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Chronic Toxicity and Carcinogenicity Studies of Chromium Picolinate Monohydrate Administered in Feed to F344/N Rats and B6C3F1 Mice for 2 Years

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Abstract

Trivalent chromium (Cr(III)) has been proposed to be an essential element, which may increase sensitivity to insulin and thus participate in carbohydrate and lipid metabolism. Humans ingest Cr (III) both as a natural dietary constituent and in dietary supplements taken for weight loss and antidiabetic effects. Chromium picolinate (CP), a widely used supplement, contains Cr(III) chelated with three molecules of picolinic acid and was formulated in an attempt to improve the absorption of Cr(III). In order to examine the potential for CP to induce chronic toxicity and carcinogenicity, the NTP conducted studies of the monohydrate form (CPM) in groups of 50 male and female F344/N rats and B6C3F1 mice exposed in feed to concentrations of 0, 2,000, 10,000 or 50,000 ppm for 2 years; exposure concentrations were selected following review of the data from NTP 3-month toxicity studies. Exposure to CPM did not induce biologically significant changes in survival, body weight, feed consumption, or non-neoplastic lesions in rats or mice. In male rats, a statistically significant increase in the incidence of preputial gland adenoma at 10,000 ppm was considered an equivocal finding. CPM was not carcinogenic to female rats or to male or female mice.

Keywords

trivalent chromium; diet; supplement; body weight; preputial gland; National Toxicology Program

Introduction

Trivalent chromium (Cr(III)) has been proposed to be an essential element, which may increase sensitivity to insulin and thus participate in carbohydrate and lipid metabolism (Anderson, 1989). The mechanism involves increased insulin binding through increasing the number of insulin receptors and increasing insulin receptor phosphorylation when the chromium is bound to a low molecular weight chromium binding substance (LMWCr; also referred to as chromodulin) and insulin is present (Anderson, 1998). In the blood, Cr(III) is bound to and

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transported to tissues by transferrin, a process regulated, at least in part, by insulin (Clodfelder et al., 2001). Cr(III) deficiency may contribute to glucose intolerance and diabetes mellitus (Type 2); however, the essentiality of Cr III and the ability of Cr III to increase insulin sensitivity have been questioned (Stearns, 2000; Stallings and Vincent, 2006). The most recent dietary guideline for adequate Cr(III) ingestion is 20 to 45 µg, set by the Institute of Medicine in 2001 (IOM, 2001). Typical serving sizes of a variety of foods and beverages, including broccoli, grape juice, whole wheat English muffins, mashed potatoes, dried garlic, dried basil, beef cubes, orange juice, turkey breast, whole wheat bread, red wine, unpeeled apple, banana, and green beans, provide 1 to 13 µg Cr(III) (NIH, 2007).

Cellular uptake of Cr(III) by cells is thought to occur by diffusion or phagocytosis, which results in very low absorption and excretion primarily in the feces. However, absorbed Cr(III) is widely distributed to tissues (Hepburn and Vincent, 2003; Anderson, et al. 1996). Chromium picolinate (CP), which contains Cr(III) chelated with three molecules of picolinic acid (Evans and Pouchnik 1993), was formulated in an attempt to increase the absorption of Cr(III) over non-chelated forms, such as chromium chloride. CP is widely used as a dietary supplement, primarily because of claims of increased metabolic (weight reducing) and antidiabetic effects. Cr(III)-containing supplements have become very popular, generating estimated annual sales in the hundreds of millions of dollars in the mid to late 1990s (Federal Trade commission, 1996; Mirasol, 2000); these supplements are available over the counter as pills, chewing gums, sports drinks, and nutrition bars (Vincent, 2001), either alone or in combination with other supplements. Numerous clinical studies have been conducted with daily doses of CP containing 200–1000 µg Cr(III) (Cefalu and Hu, 2004; Komorowski, et al. 2008) and in one study modeling human exposure to CP, a dose containing 600 µg Cr(III) was chosen (Stearns, et al. 1995a). Thus, it is likely that human exposure through consumption of supplements is in this range. Although one study suggested that the absorption of Cr following Cr(III) exposure is not enhanced by picolinic acid (Olin, et al., 1994), another study showed higher Cr tissue concentrations following exposure to chromium picolinate compared to chromium chloride (Anderson, et al. 1996). The NTP conducted absorption, distribution, metabolism, and excretion studies of [14C]-chromium picolinate monohydrate in mice and rats to differentiate the metabolic fates of chromium and [14C]-picolinic acid (NTP, 2008). The patterns of urinary and fecal excretion of chromium and picolinic acid suggest that most of the picolinic acid is not bound to chromium during absorption and that picolinic acid is more readily absorbed than chromium. These findings are consistent with previous studies (Hepburn and Vincent, 2002; Hepburn and Vincent, 2003).

Neither chromium picolinate (Anderson *et al.*, 1997) nor other Cr(III) compounds (Ivankovic and Preussmann, 1975; Anderson *et al.*, 1997; MacKenzie *et al.*, 1958; Schroeder *et al.*, 1964; Schroeder *et al.*, 1965) have displayed evidence of toxicity following oral exposure. No studies examining the carcinogenic potential of chromium picolinate in animals or humans have been reported; however, previous carcinogenicity studies of other Cr(III) compounds in rodents following oral exposure were negative (Ivankovic and Preussmann, 1975; Schroeder *et al.*, 1964; Schroeder *et al.*, 1965).

Although Cr(III) has been shown to be genotoxic in acellular test systems that permit direct contact with DNA (Snow *et al.*, 1991; Snow, 1994; Bridgewater *et al.*, 1994), Cr(III) compounds, including CP, often give negative or conflicting results in standard in vivo and in vitro genetic toxicity assays. This disparity appears to result from the very low cellular uptake of Cr(III). CP is not mutagenic in the Ames assay (Whittaker *et al.*, 2005; NTP, 2008), but some laboratories have reported increases in gene mutations or chromosomal aberrations in cultured mammalian cells treated with chromium picolinate (Stearns *et al.*, 1995b, 2002; Whittaker *et al.*, 2005). In contrast, other laboratories have reported no increases in these lesions in similar studies with chromium picolinate (Gudi *et al.*, 2005; Slesinski *et al.*, 2005). Results

of more recent *in vitro* and *in vivo* genotoxicity studies with CP were also negative. These studies included assessment of micronucleated erythrocytes in mice, DNA damage in mice, and DNA damage in cultured mammalian cells after exposure to CP (Andersson *et al.*, 2007). Other Cr(III) salts have also given negative results in a number of *in vitro* and *in vivo* assays (Zeiger *et al.*, 1992; Amrani *et al.*, 1999; Witt *et al.*, 2000; Whittaker *et al.*, 2005).

Because there is widespread human exposure to CP in dietary supplements as well as a limited body of evidence suggesting that CP is genotoxic, the NTP conducted toxicity and carcinogenicity studies of CP in the monohydrate form (CPM), in male and female F344/N rats and B6C3F1 mice. The route chosen for these studies was dosed feed, because humans are exposed in the diet and in supplements. CPM was selected for testing because it was commercially available. Three month studies were first conducted to characterize the subchronic toxicity of CPM and aid in the selection of doses for 2-year studies (NTP, 2008; Rhodes *et al.*, 2005). Rats and mice were exposed to 0, 240, 800, 2,000, 10,000 or 50,000 ppm. The highest exposure concentration of 50,000 ppm (5%) used in these studies was considered to be a limit dose in feed studies because a higher concentration of chemical is thought to alter the nutritional content of the diet. Exposure did not result in toxicity to rats or mice, as evidenced by the lack of biologically significant changes in survival, mean body weight and body weight gain, feed consumption, hematology, clinical chemistry, organ weights, histopathology and sperm morphology and vaginal cytology. A small increase in the frequency of micronucleated erythrocytes, which resulted in a significant positive trend in female mice exposed to CPM for three months was judged to be an uncertain finding. There was no evidence of an increase in micronucleus frequency in male mice. Based on these results, the three highest exposure concentrations (2,000, 10,000 and 50,000 ppm) were selected by the NTP for testing in the 2-year studies. The objective of the present report is to present the major findings from the NTP 2-year toxicity and carcinogenicity studies of CPM.

Materials and Methods

Chemical and dose formulations

Chromium picolinate monohydrate (CPM; CAS No. 27882–76–4) used in the 2-year studies was a combination of chemical obtained from TCI America (Portland, OR) and from Sigma-Aldrich (St. Louis, MO). The chemical, a reddish-purple crystalline powder, was identified as chromium picolinate monohydrate by infrared and proton nuclear magnetic resonance spectroscopy, X-ray diffraction, and electrospray ionization-mass spectrometry. The presence of approximately 1 mole of water per formula unit was confirmed by Karl Fischer titration and weight loss on drying assays. Purity was determined by elemental analyses, proton-induced X-ray emission spectroscopy, inductively coupled plasma-atomic emission spectroscopy, high-performance liquid chromatography with ultraviolet-visible detection, UV detection, or ICP-mass spectrometric detection. The overall purity of the chemical was determined to be greater than 95%.

CPM was stable as a bulk chemical for at least 2 weeks when stored in sealed amber glass containers at temperatures up to 60° C, as determined by inductively coupled plasma-atomic emission spectroscopy and high-performance liquid chromatography with ultraviolet-visible detection. To ensure stability, the bulk CPM was stored at room temperature, protected from light, in sealed plastic buckets. No degradation was detected during the 2-year studies.

The dose formulations were prepared monthly by mixing CPM with feed. Homogeneity and stability of the dose formulations were assessed by high-performance liquid chromatography with ultraviolet-visible detection. Homogeneity of the 50,000 ppm dose formulation was confirmed. The stability of this formulation was confirmed for at least 42 days at room temperature when stored in double-thick sealed plastic bags, protected from light. Periodic

analysis confirmed that all 167 dose formulations for rats and all 99 for mice were within 10% of the target concentrations.

Animals and animal maintenance

The studies were conducted at Southern Research Institute (Birmingham, AL). Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms (Germantown, NY). Animals were quarantined for 12 days prior to the initiation of the studies, and were approximately 5–6 weeks old at the beginning of the studies. Study animals were distributed randomly into groups of approximately equal initial mean body weights and identified by tail tattoo. Rats and mice were housed 1 (male mice), 3 (male rats) or 5 (female rats and mice) to a cage. The animal room was maintained at a temperature of $72 \pm 3^{\circ}\text{F}$, a relative humidity of $50\% \pm 15\%$, a twelve hour light-dark cycle, and 10 air changes per hour. Irradiated NTP-2000 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA) was available *ad libitum* and changed weekly. Tap water was available *ad libitum* via an automatic watering system. Animals were killed by asphyxiation with CO_2 .

Animal use was in accordance with the United States Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals. These studies were conducted in compliance with the Food and Drug Administration Good Laboratory Practice Regulations (21CFR, Part 58).

Study Design

Groups of 50 male and 50 female rats and mice were fed diets containing 0, 2,000, 10,000, or 50,000 ppm chromium picolinate monohydrate for 105 weeks. All animals were observed twice daily. Animals were weighed initially, once a week for the first 13 weeks, once a month thereafter, and at the end of the studies. Feed consumption was measured weekly for the first 13 weeks of the study and monthly thereafter. Clinical findings were recorded monthly. Complete necropsies and microscopic examinations were performed on all core study rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin (eyes were initially fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm , and stained with hematoxylin and eosin for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Additional details regarding the pathology data generation, quality assurance review, and NTP pathology working group are available elsewhere (NTP, 2008). Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman et al. (1985).

Statistical Methods

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). Statistical analyses for possible dose related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided. The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. Unless otherwise specified, a value of $k=3$ was used in the analysis of site-specific lesions. Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall exposure-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided.

Results

2-Year Study in F344/N Rats

In males, there was a significant trend ($P=0.041$) for decreased survival (0 ppm, 37/50; 2,000 ppm, 36/50; 10,000 ppm, 35/50; 50,000 ppm, 28/50); however, because survival was not significantly different from the control group at any exposure concentration the decreases was not considered to be related to exposure. Survival in exposed females (0 ppm, 36/50; 2,000 ppm, 35/50; 10,000 ppm, 36/50; 50,000 ppm, 40/50) was similar to that of the control group. Mean body weights of exposed groups of males and females were similar to those of the controls throughout the study. Feed consumption by exposed groups of males and females was generally similar to that of the controls throughout the study (data not shown). Body weight and feed consumption data were used to calculate average daily doses of CPM resulting from each concentration (Table 1). Average daily doses were also calculated for Cr(III) and picolinic acid, the components of the CPM complex. Based on the body weight and feed consumption data, the increases in calculated ingested dose were proportional to the increases in exposure concentration. No clinical findings or non-neoplastic lesions were attributed to exposure.

The preputial gland is an accessory sex gland in males. The incidence of preputial gland adenoma was significantly increased in males at 10,000 ppm compared to the control group (Table 2). This increase exceeded the historical control ranges for feed studies and for all routes of exposure. The incidence of preputial gland hyperplasia was not increased at any exposure concentration. Preputial gland carcinoma was not observed in control or exposed males.

Preputial gland hyperplasia was focal, characterized either by an increase in stratified squamous epithelium of the ducts or by increased numbers of sebaceous cells and possibly basal cells. Preputial gland adenomas were well circumscribed masses that grew by expansion with compression of the surrounding parenchyma. The neoplastic glands retained some resemblance of acinar structure, although there was some fusion of the acini to form solid clusters of cells (Copeland-Haines and Eustis, 1990).

The female counterpart of the preputial gland is the clitoral gland. There were no increases in the incidences of clitoral gland adenoma or hyperplasia over the control group (Table 2). Carcinomas of the clitoral gland were not observed in control or treated females.

Proliferative lesions of the preputial and clitoral glands constitute a morphological continuum, and separation of these into categories of hyperplasia, adenoma, and carcinoma is based largely on cytological features and degree of altered growth pattern (Copeland-Haines and Eustis, 1990). Lesions classified as hyperplasia are considered preneoplastic.

2-Year Study in B6C3F1 Mice

Survival of exposed groups of males (0 ppm, 46/50; 2,000 ppm, 43/50; 10,000 ppm, 38/50; 50,000 ppm, 45/50) and females (0 ppm, 45/50; 2,000 ppm, 44/50; 10,000 ppm, 44/50; 50,000 ppm, 39/50) was similar to that of the control groups. Mean body weights of exposed groups of males were generally similar to those of the controls throughout the study. In females, decreases in mean body weights of up to 10% compared to controls were observed during the middle of the study in exposed animals; however, mean body weights recovered to control values by the end of the study. Feed consumption by exposed groups of males and females was similar to that by the controls throughout the study. Body weight and feed consumption data were used to calculate average daily doses of CPM resulting from each concentration (Table 1). Average daily doses were also calculated for Cr(III) and picolinic acid, the components of the CPM complex. Based on the body weight and feed consumption data, the increases in calculated ingested dose were proportional to the increases in exposure concentration. No

clinical findings or neoplastic or non-neoplastic lesions were attributed to chromium picolinate monohydrate exposure.

Discussion

Cr(III) is consumed by the general population through its presence in many foods. In addition, there is widespread consumption of Cr(III) present in dietary supplements, such as CP, that are marketed primarily for weight loss and antidiabetic effects. Humans typically ingest 20 to 45 μg Cr(III) per day in the diet (IOM, 2001), while typical daily doses of supplements may contain 200 to 1000 μg Cr(III) (Cefalu and Hu, 2004; Komorowski, et al. 2008). These doses correspond to daily body weight adjusted doses of 0.29 to 0.64 μg Cr(III)/kg body weight in the diet and 2.86 to 14.3 μg Cr(III)/kg in supplements, in an individual of average weight (70 kg).

Average daily doses in the 2-year rodent studies of CPM were approximately three to five orders of magnitude higher than those consumed by humans ingesting typical doses of supplements. Despite exposure to concentrations of Cr that are much higher than those consumed by humans, the present studies did not result in toxicity to rats or mice. The observed lack of toxicity was consistent with the results of the NTP 3-month toxicity studies (NTP 2008; Rhodes, et al. 2005) and with other reported oral studies on compounds containing Cr (III), including chromium picolinate (MacKenzie *et al.*, 1958; Schroeder *et al.*, 1964, 1965; Ivankovic and Preussmann, 1975; Anderson *et al.*, 1997; ATSDR, 2000). The lack of an effect on body weight following exposure to CPM for 3-months or 2-years is notable because CP has been marketed as a dietary supplement for weight loss.

Exposure of male rats to CPM for 2 years resulted in a significantly increased incidence of preputial gland adenoma over the control group at the mid dose; this incidence exceeded the historical control ranges for feed studies and for all routes of exposure. This increase appeared to be treatment-related; however, because of the lack of an exposure concentration response, the lack of progression to carcinoma, the lack of preneoplastic lesions, including hyperplasia, and the lack of a neoplastic response in the clitoral gland in females, the NTP concluded that there was equivocal evidence of carcinogenic activity in male rats, indicating that the increased incidence of preputial gland adenomas in male rats may have been related to CPM exposure. In female rats and male and female mice exposed to CPM, there was no evidence of carcinogenic activity.

As part of the 2-year studies, total chromium content in excreta and selected tissues was determined in additional groups of male rats and female mice following 4, 11 or 180 days of exposure and a two day washout (NTP, 2008); these data will be reported in detail in a manuscript currently in preparation; however, the primary findings of the tissue concentration studies aid in the interpretation of the bioassay results and will be discussed briefly here. Accumulation of total chromium with exposure concentration and duration was observed in the liver and kidney of rats and mice, suggesting that Cr(III) is taken up by these tissues; this pattern was less apparent in erythrocytes, forestomach, and glandular stomach. In both rats and mice, chromium tissue concentrations were generally not proportional to exposure concentration. As a result, tissue chromium concentrations in animals exposed to 50,000 ppm CPM were similar to those in animals exposed to lower concentrations. These data suggest that the maximum achievable tissue chromium concentrations were reached in these studies and may offer a partial explanation for the lack of a higher preputial gland neoplasm incidence in male rats exposed to 50,000 ppm than was observed at 10,000 ppm.

Previous ADME studies with ^{14}C -CPM (NTP, 2008; Hepburn and Vincent 2002, 2003) suggest that following exposure to CPM, most of the picolinate was separated from the

chromium and absorbed, while the chromium was excreted in the feces, resulting in greater systemic exposure to picolinic acid compared to chromium or CPM. However, in parallel tests with CP and picolinic acid for in vivo developmental toxicity in mice (Bailey *et al.*, 2006) or in vitro genetic toxicity (Stearns *et al.*, 1995b, 2002), the observed developmental or genotoxic effects were more apparent with chromium picolinate than with picolinic acid. In another study in mice focused on the comparison of developmental neurotoxicity following exposure to CPM or picolinic acid at the same doses as were used in the previous studies, there were no statistically significant changes, however, there was the suggestion of developmental effects by picolinic acid (Bailey, et al. 2008). The exposure concentrations in these developmental toxicity studies resulted in average daily doses of 200 mg/kg for CP and 174 mg/kg for picolinic acid. These doses are similar to the dose resulting from exposure to 2,000 ppm in the present study, but are lower than the mid and high doses by factors of 5 and 25. Based on the high doses used, the high absorption of picolinic acid and the near complete lack of adverse effects in the present studies, it appears that picolinic acid is essentially nontoxic.

Although a limited body of evidence suggests that Cr(III) compounds are genotoxic in certain test systems, and interaction of Cr(III) with DNA has been shown to result in the formation of DNA adducts, DNA-protein crosslinks, and DNA interstrand crosslinks (O'Brien et al. 2003; Zhitkovich 2005; Quievryn et al. 2006; Reynolds et al. 2007), the poor ability of cells to take up Cr(III) appears to limit its access to DNA. Poor uptake may also explain the lack of toxicity or carcinogenicity observed in the present chronic oral studies and in previous reports.

In conclusion, despite exposure to very high concentrations relative to human exposures, there was very little evidence of adverse effect following dietary exposure of rats and mice to CPM for 2 years. In male rats, there was equivocal evidence of carcinogenic activity based on increased preputial gland adenomas. There was no evidence of carcinogenic activity in female rats or in male or female mice. In addition, CPM exposure did not induce in-life toxicity or increases in non-neoplastic lesion incidences.

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Abbreviations

Cr(III)	trivalent chromium
NTP	National Toxicology Program
CP	chromium picolinate
CPM	chromium picolinate monohydrate
LMWCr	low molecular weight chromium binding substance

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Table 1
Conversion of CPM exposure concentrations in feed to average daily doses (mg/kg/day) of CPM, Cr(III) and picolinic acid in F344/N rats and B6C3F1 mice exposed for 2-years.

Rats				Mice			
CPM (ppm)	CPM ^a (mg/kg/day)	Cr(III) ^b (mg/kg/day)	Picolinic Acid ^c (mg/kg/day)	CPM (ppm)	CPM (mg/kg/day)	Cr(III) ^b (mg/kg/day)	Picolinic Acid ^c (mg/kg/day)
Males							
2000	--	--	--	0	--	--	--
10000	90	10.7	79.3	2000	250	29.8	220.2
50000	460	54.9	405.1	10000	1200	143.1	1056.9
	2400	286.2	2113.8	50000	6565	783.0	5782.0
Females							
2000	--	--	--	0	--	--	--
10000	100	11.9	88.1	2000	240	28.6	211.4
50000	510	60.8	449.2	10000	1200	143.1	1056.9
	2630	313.7	2316.3	50000	6100	727.5	5372.5

^a calculated using body weight and feed consumption data;
^b calculated using the average daily dose of CPM and the percent mass of Cr(III) in CPM;
^c calculated using the average daily dose of CPM and the percent mass of picolinic acid in CPM

Table 2
Accessory sex gland neoplasms in male and female F344/N rats exposed to chromium picolinate monohydrate for two years in feed.

	Exposure Concentration (ppm)			
	0	2,000	10,000	50,000
Males				
Preputial Gland				
Number necropsied	50	50	50	50
Hyperplasia	3 ^a (2.7) ^b	1 (4.0)	0	2 (2.5)
Adenoma ^c	1 (2.2) ^d	1 (2.3)	7 (14.9) [*]	4 (9.3)
Females				
Clitoral Gland				
Number necropsied	50	50	50	50
Hyperplasia	8 ^a (2.4) ^b	10 (2.6)	11 (2.6)	4 (2.3)
Adenoma ^e	10 (21.9) ^d	2 (4.4) [*]	8 (17.7)	11 (23.4)

^a Number of animals with lesion;

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked;

^c Historical incidence for 2-year feed studies with controls given NTP-2000 diet (mean ± standard deviation): 8/250 (3.2% ± 4.2%), range 0%–10%; all routes: 43/1,193 (3.6% ± 3.5%), range 0%–10%;

^d Survival adjusted incidence (%);

^e Historical incidence: 26/200 (13.0% ± 6.2%), range 6%–20%; all routes: 104/1,096 (9.5% ± 8.6%), range 0%–34%;

^{*} Significant by Poly-3 test (P≤0.05)