



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 vivo*

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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 Analyst 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 $\pm 15\%$ 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 \pm 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 \pm 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

Table 1
Summary of Body Weights (mean \pm SD).

Test Material	Dose (mg/kg/day)	Weight (g)			
		Day -12	Day -1	Day 3	Day 4
vehicle control	0	267 \pm 8	271 \pm 5	274 \pm 12	251 \pm 8
calcium gluconate Injection, USP	2000	267 \pm 9	273 \pm 17	273 \pm 18	254 \pm 15
	3000	250 \pm 24	262 \pm 14	260 \pm 16	244 \pm 14
	4000	266 \pm 8	267 \pm 8	259 \pm 12	239 \pm 11
positive control (EMS)	200	269 \pm 2	294 ^a \pm 4	304 ^a \pm 4	278 ^a \pm 3

Vehicle control = physiological saline; EMS = ethylmethane sulfonate.

Significantly different from vehicle control value: a = $p \leq 0.05$ (Dunnett).

each group and a portion of the left lateral lobe was kept for the Comet assay evaluation. The remaining portion of the liver was preserved in 10% neutral buffered formalin and embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin. Histopathological evaluation was performed by a board-certified veterinary pathologist.

The portion of the left lateral lobe of the liver was minced into ice-cold homogenizing buffer (Hanks balanced salt solution Mg^{2+} , Ca^{2+} and phenol red free, containing 20 mM EDTA and 10% v/v dimethyl sulfoxide, pH 7.4). The obtained single cell suspension was mixed with 0.5% low melt agarose and dispensed onto two slides coated with 1.0% melt agarose. Following gelled, the slides were immersed in lysis solution (100 mM tetrasodium EDTA, 2.5 M NaCl and 10 mM Tris hydroxymethyl aminomethane with 1% v/v Triton X-100 and 10% v/v dimethyl sulfoxide) overnight at 4 °C.

The slides were rinsed in water and transferred to a dry electrophoresis tank. Alkaline buffer (300 mM NaOH and 1 mM EDTA disodium, pH > 13.0) was added to the electrophoresis tank (Cleaver Scientific model CSL-COM40), kept to below 10 °C, in order to cover the slides of about 2–3 mm above the slides. The cells were allowed to unwind its DNA in the buffer for 20 min. A voltage of 16 V was applied (0.94 V/cm) for 20 min. After electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris hydroxymethyl aminomethane, pH 7.5), placed in absolute cold ethanol for 30 min and allowed to air dry until analysis.

The slides were encoded to minimize potential operator bias. The slides were wet-mounted with propidium iodide at 20 μ g/mL and the cells visualized using a fluorescence microscope. Computerized image analysis of selected cells was performed using a Perceptive Instruments COMET ASSAY IV image analysis system. A total of 150 liver cells per animal (75 cells per slide) were evaluated. The excessively damaged cells (i.e. 'hedgehog' or 'ghost' comets) were counted but excluded from analysis.

2.10. Statistical Analysis

All statistical tests were conducted at the 5% significance level. All pairwise comparisons were conducted using two sided tests and were reported at the 0.1%, 1%, and 5% levels. Numerical data collected on scheduled occasions for body weights and clinical pathology values were analyzed as indicated according to sex and occasion. Descriptive statistics number, mean and standard deviation were reported whenever possible. Each treated group as well as the positive control group was compared to the vehicle control group.

The numerical data corresponding to the %Tail DNA was statistically evaluated using two separate datasets. The calcium gluconate treated groups were compared to the negative control group using the Levene's test, to assess the homogeneity of group variances, followed by an overall one-way ANOVA F-test, since the Levene's test was not found to be significant ($p > 0.05$). The positive control group was compared to the negative control group using the Levene's test to assess the homogeneity of group variances, followed by the Wilcoxon Rank-Sum

test, since the result of the Levene's test was found to be significant ($p \leq 0.05$).

3. Results

3.1. Dose formulation analysis

The stability of the test item in the vehicle at room temperature was demonstrated over a period of 3 days. The test item was also stable at 4 °C for 5 days. The accuracy results of all samples were within 8.2% of their mean and individual theoretical concentrations. Thus, dosing solutions were accurately prepared and the test item was distributed homogeneously. The positive control formulations were not subjected to analysis for safety reasons and because the biological response of the test system is considered to be the best measure of the appropriateness of the formulations.

3.2. Mortality, clinical signs, body weight

There were no unscheduled deaths over the course of this study.

The administration of calcium gluconate had no effect on body weights when compared to the concurrent vehicle control group (Table 1). Statistically significant higher body weights observed in the positive control group were suspected to be due to absence of surgery in this group.

On Day 4, calcium gluconate at 4000 mg/kg/day was associated with tremors and a faster respiratory rate in one male and decreased activity level (with eyes partly closed) in another one.

3.3. Clinical pathology

On Day 3, minimal increases in mean serum calcium were noted at 2000 mg/kg/day without reaching statistical significance. At 3000 and 4000 mg/kg/day, mean calcium levels reached 1.2X and 1.4X, respectively, when compared to vehicle controls. At termination, the serum calcium levels were back to control level at 2000 and 3000 mg/kg/day, and a minimal increase was still noted at 4000 mg/kg/day (Table 2). The urinary calcium on Day 4 was elevated at all calcium gluconate dose levels, reaching 27.8X at 4000 mg/kg/day when compared to vehicle control (Table 3).

On Day 4, the alkaline phosphatase (ALP) was decreased at all calcium gluconate dose levels. At 4000 mg/kg/day, calcium gluconate induced increases in urea (data not shown) and triglycerides. When calcium gluconate was given at ≥ 3000 mg/kg/day, changes included decreases in potassium, chloride and/or phosphorus. The administration of the positive control was associated with decreases in phosphorus and chloride and increases of glucose, triglyceride, total protein, and albumin.

3.4. Comet assay

Following visual examination of the slides, no evidence of overt

Table 2
Clinical Chemistry Parameters (mean + / - SD).

Test Material	Dose (mg/kg/day)	Time point	Calcium (mg/dL)	ALP (u/L)	Glucose (mg/dL)	Triglycerides (mg/dL)	Total protein (g/dL)	Phosphorus (mg/dL)	Potassium (mmol/L)	Chloride (mmol/L)	
vehicle control	0	Day 3	11.3 + / - 0.3	-	-	-	-	-	-	-	
		Day 4	10.2 + / - 0.3	96 + / - 21	100 + / - 10	33 + / - 9	5.6 + / - 0.3	9.0 + / - 0.6	4.5 + / - 0.2	108 + / - 2	
		Day 3	11.8 + / - 0.5	-	-	-	-	-	-	-	-
		Day 4	10.2 + / - 0.4	74 + / - 14 ^d	111 + / - 13	38 + / - 8	5.6 + / - 0.2	8.7 + / - 0.6	4.1 + / - 0.2	106 + / - 2	
calcium gluconate Injection, USP	2000	Day 3	13.6 + / - 1.0 ^a	-	-	-	-	-	-	-	
		Day 4	10.0 + / - 0.3	55 + / - 9 ^f	118 + / - 16	32 + / - 8	5.5 + / - 0.3	8.6 + / - 0.4	3.9 + / - 0.4 ^e	105 + / - 2 ^d	
		Day 3	15.9 + / - 1.5 ^c	-	-	-	-	-	-	-	-
		Day 4	11.0 + / - 0.8	61 + / - 10 ^f	121 + / - 15	46 + / - 9 ^a	5.4 + / - 0.3	7.3 + / - 0.8 ^f	3.7 + / - 0.2 ^f	104 + / - 2 ^f	
positive control (EMS)	200	Day 3	-	106 + / - 7	236 + / - 45 ^b	70 + / - 8 ^f	6.7 + / - 0.4 ^f	6.7 + / - 0.1 ^f	4.8 + / - 0.3	104 + / - 1 ^e	
		Day 4	10.9 + / - 0.6	-	-	-	-	-	-	-	

Vehicle control = physiological saline; EMS = ethylmethane sulfonate.

Significantly different from vehicle control value: a = p ≤ 0.05, b = p ≤ 0.01, c = p ≤ 0.001 (Dunn).

Significantly different from vehicle control value: d = p ≤ 0.05, e = p ≤ 0.01, f = p ≤ 0.001 (Dunnnett); -, Not measured.

Table 3
Calcium Levels in Urine (mean + / - SD).

Test Material	Dose (mg/kg/day)	Calcium (mg/dL)	Calcium/creatinine
vehicle control	0	9.8 + / - 6.9	0.2 + / - 0.1
calcium gluconate Injection, USP	2000	104.2 + / - 63	1.4 + / - 0.7
	3000	237.6 + / - 95.8 ^a	3.4 + / - 0.4 ^a
	4000	271.8 + / - 116.2 ^b	4.3 + / - 1.8 ^b
positive control (EMS)	200	2.8 + / - 1.1	0.1 + / - 0.0

EMS = ethylmethane sulfonate, vehicle control = physiological saline.

Significantly different from vehicle control value: a = p ≤ 0.05, b = p ≤ 0.01 (Dunn).

toxicity was noted on the slides, i.e. there was no substantial increase in background debris and/or increase in the incidence of excessively damaged cells (i.e. non-detectable cell nuclei, hedgehog or ghost comets). The group mean incidence for the negative control was 3.2%, and between 2.5 and 3.5% for the test item treated groups.

The administration of the test item had no effect on the %Tail DNA Intensity in the liver. The group mean vehicle control %Tail DNA Intensity was 0.135 + / - 0.064. Thus, the %Tail DNA Intensity group mean of the concurrent vehicle control was within, or slightly lower compared to the historical negative control range (0.1–2.1 %Tail DNA Intensity) observed in 17 experiments. The positive control showed clear, unequivocal increases in %Tail DNA Intensity (Table 4). The performance of the vehicle and positive controls were consistent with a valid assay.

3.5. Liver examination

The test item and the positive control had no influence on the macroscopic appearance of the liver. No test item- or positive control-related microscopic findings were noted. Mild increased hepatocellular mitoses were noted in 2 positive control animals.

4. Discussion and conclusion

The adequate reliability of the Comet assay to predict the genotoxic potential of a test item is well described [14–16]. Despite satisfactory performance of this assay, there are some reports of confounding factors that may compromise the confidence of the results and increase uncertainty of the risk assessment.

Some discrepancies between the sensitivity of the comet assay for short-term (1–3 daily) and long-term (2–4 weeks) administration have been demonstrated [16]. Information on target organ and on mode of genotoxicity could often explain unexpected results in the Comet assay in vivo with known genotoxins. Nevertheless, the reasons for a negative response to 2-acetyl aminofluorene remain unclear [14]. Likewise, the rodent carcinogen 4,4'-diaminodiphenyl ether produced no increase in DNA migration in liver but a decrease in DNA migration was found in stomach [17].

Table 4
Results of the Comet assay (mean + / - SD).

Test Material	Dose (mg/kg/day)	%Tail DNA
vehicle control	0	0.135 + / - 0.064
calcium gluconate Injection, USP	2000	0.092 + / - 0.058
	3000	0.127 + / - 0.037
	4000	0.080 + / - 0.038
positive control (EMS)	200	13.603 ^a + / - 1.108

Vehicle control = physiological saline; EMS = ethylmethane sulfonate.

Significantly different from vehicle control value: a = p ≤ 0.05 (Wilcoxon).

Altogether the reports demonstrate good performance of the Comet assay, but highlight the need to deepen the understanding of factors that may generate contradictory results and the necessity for careful consideration of study design when using the Comet assay as a measure of *in vivo* genotoxicity.

Calcium is reported to be associated with positive responses in the Comet assay *in vitro* and reported to induce apoptosis *in vivo* under certain circumstances [6]. In addition, administration of vitamin D caused slight hypercalcemia and increased DNA-migration in the liver of exposed rats [9]. The aim of this study was to investigate whether a mild increase in serum calcium concentration and increased calcium excretion in male rats are associated with the induction of toxicity and DNA damage in liver as measured by the Comet assay.

A slight increase in serum calcium levels was achieved after continuous intravenous infusion of calcium gluconate to male rats at dose levels of 2000, 3000, or 4000 mg calcium gluconate/kg/day. On Day 3, statistically significant increased serum calcium levels were achieved in the mid and high dose group (1.2–1.4X). In the high dose group, isolated clinical signs consisted of tremors, faster respiration and decreased activity which could be explained by the increased serum calcium concentrations. However, prominent clinical signs or effects on body weights were not seen which was expected based on Isoble et al. [10].

At termination on Day 4 which was about 2 h after the end of the infusion, serum calcium levels had already returned to normal except for the high dose group. The calcium/creatinine ratio in urine was statistically significantly increased in the mid and high dose on Day 4. This reflects the efficient excretion capacity of rats in the event of excess calcium. Patel et al. [13] reported a very short half-life of calcium. Overall, the infused amount of calcium gluconate seemed just to exceed the urinary excretion capacity of the rat kidney.

The administration of calcium gluconate by a continuous intravenous infusion for 3 days at levels of 2000, 3000, or 4000 mg calcium gluconate/kg/day did result in mild hypercalcemia but did not result in any macroscopic or microscopic findings in the liver nor in increases in %Tail DNA Intensity in the liver as compared to the vehicle control. Expected responses were noted for negative and positive control groups.

In conclusion, mild hypercalcemia did not lead to a misleading positive result in genotoxicity assessment by the Comet assay in liver of rats. Therefore, even if a non-genotoxic compound induced mild hypercalcemia *in vivo*, it is expected that the Comet assay will accurately detect it as non-genotoxic. This result is important as it confirms the reliability of this assay for regulatory evaluation of safety.

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Conflict of interest

The authors declare no conflict of interest other than employment.

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