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Chromium distribution in an oropharyngeal aspiration model for hexavalent chromium in rats

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ABSTRACT

Hexavalent chromium [Cr(VI)] is a well-known and widespread environmental contaminant associated with a variety of adverse health effects, in particular lung cancer. The primary route of exposure in humans is through inhalation. Particulate forms of Cr(VI) are the most potent but in vivo studies are difficult. Intratracheal instillation requires highly trained surgical procedures which also limits the number of repeated exposures possible and thus requires high doses. Inhalation studies can deliver lower more chronic doses but are expensive and generate dangerous aerosols. We evaluated an oropharyngeal aspiration exposure route for zinc chromate particles in Wistar rats. Animals were treated once per week for 90 days. We found chromium accumulated in the lungs, blood, and reproductive tissues of all treated animals. Additionally, we found inflammatory indicators in the lung were elevated and circulating lymphocytes had increased chromosomal damage. These results show oropharyngeal aspiration provides a practicable exposure route for chronic and sub-chronic exposures of Cr(VI) particles.

1. Introduction

Chromium is an important metal with an assortment of commercial and industrial uses. It has been used in a variety of applications for over 200 years and has led to many useful and significant advances in industry and society. Its physico-chemico-properties including solubility, valence state, and bright colors, make it an attractive and practical resource. However, this extensive use has resulted in an increasing level of environmental contamination with concerning health effects (IARC, 1990; Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Chromium, 2012).

Risks associated with Cr(VI) exposure have been reported as early as the 1800s (IARC, 1990; Newman, 1890). Cr(VI) is associated mainly with lung carcinogenesis but has also been linked to increases in asthma and other respiratory issues, reproductive and developmental effects, dermatitis, neurological, and adverse immune responses (Speer and Wise Sr., 2018; Wise Jr et al., 2022). The particulate form of Cr(VI) causes the most consequential effects and is a well-known human lung carcinogen (IARC, 1990). Epidemiological studies show these particulate forms of Cr(VI) are associated with the highest incidences of lung cancer (Langård and Vigander, 1983; Langård and Norseth, 1975). The particulate forms of Cr(VI) are able to impact and persist at lung bifurcations resulting in highly localized Cr concentrations that induce tumor formation at these sites (Ishikawa et al., 1994a, 1994b).

Despite decades of study, the mechanisms for particulate Cr(VI)-induced lung cancer are poorly understood. Cr(VI) is a well-

Abbreviations: Cr(VI), chromate or hexavalent chromium; ICM-MS, inductively coupled plasma mass spectrometry; XRF, X-ray fluorescence.

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established genotoxic compound with the ability to induce many types of DNA damage (De Flora et al., 1990; Wise et al., 2008; Wise and Wise, 2019). DNA double strand breaks are of particular importance and are induced by Cr(VI) exposure (Ha et al., 2004; Xie et al., 2005, 2009; Qin et al., 2014). DNA double strand breaks can lead to chromosomal instability if left unresolved (Masuda and Takahashi, 2002). In fact, failure of DNA double strand break repair has been demonstrated with particulate Cr(VI) exposure (Qin et al., 2014; Browning et al., 2016, Browning and Wise Sr, 2017) and leads to increased and permanent chromosome instability in human lung cells (Wise et al., 2018). Chromosome instability is also a characteristic of chromate-induced lung tumors (Maeng et al., 2003). Thus, chromosome instability appears to play a primary role in the carcinogenic mechanism of Cr(VI)-induced cancer.

These advances in our understanding of the molecular mechanisms of Cr(VI)-induced carcinogenesis are profound and important but the translation of in vitro Cr(VI) studies to mammalian organisms continues to be challenging. Several studies have investigated the effects of Cr(VI) in rats and mice and confirmed the carcinogenicity of Cr(VI) in the lung (IARC, 1990; Toya et al., 1999; Takahashi et al., 2005; Zeidler-Erdely et al., 2020). Levy et al. (1986) clearly demonstrate the particulate Cr (VI) forms are most potent, especially those with partial solubility (strontium, zinc and calcium chromates). However, most mechanistic carcinogenicity studies in animals have employed soluble forms of chromium, including sodium and potassium dichromate and chromic oxide administered either via intratracheal instillation or inhalation. Those studies that have used particulate chromate have largely treated animals via intratracheal instillation thus limiting them to a single bolus exposure (IARC, 1990; Takahashi et al., 2005). In the last decade a few studies treated mice with repeated Cr(VI) exposures intranasally to investigate long term and/or repeated exposures and their effects (Beaver et al., 2009a, 2009b; Kim et al., 2015; Zeidler-Erdely et al., 2020; Ge et al., 2022). However, intranasal exposures induce a reflux response in animals resulting in loss of material through swallowing and ultimately uneven distribution in the target lung tissue.

Inhalation studies are expensive, difficult and impractical due to the hazards of generating aerosols with a carcinogen. In addition, rats are not mouth breathers so significant animal restraint is necessary causing undue stress on the animals. Rats also have extensive nasal turbinates, which would trap Cr(VI) in their nasal passages much more than what humans would experience. Intratracheal instillation of chromate particles or pellets is also cumbersome, requiring exceptional technical skills and surgical procedures. This method is reasonable for a single exposure but to study repeated exposure scenarios would be impractical and highly stressful on the animals. Only one study used the oropharyngeal aspiration route to chronically expose mice to calcium chromate (Zeidler-Erdely et al., 2020). This study used calcium chromate as a positive control for tumor development and did not determine Cr distribution or any mechanistic effects in the animals. The purpose of our study was to establish a model of repeated low dose particulate Cr(VI) exposure in rats in order to investigate the early molecular events prior to the development of lung tumors which contribute to Cr(VI)-induced chromosome instability and carcinogenesis. Data from these studies will allow for better understanding of the key molecular mechanisms of Cr (VI)-induced chromosome instability and provide targets for early intervention and treatment in exposed populations prior to the development of cancer.

2. Methods & materials

2.1. Animals & chemical exposures

Male and female Wistar rats, aged 11 weeks, were obtained from Envigo (USA). Animals were acclimated for one week prior to the initiation of treatments. Rats were maintained 2–3 animals/cage on a 12 h light/dark cycle and fed a standard rodent chow diet with water provided ad libitum. Rodent chow and water were analyzed for Cr and Zn content and found to be below detection levels (data not shown). All animal studies were approved by the University of Louisville Institutional Animal Care and Use Committee (#18272) and applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Zinc chromate was used as a representative particulate Cr(VI) compound and was prepared as previously described except suspension of particles was in normal saline (Wise Sr. et al., 2002). Briefly, zinc chromate was washed with distilled water and acetone several times to remove water soluble compounds or organic contaminants. Dried particles were then stirred overnight with a magnetic bar to reduce the size of stock particulate chemicals. Before treatment, zinc chromate particles were suspended in normal saline on a stirring plate overnight to obtain homogeneous particle size. Dilutions were prepared with sterile normal saline. The particle size following this method ranges between 0.2 and 2.3 um with a mean size of 1.7 um (Wise Sr. et al., 2002).

Ten males and ten females were treated weekly by oropharyngeal aspiration. This technique was reported to result in minimal loss of suspended particles to the gastrointestinal tract ensuring more consistent delivery to the lungs (Rao et al., 2003). Zinc chromate solutions were vortexed for 10 s immediately prior to treatment to suspend particles. Rats were anesthetized with isoflurane, then removed from the chamber, nares pinched, and tongue held extruded, and 0, 0.4, or 0.8 mg/kg zinc chromate in normal saline was administered to the back of the animals' throat; volume was based on animal weight and did not exceed 200 ul for females or 320 ul for males. The nares and tongue were then held until the animal took 6–7 deep breaths before returning the animal to its cage. Rats were monitored for normal activity which resumed within 1–2 min.

Rats were sacrificed after 90 days of treatment with approximately 7 days between the last administered dose and time of sacrifice. Rats were euthanized with a lethal dose of ketamine and xylazine. Blood was collected from the left ventricle using a sodium citrate coated syringe and transferred to tubes containing 400 ul of sodium citrate for chromosome or metal analysis. The vasculature was perfused with sterile PBS via the left ventricle. Subsequently, organs were harvested for various endpoints and analyses. For 7 males and 7 females, the right lung was collected for protein and RNA analyses, flash frozen and stored; while the left lungs were inflated through the trachea with 10% neutral buffered formalin at a pressure of 20 cm water for 10 min at room temperature and then processed for paraffin embedding for use in histological staining. For the remaining 3 males and 3 females, lungs were separated into lobes and flash frozen for metal analyses. Other tissues were collected, weighed and either frozen for metal analyses or preserved for future analyses as needed.

2.2. Histological sample preparation and analysis

The formalin fixed right lungs were exchanged into PBS, separated into constitutive lobes and stored in 70% ethanol until processing for embedding. The lung lobes were oriented during embedding to obtain cross sections of the bronchi. Paraffin embedded lobes were cut at 5 μm on a microtome and affixed to slides.

Hematoxylin and eosin (H&E) staining provides a detailed representation of tissues with high-resolution for histopathological studies, thus allowing the identification of tissue abnormalities. Lung sections were deparaffinized with xylene and rehydrated with an ethanol gradient to distilled water. Lung sections were then stained sequentially with Gill's Hematoxylin and Eosin. All H&E staining images were captured by using a PanDesk Slide Scanner with CaseViewer software. Macrophages were identified in lung sections by their morphological appearance by an experienced pathologist. The prevalence of macrophages and immune cells in the air spaces of H&E-stained rat lung tissue was analyzed with an Olympus light microscope with 10×, 20× and 40× objectives. A scoring system was established to analyze the degree of

Table 1
Macrophages and immune cells scoring system.

Score	Condition
0	None
1	1–4 small areas
2	≥5 and < 15 small areas
3	≥ 15 small or 1 moderate area
4	≥ 15 small and 1 moderate area
5	≥ 2 moderates plus any number of small areas
6	Widespread in ≥2 different lobes

inflammation (Table 1). Each slide was scored based on the number and size of abnormal macrophages or immune cells appearance according to the scoring system by three independent reviewers who were blinded to the dose.

The recorded "small areas" are where macrophages or immune cells are restricted to a limited, "small" area of the tissue. The "moderate" areas are where macrophages or immune cells appear in larger, contiguous sections of the tissue.

2.3. Tissue metal analysis: ICP-MS

Lung tissues were separated into lobes and analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Metal measurements were performed by digesting tissues in concentrated 70% HNO_3, heating the samples at 95 °C for 1 h, adding 5 drops of 30% H_2O_2, and heating for 1 h. Samples were cooled and brought to 10 ml with ultrapure water for a final concentration of 2% UHP grade HNO_3, then vortexed thoroughly. Material was allowed to settle, then filtered with 0.2-μm or 0.45-μm filter and analyzed by ICP-MS in the direct mode with anhydrous ammonia in the Dynamic Reaction Cell (DRC) to minimize mass interferences.

Reproductive organs and blood were analyzed for total chromium and zinc using ICP-MS. For reproductive tissues whole ovary or a medial subsection of the testes was digested in 70% trace-element grade HNO_3 in metal-free tubes for 2 h at 95 °C. For blood analysis, 250 ul of whole blood was weighed and digested in 70% nitric acid for 2 h at 95 °C. Samples were diluted in ultrapure water to 2% nitric acid and analyzed on an Agilent 7900 ICP-MS in the Integrative Molecular Analysis Core (IMAC) at the University of New Mexico. Initial calibration blank (ICB) and initial calibration verification (ICV) standards were run at the start of the analysis, after every 10 samples, and at the end of the analysis. An internal standard was included in all sample runs to account for any matrix effects and recovery was between 95 and 105% for all samples. Technical replicates for a subset of samples were included to verify accuracy. All metal values are presented as ng or μg metal per g tissue wet weight.

2.4. Tissue metal analysis: XRF

A subset of animals including 3 saline treated animals (2 female, 1 male) and 3 animals treated with 0.8 mg/kg zinc chromate (2 females, 1 male) were analyzed for specific locations of Cr accumulation. Using paraffin embedded lung tissues, samples were spatially mapped for Cr distribution using a Horiba XGT-9000 with 12 um spot size. The scans were done over the 24 lung samples (4 lobes per animal) using a 50 kV 50 watt x-ray tube under vacuum with molybdenum filter and a dwell time of 100 us per pixel. For lung determination, sulfur was used as an identifying element to differentiate lung tissue from paraffin embedding material. Cr comparisons in samples were done by identifying spatially mapped areas matched to histology and averaging over on average 1122 pixels to identify the chromium level in the bronchial or alveoli areas.

2.5. Blood collection, chromosome harvest and analysis

Blood was collected at sacrifice with sodium citrate (JT Baker # 3646–01) coated syringes, and 4 ml was added to a 15 ml conical tube containing 400 ul of 3.8% sodium citrate to prevent clotting. Blood was added to RPMI (Corning #10–104-CV) media supplemented with 10% FBS (Corning #35–010-CV) and 1% penicillin/streptomycin (Corning #30–002-CI) and phytohemagglutinin (PHA-M from Gibco #10576015) in 15 ml conical tubes, placed in a slant rack (45° angle) and incubated at 37 °C for 72–96 h. One hour prior to harvest, colcemid (Gibco #15210040) was added to the cultures and harvested using standard cytogenetic methods. Slides were prepared by dropping cells onto clean, wet slides, dried and stained with 5% Giemsa (Ricca #R3250000). A total of 100 metaphases per animal were analyzed for chromosome aberrations and aneuploidy. Damage was reported as both percent of

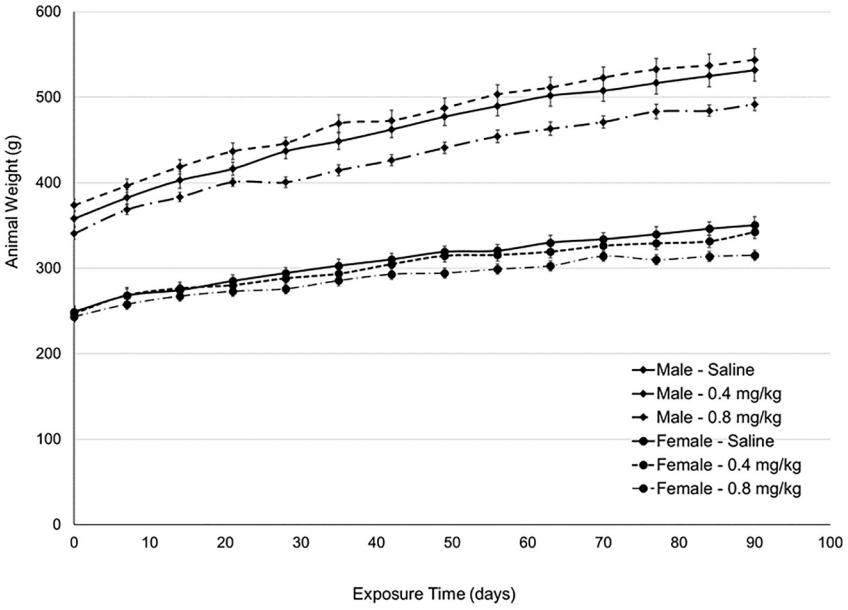

Fig. 1. Effects on Body Weight after Repeated Exposure to Zinc Chromate for 90 d. Body weight curves of male (diamonds) and female (circles) animals throughout the treatment period. Both males and females in the highest treatment group had a slight decrease in growth compared with saline and lower dose treatment animals. Results are reported as mean ± SEM (n = 10 rats/treatment group).

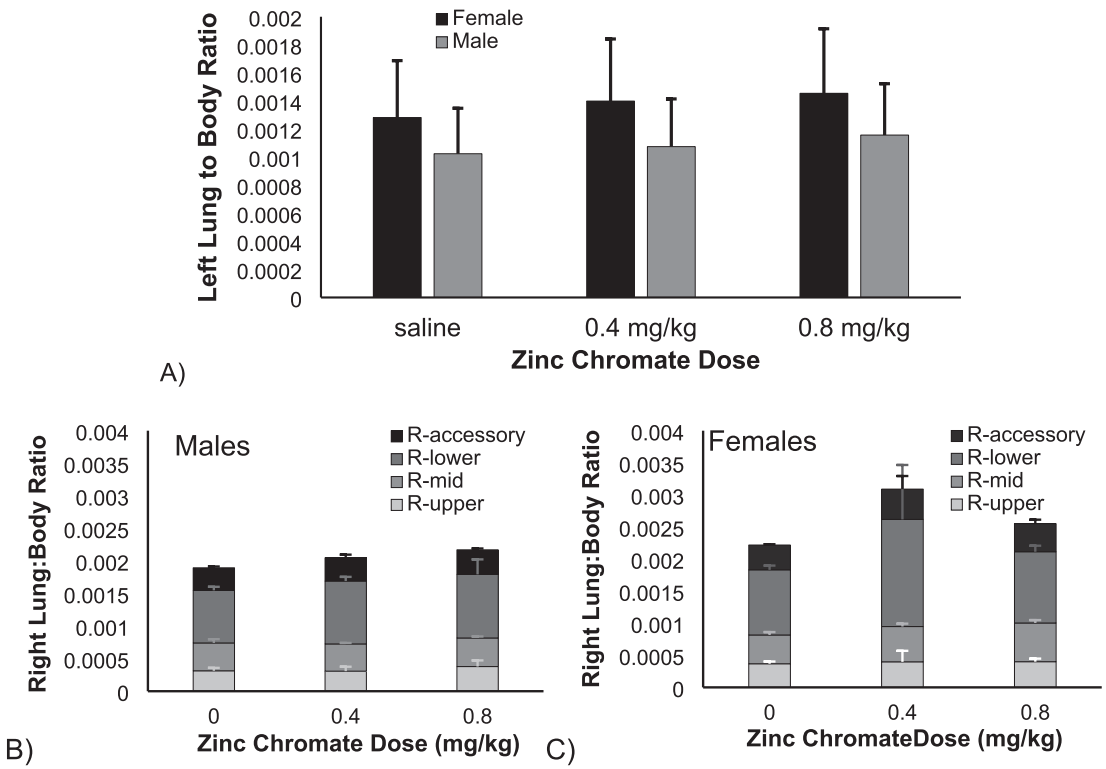

Fig. 2. 90 Day Zinc Chromate Exposure Does Not Alter Lung Weight Ratios. Lung to body weight ratios did not change after exposure to zinc chromate for 90 d; left lungs (A); right lungs for males (B) and females (C). Results are reported as mean ± SEM (n = 3 rats per treatment group). Error bars = standard error of the mean.

metaphases with damage and as the total amount of damage seen in 100 metaphases according to our published methods (Wise Sr. et al., 2002).

2.6. Statistics

Ordinary one-way ANOVA with Tukey's multiple comparisons test was used for sex differences of metals in blood and gonads. Simple linear regression analyses were performed to assess statistical significance of the dose response with increasing Cr(VI) concentrations using Prism 9 software (GraphPad Software, La Jolla, CA, USA). Two-way ANOVA with Tukey's multiple comparisons test was used for chromosome damage analysis. Two-way ANOVA was used for macrophage analysis.

3. Results

3.1. Treatment of rats with zinc chromate for 90 days does not significantly alter body and organ weights

Body weight analysis showed little to no difference between saline treated rats and rats administered 0.4 mg/kg zinc chromate but rats in the 0.8 mg/kg zinc chromate treatment group for both males and females gained slightly less weight throughout the treatment period (Fig. 1).

There was no significant change in lung weight at the time of sacrifice (data not shown). Because overall body weights did not significantly change, we compared organ weight to body weight ratios across treatment doses. Left lungs were collected for all 10 animals from each treatment group. Comparing the left lung to overall body weight at the time of sacrifice, there was no significant change in ratios (Fig. 2A).

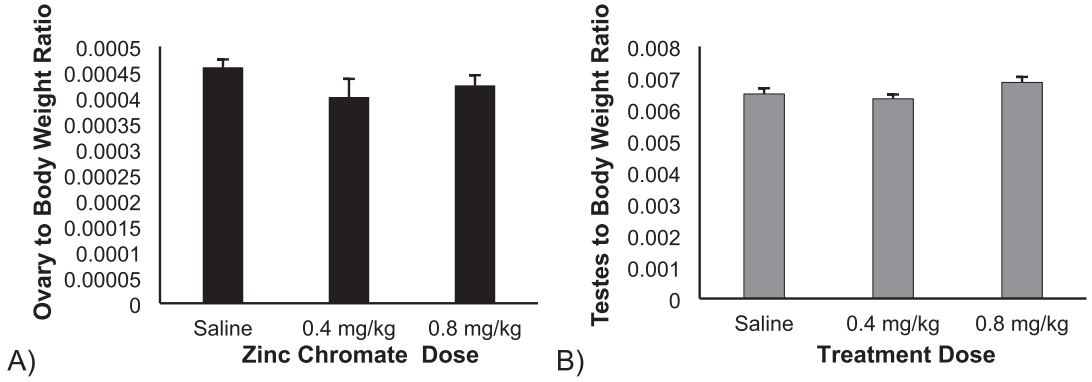

Fig. 3. 90 Day Zinc Chromate Exposure Does Not Alter Reproductive Organ Weights. Ovary to body weight ratios did not change significantly after exposure to zinc chromate for 90 d (A). Testes to body weight ratios did not change significantly after exposure to zinc chromate for 90 d (B). Results are reported as mean ± SEM (n = 10 rats per treatment group).

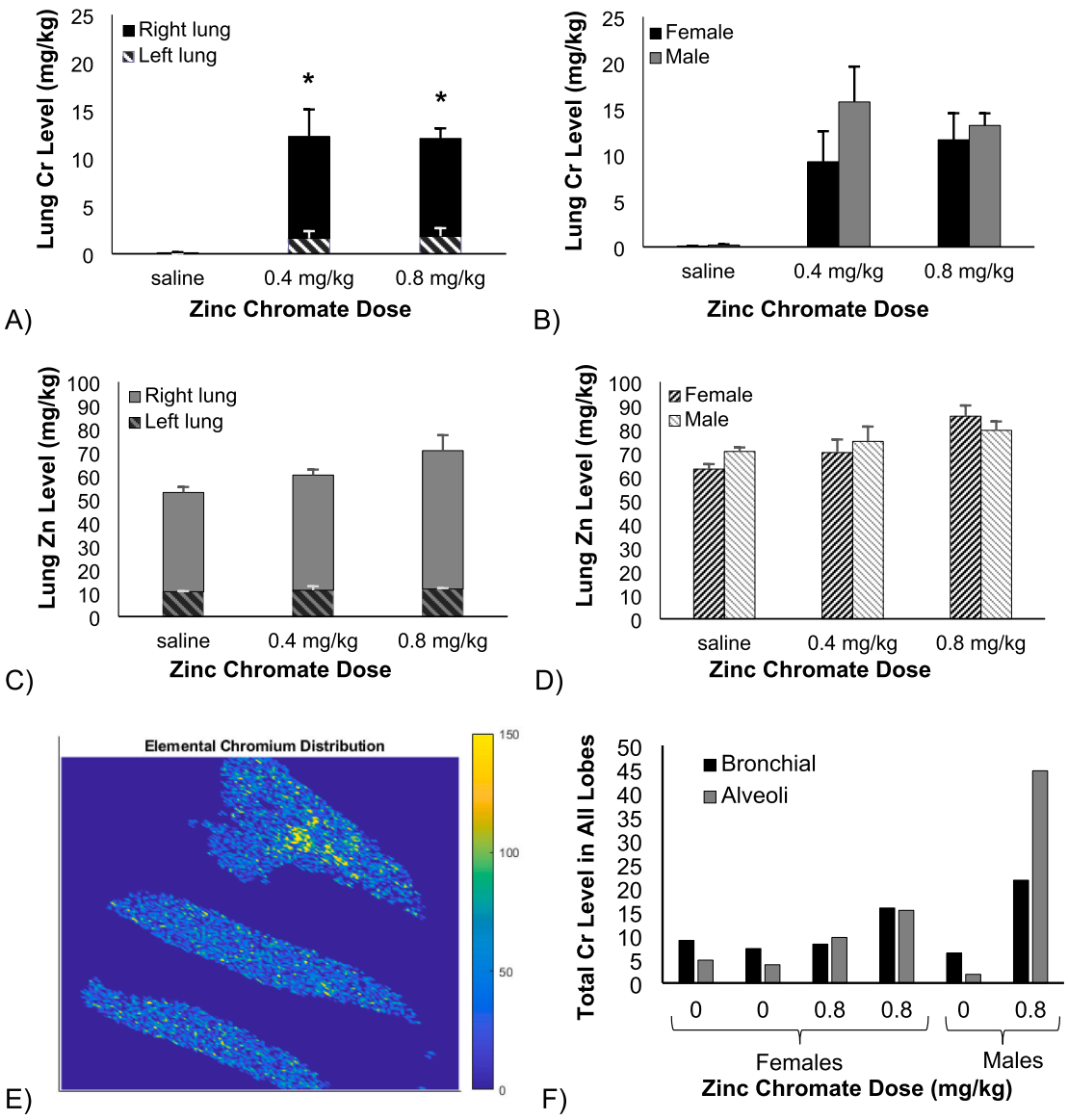

Fig. 4. Cr Accumulation in Rat Lungs. A) Repeated zinc chromate treatment for 90 d induced a significant increase in Cr concentrations in the lungs of rats (* statistically different from control, $p < 0.05$). B) There was no difference in Cr content in the lungs between males and females. C, D) Zinc levels did not significantly increase in lungs after repeated zinc chromate treatment for 90 d. E) Representative XRF image of Cr distribution in the lung from a male rat treated with 0.8 mg/kg zinc chromate. F) Quantification of Cr distribution within the lungs of 3 saline treated rats and 3 zinc chromate treated rats shows no consistent difference in the bronchial region compared to the alveolar region. Each bar represents the Cr level in the right lobes of 1 animal. Results are reported as mean ± SEM ($n = 3$). * Statistically different from control, ($p < 0.05$).

Right lungs were collected for 3 animals (the remaining right lungs were used for histological analysis) per treatment group and divided into their individual lobes for analysis. There were also no differences in the ratios when comparing the lobes of the right lung to body weight (Fig. 2B, C).

We also considered effects on reproductive organs. Reproductive organs were collected for 10 animals of each sex. We found that organ weights for ovaries and testes did not change significantly with zinc chromate treatment (data not shown). After correcting for body weight, ratios of ovaries to body weight decreased slightly (though not significantly) with zinc chromate concentration though it was not concentration dependent (Fig. 3A). There was no change in testes to body weight ratio (Fig. 3B).

3.2. *Chromium levels increased in rats treated with zinc chromate for 90 days*

We found that Cr levels increased in rat lungs after repeated exposure to zinc chromate. Cr is a naturally occurring element, thus low levels are normal in untreated tissues. In the lung, we found more Cr in the cumulative lobes of the right lung compared with single lobed left lung (Fig. 4A). At 0.4 mg/kg zinc chromate there was an average of 1.6 and 40.7 mg Cr per kg of tissue in the left and right lungs respectively; at 0.8 mg/kg zinc chromate there was an average of 1.8 and 10.2 mg Cr per kg of tissue in the left and right lung, respectively. We also found that Cr levels were similar between females and males, at 0, 0.4 and 0.8 mg/kg zinc chromate treatment we found 0.1, 9.3, and 11.6 mg Cr per kg tissue in females and 0.2, 15.8 and 13.2 mg Cr per kg tissue in males, respectively (Fig. 4B). Zinc levels did not significantly change with 90 d zinc chromate treatment and there was no difference in zinc levels between males and females (Fig. 4C, D). Next, we wanted to see if there were specific sites of Cr accumulation within the lung. We analyzed a subset of paraffin embedded lung sections using XRF technology. We found no consistent pattern between the bronchial lining of the airways compared to the alveolar regions (Fig. 4 E, F).

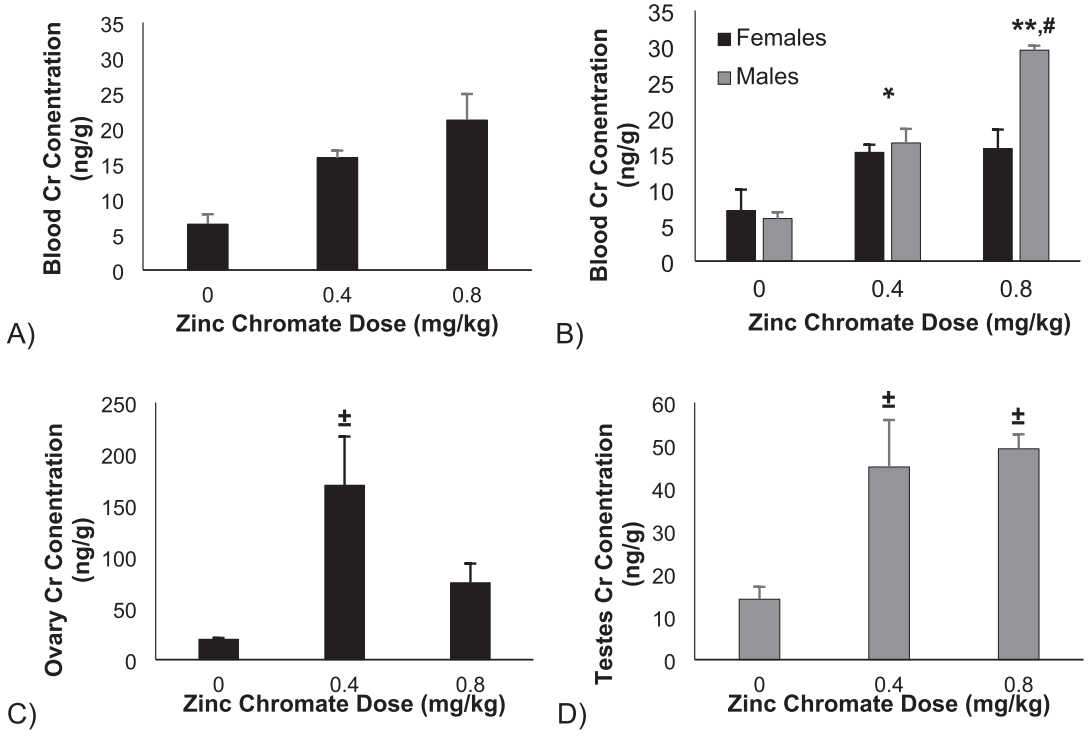

Fig. 5. Cr Accumulation in Blood and Reproductive Organs. A) Repeated zinc chromate treatment for 90 d induced a dose-dependent increase in Cr concentrations in the blood of rats, linear regression analysis: $P = 0.0002$. B) Cr levels were not dose-dependent in females but were in males. *Statistically different from control ($p < 0.01$). **Statistically different from control ($p < 0.001$). # Statistically different from 0.4 ($p < 0.005$). Repeated zinc chromate treatment for 90 d induced a significant increase in Cr concentrations in the ovaries (C) and testes (D) of rats. ± Statistically different from control ($p < 0.05$). Results are reported as mean ± SEM ($n = 3$ rats per treatment group).

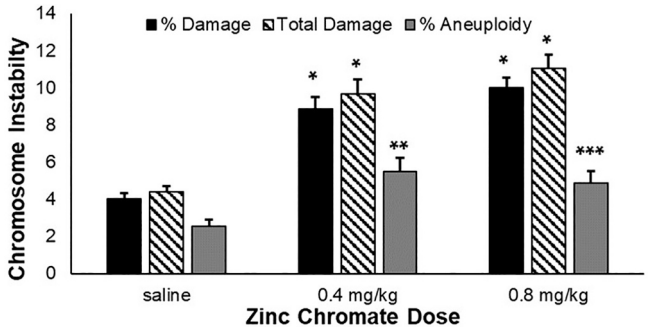

Fig. 6. Chromosome Instability in Blood of Zinc Chromate Treated Rats. Repeated zinc chromate treatment for 90 d induced dose-dependent increases in the percent of damaged metaphases (*$p < 0.0001$), the total amount of chromosome damage (*p < 0.0001), and the percent of aneuploid cells (**$p <$ 0.005, ***p,0.05) compared to control. Error bars = standard error of the mean. Data represent levels in 16–20 rats per treatment group.

We also considered Cr levels in whole blood as a measure of whole-body Cr distribution as well as reproductive organs as they are a targets of Cr exposure (Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Chromium, 2012). We found that repeated exposure of zinc chromate induced a dose-dependent increase in Cr in the blood of all animals with 6, 16, and 21 ng/g Cr after repeated exposure to 0, 0.4 and 0.8 mg/kg zinc chromate, respectively, linear regression analysis: $P = 0.0002$ (Fig. 5A). Males had higher levels of Cr in whole blood compared to controls with 6, 16 and 29 ng/g Cr in males compared to 7, 15 and 16 ng/g Cr in females (Fig. 5B). There was a statistically significant increase in Cr levels in ovaries at 0.4 mg/kg but not 0.8 mg/kg zinc chromate (Fig. 5C). In the testes, we found a dose dependent increase of Cr with 14, 45 and 49 ng/g Cr after repeated exposure to 0, 0.4 and 0.8 mg/kg zinc chromate, respectively. There was no increase of zinc levels in the blood or reproductive organs (data not shown).

3.3. Chromosome instability increased in rats treated with zinc chromate for 90 days

As a measure of whole-body Cr distribution, we considered the effects of zinc chromate on blood lymphocytes. Rats treated repeatedly for 90 d with zinc chromate exhibited a dose-dependent increase in chromosome instability in lymphocytes collected from blood. Zinc chromate induced an average of 4, 8.9 and 10% of metaphases with damage after treatment with 0, 0.4 and 0.8 mg/kg, respectively (Fig. 6). The average total amount of chromosome damage in all metaphases scored was 4.4, 9.7 and 11 at the same concentrations, respectively, indicating some metaphases had multiple chromosomes damaged. Additionally, we found an increase in aneuploidy, with 0, 0.4 and 0.8 mg/kg zinc chromate inducing 2.5, 5.5 and 4.9% of metaphases being aneuploid, respectively. No differences in chromosome damage between male and female animals were observed (data not shown).

3.4. Macrophage infiltration of the lungs increased in rats treated with zinc chromate

Cr(VI) induced macrophage aggregation in the lung after repeated exposure to zinc chromate for 90 d. Fig. 7A shows representative images of macrophage aggregation. In the saline group, there were few macrophages in the alveolar space (Fig. 7A), but in the 0.4 and 0.8 mg/kg groups, there were areas of abnormal macrophage aggregation. Fig. 7 shows significant increase of macrophages in female and male rat lungs.

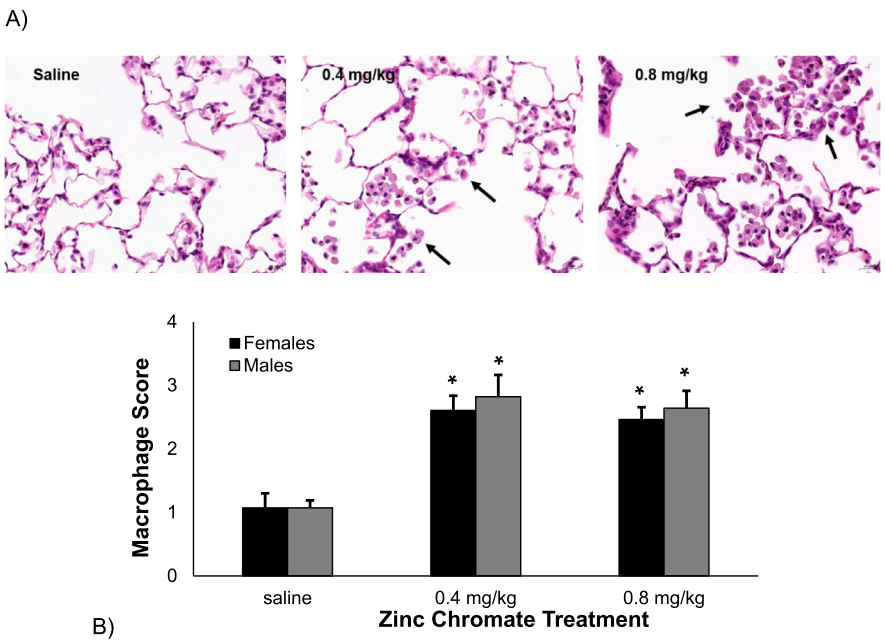

Fig. 7. Cr(VI) Induces Macrophage Aggregation in Rat Lungs After 90 Days of Exposure. A) Representative images of macrophage aggregation, arrows indicate examples of macrophages (Images are 400× magnification). B) The macrophage scores were significantly increased after exposure to 0.4 and 0.8 mg/kg zinc chromate for 90 days compared with saline treated animals (*$p < 0.0001$). There were no statistically significant differences in macrophage aggregation between female rats and male rats. Results are reported as mean ± SEM ($n = 7$ rats per treatment group).

4. Discussion and conclusion

Particulate Cr(VI) is a well-known lung carcinogen but the early and initiating carcinogenic mechanisms are still poorly understood. Establishing an accurate and relevant exposure regimen is crucial to further our understanding for environmentally relevant exposures. Few studies have investigated different mechanistic effects of repeated particulate Cr (VI) exposure in vivo, and those that have been conducted were done in various mouse models. These studies have implicated a transitional immune response and increased survival signaling (Beaver et al., 2009a, 2009b); activation of EGFR and bronchial hyperplasia (Kim et al., 2015); and aberrant remodeling of alveolar epithelial cells and increased CXCL5 and CXCR2 expression in lungs (Ge et al., 2022).

One study considered repeated oropharyngeal aspiration of calcium chromate for 26 weeks using A/J mice and reported chronic inflammation, hyperplasia and a dramatic increase in bronchiolo-alveolar carcinomas (Zeidler-Erdely et al., 2020). In the current study, we observed similar markers of inflammation, but carcinomas were not observed. This difference may be due to several reasons. First, A/J mice are more susceptible to lung cancers, which is why they are commonly used for carcinogen testing. Second, our study used a shorter Cr(VI) exposure time (13 weeks vs 26 weeks). Lastly, the amount of time following Cr(VI) exposure differed dramatically; the Ziedler-Erdely study treated animals for 26 weeks but did not conduct their analyses until the mice were 70 weeks old whereas our study considered effects one week after the final Cr(VI) treatment thus animals were still relatively young (25 weeks old). Animals from this last study were also used to investigate epitranscriptomic dysregulations as evidenced by increased levels of total RNA N6-methyladenosine (m6A) modification and RNA m6A methyltransferase like-3 (METTL3) (Wang et al., 2022). No animal studies have considered the influence of particulate Cr(VI) on the mechanisms behind chromosome instability.

Here we present a new model of exposure to particulate Cr(VI) exposure in rats. Using a weekly, repeated exposure for 90 days, we showed that this treatment regimen is minimally invasive and has no major changes on the physiology of the animals. All animals remained healthy, and no tumors developed in the treatment time frame. There were no gross changes in body mass or lung mass between saline treated and zinc chromate treated rats.

We showed that animals had increased levels of Cr in the lungs, blood and reproductive organs, showing that exposure via the respiratory tract results in widespread and consistent exposure throughout the body. We did not find a notable difference in Cr accumulation between bronchial and alveolar regions as observed in the Takahashi et al. (2005) study; this is likely due to the different treatment parameters applied and the length of exposure. In that study a single administration of a strontium chromate pellet was directly inserted into the bronchus, then tissue was analyzed around lesions which developed 9 months later. It may be that more exposure time or larger doses are required to discern difference between the lung regions. This may also be due to a higher rate of turnover in bronchial epithelial lining compared to alveolar cells in general and particularly with lung cell injury (Bowden, 1983).

We found an increase in macrophage accumulation in the lungs of all treated animals; increased macrophage infiltration is a common and expected response to many lung irritants. This is consistent with another repeated dose study performed in mice using welding fume-based metals, specifically calcium chromate once a week for 26 weeks (Zeidler-Erdely et al., 2008). It is also consistent with findings of increased macrophage infiltration in the lungs using nasal aspiration of zinc chromate particles repeated five times within 64 days (Beaver et al., 2009b). The presence of macrophages can induce a microenvironment of increased reactive oxygen and reactive nitrogen species which can in turn generate DNA damage; however given that significant DNA and chromosome damage is observed in cell culture studies without the presence of macrophages this is unlikely to be a driving factor in the mechanism of Cr(VI)-induced carcinogenesis (Speer and Wise Sr., 2018; Wise et al., 2008; Wise and Wise, 2019).

Importantly we have shown that respiratory exposure to particulate Cr(VI) induces chromosomal damage including both structural and numerical effects in circulating blood. These results are consistent with monitoring studies of chromate workers (Sbrana et al., 1990; Kortenkamp, 1997). Looking at chromosome instability within the lungs of these animals is more challenging. Future work will use chromosome probes to analyze numerical changes in these lung tissues as well as mechanisms such as centrosome amplification. Structural changes cannot be measured directly in fixed tissues; however, ongoing work in our laboratory is investigating the mechanisms underlying both structural and numerical chromosome instability including inhibition of RAD51, an important regulator in high fidelity homologous recombination repair of DNA double strand breaks. In sum, oropharyngeal

aspiration is a reliable method for investigating the early and potentially initiating mechanistic effects of prolonged and repeated particulate Cr (VI) treatment in rats.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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