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Absence of subchronic oral toxicity and genotoxicity of rice koji with *Aspergillus terreus*

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ABSTRACT

Koji products have been considered as an effective fermented food consumed in East Asia with many health benefits. Particularly, rice koji with *Aspergillus terreus* (RAT) has been reported to be able to prevent hyperlipidemia and hepatic steatosis through regulating cholesterol synthesis. Despite its biological activities, there is a lack of comprehensive information to give an assurance of its safety. Therefore, the objective of this study was to perform a series of toxicological studies (repeated dose oral toxicity and genotoxicity) according to test guidelines published by the Organization for Economic Cooperation and Development. Along with acute toxicity study using rats and beagle dogs, a 13-week toxicity study revealed no clear RAT-related toxic changes, including body weight, mortality, hematology, serum biochemistry, organ weight, and histopathology after oral administration at doses of 500, 1000, and 2000 mg/kg BW. The no-observed-adverse-effect level of RAT was considered to be more than 2000 mg/kg BW/day in rats of both genders. In addition, potential genotoxicity was evaluated using a standard battery of tests (Ames test, chromosome aberration assay, and micronucleus assay) which revealed that RAT showed no genotoxicity. Accordingly, these results suggest that RAT is a safe and non-toxic functional food for human consumption at proper dose.

1. Introduction

Traditional fermented products are considered as functional foods in Asian countries. The cultures used for fermentation of cereal grains (soybean, rice, barley, wheat) are called “koji-molds” and fungal and bacterial microorganisms consist of *Aspergillus,*

Abbreviations: NOAEL, no-observed-adverse-effect level; OECD, Organization for Economic Co-operation and Development; GLP, Good Laboratory Practice; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; BUN, blood urea nitrogen; TC, total cholesterol; TP, total protein; ALP, alkaline phosphatase; AST, aspartate transaminase; ALT, alanine transaminase; TG, triglyceride; CHL, Chinese hamster lung; MNPCEs, micronucleated polychromatic erythrocytes; NCEs, normochromatic erythrocytes.

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Monascus, Mucor, and *Rhizopus*, and *Bacillus* species (Lin and Chou, 2006; Shin et al., 2016). Koji products fermented with a variety of microorganisms such as *A. oryzae*, *R. oligosporum*, and *B. natto* have been used as the source of different traditional fermented foods such as rice wine (sake and makgeolli), red pepper paste (gochujang), and soybean paste (miso, doenjang, and cheonggukjang) in China, Japan, and Korea (Blandino et al., 2003; Kum et al., 2015; Shin et al., 2016).

During fermentation, these koji molds produce many enzymes and functional bioactive compounds (Koseki et al., 1998; Yoshizaki et al., 2014). It has been reported that koji products possess several nutritional benefits and biological activities, including anti-obesity, anti-oxidative, and anti-bacterial activities (Santiago et al., 1992; Esaki et al., 1997; Berghofer et al., 1998; Yen and Chang, 2003; Yoshizaki et al., 2014; Tamang, 2015). Although we have recently reported that rice koji fermented with *A. terreus* (RAT) can prevent hyperlipidemia and hepatic steatosis by regulating lipid metabolism (Jang et al., 2015), toxicity of RAT is a concern for its worldwide use as a functional food. Thus, it is necessary to identify potential toxicological properties of RAT although it can be available on the market as a beneficial functional food. Therefore, the objective of this study was to determine the acute and subchronic toxicity of orally administered RAT and establish its LD_{50} and no-observed-adverse-effect level (NOAEL). Also, genotoxicity studies including bacterial reverse mutation assay, *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay were performed in this study to determine the potential genotoxicity of RAT. These studies were performed according to test guidelines for toxicological studies published by the Organization for Economic Cooperation and Development (OECD), and Good Laboratory Practices (GLP).

2. Materials and methods

2.1. Test substance and animals

For preparation of rice koji according to the method of Jang et al. (2015), rice was soaked for 3 h in water and then drained for 30 min. And, yeast extract and glucose were added to the wet rice before sterilizing by autoclave for 10 min at 121 °C. After cooling to 35 °C quickly, fungal strain *A. terreus* MKDS01 was inoculated into steamed rice at concentrations from 0.2 to 2%, cultivated for 4–10 days in an incubator at 27 ± 3 °C, and dried to a moisture content of 8 ± 2% for preservation and stability. Monacolin K (also known as lovastatin) is one of the functional chemical compounds isolated from oyster mushrooms (Gunde-Cimerman and Cimerman, 1995), red fermented rice (Yoshizaki et al., 2014), and Pu-erh tea (Zhao et al., 2013). In this study, monacolin K was analyzed at Institute of Sunchang Fermented Soybean Products (Sunchang, Korea; http://www.gochujang.go.kr), and we confirmed that RAT contained 1498 mg/kg of monacolin K, which has been known to have various pharmacological activities against high blood cholesterol levels and oxidative damage (Kim et al., 2016). And, we also found that monacolin K contained in the RAT was maintained with a high degree (>90%) of stability at 4 °C during the test period (72 days).

Sprague Dawley (SD) rats, ICR mice, and beagle dogs (Orient Bio, Seongnam, Korea) were used after acclimatizing to laboratory conditions for 1–2 weeks prior to study initiation with an evaluation of health status. Animals were housed in controlled animal facilities (humidity, 40–60%; temperature, 22 ± 2 °C) accredited AAALAC International (#001169) in accordance with Guide for the Care and Use of Laboratory Animals 8th edition (NRC, 2010). A commercial rodent diet (LabDiet 5002 Certified Rodent Diet, PMI Nutrition International, St. Louis, MO, USA) and tap water were supplied to rats and mice *ad libitum*. Each dog was given 200 g of pellet feed (Cargill Agri Purina Inc., Seongnam, Korea) twice daily. The light/dark cycle was 12/12 h. This study was conducted in compliance with the guidelines published by the OECD and the GLP Regulations for Nonclinical Laboratory Studies of Korea Food Drug Administration (KFDA, 2014). The study protocol was approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital.

2.2. Experimental design for single oral dose toxicity study

For single oral dose toxicity study in beagle dogs, two male and two female beagle dogs (8 months) were weighed and orally administered RAT at 2000 mg/kg BW/day. RAT was suspended in 0.5% methylcellulose (MC). Daily application volume was 10 ml/kg of body weight. After treatment, all animals were observed every hour for 6 h and then once daily for 14 days. Body weights were measured prior to dosing, at the initiation of RAT treatment, and on day 1, 7, and 14. At the end of the study period, all dogs were anesthetized using a mixture of xylazine (46.6 mg/kg) and zoletil (50 mg/kg) and sacrificed by section of the carotid artery. Their organs were macroscopically examined.

For single oral dose toxicity study in rats, SD rats (7-week old, n = 5 male and female rats/group) were randomly divided into two groups. A vehicle control group and a high dose group (2000 mg of RAT/kg of body weight (BW)) were also included. Vehicle (0.5% MC) or RAT was orally administered once at 10 ml/kg BW. After administration, all animals were observed every hour for 6 h on the first day and then once daily for 14 days after the treatment. Body weights were measured prior to dosing, at the initiation of RAT treatment, and on day 1, 2, 3, 7, and 14. At scheduled termination, all rats were sacrificed by using isoflurane and their organs were macroscopically examined.

2.3. Experimental design for repeated oral dose toxicity study

In 14-day repeat-dose toxicity study for dose selection, male and female SD rats (7-week old, n = 5 male and female rats/group) were randomly assigned to four experimental groups, including a vehicle control group and three RAT groups (RAT 500, 1000, and 2000 mg/kg BW). Vehicle (0.5% MC) or RAT was administered once daily by oral gavage at 10 ml/kg BW for 14 days. For 13-week repeat-dose toxicity study in accordance with OECD guideline 408 (OECD, 1998), SD rats (7-week old, n = 10 male and female rats/group) were randomly assigned to four experimental groups, including a vehicle control group and three RAT groups (RAT 500, 1000, and 2000 mg/kg BW). Vehicle (0.5% MC) or RAT was administered once daily by oral gavage at 10 ml/kg of body weight for 13 weeks. During the experimental period, clinical signs (general condition, mortality, and behavioral abnormality) were recorded individually everyday up to terminal sacrifice. Body weights and food/water consumption were measured once per week. All rats that survived to the end of the experiment were sacrificed by isoflurane inhalation and their blood was taken from the posterior vena cava for hematology and serum biochemical analysis.

2.4. Urinalysis, hematology, and serum biochemistry

During the last week of the administration period in the 13-week repeat-dose oral toxicity study, fresh urine samples were collected from five male and five female SD rats per group to test the following parameters: specific gravity, pH, leukocyte, nitrite, protein, glucose, ketone body, urobilinogen, bilirubin, and occult blood using a urine analyzer (Clinitek Advantus, Siemens, Malvern, PA, USA).

In the 14-day repeat-dose oral toxicity study and the 13-week repeat-dose oral toxicity study, blood was collected from all rats