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Reduction of hexavalent chromium by fasted and fed human gastric fluid. I. Chemical reduction and mitigation of mutagenicity

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ABSTRACT

Evaluation of the reducing capacity of human gastric fluid from healthy individuals, under fasted and fed conditions, is critical for assessing the cancer hazard posed by ingested hexavalent chromium [Cr(VI)] and for developing quantitative physiologically-based pharmacokinetic models used in risk assessment. In the present study, the patterns of Cr(VI) reduction were evaluated in 16 paired pre- and post-meal gastric fluid samples collected from 8 healthy volunteers. Human gastric fluid was effective both in reducing Cr(VI), as measured by using the *s*-diphenylcarbazide colorimetric method, and in attenuating mutagenicity in the Ames test. The mean (±SE) Cr(VI)-reducing ability of post-meal samples (20.4 ± 2.6 μg Cr(VI)/mL gastric fluid) was significantly higher than that of pre-meal samples (10.2 ± 2.3 μg Cr(VI)/mL gastric fluid). When using the mutagenicity assay, the decrease of mutagenicity produced by pre-meal and post-meal samples corresponded to reduction of 13.3 ± 1.9 and 25.6 ± 2.8 μg Cr(VI)/mL gastric fluid, respectively. These data are comparable to parallel results conducted by using speciated isotope dilution mass spectrometry. Cr(VI) reduction was rapid, with >70% of total reduction occurring within 1 min and 98% of reduction is achieved within 30 min with post-meal gastric fluid at pH 2.0. pH dependence was observed with decreasing Cr(VI) reducing capacity at higher pH. Attenuation of the mutagenic response is consistent with the lack of DNA damage observed in the gastrointestinal tract of rodents following administration of ≤180 ppm Cr(VI) for up to 90 days in drinking water. Quantifying Cr(VI) reduction kinetics in the human gastrointestinal tract is necessary for assessing the potential hazards posed by Cr(VI) in drinking water.

1. Introduction

Chromium is a naturally occurring element found in the earth and all living beings, and it is also introduced into the environment by various industries including welding, electroplating, painting and priming, and production of pigments, ferrochromium/stainless steel, and chromate chemicals (ATSDR, 2012; NIOSH, 2013). Originating from both natural and anthropogenic sources, chromium is detected in groundwater, drinking water, and soil, primarily in the trivalent [Cr(III)] and hexavalent [Cr(VI)] forms (Oze et al., 2007; ATSDR, 2012; McNeil et al., 2012; U.S. EPA, 2014). Cr(III) has limited acute and chronic toxicity, and in 2-year cancer bioassays of the National Toxicology Program (NTP), it was not carcinogenic to rodents (NTP, 2008b; Stout et al., 2009a). In contrast, exposures with the drinking water to Cr(VI), as sodium dichromate dihydrate (SDD), caused an increase in small intestinal cancers in B6C3F1 mice at ≥20 mg/L and oral cavity cancers in F344/N rats at ≥60 mg/L (NTP, 2008a; Stout et al., 2009b). Notably, these carcinogenic concentrations administered to rodents were at least ~200,000 times higher than the average Cr(VI) concentrations (~0.0001 mg/L) detected in the U.S. drinking water supply (McNeil et al., 2012; U.S. EPA, 2014).

While Cr(III) enters the cell by passive diffusion, cellular uptake of Cr(VI) is facilitated through anion transporters; thus, differences in toxicity between Cr(III) and Cr(VI) are largely due to the lower cell permeability of Cr(III) (De Flora and Wetterhan, 1989; Collins et al., 2010; ATSDR, 2012). The toxicity of Cr(VI) is mitigated by extracellular reduction of Cr(VI) to Cr(III) in bodily fluids, including the gastric fluid. The earliest study suggesting Cr(VI) reduction by human gastric fluid was published 50 years ago by Donaldson and Barreras (1966) and showed that infusion of Cr(VI) into the duodenum or jejunum resulted in a greater excretion of chromium in urine, compared with oral

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administration. Thereafter, several studies demonstrated Cr(VI) reduction by human gastric fluid and, in some cases, detoxification with mitigation of mutagenicity in the Ames test. In particular, De Flora and Boido (1980) showed that pre-incubation with human gastric fluid from fasting subjects affected the mutagenicity of several chemical agents in the Ames test, either in the sense of deactivation (sodium azide and sodium dichromate) or of stabilization (captan) or even of potentiation (ICR-170) of mutagenic effects. For Cr(VI), gastric fluid samples from 5 fasting individuals sharply decreased the mutagenic response in the Ames test (*S. typhimurium* strain TA100), presumably because gastric fluid reduced Cr(VI) to the trivalent state. Heating the gastric samples did not impact the assay results, but when sample pH was increased, the decrease in positive response was attenuated (De Flora and Boido, 1980). A further study showed that fasted gastric juice samples from 4 other untreated subjects reduced 9.2 ± 0.4 μg Cr(VI)/mL (Petrilli and De Flora, 1982).

To date, De Flora et al. (1987) is the only detailed study that evaluated the circadian reduction of Cr(VI) in pre- and post-meal gastric fluid samples collected from human volunteers, including one healthy volunteer and 16 hospitalized patients of both genders, most of whom were suffering from duodenal ulcer. In that study, a nasogastric tube was positioned in the stomach for 24 h. A continuous intragastric pH monitoring was carried out, and gastric fluid samples were aspirated hourly. A total of 428 gastric fluid samples were collected, centrifuged, and a fixed amount of fluid (100 μL) was spiked *ex vivo* with 25 μL of a 200 μg/mL Cr(VI) solution (5 μg) to analyze Cr(VI) reduction within 60 min of reaction time by the *s*-diphenylcarbazide (DPC) colorimetric method. In addition, the Ames reversion test using *S. typhimurium* strain TA102 was conducted. The results showed that there was a significant decrease in Cr(VI) mutagenicity response in the presence of gastric fluid. Using the DPC colorimetric method, Cr(VI) reduction was observed to be rapid in the first 10 to 20 min of reaction time. The baseline Cr(VI) reduction quantified during the inter-digestive periods (fasted states and mainly at night) was 8.3 ± 4.7 μg Cr(VI)/mL gastric fluid, a figure similar to the results of Petrilli and De Flora (1982). During the post-prandial periods of approximately 3 to 4 h after each one of the 3 daily meals, reduction of 31.4 ± 6.7 μg Cr(VI)/mL gastric fluid (mean ± SE) was observed, with peaks of 50 to 60 μg Cr(VI)/mL gastric fluid. In parallel, post-meal samples were significantly more effective than pre-meal samples in attenuating Cr(VI) mutagenicity. As checked in 3 subjects, inhibition of Cr(VI) mutagenicity by gastric fluid was significantly enhanced by stimulating gastric acid secretion with pentagastrin. Similar to De Flora and Boido (1980), a statistically significant decrease in Cr(VI) reduction was observed when the pH of the post-meal samples was increased to 7.0 (De Flora et al., 1987).

Based on the above data, taking into account that in a fasting individual the daily gastric secretion is 1000–1500 mL (Kirsner, 1974), and in the 4-h period after each meal an average amount of approximately 800 mL is additionally secreted, De Flora et al. (1997) calculated that the overall Cr(VI) reduction by gastric fluid may be estimated to be at least 84–88 mL per day, even disregarding the amounts of Cr(VI) that are reduced by food and beverages (Kerger et al., 1996).

Zhitkovich (2005) commented that reduction measures of De Flora et al. (1987) based on the use of the DPC colorimetric method could be in error. Specifically, he stated that the colorimetric reaction with DPC to quantify Cr(VI), which is conducted in the presence of 8% sulfuric acid, would overestimate the reducing capabilities in biological systems because many organic molecules can reduce Cr(VI) in highly acid solutions. Zhitkovich (2005) disregarded the fact that Cr(VI) reduction by human gastric fluid, as assessed by colorimetric analyses, had been confirmed by the parallel inhibition of Cr(VI) mutagenicity. In addition, the hypothesis by Zhitkovich (2005) is not supported by any experimental demonstration, whereas the DPC method to analyze Cr(VI) is considered a valid analytical method that has been used for many decades in air, water, and soil samples to assess occupational and environmental Cr(VI) exposures (*e.g.*, EPA Method 3060A). Furthermore, measures of Cr(VI) reduction by the gastric fluid from fasted pre-operative patients was recently shown to be consistent with the De Flora et al. (1987) measures using speciated isotope dilution mass spectrometry (SIDMS) (Kirman et al., 2013).

The goal of the present study was to evaluate Cr(VI) reduction patterns by paired human gastric fluid samples collected, under fasted and fed conditions, from healthy individuals, in the absence of any medical treatment. We also explored the time and pH dependence of the reaction of Cr(VI) in gastric fluid. Cr(VI) reduction and mitigation of its mutagenicity were evaluated in parallel by using the DPC colorimetric method and the Ames test, and most samples were also analyzed by SIDMS (see Kirman et al., 2016). The results confirmed the ability of human gastric juice to reduce Cr(VI) and to inhibit its biological activity, with significant differences between fasted and fed samples. Understanding Cr(VI) reduction kinetics is critical for assessing the potential hazards posed by Cr(VI) in drinking water at environmentally-relevant levels.

2. Materials and methods

2.1. Collection and preparation of human gastric fluid samples

Paired pre- and post-meal gastric fluid samples were collected from 9 healthy volunteers aged 26 to 34 years (average age: 29.7 years). The study protocol was approved by the University of Genoa Ethics Committee and performed according to the Declaration of Helsinki. All study participants signed an informed consent form. Without causing harm or much discomfort, nasogastric tubes (Bicakcilar Nasogastric Cateter Levin), having 4 mm diameter and 1210 mm length, were inserted into each study participant to obtain gastric fluid samples. Pre-meal samples were collected after overnight fasting, and post-meal samples were collected 1.5 h after completion of lunch. The average caloric intake of the meal was 700 kcal, with the following standardized composition: pasta 70 g (238 kcal), roast meat or escalope 100 g (100–110 kcal), tomatoes 100 g (17 kcal), jam pastry (190 kcal), plus condiments.

All gastric fluid samples were centrifuged at 1000 ×*g* for 10 min in order to remove gross food particles, and the supernatant portions were used. The pH of each gastric fluid sample was measured with indicator strip in the ranges 0–6, 0–2.5, and 2.5–4.5 (E. Merck, Darmstadt, Germany). The centrifuged samples were then divided into 1-mL aliquots and stored at −20 °C. The samples from one study participant was too viscous to be pipetted and was discarded. Hence, a total of 16-paired gastric fluid samples (pre- and post-meal) from 8 individuals (6 males and 2 females) were tested in this study.

2.2. Measurement of Cr(VI) reduction in human gastric fluid by the DPC colorimetric method

Several preliminary experiments were conducted to identify the optimal Cr(VI) concentration and gastric fluid sample volumes to yield linear dose–response curves for Cr(VI) reduction as well as the optimal amounts of gastric juice to be challenged with the highest sodium dichromate dihydrate (SDD) dose (data not shown). These experiments showed that the optimal concentration of Cr(VI) to be tested was 6 μg SDD (Sigma, Saint Louis, MO, USA) in 100 μL deionized water. Thus, each reaction mixture contained 6 μg SDD in 100 μL deionized water, which was added to: 1) 50 μL gastric fluid; 2) 25 μL gastric fluid plus 25 μL deionized water; 3) 12.5 μL gastric fluid plus 37.5 μL deionized water; or 4) 50 μL deionized water. As such, the total volume for a gastric fluid reaction mixture was 150 μL. For most experiments, incubation of Cr(VI) in gastric fluid was conducted at 37 °C for 60 min. However, to examine time dependence of Cr(VI) reduction, various reaction times from 1 to 60 min were also tested using a pooled sample (pH 2.0) made from post-meal gastric fluid.

Several other experiments were conducted with gastric fluid samples that were either pre-heated at 56 °C for 30 min or at 70 °C for 20 min, or kept at 20 °C for 10 days to evaluate the effect of heating and storage at room temperature. The effect of freeze and thaw cycles on Cr(VI) reduction was also evaluated.

In addition, post-meal gastric fluid samples were pooled yielding pH 2.5. Varying amounts of the pooled samples (0, 12.5, 25, and 50 μL as described above) were used to investigate the pH dependence of Cr(VI) reduction in gastric fluid. Specifically, 6 μg SDD in acidified deionized water at pH 2.0 or in Mcilvaine's citrate/phosphate buffer, achieving pH values of 2.0 to 8.0, were added to the pooled samples at 37 °C for 60 min of reaction time to test the pH dependence of Cr(VI) reduction in gastric juice.

To analyze Cr(VI) reduction by the DPC colorimetric method, after 0 to 60 min of reaction time, all samples were transferred to an ice-cold bath. The content of each reaction mixture sample was divided into 2 cuvettes. The cuvettes were filled with 2.5 mL of DPC reagent (Sigma, Saint Louis, MO, USA), consisting of 40 mg DPC in 100 mL of 8% sulfuric acid, 19% ethanol, and 73% water. Another set of cuvettes, used as blanks, were filled with 2.5 mL of the same sulfuric acid-ethanol mixture, but without DPC. After 15 min at room temperature, the resulting Cr(VI)-DPC complex was measured at 540 nm in a Nano-Drop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the optical density (OD) of blanks was subtracted from the OD of gastric fluid samples.

2.3. Mutagenicity of Cr(VI) in Ames reversion test in the presence of human gastric fluid

The Ames reversion test was conducted using the *S. typhimurium* strain TA104 that carries the *hisG428* mutation (Marnett et al., 1985). All assays were performed in the absence of exogenous metabolic system (S9 mix), which is known to mitigate Cr(VI) mutagenicity (De Flora, 1978; Petrilli and De Flora, 1978; NTP, 2008b). Gastric fluid sample preparations, *i.e.*, mixtures of 6 μg SDD in 100 μL deionized water and varying amounts of gastric fluid and deionized water that were used for the DPC colorimetric method, were also applied to the Ames reversion tests. After 60 min of reaction time at 37 °C, all gastric fluid mixture samples were assayed according to the standard plate incorporation test (Maron and Ames, 1983), using 3 replicate plates per mixture. Cr(VI)-free plates were also tested to evaluate the number of spontaneous revertants. Cr(VI) added to a solution without gastric fluid was also included as a positive control in the Ames reversion test.

The results from both experimental approaches (DPC colorimetric method and Ames reversion test) were used to create regression lines for each gastric fluid sample and quantify Cr(VI) reduction capacity (see Results). For the Ames assay, the number of *S. typhimurium* his^- revertants was used instead of OD values to make the calculations (see Results). Pair-wise *t*-tests were conducted to compare Cr(VI) reduction between pre- and post-meal samples and between the two assays. Pair-wise *t*-tests were conducted using Graphpad Prism 6 for Mac (http://www.graphpad.com).

3. Results

3.1. Reduction of Cr(VI) in pre- and post-meal human gastric fluid samples

Several preliminary assays were carried out in order to assess the optimal experimental conditions (data not shown). Thereafter, in order to generate comparative data, we tested all 16 gastric juice samples in a single experiment using the colorimetric method and, in a separate aliquot, using the mutagenicity assay. The results of these analyses are shown in Table 1, along with the pH of the examined samples, which ranged between 1.6 and 2.5 for pre-meal gastric fluid samples, and between 1.9 and 3.5 for the post-meal samples. With both experimental approaches, we calculated the regression line and then derived either the Cr(VI) reducing ability or the loss of mutagenicity of each sample after 60 min of contact with varying amounts of gastric fluid (0, 12.5, 25, and 50 μL) at 37 °C. To give an example, the regression line equation for sample No. 1 is $y = -0.0003x + 0.114$, where y is the SDD dose (in μg) and x is the gastric fluid amount (in μL). For reducing all 6 μg SDD present in the mixture, the equation will be $0 = -0.0003x + 0.114$, and hence $x = 380$ μL. This means that we need 380 μL gastric fluid to reduce 6 μg SDD. By making the proportion, 1 mL of gastric juice will reduce 15.8 μg SDD. Because Cr(VI) is the 34.9% of the SDD molecule, the final result is that 1 mL of that gastric juice sample reduces 5.5 μg Cr(VI), as shown in Table 1. Similar calculations were made by using the mutagenicity assay, except that, instead of using OD values, we used the number of *S. typhimurium* his^- revertants. The regression lines generated with all samples, both using the colorimetric test and the Ames assay, are shown in Supplemental Table 1.

Table 1
Amounts of Cr(VI) reduced per mL of pre-meal and post-meal paired gastric juice samples, as assessed either by colorimetric method (DPC reagent) or by mutagenicity assay (*S. typhimurium* strain TA104, in the absence of S9 mix).

Sample	Identification code	Collection time	pH	Cr(VI) reduced (μg/mL)	
				Colorimetric method	Mutagenicity assay
No. 1	ASS	Pre-meal	1.6	5.5	7.8
No. 2	ASS	Post-meal	1.9	26.6	38.9
No. 3	GEM	Pre-meal	1.9	5.6	10.8
No. 4	GEM	Post-meal	3.5	10.9	18.8
No. 7	SAM	Pre-meal	1.9	20.2	22.0
No. 8	SAM	Post-meal	1.9	29.7	31.6
No. 11	MAR	Pre-meal	1.6	9.7	11.3
No. 12	MAR	Post-meal	2.2	21.6	20.2
No. 13	BOD	Pre-meal	1.6	4.6	13.4
No. 14	BOD	Post-meal	2.2	26.6	20.3
No. 15	BRU	Pre-meal	2.5	2.3	6.0
No. 16	BRU	Post-meal	3.0	9.3	35.0
No. 17	DEL	Pre-meal	1.6	17.6	18.1
No. 18	DEL	Post-meal	3.5	18.6	20.8
No. 19	MAZ	Pre-meal	1.6	17.1	16.7
No. 20	MAZ	Post-meal	2.0	19.7	19.3

From the observation of Table 1 it is evident that, irrespective of the experimental approach, the post-meal samples were consistently more efficient than the corresponding pre-meal samples in reducing Cr(VI) and in inhibiting its mutagenicity. The mean (±SE) Cr(VI) reducing ability of pre-meal samples, as evaluated by means of the colorimetric test, was 10.2 ± 2.39 μg Cr(VI)/mL gastric juice and that of post-meal samples was 20.4 ± 2.61 μg Cr(VI). This difference was statistically significant ($P < 0.01$). When using the mutagenicity assay, the decrease in mutagenicity produced by pre-meal samples and post-meal samples corresponded to reduction of 13.3 ± 1.91 μg Cr(VI) and 25.6 ± 2.89 μg Cr(VI), respectively. Again, this difference was statistically significant ($P < 0.01$).

In general, results by the DPC colorimetric method indicated less percent reduction than those determined by Ames reversion test, and results were significantly different for pre-meal samples ($P = 0.03$) and for all samples combined ($P = 0.04$) between methods. However, the results obtained by testing the 16 gastric juice samples by means of the colorimetric test and those obtained by using the mutagenicity assay were significantly correlated ($r = 0.675$; $P < 0.01$).

3.2. Time and pH dependence of Cr(VI) reduction in pooled post-meal human gastric fluid

Fig. 1 shows the DPC colorimetric results of varying the reaction times for a pooled post-meal sample at pH 2.0. Cr(VI) reduction was rapid; using the mass of Cr(VI) reduced in 60 min, it was observed that after 30 min of contact, 98.3% of Cr(VI) had been reduced, after 15 min, 93.3% had been reduced, after 5 min, 78.4% had been reduced, and after 1 min, 72.1% had been reduced.

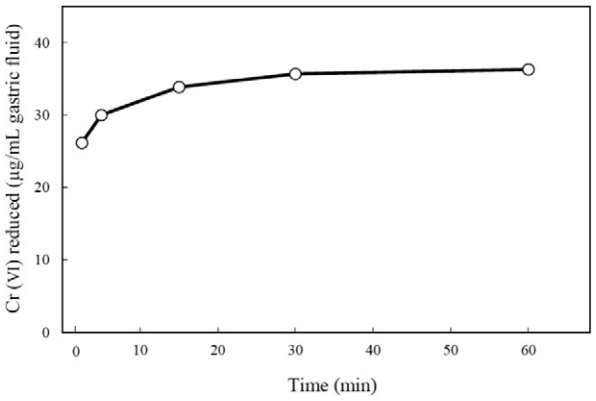

Fig. 1. Time dependence of Cr(VI) reduction following preincubation at 37 °C with a gastric juice pool (pH 2.0) for reaction times of 0 to 60 min in the DPC colorimetric method.

3.3. pH dependence of Cr(VI) reduction in pooled post-meal human gastric fluid

Fig. 2 shows the results of pH variations in gastric fluid samples. It is evident that Cr(VI) reduction capacity in human gastric fluid becomes progressively less efficient at increasing pH. On a linear scale, we observed a sharp decrease in Cr(VI) reduction starting at pH ≥ 4 (Fig. 2). At pH 8.0, reduction capacity was decreased 3.6-times as compared to that at pH 2.0 (5 μg Cr(VI)/mL gastric fluid compared to 18 μg Cr(VI)/mL gastric fluid). As discussed in Part II (Kirman et al., 2016), no pH-dependent inflection point in Cr(VI) reduction rate constant was observed.

3.4. Effect of heating, storage, and freeze-thawing the gastric fluid samples on Cr(VI) reduction

Cr(VI) reduction capacity was not affected by heating the gastric fluid samples either at 56 °C for 30 min or at 70 °C for 20 min (data not shown). Two separate experiments (not shown) provided evidence that Cr(VI) reduction was not affected even when samples were in storage for 10 days at 20 °C in the dark (not shown). Likewise, Cr(VI) reduction was not affected by freeze-thaw cycles (freezing and thawing was done 3 times for 4 gastric fluid samples, data not shown).

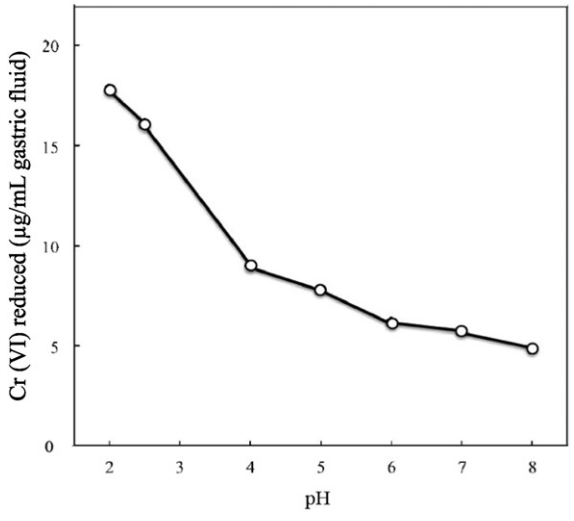

Fig. 2. pH dependence of Cr(VI) reduction after preincubation for 60 min with a gastric juice pool, having an original pH of 2.5, before colorimetric analysis.

4. Discussion

The present study evaluated for the first time Cr(VI) reduction by paired pre- and post-meal gastric fluid samples collected from healthy subjects. Our study results support the conclusions that (*a*) human gastric fluid is effective in reducing Cr(VI) and in inhibiting its mutagenic activity; (*b*) even by removing gross food residues, the Cr(VI) reducing activity is significantly increased during the periods after meals; (*c*) at relevant human exposure levels, Cr(VI) reduction is very rapid, much faster than the time of permanence of food and beverages in the stomach (see below); (*d*) Cr(VI) reduction is pH-dependent and is due to thermostable components of this biological fluid, and (*e*) the action of these reducing components is favored by low pH and becomes less pronounced at increasing pH.

In particular, the data resulting from the DPC colorimetric method indicates that the reducing ability of samples from fasted subjects accounted for 10.2 ± 2.39 μg Cr(VI)/mL gastric fluid and that of post-meal samples was 20.4 ± 2.61 μg Cr(VI)/mL gastric fluid. By using the Ames Salmonella test as a bioindicator of Cr(VI) reduction, the loss of mutagenicity produced by pre-meal samples corresponded to 13.3 ± 1.91 μg Cr(VI), and the one produced by post-meal samples corresponded to 25.6 ± 2.89 μg Cr(VI)/mL gastric fluid. These figures are comparable to those reported in our previous studies. In fact, we previously reported that fasted gastric juice samples from 4 untreated subjects reduced 9.2 ± 0.4 μg Cr(VI)/mL (Petrilli and De Flora, 1982). In 1987, the circadian evaluation of Cr(VI) reduction by gastric juice samples from hospitalized patients, mainly suffering from ulcers, showed that the reducing activity of samples from fasting individuals was 8.3 ± 4.7 μg Cr(VI)/mL, and during the 4 h after each meal Cr(VI) reduction averaged 31.4 ± 6.7 μg Cr(VI)/mL.

Five of the 8 pre-meal samples examined in the present study by using both the DPC colorimetric method and the Ames reversion test (Nos. 7, 11, 15, 17, and 19) and 7 post-meal samples (Nos. 2, 4, 8, 12, 14, 16, and 20) were also analyzed by SIDMS. The modeled 1-hour reducing capacity of Cr(VI) by SIDMS was 10.3 ± 3.0 μg Cr(VI)/mL gastric fluid for pre-meal samples and 20.4 ± 2.8 μg Cr(VI)/mL gastric fluid for post-meal samples (Kirman et al., 2016). Thus, the results obtained by using in parallel two analytical methods and a biological system were very similar. These findings demonstrate that DPC analysis may be used to quantify the capacity for Cr(VI) reduction and that the concerns raised by Zhitkovich (2005) are unfounded. In particular, several separate experiments using different methodological approaches consistently showed that the gastric fluid from fasting individuals is able to reduce about 10 μg Cr(VI)/mL. Because daily gastric secretion in a fasting individual is 1000–1500 mL, it can be calculated that the baseline reduction in the human stomach is 10–15 mg Cr(VI)/day. The reducing capacity of gastric fluid collected from the same healthy subjects was doubled when it was evaluated 1.5 h after a light meal. This figure cannot be compared to the values recorded in ulcer patients. In the previous study (De Flora et al., 1987), Cr(VI) reduction had been monitored at multiple time points, and the peak of reduction was found to vary from individual to individual during the 3–4-h period after each meal. In any case, taking into account that approximately 800 mL gastric fluid is secreted during the 4-h period after each meal, the data generated in healthy subjects suggest that about 16 mg Cr(VI) are reduced in the gastric environment after each meal.

Similar to the findings of previous studies (De Flora and Boido, 1980; Petrilli and De Flora, 1982; De Flora et al., 1987), our study results also indicate that human gastric fluid is effective in attenuating response in the Ames test. Mitigation of Cr(VI) mutagenicity by gastric fluid in the Ames assays are consistent with the lack of positive results in the *in vivo* genotoxicity studies where Cr(VI) was administered by physiologically-relevant routes of exposure (*e.g.*, ingestion) (De Flora et al., 2008; NTP, 2007; O'Brien et al., 2013; Thompson et al., 2015a, 2015b, 2015c). It is also notable that Cr(VI) reduction in our gastric fluid samples was very rapid, and at relevant exposure is expected to be nearly complete

prior to emptying in to the small intestine. The half-time for saline emptying from the stomach is 12 min in fasting individuals, while the transit time for a meal in the stomach is approximately 4 h (Granger et al., 1985). Even though pH is higher in the post-meal samples and large food particles were removed by centrifugation, Cr(VI) reduction was remarkably increased (~50%) compared to the pre-meal samples. Hence, our study results affirm that Cr(VI) reduction capacity is affected largely by the thermostable reducing agents present in the gastric fluid.

The Ames results herein demonstrate the role that gastric fluid has in protecting cells from Cr(VI) toxicity. Consistently, DNA damage measured by γ-H2AX immunostaining, micronucleus induction, and k-*ras* codon 12 mutations are negative in the duodenum of B6C3F1 mice exposed to ≤180 ppm Cr(VI) for up to 90 days (O'Brien et al., 2013; Thompson et al., 2015a, 2015b). X-ray fluorescence imaging of chromium distribution within the intestinal mucosa indicates Cr signal in the villi, but not in the crypt region of mice exposed to 180 mg/L Cr(VI) (Thompson et al., 2015a, 2015b). This suggests that any unreduced Cr(VI) poses little genotoxic risk to the long-lived stem cells of the small intestine.

It is evident that, due to the lack of toxicity of Cr(III), reduction of Cr(VI) in the stomach, and in other body compartments, represents detoxification and introduces a threshold or nonlinear mechanism limiting Cr(VI) toxicity, genotoxicity and carcinogenicity *in vivo* (De Flora, 2000). This longstanding observation was developed based on earlier gastric reduction data and confirmed with the current findings. With reference to the De Flora et al. (1987) study, the IARC Working Group "interpreted these findings as indicating mechanisms that limit the activity of chromium(VI) compounds *in vivo*" (IARC, 1990). When setting new standards for drinking water, the U.S. EPA acknowledged that "the body's normal physiology provides detoxification for chromium(VI), which provides protection from the oral toxicity of Cr(VI)" (U.S. EPA, 1991). The Agency for Toxic Substances and Disease Registry indicated that these "mechanisms limit the bioavailability and attenuate the potential effects of chromium(VI) compounds *in vivo*" (ATSDR, 1993). The European Food Safety Authority (EFSA) reported that "the determinant of the genotoxic effects of Cr(VI) *in vivo* is the reductive capacity of the gastrointestinal tract that may significantly limit or fully prevent Cr(VI) uptake in the blood and/or distribution to the target tissues when administered orally" (EFSA, 2014).

Based on these mechanisms, it is very unlikely that drinking water exposure to Cr(VI) poses a carcinogenic hazard, even at doses largely exceeding the amounts found in drinking water supplies, which typically occur at average levels <0.001 mg/L. It is recognized that individuals using PPIs have higher pH stomach conditions and thus reduce Cr(VI) at lower rates as compared to non-users. Kirman et al. (2016) reported stomach reduction data for three fasting PPI users with stomach pH of 5 to 7.5. The pH-dependent reduction rate model was able to predict reduction rates in PPI users, and as such, this subpopulation can be quantitatively evaluated in future risk assessments (Kirman et al., 2016).

Regarding incidental ingestion of Cr(VI) in the workplace from airborne exposures, a literature review and meta-analysis of oral cavity, esophageal, stomach, small intestine, colon, and rectal cancers among workers occupationally exposed to Cr(VI) during the period 1950–2009 provided evidence that Cr(VI)-exposed workers are not at a greater risk of cancers of these tissues than reference populations (Gatto et al., 2010). This finding is supported by two recent updates of mortality among very highly exposed cohorts of US chromate production workers (Proctor et al., 2016; Gibb et al., 2015), neither of which found a significant increase in stomach cancer risk. In contrast, the meta-analysis by Welling et al. (2015) among exposed workers concluded: "Overall, these results suggest that Cr(VI) is a stomach carcinogen in humans, which is consistent with the tumour results reported in rodent studies". The differences in findings between the Gatto et al. (2010) and Welling et al. (2015) meta-analyses deserves further study, but likely results from differences in inclusion criteria. Further, the statement by Welling et al. (2015) that Cr(VI) is a stomach carcinogen in rodents is not documented by any study. In fact, the only study that reported positive results in the forestomach (Borneff et al., 1968) suffered from a number of inadequacies, as admitted by the authors themselves, to such an extent that it was not even mentioned in the IARC (1990) Monograph. On the other hand, the NTP (2008a) study did not observe any tumorigenic effects in either the glandular stomach or forestomach of rats and mice, even at very high Cr(VI) exposures (NTP, 2008a; Stout et al., 2009b).

The conclusion of the NTP (2008a) study was that "Cr(VI) exposure resulted in increased incidences of rare neoplasms of the squamous epithelium that lines the oral cavity (oral mucosa and tongue) in male and female rats, and of the epithelium lining the small intestine in male and female mice" (NTP, 2008a; Stout et al., 2009b). In particular, an increase of small intestine tumors only occurred at 20 mg Cr(VI)/L and higher. Increased carcinogenicity was not observed at the lowest concentrations tested in the NTP (2008a) study, corresponding to 5–10 mg Cr(VI)/L water. As Cr(VI) reduction in stomach fluids has been shown to be concentration-dependent (Proctor et al., 2012; Kirman et al., 2012, 2013; Schlosser and Sasso, 2014) and the exposures that caused cancer exceed the drinking water exposures by orders of magnitude, mouse intestinal tumors are not expected to be relevant for human exposures. This is in agreement with negative genotoxicity in the forestomach, glandular stomach and duodenum of mice receiving sodium dichromate with the drinking water at 5 and 20 mg Cr(VI)/L (De Flora et al., 2008).

Studies have demonstrated inter-species differences in the gastric reducing capacity and rate with mice < rats < humans (Kirman et al., 2013; Sasso and Schlosser, 2015). Differences in the anatomo-histological structures and functional properties of the stomach also contribute to differences between rodents and humans species. First, the pH of the gastric environment is higher in rodents than in humans. For the purposes of developing a rodent physiologically-based pharmacokinetic (PBPK) model, the pH of stomach contents (forestomach and stomach combined) of rats and mice, of the same strains and in the conditions of the NTP study, was reported as 4.25–4.5 (Proctor et al., 2012). Although the pH of the rodent aglandular forestomach and glandular stomach are likely to vary, the gastric pH of healthy volunteers in our study was considerably lower, 1.6–2.5 in pre-meal samples and 1.9–3.5 in post-meal samples. As reduction capacity is significantly reduced at higher pH, Cr(VI) reduction is expected to be less efficient in rodents than in humans at baseline pH. Second, there are major differences in the anatomical and histological structure of the stomach of rodents and humans, and diverging physiology concerning digestion of food and transformation of ingested toxic substances. In humans the entire stomach is secretory and has numerous prominent folds (*rugae*), whereas in rodents the interior of the stomach has two distinctive regions separated by a prominent limiting ridge. The forestomach has a hard and stratified squamous epithelium, which is heavily cornified, 2–3 layers thick, and devoid of glands and *muscularis mucosa*. Conversely, the glandular stomach, which empties into the duodenum, has a delicate secretory epithelium and *rugae* that are noticeable when the stomach is empty (DeSesso and Jacobson, 2001). In fasting humans, the half-time for saline emptying from the human stomach is 12 min, while the transit time for a meal in the stomach is approximately 4 h (Granger et al., 1985). In rodents, the forestomach, which constitutes the 60% of the stomach area, functions as a reservoir of food, which is transferred to the glandular stomach in case of metabolic necessities. The quantity of chyme delivered to the duodenum is independent of stomach filling and corresponds to the rodent actual energy requirements. Because mice are continuous feeders (de Zwart et al., 1999), there are no post-meal peaks of gastric juice secretion that in humans provide the bulk of Cr(VI) reduction.

It is well recognized that the detoxifying capacity of the gastric environment is not infinite (Proctor et al., 2012; Kirman et al., 2013). The finding that small intestine lesions and tumors were detected in mice exposed to Cr(VI) in drinking water (NTP, 2008a; Stout et al., 2009b) suggests that, at high concentrations, the reducing capacity of the

mouse GI tract was likely exceeded allowing for high concentrations of Cr(VI) to reach the intestine. Due to interspecies differences, the results of the NTP (2008a) study cannot be transferred to the human situation. Incidentally, tumors affecting the small intestine are extremely rare in humans (NCI, 2016).

In conclusion, the results of the present study provide further evidence and quantitative data that, as previously demonstrated in ulcer patients, Cr(VI) reducing ability is remarkably increased after meals in healthy individuals. Such a pH-dependent mechanism is quite efficient in humans and is expected to provide a formidable barrier limiting the toxicity, genotoxicity, and carcinogenicity of Cr(VI) introduced by the oral route. The data generated in the present study may contribute to assess the potential hazards posed by Cr(VI) in drinking water at environmentally relevant levels and to develop pharmacokinetic models predicting the Cr(VI) fate in the human stomach.

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Transparency Document

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

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