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ORIGINAL PAPER

$[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3{\cdot}H_2O$ (Cr3) Toxicity Potential in Bacterial and Mammalian Cells

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Abstract Chromium(III) has generally been considered to be essential for proper carbohydrate and lipid metabolism, and, despite recent evidence to the contrary, chromium(III)-containing compounds remain one of the most popular commercial dietary supplements. Cr3, or $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3{\cdot}H_2O$, is a trivalent chromium compound that is a promising chromium nutritional supplement. Studies with Cr3 have indicated that it is non-toxic in developmental and short- and long-term exposure studies in rodents, but the safety of this compound to chromosomes and cells has not been explored. The current study evaluates the mutagenicity, cytotoxicity, and clastogenicity of Cr3 in bacterial and mammalian cells and compares these results with similar studies using the bestselling Cr(III) nutritional supplement, chromium picolinate (CrPic). The mutagenicity of CrPic and Cr3 was tested in *Escherichia coli* FX-11 and *Salmonella typhimurium* (TA 98 and TA 100). Cytotoxicity was measured as a decrease in plating efficiency relative to controls after treatment with Cr3 and CrPic for 24 h in CHO K1 cells. Clastogenicity was measured by counting the number of metaphases damaged and of the total number chromosomal aberrations in CHO K1 cells. Mutagenesis assays in *E. coli* and *S. typhimurium* were negative. All treatments of Cr3 produced ≥ 84% plating efficiency except 80 μg/cm^2, which reduced the plating efficiency to 62%. Cr3 at any treatment level did not produce a significant increase in the number of cells with abnormal metaphases, while treatments using ≥ 40 μg/cm^2 of CrPic elevated the number significantly. These data suggest that Cr3 is significantly less mutagenic in bacteria cells and less clastogenic in CHO K1 cells, while CrPic is clastogenic in CHO K1 cells.

Keywords Cr3 · $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ · CHO · Cytotoxicity · Chromium picolinate

Introduction

The element chromium (Cr) in its trivalent oxidation state has been thought to be essential for proper carbohydrate and lipid metabolism for nearly 40 years [1–3]. Despite recent evidence that Cr should not be considered an essential element [4] and the determination by the European Food Safety Authority [5, 6] that Cr is not an essential element for animals or humans and dietary supplementation is therefore unnecessary, the sale of Cr(III)-containing compounds, mainly in the form of chromium picolinate (CrPic), has been a multimillion dollar business [7]. Yet, rodent studies suggest that Cr(III) can have beneficial effects on insulin sensitivity, particularly in models of insulin resistance and diabetes, at large, pharmacologically relevant doses [8]. CrPic has been the focus of numerous clinical studies; however, CrPic and other forms of Cr(III) have not been convincingly demonstrated to have beneficial effects in humans [9]. However, none of the human clinical trials have approached the dose (when scaled) administered to rodents and leading to beneficial effects. In addition, the safety of CrPic has been questioned [10], as it was reported to cause

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chromosomal aberrations 3-fold to 18-fold above control levels for concentrations from 0.05 to 1.0 mM in Chinese hamster ovary (CHO) cells after 24 h of exposure [11]. CrPic also induced mutagenesis in L5187Y mouse lymphoma cells at relatively non-toxic levels [12]. Previous studies have indicated that CrPic may induce vertebral malformations in mouse fetuses exposed to high levels of the supplement [13]. The results of a behavioral study with picolinic acid, the ligand in CrPic, suggest that it may cause behavioral deficits in mouse pups exposed through gestation and weaning, although the results of that study were not statistically significant [14].

As CrPic may have adverse effects, an alternate form of Cr(III) for use as an oral pharmaceutical agent and as part of a potential treatment for diabetes might prove valuable. A low-molecular-weight chromium-binding substance (LMWCr), an oligopeptide, has been proposed to be the active form of Cr, as it enhances the tyrosine kinase activity of insulin receptors up to 8-fold in the presence of insulin in vitro [15]. However, LMWCr is susceptible to hydrolysis under acidic conditions and would be degraded when taken orally [16]. Davis and coworkers demonstrated that a propionate complex of Cr(III), [$Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3$]$NO_3$·$H_2O$, or Cr3, could activate the tyrosine kinase activity of insulin receptor as well, and Cr3 has been shown to be stable in aqueous and acidic solutions [17]. Cr3 lowers the levels of fasting plasma triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, and plasma insulin in healthy and type 2 diabetic rats [18]. Cr3 has not been shown to increase the incidence of vertebral abnormalities [19], and long-term administration of the compound to adult male Wistar rats did not reveal any adverse effects [6].

Not only do multiple animal studies indicate that Cr3 causes no adverse effects; the compound also provides more bioavailable chromium than any other supplement currently on the market. When ingested orally, Cr3 can be absorbed with up to 60% efficiency, which is greater than chromium chloride or CrPic (0.5–2%) [20]. As a promising supplement, regulatory agencies such as the US Food and Drug Administration require assessments of cytotoxicity and mutagenicity before a product is made available for human consumption. To date, few such studies of Cr3 have been published. As a dietary supplement, this compound would generally be outside the jurisdiction of most regulatory agencies, but nevertheless, information about the cytotoxicity and mutagenicity of Cr3 is helpful. Thus, the current study investigated the mutagenicity of Cr3 in *Escherichia coli* FX-11 and *Salmonella typhimurium* cells in the presence and absence of S9 metabolism. Additionally, the cytotoxicity and clastogenicity of both Cr3 and CrPic were examined in Chinese hamster ovary (CHO) K1 cells.

Materials and Methods

Bacterial Strains and Test Chemicals

The *E. coli* FX-11 bacteria were kindly provided by Dr. Yixin Yang from Emporia State University (Emporia, KS). Two strains (TA 98 and TA 100) of *S. typhimurium* and 10% S9 rat liver mixtures were purchased from Moltox, Inc. (Boone, NC). The 10% S9 rat liver mixture was prepared by mixing Aroclor 1254-induced Sprague Dawley male rat liver S9 with NADPH-regenerating system cofactors and phosphate buffer by the company. Chinese hamster ovary (CHO) K1 cells were originally purchased from American Type Culture Collection (Manassas, VA, no. CCL-61).

Cr3 ([$Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3$]$NO_3$·$H_2O$; molar mass 744.5) was synthesized according to the methods of Earnshaw et al. [21]. CrPic ([Cr(picolinate)$_3$·H_2O]; molar mass 436.3) was synthesized according to the methods of Press et al. [22]. The authenticity of both was established by high-resolution electron impact mass spectrometry [23, 24]. Deionized (DI) water was used as the solvent for Cr3 and CrPic in all assays. *N*-ethyl-*N*-nitrosourea (ENU) was also generously provided by Dr. Yixin Yang.

E. coli Mutagenicity Assay

The assay was carried out as described by Yang and Fix [25]. Due to a UAA defect in the messenger RNA (mRNA) encoding amino acid number 161 of the *tyrA* gene product, *E. coli* FX-11 are auxotrophic for tyrosine [26].

In this mutagenicity assay, *E. coli* cultures were grown overnight in A-O minimal media [27] at 37 °C to the density of OD_{450} = 0.5. Cells were then divided into two groups based on treatment time (1 h treatment or 48 h treatment). In both assays, *E. coli* cells were treated with either 0.025, 0.05, 0.1, 0.5, or 1.0 mM solutions of Cr3 or CrPic. All solution concentrations refer to the concentration of the compound, and not to the Cr(III) only.

One-Hour Assay

In this assay, mutagenesis and viability were assayed to determine mutation frequency. After 1 h of treatment, the cells were centrifuged and resuspended in A-O buffer. ENU was applied as the positive control at a final concentration of 0.5 mM. To test viability, cells were diluted 100,000 times and were plated on A-O semi-enriched minimal agar. The plates were then incubated at 37 °C for 24 h, and the number of colony-forming units (CFUs) was counted. Mutagenesis was determined by plating cells directly onto A-O semi-enriched minimal agar plates lacking tyrosine, and the number of revertant colonies was counted after 48 h incubation at 37 °C.

Mutation frequency was calculated as the number of revertants divided by the number of viable cells.

Forty-Eight-Hour Mutagenicity Assay

In this experiment, test chemicals were not removed prior to plating. A mixture of the appropriate test chemicals (at the concentrations used in the 1-h assay) and cells was applied directly onto the surface of tyrosine(−) A-O semi-enriched minimal agar plates to test mutagenesis. After 48 h incubation at 37 °C, visible colonies were counted as revertants.

Ames Assay

This assay was performed as described by Mortelmans and Zeiger with or without S9 mixture [28]. *Salmonella* strains TA 98 and TA 100 are both histidine auxotrophs and detect frameshift mutations and substitution mutations, respectively. They were grown overnight in oxoid nutrient broth no. 2 (Sigma) at 37 °C to the density of 1–2 × 10^9 cells/ml. To the sterile glass tubes, 0.5 ml A-O buffer or S9 mixture, 0.05 ml of DI water or test chemical at the concentration of interest, and 0.1 ml bacteria culture were added. After a 20-min incubation, 2.0 ml of molten top agar was added to each tube, and the mixture was poured onto the surface of glucose minimum agar plates that contained no histidine. Five concentrations from 100 to 10,000 μg/cm^2 of Cr3 and CrPic were tested in duplicate for each strain. After 48 h incubation at 37 °C, the number of CFUs was counted (revertants). The criteria for a tested compound to be considered mutagenic were the mean number of revertants (over the solvent control) must increase 2–3-fold per plate and the increase in number must be dose-dependent [29].

Colony-Forming Cell Assay

Chinese hamster ovary (CHO) K1 cells were grown in McCoy's 5A culture medium supplemented with 10% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin (Sigma). In each T25 flask, 5 × 10^5 cells were seeded and incubated at 37 °C in a 5% CO_2 atmosphere for 20 h. Cells were treated with 4.0, 20, 40, or 80 μg/cm^2 of Cr3 or CrPic for 24 h. Deionized (DI) water was used as the solvent for Cr3 and CrPic. After 24 h, cells were rinsed with Hank's balanced salt solution (HBSS, Sigma) and harvested with 0.25% trypsin/EDTA (Sigma). After enumeration with a hemocytometer, each treatment group was reseeded in quadruplicate, with 200 cells in a 6-well plate with 3 ml culture medium. Cells were incubated at 37 °C in 5% CO_2 grown for 7 days, and the medium was changed every 3 days. After 7 days, cells were fixed with freshly prepared Carnoy's fixative (3:1 methanol/acetic acid) and stained with crystal violet. Colonies were counted and averaged for quadruplicate doses. Plating efficiency was calculated as percent colonies in treated groups relative to water control (125/200 colonies on average).

Chromosomal Aberration Assay

The chromosomal aberration assay was conducted using a standard assay as described by Klein et al. [30]. CHO cells were seeded and treated in the same way as in the cytotoxicity assay at 37 °C in a 5% CO_2 incubator for 24 h. Two hours before harvesting cells, demecolcine solution (Sigma) was added to cell cultures at a concentration of 0.1 μg/ml. Following the 2-h demecolcine treatment phase, cells were trypsinized and transferred to 15 ml tubes and centrifuged. Cell pellets of each treatment group were resuspended in 4 ml of 0.075 M KCl. After a 10-min period, 5 ml of a Carnoy's fixative solution was added to cell suspensions, refrigerated at 4 °C for 30 min, and centrifuged 10 min at 300×*g* at 4 °C. The 40 min KCl/Carnoy's fixation process was repeated three times. After centrifuge at the third time, cells were resuspended in 1 ml of Carnoy's fixative for slide preparation.

Glass slides were chilled overnight at 4 °C. To each slide, 3–4 drops of the cell suspension were applied. Fixed cells were stained with 10% Giemsa solution for 1 min. The Giemsa solution consisted of 9 ml Sorenson's phosphate buffer and 1 ml Giemsa stain. For each treatment group, 100 cells in metaphase were examined under oil immersion for chromosomal aberrations. Cell samples were coded by a person not involved in the project and scored by the author without any knowledge of treatment condition. The percentage of metaphases with chromosomal damage and total aberrations per 100 metaphases population were analyzed for each group before the code was revealed. Experiments were carried out in triplicate, with 100 metaphase cells examined for each dose level. A total of 300 metaphase cells were analyzed for each treatment group.

Statistical Methods

Data were analyzed by one-way ANOVA using SPSS (SPSS, Inc., Chicago, IL) followed by a two-way Dunnett's *t* test to determine specific significant differences ($p \leq 0.05$).

Results

Mutagenicity Assays

In the *E.coli* mutagenicity assay, mutagenesis was measured as an increase in the number of revertants relative to the solvent control after 1 or 48 h exposure to varying concentrations of Cr3, CrPic, or ENU. After 1 h of treatment, no significant

differences ($p \leq 0.05$) were present among the groups with regard to mutation frequency except for the ENU 0.5 M group (positive control) (Fig. 1). After 48 h of exposure, no major difference existed in the number of revertants between Cr3 and CrPic groups, and neither of them caused a significant increase in the number of revertants compared to untreated controls (Fig. 1). Thus, the results of the *E. coli* mutagenicity assay show that treatment with Cr3 and CrPic up to 1.0 mM did not produce mutagenesis. Cr3 and CrPic were tested in the *S. typhimurium* Ames assay with tester strains TA 98 and TA 100 at dose levels up to and including 10,000 μg/plate with and without S9 metabolic activation. Both Cr3 and CrPic were clearly non-mutagenic in the *Salmonella* strains tested (Table 1).

Colony-Forming Cell Assays

CHO K1 cells were exposed to solvent (DI water) alone or to four concentrations of Cr3 and CrPic ranging from 4.0 to 80 μg/cm^2 for a 24-h period. Toxicity was measured as a reduction in plating efficiency relative to the solvent control. According to the criteria established by the World Health Organization (WHO), a concentration giving rise to > 50% plating efficiency can be considered non-toxic [31]. Recently, a new limit of 40% cytotoxicity (60% plating efficiency) was suggested to prevent the observation of false positive results [32].

In the current study, the cutoff between cytotoxic and non-cytotoxic was defined as ≤ 60% plating efficiency. As shown in Table 2, treatments of 4.0, 20, and 40 μg/cm^2 Cr3 produced colonies > 84% of control. Cr3 at 80 μg/cm^2 decreased the plating efficiency to 62%, which, although statistically significant, is still considered non-toxic based on the criteria set forth by the WHO. In contrast, CrPic concentrations over 40 μg/cm^2 produced a significant cytotoxicity (plating efficiency < 60%).

Clastogenicity Assays

Chromosomal aberrations were measured for CHO K1 cells treated for 24 h with the same concentrations of Cr3 and CrPic as in colony-forming cell assay described above. The number of chromosomal aberrations produced by Cr3 ranged from 2 ± 1 to 5 ± 2 per 100 cells in metaphase (Table 3), and there

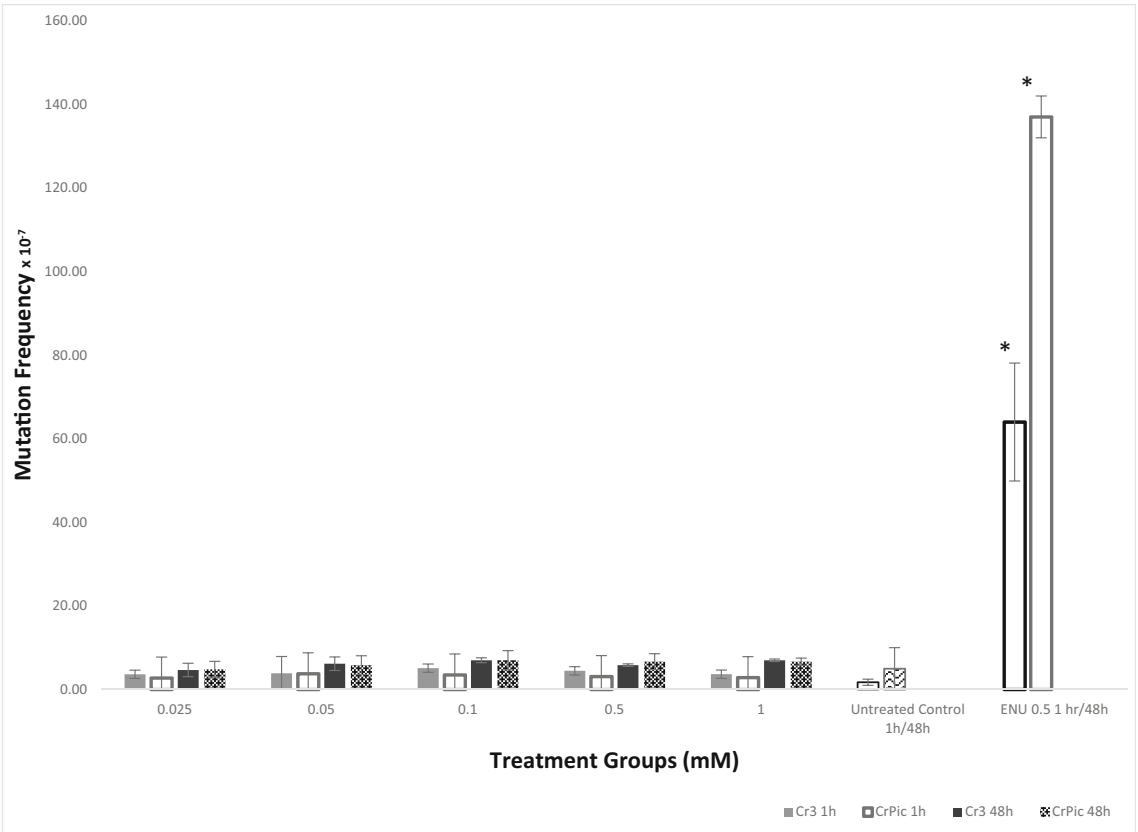

Fig. 1 Effect of various concentrations of Cr3 and CrPic on mutation frequency in *E. coli* FX-11after 1 and 48 h treatments. Mutation frequency (×10^{-7}) = number of revertants/number of viable cells. Data are presented as mean ± standard deviation. *$p < 0.05$ as compared to all other treatment groups

Table 1 Mutagenicity of Cr3 and CrPic in *Salmonella typhimurium*

Dose (μg/plate)	TA 98 (−) S9	(+) S9	TA 100 (−) S9 S9	(+)
DI water	38 ± 3	36 ± 10	23 ± 2	33 ± 8
Cr3 100	41 ± 3	42 ± 4	28 ± 8	33 ± 3
Cr3 333	45 ± 4	48 ± 9	26 ± 6	33 ± 7
Cr3 1000	49 ± 9	45 ± 7	28 ± 5	34 ± 8
Cr3 3333	47 ± 7	46 ± 7	26 ± 5	35 ± 7
Cr3 10,000	51 ± 9	62 ± 8	29 ± 6	38 ± 11
CrPic 100	51 ± 9	41 ± 4	23 ± 8	34 ± 9
CrPic 333	49 ± 5	40 ± 6	37 ± 23	41 ± 13
CrPic 1000	47 ± 7	43 ± 8	30 ± 11	32 ± 5
CrPic 3333	52 ± 13	44 ± 6	33 ± 6	39 ± 9
CrPic 10,000	55 ± 8	53 ± 7	39 ± 8	37 ± 10
Positive control[a]	402 ± 19	385 ± 14	457 ± 30	487 ± 9

Average revertants/plate ± standard deviation

[a] The positive controls without S9 were 2-nitrofluorene (1.0 μg/plate) for TA98, sodium azide (1.0 μg/plate) for TA100. With S9 mixture, 1-aminoanthracene (1.0 μg/plate) was used for both tested strains

was no significant difference among any of the Cr3 doses and the solvent control cells. The 4.0 μg/cm^2 group of CrPic yielded 5 ± 1 chromosomal aberrations, the clastogenicity of which was almost the same as that of the highest dose of Cr3. Though 4.0 and 20 μg/cm^2 doses of CrPic did not produce significant clastogenicity compared to untreated controls, the number of chromosomal aberrations was still higher than equivalent doses of Cr3. For the 80 μg/cm^2 CrPic, the number of chromosomal aberrations was 15 ± 2, which was 3 times higher than the number produced at the equivalent dose of Cr3 (Table 3). None of the Cr3 doses produced a significant increase in the percentages of damaged metaphases, while the percentages for CrPic at 40 and 80 μg/cm^2 were significantly elevated compared to the cells exposed to solvent alone (Table 3). Therefore, CrPic appeared to be more cytotoxic and clastogenic than Cr3 at all dose levels in CHO K1 cells, and both of the effects were dose-dependent.

Table 2 Cytotoxicity of Cr3 and CrPic in CHO K1 cells after 24 h treatment

Treatment	Dose (μg/cm^2)	Plating efficiency (%)	*P* value	SD
DI water		100		5.80
Cr3	4.0	98	> 0.05	5.32
	20	96	> 0.05	5.39
	40	84	> 0.05	4.87
	80	62	< 0.0001	7.40
CrPic	4.0	79	< 0.0001	7.09
	20	74	< 0.0001	6.67
	40	57	< 0.0001	5.18
	80	29	< 0.0001	5.04

Each data point represents the average of three independent experiments

Discussion

The mutagenicity of CrPic in *S. typhimurium* TA 98 and TA 100 with and without S9 metabolism was tested and reported negative by the National Toxicology Program (NTP) [12]. The solvent used was dimethyl sulfoxide (DMSO). The extreme water solubility of Cr3 meant that DI water, rather than a solvent such as DMSO, was able to be used to solubilize the compound for testing. Chromium supplements have been shown to generate reactive oxygen species in cells, such as superoxide anion, hydroxyl radicals, and hydrogen peroxide [33–35]. Considerable evidence indicates that reactive oxygen species play an important role in the process of cellular injury which can lead to cancer. Of all the Cr supplements, CrPic was found to enhance the production of hydroxyl groups [34]. DMSO is known to be a radical scavenger with high affinity for hydroxyl radicals [36], so it should decrease the reactive oxygen species produced through Fenton-type chemistry [33], thus decreasing the DNA damage that leads to mutations. The exclusion of DMSO in this study ensures that the negative results reported in this study are because Cr3 is not mutagenic and not because of solvent interference. It should be noted, however, the reported solubility of CrPic in water is 0.6 mM at room temperature, so the 1.0 mM of CrPic was partially suspended in DI water, but at all other concentrations, CrPic was dissolved. The mutagenicity of Cr3 has not been previously reported, and our results indicate that indeed it is not mutagenic based on results of the tester strains used in this study.

The data in the present study strongly suggest that CrPic is cytotoxic at far lower levels than Cr3. A 4.0-μg/cm^2 dose of CrPic produced a plating efficiency of 79%, which was a statistically significant decrease compared to the 98% in Cr3

Table 3 Chromosomal aberrations in CHO K1 cells after 24-h exposure to Cr3 and CrPic

Treatment	Dose (μg/cm^2)	Metaphases with damage (%)	Total aberrations (per 100 metaphases)
DI water	0	1 ± 1	1 ± 1
Cr3	4.0	2 ± 1	2 ± 1
	20	4 ± 1	4 ± 1
	40	3 ± 1	3 ± 1
	80	5 ± 1	5 ± 2
CrPic	4.0	5 ± 1	5 ± 1
	20	5 ± 1	6 ± 1
	40	9 ± 1[a]	10 ± 1[a]
	80	13 ± 2[a]	15 ± 2[a]
Mitomycin C	0.016	58 ± 4[a]	83 ± 3[a]

Data is presented as mean ± standard deviation. Three hundred metaphases (100 per experiment) were analyzed for each treatment group

[a] Indicates significant difference of metaphases with damage or of chromosomal aberrations ($p < 0.05$)

4.0 μg/cm^2 group (Table 2). Similar results are reported for other doses of CrPic, and only the 4.0 and 20 μg/cm^2 concentrations were considered acceptably non-cytotoxic according to the criteria (> 60% plating efficiency). Cytotoxicity for concentrations of 40 and 80 μg/cm^2 of CrPic produced 57 and 29% plating efficiency, respectively, which fall below the cutoff limits. The plating efficiency of 80 μg/cm^2 dose is consistent with the results about CrPic reported by Stearns et al., who produced 24 ± 11% plating efficiency in CHO AA8 cells although the CrPic was also suspended in acetone in their study [37]. CrPic treatment at all levels was more cytotoxic than the corresponding concentrations of Cr3. This difference in plating efficiency between Cr3 and CrPic suggests that Cr3 can be considered safer in mammalian cells. If CrPic could completely dissolve at this level, the cytotoxicity produced possibly would be even higher.

Concerns about the potential toxicity of CrPic were first raised in studies by Stearns and co-workers that demonstrated the compound as a solid suspension in acetone or the mother liquor from the synthesis of CrPic-generated chromosomal aberrations in CHO AA8 cells [11]. Particulate doses of CrPic of 8.0 and 40 μg/cm^2, but not 4.0 μg/cm^2, were found to lead to more total aberrations than controls treated with only acetone. The number of aberrations was dose responsive. Subsequent studies by this group demonstrated that CrPic was mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase locus in CHO AA8 cells [37] and generates mitochondrial damage and apoptosis [38].

The clastogenicity of CrPic has been studied previously in CHO K1 cells, and no chromosome damage was found with doses up to 770 μg/mL, which is equivalent to 123 μg/cm^2 [39]. Related studies in CHO K1 cells found CrPic was not mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase locus [40]. These studies, funded by the major commercial provider of CrPic, contrast starkly with the results of Stearns and co-workers. However, the hydroxyl radical scavenger DMSO was used as the solvent for CrPic, increasing the possibility the free radicals released by CrPic were trapped by DMSO. Coryell and Stearns [41] have actually demonstrated this quenching of CrPic mutagenicity by DMSO. They also found that substitutions comprised 33% of CrPic-derived DNA mutations, with transversions being predominate; 62% were deletions with one-exon deletions predominating. Insertions of 1–4 base pairs comprised 5%. CrPic appears to be mutagenic in mammalian cells. In 2003, the UK's Expert Group on Vitamins and Minerals [42] found that doses of Cr(III) up to 10 mg/day were expected to be without adverse health effects but CrPic was excluded from this guidance as it had been shown to cause DNA damage in mammalian cells in vitro. This group put out a subsequent statement on chromium and chromium picolinate that removed the exclusion of CrPic; the change was based largely on the flawed in vitro studies using CrPic dissolved in DMSO [43].

In the current study, clastogenicity was observed after treatments with 40 and 80 μg/cm^2 of CrPic, and the response was concentration dependent from 4.0 to 80 μg/cm^2 (Table 3). The current results were again consistent with the work by Stearns et al. [11], who reported that exposure to CrPic at 40 μg/cm^2 produced chromosomal aberrations 16-fold above control levels [11]. Increases in DNA fragmentation from CrPic treatment have been observed in murine macrophages [34, 35]. Andersson et al. have found concentration-dependent DNA damage from the supplement in human lymphocytes using Comet assays [44]; no effect was observed if CrPic was dissolved in DMSO in either human lymphocytes or L5178Y mouse lymphoma cells. These chromosome aberrations are only significant if the body is unable to repair this damage. Hepburn and co-workers using *Drosophila* as a model organism have found that CrPic, but not Cr3, at 260 μg Cr/kg food generated developmental delays and decreases in success rates of hatching and eclosion [45]. CrPic was found to generate

approaching one mutation per chromosome per individual and 12% sterility. Subsequently, the ability of CrPic to generate chromosomal aberrations in polyene chromosomes of the salivary glands of *Drosophila* larvae was examined. In the CrPic-treated group, 53% of the identified chromosomal arms were found to contain one or more aberrations, while no aberrations were observed for the identified chromosomal arms of the control group [46]. Thus, CrPic is clastogenic in mammalian cells and in *Drosophila*. In contrast to CrPic, a 24-h exposure to any of the tested concentrations of Cr3 did not produce any significant increase in the number of cells with abnormal metaphase. Cr3 does not appear to be clastogenic.

According to previous studies, people who took Cr in the form of CrPic were found to have a serum Cr level of 16 nM after 2 months [47], and the tissue Cr levels may have as much as 100 times higher than serum Cr levels [48]. The highest concentration for Cr3 in our study is 80 μg/cm^2, which is equivalent to 0.7 mM, exceeding the Cr amount that is likely to exist in human tissues by over 400-fold. However, the highest dose that did not increase the number of chromosomal aberrations significantly for CrPic was 20 μg/cm^2 (Table 3).

The results of this study are consistent with several other previous studies [12, 31–34, 38, 40, 44] on CrPic demonstrating clastogenic and toxic effects on mammalian, but not bacterial, cells. These results probably suggest an inability of CrPic to penetrate bacterial cells, as previously proposed [49]. However, in a study commissioned by the National Toxicology Program when given orally to male and female rats and mice in diets containing up to 5% CrPic for up to 2 years, no effects on body mass, food intake, survival, or non-plastic lesions have been observed [50]. (A statistically significant increase in the incidence of preputial gland adenoma in male rats at 1% CrPic was considered an equivocal finding). These results appear to have been reconciled [49]. CrPic readily breaks down in the gastrointestinal tract so that only ~ 1% of the absorbed dose is probably in the form of intact CrPic, while CrPic also breaks down readily after being absorbed. Thus, cells of the body are not exposed to CrPic, a form of Cr(III) capable of leading to oxidative damage and DNA cleavage if it entered cells. Yet, potential harm from the supplement cannot entirely be ruled out. The gastrointestinal tract of mammals varies greatly from, for example, primates and rodents to ungulates. Curiously, a daily dose of 200 or 400 μg Cr as CrPic to calves has been shown to result in increased lymphocyte micronucleus frequency, malondialdehyde levels, and frequency of apoptotic cells [51].

The results of in vivo studies provide no evidence that Cr3 causes adverse effects. Cr3 appears non-cytotoxic to bacterial and mammalian cells at the concentrations tested. This is not surprising based on in vitro studies. While Lay and co-workers [52, 53] have shown that Cr3, similar to other trinuclear basic Cr(III) carboxylates [54], can react at non-physiologically relevant concentration with hydrogen peroxide to produce toxic high-valent Cr species, Cr3 is poor at cleaving DNA in comparison to CrPic, and under physiologically relevant conditions does not produce sufficient amounts of either high-valent Cr or reactive oxygen species to cleave detectable amounts of DNA [33]. No deleterious effects on *Drosophila* were found under conditions that CrPic resulted in chromosomal aberrations, delayed development, or reductions in hatching or eclosion success. Cr3 has been shown to have an oral LD_{50} greater than 2 g/kg body mass in male and female rats, measured by the OECD procedure [55], but further studies would be needed to unequivocally establish its safety in humans.

Conclusion

The current study evaluated the mutagenicity, cytotoxicity, and clastogenicity of Cr3 in bacterial and mammalian cells and compared these results with similar studies using the bestselling Cr(III) "nutritional" supplement CrPic. The mutagenicity of CrPic and Cr3 was tested in *Escherichia coli* FX-11 and *Salmonella typhimurium* (TA 98 and TA 100). Cytotoxicity was measured as a decrease in plating efficiency relative to controls after treatment with Cr3 and CrPic for 24 h in CHO K1 cells. Clastogenicity was measured by counting the number of metaphases damaged and of the total number chromosomal aberrations in CHO K1 cells. Mutagenesis assays in *E. coli* and *S. typhimurium* were negative. All treatments of Cr3 produced ≥ 84% plating efficiency except 80 μg/cm^2, which reduced the plating efficiency to 62%. Cr3 at the above treatments did not produce a significant increase in the number of cells with abnormal metaphases, while treatments using ≥ 40 μg/cm^2 of CrPic elevated the number significantly. These data suggest that Cr3 is significantly less mutagenic in bacteria cells than CrPic and less clastogenic in CHO K1 cells, while CrPic is clastogenic in CHO K1 cells.

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Compliance with Ethical Standards

Conflict of Interest J.B.V. is the inventor or co-inventor of 7 patents involving the use of Cr compounds as nutritional supplements or therapeutic agents, including 6 patents involving Cr3; The University of Alabama is currently not licensing any of these patents nor producing any Cr compound for sale.

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