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High concentrations of hexavalent chromium in drinking water alter iron homeostasis in F344 rats and B6C3F1 mice ☆

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ABSTRACT

Hexavalent chromium [Cr(VI)] induces hematological signs of microcytic anemia in rodents. Considering that Cr(VI) can oxidize ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron, and that only the former is transported across the duodenum, we hypothesize that, at high concentrations, Cr(VI) oxidizes Fe^{2+} in the lumen of the small intestine and perturbs iron absorption. Herein we report that 90-day exposure to Cr(VI) in drinking water resulted in dose-dependent decreases in Fe levels in the duodenum, liver, serum, and bone marrow. Toxicogenomic analyses from the duodenum indicate responses consistent with Fe deficiency, including significant induction of divalent metal transporter 1 (DMT1, *Slc11a2*) and transferrin receptor 1 (TFR1, *Tfr1*). In addition, at ⩾20 mg Cr(VI)/L in drinking water, Cr RBC:plasma ratios in rats were increased and exceeded unity, indicating saturation of reductive capacity and intracellular absorption of Cr(VI) into red blood cells (RBCs). These effects occurred in both species but were generally more severe in rats. These data suggest that high concentrations of Cr(VI) in drinking limit Fe absorption and alter iron homeostasis. Furthermore, some effects observed at high doses in recent Cr(VI) chronic and subchronic bioassays may be explained, at least in part, by iron deficiency and disruption of homeostasis.

1. Introduction

In a recent 2-year cancer bioassay conducted by the National Toxicology Program (NTP), hexavalent chromium [Cr(VI)] administered in drinking water induced small intestinal tumors in B6C3F1 mice, and tumors of the oral cavity in Fisher 344 rats; no other treatment-related tumors were reported in either species (NTP, 2008b; Stout et al., 2009a). In the small intestine, there were clear differences between mice and rats with respect to non-neoplastic lesions (NTP, 2008b). The mode of action (MOA) for the intestinal tumors is posited to involve chronic intestinal injury leading to long-term intestinal regenerative hyperplasia (Thompson et al., 2013). In contrast to the intestine, the oral mucosae of mice and rats exhibited no non-neoplastic or pre-neoplastic lesions; and to date, there is no published MOA describing Cr(VI)-induced oral tumors in rats.

As part of our research on the toxicity and carcinogenicity of Cr(VI) by the oral route of exposure (Kirman et al., 2012; Kopec et al., 2012a,b; Proctor et al., 2012; Thompson et al., 2011, 2012), we collected data to follow up on previous reports that Cr(VI) causes microcytic hypochromic anemia (NTP, 1996, 1997, 2007, 2008b). In the 90-day NTP study with drinking water concentrations of ~22–300 mg/L Cr(VI), microcytic hypochromic anemia was observed in rats and mice; anemia was reported to be more severe in rats than mice, but no significant sex differences in hematological effects were reported (NTP, 2007). In the 2-year bioassay, NTP reported hematological effects (including hemoglobin, mean cell volume, erythrocytes) in male rats and female mice at multiple time points, which NTP concluded were more severe and prolonged in rats than mice (Supplemental Fig. S1) (NTP, 2008b). Because female rats and male mice were not evaluated, sex differences in regard to hematological effects could not be assessed in the 2-year NTP bioassay. The anemia did ameliorate with time, but significant changes in some hematological markers were still

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evident in the high dose group rats at one year, which was the final time point at which hematology was evaluated in the 2-year NTP bioassay.

These anemic responses to high concentrations of Cr(VI) suggest interference with iron (Fe) absorption and/or homeostasis. In this regard, it is known that Cr(VI), Cr(V), and Cr(IV) can oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) (Buerge and Hug, 1997; Fendorf and Li, 1996):

$$Cr(VI) + Fe^{2+} \leftrightarrow Cr(V) + Fe^{3+} \quad \text{(i)}$$

$$Cr(V) + Fe^{2+} \leftrightarrow Cr(IV) + Fe^{3+} \quad \text{(ii)}$$

$$Cr(IV) + Fe^{2+} \leftrightarrow Cr(III) + Fe^{3+} \quad \text{(iii)}$$

Further, it is well established that the major form of non-heme dietary iron is Fe^{3+}, which must be reduced to Fe^{2+} prior to absorption by enterocytes in the proximal small intestine (Kumar et al., 2005; Lonnerdal, 2010). Therefore, we hypothesize that high concentrations of ingested Cr(VI) may oxidize Fe^{2+} to Fe^{3+} and hinder Fe^{2+} absorption from the intestinal lumen. Notably, anemic effects were not observed in rodents chronically exposed to trivalent chromium [Cr(III)] (NTP, 2008a; Stout et al., 2009b)—further suggesting that the chemistry in formulas i, ii, iii may, at least partially, explain the anemic effects of Cr(VI).

In addition to this extracellular chemistry, Cr(VI) absorbed to the bloodstream may be taken up into red blood cells (RBCs) and reduced to Cr(III). Once inside the cell, Cr(III) can bind to ferritin in competition with intracellular Fe (Ani and Moshtaghie, 1992; Wauters et al., 1978). In contrast, Cr(III) from extracellular reduction of Cr(VI) in the blood does not readily cross the RBC membrane and is thus free to compete with Fe binding to transferrin (Ducros, 1992; Hopkins and Schwarz, 1964; Sayato et al., 1980). Once Cr(VI) is absorbed into the RBCs, Cr(III) is produced from intracellular reduction of Cr(VI), and becomes trapped in the RBC, increasing the total Cr RBC:plasma ratio. As such, there are several ways in which high doses of Cr(VI) can cause disruption Fe metabolism and homeostasis.

Iron plays an important role in many metabolic pathways because it is a functional component of oxygen-carrying globin proteins including hemoglobin and myoglobin, cytochromes, and enzymes that transfer electrons (Andrews and Schmidt, 2007; Recalcati et al., 2012). Further, Fe is used for DNA synthesis and energy production, and as such, Fe homeostasis is tightly controlled to ensure coordinated absorption by enterocytes, reutilization in the macrophages, and proper distribution for erythropoiesis or storage in hepatocytes (Andrews and Schmidt, 2007; Recalcati et al., 2012). Iron is also an essential element for proper function of the immune system; in fact, macrophages sequester Fe by different mechanisms to contain microbial infection (Huang, 2003; Recalcati et al., 2012). It has also been suggested that disruption of Fe metabolism can potentiate oral carcinogenicity (Binnie et al., 1983; Prime et al., 1983; Rennie et al., 1984; Richie et al., 2008). Given the importance of Fe in basic physiological processes, disruption of Fe status and homeostasis could play a role in several of the adverse outcomes observed with high dose Cr(VI) exposure.

Recently, the United States Environmental Protection Agency (U.S. EPA) released drinking water supply monitoring data for its third Unregulated Contaminant Monitoring Rule (UCMR3) program; Cr(VI) was detected in 5260 drinking water samples, of 6929 samples analyzed, at concentrations ranging from 0.00003 mg/L, the minimal reporting level, to 0.091 mg/L, with mean and median values of 0.0014 and 0.00019 mg/L, respectively (USEPA, 2013). Thus, drinking water exposures to Cr(VI) in humans are several orders of magnitude lower than those that disrupted Fe absorption and homeostasis in rodents of the NTP bioassays (NTP, 2007, 2008b; Stout et al., 2009a).

Herein we evaluate the biochemical and toxicogenomic evidence that Cr(VI) disrupts Fe absorption and homeostasis, as well as evaluate pharmacokinetic data to assess dose-dependent transitions in chromium disposition. This study extends our previous analyses (Kopec et al., 2012a,b; Thompson et al., 2011, 2012) which described the effects of 7 and 90 days of exposure to Cr(VI) on gene expression in the duodena of rats and mice (Kopec et al., 2012a,b). Because most dietary Fe is absorbed in the proximal small intestine and Fe homeostasis is regulated at the level of intestinal absorption (Kumar et al., 2005; Lonnerdal, 2010; Wood and Han, 1998), toxicogenomic data from the duodenum are useful for assessing Fe homeostasis.

2. Material and methods

2.1. Animal husbandry and study design

Details of the test substance, animal husbandry, and study design have been described previously (Thompson et al., 2011, 2012). Briefly, Southern Research Institute (Birmingham, AL) acclimatized 4–5 week old female Fischer F344 rats and female B6C3F1 mice (Charles Rivers Laboratories International, Stone Ridge, NY) for a minimum of 7 days with free access to irradiated NTP-2000 wafers (Zeigler Bros, Gardners, PA). Animals were continuously exposed to Cr(VI), administered as sodium dichromate dihydrate (SDD), dissolved in tap water at 0, 0.3, 4, 14, 60, 170 and 520 mg/L, corresponding to 0, 0.1, 1.4, 5, 20, 60, and 180 mg/L Cr(VI), for 7 and 90 days (note: rats were not exposed to 14 mg/L SDD). Based on animal body weight, water intake, and administered drinking water concentrations of Cr(VI), 90-day average daily doses for rats and mice were calculated (Table 1). Rats and mice were euthanized using CO_2. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee at Southern Research Institute.

2.2. Assessment of Fe levels in serum and bone marrow

Serum Fe levels were measured in 10 animals from each dose group. Samples (~0.4 ml) were collected from the retro-orbital plexus into tubes containing no anticoagulant. The contents of the tubes were centrifuged to separate serum. Serum Fe was measured using the FerroZine method and Cobas c501 Clinical Chemistry Analyzer (Version 04-02; Roche Diagnostics; Indianapolis, IN). The Fe content in bone marrow smears was also assessed in 5 animals per dose group. Gale scores were used as semi-quantitative grading scheme to assess Fe content in bone marrow (Gale et al., 1963). In each dose group, one bone marrow smear from each animal was prepared and stained with Prussian blue to assess the presence of Fe.

Table 1
Average daily doses of ingested Cr(VI) for rats and mice administered Cr(VI) as SDD in drinking water for 90 days.

	Administered drinking water concentration, Cr(VI) (mg/L)					
	0.1	1.4	5	20	60	180
	90-Day average daily dose, Cr(VI) (mg/kg)[a]					
Rat	0.02	0.21	–[b]	2.90	7.20	20.5
Mouse	0.02	0.30	1.10	4.60	11.7	31.1

[a] Average daily doses were calculated from animal bodyweight, water intake, and administered drinking water concentrations of Cr(VI). Data for bodyweight, intake, and doses are taken from Thompson et al. (2011, 2012).

[b] Rats were not administered drinking water concentration of 5 mg/L Cr(VI).

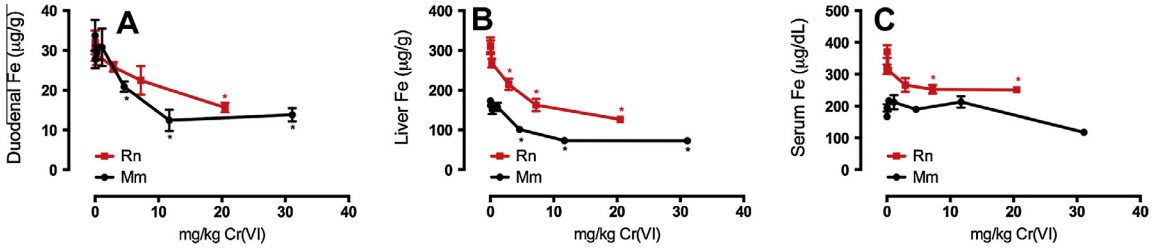

Fig. 1. Comparison of effects of Cr(VI) on Fe in rats and mice after 90-days of exposure in drinking water. Effects are shown for total Fe in the duodenum (A), liver (B), and serum (C) in rats and mice by their 90-day average daily doses of Cr(VI). The average daily doses of Cr(VI) were based on animal bodyweight, water intake, and administered drinking water concentrations of Cr(VI). Dose-dependent decrease in Fe was observed for duodenum, liver, and blood. *Significantly different from that of control animals ($p < 0.05$) using the Shirley's test. Rn, rats; Mm, mice.

2.3. Assessment of Fe and Cr levels in tissues

Tissue levels of total Cr and Fe were measured in 5 animals per dose group. Samples were shipped frozen to Brooks Rand Laboratories where approximately 100 mg of tissue was digested in nitric acid in a controlled microwave digestion program. Samples were then brought to a final volume of 8 mL with deionized water. Analysis was performed using EPA Draft Method 1638 (modified) using inductively coupled plasma-mass spectrometry (ICP-MS) with Dynamic Reaction Cell (DRC™) technology. Digested samples were analyzed utilizing internal standardization with rhodium. This method incorporates ionization of the sample in an inductively-coupled RF plasma, with detection of the resulting ions by mass spectrometer on the basis of their mass-to-charge ratio. Results were presented for total Fe in duodenum and liver and total Cr in the RBCs and plasma. The limit of detection was 0.02 µg Cr/g tissue. Iron levels were simultaneously measured in these tissue samples, and the limit of detection was 0.2 µg Fe/g tissue.

2.4. Microarray analysis

For toxicogenomic analyses, duodenal samples were scraped and processed as described previously (Kopec et al., 2012a). Briefly, intestinal sections were collected and flushed with ice-cold phosphate buffered saline. Duodenal sections were cut longitudinally and the epithelium was scraped using disposable sterile plastic spatulas (VWR International) into vials containing ~1 ml of TRIzol (Invitrogen, Carlsbad, CA) and snap-frozen in liquid nitrogen. The samples were stored at −80 °C and shipped on dry ice to Michigan State University for gene expression analysis. Total RNA was isolated according to the manufacturer's protocol with an additional acid phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}), and quality of each sample was assessed by evaluation of the A_{260}/A_{280} ratio and by visual inspection of 1 µg total RNA on a denaturing gel. Gene expression was examined using rat 4 × 44 K Agilent whole-genome oligonucleotide microarrays (version 1, Agilent Technologies, Inc., Santa Clara, CA). Microarrays were scanned at 532 nm (Cy3) and 635 nm (Cy5) on a GenePix 4000B scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 software (Molecular Devices).

2.5. Statistical analyses

Dose-related trends for tissue concentrations of total Fe were analyzed initially using Jonckheere's test (Jonckheere, 1954). Then, data with a significant dose-related trend were analyzed by William's tests (parametric) or Shirley's tests (nonparametric) (Shirley, 1977). If no monotone trend was observed, Dunnett's tests (parametric) or Dunn's (nonparametric) tests were conducted. Statistical packages used included Prism 5 for Mac (GraphPad Software, San Diego California USA, www.graphpad.com).

Microarray data were normalized using a semi-parametric approach (Eckel et al., 2005) and the posterior probability P1(t) values were calculated using an empirical Bayes method based on a per gene and dose basis using model-based t values (Eckel et al., 2004). Gene expression data were ranked and prioritized using |fold change| > 1.5 and statistical P1(t) value >0.999 criteria to identify differentially expressed genes.

3. Results

3.1. Effects on iron status

As shown in Fig. 1, there were dose-dependent decreases of the total Fe in the duodenum, liver, and serum of both species. Dose-dependent changes in liver and serum were slightly more pronounced in rats than mice (Fig. 1B and C). We also examined the bone marrows for Fe content by Prussian blue staining. Overall, there were no changes in Fe content of bone marrows for rats administered drinking water concentrations of 0–20 mg/L Cr(VI), in one animal of the 20 mg/L Cr(VI) group and in one of the control

Table 2
Iron content in bone marrow of rats administered Cr(VI) as SDD in drinking water for 90 days. Gale scores were used as semi-quantitative grading scheme to assess Fe content in bone marrow (Gale et al., 1963).

Cr(VI), mg/L	Animal (n = 5 per dose group)	Fe content in bone marrow smear	
		Grades 0–6[a]	Fe content description[b]
0	1a	2–3	Slight to moderate
	2a	3	Moderate
	3a	3	Moderate
	4a	3	Moderate
	5a	3	Moderate
0.1	1b	3	Moderate
	2b	3	Moderate
	3b	3	Moderate
	4b	3	Moderate
	5b	3	Moderate
1.4	1c	3	Moderate
	2c	3	Moderate
	3c	3	Moderate
	4c	3	Moderate
	5c	3	Moderate
5	1d	3	Moderate
	2d	3	Moderate
	3d	3	Moderate
	4d	3	Moderate
	5d	3	Moderate
20	1e	3	Moderate
	2e	3	Moderate
	3e	3	Moderate
	4e	3	Moderate
	5e	2–3	Slight to moderate
60	1f	2–3	Slight to moderate
	2f	2–3	Slight to moderate
	3f	2–3	Slight to moderate
	4f	3	Moderate
	5f	3	Moderate
180	1g	2	Slight
	2g	2	Slight
	3g	2	Slight
	4g	2	Slight
	5g	2	Slight

[a] Based on Gale et al. (1963), Grades 0–6 were used. A score of 0 indicated no iron visible under high power magnification (100×), and a score of 6 indicated very large intra- and extra-cellular Fe deposits present throughout the marrow fragments obscuring cellular detail.

[b] Iron content that was slight (Grade 2) indicated small Fe particles that were sparsely distributed in the bone marrow (100×). Moderate Fe content (Grade 3) indicated that many small Fe particles were present in reticulum cells throughout bone marrow fragments (100×).

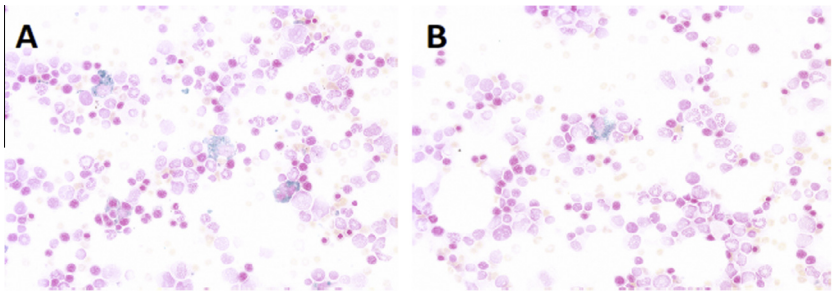

Fig. 2. Prussian blue staining of bone smears from F344 rats exposed to 0 (A) and 180 mg/L Cr(VI) (B) for 90 days. Blue color represents staining of Fe in macrophages. Note that it was difficult to find multiple blue macrophages in one field in the 180 mg/L Cr(VI) treatment group.

animals, the Fe content was slight to moderate (grade 2–3) (Table 2). Iron content that was slight (grade 2) indicated small Fe particles that were sparsely distributed in the bone marrow, which were observed by 100× magnification. Moderate Fe content (grade 3) indicated that many small Fe particles were present in reticulum cells throughout bone marrow fragments. In several rats administered 60 mg/L Cr(VI), the Fe content was slight to moderate (grade 2–3), in all animals of the highest dose group (180 mg/L Cr(VI)), the Fe content was slight (grade 2) (Table 2). As shown in Fig. 2, there were fewer bone marrow macrophages with blue staining (i.e. Fe) in the 180 mg/L Cr(VI)-treated rat as compared to the control animal. In contrast, Prussian blue staining of bone marrow from Cr(VI)-treated mice were unremarkable (data not shown).

3.2. Effects of Cr(VI) on genes involved in iron homeostasis

Three genes involved in Fe transport and absorption from the intestinal lumen were significantly altered by Cr(VI) exposure. Duodenal cytochrome b (DCYBT, *Cybrd1*) and divalent metal transporter 1 (DMT1, *Slc11a2*) are expressed on the apical membrane of villus enterocytes and function together to absorb non-heme Fe in the diet. DCYBT is responsible for reducing luminal Fe^{3+} to Fe^{2+}, which is subsequently transported into the enterocyte via DMT1. Heme-based Fe is acquired by transport of dietary heme across the villus enterocyte via heme carrier protein 1 (HCP1, *Slc46a1*). *Slc11a2* was elevated in both species at both time points in a dose-dependent manner (Fig. 3). After 90 days of exposure, *Slc11a2* was induced ~4-fold in rats at 180 mg/L Cr(VI), and ~1.6-fold in mice. *Cybrd1* expression was decreased in mice at both day 8 and 91, but was only significantly reduced in rats at day 8. *Slc46a1* expression was reduced in both species at both time points (Fig. 3).

Transferrin receptor 1 (TFR1, *Tfr1*) is primarily involved in Fe uptake from the blood and is thus usually expressed on the basolateral membrane. Ferrotransferrin (i.e. Fe-bound transferrin) in the blood binds to TFR1 thereby stimulating endocytosis and cellular Fe absorption. Like *Slc11a2*, *Tfr1* expression was increased in a dose-dependent manner in both species at day 8 and day 91. At day 8, the expression of *Tfr1* was quite robust in both species with increases ranging 4- to 5-fold (Fig. 3). By day 91, *Tfr1* induction was higher in rats than mice.

Two genes expressed on the basolateral enterocyte membrane that are involved in the export of duodenal Fe were also altered by Cr(VI). Ferroportin (*Slc40a1*) is responsible for the export of Fe^{2+} into the blood where it is subsequently oxidized to Fe^{3+} by hephaestin (*Heph*) so that it can bind to transferrin. *Slc40a1* was down-regulated in both species at day 8, unchanged in rats at

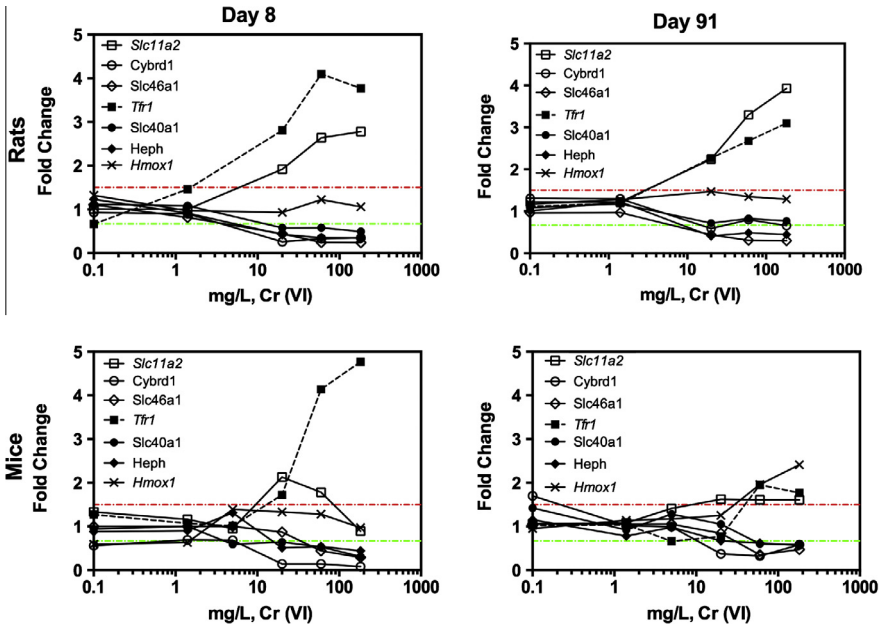

Fig. 3. Fold change expression of select genes in the rat and mouse duodena after 7 and 90 days of exposure to Cr(VI) in drinking water. Open and filled symbols represent genes typically expressed on the apical and basolateral epithelial surfaces, respectively. Red and green lines mark 1.5-fold increases or decreases in gene expression, respectively. See text for details on gene/protein nomenclature. See Kopec et al. (2012a,b) for methods related to microarray analysis.

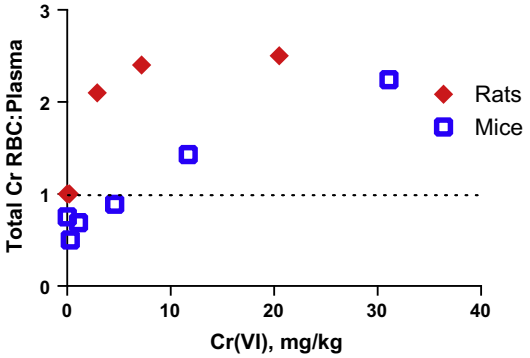

Fig. 4. RBC to plasma ratios of total Cr in rats and mice by their 90-day average daily doses of Cr(VI). The average daily doses of Cr(VI) were based on animal bodyweight, water intake, and administered drinking water concentrations of Cr(VI). RBC:plasma ratio was above unity (>1) in rats at drinking water concentrations ⩾20 mg/L Cr(VI) (equivalent to ⩾2.90 mg/kg). In mice, the RBC:plasma ratio was > 1 at drinking water concentrations ⩾60 mg/L Cr(VI) (equivalent to ⩾11.70 mg/kg).

day 91, and down-regulated in mice at day 91 (albeit insignificantly). *Heph* was down-regulated in both species at both time points (Fig. 3).

In addition to these membrane-bound enzymes/genes, heme oxygenase 1 (*Hmox1*), which is also involved in Fe regulation, was elevated at day 91 in mice but not rats (Fig. 3). Heme oxygenase is involved in the catabolism of heme to CO, Fe^{2+}, and biliverdin. Thus, elevation in *Hmox1* might have led to increased cellular Fe, which might partially explain why *Slc11a2* and *Tfr1* expression was less affected in mice than rats at day 91 (Fig. 3). Diagram 1 summarizes the aforementioned gene changes in rats and the potential influence of Cr(VI) on Fe homeostasis.

3.3. Cr(VI) disposition

Based on concentrations of total Cr measured in RBCs and plasma, RBC:plasma ratios were assessed in rats and mice for the different dose groups. The ratio of Cr concentration in RBCs and plasma has long been used in studies as a biomarker of systemically absorbed Cr(VI) (Alexander and Aaseth, 1995; Buttner and Beyersmann, 1985; Gray and Sterling, 1950). As shown in Fig. 4, the RBC:plasma ratio increased in both species in a dose-dependent manner, but the increase appears at lower doses in rats than in mice. The RBC:plasma ratio was above unity (>1) in rats administered drinking water concentrations of 20 mg/L Cr(VI) and higher (dose of ⩾2.90 mg/kg). In mice, the RBC:plasma ratio was >1 at drinking water concentrations of 60 mg/L Cr(VI) and higher (dose of ⩾11.70 mg/kg).

4. Discussion

Previous studies have shown that high concentrations of Cr(VI) induce anemia in rodents (NTP, 1996, 1997, 2007, 2008b), which may be explained by the disruption of Fe homeostasis by Cr(VI). This current study is the first to demonstrate Fe depletion from the duodenum, liver, serum, and bone marrow following exposure to high concentrations of Cr(VI) in drinking water (Figs. 1 and 2; Table 2). Moreover, Cr(VI) altered the expression of several key genes involved in Fe homeostasis (Fig. 3; Diagram 1). Many of the duodenal gene changes in response to Cr(VI) are consistent with previously reported duodenal gene changes in rats placed on Fe-deficient diets. Collins et al. (2005) studied genomic responses in the duodena of rats placed on Fe-deficient diets at different stages of development (suckling through adulthood), and

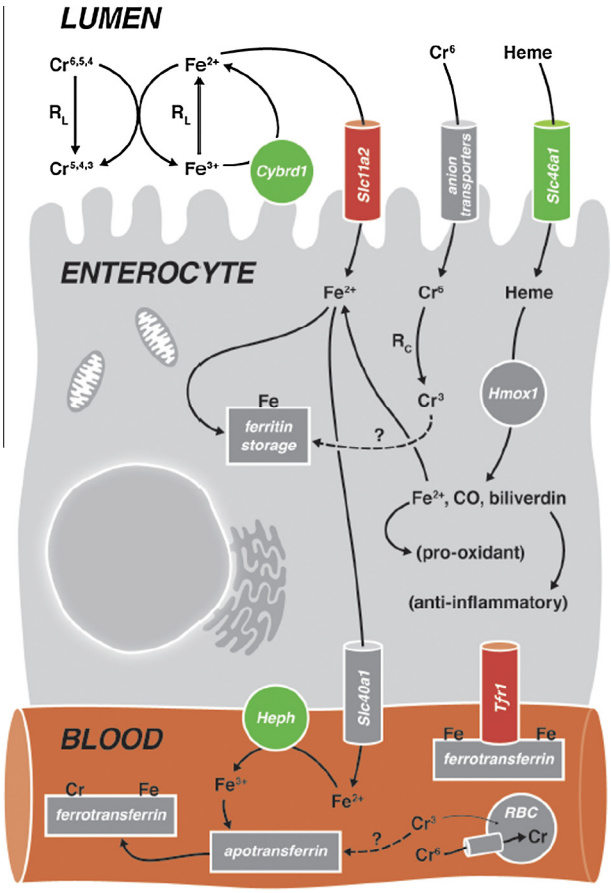

Diagram 1. Summary of gene expression changes in rats and potential influence of Cr on Fe Homeostasis. Ferric iron (Fe^{3+}) in the intestinal lumen is reduced to ferrous iron (Fe^{2+}) by cytochrome b reductase (DCYBT, *Cyprd1*). Fe^{2+} then enters the apical membrane of the enterocyte via divalent metal transporter 1 (DMT1, *Slc11a2*). Fe^{2+} can then be stored in ferritin or enter the bloodstream at the basolateral membrane via ferroportin (*Slc40a1*). Fe^{2+} is then oxidized to Fe^{3+} by hephaestin (*Heph*) so that it can bind to transferrin. Diferric transferrin binds transferrin receptor 1 (Tfr1) and mediates Fe uptake via endocytosis. Heme is taken up by heme carrier protein 1 (HCP1, *Slc46a1*) and subsequently catabolized to Fe^{2+}, CO, and the antioxidant biliverdin by heme oxygenase 1 (*Hmox1*). Genes that were generally up or down regulated in F344 rats by Cr(VI) are colored red or green, respectively. The presence of Cr(VI, IV, and V) can oxidize Fe^{2+} to Fe^{3+} (shown in the lumen), which may hinder Fe^{2+} absorption. Once transported into the enterocyte through anion transporters, Cr(VI) can be reduced by cytoplasmic reductants (R_C, e.g. ascorbate, GSH) to Cr(III). Cr(III) can bind to certain Fe-binding proteins. Cr(VI, V, and IV) may also compete with Fe^{3+} for reductants in the lumen (R_L, e.g. ascorbate, cysteine).

reported increases in *Slc11a2*, *Cybrd1*, *Tfr1* and *Hmox1*, decreases in *Heph*, and no change in *Slc40a1*. Among those genes induced by an Fe-deficient diet, *Slc11a2* and *Tfr1* were induced by Cr(VI) in both species; and *Hmox1* was induced by Cr(VI) in mice. The notable exception was *Cybrd1*, which was decreased by Cr(VI) in both species. Consistent with changes in rats fed an Fe-deficient diet for 9 weeks (Collins et al., 2005), mRNA levels of *Heph* were decreased in both rats and mice exposed to Cr(VI). With regard to ferroportin (*Slc40a1*), the lack of change in rats and mice at day 91 is also consistent with the findings of Collins et al. (2005). Thus, with the notable exception of *Cybrd1*, the gene changes in rats and mice were generally consistent with dietary Fe deficiency. These findings support the hypothesis that high concentrations of Cr(VI) in the intestinal lumen can oxidize luminal Fe^{2+} to Fe^{3+} thereby hindering Fe absorption. Indeed, we have shown that the high concentrations of Cr(VI) used in the 2-year NTP bioassay are not entirely reduced to Cr(III) by gastric fluid and thus high levels of Cr(VI) are expected to be present in the intestinal lumen (Proctor

Table 3
Summary of effects potentially associated with iron homeostasis observed in 2-year NTP bioassay (NTP, 2008b) and 90-day mode of action (MOA) drinking water study (Thompson et al., 2012).[a]

Endpoint	Study	Dose-related trend observed		Evidence for potential association with iron
		Rats	Mice	
Histiocytic infiltration	NTP (2008b)	Increased in liver, duodenum, and mesenteric lymph nodes: males (⩾20 mg/L) females (⩾60 mg/L)	Increased in males and females for duodenum, mesenteric lymph nodes Increased in liver of females only	Iron can be displaced from iron-binding proteins by Cr(III). Histiocytic infiltration may indicate adaptation to oxidative stress induced by free heme and iron (Grom, 2010) Systemic iron sequestration by macrophages can lead to anemia of chronic disease and promote pro-inflammatory activation (Recalcati et al., 2012)
	Thompson et al. (2012)	Duodenum and jejunum (⩾60 mg/L)	Duodenum and jejunum: (⩾60 mg/L)	Iron plays a role in the contribution of macrophages toward either wound healing or chronic inflammation (Recalcati et al., 2012)
Fatty liver	NTP (2008b)	Increased in females (⩾20 mg/L) No dose-related trend in males	No dose-related trend	Higher total hepatic lipid content observed in rats on iron-deficient diet compared to rats on normal diet (Hirosue and Hosogai, 1993) Fatty livers observed in pups of iron-deficient rats (Bartholmey and Sherman, 1985)
Chronic liver inflammation	NTP (2008b)	Increased in females (⩾5 mg/L) No dose-related trend in males	No dose-related trend	Hepatitis observed in Long-Evans Cinnamon rats induced by iron-deficient diet (Sugawara and Sugawara, 1999)
Salivary gland atrophy	NTP (2008b)	Increased in females (⩾60 mg/L) No dose-related trend in males	No dose-related trend	Impairment of salivary peroxidase activity and decreased secretion rate of saliva observed in growing rats on iron-deficient diet (Johansson and Fagernas, 1994)
Oral squamous cell carcinoma	NTP (2008b)	Increased in males and females at 180 mg/L[b]	Not observed	Tumor development observed earlier in iron-deficient rats compared to control animals (both groups were treated with 4-nitroquinoline 1-oxide (4NQO)) (Prime et al., 1983)
Bone marrow smears	Thompson et al. (2012)	Decreased iron content and storage (180 mg/L)	Not observed	Decreased bone marrow content observed with iron deficiency (Harvey, 2000) Bone marrow smears in normal rats showed abundant Fe, and decreased or trace level of Fe observed in anemic rats (Prime et al., 1983)

[a] 90-Day drinking water study was conducted in female rats and mice only (Thompson et al., 2012).
[b] Increase compared to historical controls in females at 60 mg/L (NTP, 2008b).

et al., 2012). There are other important genes expressed primarily in the liver that regulate Fe homeostasis including hepcidin and transferrin receptor 2 (TFR2). In this study, toxicogenomic analyses were conducted on duodenal samples and thus hepcidin and TFR2 expressions were not quantified. Studies that assess gene expression in liver samples of animals treated with Cr(VI) will be informative to further describe Fe perturbations from Cr(VI) exposures.

In addition to the apparent disruption of Fe absorption from the intestinal lumen, we observed dose-dependent changes in Cr disposition based on Cr RBC:plasma ratios calculated for both species (Fig. 4). If Cr(VI) enters the bloodstream, it can be transported into RBCs, reduced to Cr(III), and subsequently bind to heme. Hence, intracellular Cr(III) will be trapped for the lifespan of the RBC. The RBC lifespan is 120, 66, and 42 mean days in humans, rats, and mice, respectively (Derelanko, 1987; Shemin and Rittenberg, 1946; Van Putten, 1958). If Cr(VI) is reduced extracellularly to Cr(III) in the blood or if Cr(III) enters the blood, Cr will not readily accumulate in RBCs; thus, concentrations of Cr in plasma and RBCs remain approximately equal. We observed concentrations of total Cr in RBCs exceeding that of plasma at ⩾20 and ⩾60 mg/L Cr(VI) in rats and mice, respectively (Fig. 4). Our previous kinetics research suggests that the reducing capacity of rat stomach contents reaches saturation at ~20 mg Cr(VI)/L in drinking water, and is greatly exceeded at 60 and 180 mg/L (Proctor et al., 2012). We have previously shown that plasma GSH/GSSG ratios of rats and mice were significantly decreased following 90 days of exposure at ⩾60 mg/L Cr(VI) (Thompson et al., 2012), which may further suggest the presence of Cr(VI) in the blood. Taken together, multiple lines of evidence suggest a dose-dependent change in Cr disposition occurring at approximately 20–60 mg/L in rodents.

Disruption of Fe homeostasis by Cr(VI) may play a role in the toxic responses observed in Cr(VI) drinking water bioassays. Although the relationship between Fe depletion and adverse outcomes has not been evaluated specifically in chronic rodent studies, we examined the findings of the 2-year NTP bioassay and our 90-day study (NTP, 2008b; Thompson et al., 2012) which may be associated with Fe deficiency (Table 3). In both studies, many outcomes potentially associated with Fe deficiency as outlined in Table 3 were observed; effects potentially related to Fe deficiency were more consistently observed in female as compared to male rats, and in rats compared to mice. In addition, these effects potentially related to Fe were mostly all high dose effects, occurring at ⩾20 mg/L Cr(VI). Although the incidence of chronic liver inflammation, which might be related to Fe depletion, was significantly increased at 5 mg/L in females, the severity was increased at ⩾60 mg/L. Thus, the perturbation of Fe homeostasis by Cr(VI) and the associated health effects appear to be limited to high dose exposure.

The MOA for rat oral tumors observed in the 2-year NTP bioassay of Cr(VI) has not been described specifically in the scientific literature. There is some evidence that nutritional status that can affect saliva, salivary glands, oral health, and possibly the risk of oral carcinogenesis (Lingstrom and Moynihan, 2003;

Moynihan and Lingstrom, 2005). Moreover, saliva may play an important role in oral carcinogenesis (pull refs from Section 5: Wallenius 1965, 1966; Naglar and Dayan (2006), Vered et al. (2010), Smiler (1970), Stich et al. (1982)). It is possible that Fe-deficiency reported herein may have played a role for the rat oral tumors. Experimentally-induced anemia, for example, has been shown to potentiate the oral carcinogenicity of 4-nitroquinoline 1-oxide (Prime et al., 1983), and several chemicals that induce oral cancers in rats, also cause microcytic anemia. For example, Ramot et al. (2012) recently observed that gavage doses of 3,3′-4,4′-tetrachloroazobenzene (TCAB) induced oral tumors in rats in the oral mucosa adjacent to molar teeth in nasal section III, which is the same location as oral tumors induced by Cr(VI) in the 2-year NTP bioassay. TCAB has been shown to cause microcytic normochromic responsive anemia in male Sprague–Dawley rats (NTP, 2010). Although TCAB and Cr(VI) are not known to act by the same MOA, microcytic anemia might contribute to formation of rat oral tumors observed following exposure to both chemicals. Also in humans, conditions of anemia such as Plummer-Vinson syndrome have been linked to oral cancer (Kumar et al., 2005; Lucenteforte et al., 2009; Richie et al., 2008). Yet based on the currently available data, it is not feasible to discern effects caused by Cr(VI) directly from those that may be induced or exacerbated by Fe deficiency at high doses. It may be useful to consider future studies in rodents that investigate the potential role of long-term Fe disruption and depletion in context of Cr(VI) exposure. Finally, it cannot be ruled out that other general signs of poor health such as reduced bodyweight gain and water intake might have contributed to the outcomes observed in the 2-year NTP bioassay (Supplemental Fig. S2).

Herein we have described biochemical and molecular support for previously observed Cr(VI)-induced microcytic anemia in rodents. Moreover, Cr(VI)-induced Fe deficiency may have caused or contributed to other adverse effects of Cr(VI) observed in animal bioassays. Most adverse effects potentially related to Fe deficiency occur at Cr(VI) drinking water concentrations that correspond with a shift in the toxicokinetics of Cr(VI) absorption, occurring in rats at $\geqslant$20 mg/L. Because Fe depletion mainly occurred at very high concentrations of Cr(VI) in drinking water, adverse outcomes associated with disruption of Fe homeostasis are not likely at typical U.S. drinking water exposures, recently reported to range from ~0.00003 mg/L to 0.091 mg/L, with median value of 0.0002 mg/L (USEPA, 2013). Based on these data, this study provides evidence of perturbations in Fe homeostasis from exposures to high concentration of Cr(VI) ($\geqslant$20 mg/L) in drinking water. The findings of this study may be useful to guide experimental designs of future studies to evaluate Fe homeostasis disturbance and potentially related adverse effects from prolonged exposures to Cr(VI).

Conflict of Interest

The authors employment affiliation is as shown on the cover page. Both ToxStrategies and Summit Toxicology are private consulting firms providing services to private and public organizations on toxicology and risk assessment issues. The authors [CT, MH, DP, CK, LH] have presented study findings in meetings with regulators including public meetings on behalf of the Cr(VI) Panel of the ACC. DP has also been an expert in litigation involving Cr(VI), which was unrelated to this research or ACC.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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This work was supported by the Cr(VI) Panel of the American Chemistry Council (ACC). The funders were given the opportunity to review the draft study design, as it went through an external peer review process, and draft manuscripts at the time of external peer review. The purpose of the review was to allow input on the clarity of the science presented but not in interpretation of the research findings. The contents of this review reflect solely the view of the authors.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2014.01.009.

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