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Investigating the Effect of Arsenic on Chromium(VI)-Induced Genotoxicity in Human Lung Fibroblasts

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Investigating the Effect of Arsenic on Chromium(VI)-Induced Genotoxicity in Human Lung Fibroblasts

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A THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF APPLIED MEDICAL SCIENCES – TOXICOLOGY

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We hereby recommend that the thesis of Jason Stewart entitled

Investigating the effects of Arsenic on Hexavalent Chromium(VI)-induced Genotoxicty in Human Lung Fibroblasts

be accepted as partial fulfillment of the requirements for the Degree of Master of Science in Applied Medical Sciences

Advisory Committee:

Chairperson

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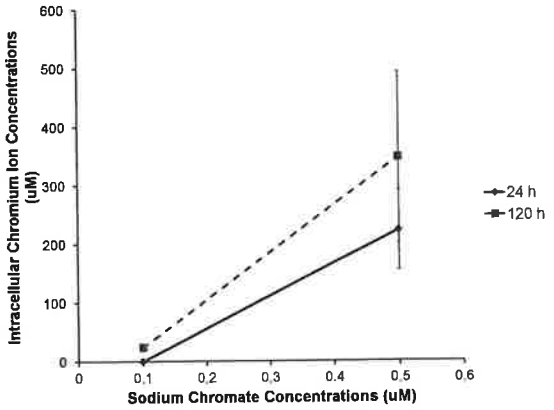

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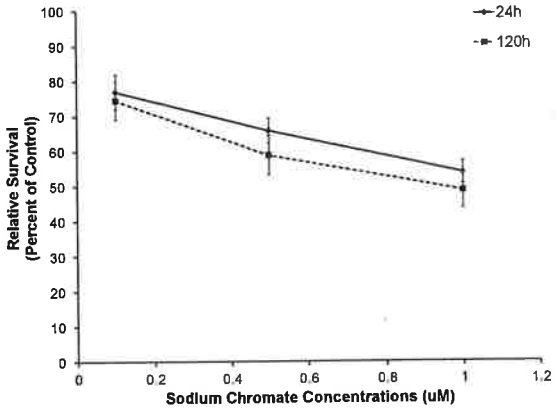

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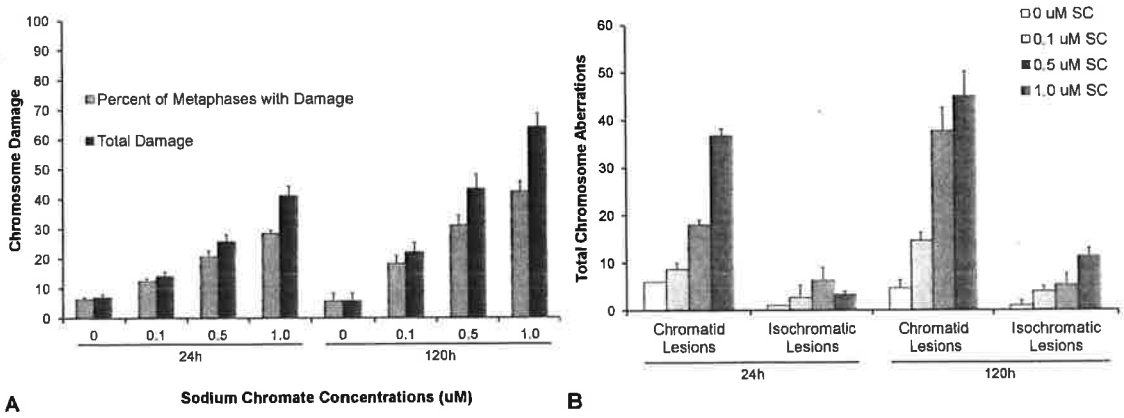

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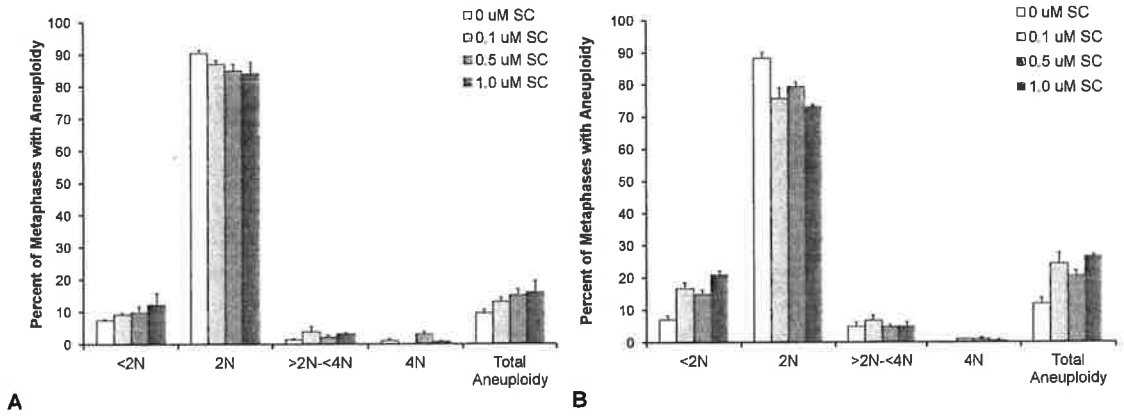

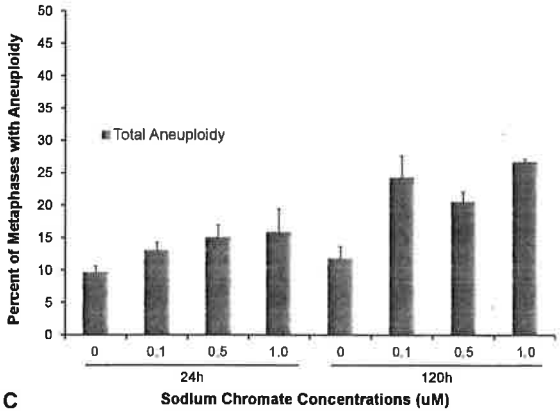

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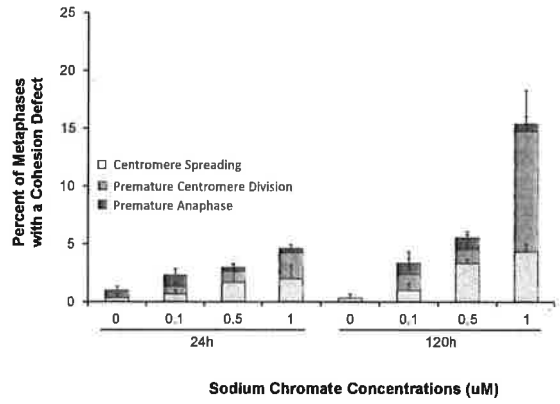

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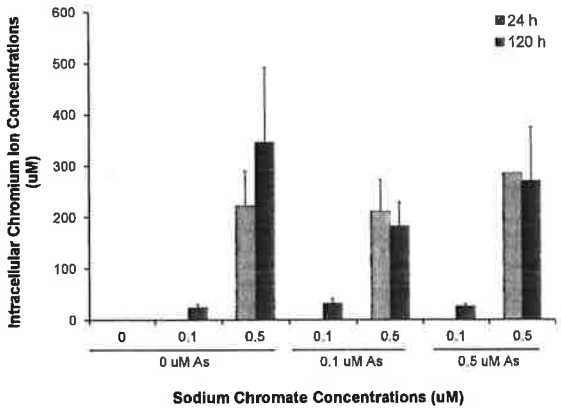

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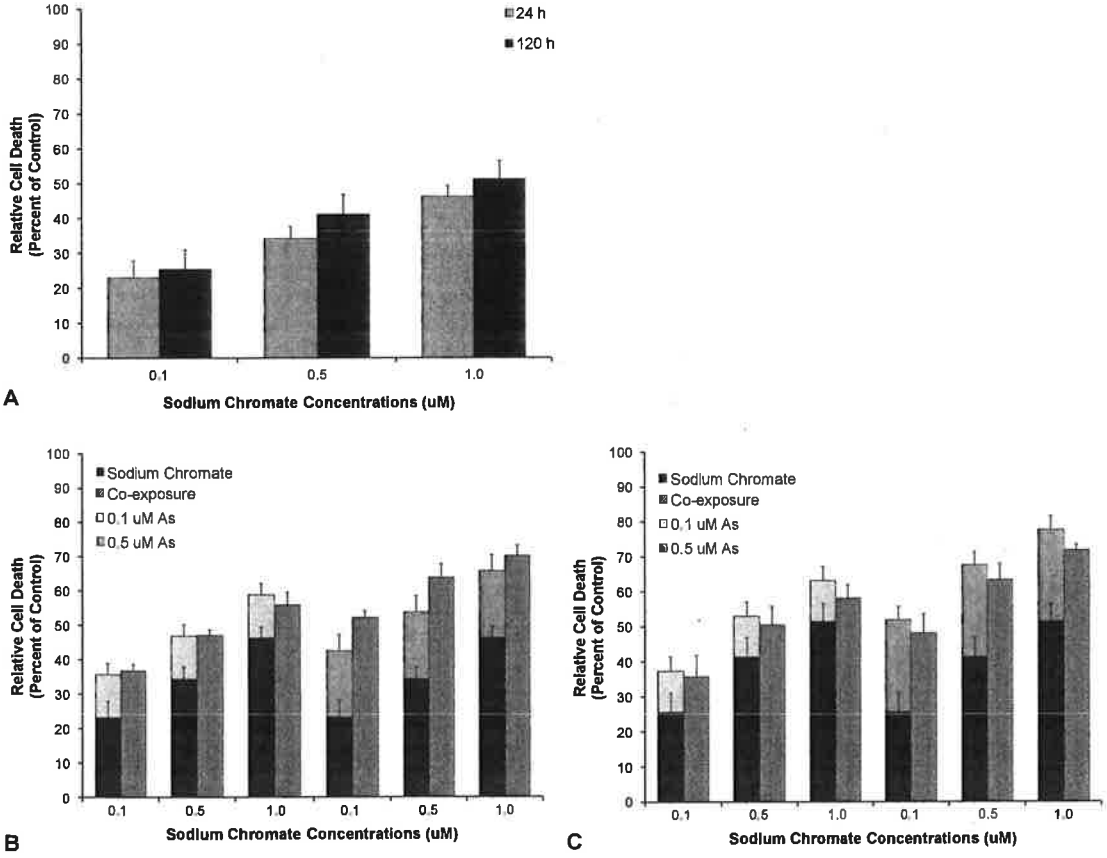

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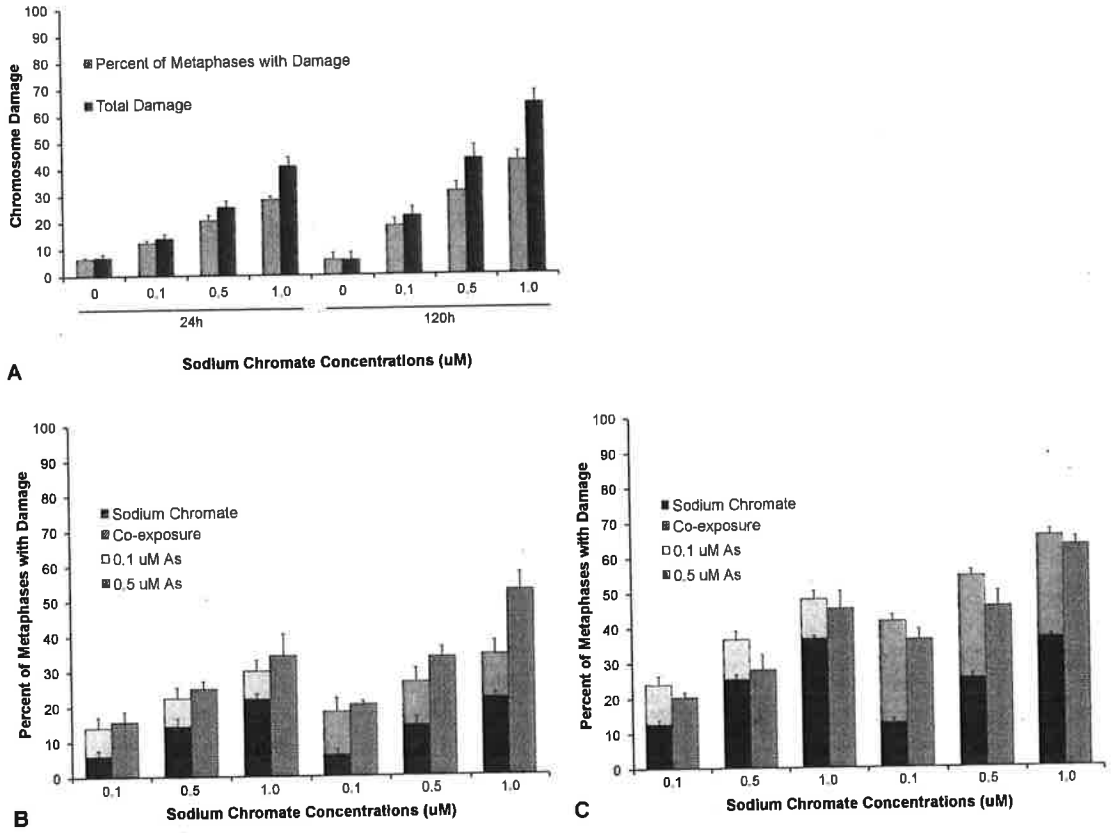

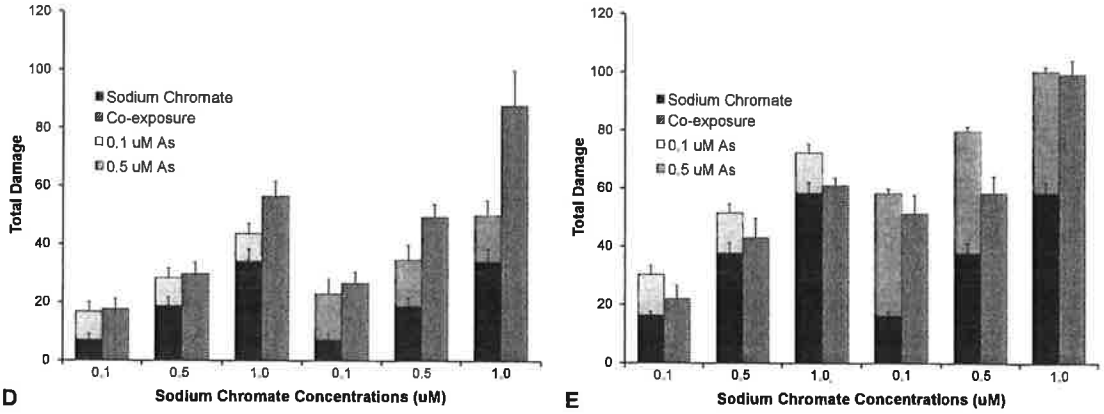

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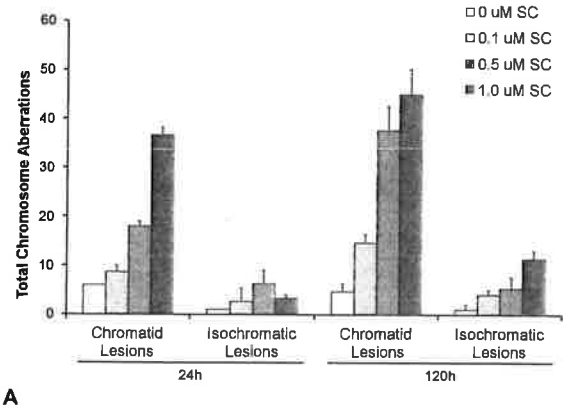

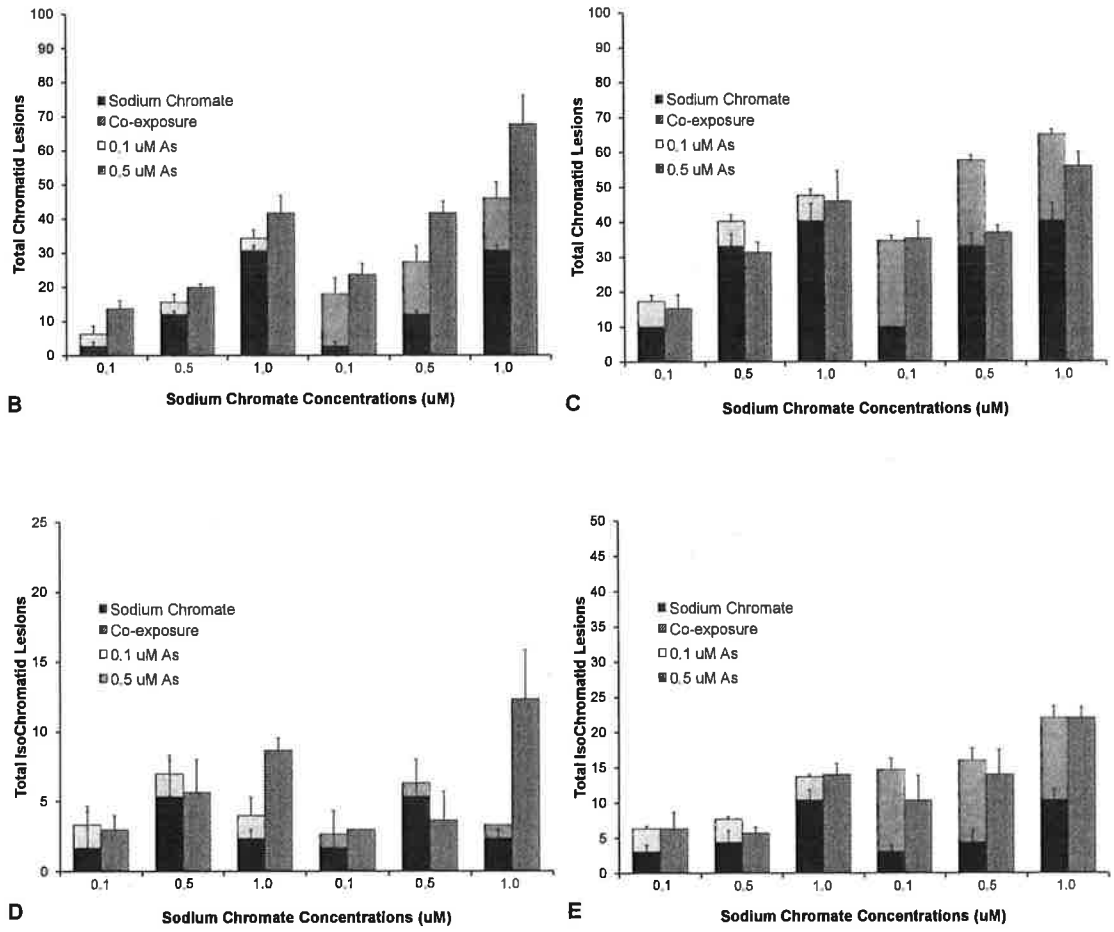

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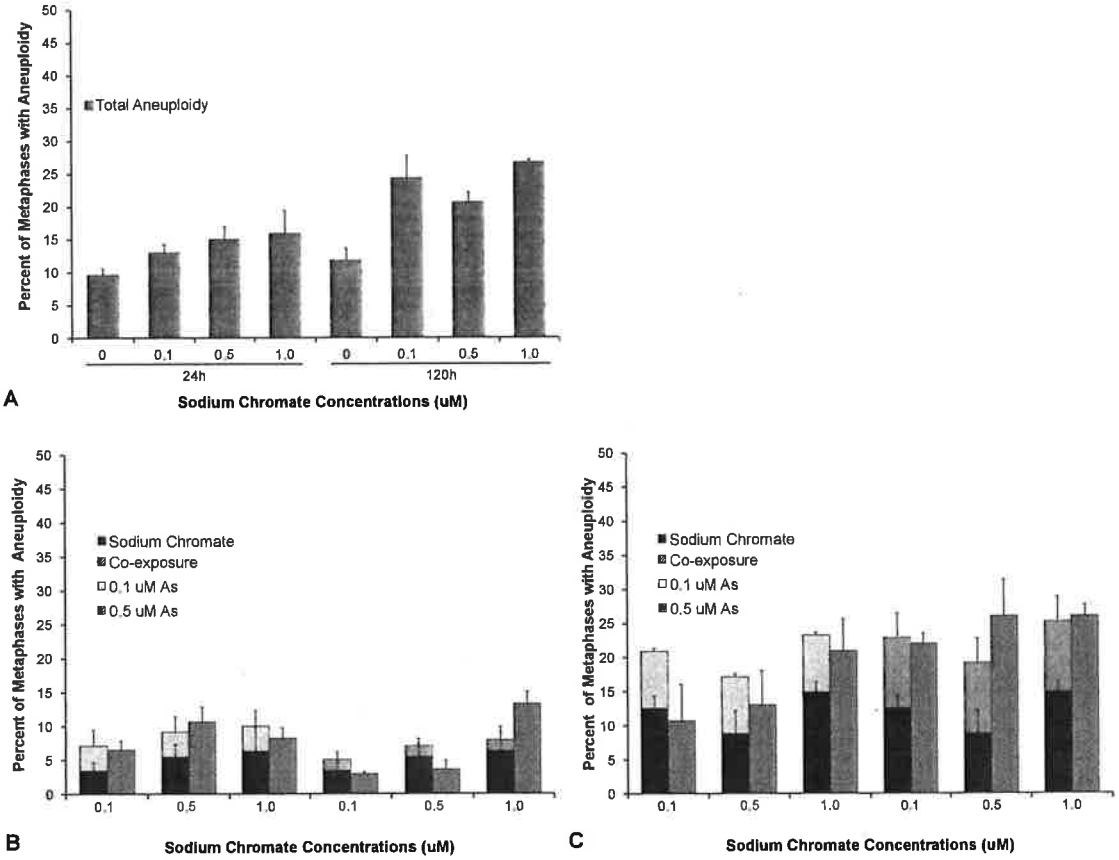

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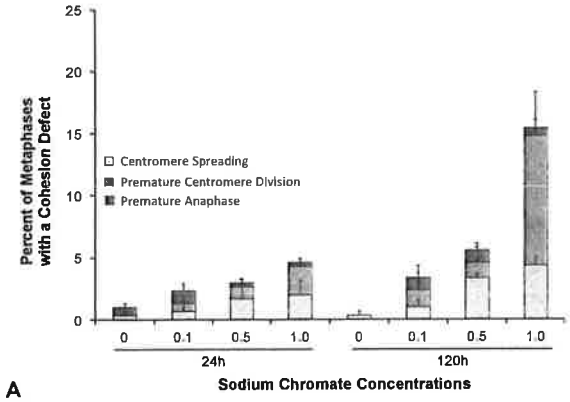

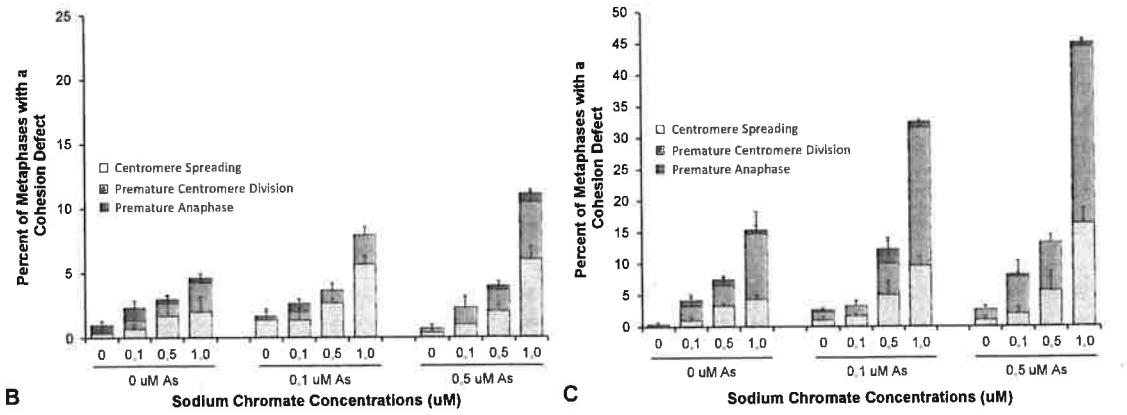

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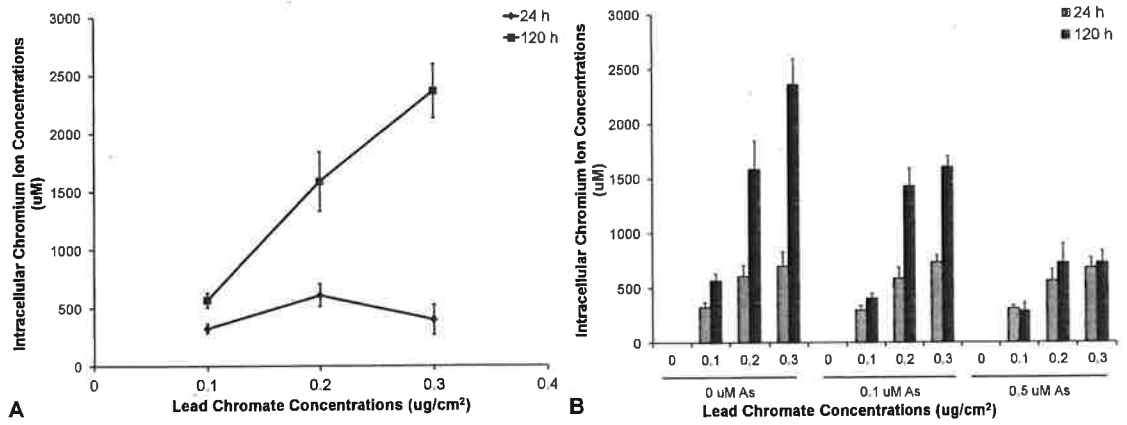

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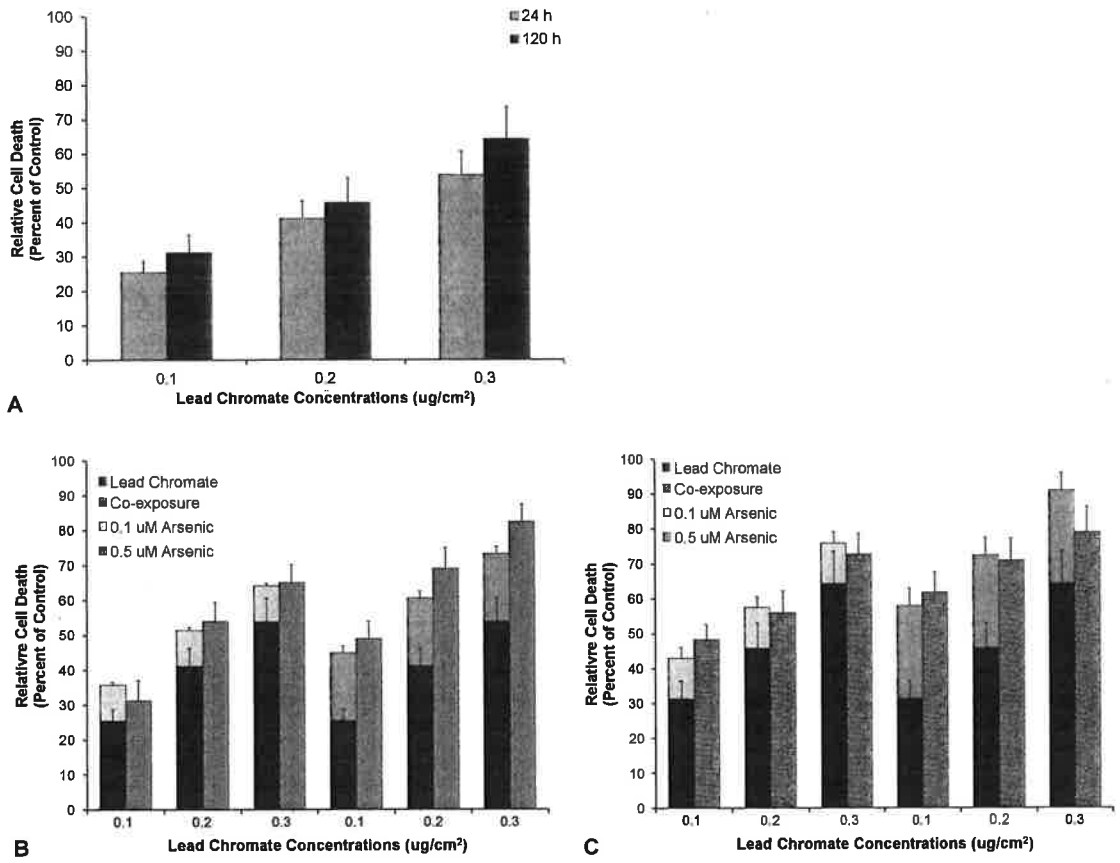

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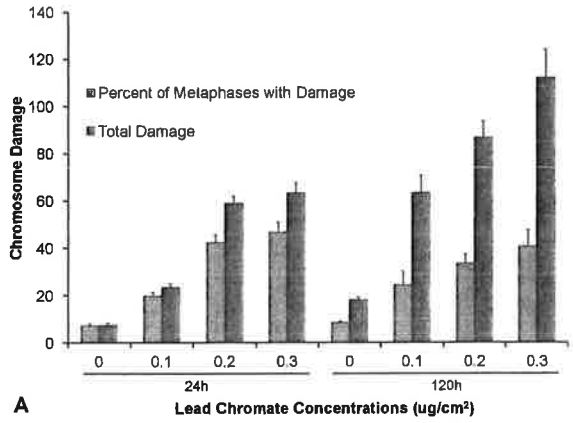

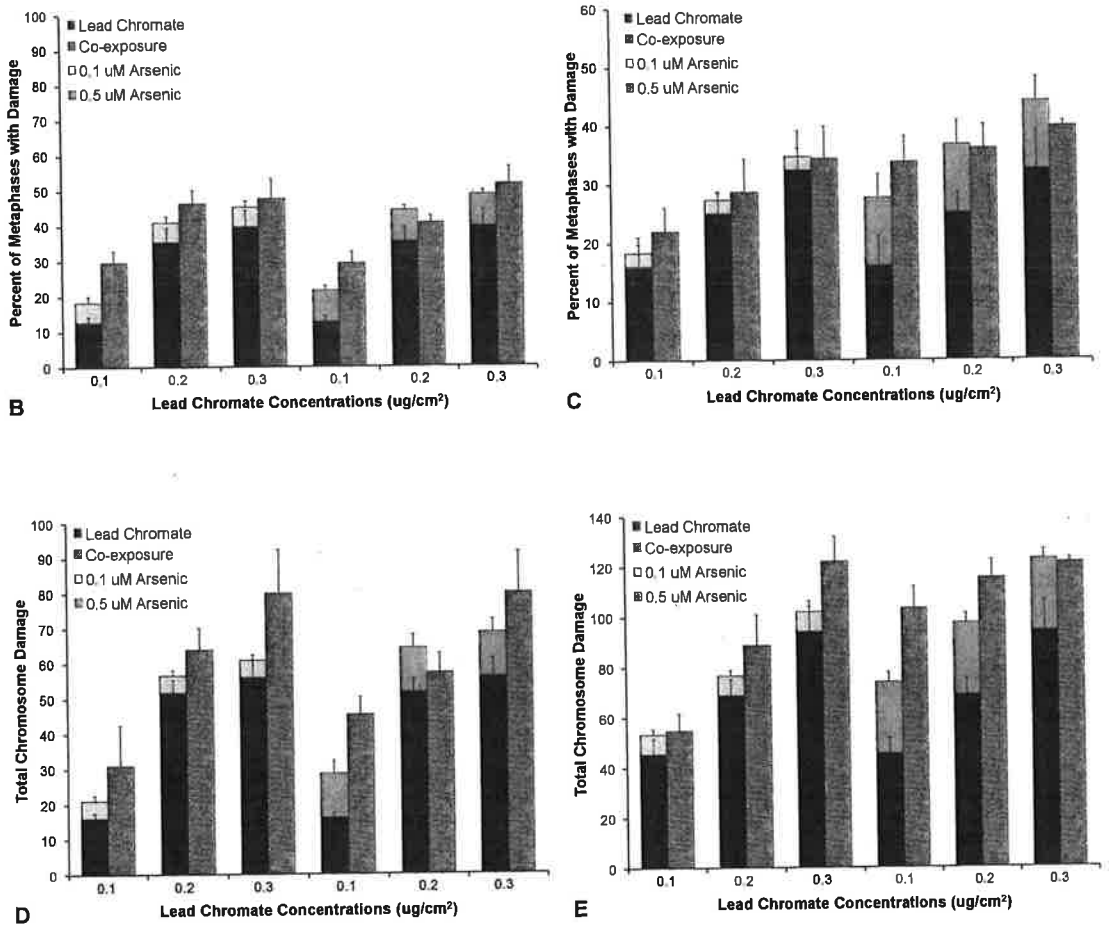

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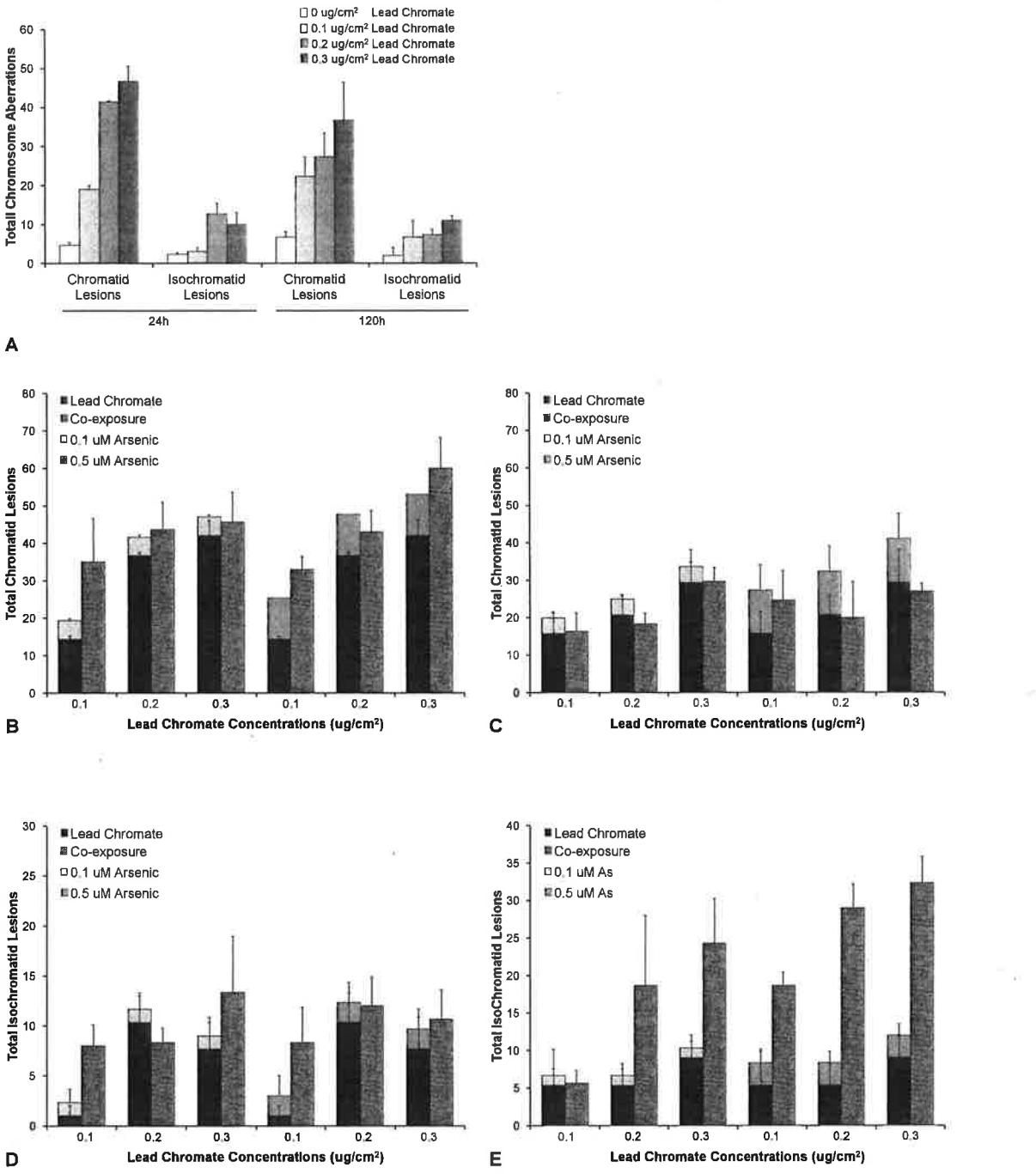

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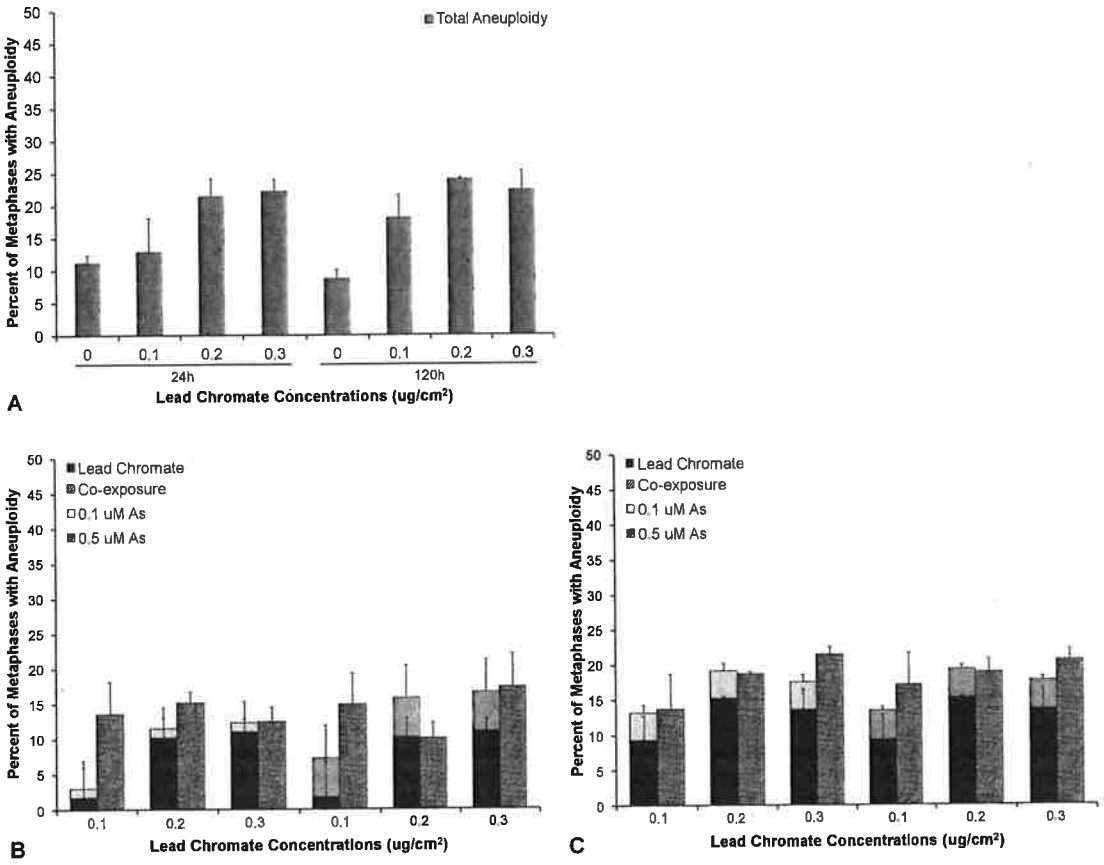
Panels **D)** and **E)** show there was a time and concentration-dependent increase in total isochromatid lesions in cells co-exposed to LC and As after acute and prolonged exposure (respectively). In regards to isochromatid lesions, there was a greater-than-additive effect induced after prolonged co-exposure to CL and As concentrations. Solid black bars represent LC concentrations alone. Light and dark grey bars represent 0.1 and 0.5 uM As, respectively. Black/White hashed bars represent co-exposed cells. Data represents an average of three experiments ± standard error of the mean.



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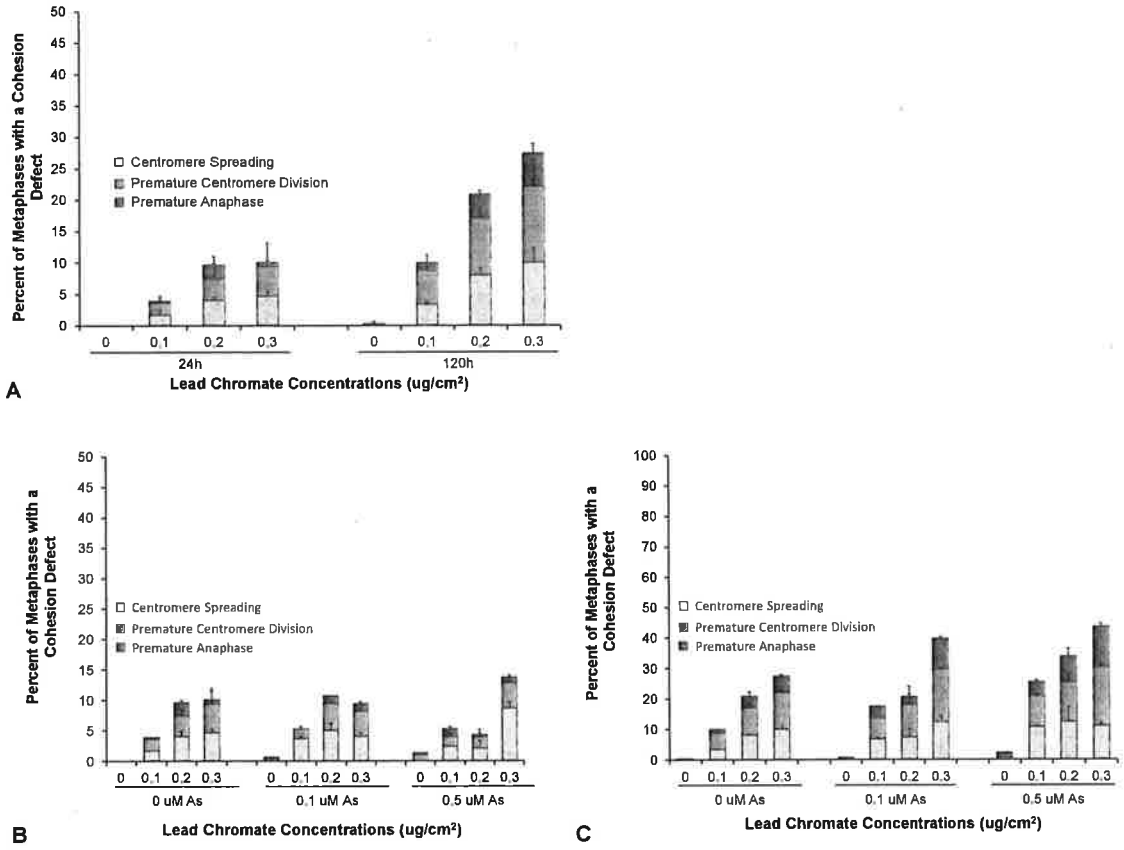

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Investigating the Effect of Arsenic on Chromium(VI)-Induced Genotoxicity in Human Lung Fibroblasts

1. Introduction

Hexavalent chromium (Cr(VI)) compounds are well-established human lung carcinogens. Arsenic (As) is a well documented human carcinogen, with long term exposure to As in drinking water being linked to lung, kidney and liver cancer, among others. Co-exposure studies are now becoming more common as humans are not exposed to just one toxicant at a time. This is the first study to investigate the effects of co-exposure with arsenic and soluble or particulate Cr(VI) compounds in human lung fibroblasts.

Solubility of Cr(VI)

There are many Cr(VI) compounds ranging in solubility from water-soluble to almost entirely water-insoluble. Solubility plays a key role in the potency of the various Cr(VI) compounds with the most potent compounds identified as the water-insoluble or 'particulate' Cr(VI) compounds (1-4). Exposure to particulate and soluble Cr(VI) compounds have been investigated and compared out to 72 h (5) of exposure, however no study has investigated the effects of the soluble Cr(VI) compound sodium chromate (SC) out to 120 h of exposure.

Sources of Cr(VI)

Cr(VI) is used industrially in paints, dyes and inks; it is also used for chrome plating, leather tanning and wood preserving (6). It is also used as an anticorrosive in industrial cooling towers and military vehicles such as tanks and helicopters (6). Cr(VI) is found in the combustion of fossil fuels, the exhaust of catalytic converters, in the dust from cement and brake linings and also present in cigarette smoke (6-7). Primarily, Cr(VI) enters the environment through anthropogenic sources. Soluble Cr(VI) compounds are released into waste streams from electroplating, leather tanning and textile industries.

Human Exposure to Cr(VI)

Human exposure to Cr(VI) is primarily through industrial settings like chromate production, chrome pigment production and chrome plating. In 2006, OSHA lowered the permissible occupational exposure limit of Cr(VI) from 52 ug/m^3 to 5 ug/m^3 although this level still carries significant carcinogenic risk (8).

Carcinogenesis of Cr(VI)

Cr(VI) readily enters the cell through the anion transport system (9). Once in the cell, Cr(VI) is rapidly reduced by reducing agents like glutathione and ascorbate (8) to the trivalent form of chromium, Cr(III). The reduction of Cr(VI) to Cr(III), and the production of the highly reactive intermediaries Cr(IV) and Cr(V), also releases reactive oxygen species, which are also known to cause DNA damage (10). The

ultimate carcinogenic agent is unknown as any of these intermediates as well as Cr(III) have the ability to react with and damage DNA.

Human Exposure to As

As is an abundant naturally occurring element found in the earth's crust (11). It is also released into the environment from human activities such as mining, electronics manufacturing and farming. These human activities have lead to increased arsenic levels in groundwater and food which resulted in the United States Environmental Protection Agency (EPA) to lower the drinking water standard from 50 ug/L to 10 ug/L to protect populations from the long-term effects of arsenic exposure (12). Arsenic has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC). Studies show that chronic inorganic arsenic exposure leads to the development of lung, skin, liver, kidney and urinary bladder cancers (13). Among these cancers, lung cancer is a major public health concern due to its high incidence rate and mortality (14). Though inhalation is one pathway by which arsenic can induce lung cancer, a significant dose-response relationship between the ingestion of inorganic arsenic in drinking water and increased lung cancer risks was found in Bangladesh (15),Taiwan (16-17) and Chile (18). It has also been determined (19) that moderate concentrations (< 7.5 ppm) of arsenic significantly impact lung cancer incidence which suggests non-occupational exposures, like environmental, to arsenic could be a contributor and concern for lung cancer.

As Carcinogenesis

The mechanism of arsenic-induced lung cancer is uncertain; however several hypotheses have proposed that genotoxicity, induction of oxidative stress and inhibition of DNA repair (20) may play integral parts of the carcinogenic mechanism. In regards to genotoxicity it has been determined that (22-23) arsenic induced both DNA single strand breaks and DNA-protein crosslinks in human fetal lung fibroblasts. This ability of arsenic to induce DNA aberrations is proof for further investigation into the mechanism of arsenic induced carcinogenesis.

Co-exposure Studies

Co-exposure studies are now becoming more common as humans are not exposed to just one toxicant at a time. Cr(VI) and As are both well known environmental and occupational hazards and are often found as mixed contaminants and wastes. A study into the co-exposure of mouse skin cells with benzo[*a*]pyrene and arsenic saw an increase in DNA adduct levels in co-treated cells (23). Despite that people are exposed to Cr(VI) and As simultaneously under most conditions and the potential to cause lung cancer is greater, little is known of the potential impact of co-exposure. Accordingly, the objective of this study was to determine the cytotoxicity and genotoxicity of co-exposure to Cr(VI) and arsenic in human lung fibroblast cells.

2. Materials and Methods

2.1 Chemicals and Reagents

Lead chromate. sodium chromate, sodium metaarsenite, demecolchicine and potassium chloride were purchased from Sigma/Aldrich (St. Louis, MO). Giemsa stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Sodium dodecyl sulfate (SDS) was purchased from American Bioanalytical (Natick, MA). Trypsin/EDTA, sodium pyruvate, penicillin/streptomycin, L-glutamine and Gurr's buffer were purchased from Invitrogen Corporation (Grand Island, NY). Methanol, acetone and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Dulbecco's minimum essential medium and Ham's F-12 (DMEM/F-12) 50:50 mixture was purchased from Mediatech Inc. (Herndon, VA). Cosmic calf serum (CCS) was purchased from Hyclone (Logan, UT). Tissue culture dishes, flasks and plasticware were purchased from Corning Inc. (Acton, MA).

2.2 Cells and Cell Culture

WTHBF-6 cells, a clonal cell line generated from normal human bronchial fibroblasts transfected with hTERT, were used in this study. These cells have similar clastogenic and cytotoxic responses to metals compared to their parent cells (24). Cells were maintained as subconfluent monolayers in DMEM/F-12 supplemented with 15% CCS, 2 mM L-glutamine, 100 U/ml penicillin/100 ug/ml streptomycin and 0.1 mM sodium pyruvate and incubated in a 5% CO_2 humidified environment at 37°C. They were fed three times a week and subcultured at least once a week using 0.25% trypsin/1mM EDTA solution. All experiments were performed on logarithmically growing cells.

2.3 Preparation of Chemicals

Lead chromate (LC), a model particulate Cr(VI) compound, was administered as a suspension in cold, sterile filtered water as previously described (25). Sodium chromate (CAS #7775-11-3, ACS reagent minimum 98% purity, Lot: MKBJ9938V) and sodium metaarsenite (As) were used as soluble compounds and were administered as a solution in water as previously described (25).

2.4 Determination of Intracellular Chromium and Arsenic Ion Levels

Cells were prepared for determination of intracellular Cr and As ion levels as previously described (27-28). Briefly, a monolayer of cells was treated with 0.1, 0.5 uM and 1.0 SC and 0.1 and 0.5 uM As or 0.1, 0.2 and 0.3 ug/cm^2 LC and 0.1 and 0.5 uM As (single and co-exposure of each compound for comparison) 24 and/or 120 h. Cells were then harvested and swelled with hypotonic solution followed by

2% SDS to degrade the cell membrane. This solution was then sheered through a needle seven times and filtered. Cr ion concentrations of the samples were then measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) as described previously (28).

2.5 Cytotoxicity

Cytotoxicity was determined by a clonogenic assay, which measures the reduction in plating efficiency in treatment groups relative to the controls as previously described (25). Briefly, cells were seeded into a 6 well tissue culture plate and allowed to grow for 48 h. The cultures were then treated for 24 h or 120 h with sodium chromate (SC), SC + As, LC or LC + As. Dishes in the 120 h SC or SC + As co-exposure experiments were retreated every 24 h with SC or SC + As after renewing media. After the exposure time, the treatment medium was collected (to include any loosely adherent mitotic cells); the cells were rinsed with PBS; and then removed from the dish with 0.25 % trypsin/1mM EDTA solution. Cells were centrifuged at 1000 rpm, 4°C for 5 min. The resulting pellet was re-suspended in 5 ml of medium, counted with a Coulter Multisizer III, and reseeded at colony forming density. Colonies were allowed to grow for 10 days, fixed with 100% methanol, stained with crystal violet, and the colonies counted. There were four dishes per treatment group and each experiment was repeated at least three times.

2.6 Clastogenicity

Clastogenicity was determined by measuring the production of chromosomal aberrations based on our published methods with minor modifications (25). Briefly, logarithmically cells were seeded in 100 mm dishes; allowed 48 h to plate and resume normal cycling; and then treated for 24 or 120 h with SC, SC + As, LC, or LC + As. Dishes in the 120 h SC or SC + As co-exposure experiments were retreated every 24 h with SC or SC + As after renewing media. Shortly before the end of the treatment time, 0:1 g/ml demecolchicine was added to block the cells in metaphase. At the end of treatment, the culture medium and adherent cells were collected. Cells were then resuspended in 0.075M KCl hypotonic solution for 17 min to swell the cells and the nuclei. Next, cells were fixed in 3:1 methanol:acetic acid and the fixative was changed twice. Finally, cells were dropped onto clean wet slides and uniformly stained with 5% Giemsa stain in Gurr's buffer.

Chromosomal aberrations were scored blind using standard criteria by two different scorers (25). Because deletions can only be unequivocally distinguished from achromatic lesions if the distal acentric fragment is displaced, these aberrations were pooled. This approach also helps avoid artificial discrepancies between scorers due to different perceptions of the width of an achromatic lesion relative to the width of its chromatid. Furthermore, recent data show a much stronger correlation between DNA

strand breaks and chromosomal aberrations if these achromatic lesions and deletions are pooled (26). Accordingly, chromatid deletions and achromatic lesions were pooled as chromatid lesions and isochromatid deletions and isochromatid achromatic lesions were pooled as isochromatid lesions. One hundred metaphases per data point were analyzed in each experiment. Each experiment was repeated at least three times.

3. Results

3.1 Intracellular Levels of Sodium Chromate in Human Lung Fibroblasts

To determine whether intracellular chromium ion concentrations increased with prolonged exposure to Cr(VI), we performed uptake experiments. We found that intracellular chromium ion concentrations increased in a time and concentration-dependent manner after prolonged exposure to sodium chromate (Figure 1). Specifically, cells exposed to 0.1 and 0.5 uM SC after 24 h saw intracellular chromium ion concentrations of 0 and 223 uM respectively. Then after 120 h of exposure to SC, intracellular chromium ion concentrations increased to 24 and 347 uM, respectively. Cells were not exposed to 1.0 uM SC for 24 or 120 h timeframes due to a communication error and time constraints.

3.2 Sodium Chromate is Cytotoxic to Human Lung Fibroblasts

We used the clonogenic survival assay as a cytotoxicity marker of the WTHBF-6 human fibroblast cell line treated with SC at concentrations of 0.1, 0.5 and 1.0 uM for 24 and 120 h to mimic acute and prolonged exposure. After exposure, SC induced a concentration and time dependent decrease in relative survival. Specifically, after 24 h the relative survival of cells exposed to 0.1, 0.5 and 1.0 uM SC was 77, 66 and 54 percent, respectively. Cells exposed for 120 h saw a reduction of relative survival to 75, 59 and 49 percent, respectively. (Figure 2).

3.3 Sodium Chromate Induces Chromosome Damage in Human Lung Fibroblasts

Next we used the clastogenicity assay as a marker for genotoxicity in the WTHBF-6 cell line. We considered two measures of damage for our chromosome damage studies. We measured the percent of metaphases with damage as a determination of the frequency of damaged cells. We also measured the total aberrations per 100 metaphases as a determination of the extent of damage within cells. After 24 and 120 h exposure to SC we saw a concentration and time-dependent increase in percent of metaphases with damage and total aberrations (Figure 3A). Specifically, after 24 h of exposure to 0, 0.1,

0.5 and 1.0 uM of SC there was 6, 12, 21 and 28 percent of metaphases with damage. After 120 h of exposure to the same concentrations we saw an increase to 6, 18, 31 and 43 percent of metaphases with damage, respectively. When considering total chromosome aberrations, 24 h of exposure to the concentrations of SC saw 7, 14, 26 and 41 total damage, respectively. 120 h of exposure saw 6, 22, 43 and 54 total chromosome aberrations.

With chromosome damage results we also wanted to determine specific damage caused to cells by SC. To investigate specific damage we designated damage as chromatid lesions and isochromatid lesions, with the latter being the rarer of the two specific damages. After 24 and 120 h exposure to SC, WTHBF-6 cells we saw a concentration and time-dependent increase in specific chromosome aberrations (Figure 3B). After 24 h exposure to SC concentrations there was 6, 9, 18 and 37 chromatid lesions, respectively; and 1, 3, 6 and 3 isochromatid lesions per 100 cells, respectively. Then after 120 h exposure we saw 5, 15, 38 and 50 chromatid lesions, respectively; and 1, 4, 5 and 11 isochromatid lesions in 100 metaphases, respectively.

3.4 Prolonged Sodium Chromate Exposure Induces Aneuploidy in Human Lung Fibroblasts

Numerical aberrations manifested as Aneuploidy, polyploidy and tetraploidy. Cells were grouped based on chromosome number into diploid (46 chromosomes), hypodiploid (<46 chromosomes), hyperdiploid (between 47 and 91 chromosomes), and tetraploid (92 chromosomes). Prolonged exposure to 0.1, 0.5 and 1.0 uM SC induced aneuploidy in human lung fibroblasts (Figure 4B-C). 24 h of exposure to the same concentrations of SC did not induce aneuploidy. Specifically, after 120 h of exposure to 0, 0.1, 0.5 and 1.0 uM SC we saw 12, 24, 21 and 27 percent total aneuploidy in 100 metaphases, respectively. Hypodiploid cells jumped from 7 percent in the control to 17, 15 and 21 percent in cells treated with 0.1, 0.5 and 1.0 uM SC respectively. There was very little increase in hyper-and tretraploidy found in SC treated cells.

3.5 Prolonged Sodium Chromate Exposure Induces Metaphase Damage in Human Lung Fibroblasts

Chromosomal aberrations and aneuploidy can result from a defective mitotic checkpoint (28-29). Thus, we investigated whether SC induced mitotic abnormalities in WTHBF-6 cells. We found that SC does not induce metaphase damage after 24 h exposure; however after 120 h exposure SC induces a concentration-dependent increase in metaphase damage in fibroblasts (Figure 5). Specifically, after 120 h exposure to 0, 0.1, 0.5 and 1.0 uM SC we saw 0.3, 3, 6 and 15 percent of 100 metaphases with a cohesion defect, respectively. Specific metaphase damage seen include centromere spreading, premature centromere division and premature anaphase. Centromere spreading was defined as a cell in

which centromere separated in at least one chromosome. Premature centromere division was defined as a cell in which at least one chromosome was still attached to its sister chromatid and at least one chromosome was completely separated from its sister chromatid. Premature anaphase was defined as cells in which all of the sister chromatids were completely separated from each other.

3.6 Arsenic Does Not Inhibit Intracellular Chromium Ion Uptake after Co-exposure with Sodium Chromate in Human Lung Fibroblasts

Next we wanted to determine whether arsenic inhibited the cellular uptake of chromium ions in human lung fibroblasts after co-exposure with SC, thus we performed uptake experiments as before. We found that intracellular chromium ion uptake was not inhibited by arsenic after co-exposure with the SC and As (Figure 6). Specifically, cells initially exposed to 0.1 and 0.5 uM after 24 saw intracellular chromium ion concentrations of 0 and 223 uM, respectively. Co-exposure of cells with the above mentioned SC concentrations with 0.1 uM As intracellular chromium ion concentrations were 0 and 211 uM, respectively. Co-exposure of cells with SC concentrations and 0.5 uM As after 24 h were 0 and 285 uM, respectively.

Next we analyzed for prolonged (120 h) of exposure to SC and then co-exposure to SC and As (Figure 6). Initially after 120 h of exposure to 0.1 and 0.5 uM SC we found the intracellular chromium ion concentrations increased to 24 and 347 uM, respectively. After cells were co-exposed to SC and 0.1 uM As ion uptake was 32 and 183 uM, respectively. Co-exposure of cells with SC concentrations and 0.5 uM As after 120 h were 26 and 270 uM, respectively. Cells were not exposed to 1.0 uM SC, and subsequent co-exposure with 0.1 or 0.5 uM As due to a communication error and time constraints.

3.7 Arsenic Increases Sodium Chromate Induced Cytotoxicity to Human Lung Fibroblasts

We used the clonogenic survival assay as a cytotoxicity marker of the WTHBF-6 human fibroblast cell line treated with SC at concentrations of 0, 0.1, 0.5 and 1.0 uM for 24 and 120 h to mimic acute and prolonged exposure. After exposure, SC induced a concentration and time dependent decrease in relative survival. Specifically, after 24 h the relative cell death of cells exposed to 0.1, 0.5 and 1.0 uM SC was 23, 34 and 46 percent, respectively. Cells exposed for 120 h saw an increase cell death to 26, 41 and 51 percent, respectively. (Figure 7A).

Next we treated cells with 0.1 and 0.5 uM As alone, each producing 13 and 20 then 12 and 26 percent cell death for 24 and 120 h of exposure, respectively. When cells were co-treated with the above mentioned SC concentrations and 0.1 uM As, cell death percentages after 24 h of exposure were increased to 37, 47 and 56 percent, respectively. Co-exposure with SC and 0.5 uM As after 24 h saw an

increase to 52, 64 and 70 percent, respectively (Figure 7B). Cells co-treated with SC and 0.1 uM As after 120 h saw an increase in relative cell death to 36, 51 and 58 percent, respectively; cells co-exposed with SC and 0.5 uM As for 120 h saw an increase to 48, 63 and 72 percent, respectively (Figure 7C).

3.8 Arsenic Increases Sodium Chromate Induced Chromosome Damage in Human Lung Fibroblasts

Next we used the clastogenicity assay as a marker for genotoxicity in the WTHBF-6 cell line. We considered two measures of damage for our chromosome damage studies. We measured the percent of metaphases with damage as a determination of the frequency of damaged cells. We also measured the total aberrations per 100 metaphases as a determination of the extent of damage within cells. After 24 and 120 h exposure to SC we saw a concentration and time-dependent increase in percent of metaphases with damage and total aberrations (Figure 8A). Specifically, after 24 h of exposure to 0, 0.1, 0.5 and 1.0 uM of SC there was 6, 12, 21 and 28 percent of metaphases with damage. After 120 h of exposure to the same concentrations we saw an increase to 6, 18, 31 and 42 percent of metaphases with damage, respectively. When considering total chromosome aberrations, 24 h of exposure to the concentrations of SC saw 7, 14, 26 and 41 total damage, respectively. 120 h of exposure saw 6, 22, 43 and 64 total chromosome aberrations. (Figure 8A)

Next we co-exposed cells to the above concentrations of SC with 0.1 or 0.5 uM As. After 24 h of exposure cells exposed to 0, 0.1, 0.5 and 1.0 uM SC and 0.1 uM As saw an increase in percent of metaphases with chromosome damage to 14, 22, 31 and 41 percent, respectively. After 120 h of co-exposure to the SC and 0.1 uM As we saw a jump to 17, 26, 34 and 51 percent damage, respectively. WTHBP-6 cells exposed for 24 h to SC and 0.5 uM As saw an increase to 19, 27, 40 and 59 percent damaged metaphases, respectively. After 120 h of co-exposure to the two metals, we saw a jump to 35, 42, 51 and 69 percent of metaphases with damage, respectively. (Figure 8B-C)

When considering total chromosome aberrations, cells exposed to just SC for 24 and 120 h saw 7, 14, 26 and 41 then 6, 22, 43 and 64, respectively. When cells were co-exposed with SC and 0.1 uM As for 24 and 120 h, total aberrations jumped to 17, 25, 37 and 63; and 20, 28, 49 and 67, respective of concentration and time. Cells that were co-exposed with SC and 0.5 uM As for 24 and 120 h, there was an increase in total aberrations to 23, 34, 56 and 95; and 48, 57, 64 and 105, respective of concentration and time. (Figure 8D-E)

With chromosome damage results we also wanted to determine specific damage caused to cells by SC and As co-exposure. To investigate specific damage we designated damage as chromatid lesions and isochromatid lesions, with the latter being the rarer of the two specific damages. After 24 and 120 h

exposure to SC, WTHBF-6 cells saw a concentration and time-dependent increase in specific chromosome aberrations (Figure 9A). After 24 h exposure to SC concentrations there was 6, 9, 18 and 37 chromatid lesions, respectively; and 1, 3, 6 and 3 isochromatid lesions per 100 cells, respectively. Then after 120 h exposure we saw 5, 15, 38 and 50 chromatid lesions, respectively; and 1, 4, 5 and 11 isochromatid lesions in 100 metaphases, respectively.

Next we co-exposed WTHBF-6 cells with SC and 0.1 or 0.5 uM As and analyzed for both chromatid and isochromatid lesions (Figure 3B-E). After 24 and 120 h of co-exposure to 0, 0.1, 0.5 and 1.0 uM SC and 0.1 uM As we saw 10, 20, 26 and 48; and 12, 20, 36 and 51 total chromatid lesions, respectively. Cells co-exposed to the same SC concentrations and 0.5 uM As after 24 and 120 h saw a jump to 21, 30, 48 and 74; and 29, 40, 42 and 61 chromatid lesions, respectively (Figure 9B-C).

With regards to isochromatid lesions, cells exposed for 24 and 120 h to 0.1, 0.5 and 1.0 uM SC and 0.1 uM As saw 3, 4, 7 and 10; and 4, 7, 7 and 15 total isochromatid lesions, respectively. Cells that were co-exposed with SC and 0.5 uM As for 24 and 120 h saw an increase to 2, 4, 6 and 13; and 13, 11, 15 and 23 total isochromatid lesions, respectively. (Figure 9D-E).

3.9 Arsenic Increases Prolonged Sodium Chromate Induced Aneuploidy in Human Lung Fibroblasts

Prolonged exposure to 0.1, 0.5 and 1.0 uM SC induced aneuploidy in human lung fibroblasts (Figure 10A). 24 h of exposure to the same concentrations of SC did not induce aneuploidy. Specifically, after 120 h of exposure to 0, 0.1, 0.5 and 1.0 uM SC we saw 12, 24, 21 and 29 percent total aneuploidy in 100 metaphases, respectively. Hypodiploid cells jumped from 7 percent in the control to 17, 15 and 23 percent in cells treated with 0.1, 0.5 and 1.0 uM SC respectively. There was very little increase in hyper- and tretraploidy found in SC treated cells.

Next we co-exposed cells with 0, 0.1, 0.5, and 1.0 uM SC with either 0.1 or 0.5 uM As. We found the same trend with co-exposure as with SC solo exposure, that only prolonged co-exposure of human lung fibroblasts saw an increase in aneuploidy (Figure 10B-C). Cells exposed for 120 h to 0.1, 0.5 and 1.0 uM SC and 0.1 uM As saw 20, 23, 25 and 33 percent of metaphases with aneuploidy, respectively. Cells exposed to 0.1, 0.5 and 1.0 uM SC and 0.5 uM As for 120 h saw an increase in total aneuploidy to 22, 29, 34 and 38 percent, respectively.

3.10 Arsenic Increases Prolonged Sodium Chromate Induced Metaphase Damage in Human Lung Fibroblasts

We found that SC does not induce metaphase damage after 24 h exposure; however after 120 h exposure SC induces a concentration-dependent increase in metaphase damage in fibroblasts (Figure 11A). Specifically, after 120 h exposure to 0, 0.1, 0.5 and 1.0 uM SC we saw 0.3, 3, 6 and 15 percent of 100 metaphases with a cohesion defect, respectively.

Next we co-exposed cells with 0, 0.1, 0.5, and 1.0 uM SC with either 0.1 or 0.5 uM As. We found the same trend with co-exposure as with SC solo exposure, that only prolonged co-exposure of human lung fibroblasts saw an increase in metaphase damage (Figure 11B-C). After 120 h of co-exposure to SC and 0.1 uM As, cells saw an increase in metaphase damage to 3, 3, 17 and 25 percent, respectively. Cells co-exposed with SC and 0.5 uM As saw a further increase in percent of cells with a cohesion defect of 3, 8, 13 and 45 percent, respectively.

3.11 Arsenic Does Not Inhibit Intracellular Chromium Ion Uptake after Co-exposure with Lead Chromate in Human Lung Fibroblasts

Next we wanted to determine whether arsenic inhibited the cellular uptake of chromium ions in human lung fibroblasts after co-exposure with LC, thus we performed uptake experiments as before. We found that intracellular chromium ion uptake was not inhibited by arsenic after co-exposure with LC and As (Figure 12). Specifically, cells initially exposed to 0.1, 0.2 and 0.3 ug/m^2 LC after 24 saw intracellular chromium ion concentrations of 325, 609 and 698, respectively. Co-exposure of cells with the above mentioned LC concentrations with 0.1 uM As intracellular chromium ion concentrations were 294, 587 and 732, respectively. Co-exposure of cells with SC concentrations and 0.5 uM As after 24 h were 309, 563 and681, respectively.

Next we analyzed for prolonged (120 h) of exposure to LC and then co-exposure to LC and As. Initially after 120 h of exposure to 0.1, 0.2 and 0.3 ug/m^2 LC we found the intracellular chromium ion concentrations increased to 568, 1587 and 2363, respectively. After cells were co-exposed to LC and 0.1 uM As ion uptake was 402, 1429 and 1603., respectively. Co-exposure of cells with LC concentrations and 0.5 uM As after 120 h were 288, 727 and 726, respectively.

3.11 Arsenic Induces an Additive Effect to Lead Chromate Induced Cytotoxicity in Human Lung Fibroblasts

We used the clonogenic survival assay as a cytotoxicity marker of the WTHBF-6 human lung fibroblast cell line treated with 0.1, 0.2 and 0.3 ug/m^2 LC and 0.1 and 0.5 uM As concentrations for 24 and 120 h to mimic acute and prolonged exposure. After exposure, LC induced a concentration and time dependent increase in relative cell death. Specifically, after 24 h the relative cell death exposed to 0, 0.1, 0.2 and 0.3 ug/cm^2 LC was 0, 25, 41 and 54 percent, respectively. Cells exposed for 120 h saw an increase in cell death to 0, 31, 46 and 64 percent, respectively. (Figure 13A).

After 24 h of co-exposure of the above mentioned LC concentrations to 0.1 uM As, the relative cell death increased to 10, 31, 54 and 65 percent, respectively. 120 h of co-exposure saw an increase to 12, 48, 56 and 73 percent, respectively. When cells were co-exposed for 24 h to LC and 0.5 uM As, relative cell death was increased to 20, 49, 69 and 82 percent, respectively. After 120 h of co-exposure, we saw an increase to 27, 62, 71 and 79 percent, respectively. (Figures 13B-C). These increases in relative cell death indicate that an additive effect was seen when cells were co-exposed to LC and As.

3.13 Arsenic Induces an Additive Effect in Lead Chromate Induced Chromosome Damage in Human Lung Fibroblasts

Next we used the clastogenicity assay as a marker for genotoxicity in the WTHBF-6 cell line. We considered two measures of damage for our chromosome damage studies. We measured the percent of metaphases with damage as a determination of the frequency of damaged cells. We also measured the total aberrations per 100 metaphases as a determination of the extent of damage within cells. After 24 and 120 h exposure to LC we saw a concentration and time-dependent increase in percent of metaphases with damage and total aberrations (Figure 14A). Specifically, after 24 h of exposure to 0, 0.1, 0.2 and 0.3 ug/cm^2 LC there was 7, 20, 42 and 47 percent of metaphases with damage. After 120 h of exposure to the same concentrations we saw 8, 24, 33 and 41 percent of metaphases with damage, respectively. When considering total chromosome aberrations, 24 h of exposure to the concentrations of LC saw 7, 23, 59 and 63 total damage, respectively. 120 h of exposure saw an increase to 9, 32, 43 and 56 total chromosome aberrations. (Figure 14A)

Next we analyzed cells co-exposed to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.1 uM As. After 24 h of co-exposure we saw 13, 37, 53 and 55 percent of metaphases with damage, respectively. After 120 h of co-exposure we saw 11, 30, 37 and 43 percent of metaphases with damage, respectively (Figure 14B-C). Analysis of total chromosome aberrations after 24 h of co-exposure saw 13, 60, 68 and 73 total aberrations, respectively. After 120 h of co-exposure we saw 13, 36, 53 and 70 total aberrations, respectively (Figure 14 D-E).

Cells co-exposed to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.5 uM As after 24 h saw 16, 36, 48 and 59 percent of metaphases with damage, respectively. After 120 h of co-exposure we saw 20, 42, 44 and 48 percent of metaphases with damage, respectively (Figure 14B-C). Analysis of total chromosome aberrations after 24 h of co-exposure saw 20, 53, 65 and 87 total aberrations, respectively. After 120 h of co-exposure we saw 23, 61, 67 and 70 total aberrations, respectively (Figure 14 D-E).

With chromosome damage results we also wanted to determine specific damage caused to cells by LC and As co-exposure. To investigate specific damage we designated damage as chromatid lesions and isochromatid lesions, with the latter being the rarer of the two specific damages. After 24 and 120 h exposure to LC concentrations, WTHBF-6 cells saw a concentration and time-dependent increase in specific chromosome aberrations (Figure 15A). After 24 h exposure to LC concentrations there was 5, 19, 41 and 47 chromatid lesions, respectively; and 2, 3, 13 and 10 isochromatid lesions per 100 cells, respectively. Then after 120 h exposure we saw 7, 22, 27 and 37 chromatid lesions, respectively; and 2, 7, 7 and 11 isochromatid lesions in 100 metaphases, respectively (Figure 15A).

Cells co-exposed to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.1 uM As after 24 h saw 10, 40, 48 and 50 chromatid lesions, respectively; and 3, 10, 11 and 16 isochromatid lesions per 100 cells, respectively. Then after 120 h exposure we saw 11, 23, 24 and 35 chromatid lesions, respectively; and 2, 8, 21 and 27 isochromatid lesions in 100 metaphases, respectively (Figure 15B-E).

Cells co-exposed to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.5 uM As after 24 h saw 16, 38, 48 and 65 chromatid lesions, respectively; and 3, 11, 14 and 13 isochromatid lesions per 100 cells, respectively. Then after 120 h exposure we saw 18, 31, 27 and 27 chromatid lesions, respectively; and 3, 21, 31 and 34 isochromatid lesions in 100 metaphases, respectively (Figure 15B-E).

3.14 Prolonged Lead Chromate Exposure Induces Aneuploidy in Human Lung Fibroblasts

Prolonged exposure to 0, 0.1, 0.2 and 0.3 ug/cm^2 LC induced aneuploidy in human lung fibroblasts (Figure 16A). 24 h of exposure to the same concentrations of LC did not induce aneuploidy. Specifically, after 120 h of exposure to 0, 0.1, 0.2 and 0.3 ug/cm^2 LC we saw 9, 18, 24 and 23 percent total aneuploidy in 100 metaphases, respectively. Hypodiploid cells jumped from 6 percent in the control to 12, 15 and 17 percent in cells treated with 0, 0.1, 0.2 and 0.3 ug/cm^2 LC, respectively. There was very little increase in hyper-and tretraploidy found in LC treated cells.

In cells co-exposed to LC and both As concentrations we found that only prolong co-exposure induced aneuploidy (Figure16C). Cells co-exposed to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.1 uM As after 120 h saw 13, 22, 27 and 30 percent total aneuploidy, respectively. Cells co-exposed to LC concentrations and 0.5 uM As saw an increase in percent total aneuploidy to 13, 26, 28 and 30 percent, respectively (Figure 16C).

3.15 Arsenic Induces an Increase in Prolonged Lead Chromate Induced Metaphase Damage in Human Lung Fibroblasts

We found that LC does not induce metaphase damage after 24 h exposure; however after 120 h exposure LC induces a concentration-dependent increase in metaphase damage in fibroblasts (Figure 17). Specifically, after 120 h exposure to 0, 0.1, 0.2, 0.3 ug/cm^2 LC we saw 0.3, 10, 21 and 27 percent of 100 metaphases with a cohesion defect, respectively.

Cells co-exposed to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.1 uM As after 120 h saw an increase to 0.7, 18, 21 and 40 percent of metaphases with a cohesion defect, respectively. After co-exposure to 0, 0.1, 0.2, 0.3 ug/cm^2 LC and 0.5 uM As we saw a further increase to 2, 25, 34 and 44 percent of metaphases with a cohesion defect, respectively (Figure 17C).

4. Discussion

Hexavalent chromium (Cr(VI)) is a known human lung carcinogen and extensive research into the various Cr(VI) compounds, like zinc, lead, barium and sodium chromate, has been done. This is the first experiment to investigate the effects of the soluble Cr(VI) compound, sodium chromate (SC), in human lung fibroblasts in prolonged, e.g.120 h, exposures, as humans and animal species are rarely exposed to a toxicant for just acute, e.g 24 h, time frames. This study has shown that there are time and concentration-dependent increases in intracellular chromium ion concentrations, cytotoxic response and clastogenicity after SC exposure to human lung fibroblasts. We have also shown that prolonged exposure to sodium chromate induces both aneuploidy and damage indicative of cohesion defects (Figs. 1-5).

Based on the chromium ion uptake data we can conclude that the intracellular chromium ion levels increased with both increasing time and SC concentrations. It is also important to note that we refreshed the sodium chromate treatment every 24 h for prolonged experiments to ensure that cells were continually exposed to Cr(VI), as previously described (30). Our ion uptake data using human lung fibroblasts was consistent with a previous study (31) that used human bronchial cells that saw an increase in sodium chromate concentrations after 24 h of exposure. Our cytotoxicity results show that sodium chromate induced a decrease in relative survival in a time and concentration-dependent manner.

Our 24 h results were consistent with previous studies (31), which observed relative survival percentages of 83, 67 and 68 after treatments with 0.1, 0.5 and 1.0 uM SC. We found 77, 66 and 54 percent relative survival, respectively. Our prolonged data, again, is the first of its kind with sodium chromate treatments.

When investigating for genotoxic effects of prolonged exposure to SC we found there was an increase in percent of metaphases with damage as well as total aberrations per 100 metaphases. This data are also consistent with previous data with human lung and human bronchial cells (5, 31). We also investigated for specific damage to chromosomes. When reporting chromatid and iso-chromatid lesions, which can lead to downstream effects such as a defective mitotic checkpoint (28, 29), we saw an increase in both chromosome aberrations after acute and prolonged exposure.

Chromosome instability (CIN), which is a hallmark of cancer (32), is brought on by numerical or structural alterations. When considering numerical chromosomal abnormalities, like aneuploidy, we noted that only after prolonged exposure with 1.0 uM SC did we see a marked increase in aneuploidy. We did find a decrease in total aneuploidy from cells treated for 120 h with 0.1 to 0.5 uM SC. Though it is only a small decrease it is interesting to note the difference. We also noted the same trend when considering damage indicative of cohesion defects; only prolonged treatment with 1.0 uM SC saw a marked increase when compared to 0.1 and 0.5 uM SC treatments, with percent of metaphases with a cohesion defect of cells treated with 1.0 uM SC nearly double that of 0.5 uM SC treated cells (8 vs. 15 percent). These results suggest that at potentially high doses we may see an even stronger response in CIN. Persistent CIN has been shown to induce permanent changes in the genome (32).

This is the first study to explore the effects of the soluble form of Cr(VI), sodium chromate (SC), and arsenic (As) co-exposure in human lung fibroblasts. We found that As did not inhibit the intracellular chromium ion uptake after co-exposure with SC; that As induced an additive effect in SC induced cytotoxicity and clastogenicity after acute and prolonged exposure (Figs. 6-11). We also found that after prolonged co-exposure, As induced an increase in SC induced CIN, specifically aneuploidy and cohesion defects, at our highest doses of SC and As (1.0 and 0.5 uM, respectively), a similar trend we saw when treating cells with SC alone.

We did not see a significant difference in intracellular chromium ion concentrations between cells treated with SC alone and cells co-exposed to SC and As concentrations after either acute or prolonged exposures. Thus we were able to confirm that As did not inhibit the uptake of chromium ion into the cell and could carry on further co-exposure experiments knowing that As would not affect chromium getting into the cells. Our cytotoxicity data showed that As induced an additive effect in SC induced cell death. We define an additive effect as the combined effect of two chemicals is equal to their individual effects (33). We also saw an additive effect induced after co-exposure in our chromosome damage and specific

aberrations, like chromatid lesions. When considering chromosome instability we noted that only after prolonged co-exposure to 1.0 uM SC and As concentrations was there an increase in aneuploidy and damage indicative of cohesions defects. An explanation for this may be that lower concentrations of soluble Cr(VI) (specifically 0.1 and 0.5 uM SC) can be metabolized by the cell by glutathione and ascorbate (8), even after retreatment with SC and As every 24 h. Previous *in vitro* and epidemiological co-exposure studies (34-36) suggest that an additive effect or a greater-than-additive effect is seen after cells and humans are co-exposed to two or more heavy metals, supporting our co-exposure results. Specifically, Patel et al. (34) saw enhanced cytotoxicity in human lung epithelial cells after co-exposure to the soluble compounds nickel chloride and cobalt chloride at relatively nontoxic concentrations. This study also saw an increase in chromatid lesions, similar results to our observations.

This is also the first study to explore the effects of the water insoluble or 'particulate' Cr(VI) compound, lead chromate (LC) and As co-exposure in human lung cells (Figs. 12-17). Insoluble forms of Cr(VI) (ie lead or potassium chromate) are known to be more potent (1-4) than water soluble compounds, like sodium chromate. We found that As did not significantly inhibit the intracellular chromium ion concentrations in cells co-exposed with both LC and As. As also induced an additive effect in LC induced cytotoxicity and clastogenicity in cells. Chromosome instability (CIN) was seen in the form of aneuploidy and damage indicative of cohesion defects within cells after prolonged exposure to both solo LC exposure and co-exposure to LC and As concentrations.

We found slightly confounding results when considering the intracellular chromium ion uptake data (Fig 12B). After acute co-exposure the results showed little to no change. However, after prolonged co-exposure to 0.1 uM As and 0.1, 0.2 and 0.3 ug/cm^2 LC, the chromium ion concentrations were reduced by 166, 158 and 760 uM, respectively. Cells exposed to 0.5 uM As and LC concentrations saw chromium ion concentrations reduced by 280, 860 and 1637 uM, respectively. These results are interesting because they may suggest that arsenic, at higher doses (0.5 uM or higher), may in fact inhibit chromium uptake into the cell. Another explanation could be that glutathione (GSH) is incorporated in the reduction and metabolism of both Cr(VI) and As within the cell and with increasing levels of As, GSH activity thereby is increased and readily available to metabolize Cr(VI) within the cell. Previous studies (37, 38) have shown that upon an increase in As concentrations there is an increase in GSH activity. Yeh et al. (37) have shown that in porcine endothelial cells, that exposure to three different As compounds (arsenic trioxide, sodium arsenite and sodium arsenate) induced an increase in GSH and other reducing agents within the cells. This could suggest why we saw a decrease in intracellular chromium ion concentrations after prolonged co-exposure. Even more interesting is that our subsequent experiments with LC and As co-exposure yielded results similar to or greater than the previous soluble Cr(VI) results described above.

Analysis of our clastogenicity data showed that As induced an additive effect in LC induced chromosome damage and chromosome aberrations. We noted an interesting trend when specifically investigating total chromosome aberrations. Results from prolonged co-exposure saw a decrease in total chromatid lesions, however there was a sharp increase in total isochromatid lesions after prolonged co-exposure. The effect seen after prolonged exposure (Fig. 14E) is a greater-than-additive effect, further proving the potency of particulate Cr(VI) compounds and suggesting a much greater response after co-exposure of two heavy metals. Chromosome instability (CIN) in the forms of aneuploidy and cohesion defects were only seen in cells treated for prolonged co-exposure. Previous studies (28, 39) have shown that particulate Cr(VI) compounds, including LC, induce CIN after prolonged exposure, further supporting our data. Cells only seeing a prolonged exposure to 0.1, 0.2 and 0.3 ug/cm^2 LC saw 0, 10, 21 and 27% of cells with a cohesion defect (Fig. 17C); these percentages jumped to 25, 34 and 44% after co-exposure with 0.5 uM As, further asserting that the co-exposure of human lung fibroblasts to LC and As can result in an additive or a greater-than-additive effect.

The goal of this study was to explore the prolonged effects of SC exposure in human lung fibroblasts. We also sought to explore the effects of arsenic co-exposure on soluble and particulate Cr(VI) compounds in human lung fibroblasts. We have shown that after prolonged exposure, SC is cytotoxic, induces clastogenicity and at high doses induces aneuploidy and CIN in human lung fibroblasts. This is the first study to show that As induces an additive effect in soluble and particulate induced cytotoxicity and clastogenicity. As also induced a greater-than-additive effect in soluble and particulate induced specific chromosome aberrations (ie isochromatid breaks) and in chromosome instability, specifically damage indicative to a cohesion defect. CIN is a hallmark of cancer (32) and is characteristic of numerical and structural alterations to the chromosome. These structural alterations and CIN caused by Cr(VI) and As co-exposure could possibly contribute to the initiation of lung cancer. Our results are further supported by previous heavy metal co-exposure studies (34-36) suggesting that additive and greater-than-additive effects can be seen after co-exposure. Other mechanisms at play that should be further investigated when considering co-exposure, specifically damage caused by Cr(VI) and As, are the production of reactive oxygen species (ROS) during metabolism of each heavy metal that causes additional damage to the cell and the disruption of DNA repair methods that the damage caused by either Cr(VI) and As and the subsequent downstream effects of DNA repair loss.

6. References

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