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Evaluation of the repeated-dose liver, bone marrow and peripheral blood micronucleus and comet assays using kojic acid

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ABSTRACT

The repeated-dose liver micronucleus assay has the potential to detect liver carcinogens and could be integrated into general toxicological studies. To assess the performance of this assay, kojic acid was tested in 14-day and 28-day liver micronucleus assays. We evaluated the incidence of micronucleated cells in liver, bone marrow and peripheral blood and performed comet assays in both the liver and peripheral blood (comet assay was performed only for 14-days). Kojic acid, a skin-whitening agent used in cosmetic products, was orally dosed in six-week-old male rats at 250, 500 and 1000 mg/kg/day for 14 days, and at 125, 250 and 500 mg/kg/day for 28 days. Organ weight and histopathology were examined at the end of the experiment. Neither a clear, positive response in micronucleus (MN) incidence nor changes in the percent of tail DNA in the comet assays was noted in liver and bone marrow. An increase of relative liver weight was observed in 1000 mg/kg/day for 14 days. In histopathology, minimal hypertrophy of hepatocytes was found at 1000 mg/kg/day for 14 days. The results of both the micronucleus assay and the comet assay indicate that 14-day and 28-day repeated dosing of kojic acid are non-genotoxic in the liver and bone marrow.

Kojic acid has been known to act as a tumor-promoter in thyroid carcinogenesis but has not been shown to have initiation activities in liver carcinogenesis. Findings in this study are consistent with the evidence that kojic acid is not an apparent initiator of liver carcinogenesis. Therefore, the liver micronucleus assay is simple and sensitive to detect genotoxic liver carcinogens.

1. Introduction

This collaborative study by the Mammalian Mutagenicity Study (MMS) Group, a subgroup of the Japanese Environmental Mutagen Society (JEMS), was conducted to evaluate the suitability to detect liver carcinogens of the repeated-dose liver micronucleus assay using young adult rats. This study, using kojic acid (KA), was performed as a part of this collaborative study.

KA is a natural substance produced by various strains of *Aspergillus, Penicillium* and *Acetobacter*. It has been widely used as a skin-whitening agent in a category of quasi-drugs or cosmetics. There are many reports of genotoxicity and carcinogenicity in studies with KA.

KA tested positive for genotoxicity in a bacterial reverse mutation test (with and without metabolic activation) and a chromosome aberration study in V79 cells (only cytotoxic dose without metabolic activation) [1]. However, negative results were obtained for KA genotoxicity in a gene mutation assay in V79 cells and in a mouse lymphoma assay [1]. In *in vivo* studies, KA treatment tested negative for genotoxicity in an unscheduled DNA synthesis study in rat hepatocytes, and negative results were reported in mouse bone marrow micronucleus assays [1]. In the *LacZ* transgenic mouse study, KA treatment was negative for genotoxicity in the liver [1]. In a previous collaborative study by MMS Group, KA was judged as negative in a liver micronucleus assay using a single administration to young rats [2].

Although positive results for KA carcinogenicity were obtained in the rodent thyroid [3,4], these could be attributed to KA's interference with iodine uptake and organification, which causes a negative feedback of the pituitary–thyroid axis following the inhibition of thyroxin synthesis [5–7]. Other studies reported that

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KA treatment increased the number and area of glutathione-*S*-transferase placental-form positive foci in rat liver through repeated dosing toxicity studies. However, clear tumor induction was not observed in these livers [8–11]. It was suggested that KA may act as a tumor promoter rather than a tumor initiator in the liver [7–12].

In this study, we evaluated whether KA has the potential to induce micronuclei in the liver or bone marrow of rats after repeated dosing. Since it is very useful to evaluate multiple genotoxic endpoints in the same animals so as to reduce animal usage, we also tried to evaluate KA using the comet assay in the liver and peripheral blood.

2. Materials and methods

2.1. Chemicals

Kojic acid (KA, CAS No. 501-30-4, >98% purity) was purchased from Sigma–Aldrich (St. Louis, USA). KA was suspended in 0.5% sodium carboxymethylcellulose (0.5% CMC-Na, Wako Pure Chemical Industries, Ltd., Osaka, Japan) before use.

2.2. Animals

Male Crl:CD (SD) rats were purchased from Charles River Japan Inc. (Tsukuba, Japan), and they were six weeks old and weighed approximately 190–240 g at the beginning of the administration. The animals were housed one per cage in an air-conditioned room with a 12-h light/dark cycle. Food pellets and water were given *ad libitum* throughout the acclimatization and experimental periods. This study was conducted in accordance with the "Guideline for Animal Experimentation" specified by Research Center, Taisho Pharmaceutical Co., Ltd.

2.3. Dose levels and treatment

In the three-month oral toxicity study in rats, only one of the 10 animals from the 1000 mg/kg/day group died in week three [13], allowing for 1000 mg/kg/day to be set as the highest dose and 500 and 250 mg/kg/day to be set as lower doses for the 14-day toxicity study, and 500 mg/kg/day to be set as the highest dose and 250 and 125 mg/kg/day to be set as lower doses for the 28-day toxicity study. Administration volume was 10 mL/kg. Animals receiving only the vehicle served as the negative control group, and no positive control group was established. KA was routinely administered orally by gavage once a day for 14 or 28 consecutive days.

2.4. Micronucleus (MN) assays

2.4.1. Liver MN assay

Approximately 24 h after the 14th (14-day toxicity study) or 28th (28-day toxicity study) administration of KA or vehicle control, the rats were euthanized under anesthesia using isoflurane. The hepatocyte preparations were performed using a modified method of Narumi et al. [14]. The livers were excised, and approximately 1-g portions of the left lateral lobe were sliced into several 1-mm strips with a razor blade. These strips were rinsed with Hanks' balanced salt solution (HBSS; GIBCO-Invitrogen, Carlsbad, CA, USA) and treated with HBSS containing 100 units/mL of collagenase (Collagenase Yakult-S, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) in a screw-capped flask with shaking at 37 °C for 1 h. They were then shaken hard by hand approximately 50 times every half hour. The HBSS containing collagenase was removed, and fresh HBSS was added. The liver suspended in fresh HBSS was shaken hard to break apart cell clumps and forced through a cell strainer. The obtained cell suspension was then rinsed with 10% neutral buffered formalin and centrifuged at 50 × *g* for 2 min. The hepatocyte pellet was resuspended in 10% neutral buffered formalin and stored in a refrigerator until analysis.

The hepatocyte suspension was mixed with an equal volume of an acridine orange (AO: 500 μg/mL) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI: 10 μg/mL) staining solution immediately before observation. Small aliquots (10-μL) of this mixture were dropped onto glass slides and covered with a coverslip. The specimens were observed under a fluorescent microscope (BX51-34FL-1 and BX60-34FLB-1, Olympus Co., Ltd., Tokyo, Japan) with an ultraviolet excitation filter at 400× magnification and the number of MNHEPs per 2000 parenchymal hepatocytes (HEPs), including mono-, bi- and multi-nucleated cells, was counted for each animal. The number of mitotic phase cells among the 2000HEPs was also counted to determine the mitotic index (MI).

2.4.2. Bone marrow MN assay

After removing the livers as described in the liver MN assay, the femurs were removed from the same animals, and the bone marrow cells were collected by washing these femurs with fetal bovine serum (FBS; GIBCO-Invitrogen). This mixture of bone marrow cells and FBS was centrifuged at 180 × *g* for 5 min, and the resulting supernatant was removed. The bone marrow cells were then suspended in a small amount of the remaining supernatant, and 4-μL aliquots of this suspension were dropped and smeared onto glass slides. After the specimens had air-dried, they were fixed in methanol for 5 min. The specimens were stained with 40 μg/mL AO solution, washed with Sörenzen's phosphate buffer and covered with a cover slip. They were immediately observed under a fluorescent microscope (BX51-34FL-1, BX53-34FL-1 and BX60-34FLB-1, Olympus Co., Ltd., Tokyo, Japan) with a blue excitation filter at 1000× magnification. A total of 800 erythrocytes [immature erythrocyte (IME) + mature erythrocyte (ME)] from each animal were observed to determine the proportion of IMEs among erythrocytes. A total of 2000 IMEs from each animal was examined for micronucleated immature erythrocytes (MNIMEs), and the incidence of MNIMEs was determined.

2.4.3. Peripheral blood MN assay

Peripheral blood was collected by piercing the ventral tail vein before the 4th day of KA administration (14-day toxicity study only) and again after the last administration of KA. A total of 5 μL of peripheral blood was placed on an AO-coated glass slide and covered immediately with a cover slip. Erythrocytes were observed under a fluorescent microscope (BX51-34FL-1 and BX53-34FL-1, Olympus Co., Ltd., Tokyo, Japan) with a blue excitation filter at 1000× magnification. A total of 1000 erythrocytes [reticulocyte (RET) + mature erythrocyte (ME)] from each animal was observed to determine the proportion of RETs among erythrocytes. A total of 2000 RETs from each animal was examined for micronucleated reticulocytes (MNRETs), and the incidence of MNRETs was determined.

2.5. Comet assay

Comet assays were conducted (14-day toxicity study only) according to the OECD guideline [15]. An extra administration was performed 21 h after the last administration (3 h before necropsy) prior to the comet assay. Following sampling for MN assays, a small piece of liver and a small volume of peripheral blood were collected from each animal. The livers were placed into an ice-cold mincing buffer (20 mM EDTA (disodium) and 10% dimethylsulfoxide in Hank's balanced salt solution (HBSS) (Ca^{++}, Mg^{++} and phenol red free)), rinsed sufficiently with the cold mincing buffer to remove residual blood and minced with a pair of fine scissors to release the cells. The cell suspensions were strained through a