Genotoxicity of Tri- and Hexavalent Chromium Compounds In Vivo and Their Modes of Action on DNA Damage In Vitro

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Abstract
Chromium occurs mostly in tri- and hexavalent states in the environment. Hexavalent chromium [Cr(VI)] compounds are extensively used in diverse industries, and trivalent chromium [Cr(III)] salts are used as micronutrients and dietary supplements. In the present work, we report that they both induce genetic mutations in yeast cells. They both also cause DNA damage in both yeast and Jurkat cells and the effect of Cr(III) is greater than that of Cr(VI). We further show that Cr(III) and Cr(VII) cause DNA damage through different mechanisms. Cr(VI) intercalates DNA and Cr(III) interferes base pair stacking. Based on our results, we conclude that Cr(III) can directly cause genotoxicity in vivo.

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Introduction
Chromium (Cr) is a heavy metal and a well-known environmental contaminant mainly exists in hexavalent [Cr(VI)] and trivalent [Cr(III)] states [1]. High levels of exposure to Cr(VI) occurs in chromate manufacturing, chrome plating, ferrochrome production and stainless steel welding. Occupational exposure to Cr(VI) compounds is a well-documented cause of respiratory cancers [2], [3], and Cr(VI) is a proven toxin, mutagen and carcinogen [4–9]. Many types of structural genetic lesions produced by Cr(VI) have been observed in vivo and in vitro, including interstrand cross-links, DNA protein cross-links, strand breaks and Cr-DNA adducts [10–14]. Association with the phosphodiester backbone of DNA (chromium-DNA adduct) is one of the most abundant genetic lesions induced by chromium in mammalian cells and is thought to be a primary cause of Cr(VI) mutagenicity [7]. However, the molecular mechanism of chromium carcinogenicity is still unclear.

In contrast, Cr(III) was initially considered a relatively nontoxic agent that plays an important role in regulating blood glucose levels and is regarded as a dietary supplement [15–18]. Studies in cell-free systems demonstrated that Cr(III) does bind to DNA, leading to a decrease in the fidelity and an increase in the processivity of DNA polymerases, which may ultimately lead to increased mutations [19]. However, a mutagenic effect of Cr(III) in vivo remains to be investigated. It is also unclear whether Cr(III) and Cr(VI) act on DNA through the same or different mechanisms.

In this study, we compared the effects of Cr(VI) [i.e., CrO3] and Cr(III) [i.e., CrCl3] on DNA damage both in vivo and in vitro. We found that they both increase mutational rates and cause DNA degradation. However, we found that CrCl3 is surprisingly more genotoxic than CrO3 in both yeast and animal cells. We also found that these two compounds interact with DNA differently. CrO3 binds to DNA in an intercalative manner and irreversibly destroys the configuration of DNA. In contrast, CrCl3 interferes with the stacking mode of the base pairs. Taken together, our results suggest that both trivalent and hexavalent chromium compounds are genotoxic and that they cause DNA damage through different modes of action.

Materials and Methods

Chemical Reagents
Chromium trioxide (CrO3) and chromium chloride (CrCl3) were purchased from Sigma. Stock solutions were freshly prepared before each experiment in sterile water. In all experiments, an equal volume of water was used as the vehicle control.

Mutational Rate Analysis in Yeast
The S. cerevisiae strain used in this work was SJR576 (MATa ade2-1oc can1-100oc leu2-K lys2-1oc ura3-1Nco), ade2-1oc, can1-100oc and lys2-1oc are ochre alleles of the respective genes suppressible by SUP4-o, an ochre suppressor allele of the yeast tyrosine tRNA gene. This SUP4-o allele is carried on pRS179, a centromeric vector that mimics chromosome behavior in yeast.
cells [20]. This plasmid was transformed into SJR576 to generate strain SJR576-p.

The yeast growth media was a synthetic complete medium lacking uracil (SC-Ura). The media for selecting sup4-o mutants was a minimal medium consisting of yeast nitrogen base without amino acids (1.5 g/L), ammonium sulfate (3 g/L), glucose (20 g/L), leucine (0.262 g/L), lysine (0.182 g/L), adenine (6.65 mg/L) and canavanine (60 mg/L). YEPD medium containing yeast extract (10 g/L), bacto peptone (20 g/L), dextrose (20 g/L) and adenine (250 mg/L) was used to determine the total number of viable cells being assayed in each experiment.

SJR576-p yeast cells were inoculated in liquid SC-Ura and grown at 30°C with shaking for an overnight. The starter culture was used to inoculate 3 mL fresh liquid SC-Ura with an initial cell density of 2×10⁶ cells/mL. The cells were then incubated in the presence or absence of 300 μM CrO₃ or 150 μM CrCl₃ for 24 hours, diluted, and plated on a minimal medium to select for sup4-o mutants. The plates were incubated at 30°C for three days and placed at 4°C for about 20 days. Red colonies that emerged on canavanine-containing medium were scored as sup4-o mutants. The mutation frequency for each independent culture was determined by calculating the percentage of red and canavanine-resistant colonies of all colonies grown on YPD medium.

Cell Culture

The human T cell leukemia Jurkat cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). They were cultured in RPMI 1640 supplemented with 10% FBS (Sigma), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in humidified air supplemented with 5% CO₂.

Single-Cell Gel Electrophoresis (SCGE) Assay

The S. cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ1 met15Δ0 ura3Δ0) was treated with 300 μM CrO₃ or 150 μM CrCl₃ for 24 hours. The Jurkat cell line was treated with 150 μM CrO₃ or 150 μM CrCl₃ for 24 hours. Slides were prepared in duplicate per sample. Fully frosted microscopic slides were covered with 0.8% normal melting agarose (NMA). After the application of a coverslip, the slides were allowed to gel at 4°C for 10 minutes. In the meanwhile, cell suspensions (2×10⁶ cells/mL) were added to 0.65% of low melting agarose (LMA). After carefully removing the coverslips, a second layer of the sample mixture was added onto the pre-coated slides and allowed to solidify at 4°C for 10 minutes. The cover slips were removed, and a third layer of LMA was added onto the slides and allowed to gel at 4°C for 10 minutes.

A slide was immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, pH 10, 1% sodium N-lauroyl sarcosinate, 1% Triton X-100 and 10% DMSO [added just before use]) and refrigerated for 1 hour. The slide was then placed in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 30 minutes to allow unwinding of DNA. Electrophoresis was conducted for 30 minutes at 18 V. The slide was then drained, placed on a tray and washed slowly three times with 1xPBS buffer every 5 minutes. DNA was precipitated and the slide was dehydrated in absolute methanol for 10 minutes and before being left at room temperature to dry. Finally, the slide was stained with ethidium bromide and visualized under fluorescent microscope (Olympus BX51, Tokyo, Japan).

Degradation of Plasmid DNA Induced by Chromium

Supercoiled YEplac195 plasmid DNA was purified from DH5α cells using an EndoFree Plasmid Kit (QIAGEN, USA). Purified plasmid was treated with different concentrations of CrO₃ or CrCl₃ at 37°C for 2 hours. All samples were resolved with agarose (1%) electrophoresis to monitor DNA cleavage/degradation induced by the chromium compounds. Agarose gel images were analyzed with the Tanon-3500 gel imaging system (Tanon Science & Technology Co., Ltd. Shanghai, China).

Supercoiled plasmid DNA was also digested with the linearized by endonuclease HinIII. Linearized DNA was incubated in the absence or presence of various concentrations of CrO₃ or CrCl₃ as described above. The cleavage products were extracted with a DNA extraction kit (TIANGEN Midi Purification Kit, China) and subjected to ligation reactions using T4 DNA ligase.

To evaluate the effects of buffer and temperature on Cr-induced DNA degradation, CrO₃ and CrCl₃ were dissolved in Tris-HCl buffer (0.1M) or PBS buffer (0.5M) of different pH. Purified plasmid DNA was incubated with CrO₃ and CrCl₃ at 37°C for 2 hours at indicated pH or indicated temperature (i.e., 0, 20, 30, and 37°C). To evaluate the effects of DTT on Cr-induced DNA degradation, purified plasmid DNA was treated with DTT (10 mM) or chromium solution (30 μM) containing different concentrations DTT (i.e., 0.1, 0.5, 1.0, 5.0 and 10 mM) at 37°C for 2 hours.

Circular Dichroism Measurement

Circular dichroism (CD) spectroscopy was performed using a Chirascan CD spectrometer equipped with a temperature-controlled water bath (Applied Photophysics, Leatherhead, UK). A sample was loaded into a 10 mm quartz cuvette. A spectrum was recorded from 200 to 320 nm with a 1 nm bandwidth at 22°C. Each spectrum was averaged from five successive accumulations at a scan rate of 50 nm/minute.

Fluorescence Competition Binding Assay

A DNA sample (6 μg/mL) was first incubated with or without CrO₃ or CrCl₃ (150 μM) and then with an equal volume of ethidium bromide (12 μg/mL). The fluorescence intensity was measured at an excitation wavelength at 520 nm and an emission wavelength at 610 nm using a Genios multifunction-reader (Tecan GENios Pro, Tecan Group Ltd. Maennedorf, Switzerland).

Melting Temperature-Based SYBR Green I Assay

Total RNA from SH-SY5Y cells was extracted using RNAiso Plus (Takara). The PCR forward and reverse primers for amplifying GADPH were 5′-AGAAGGCTGGGGCT-CATTGG-3′ and 5′-AGGGCCATCAGCTCTTCTC-3′, respectively. Real-time RT-PCR was performed using the primeScript RT reagent kit with gDNA Erase and SYBR Premix Ex Taq kit (Takara) in a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA). The products were treated with 300 μM CrO₃ or 150 μM CrCl₃. Melting of DNA was performed from 60 to 95°C at 0.1°C/s with a smooth curve setting. The melting peaks were visualized by plotting the first derivative against the melting temperature. A melting temperature (Tm) was defined as the peak of the curve.

Results and Discussion

Both Cr(VI) and Cr(III) Induce Mutations in Yeast

To evaluate the potential effects of Cr(III) and Cr(VI) in causing mutations, we assayed the well-defined SUP4-o allele. This allele encode a mutant tRNA that suppresses ochre stop codons by inserting a tyrosine. Two ochre alleles, ade2-1oc and can1-100oc, were used to monitor the loss of SUP4-o function. The ade2-1oc mutation causes adenine auxotrophy as manifested as red colony color. The can1-100oc mutation causes resistance to canavanine.
The presence of a functional \( SUP4-o \) allele renders cells containing the \( ade2-10c \) and \( can1-100oc \) form white colonies that are sensitive to canavanine. Mutations that inactivate \( SUP4-o \) can be identified by the simultaneous loss of suppression of both \( ade2-1 \) and \( can1-100 \) alleles, resulting in red and canavanine resistant colonies. Using this system, we tested whether \( Cr(VI) \) and \( Cr(III) \) might increase mutational frequency in yeast. In untreated cells, the frequency of loss of \( SUP4-o \) function was about \( 6.32 \times 10^{-6} \). In \( CrO_3 \) (at 300 \( \mu M \)) and \( CrCl_3 \) (at 150 \( \mu M \)) treated cells, the frequency was increased to \( 31.6 \times 10^{-6} \) and \( 33.86 \times 10^{-6} \), respectively (Fig. 1). Therefore, \( Cr(VI) \) and \( Cr(III) \) significantly induce the loss of \( SUP4-o \) function in yeast cells (\( P < 0.001 \)) (Fig. 1).

Both \( Cr(VI) \) and \( Cr(III) \) Induce DNA Damage in Yeast and Jurkat Cells

To explore the possibility that the loss of \( SUP4-o \) function induced by \( Cr \) was at least partly due to DNA damage, we employed a SCGE (or Comet) assay in both yeast and Jurkat cells. In this assay, increased DNA damage is manifested as enlarged comet tails as seen in the positive controls (i.e., hydrogen peroxide in yeast cells and UV irradiation in Jurkat cells) (Fig. 2). We observed increased DNA damage in samples treated with either \( CrO_3 \) or \( CrCl_3 \) when compared to the untreated samples (Fig. 2). Interestingly, the extent of DNA damage caused by \( CrCl_3 \) (150 \( \mu M \)) was greater than that caused by \( CrO_3 \) (300 \( \mu M \)) (Fig. 2). This was a surprise to us given that the genotoxic effects of \( Cr(III) \) was not previously appreciated as much as that of \( Cr(VI) \).

Most previous studies on chromium have primarily focused on \( Cr(VI) \) compounds because they readily penetrate the cellular membrane and can be reductively metabolized to \( Cr(III) \) [3], [21], [22]. Our previous studies on \( Cr(VI) \) and \( Cr(III) \) cytotoxicity and their effects on oxidative state of yeast cells showed that they both can enter into the yeast cells and induce cytotoxicity and oxidative stress [23]. Our comparative results on the genotoxicity of \( CrO_3 \) and \( CrCl_3 \) presented so far provided further evidence that \( CrCl_3 \) can also be taken up by living cells and inflict DNA damage. Other previous reports showed that the rate of absorption of \( Cr(III) \) may be affected by several factors, including the chemical and physical properties of the compound and the primary exposure route [24–

**Figure 1.** Both \( Cr(VI) \) and \( Cr(III) \) induce mutations in yeast. Cells of the yeast strain SJR576 carrying the \( SUP4-o \) plasmid were treated with or without \( CrO_3 \) or \( CrCl_3 \) for 24 hours and plated on indicator plates to select for \( sup4-o \) mutants. Mutational rates were calculated and plotted. The results are the means of three independent experiments. The error bars represent the means ± SD.

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Therefore, CrCl₃ can function as a genotoxic compound in at least some type of cells, including the yeast and mammalian Jurkat cells used in this study. These together lend further support of a previous finding that Cr(III) compounds, including CrCl₃, can be toxic as nutrient supplements [27].

Both Cr(VI) and Cr(III) Induce DNA Damage in vitro

We next investigated whether both CrCl₃ and CrO₃ can cause DNA damage in vitro using cleavage of supercoiled plasmid DNA as the assay [28]. We found that treatment with increasing concentrations of both Cr(VI) and Cr(III) leads to the disappearing of supercoiled DNA (Band I) (Fig. 3A and 3B). This was accompanied by the appearing of nicked circular or linear DNA. Eventually, all DNA molecules in a sample could be cleaved (Fig. 3A and 3B). These results suggested that both Cr(VI) and Cr(III) can induce DNA cleavage in vitro. To test whether Cr compounds might only interact with supercoiled DNA, we next evaluated their effects on linearized plasmid DNA. We found that linear plasmid DNA is quickly degraded upon incubation with CrCl₃ but the effect of CrO₃ was not as obvious (Fig. 3A and 3B). We also investigated whether the degraded DNA can be re-ligated back to circular forms to produce transforming units in bacterial cells. Such reactions were compromised when DNA molecules are pre-treated with both chromium compounds (Fig. 3C, 3D, 3E and 3F). These results together suggest that both CrCl₃ and CrO₃ can cleave both supercoiled DNA and linearized DNA molecules, and induce their degradation. Notably, the ability of CrCl₃ to induce DNA cleavage and degradation in vitro is greater than that of CrO₃. This observation is consistent with the in vivo results described in the previous section.

Effects of Buffer and Temperature on Degradation of DNA by Cr(VI) and Cr(III)

The normal pH values of the CrO₃ and CrCl₃ solutions are 4.18 and 4.12, respectively. We next investigated whether the effects of Cr(VI) and Cr(III) on DNA degradation might be related to the low pH of these solutions. We found that Tris buffers at pH 5, pH 7, and pH 10.58 all prevented DNA degradation by both Cr(VI) and Cr(III) (Fig. 4A and 4B, compared to Fig. 3A and 3B). From these results, it was hard to conclude whether the preventive effects are caused by Tris or by the higher pH. To investigate this further, we next tested the effects of PBS buffers at pH 4, pH 5, pH 7, and pH 10 on DNA degradation by Cr(VI) and Cr(III). Under the conditions used, PBS at all four pH values blocked or reduced DNA degradation by CrCl₃ (at 80 uM) (Fig. 4C). However, the protective effect offered by the solution at pH 10 was smaller than that by those solutions at lower pH values. These results suggest that Cr(III)-induced DNA degradation is not caused by the low pH of the solutions. We also investigated possible effects of different temperatures (i.e. 0, 20, 30 and 37°C) on Cr-induced DNA degradation. We found that a higher temperature (37°C) favors Cr(VI)-induced DNA degradation and a lower temperature (0°C) favors Cr(III)-induced DNA degradation (Fig. 4D). These results also suggest that the modes of action on DNA are likely different between Cr(VI) and Cr(III).

Effects of DTT on Cr-induced DNA Degradation

Previous studies have suggested that reduction of chromate by intracellular reductants results in the formation of reactive oxidative species, which cause various DNA lesions [29], [30]. We thus next investigated whether Cr-induced DNA damage...
might be related to stimulation of oxidation. To this end, we tested the effects of dithiothreitol (DTT), a very strong reducing agent, on Cr(VI)- and Cr(III)-induced DNA degradation. We found that, DTT promoted DNA cleavage and degradation caused by CrO$_3$ (Fig. 4E). This is consistent with the model that, when encountering reducing agents, CrO$_3$ stimulates the formation of reactive oxygen species and to cause DNA damage [29], [30]. However, we also cannot rule out the possibility that DTT promote DNA degradation by converting Cr(VI) to the more genotoxic Cr(III). In contrast, DTT slightly reduced CrCl$_3$-induced DNA degradation at the highest concentration tested (i.e., 10 mM) (Fig. 4F). These results further suggest that Cr(VI) and Cr(III) cause DNA damage likely through different mechanisms.

Both Cr(VI) and Cr(III) Cause Structural Changes in DNA Molecules

Both Cr(VI) and Cr(III) are previously shown to directly bind to DNA. We next investigated whether they might bind to DNA in different manners using circular dichroism (CD) spectroscopy.

Consistent with the model that both Cr(VI) and Cr(III) directly bind to DNA, we found that they both alter the CD spectrum of DNA molecules. We also observed significant difference between the treatment of DNA with CrO$_3$ and with CrCl$_3$. CrO$_3$ reduced the intensity of both positive and negative ellipticity bands (Fig. 5), suggesting alterations in both the stacking mode and the orientation of the base pairs in DNA molecules. This is characteristic of an intercalative interaction between a compound and DNA [31]. On the other hand, CrCl$_3$ reduced the intensity of the positive ellipticity band but had little effect on the negative ellipticity band (Fig. 5), suggesting interference with the base pair stacking only. Therefore, CrO$_3$ likely intercalates into the planes between the base pairs of the DNA and CrCl$_3$ likely only alters the DNA stacking mode.

To further corroborate these models, we measured binding of ethidium bromide (EB) to DNA molecules pre-incubated with or without Cr(VI) or Cr(III) using fluorescence spectroscopy. EB intercalates with DNA and gives rise to fluorescence emission [32]. Neither Cr(VI) nor Cr(III) caused obvious DNA degradation.
under the experimental conditions, yet we found that Cr(VI) only partially block EB-DNA binding, as reflected by reduced but not completely abolished fluorescence intensity (Fig. 6). This is consistent with the model that both EB and Cr(VI) intercalate DNA molecules and that they compete with each other. In contrast, Cr(III) completely blocked the binding of EB to DNA (Fig. 6), consistent with a model that Cr(III) and EB likely do not compete with each other for binding to DNA. Alternatively, these
results could suggest that Cr(III) is more effective in inflicting DNA structural distortion than Cr(VI) in vitro.

We also investigated possible effects of both CrO₃ and CrCl₃ on the melting temperature (Tₘ) of DNA molecules. We did not find an obvious effect of either compound on the Tₘ of DNA (Fig. 7). However, both compounds induced hypochromicity (Fig. 7), which indicates more closed DNA structures than in the control samples. Again, the effect of Cr(III) was slightly greater than that of Cr(VI) (Fig. 7), consistent with the observations that Cr(III) is more genotoxic than Cr(VI).

In conclusion, our in vivo results suggest that both Cr(VI) and Cr(III) significantly induce genetic mutation in yeast and cause DNA damage within both yeast and Jurkat cells and that both can act as genotoxic compounds. In addition, the ability of CrCl₃ to

Figure 5. Effects of Cr(VI) and Cr(III) on the CD spectra of plasmid DNA. Plasmid DNA (25 ng/µL) was measured in the presence or absence of 150 µM CrO₃ or CrCl₃.
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Figure 6. Effects of Cr(VI) and Cr(III) on binding of ethidium bromide (EB) to DNA molecules. DNA samples (6 µg/mL) were first incubated in the presence or absence of 150 µM CrO₃ or CrCl₃ and then with equal volume of EB (12 µg/mL). Binding of EB to DNA under each condition was measured as the relative fluorescence intensity unit (RFU).
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generate DNA damage is significantly greater than that of CrO3. The results provide unambiguous evidence for the significance of Cr(III) compounds in the generation of genotoxic damage. Our results also indicate that the mode of CrCl3 interaction with DNA is different from that of CrO3. Previous studies on chromium genotoxicity mainly focused on the formation of chromium-DNA adducts [33–35]. There are evidences that Cr(III) can interact electrostatically with the DNA phosphate backbone [36], [37]. Cr(III) was also suggested to form covalent bonds with phosphate on the backbone of DNA [38–41] and with the endocyclic nitrogen atoms of the DNA bases[42–46]. In this study, we demonstrated that Cr(III) likely also interferes with the stacking mode of DNA base pairs and causes DNA cleavage and degradation of DNA. Cr(VI) was previously suggested to interact with structural changes via two modes of interaction: inducing oxidative changes and DNA compaction [11]. Our results further suggested that Cr(VI) can intercalate into the planes between DNA base pairs and cause DNA structural changes and degradation.

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Author Contributions

Conceived and designed the experiments: PS ZH. Performed the experiments: ZF MZ HZ LC. Analyzed the data: ZF MZ HZ LC PS ZH. Contributed reagents/materials/analysis tools: PS ZH. Contributed to the writing of the manuscript: PS ZH. Obtained permission for use of plasmid PRS179 and yeast strain SJR576: SJ.

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