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Slight hypercalcemia is not associated with positive responses in the Comet Assay in male rat liver

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ABSTRACT

Maintenance of physiological levels of intracellular and extracellular calcium is essential for life. Increased intracellular calcium levels are involved in cell death (apoptosis and necrosis) and are associated with positive responses in the Comet assay in vitro. In addition, high calcium and vitamin D intakes were reported to induce apoptosis in adipose tissue in obese mice and to increase DNA-migration in the Comet assay. To investigate increased serum concentration of calcium as a potential confounding factor in the regulatory Comet assay in vivo, we induced mild hypercalcemia in male Wistar rats by 3-day continuous intravenous infusion of calcium gluconate and performed the Comet assay in the liver in line with regulatory guidelines.

The results of the study showed that mild increases in serum calcium concentration (up to 1.4 times above the concurrent control) and increased urinary calcium concentration (up to 27.8 times above the concurrent control) results in clinical signs like mild tremor, faster respiration rate and decreased activity in a few animals. However, under the conditions of the study, no increase in the %Tail DNA in the Comet assay and no indication of liver damage as determined by histopathological means were observed. Thus, mild increases in plasma calcium did not lead to positive results in a genotoxicity assessment by the Comet assay in the rat liver. This result is important as it confirms the reliability of this assay for regulatory evaluation of safety.

1. Introduction

Calcium homeostasis is ensured by the interaction of three calciotropic hormones (parathormone, calcitonin and 1,25-dihydroxy vitamin D3). Hypercalcemia is a high level of calcium (Ca^{2+}) in the blood serum. In adult humans, the normal range of total calcium is 2.14–2.60 mmol/L [1]. Slight hypercalcemia (levels not exceeding 0.25 mmol/L above normal range) is usually without symptoms. In those with greater levels (> 3.5 mmol/L), symptoms may include weakness, lethargy, dehydration, depression, renal, cardiovascular and/or gastrointestinal disorders. Hypercalcemia is multifactorial due to hyperparathyroidism, malignancy-associated hypercalcemia, endocrine disorders, or vitamin D intoxication [1,2].

Calcium is an intra- and extracellular messenger. Specifically, cytosolic calcium is involved in contraction of myofilaments, secretion of messengers such as hormones or neurotransmitters. Calcium can regulate mitotic division of cells but also regulates cell death via necrosis and apoptosis [3]. It was reported that increased nuclear or intracellular calcium concentrations increased the incidence of DNA damage in vitro [4,5]: Costunolide, a sesquiterpene lactone, induced G1-phase arrest and subsequent apoptosis. This effect was due to a Costunolide induced overload of nuclear calcium and DNA strand breaks (measured by the Comet assay) in PC-3 cells in vitro [4]. Likewise, acidified incubation medium caused increased intracellular calcium in FLO cells in vitro. This increased intracellular calcium mediated DNA-damage measured by the Comet assay [5]. High calcium and vitamin D intakes induced apoptosis in adipose tissue of obese mice [6]. The JACVAM validation trial [7] has demonstrated that amongst others apoptotic or necrotic changes have been associated with increases in DNA migration in the Comet assay.

As calcium mediates the formation of DNA strand breaks in vitro and apoptosis in vivo, it was hypothesized that hypercalcemia could be a confounding factor to genotoxic potential determination. Vitamin D Receptor agonists, low digestible carbohydrates (polyols or lactose), diuretics, or lithium are known to increase calcium levels [1,8]. Administration of vitamin D caused a slight increase in circulating calcium approximately 30–40% higher compared to controls, increased serum AST, and increased the tail intensity in rat liver [9]. The aim of this study was to examine whether the association of increased calcium and positive response in the Comet assay in vitro is also relevant for the in

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vivo situation. This would help in the discussion on potential confounding factors in the in vivo Comet assay.

We therefore induced slight hypercalcemic condition in male rats by means of continuous infusion of calcium gluconate as described by Isobe et al. [10] to study whether such condition affects liver tissue and causes DNA damage in the liver. For the latter endpoint, the Comet assay was used.

2. Materials and methods

2.1. Test and control items

The test item, calcium gluconate Injection, USP 10% (calcium gluconate) was manufactured by Fresenius Kabi at a concentration of 100 mg/mL. The vehicle, 0.9% Sodium Chloride for Injection, USP (physiological saline) was supplied by Baxter. The positive control, Ethylmethane sulfonate (EMS), was supplied by Sigma-Aldrich.

2.2. Verification of dose levels by analytical chemistry

Dose level verification of the test item calcium gluconate was done using calcium as the marker. Calcium was determined in the dosing solution by flame atomic absorption spectroscopy using a Perkin Elmer Aanalyst 800 instrument with a Calcium lamp set to the wavelength of 422.7 nm and the flame type of air/acetylene. The analytical procedure was validated with respect to selectivity, calibration range, precision, accuracy, injection medium stability and matrix stability and covered the concentration range of 25.0–100 mg/mL of calcium gluconate. The accuracies of the three treatment groups (50, 75 and 100 mg calcium gluconate/mL) as well as in the vehicle control group were performed in duplicate.

2.3. Guidelines and GLP compliance

The study was performed in accordance with the OECD Principles of Good Laboratory Practice. The design of this study was based on the study objectives, the ICH Harmonised Tripartite Guideline S2 (R1) [11], and OECD Guideline 489 [12].

2.4. Animal housing

The study was performed in an Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC) and a Canadian Council of Animal Care (CCAC) accredited test facility.

Twenty-seven (27) Crl:WI (Han) Wistar Hannover male rats were obtained from Charles River Kingston, Stone Ridge, NY. At initiation of treatment, the animals were 10 weeks old and weighed between 244 and 299 g. Animals were allowed an acclimation period to 2 weeks. Healthy animals were assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights.

Animals were individually housed in stainless steel perforated floor cages equipped with an automatic watering valve. Target temperatures of 19 °C to 25 °C with a relative target humidity of 30% to 70% and a 12 h light/12 h dark cycle were maintained. PMI Nutrition International Certified Rodent Chow No. 5CR4 (14% protein) was provided ad libitum throughout the study, except during designated procedures. Municipal tap water after treatment by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system, except during designated procedures.

2.5. Experimental design and treatment

The increased calcium levels were achieved by administering calcium gluconate by continuous intravenous infusion via a surgically implanted femoral indwelling catheter for 3 days (72 h) to Wistar Hannover rats: 4 groups of six male rats per group were treated by continuous infusion at a volume of 40 mL/kg/day. A positive control group, consisting of 3 rats, was also included in the study, and received two oral doses of 200 mg EMS/kg/day with an interval of 21 h, with the last administration 3 h prior to necropsy.

All animals in vehicle control and test item groups received within ± 15% of their overall targeted dose volumes over the course of the dosing period.

Throughout the study, animals were observed for general health/mortality and morbidity twice daily, once in the morning and once in the afternoon. Detailed clinical observations were performed prior to initiation and at completion of dosing. The animals were removed from the cage for the observations. Animals were weighed individually prior to randomization and on Days −1 and 3. A fasted weight was recorded on the day of necropsy.

2.6. Justification of route and dose levels

The intravenous route of exposure was selected as this is the appropriate route to obtain sustained increased calcium levels in the blood. This is necessary because rats can excrete excess calcium via urine within a short period of time (half-life 23 min [13]) which makes it difficult to achieve and maintain hypercalcemia by bolus administration of calcium.

Dose levels for this study were chosen based on a study conducted by Isobe et al. [10] in which the effect of hypercalcemia was investigated on another endpoint. The positive control EMS is typically administered for the Comet assay to show the laboratory proficiency and the dose levels were based on validated methodologies.

2.7. Blood and urine sampling and analysis

Blood was collected via the jugular vein on Day 3 and from the abdominal aorta following isoflurane anesthesia at termination (2 h ± 30 min post end of infusion). Urine was collected overnight from individually housed animals just before termination. Animals were fasted overnight before blood sampling and urine collection. Animals were not deprived of water during the urine collection procedure.

Blood samples were processed for serum, and the serum was analyzed for sample appearance/quality, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, creatine kinase, total bilirubin, urea nitrogen, creatinine, calcium, phosphorus, total protein, albumin, globulin, albumin/globulin ratio, glucose, cholesterol, triglycerides, sodium, potassium, and chloride.

Urine samples were processed and analyzed for the following parameters: color, appearance/clarity, specific gravity, pH, protein, glucose, bilirubin, ketones, blood, calcium, creatinine, and calcium/creatinine ratio.

2.8. Necropsy and sample collection

All animals were euthanized by exsanguination by incision from the abdominal aorta following isoflurane anesthesia and completion of blood collection. The animals were euthanized rotating across dose groups such that similar numbers of animals from each group, including controls, were necropsied throughout the day. Animals were fasted overnight before their scheduled necropsy. Vehicle control and test item treated animals were necropsied 2 h ± 30 min post end of infusion on Day 4 while the positive control animals were necropsied 3 h post last dose. Animals were subjected to a limited necropsy examination, which only included evaluation of the liver.

2.9. Liver sampling and Comet assay

At scheduled necropsy, the liver was dissected from all animals from