Hexavalent Chromium Induces Chromosome Instability in Human Urothelial Cells

Sandra S. Wise[1,2], **Amie L. Holmes**[1,3], **Louis Liou**[4], **Rosalyn M. Adam**[5], and **John Pierce Wise Sr.**[1,2,**]

[1]Wise Laboratory of Environmental and Genetic Toxicology, Maine Center for Toxicology and Environmental Health, Department of Applied Medical Science, University of Southern Maine, Science Building, 96 Falmouth Street, Portland, ME 04103, USA

[3]Department of Radiation Oncology, Dana Farber Cancer Institute, 450 Brookline Ave., Boston, MA 02215, USA

[4]Department of Pathology, Boston University School of Medicine, 670 Albany St., Boston, MA 02118, USA

[5]Department of Surgery, Harvard Medical School, Boston, MA 02115

Abstract

Numerous metals are well-known human bladder carcinogens. Despite the significant occupational and public health concern of metals and bladder cancer, the carcinogenic mechanisms remain largely unknown. Chromium, in particular, is a metal of concern as incidences of bladder cancer have been found elevated in chromate workers, and there is an increasing concern for patients with metal hip implants. However, the impact of Cr(VI) on bladder cells has not been studied. We compared chromate toxicity in two bladder cell lines; primary human urothelial cells and hTERT-immortalized human urothelial cells. Hexavalent chromium (Cr(VI)) induced a concentration- and time-dependent increase in chromosome damage in both cell lines, with the hTERT-immortalized cells exhibiting more chromosome damage than the primary cells. Chronic exposure to Cr(VI) also induced a concentration-dependent increase in aneuploid metaphases in both cell lines which was not observed after a 24 h exposure. Aneuploidy induction was higher in the hTERT-immortalized cells. When we correct for uptake, Cr(VI) induces a similar amount of chromosome damage and aneuploidy suggesting that the differences in Cr(VI) sensitivity between the two cells lines were due to differences in uptake. The increase in chromosome instability after chronic chromate

[**]Corresponding Author. Wise Laboratory of Environmental and Genetic Toxicology, Department of Pharmacology and Toxicology, University of Louisville, 505 S. Prescott St, Louisville, KY., 40292, USA. Tel.: +1 502 852 8524; Fax: +1 502 852 7868. ; Email: john.wise@louisville.edu (J.P. Wise. Sr.)
[2]Present Address: Wise Laboratory of Environmental and Genetic Toxicology, Department of Pharmacology and Toxicology, University of Louisville, 505 S. Prescott St, Louisville, KY., 40292, USA

treatment suggests this may be a mechanism for chromate-induced bladder cancer specifically and may be a mechanism for metal-induced bladder cancer in general.

Keywords

Chromium; chromate; urothelial; bladder cancer; aneuploidy; genotoxicity

Introduction

Hexavalent chromium [Cr(VI)] is a well-established human carcinogen with most of the focus and attention has been on Cr(VI) acting as a lung carcinogen (IARC, 1990). This primary focus is based, in part, on epidemiology studies, which consistently show Cr(VI) to be a human lung carcinogen; and human pathology studies, which show lung tumors form where Cr(VI) particles impact and persist (Cohen, et al 1993; Ishikawa, et al, 1994). But, this focus is also based, in part, on the toxicology of Cr(VI) which indicates that when reduced extracellularly to Cr(III) there is a loss in toxicity, especially genotoxicity (Xie et al., 2004). In other words, Cr(VI) has been thought to be a site-of-exposure carcinogen because the expectation has been that Cr(III) cannot induce carcinogenesis as it is too poorly absorbed and any Cr(VI) that enters the body would simply be reduced after penetrating the site of exposure.

However, while epidemiology studies have focused attention on the lung, some also consistently show that metal workers with Cr(VI) exposure also have an elevated rate of bladder cancer (IARC 1990). For example, a large study of welders showed an elevation of lung and bladder cancer, that correlated with Cr and nickel (Ni) exposure (Milham, 1983). A study of chrome tannery workers also showed an elevated bladder cancer risk (Montanaro et al., 1997). In addition, two other studies found correlations with occupations involving chromate and bladder cancer (Claude, et al., 1986; Claude et al., 1988). To be clear, these studies do not definitively show Cr(VI) causes bladder cancer. There are power limitations and co-exposures to additional carcinogens that confound their full interpretation, however, at the same time, these confounders do not rule out the possibility that Cr(VI) can cause bladder cancer, and they do suggest such an outcome is possible.

This possibility has become more urgent with the increased use and emerging health concerns of metal-on-metal hip implants. There are several types of metal-on-metal implants with cobalt chrome, titanium-based alloy or stainless steel in the stem. The epidemiology on whether a specific implant confers a greater cancer risk than another is currently poorly developed. However, concern is focused on the implants made of cobalt/chromium (CoCr) alloy, because they are extensively used and the CoCr alloy wears over time and releases a known carcinogen [chromium (Cr)] and a suspected carcinogen [cobalt (Co)] along with CoCr nanoparticles (Keegan, et al., 2007, 2008; Case, et al., 1994). Moreover, the new generation of these CoCr implants are failing in very high numbers, perhaps as high as 50% in some cases (Langton, et al., 2010). Clinical data clearly show the bladder in these patients are directly exposed to high levels of Cr based on blood and urinary excretion levels (Keegan, et al., 2007, 2008; Lhotka, et al., 2003; Pilger, et al., 2002). The valence of Cr

released is unclear at this time, but some studies do indicate that Cr(VI) might be released (Keegan, G.M., et al., 2007, 2008; Case, et al., 1994).

Thus, this new public health concern combined with the previous possibility that Cr(VI) causes bladder cancer indicates a need to better understand the impact of Cr(VI) on the bladder. Moreover, our recent data showing a carcinogenic mechanism of Cr(VI)-induced lung cancer that involves chromosome instability (Xie et al., 2007, 2009; Holmes et al., 2006a, 2008, 2010; Wise and Wise, 2010, 2012) is consistent with the fact that chromosome instability is considered a key mechanistic and diagnostic event in bladder carcinogenesis (Florl and Schulz, 2008). Currently, there appears to be no data on the effects of Cr(VI) in bladder cells. Thus, the focus of this study was to investigate the ability of Cr(VI) to induce chromosomal instability in human urothelial cells.

Materials and Methods

Chemicals and Reagents

Sodium chromate, Triton X-100, demecolcine and potassium chloride (KCl) were purchased from Sigma Chemical (St. Louis, MO). Giemsa stain was purchased from Ricca Chemical Company. (Arlington, TX). Crystal violet, sodium dodecyl sulfate (SDS), and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Nitric acid and methanol were purchased from BDH Chemicals (Radnor, PA). Gurr's buffer, Keratinocyte-SFM, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Corporation (Grand Island, NY). Tissue culture dishes, flasks and plasticware were purchased from BD (Franklin Lakes, NJ). Urothelial cell medium, trypsin/EDTA and trypsin neutralizing solution were purchased from ScienCell Research Laboratories (Carlsbad, CA). Penicillin/streptomycin was purchased from Mediatech (Manassas, VA). Paraformaldehyde 4% was purchased from Alfa Aesar (Ward Hill, MA). Phospho-histone H2A.X (Ser139) antibody was purchased from Cell Signaling (Beverly, MA). AlexaFluor 488-conjugated goat anti-rabbit IgG secondary antibody was purchased from ThermoScientific (Rockford, IL).

Cells and cell culture

Normal human urothelial cells (HUC) were purchased from ScienCell. These cells were maintained in urothelial cell medium supplemented with 1% penicillin/streptomycin and urothelial cell growth supplement and incubated in a 5% CO_2 humidity environment at 37°C. hTERT expressing human urothelial cells, hTU1, were obtained as a generous gift from Dr. Louis Liou (described in Kim et al., 2011). hTU1 cells were further subcloned and a stable clone was selected (hTUC1-38). Cells were maintained in keratinocyte-SFM and incubated in a 5% CO_2 humidified environment at 37°C. Cells were maintained as adherent subconfluent monolayers, fed twice weekly and subcultured at least once weekly. Cells were routinely tested for mycoplasma contamination.

Chemical treatments

Sodium chromate (CAS #7775-11-3, ACS reagent minimum 98% purity), a soluble hexavalent Cr compound was administered as a solution in water as previously described (Wise J et al., 2002). A stock solution of sodium chromate was suspended in double distilled

sterile water the day of treatment. Dilutions were made from the stock solution and administered to the cells for final concentrations ranging from 1–5 uM chromate. Cells were exposed to Cr(VI) for 24 and 120 h.

Intracellular Chromium Ion Measurement

Intracellular chromium ion levels were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) using standard methods (Holmes, et al., 2005). Briefly, a monolayer of cells was treated for 24 or 120 h with varying concentrations of sodium chromate. The cells were harvested by trypsinization and treated with a hypotonic solution followed by 2% SDS. This solution was sheered through a needle seven times and filtered into a vial. Samples of intracellular fluids were diluted 5x in 0.16 M aqueous HNO_3 prior to analysis. Chromium concentrations in the intracellular samples were then measured using a Perkin Elmer Optima 2000, equipped with a gem cone low flow nebulizer. Solutions were introduced to the nebulizer using a peristaltic pump operating at 2 mL/min. Chromium was determined using emission wavelength at 267.716 with a minimum detection limit of 2 ppb. Yttrium (Y) was used as an internal standard for chromium determinations. The intracellular concentrations were corrected by converting from ug/L to uM by dividing by the volume of the sample, the atomic weight of the chemical, the number of cells in the sample and the average cell volume for the given cell line.

Immunofluoresence for Gamma-H2A.X Foci Formation

Immunofluorescent staining was performed as previously published (Wise S et al., 2010). Briefly, after a 24 or 120 h sodium chromate treatment, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were then incubated with an anti-gamma-H2A.X antibody, washed, and incubated with an AlexaFluor 488-conjugated goat anti-rabbit IgG secondary antibody. DNA was counterstained with DAPI. Slides were then coverslipped and analyzed using a laser scanning confocal microscope. One hundred cells were analyzed per concentration using Image J software.

Chromosome Instability Analysis

Structural and numerical chromosome instability was determined using a chromosomal aberration assay based on our published methods (Wise et al., 2002). Briefly, log phase cells were seeded into 100 mm dishes and treated for 24 and 120 h with sodium chromate. Demecolcine (0.1 g/ml) was added 1 h before the end of treatment to arrest the cells in metaphase. Cells were then collected by trypsinization, spun down and resuspended in a 0.075M KCl hypotonic solution for 17 min followed by fixation with 3:1 methanol:acetic acid. The fixative was changed twice then cells were dropped onto clean wet slides and stained with 5% Giemsa stain in Gurr's buffer. A minimum of one hundred metaphases per treatment were analyzed for aneuploidy and chromosome aberrations scored using standard criteria (Wise J. et al., 2004; Holmes et al., 2010). Each experiment was repeated three times.

Statistical Analysis

We used a one-way ANOVA and SNK method to compare each dose to the control and to compare each dose to each other. P-values of < 0.05 are considered statistically significant.

Results

Intracellular Cr Ion Concentrations Are Higher in hTERT-Immortalized Urothelial Cells

Hexavalent chromium induced a time- and concentration-dependent increase in intracellular Cr ion levels in both primary and hTERT-immortalized urothelial cells (Fig. 1). The hTERT-immortalized cells take up less chromium than primary cells. Following 24 h treatment, concentrations of 0, 1, 2.5 and 5 uM sodium chromate resulted in intracellular Cr ion concentrations of 20, 81, 206, and 712 uM, respectively, in HUCs. At the same time point, hTUC1-38 cells had intracellular Cr ion concentrations of 0, 130, 212, and 324 uM, respectively, with treatment concentrations of 0, 1, 1.5 and 2 uM sodium chromate. After 120 h treatment with concentrations of 0, 1, and 2.5 uM sodium chromate, HUCs had intracellular Cr ion concentrations of 0, 84, and 709 uM, respectively. After 120 treatment with concentrations of 0, 1, 1.5, and 2 uM sodium chromate, hTUC1-38 cells had intracellular Cr ion concentrations of 0, 315, 872, and 1454 uM, respectively.

Hexavalent Chromium Induces DNA Damage in Urothelial Cells

Hexavalent chromium induces a concentration- and time-dependent increase in gamma-H2A.X foci in both primary and hTERT-immortalized urothelial cells. As administered, sodium chromate induced more DNA damage in hTUC1-38 cells than in HUCs (Fig. 2A and 2B). At concentrations of 0, 1, 2.5, and 5 uM sodium chromate there were 7.3, 22.3, 25.7 and 48 percent of cells with greater than 5 foci, respectively, after 24 h exposure; and 8.6, 25.8, and 38 percent of cells with greater than 5 foci with treatments of 0, 1, and 2.5 uM sodium chromate, respectively, after 120 h exposure in HUCs (Fig. 2A). In hTUC1-38 cells, concentrations of 0, 1, 1.5, and 2 uM sodium chromate induced 7.7, 16, 22, and 29.3 percent of cells with greater than 5 foci, respectively, after 24 h of exposure, and concentrations of 0, 1, and 1.5 uM sodium chromate induced 6.8, 30, and 35 percent of cells with greater than 5 foci, respectively, after 120 h of exposure (Fig. 2B). When corrected for intracellular Cr ion levels, both primary and hTERT-immortalized cells had similar amounts of DNA damage (Fig. 2C).

Hexavalent Chromium is Clastogenic to Urothelial Cells

Hexavalent chromium induces a concentration- and time-dependent increase in chromosome damage in both primary and hTERT-immortalized urothelial cells. Based on administered concentration, sodium chromate induces more chromosome damage in hTERT-immortalized cells than in primary cells (Fig. 3A and 3B). HUCs had 2.5, 11.5, 20.5, and 35.3 percent of cells with damage when treated with 0, 1, 2.5 and 5 uM sodium chromate for 24 h and 3.8, 19.3, and 33.8 percent of cells with damage when treated with 0, 1, and 2.5 uM sodium chromate for 120 h. hTUC1-38 cells had 3.3, 19.3, 23, and 27 percent of cells with damage when treated with 0, 1, 1.5 and 2 uM sodium chromate for 24 h and 2, 28.5, and 62.3 percent of cells with damage when treated with 0, 1, and 1.5 uM sodium chromate for 120 h. At

concentrations higher than 1.5 uM for 120 h, no metaphases were observed due to cell cycle arrest. Based on intracellular Cr concentrations, sodium chromate induces similar levels of chromosome damage in primary and hTERT-immortalized human urothelial cells (Fig. 3C). Interestingly, there was no effect of time on the amount of damage seen after correcting for chemical uptake in the primary urothelial cells.

Hexavalent Chromium is Aneugenic to Urothelial Cells

Chronic exposure to Cr(VI) induces a concentration-dependent increase in aneuploidy in both primary and hTERT-immortalized urothelial cells. After 24 h treatment with sodium chromate, no increase in aneuploidy was observed for any concentration in either cell line (Fig. 4 A and B). However, after 120 h, we observed that 8, 19.7, and 31.2 percent of metaphases were aneuploid with treatments of 0, 1, and 2.5 uM sodium chromate in HUCs. In hTUC1-38 cells, we found 9.2, 19.8, and 40.3 percent of metaphases were aneuploid after 120 h treatment with 0, 1, and 1.5 uM sodium chromate for 120 h. Based on intracellular Cr concentrations, there were similar amounts of aneuploidy in both primary and h-TERT-immortalized urothelial cells (Fig. 4C).

Discussion

Cr(VI) is a potent and known human lung carcinogen and some epidemiologic data suggest that it may also have a role in bladder cancer (IARC, 1990). Recently there has been increased concern for metal-exposed individuals; specifically for patients receiving total hip replacement and other osteo-replacement therapies. While there is, as yet, limited evidence for carcinogenic outcomes for these patients, there is evidence of exposure to known and suspected carcinogens (Cr and Co) as indicated by increased metal levels in urine. Thus, there is increased concern for possible carcinogenic outcomes. This is the first study to investigate the impact of Cr(VI) exposure specifically in human urothelial cells.

We found that Cr(VI) induced genotoxicity in two human urothelial cell lines. We used primary urothelial cells and h-TERT immortalized urothelial cells to establish that these cells have the same response to Cr(VI). We found that the hTERT-immortalized human urothelial cells were more sensitive to Cr(VI). Sodium chromate induced more aneuploidy, chromosome damage and DNA double strand breaks in hTERT-immortalized human urothelial cells compared to primary cells based on the administered sodium chromate doses. However, when we corrected for differences in chromium uptake between the two cell lines, we found that Cr(VI) induced a similar amount of aneuploidy, chromosome damage and DNA double strand breaks indicating that the differences in Cr(VI) sensitivity between the two cells lines were due to differences in uptake. This serves as confirmation that the induction of chromosome instability is a universal mechanism as well as confirming that the hTERT-immortalized cell line had a similar response to metals and will serve as a good tool for studying metal-induced bladder carcinogenesis in future investigations.

Our study is the first study to show that Cr(VI) induces DNA damage in urothelial cells. Zinc chromate and lead chromate have been show to induce DNA double strand breaks in human lung and skin cells using gamma-H2A.X foci formation and single cell gel electrophoresis (Comet assay) (Ha et al., 2004, Xie et al., 2005, 2009). DNA double strand

breaks are one of the most dangerous forms of cellular damage. Unrepaired DNA double strand breaks are lethal to the cell and mis-repaired double strand breaks can lead to mutations or chromosome instability.

This study is the first to show that Cr(VI) induces chromosome damage in human urothelial cells. These data are not surprising and are consistent with historical literature showing Cr(VI) is a clastogen ([IARC 1990], De Flora et., 1990). Our study is also consistent with current studies using primary and immortalized lung and skin cell lines ([Wise, J. et al., 2002]; Wise, S. et al. 2004, 2006a, Holmes et al, 2006a, 2006b, Xie et al., 2015). Difficulties arise in trying to compare the urothelial cells to these studies as different cell types require different media which can affect Cr(VI) reduction and dissolution. For example, there was more chromosome damage seen in primary human lung fibroblasts treated with 1 and 2.5 uM sodium chromate, than in the primary urothelial cells; 18 and 33 percent of cells were damaged, respectively, in human lung cells compared to 12 and 20 percent of cell damaged, respectively, in human urothelial cells ([Wise, S. et al, 2002]). The reasons for these differences are unclear without further investigation but could be due to differences in the cell type (epithelial vs fibroblast), origin (lung vs bladder), or due to differences in culture media (with or without serum). One study used a lung epithelial model using serum free media ([Wise, S. et al., 2006a]) and showed cells treated with 1, 2.5 and 5 uM sodium chromate induced 25, 34, and 41 percent of metaphases with damage whereas our current study found 12, 20, and 35 percent of metaphases with damage at the same administered concentrations of sodium chromate in primary urothelial cells. Again, the reason for the difference are unclear without further investigation but could be due to differences in cell origin (lung vs bladder) or differences in the model (primary vs immortalized).

Our data are the first to show that Cr(VI) induces aneuploidy in human urothelial cells. These data are consistent with data in human lung cells showing that chronic exposure to Cr(VI) induces chromosome instability and aneuploidy ([Wise, S. et al., 2006b]; Holmes et al., 2006, 2010; Rodrigues et al., 2009). Our data are also consistent with a study using hTERT-immortalized skin cells ([Figgitt et al., 2010]) which showed increases in complex aneuploidy following chronic treatment with physiologically relevant doses of Cr(VI) that were equivalent to levels of Cr found in blood of patients with worn metal on metal orthopedic implants. Interestingly, this increased aneuploidy was exacerbated by co-exposure to cobalt, another metal of concern for joint replacement patients.

In sum, we found that Cr(VI) induced DNA double strand breaks and chromosome damage in both urothelial cell lines. We also found that Cr(VI) was aneugenic with prolonged exposures. Chromosome instability is of particular concern as this is considered an initiating event for carcinogenesis. More work will be needed to fully elucidate this potential mechanism for Cr(VI)-induced urothelial cancer, including the role of DNA repair as well as the possible role of centrosome amplification in the induction of aneuploidy and further chromosome instability. However, these data demonstrate that Cr(VI) has a strong likelihood of initiating carcinogenic events leading to bladder cancer.

Acknowledgments

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Highlights

- Hexavalent chromium is genotoxic to human urothelial cells

- Hexavalent chromium induces aneuploidy in human urothelial cells

- hTERT-immortalized human urothelial cells model the effects seen in primary urothelial cells

- Hexavalent chromium has a strong likelihood of being carcinogenic for bladder tissue
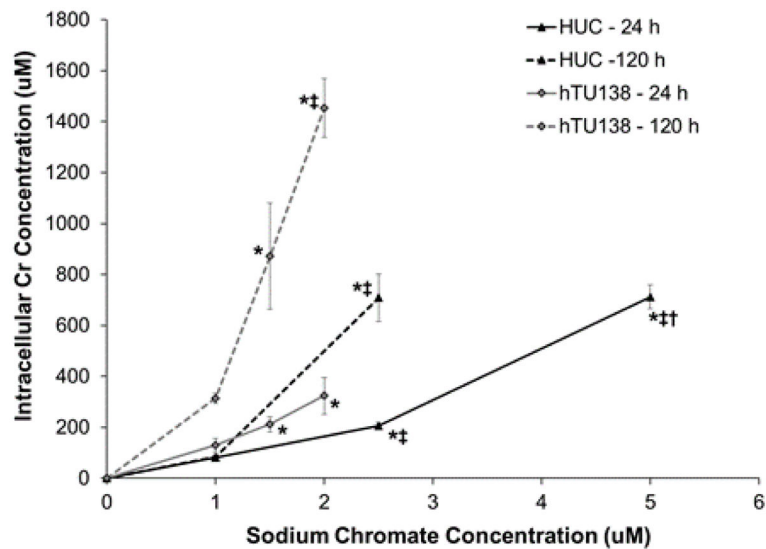
Figure 1.
hTERT-Immortalized Human Urothelial Cells Take Up More Chromium Than Primary Cells.

This figure shows sodium chromate induced a concentration- and time-dependent increase in intracellular Cr ion concentrations in human urothelial cells with hTERT-immortalized cells taking up more chromium than primary cells. Data represent an average of three experiments ± the standard error of the mean. Statistics: * indicates statistically different from control ($p < 0.05$); ‡ indicates statistically different from 1 uM sodium chromate ($p < 0.05$); † indicates statistically different from 2.5 uM sodium chromate ($p < 0.001$).
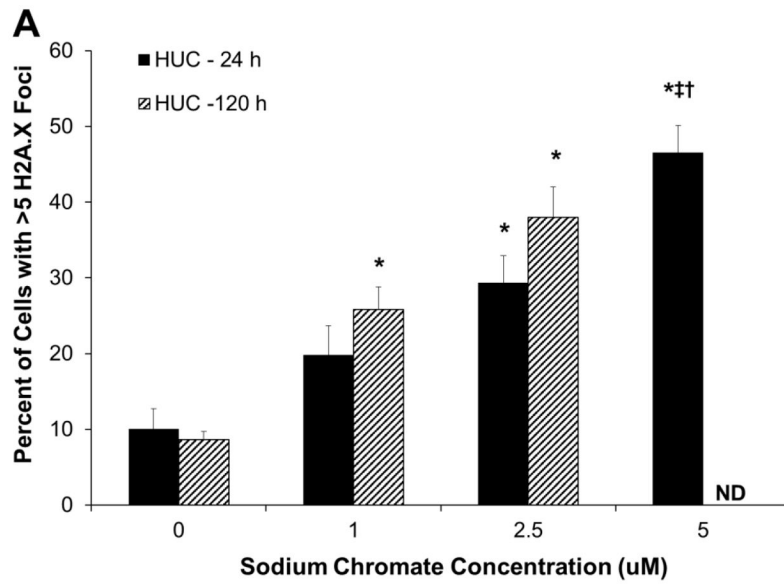
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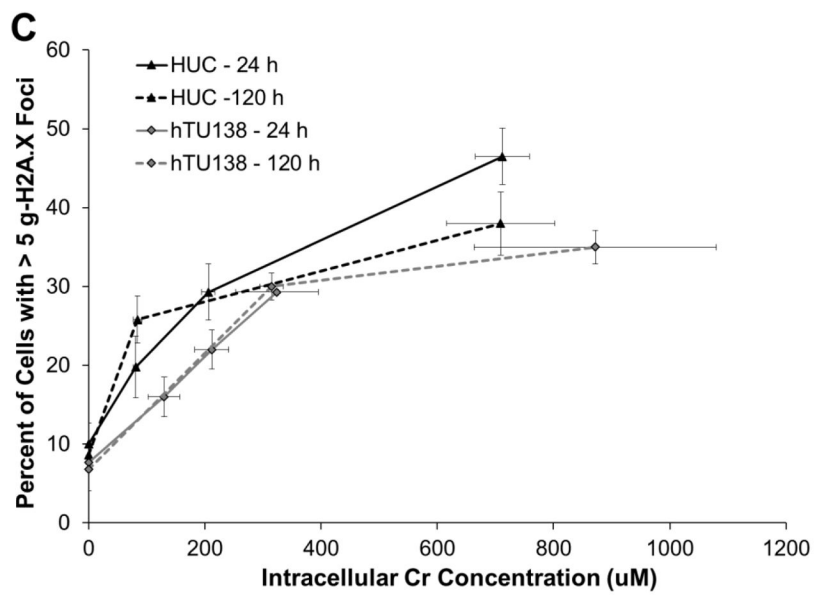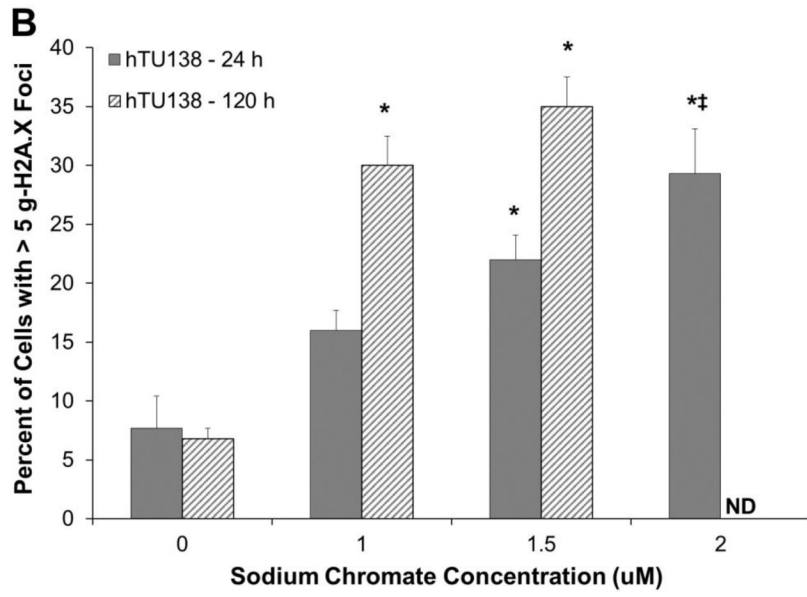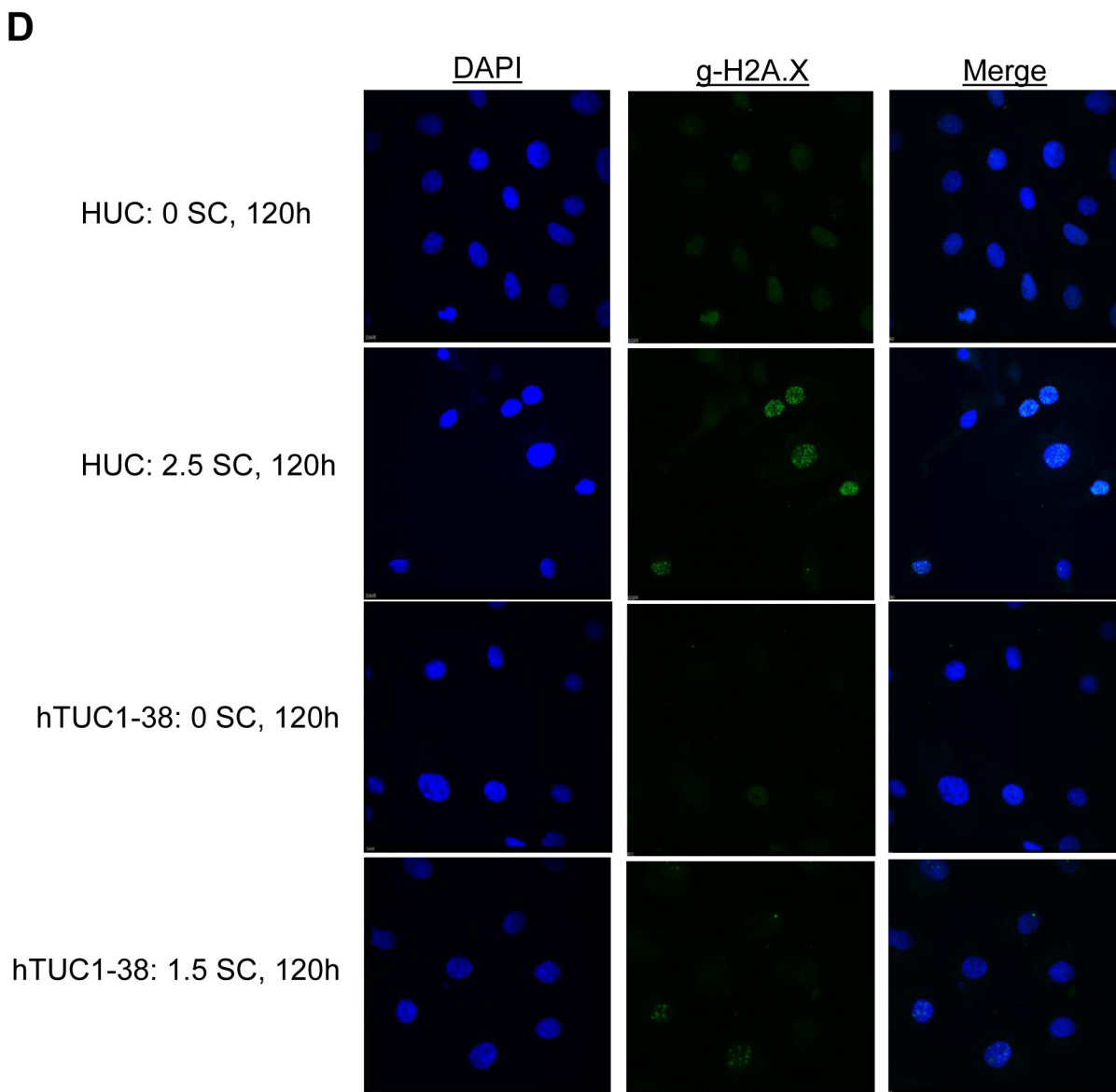
Figure 2.
Hexavalent Chromium Induces DNA Double Strand Breaks in Human Urothelial Cells.
This figure shows acute and chronic exposure to sodium chromate induces DNA double
strand breaks in human urothelial cells. A) Primary human urothelial cells. B) hTERT-
immortalized human urothelial cells. Based on administered concentration, sodium chromate
induces more DNA double strand breaks in hTERT-immortalized cells than in primary cells.
ND = Not done. C) When corrected for uptake differences, sodium chromate induces similar
levels of gamma-H2A.X foci in primary and hTERT-immortalized human urothelial cells.
D) Representative images of gamma-H2A.X foci in primary and hTERT-immortalized
human urothelial cells. Data represent an average of at least three experiments ± the standard
error of the mean. Statistics: * indicates statistically different from control (p < 0.05); ‡

indicates statistically different from 1 uM sodium chromate (p < 0.05); † indicates statistically different from 2.5 uM sodium chromate (p < 0.01).
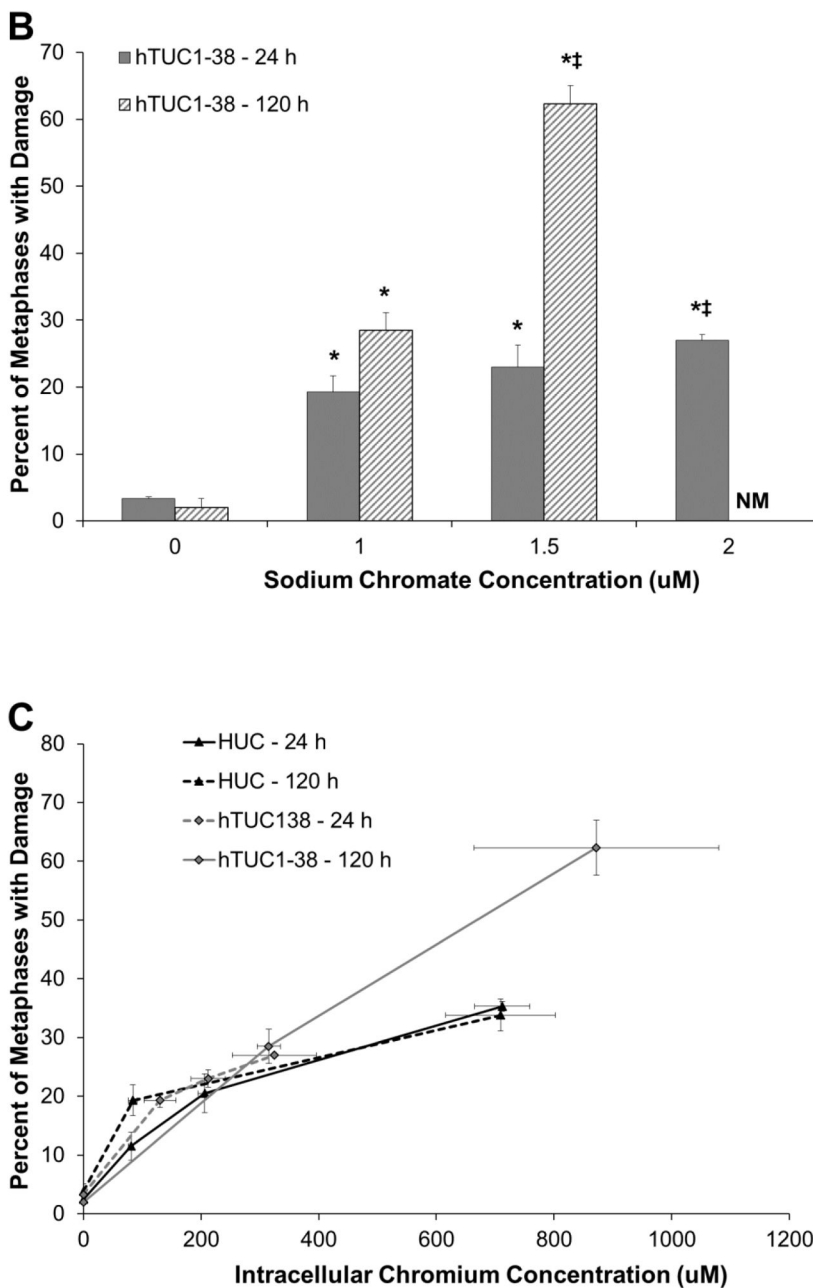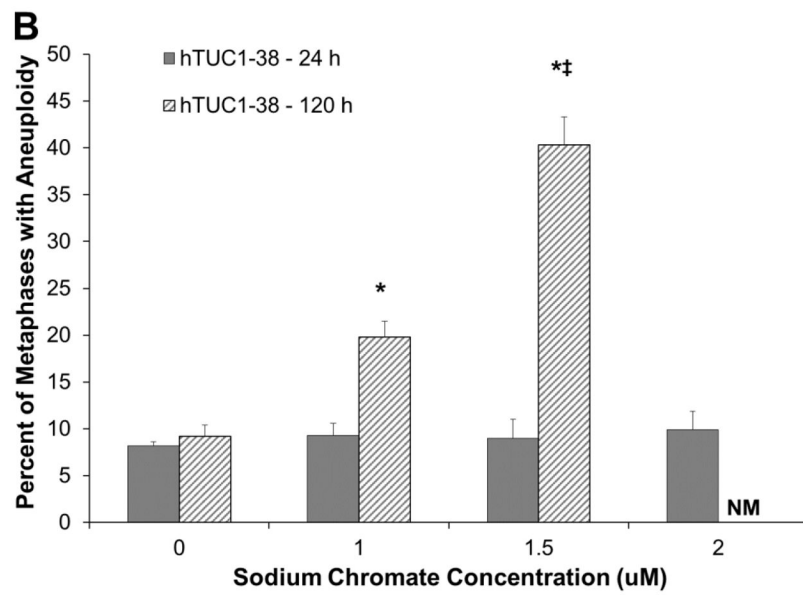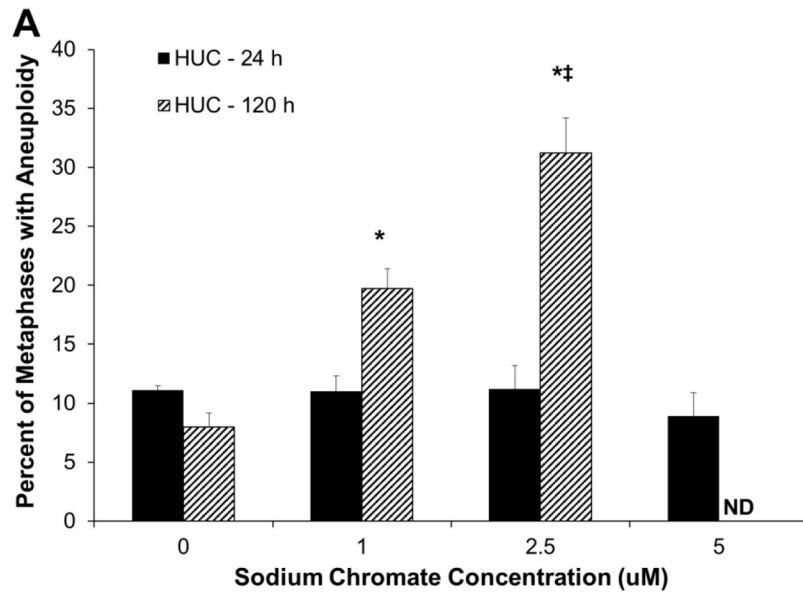
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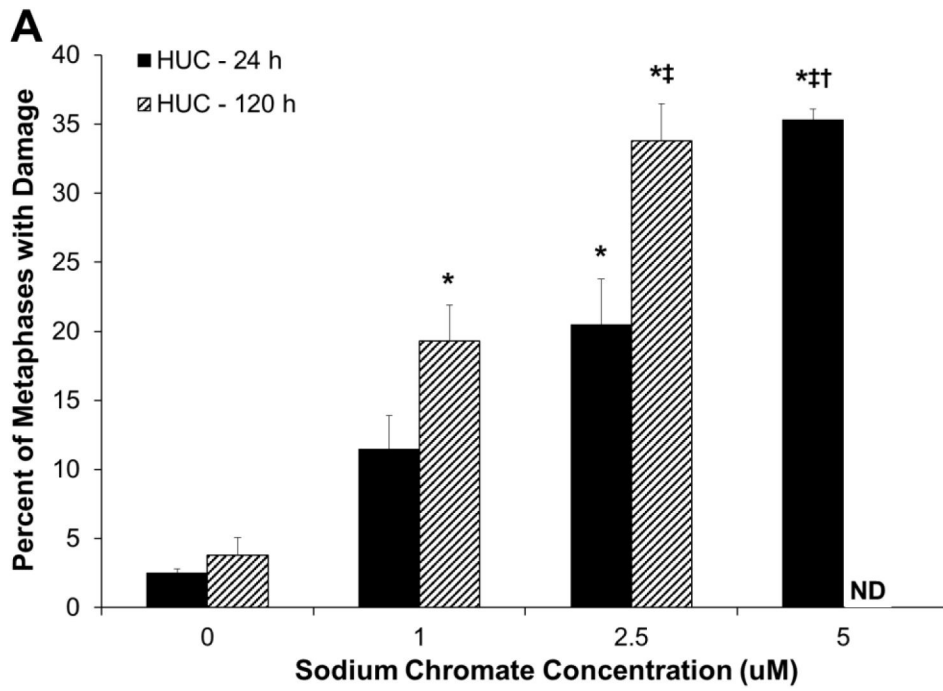
Figure 3.

Hexavalent Chromium Induces Chromosome Damage in Human Urothelial Cells.
This figure shows acute and chronic exposure to sodium chromate induces chromosome
damage in human urothelial cells. A) Primary human urothelial cells. B) hTERT-
immortalized human urothelial cells. Based on administered concentration, sodium chromate
induces more chromosome damage in hTERT-immortalized cells than in primary cells. ND
= not done. NM = No metaphases. C) Based on intracellular Cr concentrations, sodium
chromate induces similar levels of chromosome damage in primary and hTERT-
immortalized human urothelial cells. Data represent an average of at least three experiments

± the standard error of the mean. Statistics: * indicates statistically different from control (p < 0.005); ‡ indicates statistically different from 1 uM sodium chromate (p < 0.05); † indicates statistically different from 2.5 uM sodium chromate (p < 0.005).
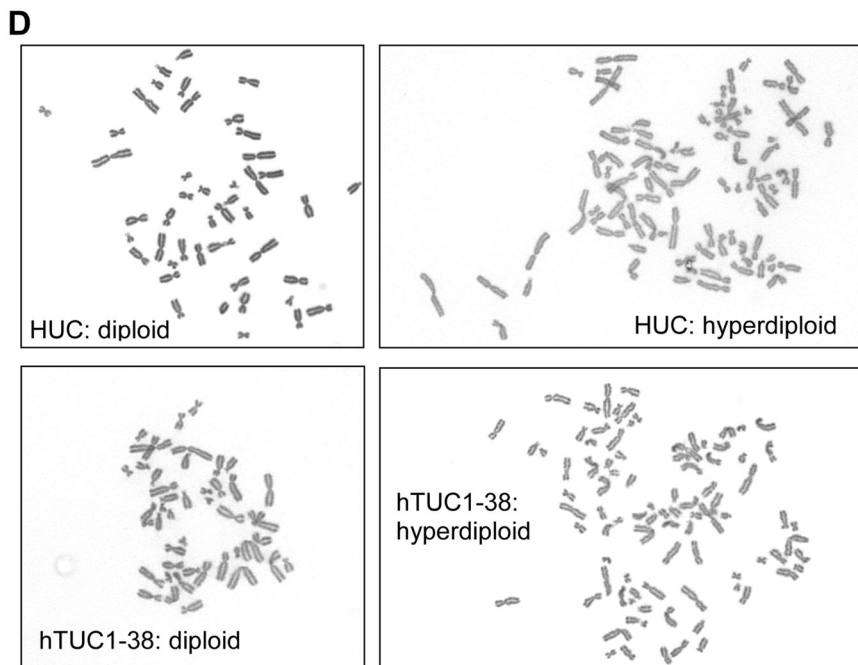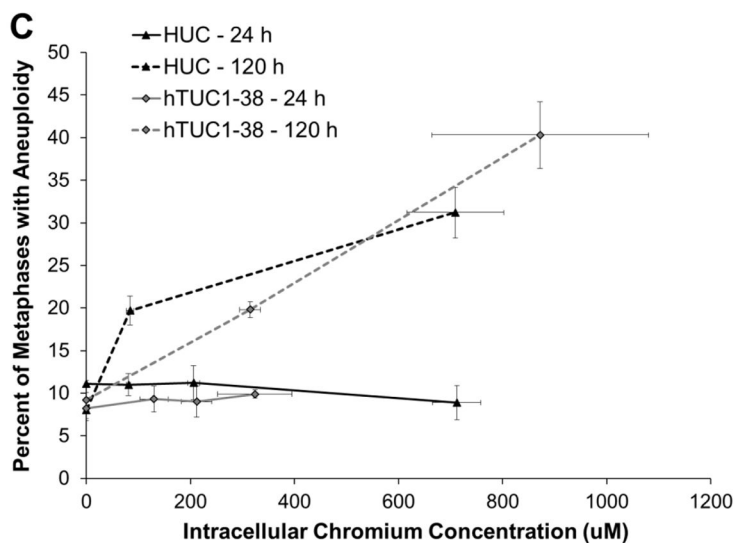
Figure 4.
Hexavalent Chromium Induces Aneuploidy in Human Urothelial Cells.
This figure shows chronic exposure to hexavalent chromium induces aneuploidy in human
urothelial cells. Exposure to sodium chromate for 24 h does not induce aneuploidy; however,
a 120 h exposure induces a concentration-dependent increase in aneuploidy. A) Primary
human urothelial cells. B) hTERT-immortalized human urothelial cells. Based on
administered concentration, sodium chromate induces more aneuploidy in hTERT-
immortalized cells than in primary cells. ND = not done. NM = No metaphases. C) Based on
intracellular Cr concentrations, sodium chromate induces similar levels of aneuploidy in
primary and hTERT-immortalized human urothelial cells. D) Representative images of
aneuploidy in primary and hTERT-immortalized human urothelial cells. Data represent an

average of at least three experiments ± the standard error of the mean. Statistics: * indicates statistically different from control ($p < 0.05$); ‡ indicates statistically different from 1 uM sodium chromate ($p < 0.05$).