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Short Communication

Chromium (VI) potentiates the DNA adducts (O^6-methylguanine) formation of *N*-nitrosodimethylamine in rat: Implication on carcinogenic risk

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HIGHLIGHTS

- Cr(VI) synergistically enhanced the O^6-MeG formation of NDMA in rat hepatic tissues.
- Cr(VI) did not alter the CYP 2E1 enzyme activity.
- Cr(VI) exposure decreased the GSH content in rats.

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ABSTRACT

Chromium (VI) [Cr(VI)] and nitrosamines such as *N*-nitrosodimethylamine (NDMA) exist commonly in the environment. To evaluate the potential influence of Cr(VI) co-exposure on the carcinogenic risk of NDMA, Female Wistar rats were treated with various concentrations of Cr(VI) and/or NDMA via drinking water for 15 days and the DNA adducts (O^6-methylguanine, O^6-MeG) of NDMA in liver tissue was used as a bioindicator. The results showed that Cr(VI) synergistically enhanced the O^6-MeG formation, which could lead to an increase in DNA damage and carcinogenic potential. Although Cr(VI) did not alter the CYP 2E1 enzyme activity, it decreased GSH content, which would be an potential mechanism for the potentiated O^6-MeG formation by Cr(VI) co-exposure. These results would contribute to the development of quantitative risk assessment of NDMA or even for a group of nitrosamines under environmental mixture exposure.

1. Introduction

Nitrosamines and hexavalent chromium [Cr(VI)] are toxicologically important compounds that are widely distributed in the environment. Nitrosamines are present in large quantities in cigarette smoke (Ashley et al., 2010), cured meats and smoked fish (Lijinsky, 1999), and can be endogenously formed from interaction of nitrate or nitrite with secondary or tertiary amines and amides in human stomach (Eichholzer and Gutzwiller, 1998). In recent years, nitrosamines ingested via drinking water has been found to be a new route of human exposure (Mitch and Sedlak, 2002; Ma et al., 2012). Cr(VI) is widespread in cigarette smoke and automobile emissions, and also commonly used in chemical industries (IARC, 1990). There are also concerns about Cr(VI) exposure to the general population through consumption of Cr(VI)-contaminated drinking water (Pellerin and Booker, 2000; Sharma et al., 2012). Recent studies reported that high concentrations of nitrosamines and Cr(VI) were both detected in groundwater of a district with high incidence of digestive system cancer (Ma et al., 2012; Zhang et al., 2011). Thus, human would be simultaneously exposed to the two contaminants.

Cr(VI), at physiological pH, exists predominantly as the chromate anion, and can be rapidly taken up into cells through sulfate and phosphate ion channels. Once entering into the cells, Cr(VI) can be rapidly reduced to Cr(III) by sulfhydryl groups, and glutathione (GSH) can protect against Cr(VI)-mediated toxicity (Valko et al., 2006). As one of the most important environmental carcinogen of nitrosamines, *N*-nitrosodimethylamine (NDMA) is metabolized primarily in the liver by CYP 2E1 to a methylating reactive intermediate (probably the methyldiazonium ion) which methylates cellular macromolecules (Yoo et al., 1990). GSH is also important for cellular protection against electrophiles, serving as the primary source of conjugation of NDMA-derived methylating metabolites (Limón-Pacheco and Gonsebatt, 2009). Both NDMA

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metabolites and Cr(VI) bind to GSH, suggesting that depletion of GSH by Cr(VI) might serve to decrease NDMA metabolite conjugation, and therefore increasing the potential for reaction of NDMA metabolite with DNA; however, no paper has investigated the molecular interactions of Cr(VI) and nitrosamines co-exposure.

O^6-methylguanine (O^6-MeG) is one of major methylation adducts of NDMA metabolite interacting with DNA, plays a major role in mutagenesis and carcinogenesis of NDMA (Souliotis et al., 1998). In this study, the influence of Cr(VI) on the O^6-MeG formation of NDMA in rat hepatic tissues was tested. Subsequently, CYP 2E1 enzyme activities and GSH levels were also evaluated to better understand the enhancement of Cr(VI) exposure on the O^6-MeG formation of NDMA. The results in this study are expected to contribute to the risk assessment for chemical mixtures of Cr(VI) and NDMA.

2. Materials and methods

2.1. Chemicals

Potassium dichromate, NDMA, O^6-MeG, and GSH were from Sigma (St Louis, MO, USA), and [CD_3] O^6-methylguanine (O^6-MeG-D_3) was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). HPLC-grade acetonitrile and methanol were purchased from Fisher Chemical Co. (USA). HPLC-grade formic acid was provided by Dima Technology (USA). HCl (37%), NaOH, and *p*-nitrophenol were obtained from Beijing Chemical Co. (China). Stock solutions for all standard substances were stored at −20 °C.

2.2. Treatment of animals

Female Wistar rats, aged 6–7 weeks and weighing 170–190 g, were obtained from the Experimental Animal Tech. Co. of Weitonglihua (Beijing, China). The rats were housed in stainless steel wire-mesh cages in a temperature-controlled room on a 12-h light/dark cycle in the Laboratory Animal Center of Peking University. Rats were fed a diet prepared by the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Peking University.

The animals were divided randomly into 9 groups (n = 6/group, three per cage) and exposed to Cr(VI) and NDMA at various concentrations (Table 1). Cr(VI) and NDMA were introduced to rats via drinking water. Drinking water solutions were prepared daily. Water bottles containing Cr(VI) and NDMA were shielded from light with foil. Body weight, growth rate, fur condition, food consumption, and water consumption of all animals were monitored daily. Animals were sacrificed by cervical dislocation on the 15th day of the experiment, and their livers were sampled, weighed, and immediately frozen in liquid N_2.

Table 1
Experimental design.

Group	NDMA (mg/L)	Cr(VI) (mg/L)
1	0	0
2	0	5
3	0	20
4	0.5	0
5	0.5	5
6	0.5	20
7	1.5	0
8	1.5	5
9	1.5	20

2.3. Determination of O^6-MeG in liver tissue

DNA was isolated from liver tissue according to the method reported previously (Peterson and Hecht, 1991). The analysis of O^6-MeG followed the method as previously described (Upadhyaya et al., 2009). Briefly, each DNA sample spiked with O^6-MeG-D_3 was dissolved in 1 ml of HCl (0.1 N) and heated at 80 °C for 30 min, cooled, and neutralized with 1 N NaOH to pH 7.0. A portion of the hydrolysate (50 μL) was reserved for the determination of guanine concentration, and the rest was extracted using Strata-X cartridge (Phenomenex, Torrance, CA) which was preconditioned with 6 mL of methanol and 6 mL of ultrapure water. The cartridge was eluted with 6 mL of methanol, and the extract was evaporated to dryness under a gentle stream of nitrogen and dissolved in 0.5 mL NH_4OAc (2%).

Chromatographic analysis was carried out on an ACQUITY ultraperformance liquid chromatography (UPLC) BEH C_8 column (2.1 × 100 mm, 1.7 μm, Waters) using a Waters ACQUITY UPLC system. The column was maintained at 40 °C at a flow rate of 0.2 mL/min and isocratically eluted with 88% NH_4OAc (25 mM) and a 12% mixture of methanol and acetonitrile (75:25). The injection volume was set to 5 μL. Mass spectrometry was performed using a Waters Premier XE detector, which was operated with electrospray ionization (ESI) in the positive ion mode. The MS parameters were as follows: capillary voltage, 3.6 kV; source temperature, 110 °C; desolvation temperature, 350 °C; source gas flow, 50 L/h; and desolvation gas flow, 800 L/h. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode with the ion transitions m/z 166.0 > 149.1 with a collision energy of 19 eV for O^6-MeG and m/z 169.0 > 152.1 (40 eV) for O^6-MeG-D_3. The amount of guanine in each sample was determined by high-performance liquid chromatography according to a method reported previously (Reh et al., 2000), and adduct levels were expressed as μmol/mol guanine (μmol/mol G).

2.4. CYP 2E1 enzyme activity assay

The livers were excised, weighed and homogenized in pH 7.4 sodium phosphate buffer with 10% glycerol, and then centrifuged at 20000 g for 20 min. The supernatant was stored at −80 °C. CYP 2E1 enzyme activity was determined by measuring the hydroxylation of *p*-nitrophenol according to the procedure described previously (Koop, 1986).

2.5. Determination of GSH levels

The hepatic GSH content was determined by the method reported previously (Norris et al., 2001) and was expressed as mg/g protein. The livers were excised, weighed and homogenized in 1 mL of methanol which was kept on ice. The homogenate was then centrifuged at 800 g for 5 min. A 100 μL sample of the supernatant was evaporated to dryness under a gentle stream of nitrogen gas at room temperature and dissolved in 1.5 mL of ultrapure water.

Chromatographic analysis of GSH was carried out on an ACQUITY ultraperformance liquid chromatography (UPLC) BEH C_{18} column (2.1 × 100 mm, 1.7 μm, Waters) at a flow rate of 0.3 mL min^{-1} at 40 °C, using a Waters ACQUITY UPLC system. Mobile phase A was methanol, and mobile phase B was 0.1% formic acid in ultrapure water. The gradient (with respect to mobile phase A) was as follows: 0–3 min, 2–10% A; 3–6 min, 10% A; 6–8 min, re-equilibration with 2% A. The injection volume was set to 5 μL. Mass spectrometry was performed using a Waters Premier XE detector, which was operated with electrospray ionization (ESI) in the positive ion mode. Quantitative analysis was performed in the MRM mode with the ion transitions m/z 308.1 > 162.0 with a

collision energy of 40 eV. The optimized parameters were as follows: capillary voltage, 3.40 kV; cone voltage, 25.00 V; extractor, 5.00 V; RF lens, 0.5 V; source temperature, 110 °C; desolvation temperature, 350 °C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; multiplier voltage, 650 V.

2.6. Statistical analysis

Statistical analyses were conducted using SPSS 17.0 (SPSS, Chicago, IL, U.S.A.). Shapiro–Wilk test was used to test the normality of the data, and all data follow normal distribution. Levene test was used to test equal variances of the data, but equality of variances were not satisfied ($p < 0.05$). Thus, differences among the exposure groups were analyzed by one-way ANOVA followed by the Tamhane's T2 (M) test. Differences with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. General health

Administration of Cr(VI) and/or NDMA in drinking water for 15 days had no effects on the survival of the rats and any clinical signs of treatment-related toxicity in this study. Food consumption, daily water consumption, body weight and growth rates of all animals were monitored throughout the experiment and showed no statistical differences between the Cr(VI) and/or NDMA-treated animals compared to controls. These results were consistent with observations in rats exposed to ⩽20 mg/L Cr(VI) or ⩽2.64 mg/L NDMA as reported previously (Souliotis et al., 1995; Stout et al., 2009). The liver weight of each group also showed no statistical differences. These findings indicate that all the animals under our experimental conditions were in good health.

3.2. Effect of Cr(VI) on DNA adducts (O^6-MeG) formation

Souliotis et al. (1995) has reported that O^6-MeG reached steady-state levels after 15 day of NDMA exposure. Fig. 1 shows the effect of Cr(VI) on O^6-MeG formation in rat after 15-day exposure to NDMA. No variation in O^6-MeG formation was observed in animals treated with Cr(VI) alone compared control animals. When the animals were treated with NDMA alone, O^6-MeG formation was dependent on the concentration of NDMA, showing dose-dependent increase. O^6-MeG yields in the livers of animals exposed to 0.5 mg/L and 1.5 mg/L NDMA were 0.15 μmol/mol G and 0.40 μmol/mol G, 10-fold and 26-fold, higher than that (0.015 μmol/mol G) of control, respectively. The results was consistent with that reported previously (Souliotis et al., 1995). It should be noted that Cr(VI) co-exposure significantly increased the net formation of O^6-MeG in a concentration-dependent manner in the each NDMA exposure group ($p < 0.05$). The formation of O^6-MeG in the livers of animals co-exposed to 0.5 mg/L NDMA and 20 mg/L Cr(VI) was up to 0.45 μmol/mol G, even higher than that (0.40 μmol/mol G) treated with 1.5 mg/L NDMA alone ($p < 0.05$), showing that Cr(VI) synergistically enhanced the NDMA-induced hepatic DNA adducts, which may further promote an increase in DNA damage and carcinogenic potential.

3.3. Possible mechanism

CYP 2E1 enzyme is primarily responsible for the metabolic activation of NDMA. In a recent study, when tilapia and zebrafish were orally treated with Cr(VI) for 30 consecutive days (4.37 mg/kg bw for tilapia or 1.74 mg/kg bw for zebrafish), a significant increase in hepatic mRNA level of CYP 2E1 were found in both fish species compared to the control (Khalil et al., 2011). However, to the best of our knowledge, there was no report that Cr(VI) exposure could induce the changes of CYP 2E1 enzyme activity in rodents. To investigate the enhancement of O^6-MeG formation, CYP 2E1 enzyme activities of each exposure group were compared. In 5 mg/L or 20 mg/L Cr(VI) alone treated group, no statistical differences of the CYP 2E1 enzyme activity was observed compared with control group (Fig. 2). The CYP 2E1 enzyme activity of 0.5 mg/L NDMA with or without Cr(VI) co-exposed group also showed no difference from that of the control group. The CYP 2E1 enzyme activities of 1.5 mg/L NDMA exposed group was lower than that of the control group, consistent with a previous study that *N*-nitrosodiethylamine treatment significantly reduced the CYP 2E1 enzyme activities in mice liver (Zhang et al., 2013). However, Cr(VI) had no effect on the CYP 2E1 enzyme activity of 1.5 mg/L NDMA co-exposed group. These results suggest that the

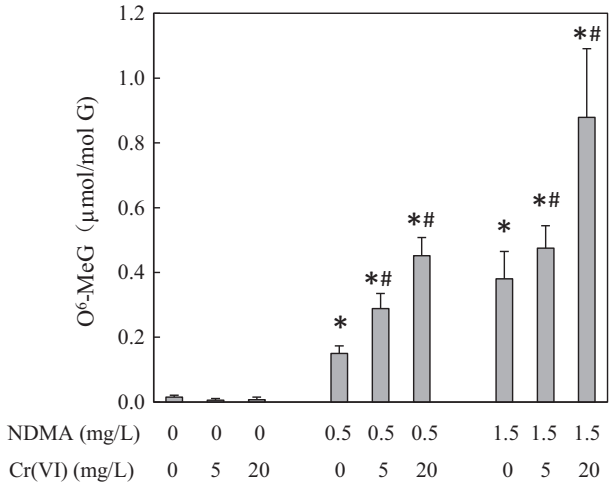

Fig. 1. Effect of Cr(VI) on hepatic levels of O^6-MeG formation. Female Wistar rats were treated with different concentrations of NDMA (0, 0.5, and 1.5 mg/L) and Cr(VI) (0, 5, and 20 mg/L) for 15 days via drinking water. Results are presented as mean ± standard deviation. Mean was the average of 6 replicates. $^*p < 0.05$ compared with controls. $^\#p < 0.05$ compared with NDMA alone treated rats.

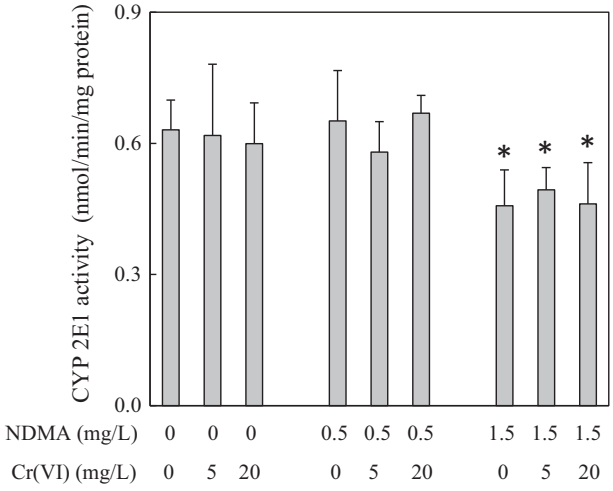

Fig. 2. Effect of Cr(VI) on CYP 2E1 activities in Female Wistar rats. Animals were treated with different concentrations of NDMA (0, 0.5, and 1.5 mg/L) and Cr(VI) (0, 5, and 20 mg/L) for 15 days via drinking water. Results are presented as mean ± standard deviation. Mean was the average of 6 replicates. $^*p < 0.05$ compared with controls.

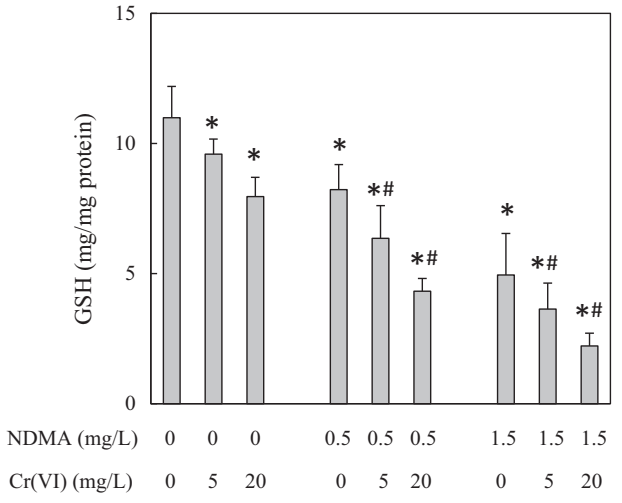

Fig. 3. Effect of Cr(VI) on hepatic GSH contents in Female Wistar rats. Animals were treated with different concentrations of NDMA (0, 0.5, and 1.5 mg/L) and Cr(VI) (0, 5, and 20 mg/L) for 15 days via drinking water. Results are presented as mean ± standard deviation. Mean was the average of 6 replicates. $^{*}p < 0.05$ compared with controls. $^{\#}p < 0.05$ compared with NDMA alone treated rats.

enhancement of O^6-MeG adduct formation in rat co-exposed to Cr(VI) and NDMA did not contributed by the induction of CYP 2E1 enzyme activity.

Although we did not observe an effect of Cr(VI) on phase I metabolism of NDMA, Cr(VI) may affect the fate of DNA reactive metabolites of NDMA by altering phase II conjugation reactions. Considering that GSH is a primary reductant that reduces Cr(VI) to Cr(III) in cells, and also the primary source of conjugation of NDMA-derived methylating metabolites (Limón-Pacheco and Gonsebatt, 2009), GSH contents of each exposure group were also determined in this study. When the animals were treated with NDMA alone or Cr(VI) alone, GSH contents both significantly decreased compared to control (Fig. 3). It should be noted that for each NDMA concentration tested, Cr(VI) significantly decreased the GSH contents in a concentration-dependent manner ($p < 0.05$). The concentration of GSH in the livers of animals co-exposed to 0.5 mg/L NDMA and 20 mg/L Cr(VI) (4.32 mg/mg protein) was even less than that treated with 1.5 mg/L NDMA alone (4.95 mg/mg protein), The concentration of GSH in the livers of animals co-exposed to 1.5 mg/L NDMA and 20 mg/L Cr(VI) was 2.22 mg/mg protein, less than half of that (4.95 mg/mg protein) treated with 1.5 mg/L NDMA alone, showing additive effects of Cr(VI) and NDMA on the decrease of GSH content in the liver, which in turn increased the formation of O^6-MeG. It should be noted that Cr(VI) can decrease the affinity of a chemotherapeutic drug mitoxantrone to DNA (Nowicka et al., 2013). However, we could not conclude that similar mechanism may also exist between Cr(VI) and NDMA, considering the synergistic effect of Cr(VI) on DNA methylation with NDMA observed in this study.

In summary, this study demonstrates that Cr(VI) can greatly enhance NDMA-induced O^6-MeG formation in hepatic tissues which would induce higher carcinogenic potential. These results would contribute to the development of quantitative risk assessment of NDMA under environmental co-exposure with another environmentally important chemical, Cr(VI).

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