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Evaluation of repeated dose micronucleus assays of the liver and gastrointestinal tract using potassium bromate: A report of the collaborative study by CSGMT/JEMS.MMS

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ABSTRACT

The food additive potassium bromate ($KBrO_3$) is known as a renal carcinogen and causes chromosomal aberrations *in vitro* without metabolic activation and *in vivo* in hematopoietic and renal cells. As a part of a collaborative study by the Mammalian Mutagenicity Study group, which is a subgroup of the Japanese Environmental Mutagen Society, we administered $KBrO_3$ to rats orally for 4, 14, and 28 days and examined the micronucleated (MNed) cell frequency in the liver, glandular stomach, colon, and bone marrow to confirm whether the genotoxic carcinogen targeting other than liver and gastrointestinal (GI) tract was detected by the repeated dose liver and GI tract micronucleus (MN) assays. In our study, animals treated with $KBrO_3$ showed some signs of toxicity in the kidney and/or stomach. $KBrO_3$ did not increase the frequency of MNed cells in the liver and colon in any of the repeated dose studies. However, $KBrO_3$ increased the frequency of MNed cells in the glandular stomach and bone marrow. Additionally, the MNed cell frequency in the glandular stomach was not significantly affected by the difference in the length of the administration period. These results suggest that performing the MN assay using the glandular stomach, which is the first tissue to contact agents after oral ingestion, is useful for evaluating the genotoxic potential of chemicals and that the glandular stomach MN assay could be integrated into general toxicity studies.

1. Introduction

The International Conference on Harmonisation S2(R1) guideline recommends that the choice of tissue in the second part of an *in vivo* assessment should be based on factors such as the potential mechanism, *in vivo* metabolism, or the nature of the exposed tissues [1]. The liver is typically the preferred tissue because of its exposure and metabolizing capacity, and the liver micronucleus (MN) assay has the potential to detect hepatocarcinogens [2–6]. Additionally, the gastrointestinal (GI) tract MN assay is effective for assessing the genotoxicity of substances administered orally; therefore, we have developed a short-term MN assay using the glandular stomach and colon [7–11].

A collaborative study by the Mammalian Mutagenicity Study (MMS) group, which is a subgroup of the Japanese Environmental Mutagen Society (JEMS), was conducted to evaluate the suitability of the repeated dose liver and GI tract MN assays using young adult rats. The present study was performed at the Yakult Central Institute as a part of this collaborative study, and we selected the food additive potassium bromate ($KBrO_3$) as the test chemical to examine whether the genotoxic carcinogen which does not target the liver and GI tract could be detected by the repeated dose liver and GI tract MN assays.

$KBrO_3$, which is used as a flour-improving agent, is a strong oxidizing agent and is carcinogenic in the kidney, peritoneal cavity, and thyroid gland, but not in the liver, stomach, or colon of rats [12–14]. $KBrO_3$ has been classified as a genotoxic carcinogen based on positive results in the *in vitro* Ames, chromosomal aberration, and MN tests, with and/or without metabolic activation, and in the *in vivo* bone marrow MN assay [12,15–18]. It was also reported that oral administration of $KBrO_3$ induced DNA fragmentation and increased the frequency of micronucleated (MNed) cells, the concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG), the mutant- and mutation-frequencies, and the frequency of GC to TA

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transversions in the kidney of normal or Big Blue rats [19–21]. Regarding genotoxic effects in the rat liver and GI tract, Kasai et al. reported that oral administration of $KBrO_3$ did not increase the concentration of 8-OHdG in the liver [20]. Additionally, we confirmed that $KBrO_3$ increased the frequency of MNed cells in the glandular stomach but not in the colon after a 4-day repeated oral administration [11]. However, MN induction after long-term administration of $KBrO_3$ has been assessed in neither the liver nor GI tract.

To assess the suitability of the repeated dose liver and GI tract MN assays, this study focused on the effects of oral $KBrO_3$ administration to rats for 4, 14, and 28 days on the frequency of MNed cells in the liver, glandular stomach, colon, and bone marrow.

2. Materials and methods

2.1. Animals

Male Crl:CD (SD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), acclimated for 10 days, and used at 6 weeks of age. Either two or three rats were housed in a cage with wood-chip bedding under constant temperature (20–26 °C) and humidity (30–70%) with alternating 12 h intervals of light and dark throughout the acclimation and experimental periods. The rats were given food and water *ad libitum*, but no food was given for 18 h before euthanasia. All experiments were performed according to the guidelines for the care and use of laboratory animals in the Institutional Animal Care and Use Committee of Yakult Central Institute, and the protocols were approved by the committee.

2.2. Chemicals

$KBrO_3$ [CAS 7758-01-2] (Wako Pure Chemical Industries, Ltd., Osaka, Japan, Lot No. PEM1558, ≥99.8% purity) was dissolved in distilled water (DW) and given to rats immediately after preparation.

2.3. Dose levels and treatment

Either the dissolved $KBrO_3$ or DW was administered to rats once a day for 4, 14, and 28 days. The administered dosage for the 4-day treatment group was 80 mg/kg body weight/day, whereas the dosages for the 14- and 28-day treatment groups were 40, 60, and 80 mg/kg body weight/day. These dosages were selected from the literature [18] and preliminary experiments based on toxicological signs (*e.g.*, decreased body weight). All treatments were *via* oral gavage in a volume of 10 mL/kg. Each treatment group consisted of five randomly selected rats. During the treatment period, the animals were weighed on every day in the 4-day experiment, on days 1 (the first day of dosing), 4, 8, 11, and 14 in the 14-day experiment, and on days 1, 4, 8, 11, 15, 18, 22, 25, and 28 in the 28-day experiment. The general condition of the animals was observed twice a day, before and after dosing, and once on the day of necropsy.

Twenty-four hours after the final administration, rats were terminally anaesthetized with pentobarbital and euthanized by exsanguination *via* the abdominal aorta. The liver, glandular stomach, colon, right femur, and kidneys were obtained; however in the 40 mg/kg treatment groups, the colon was not collected. The liver and kidneys were weighed.

2.4. Histopathological examination

The liver (left lateral lobe), glandular stomach (containing the fundus, antrum, and pyloric regions), colon (proximal and middle regions), and left kidney were used for histopathological examination; the residual parts of the liver, glandular stomach, and colon were used for MN assays. The tissues used for histopathological examination were fixed with 10% neutral-buffered formalin and embedded in paraffin. The paraffin-embedded tissues were cut into 4 μm sections and then stained with hematoxylin and eosin (HE). Histopathological examination was performed under a light microscope.

2.5. MN assays

MN assays using the liver, glandular stomach, colon, and bone marrow were performed according to the protocol described in the summary report of this collaborative study [22]. Briefly, the liver, approximately 1 g portion of the left lateral lobe, was sliced and incubated in a flask containing 100 units/mL of collagenase (Collagenase Yakult-S, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) to isolate hepatocytes. The glandular stomach and colon were each everted on a glass rod and incubated in a tube containing 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) + 2 mM dithiothreitol in Mg^{2+}/Ca^{2+}-free Hanks' balanced salt solution (HBSS) for stomach and in a tube containing 1 mM EDTA in Mg^{2+}/Ca^{2+}-free HBSS for colon, to isolate the epithelium. The bone marrow cells were collected by washing the femur cavity with 1 mL of 10% neutral-buffered formalin. The cell suspensions of the liver, glandular stomach, colon, and bone marrow were centrifuged, and the collected cells were fixed with 10% neutral-buffered formalin and stored at 4 °C until analysis.

Immediately before observation, the cell suspensions (5–10 μL) were mixed with an equal volume of staining solution containing 500 μg/mL of acridine orange (AO) and 10 μg/mL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for liver, 50 μg/mL AO and 2.5 μg/mL DAPI for glandular stomach, 125 μg/mL AO and 2.5 μg/mL DAPI for colon, and 40 μg/mL AO for bone marrow on glass slides. Scoring was carried out under blinded conditions by use of a fluorescence microscope (magnification: 400×; liver, 600×; glandular stomach, colon, and bone marrow) with UV excitation (365 nm) for liver, glandular stomach, and colon and with blue excitation (490 nm) for bone marrow. Two thousand cells or immature erythrocytes (IMEs) were scored per tissue per animal to determine the frequency of MNed cells. Additionally, 2000 hepatocytes and 1000 erythrocytes from each rat were analyzed to determine the mitotic index (MI) and the percentage of IMEs to total erythrocytes (%IME), respectively.

2.6. Ki-67 Immunohistochemistry

Cell proliferation in the GI tract was assessed using Ki-67-positive cells as a marker. This analysis was performed according to our previous report [10] using the paraffin-embedded tissue sections of the stomach and colon from three rats in each dosage group. Briefly, immunohistochemical staining with a monoclonal mouse anti-rat Ki-67 antigen (clone MIB-5, Dako/Agilent Technologies Inc.) was performed using the streptavidin-biotin complex method. The staining was developed with diaminobenzidine, and the sections were counterstained with hematoxylin, dehydrated, and mounted. Scoring was carried out by use of a light microscope (600×). Thirty glands (*i.e.*, three parts of ten well-oriented glands) in the gastric fundus and twenty crypts in the proximal colon were scored to determine the number of Ki-67-positive cells per gland or crypt. A cell was scored positive for Ki-67 when the nucleus of the cell was distinctively brown.

2.7. Statistical analysis

Differences of MNed cell frequency between the $KBrO_3$ and DW control groups were analyzed statistically using Kastenbaum and Bowman's tables with an upper-tailed significance level of 0.05. When the frequency of MNed cells increased, the Cochran-Armitage test for a dose-related trend was also performed with an one-sided significance level of 0.05.

The other quantitative data were analyzed for their statistical significance with two- or multiple-comparison tests. Briefly, the statistical significance between two groups was determined using Student's *t*-test for homogenous data or Aspin-Welch test for non-homogenous data, whereas the statistical significance between multiple groups was determined using Dunnett's test for homogenous data or Kruskal–Wallis test for non-homogenous data (significance level of 0.05). The variance homogeneity of two groups or multiple groups was examined using *F*-test or Bartlett's test, respectively (significance level of 0.05). All analyses were performed using SAS, Version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Body weight and general condition

A slight decrease in body weight was observed in the $KBrO_3$ treatment groups compared to the DW control group although there was no statistical significance (Fig. 1), and a significant decrease in body weight gain was observed at doses of 60 mg/kg (during days 25–28) and 80 mg/kg (during days 11–15, 22–25, and 25–28) compared to the DW control group in the 28-day experiment (data not shown). $KBrO_3$-related changes in general condition were not observed.

3.2. Organ weight

A significant increase in the relative kidney weight was observed at doses of 60 mg/kg over a period of 28 days and 80 mg/kg over a period of 4, 14, and 28 days (Table 1). $KBrO_3$-related changes in the liver weight were not observed.

3.3. Histopathological examination

After 14 days of $KBrO_3$ treatment, nuclear karyorrhexis/pyknosis in the epithelium of the glandular stomach near the proliferative zone and eosinophilic body/hyaline droplets

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