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Prolonged Particulate Chromate Exposure Does Not Inhibit Homologous Recombination Repair in North Atlantic Right Whale (*Eubalaena glacialis*) Lung Cells

Cynthia L. Browning^{a,b,1}, Catherine F. Wise^c, and John Pierce Wise Sr.^{a,b,*}

^aWise Laboratory of Environmental and Genetic Toxicology, Department of Pharmacology and Toxicology, University of Louisville School of Medicine, 505 S Hancock St, CTRB, Louisville KY 40292

^bGraduate School of Biomedical Science and Engineering, University of Maine, 42 Stodder Hall, Orono ME 04469

^cProgram in Environmental and Molecular Toxicology, Department of Biological Sciences, North Carolina State University, Raleigh, North Carolina 27695, USA

Abstract

Chromosome instability is a common feature of cancers that forms due to the misrepair of DNA double strand breaks. Homologous recombination (HR) repair is a high fidelity DNA repair pathway that utilizes a homologous DNA sequence to accurately repair such damage and protect the genome. Prolonged exposure (>72 h) to the human lung carcinogen, particulate hexavalent chromium (Cr(VI)), inhibits HR repair, resulting in increased chromosome instability in human cells. Comparative studies have shown acute Cr(VI) exposure induces less chromosome damage in whale cells than human cells, suggesting investigating the effect of this carcinogen in other species may inform efforts to prevent Cr(VI)-induced chromosome instability. Thus, the goal of this study was to determine the effect of prolonged Cr(VI) exposure on HR repair and clastogenesis in North Atlantic right whale (*Eubalaena glacialis*) lung cells. We show particulate Cr(VI) induces HR repair activity after both acute (24 h) and prolonged (120 h) exposure in North Atlantic right whale cells. Although the RAD51 response was lower following prolonged Cr(VI) exposure compared to acute exposure, the response was sufficient for HR repair to occur. In accordance with active HR repair, no increase in Cr(VI)-induced clastogenesis was observed with increased exposure time. These results suggest prolonged Cr(VI) exposure affects HR repair and genomic stability differently in whale and human lung cells. Future investigation of the differences in how human and whale cells respond to chemical carcinogens may provide valuable insight into mechanisms of preventing chemical carcinogenesis.

*Corresponding author at: Wise Laboratory of Environmental and Genetic Toxicology, Department of Pharmacology and Toxicology, University of Louisville, 505 S. Hancock St, CTRB Room 522, Louisville, KY 40292, USA.; Phone: (502) 852-8524; Fax: (502) 852-7868; john.wise@louisville.edu.

¹Present address: Department of Pathology & Laboratory Medicine, Brown University, 70 Ship St, Box G-E5, Providence, RI, 02906

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Graphical Abstract

Keywords

hexavalent chromium; homologous recombination repair; genomic instability; North Atlantic right whale lung fibroblasts; Rad51

1. Introduction

Utilizing high fidelity DNA repair to maintain genomic stability is crucial as genomic instability drives carcinogenesis and is a feature of almost all cancers (Pikor et al., 2013). Homologous recombination (HR) repair is a high fidelity DNA repair pathway that repairs dangerous DNA double strand breaks in a manner that maintains genomic stability. The defining step of the HR repair pathway, the formation of the RAD51 nucleofilament, facilitates the identification of a homologous DNA sequence, which is replicated to repair the double strand break without introducing errors into the genetic sequence (Bauman et al., 1996; Sung and Robberson, 1995). RAD51 nucleofilament formation inhibits mutagenic repair pathways such as single strand annealing and alternative end-joining (Bennardo et al., 2008; Stark et al., 2004).

HR repairs double strand breaks induced by a variety of carcinogenic heavy metals including: arsenic, cadmium, hexavalent chromium (Cr(VI)), lead and nickel (Bryant et al., 2006; Gastaldo et al., 2007; Helleday et al., 2000; Stackpole et al., 2007; Ying et al., 2009). Moreover, HR repair protects against particulate Cr(VI)-induced chromosome instability (Bryant et al., 2006; Stackpole et al., 2007). However, genomic instability has been proposed as the underlying mechanism of particulate Cr(VI)-induced carcinogenesis, based on a wealth of data demonstrating Cr(VI) induces chromosome instability (Holmes et al., 2008; Qin et al., 2014; Wise et al., 2012; Wise et al., 2016; Xie et al., 2009).

Recent studies show a temporal correlation between increased particulate Cr(VI)-induced chromosome instability and an inhibition of HR repair (Browning et al., 2016; Qin et al., 2014). Specifically, acute (24 h) Cr(VI) exposure induces double strand breaks, and an increase in HR repair activity (Browning et al., 2016; Qin et al., 2014; Xie et al., 2009). In contrast, after prolonged (>72 h) Cr(VI) exposure, chromosome damage levels increase and levels of double strand breaks remain consistent over time, however, HR repair is reduced, through the inhibition of RAD51 (Browning et al., 2016; Qin et al., 2014). As particulate Cr(VI) is a human lung carcinogen and a major health hazard, it is important to determine how a Cr(VI)-induced chromosome damage and inhibition of HR repair can be avoided.

The unconventional approach of investigating the effect of Cr(VI) in whale cells may provide insight into how to prevent Cr(VI)-induced chromosome damage. Several studies have shown whale cells are more resistant to Cr(VI)-induced chromosome instability than human cells (Li Chen et al., 2009a; Li Chen et al., 2009b; Li Chen et al., 2012; Wise et al., 2015). Increased resistance to acute (24 h) Cr(VI) exposure has been demonstrated in fin whale (*Balaenoptera physalus*) skin fibroblasts, sperm whale (*Physeter macrocephalus*) skin fibroblasts and North Atlantic right whale (*Eubalaena glacialis*) lung fibroblasts (Li Chen et al., 2009a; Li Chen et al., 2012; Wise et al., 2015). HR repair is highly evolutionarily conserved (Ladoukakis & Zouros, 2001; Liang et al., 1998; Rocha et al., 2005). Thus, this protective DNA repair pathway may play a role in the increased Cr(VI) resistance observed in whale cells. However, no data are available describing HR repair activity in response to Cr(VI) exposure in whale cells. In addition, no studies to date describe whether the time related effects of Cr(VI) observed in human cells, specifically increased clastogenesis and the inhibition of HR repair, occur in whale cells. Identifying differences in the response of whale and human cells to particulate Cr(VI) provides valuable insight into how Cr(VI)-induced carcinogenesis may be prevented or treated. The goal of this study is to investigate the effect of exposure time on particulate Cr(VI)-induced cytotoxicity, structural chromosome damage and HR repair in North Atlantic right whale lung cells.

2. Materials and Methods

2.1. Chemicals and reagents

DMEM and Ham's F12 50:50 mixture and GlutaGRO (L-alanyl-L-glutamine solution) were purchased from Mediatech Inc (Herndon, VA). Cosmic Calf Serum was purchased from Hyclone (Logan, UT). Zinc chromate (CAS#13530-65-9) was purchased from Pfaltz and Bauer (Z00277, Waterbury, CT). Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin, sodium pyruvate, trypsin/EDTA, goat serum, Prolong Gold Antifade Reagent with DAPI and Alexa Fluor 488 secondary antibody were purchased from Life Technologies (Grand Island, NY). All plasticware was purchased from BD Biosciences (Franklin Lakes, NJ). Potassium chloride and 4% paraformaldehyde in PBS were purchased by Alfa Aesar (Ward Hill, MA). Triton X-100, demecolchicine, 5-bromo-2'-deoxyuridine (BrdU) and gelatin were purchased from Sigma-Aldrich (St. Louis, MO). Nunc Lab Tek II glass chamber slides, Scientific Super Up-Rite microscope slides, cyto seal 60 and Gurr's buffer tablets were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). FNC coating mix was purchased from AthenaFS (Baltimore, MD). Acetone, methanol and crystal violet were purchased from JT Baker (Phillipsburg, NJ). Giemsa stain was purchased from Ricca Chemical Company (Arlington, TX) and Hoechst 33258 pentahydrate was purchased from Invitrogen (Grand Island, NY).

2.2. Cell culture

For these studies we used primary North Atlantic right whale lung fibroblasts derived from tissues collected from a stranded animal as previously described (Wise et al., 2008). Cells were cultured as adherent monolayers in DMEM/F12 50:50 mixture, supplemented with 15% cosmic calf serum, 1% L-alanyl-L-glutamine, 1% penicillin/streptomycin, and 0.1 mM sodium pyruvate. Cells were maintained in a 5% CO₂-humidified environment at 33°C.

Cells were seeded for experiments at different seeding densities, depending on their designated exposure time. This approach ensured all cells were at ~80–90% confluency at the completion of their exposure times and eliminated the need to split cells during prolonged exposures.

2.3. Treatment with particulate Cr(VI) compound

Zinc chromate was administered as a suspension of particles in cold, sterile water as previously described (Xie et al., 2009). Cells were treated for 24 and 120 h with concentrations of 0.1–1 $\mu\text{g}/\text{cm}^2$ zinc chromate. We selected 24 and 120 h to represent acute and prolonged Cr(VI) exposure, respectively, because RAD51 and HR repair activity differ in human lung cells after 24 and 120 h exposures to 0.1–0.3 $\mu\text{g}/\text{cm}^2$ zinc chromate (Browning et al., 2016; Qin et al., 2014). Since no data are available on chromium levels in whale lungs, we compared our experimental concentrations with previously reported chromium levels of skin biopsy samples acquired from free-swimming North Atlantic right whales to demonstrate they are representative of real-world exposure. Wise et al. (2008) reported mean dermal chromium level of 7.1 $\mu\text{g Cr/g}$ tissue (range 4.9–10 $\mu\text{g/g}$). As the concentrations utilized in this study equate to 0.1–1 $\mu\text{g/g}$, the highest concentration used in this study is seven times lower than the mean level of chromium detected in live right whales.

2.4. Clonogenic survival assay

Cytotoxicity was assessed using a clonogenic survival assay based on our published methods (Wise et al., 2002). Briefly, cells were seeded into 6-well plates, allowed to enter log phase growth and treated with zinc chromate for 24 or 120 h. Following treatment, cells were harvested, counted and reseeded at a density of 1,000 cells/dish in 100 mm dishes coated with 0.1% gelatin (4 dishes per treatment). Cells were allowed to grow to form colonies. Once colonies formed, they were fixed with methanol, stained with crystal violet and counted. At least three independent experiments were conducted.

2.5. Chromosome aberration assay

Clastogenicity was determined by measuring chromosomal aberrations according to our published methods (Wise et al., 2002). Briefly, cells were seeded into 100 mm dishes and allowed to rest for 48 h before treatment with zinc chromate for 24 or 120 h. Five hours prior to the end of treatment, 0.1 $\mu\text{g}/\text{ml}$ demecolcine was added to arrest cells in metaphase. Cells were then harvested, resuspended in 0.075 M KCl and fixed with 3:1 methanol:acetic acid. Cells were then dropped on clean, wet slides, stained with 5% Giemsa stain and scored for chromosome aberrations using previously defined criteria (Wise et al., 2002). One hundred metaphases were analyzed for each treatment and three independent experiments were conducted. Results were expressed as the percent of metaphases with damage and as total aberrations which considers the metaphase and each chromosome as the unit, respectively.

2.6. Immunofluorescence

Immunofluorescence staining was conducted as previously described with minor alterations (Xie et al., 2005). Briefly, cells were seeded on glass chamber slides coated with FNC,

allowed to re-enter logarithmic growth and treated with zinc chromate for either 24 or 120 h. At harvest, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with 10% goat serum and 5% BSA in PBS for 1 h. Cells were incubated with anti-RAD51 antibody (Santa Cruz sc-8349; 1:200) overnight, washed with PBS and incubated with Alexa Fluor 488 (1:3000) for 1 h. Coverslips were mounted with DAPI and nuclear foci were scored in one hundred cells per concentration/timepoint using fluorescence microscopy. Results were expressed as the percentage of cells with >5 foci based on background levels such that negative controls had 5% or less of cells with this level.

2.7. Sister chromatid exchange

Sister chromatid exchanges (SCEs) were quantified as a measure of homologous recombination repair activity. Cells were seeded into 100 mm dishes, allowed to enter log phase growth and treated with zinc chromate for 24 or 120 h. Concurrently, cells were incubated with 0.6 $\mu\text{g/ml}$ BrdU for 96 h prior to harvesting. After treatment, cells were harvested and slides prepared according to the chromosome aberration assay protocol described above. Slides were aged overnight, soaked in PBS for 5 min and stained with 0.5 $\mu\text{g/ml}$ Hoechst 33258 pentahydrate solution for 10 min at room temperature. Slides were incubated in 25 $\mu\text{g/ml}$ Hoechst 33258 pentahydrate under 27 W fluorescent lights for 11 h in a humidity chamber. After incubation, slides were washed with distilled water and slides were immersed in 2x sodium chloride/sodium citrate solution for 15 min at 60°C. Finally, slides were washed with distilled water, stained with 4% Giemsa stain in Gurr's buffer and coverslipped. The total number of SCEs was counted in fifty harlequin stained metaphases per concentration/timepoint.

2.8. Statistics

Results are expressed as the mean $\pm$ SEM (standard error of the mean) of at least three independent experiments. A two-way ANOVA was used to determine the effect of exposure time and Cr(VI) concentration. Tukey's post-hoc analysis or Dunnett's multiple comparisons test was performed to identify differences between individual means while correcting for multiple comparisons. A 95% confidence interval was constructed for the difference in means of each pair of concentrations. The criterion for statistical significance was $p < 0.05$. All analyses were conducted using GraphPad.

3. Results

3.1. Exposure Time Does Not Increase Cr(VI)-induced Cytotoxicity in North Atlantic Right Whale Lung Cells

Particulate Cr(VI) induced a significant concentration-dependent decrease in relative survival of cells after both 24 and 120 h exposure ($p < 0.0001$) (Fig. 1). Cr(VI)-induced cytotoxicity was slightly higher after 120 h exposure than 24 h, but the difference between the two exposure times was not significant. For example, 24 h exposure to 0.2, 0.4, 0.6, 0.8 and 1 $\mu\text{g/cm}^2$ zinc chromate induced 78, 48, 33, 18 and 13 percent relative survival, while 120 h exposure induced 67, 45, 23, 7 and 2 percent relative survival, respectively.

3.2. Exposure Time Does Not Increase Cr(VI)-induced Chromosome Damage in North Atlantic Right Whale Lung Cells

Cr(VI)-induced clastogenesis was determined by quantifying chromosome aberrations. Prolonged (120 h) exposure to zinc chromate concentrations higher than 0.5 $\mu\text{g}/\text{cm}^2$ resulted in a low mitotic index that prevented chromosome scoring. Thus, chromosome aberrations were quantified after acute and prolonged exposure to 0–0.5 $\mu\text{g}/\text{cm}^2$ zinc chromate. The data show Cr(VI) induced a significant concentration-dependent increase in chromosome damage levels after both 24 and 120 h exposure ($p < 0.001$) (Fig. 2A and 2B). However, there was not a significant difference in the amount of chromosome damage between the two exposure times. For example, 24 h exposure to 0.1, 0.3 and 0.5 $\mu\text{g}/\text{cm}^2$ induced 9, 11 and 17 percent of metaphases with damage, while 120 h exposure induced 7, 9 and 16 percent, respectively (Fig. 2A). Likewise, 24 h exposure to 0.1, 0.3 and 0.5 $\mu\text{g}/\text{cm}^2$ induced 10, 14 and 20 total chromosome aberrations, while 120 h exposure induced 7, 9 and 16 total chromosome aberrations, respectively (Fig. 2B).

3.3. Homologous Recombination Repair Is Active in North Atlantic Right Whale Lung Cells After Cr(VI) Exposure

Since HR repair protects against Cr(VI)-induced chromosome damage and we have shown exposure time does not increase Cr(VI)-induced chromosome damage, we hypothesized HR repair is functional in whale cells following prolonged Cr(VI) exposure. To test this hypothesis, we determined the effect of Cr(VI) on HR repair in North Atlantic right whale lung cells following acute and prolonged exposure.

First we considered RAD51 nuclear foci. The data show Cr(VI) induced a significant increase in RAD51 nuclear foci formation after 24 h exposure ($p = 0.0014$) (Figure 3A and 3B). Specifically, the percent of cells with RAD51 foci increased from a control level of 5 to 18, 17 and 23 percent after 24 h exposure to 0.1, 0.3 and 0.5 $\mu\text{g}/\text{cm}^2$ zinc chromate, respectively. However, prolonged Cr(VI) exposure of 120 h only induced a slight increase in RAD51 foci formation. Specifically, the percent of cells with RAD51 foci increased from a control level of 4 to 11, 7 and 11 percent after 120 h exposure to 0.1, 0.3 and 0.5 $\mu\text{g}/\text{cm}^2$ zinc chromate, respectively. The percent of cells with RAD51 foci following 24 and 120 h exposures differed significantly ($p < 0.0001$). For example, 24 h exposure to 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate produced RAD51 foci formation in 23 percent of cells, while 120 h exposure to the same concentration only produced RAD51 foci in 9 percent of cells.

We then determined whether the observed decrease in RAD51 response after prolonged Cr(VI) exposure results in the inhibition of HR repair. SCEs, which form as a result of crossover between sister chromatids during the resolution step of HR, were quantified as a measure of HR repair activity (Fig. 4A). Cr(VI) induced a significant concentration-dependent increase in SCE formation significantly after both 24 and 120 h exposure ($p < 0.0001$) (Figure 4B and 4C). Specifically, 24 h exposure to 0, 0.2 and 0.4 $\mu\text{g}/\text{cm}^2$ zinc chromate induced an average of 2, 5.5 and 8 SCEs per metaphase, respectively; and 120 h exposure induced an average of 0.7, 4.3 and 6.9 SCEs per metaphase, respectively. These data were then considered as the number of SCEs per chromosome to control for any variation in chromosome number after Cr(VI) treatment. Again, Cr(VI) induced a

significant, concentration-dependent increase in the number of SCEs per chromosome after both 24 and 120 h exposures ($p < 0.0001$) (Figure 4C). For example, 24 h exposure to 0, 0.2 and 0.4 $\mu\text{g}/\text{cm}^2$ zinc chromate induced an average of 0.05, 0.13 and 0.19 SCEs per chromosome, respectively; and 120 h exposure induced an average of 0.02, 0.10 and 0.17 SCEs per chromosome, respectively. No significant difference in SCE formation was observed between exposure times.

4. Discussion

HR repair is crucial to maintaining genomic stability and is activated in response to DNA damage induced by a variety of carcinogenic heavy metals. Recent studies demonstrate prolonged Cr(VI) exposure inhibits HR repair, resulting in increased chromosome instability in human cells (Browning et al., 2016; Stackpole et al., 2007; Qin et al., 2014). This is an important component of the molecular mechanism of Cr(VI)-induced carcinogenesis. Interestingly, comparative studies suggest investigating the effect of this carcinogen in other species may shed light on how to prevent Cr(VI)-induced chromosome instability. Several studies show whale cells are more resistant to Cr(VI)-induced chromosome instability than human cells (Li Chen et al., 2009a; Li Chen et al., 2009b; Li Chen et al., 2012; Wise et al., 2015). However, these studies focused on acute exposure (24 h) and no data are available determining the effect of prolonged exposure time on clastogenesis in whale cells. In addition, the effect of Cr(VI) on HR repair has yet to be determined in whale cells.

This report is the first to show HR repair responds to a chemical carcinogen in whale lung cells. Acute (24 h) Cr(VI) exposure induced a concentration-dependent increase in HR repair activity. Further, RAD51 responded to acute Cr(VI) exposure, which agrees with our finding of active HR repair. These observations are in agreement with previously reported induction of HR repair after 24 h Cr(VI) exposure in human lung cells (Browning et al., 2016; Helleday et al., 2000).

Interestingly, our data show HR repair activity increased comparably following acute and prolonged Cr(VI) exposure in whale lung cells. Although prolonged Cr(VI) exposure induced a significantly lower increase in RAD51 response compared to acute Cr(VI) exposure, the observed induction of SCEs following prolonged Cr(VI) exposure suggests the RAD51 response observed after prolonged Cr(VI) exposure is still sufficient for HR repair to occur. These data conflict with previously reported time-dependent inhibition of HR repair in human lung cells (Browning et al., 2016; Qin et al., 2014), indicating a different response for whales than human cells. In addition, RAD51 response was reduced to less than control levels following prolonged Cr(VI) exposure, evidenced by RAD51 foci and nucleofilament formation in human cells (Browning et al., 2016; Qin et al., 2014).

One potential explanation, albeit untested, for the difference in the effect of prolonged Cr(VI) exposure on HR repair in human and whale lung cells is that whales have evolved more efficient regulation of DNA repair. While HR repair is highly evolutionarily conserved (Ladoukakis & Zouros, 2001; Liang et al., 1998; Rocha et al., 2005) recent studies document genetic alterations that may result in enhanced DNA repair in some species. For example, analysis of the bowhead whale genome revealed duplication of the PCNA gene, which is

involved in the re-synthesis of DNA during HR repair (Keene et al., 2015; Sebesta et al., 2013). If HR repair is more efficient in whale cells, this would result in a protective mechanism against Cr(VI)-induced chromosome instability. Further investigation is needed to compare the response of HR repair in human and whale cells.

Our data show increased exposure time did not increase chromosome damage levels in whale lung cells. This observation is not surprising since HR repair was active after both acute and prolonged Cr(VI) exposure in this cell line and HR repair protects against Cr(VI)-induced clastogenesis (Bryant et al., 2006; Stackpole et al., 2007). The concentration-dependent increase in chromosome aberrations we observed following acute particulate Cr(VI) exposure agrees with previous reports of Cr(VI)-induced clastogenesis in whale cells (Li Chen et al., 2009a; Li Chen et al., 2009b; Li Chen et al., 2012; Wise et al., 2015). However, this is the first study to consider the effect of prolonged Cr(VI) exposure in whale cells.

The lack of a time-dependent increase in Cr(VI)-induced chromosome damage could be attributed to increased apoptosis removing cells with large amounts of damage from the population analyzed for chromosome aberrations. However, our clonogenic survival data does not support this possibility, revealing no significant difference between the percent of cells that survive and proliferate after 24 and 120 h of exposure.

In summary, our data show Cr(VI) does not inhibit HR repair in North Atlantic right whale cells. In accordance with active HR repair, no increase in Cr(VI)-induced clastogenesis was observed with increased exposure time. These results underline the importance of HR repair in maintaining genomic stability after Cr(VI) exposure. Prolonged Cr(VI) exposure appears to affect HR repair and genomic stability differently in whale and human lung cells. Further exploration of these inherent differences could enhance our understanding of HR repair response to chemical carcinogens and provide innovative methods to evade chemical induced carcinogenesis.

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Research Highlights

- Sister chromatid exchanges increased after acute and prolonged chromate exposure
- Acute chromate exposure induced more RAD51 foci formation than prolonged exposure
- Acute and prolonged chromate exposure are comparably genotoxic to right whale cells

Fig. 1. Exposure Time Does Not Increase Cr(VI)-induced Cytotoxicity in Northern Right Whale Cells

This figure shows the concentration-dependent decrease in the relative survival of cells induced by zinc chromate was similar after 24 and 120 h exposures. Data represent the average of at least 3 independent experiments. Error bars = standard error of the mean. All concentrations were statistically different from controls ($p < 0.05$ for 0.1 and 0.2 µg/cm² zinc chromate; $p < 0.0001$ for all other concentrations). 120 h exposure to zinc chromate was not significantly more cytotoxic than 24 h exposure.

Fig. 2. Exposure Time Does Not Increase Cr(VI)-induced Chromosome Damage in Northern Right Whale Cells

This figure shows the amount of Cr(VI)-induced chromosome damage detected after 24 and 120 h exposures is not statistically different. Data represent 3 independent experiments.

Error bars = standard error of the mean. (A) Percent of Metaphases with Damage. (B) Total Damage in 100 metaphases. Statistically different from control: * $p < 0.05$; # $p < 0.01$; ## $p < 0.001$. NM = no metaphases.

Fig. 3. Cr(VI) Induces RAD51 Foci Formation in North Atlantic Right Whale Lung Cells

This figure shows the percent of cells with >5 RAD51 foci increased significantly after both 24 h exposure, while increasing to a lesser extent after 120 h. (A) Percent of cells with >5 RAD51 foci. The percent of cells with >5 RAD51 foci was significantly different from control after 24 h exposure to 0.2, 0.4 and 0.5 $\mu\text{g}/\text{cm}^2$ zinc chromate (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$). RAD51 foci formation after 120 h was not significant. Data represent an average of at least three experiments. Error bars = standard error of the mean. (B) Representative images of RAD51 immunofluorescence.

Fig. 4. Cr(VI) Exposure Induces Sister Chromatid Exchange Formation in North Atlantic Right Whale Lung Cells

This figure shows both 24 and 120 h exposure to zinc chromate induced a concentration-dependent increase in sister chromatid exchange formation. Data represent an average of three experiments Error bars = standard error of the mean. (A) Representative Images of Sister Chromatid Exchanges. (B) Average SCEs per metaphase. All concentrations induced a significant increase in the number of SCEs per metaphase (### $p < 0.0001$). (C) Average SCEs per chromosome. All concentrations induced a significant increase in the number of SCEs per chromosome (** $p < 0.005$; ## $p < 0.001$; ### $p < 0.0001$).