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Genotoxicity of Hexavalent Chromium Combined with Chloroethylnitrosourea at Low Levels by Ames Assay

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Chromium contaminated drug capsules drew much of world's attentions. Although trace amount of chromium allowed to existence in the drug capsules, but the combined effects of chromium with other drug chemicals remain unclear. Therefore, the combined genotoxicity of hexavalent chromium [Cr (VI)] with nitroso drug chloroethylnitrosourea (ACNU) was examined using the *Salmonella* mutagenicity assay. The results showed that Cr (VI) and ACNU produced a dose-dependent increase in the revertants in TA 98 and TA 100 strains. In the individual chemical mutation assay, the positive mutagenicity response for Cr (VI) doses was above 2 mg per plate, and for ACNU above 5 mg per plate. In binary chemical mutation assay, the straightforward positive results could even be detected when the dose of Cr (VI) and ACNU both down to 1 mg per plate. It was indicated that neither Cr (VI) nor ACNU individually caused more genotoxicity to bacterial DNA at low levels. However, the two, working together, had significant revertant mutation effects. This means the combined genotoxicity effects of Cr (VI) and ACNU are more harmful than the individual exposure.

Key words: Hexavalent chromium, Nitrosourea, Genotoxicity, Combined effect.

Exposure to chemical contaminants remains a major source of human health risk throughout the world. Nowadays, we tend to give more attention to overdose chemical exposures in industry, agriculture, medicine, food and household products. However, one of the most important contaminants for humans is the low-level and complex exposure[1]. Chromium is in widespread industrial use and is a very persistent anthropogenic pollutant[2]. The hexavalent chromium [Cr (VI)] compounds were among the earliest chemicals to be classified as carcinogens[3]. Such Cr (VI) induced occupational cancers are of considerable concern because of the epidemiological evidence that has established links between the wide use of Cr in the industry and respiratory cancers[4,5]. In April 2012, a notice issued by the China Central Television revealed that several commonly used drug capsules were made from industrial gelatin, which remained some degree of chromium residues[6]. Although the standard chemical residue limit for chromium has been strictly established, for example, the drug capsules are not allowed to exceed 2 mg per kilogram of capsules according to the latest Chinese Pharmacopoeia[7]. However, there is no reference to the combined effects of chromium with other chemical drug elements. It has been reported that a considerable fraction of drugs are N-nitroso compounds (NOCs) or those that can be theoretically nitrosatable due to the presence of amine, amide or other groups which by reacting

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with nitrite in the gastric environment to give rise to the formation of NOCs[8]. The large majority of NOCs can produce genotoxic effects and tumor development in the laboratory animals[9]. Therefore, the above paradigm is significant in light of the hypothesis that the combined risks might be increased in the process of medication, especially for some long-term drug-taking patients. Up to now, little research had been conducted to study the combined mutation effects of chromium with chemical drug elements.

The *Salmonella* mutagenicity test (Ames assay) was specifically designed to detect chemically induced mutagenesis[10]. Over the years, the test is widely used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs. There is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained[11]. Furthermore, the Ames test is rapid, inexpensive and relatively easy to perform. These characters indicate its potentials in performing combined mutation tests with two or more chemical factors. In this research, the Ames assay was applied to study the combine mutagenic effects of Cr (VI) with chloroethylnitrosourea urea (ACNU), one kind of nitroso drugs used to treat with malignant gliomas in active clinical use. The results showed that Cr (VI) and ACNU could produce genetic damage that induced both frameshift mutation and base-pair substitutions. At low levels, Cr (VI) and ACNU individually caused less mutation to bacterial DNA. However, the two, working together, had significant revertant mutational effects.

MATERIALS AND METHODS

Materials

Cr (VI) (K_2CrO_4) standard solution was purchased from National Institute of Metrology (Beijing, P. R. China). 4- Nitroquinoline-1-oxide (4-NQO) was purchased from Alfa Aesar (Ward Hill, MA, USA). ACNU, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, L-histidine, D-biotin and 2-aminofluorene were purchased from Sigma (St. Louis, MO, USA). Other chemicals used in this study were of reagent grade.

Bacterial strains

Two tester strains of *Salmonella typhimurium* histidine-dependent (*his*$^-$) auxotrophs TA98 and TA 100 were obtained from Molecular Toxicology, Inc. (Moltox™, USA), whose strains were collected from the library of Dr. B. N. Ames, University of California, Berkeley, USA. The genetic characteristics of TA 98 and TA 100 were routinely identified before the Ames assay. The strains are known to be defective in DNA excision repair, carry the *rfa* mutation and the plasmid pKM101. In all cases, frozen bacterial cultures with 10% of DMSO (spectral grade) stored at -80°C were employed.

Preliminary toxicity assay

A preliminary toxic dose range experiment was performed to determinate the appropriate dose range for the mutagenicity assay by turbidimetric method. The toxicities of chemicals on the bacterial growth were performed by inoculating TA100 tester strains in nutrient broth medium with different concentrations of Cr (VI) or ACNU. The frozen stock cultures of TA 100 were applied, and thawed at room temperature before the beginning of each experiment. In each culturing flask, culture solution gave rise to a total volume of 20 mL, and the initial cell density was the same 10^7 CFU/ mL (1:100 dilutions). The treated concentration of Cr (VI) with bacteria were 1.67, 3.33, 8.33, 16.67 and 33.33 mg/L; and ACNU concentrations were 1.67, 8.33, 16.67, 41.67, 83.33 and 250.00 mg/L, respectively. The conical flasks were wrapped in aluminum foil and gently shaken (100 rpm) for 12 hrs at 37°C. Microbial growth reflected by the optical density (OD) values was monitored with an ultraviolet spectrophotometer (Hitachi Ltd., Japan) at the wavelength of 600 nm. The bacterial growth curves had been drawn according to the OD values at regular culturing intervals. TA100 tester strains incubated with nutrient broth alone served as the blank control.

Mutagenicity assay

The mutation assay was conducted with *Salmonella typhimurium* TA98 and TA100 strains using preincubated plate incorporation procedure described by Maron and Ames with minor modification[10,12]. Firstly, the tester trains (0.1 mL, about 10^8 bacteria cells) were exposed directly to tested chemical (0.1 mL) in a small volume (0.5 mL) of phosphate buffer solution (pH 7.4). Secondly, 2 mL of molten top agar (maintaining at 46°C to 48 °C) containing biotin and a trace amount of histidine was transferred into the above reaction

solutions after shaking (100 rpm) for 45 minutes at 37 °C. The mixture was gently mixed and poured onto the minimal glucose agar plate (GM plate). Then the petri dish was quickly swirled to ensure an even distribution of the top agar on the surface of GM plate. When the top agar had solidified, the plates were incubated in an inverted position at 37 °C for 48 hours at which time the histidine revertant colonies were counted. The test chemical was regarded as positive mutagen when it produced a reproducible, dose-related increase in the numbers of revertant colonies in one or two strains, and with a mutagenic index (MI) of 2.0 or higher. Equation (1) describes the calculation method of MI.

MI= number of *his*$^+$ induced in the sample/number of spontaneous *his*$^+$ in the solvent control (eq.1)

Furthermore, at the time the plates were scored, it was important to examinant their background lawn by using a 40× dissecting microscope, which further confirmed the positive results, or indicated the toxicity and the presence of precipitates. The results were reported as the mean number of revertant colonies per plate, and the data presented as mean revertants ± s.d. of three replicates for the test chemical and the control. The 4-NQO (0.5 µg per plate) and sterilized H_2O (500 µL per plate) were set as a positive control and solvent control respectively. All experiments were performed twice under similar conditions. The samples were analyzed without the hepatic S_9 fraction because it had been reported that Cr (VI) and ACNU were the mutagens without metabolic activation[13, 14].

RESULTS

Applying the preliminary toxicity assay to select the top dose of test chemicals for the Ames test

Growth curves of *S. typhimurium* TA 100 at 37 °C in the presence of different concentrations of Cr (VI) and ACNU are shown in Fig. 1. Most of them showed, more or less, lag phases and log phases in 12 hours of culturing times. During the lag phases and the log phases, Cr (VI) and ACNU inhibited their growth to different extents and the inhibitory effects increased with the treated concentrations. When the concentration of Cr (VI) and ACNU reached above 16.67 mg/ L and 83.33 mg/ L respectively, the lag phases and log phases were significantly different from those of control or low concentrations. From the prolonged lag phases and retarded log phases, it was indicated that the inoculated seed bacteria were inhibited or even 'killed' more or less by the high concentration of test chemicals. When the concentration of Cr (VI) reached 33.33 mg/ L, and ACNU reached 250.00 mg/ L, the bacterial growth was completely inhibited by the toxicity of Cr (VI) and ACNU (data not shown). In most cases, the toxicity and mutagenicity of tested mutagens coexists competitively. The toxicities of chemicals are inevitable and sometimes have significant influence on the mutagenic results in the Ames assay. Therefore, to select an appropriate dose range for the mutagenicity assay is a most important task. In this study, it was easy to observe that the top dose for Cr (VI) and ACNU should not exceed 33.33 mg/ L and 250.00 mg/ L, which means no more than

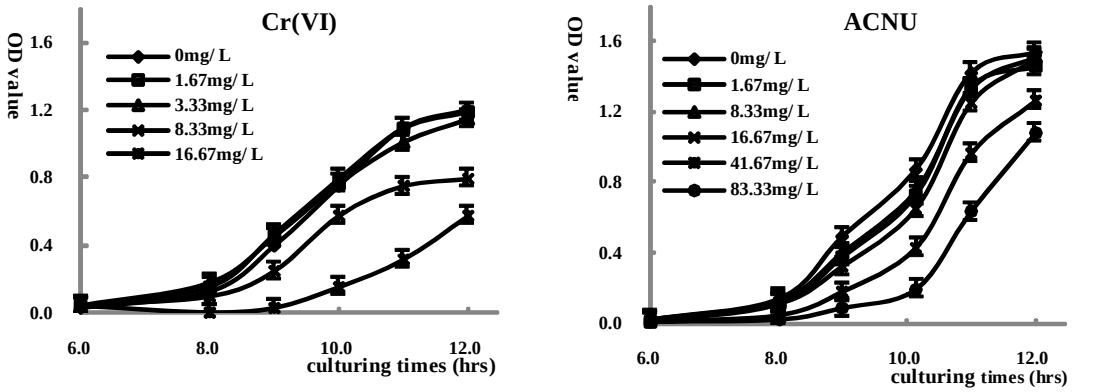

Fig. 1. Growth curves of TA100 affected by various concentrations of Cr (VI) or ACNU at 37°C

20 µg per plate and 150 µg per plate in plate incorporation assay, respectively. The dosage starting to show antibacterial effects in the toxic assay needs to be combined with Ames assay to score the final top toxic dose.

Mutagenecities of Cr (VI) and ACNU in the individual test manner

Two histidine-dependent *Salmonella* TA98 and TA100 were employed as tester strains to exam the mutation effects in separate and combined treated manner. In the individual chemical mutation test, Cr (VI) and ACNU caused a dose-dependent increase in the number of *his*$^+$ revertants in both TA98 and TA100 strains without metabolic action. Compared with the mutagenic index (MI) values showed in table 1, the minimal mutagenic dose of Cr (VI) and ACNU for TA98 was 5 mg per plate and 50 mg per plate; at these dosages an increase of 2.95-fold and 2.35-fold over the solvent control value was observed. While for TA 100, the minimal mutation dose of Cr (VI) and ACNU was down to 2mg per plate and 10 mg per plate. Both Cr (VI) and ACNU induced more revertants in TA100 than in TA98. It was seemed that TA 100 tester strains were more sensitive than TA 98 to induce DNA mutation by Cr (VI) and ACNU. Since TA98 and TA100 were specifically designed to carry different mutations in various genes in the histidine operation, these mutations acted as hot spots to detect chemical mutagens that caused frameshift mutation in TA98 and base-pair substitution in TA100. Therefore, it could conclude that Cr (VI) and ACNU produced genetic damage to lead to both frameshift mutations and base-pair substitutions. When the dose of Cr (VI) increased up to 10 mg per plate, and ACNU to 150 mg per plate, numbers of revertant colonies declined, and numerous pinpoint colonies in conjunction with thinning background lawn were appeared. This phenomenon indicated the chemical toxicities. Because high dose of chemical killed or inhibited most of bacterial growth, the survival part of bacteria in the support of a small amount of histidine added in the top agar allowed these survived bacteria to undergo several cell divisions before the histidine was depleted. In this case, pinpoint colonies would be formed from the limited growth of surviving bacteria and not extend any more with culture times prolonged.

Table 1. Separate induction of *his*$^+$ revertants TA 98 and TA 100 by Cr (VI) and ACNU

Dose	TA 98		TA 100	
	Mean revertants ± s.d.	MI	Mean revertants ± s.d.	MI
Blank Control	25±3	1.25	92±9	1.05
H_2O(500 µl/plate)	20±4	1.00	88±8	1.00
4-NQO(0.5µg/plate)	448±64	22.40	1150±224	13.07
Cr(VI) 1 µg/plate	30±7	1.50	137±11	1.56
Cr(VI) 2 µg/plate	37±8	1.85	205±6	2.33
Cr(VI) 5 µg/plate	59±5	2.95	311±11	3.53
Cr(VI) 10 µg/plate	*[a]	—	187±10	2.13
ACNU 1 µg/plate	14±3	0.70	103±13	1.17
ACNU 5 µg/plate	20±5	1.00	172±13	1.95
ACNU 10 µg/plate	31±2	1.55	302±15	3.43
ACNU 25 µg/plate	36±5	1.80	691±91	7.85
ACNU 50 µg/plate	47±3	2.35	1066±66	12.11
ACNU 150 µg/plate	29±5	1.45	611±58	6.93

a.Asterisk means numerous “pinpoint colonies” in the treated plates.

Mutagenecity of Cr (VI) in combination with ACNU

Due to the trace chromium allowed to residue in the capsules, the combined mutagenicity of Cr (VI) with ACNU was studied mainly at the ‘low’dose range. Apparently, the mutagenesis was increased when test strains undergo binary chemical exposures (Table 2). It was worth noticing that, at 1µg per plate, neither Cr (VI) nor ACNU caused more revertant colonies than solvent

control, but their interactive effects caused significant mutagenesis in TA 100, and the MI value was more than twice higher than the solvent's. Analogues existed in Cr(VI) at 2 µg with ACNU at 5µg or 10 µg per plate tested by TA98. The results indicated that Cr (VI) and ACNU at low levels individually caused less mutation to bacterial DNA, but working together, had significant revertant mutation effects. That means the cooperative mutation effects of heavy metal Cr (VI) with nitroso compound ACNU are more genotoxic than the individual exposure.

Table 2. Separate induction of *his*$^+$ revertants TA 98 and TA 100 by Cr (VI) and ACNU[b]

Dose	TA 98		TA 100	
(¼g/ plate)	Mean revertants ± s.d.	MI	Mean revertants ± s.d.	MI
Cr(VI) 1 + ACNU 1	30±5	1.50	179±12	2.03
Cr(VI) 2 + ACNU 5	45±2	2.25	336±34	3.82
Cr(VI) 2 + ACNU 10	52±7	2.60	491±25	5.58
Cr(VI) 5 + ACNU 25	94±9	4.70	865±112	9.83

b. Blank control, solvent control and positive control were the same as the separate reverse mutation assays in table 1.

DISCUSSION

In the Ames test, the top dose to be used is mainly dependent on its cytotoxicity and solubility[15,16]. If not restricted by the insolubility, the cytotoxicity is evaluated mainly according to the results of Ames test itself, which determined by evaluation of characteristics of the final population on the GM agar plate after the 48-h incubation. These characteristics include a reduction in the number of revertants, and / or clearing or diminution of the background lawn[15,16]. In such cases, if had no idea with the properties of unknown chemicals, it would need several times of pilot experiments to determine the appropriate dose range. In 1982, Waleh *et al.,* proposed a toxicity assay to couple with the Ames test for evaluating the toxicities and mutagenicities of mutagens simultaneously[17]. However the sophisticated experimental procedures restricted its applications in the worldwide. Up to now, it is important to develop an easy-performing toxicity assay to score the highest amount of test substance to be used in the Ames assay. In this research, the turbidimetric method was applied for analysis of the preliminary toxicity. Although the quantitative survival determination could not be accurately calculated by this method, but the top dose range could be easily selected according to the different features showed on the lag and log phases. Furthermore, it gives an important hint for the doses starting to show antibacterial effects in the Ames assay. Combining examinations of the background lawns under microscopy further determined the real cytotoxicity. For instance, when the concentration of Cr (VI) reached 8.33 mg/ L(amount to 5 µg per plate in the Ames test) and ACNU reached 41.67 mg/ L (25 µg per plate), 'weak' toxicities were observed during the late log phases culturing with tested chemicals. However the background lawns showed no significant differences compared to the blank or solvent control in the Ames test. While Cr (VI) at 16.67 mg/ L (10 µg per plate), and ACNU at 250 mg/ L (150 µg per plate), the cytotoxicities were confirmed by the Ames assay according to the reduction in the number of revertants and diminution of the background lawn. In this case, the decrease in the number of *his*$^+$ colonies at higher doses of the chemicals may mask the mutagenic properties. Therefore, the top toxic dose for Cr (VI) was finally adjusted to 10 µg per plate in the Ames assay. Consequently application of the preliminary toxic assay to be coupled to the Ames assay could easily and quickly provide the mutagenicity and cytotoxicity valuations.

Exposure to harmful substances that most people encounter tends not to be in the form of a single agent. Considering the presence of mutagens alone in complex mixtures presents a challenge to the genetic toxicologist in terms of determining the genotoxicity of mixtures.

Mutagenic assays such as the *S. typhimurium* test have been widely used to assess the mutagenic of various compounds[18]. In this study, the mutagenicity of binary chemical mixtures was investigated by the Ames test. Most of the results indicated that mutagenesis was increased when test strains underwent complex exposures. More attention should be given to the genotoxicity of mixtures at low concentrations, which are common in the daily life. At low levels, neither Cr (VI) (at 1µg per plate) nor ACNU (at 1µg per plate) induced significant mutagenic revertants, but their interactive effect was positive through mutation index in TA 100. Analogues existed in doses of Cr (VI) at 2 µg per plate with ACNU at 5 or 10µg per plate to TA 98. It means the synergistic effects of heavy metal Cr (VI) and nitroso compound ACNU are significantly more genotoxic than the individual exposure.

According to the latest Chinese Pharmacopoeia[7], the empty capsules allow to contain no more than 2 mg/kg chromium which amount to about 1 to 2µg per plate of Cr (VI) in the Ames assay. If excessive chromium exists in capsules, patients with long-term prescriptions will be up against an increased genotoxic risk. ACNU is an important chloroethylnitrosourea used to treat with various neoplastic diseases and a recent meta-analysis has suggested a significant survival gain using ACNU for newly diagnosed high-grade gliomas[19]. Unfortunately, secondary tumor formation is a severe threat in chemotherapeutic regimens with nitrosourea drugs which have neurotoxicity and produce alkylation in tumor cells as well as in normal human cells[20]. Although guidelines for genotoxicity testing of pharmaceuticals indicate the safety of drugs, the interactions between drugs and chemicals possible coexisting are unidentified. As the synergistic effects exist, the potential hazards of the chemicals should be re-evaluated, taking their synergistic properties into consideration. Therefore, it needs to promote further research on the chemical mixtures, especially for chemicals that directly affect health safety.

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REFERENCES

1. Howard V. Synergistic effects of chemical mixtures—can we rely on traditional toxicology? *Ecologist*, 1997; **27**(2): 192–5.
2. Langård S.One hundred years of chromium and cancer: A review of epidemiological evidence and selected case reports. *Am. J. Ind. Med.*, 1990; **17**(2): 189–214.
3. Costa M. Toxicity and carcinogenicity of Cr (VI) in animal models and humans. *Crit. Rev. Toxicol.*, 1997; **27**(5): 431-42.
4. International Agency for Research on Cancer (IARC): Chromium and chromium compounds. In: IARC Monographs on the Evaluation of Carcinogenic Risks in Humans: Chromium, Nickel, and Welding (IARC ed). Lyon: 1990; pp 49.
5. Kerger B.D., Butler W.J., Paustenbach D.J., et al. Cancer mortality in Chinese populations surrounding an alloy plant with chromium smelting operations. *J. Toxicol. Environ. Health A.*, 2009; **72**(5): 329-344.
6. Xinhua Net: China issues alert on 'chromium-contaminated' capsules. 2012, http://news.xinhuanet.com/english/china/2012-04/16/c_131528301.htm
7. The Pharmacopoeia Commission of P. R. China (ed): Pharmacopoeia of the People's Republic of China: Part 1,1st ed. Beijing: Chemical Industry Press, 2010; pp 1204-6.
8. Brambilla G., Martelli A. Genotoxic and carcinogenic risk to humans of drug–nitrite interaction products. *Mutat. Res.*, 2007; **635**(1): 17–52.
9. Bogovski P., Bogovski S. Animal species in which N-nitroso compounds induce cancer. *Int. J. Cancer*,1981; **27**(4): 471–4.
10. Ames B.N., McCann J., Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella* / mammalian microsome mutagenicity test.*Mutat. Res.*,1975; **31**(6):347–64.
11. Zeiger E., Haseman J.K., Shelby M.D., et al.Evaluation of four *in vitro* genetic toxicity tests for predicting rodent carcinogenicity: confirmation of earlier results with 41 additional chemicals.*Environ. Mol. Mutagen.*,1990; **16**(Suppl. 18): 1–14.
12. Maron D., Ames B.N. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*,1983;

113(3-4):173–215.

13. Codina J.C., Pérez-Torrente C., Pérez-García A., et al. Comparison of microbial tests for the detection of heavy metal genotoxicity. *Arch. Environ. Contam. Toxicol.*, 1995; **29**(2): 260-5.
14. Franza B.R., Oeschger N.S., Oeschger M.P., et al. Mutagenic activity of nitrosourea antitumor agents. *J. Natl. Cancer. Inst.*, 1980; **65** (1): 149-54.
15. OECD: No. 471: bacterial reverse mutation test. In: OECD guidelines for the testing of chemicals Section 4: Health Effects Test. OECD publishing, 1997; pp1-11.
16. ICH Steering Committee: Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use. In:ICH Harmonised Tripartite Guideline S2R1 (ICH Steering Committee, ed). 2011; pp 1-29.
17. Waleh N.S., Rapport S.J., Mortelmans K.E. Development of a toxicity test to be coupled to the Ames *Salmonella* assay and the method of construction of the required strains. *Mutat. Res.*, 1982; **97**(4): 247–56.
18. Asita A.O., Matsui M., Nohmi T., et al. Mutagenicity of wood smoke condensates in the *Salmonella*/microsome assay. *Mutat. Res.*, 1991; **264**(1): 7-14.
19. Wolff J.E.A., Berrak S., Webb S.E.K.,et al. Nitrosourea efficacy in high-grade glioma: a survival gain analysis summarizing 504 cohorts with 24193 patients. *J. Neuro-oncol.*, 2008; **88**(1): 57-63.
20. Yamashima T., Yamashita J.,Shoin K. Neurotoxicity of local administration of two nitrosoureas in malignant gliomas. *Neurosurgery*, 1990; **26**(5): 794-9.