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Lanthanum nitrate genotoxicity evaluation: Ames test, mouse micronucleus assay, and chromosome aberration test

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ABSTRACT

The increasing use of rare-earth elements (REE) and their compounds has led to their accumulation in the environment and has raised concern about their safety. The toxic effects of REE such as lanthanum are largely unknown; genotoxicity studies have been limited and results are controversial. We evaluated the genotoxicity of lanthanum nitrate ($La(NO_3)_3$) in several in vitro and in vivo tests, including bacterial reverse mutation assay (Ames test), mouse bone marrow micronucleus assay, and chromosome aberration assay. $La(NO_3)_3$ was not mutagenic in the Ames test. $La(NO_3)_3$ did not increase the frequencies of bone marrow micronuclei or chromosome aberration in the mouse after repeated treatments at oral doses up to 735 (females) and 855 mg/kg (males). The compound did not increase the frequency of chromosome aberrations in CHO cells in vitro. These results indicate that lanthanum is not a genotoxic hazard.

1. Introduction

In plants, rare-earth elements (REE) affect many physiological and biochemical responses, with beneficial effects on growth and development [1]. REE are widely used in agriculture as plant growth stimulants and as feed additives for livestock, poultry, and aquaculture [2]. The increasing use of REE has caused them to accumulate in the ecosystem (soil, atmosphere, water), resulting in increased human ingestion through the food chain [1,3]. A recent survey of 16 REE in major foods in China (the predominant supplier of REE), indicated that certain elements, including La, were found at much higher levels than others. La is not an essential element for humans. The average contents of La in major foods and fresh vegetables were 29 and 48 μg/kg, respectively, with a maximum level of 4 mg/kg; this may reflect the abundance of this element in soil [4]. La and its compounds warrant attention with regard to food safety.

Available data on the toxic effects of La are limited. Subchronic toxicity of lanthanum nitrate ($La(NO_3)_3$) was studied in rats [5]. Some surveys reported that REE may impair cognitive functions in children, and research on possible toxic consequences of La exposure have focused on neurotoxicity [6–8]. Lanthanum carbonate ($La_2(CO_3)_3$) is a safe and effective phosphate-binding agent. Although its bioavailability is extremely low, limited retention in bone, liver, and the gastrointestinal tract occurs with prolonged use [9]. To ensure that any La retained in tissues does not present a latent genotoxic or carcinogenic hazard to patients, $La_2(CO_3)_3$was subjected to a range of genotoxicity tests, concluding that therapeutic use of the compound is unlikely to present a hazard [10]. However, the literature on La genotoxicity contains some contradictory results. For example, $La(NO_3)_3$ was reported to cause increases in micronucleus frequency, DNA single-strand breaks, and unscheduled DNA synthesis in cultured human lymphocytes [11]. Recent studies showed that exposure to lanthanoids elicits oxidative injury of the lungs and kidneys in animals and humans [12,13], suggesting that La-induced oxidative stress and peroxidation might elicit genotoxicity. Therefore, it is important to test whether La, as a representative of REE, is genotoxic, so as to provide essential data for food safety assessment. We have evaluated the genotoxicity of $La(NO_3)_3$ in a battery of in vitro and in vivo genotoxicity tests, including a bacterial reverse mutation assay (Ames test), mouse bone marrow micronucleus assay, and in vivo/vitro chromosome aberration test.

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2. Materials and methods

2.1. Chemicals

Lanthanum was provided by the General Research Institute for Nonferrous Metals (Beijing, China). The element was dissolved in nitric acid to form a lanthanum nitrate ($La(NO_3)_3$) stock solution, 324.8 g/l. The stock was diluted to deliver the required dose in a volume of 5 ml/kg. Sodium azide (NaN_3), 2-aminofluorene (2-AF), 4-nitro-*o*-phenylenediamine (4NOPD), and 8-dihydroxyanthraquinone (DHAQ) were obtained from Sigma Aldrich (St. Louis, MO, USA). Mitomycin C (MMC), cyclophosphamide (CPA), and colchicine were purchased from Tocris Bioscience (UK).

2.2. Animals

BABL/c mice (6-week old) were provided by the Laboratory Animal Center of the Academy of Military Medicine Science (Beijing, China). The animals were kept in plastic boxes in barrier environment under controlled conditions of temperature ($22 \pm 2\,^{\circ}C$) and humidity ($50 \pm 10\%$) on a 12-h light/dark cycle, with standard mouse chow and water provided *ad libitum*. All aspects in this project involving animal care, use, and welfare were performed in compliance with the Food and Drug Administration (FDA) principles of GLP and in accordance with the FDA Guidance for Industry and Other Stakeholders, "Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000" [14]. All animal study protocols were approved by the Office of Laboratory Animal Welfare, China National Center for Food Safety Risk Assessment (Beijing, China).

2.3. Bacterial reverse mutation assay (Ames test)

Four histidine-dependent stains of *Salmonella typhimurium* (TA97, TA98, TA100, and TA102) were used for the bacterial reverse mutation assay and the experiment was performed according to the guidelines recommended by OECD [15]. Rat liver S9 was prepared according to previous method [16]. Bacteria were treated with $La(NO_3)_3$ doses = 0, 8, 40, 200, 1000, and 5000 μg/plate, with or without S9. Standard mutagens were used as positive controls in the experiments: NaN_3 (1.5 μg/plate) for TA100 without S9, 2-AF (10 μg/plate) for TA97, TA98, and TA100 with S9, 4NOPD (20 μg/plate) for TA97 and TA98 without S9, MMC (2.5 μg/plate) for TA102 with S9, and DHAQ (50 μg/plate) for TA102 without S9. After incubation for 48 h at 37 °C, revertant colonies were counted manually. The experiment was repeated twice.

2.4. Mouse bone marrow micronucleus assay

BABL/c mice (50) were randomly divided into five groups, ten mice per group and five mice for each sex. In a preliminary range-finding study, the oral LD_{50} for $La(NO_3)_3$ was determined to be 1710 mg/kg BW for male and 1470 mg/kg BW for female animals. According to the ICH guideline, the top dose was selected as ½ LD50 (855 mg/kg BW for male and 735 mg/kg BW for female), which was close to the minimum lethal dose. Animals were treated with the test compound at 0, 183.8, 367.5, 735.0 mg/kg BW (female mice) and 0, 213.8, 427.5, 855.0 mg/kg BW (male mice), by gavage, twice, with a 24 h interval. Cyclophosphamide (40 mg/kg BW) was used as positive control and purified water as negative control. 6 h after the second gavage, the animals were euthanized and the sternum was removed aseptically. The contents of the spinal canal were squeezed out, diluted with calf serum, and smeared onto the slides. After fixation with methanol and Giemsa staining, red blood cells (RBC) and polychromatic erythrocytes (PCE) were observed under microscopy. The number of PCE was counted from 200 RBC in each animal and the ratio of PCE/RBC was calculated. For each animal, 2000 PCE were examined for the incidence of micronucleated PCE.

2.5. Chromosome aberrations in mouse bone marrow cells

BABL/c mice (70) were randomly divided into five groups, seven female and seven male mice per group. Similarly, based on the LD_{50} in a preliminary range-finding study, animals were treated with the test compound at 0, 183.8, 367.5, 735.0 mg/kg BW (female mice) and 0, 213.8, 427.5, 855.0 mg/kg BW (male mice), or with cyclophosphamide, 40 mg/kg BW, by gavage, twice, with a 24 h interval. 14 h after the second gavage, the animals were intraperitoneally injected with colchicine, 4 mg/kg BW. 4 h later, all animals were euthanized and the femurs removed aseptically. Mouse bone marrow cells were immediately collected and suspended in KCl solution, 75 mM. After 30 min at room temperature, cells were fixed in fixative (methanol: glacial acetic acid = 3:1, v/v) and then dropped onto slides. Slides were stained with 5% Giemsa solution. 100 metaphase cells were analyzed for each animal. Any structural aberrations were recorded; the number of total aberrations and the aberration rate were calculated.

2.6. Chromosome aberrations in Chinese hamster ovary (CHO) cells

The CHO cell line used in the experiment was obtained from American Type Culture Collection (ATCC, CCL-61). Cells were cultured in ATCC-formulated F-12K Medium supplemented with fetal bovine serum (FBS, Gibco) to a final concentration of 10% at 37 °C in 95% air/5% CO_2. The chromosome aberration study was carried out in accordance with the methods described in OECD [17] and International Council for Harmonization (ICH) Guidance [18]. We preliminarily measured the cytotoxicity of the test compound at 0.10–10 mM under a 24 h-treatment with and without S9, using a Cell Counting Kit (CCK-8, sigma), and $La(NO_3)_3$ LC_{50} was estimated = 3.29 mM (Supplementary Fig. S1 in the onlin version at DOI: 10.1016/j.mrgentox.2016.09.008). However, due to the limit of solubility, we selected 2.5 mM (about 20% reduction in cell growth) as the highest concentration and two-fold dilutions for the other two concentrations, in our experiment. CHO cells were seeded at 5×10^4 cells/mL in plastic dishes and were treated with the test compound at 0, 0.62, 1.25, and 2.5 mM in the presence or absence of S9. CPA (10 μg/ml) and MMC (1.0 μg/ml) were the positive controls. Approximately 22 h after the initial treatment, colchicine (final concentration, 1 μM) was added to each culture and incubated for an additional 3 h. After 24 h from the start of treatment, the cells of each flask were washed with phosphate-buffered saline (PBS), dissociated and counted for Relative Increase in Cell Counts (RICC), as the index of cytotoxicity [17]. Cells were collected by centrifugation and resuspended in KCl solution, 75 mM. After incubation for 30 min at 37 °C, cells were fixed in fixative (methanol:glacial acetic acid = 3:1, v/v) and then dropped onto slides. Slides were stained with 5% Giemsa solution. Analyzable metaphases were identified with Axio Imager 2 & Metafer Ultra High Throughput Scanning System (Zeiss, Germany). Chromosomal aberrations were examined morphologically according to the principles described in the "Atlas of Genetics and Cytogenetics in Oncology and Haematology" [19,20]. Any structural aberrations were recorded, including chromosome-type breaks and exchanges calculated as incidence per 100 metaphase cells.

2.7. Statistical analysis

Ames test data were expressed as mean ± standard deviation and evaluated using one-way analysis of variance (ANOVA).