substances such as natural products, pharmaceuticals, and industrial chemicals. Most of these studies have been widely used laboratory animals (e.g. mice, rats and rabbits) to determine toxicity (Borja et al., 2016). Toxicity studies include assessment of acute (14 day), subacute (28 day), subchronic (90 day), or chronic (6–12 month) toxicity according to a specific date period. These studies are aimed at investigating the toxicity, dose–response relationships, and major target organs during the specified period (Prieto et al., 2005).

Drug metabolism plays an important role in the metabolic disposition of xenobiotics including therapeutic drugs or environmental pollutants, and endogenous/exogenous substances (Kim and Novak, 2007). Drug-metabolizing enzymes are responsible for the activation and deactivation of drugs, including a number of cytotoxic drugs and can be categorized into two main groups: phase I (cytochrome P450s, CYP450s) and phase II enzymes (UDP-glucuronosyltransferases, UGTs), respectively. CYP450s and UGTs are the most important enzyme systems for detoxication (Michael and Doherty, 2005).

Herbal formulas are widely used worldwide, and consist of various herbs with broad pharmacological properties (Isohama et al., 1997). As herbal formulas usage has become increasingly popular as health food supplements, investigations of their safety and efficacy are paramount. *Oryeong-san*, a traditional herbal prescription of Korea (known as *Wulingsan* in China and *Gorei-san* in Japan) is an herbal formula consisting of various herbs and has long been used for the treatment of kidney diseases manifesting as dysuria, oliguria, and edema (He et al., 2008).

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Oryeong-san has been reported to have pharmacological activities such as antioxidant (Shin et al., 1996), antihypertensive (Kiga et al., 2008), and antidiabetic (Liu et al., 2009) activities. However, toxicity level and safety studies of treatment with *Oryeong-san* has yet to be demonstrated. Our research group reported toxicological safety assessment on acute toxicity (Jeon et al., 2012) and genotoxicity (Lee et al., 2015) of an *Oryeong-san* water extract (OSWE) *in vivo*. However, a subacute toxicity study is necessary to determine the toxicity and safety of OSWE.

Therefore, the present studies were performed to assess the toxicological safety of daily doses of OSWE for 4 weeks in Crl:CD Sprague Dawley (SD) rats. This study was conducted in accordance with the guidelines established by the Organization for Economic Cooperation and Development (OECD, 2002) for the testing of chemicals, and recent Good Laboratory Practice (GLP) regulations. Also, we examined the effects of a OSWE on the activities change of the major human CYP450 (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) and UGT (UGT1A1, UGT1A4, and UGT2B7) isozymes *in vitro*.

2. Materials and methods

2.1. Preparation of OSWE

The crude herbs forming the herbal formula of *Oryeong-san* were purchased from a traditional herb markets, Kwangmyungdang Medicinal Herbs Co.,Ltd. (Ulsan, Korea) in February 2013. Professor Je-Hyun Lee of Dongguk University, Gyeongju, Republic of Korea, confirmed the taxonomic identity of each crude herb. Voucher specimens of *Oryeong-sa*n (2013-KE17-1–KE17-5) are available at the K-herb Research Center, Korea Institute of Oriental Medicine (KIOM). *Oryeong-san* was prepared as described in Table 1 and its extract was obtained by boiling the herbs in distilled water at 100 °C for 2 h using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The extract was freeze-dried (PVTFD10RS, IlShinBioBase, Yangju, Korea) after filtration using a standard sieve (No. 270, 53 μm). The amount of OSWE was 21.8 kg (yield, 18.17%). The established high-performance liquid chromatography (HPLC) analytical method was applied for the simultaneous quantification of the OSWE for 1 and 4 week, respectively. The contents of active components were 0.37 mg/g (coumarin) and 0.05 mg/g (cinnamaldehyde), which are characteristic compounds of *Oryeong-san* (Lee et al., 2015).

2.2. Reagents

Vivid® CYP450 screening kits (Vivid® CYP1A2 Blue, Vivid® CYP2B6 Blue, Vivid® CYP2C9 Blue, Vivid® CYP2C19 Blue, Vivid® CYP2D6 Blue, Vivid® CYP2E1 Blue, and Vivid® CYP3A4 Green) were purchased from Invitrogen Co. (Camarillo, CA, USA). These kits use 7-ethoxy-methyloxy-3-cyanocoumarin as a substrate for CYP1A2, CYP2D6, CYP2C19, and CYP2E1. In addition, di-(benzyloxymethoxy) fluorescein was used as a substrate for CYP3A4, and 7-benzyloxy-4-trifluoromethylcoumarin was used as a substrate for CYP2B6 and CYP2C9. UGT-Glo™ UGT1A1 and UGT2B7 Screening Systems were purchased from Promega (Madison, WI, USA). Recombinant human UGT1A4 enzyme was purchased from Corning Inc. Life Science (Tewksbury, MA, USA). α-Naphthoflavone, diclofenac, ketoconazole, lopinavir, miconazole, quinidine, sodium diethyldithiocarbamate trihydrate, and sulfaphenazole were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.3. Experimental animals

The animal studies of subacute toxicity were performed according to the guidance of the Institutional Animal Care and Use Committee in Korea Institute of Toxicology under the Good Laboratory Practice Regulations for Non-Clinical Laboratory Studies and approved by Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee (Daejeon, Korea); approval number JN13001.5-week-old Specific pathogen-free Crl:CD (SD) rats (n = 20/per sex) were obtained from the Orient Bio Inc. (Seongnam, Korea) and used after quarantine and acclimatization for 8 days. The animals were housed in rooms with a controlled environment maintained at 22 ± 3 °C with a relative humidity of 50 ± 20% with 12–15 air changes/h and a conventional dark–light cycle at 12:12 h (artificial lighting from 08:00 to 20:00). All rats were kept in stainless-steel wire-mesh cages and allowed sterilized tap water and commercial laboratory rodent food (PMI Nutrition International, Richmond, VA, USA) *ad libitum* as described previously (Shin et al., 2012; Jeong et al., 2015).

2.4. Grouping and dose selection

OSWE did not cause toxicological changes including death, abnormal responses and body weight change following a single dose (2000 mg/kg) in the acute toxicity study (Jeon et al., 2012). For subacute toxicity studies, we chose the maximum dose of OSWE to be 5000 mg/kg/day with consideration for doses used in clinical treatment. Therefore, the middle and low dose with a common ratio of 2 (1000 and 2000 mg/kg/day, approximately) was selected for this study. The design of this experiment is outlined in Fig. 1. Normal rats were assigned to four groups (n = 5 male and 5 female rats per group) using a Path/Tox System, version 4.2.2 (Xybion Medical Systems Corporation, Cedar Knolls, NJ, USA). OSWE was dissolved in distilled water for injection (Choong-wae Pharmaceutical Co., Seoul, Korea) and administered by oral gavage at 0, 1000, 2000, and 5000 mg/kg once daily for 4 weeks. Vehicle-treated control rats (0 mg/kg/day) were given to the distilled water an equal volume as OSWE-administrated groups. The daily dose (10 mL/kg body weight) of OSWE was calculated based on the most recently recorded body weight of rats.

2.5. General observations

The mortality, clinical signs and body weight of rats were monitored for 28 days. Mortality and clinical signs were recorded twice a day (before and after administration) during the period of the study. All clinical signs were recorded individually for type, observation day/time, and duration using the Path/Tox System. The body weight of each rat was measured at the initiation of administration, and once a week throughout the study period. Cumulative food consumption was

Table 1
The composition of OSWE.

Scientific name	Latin name	Plant parts	Wild or cultivated species	Family	Amount (g)	Ratio	Source
Alismatis Rhizoma	*Alisma orientale* Juzepzuk	Tuber	Cultivated	Alismataceae	9.375 (33.3%)	5	Yeongcheon, Korea
Poria Sclerotium	*Poria cocos* Wolf	Sclerotium	Cultivated	Polyporaceae	5.625 (20%)	3	Pyeongchang, Korea
Atractylodis Rhizoma Alba	*Atractylodes macrocephala* Koidzumi	Rhizome	Cultivated	Compositae	5.625 (20%)	3	China
Polyporus	*Polyporus umbellatus* Fries	Sclerotium	Wild	Polyporaceae	5.625 (20%)	3	China
Cinnamomi Cortex	*Cinnamomum cassia* Presl	Bark	Cultivated	Lauraceae	1.875 (6.7%)	1	Vietnam
Total amount					28.125 (100%)		