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Chronic Arsenic Exposure in Nanomolar Concentrations Accelerates Senescent Phenotypes *in vitro*

An honors thesis presented to the
Department of Biological Sciences
University at Albany, State University Of New York
in partial fulfillment of the requirements for graduation with
Honors in Biological Sciences and graduation from
The Honors College.

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Research Advisor: Melinda Larsen Ph. D

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Abstract

Arsenic is recognized as an environmental carcinogen, in which over 100 million individuals worldwide experience chronic exposure through contaminated drinking water. Arsenic exposure is linked to several health conditions, such as cancer, diabetes, skin lesions, immune dysfunction, and cardiovascular disease. Here we test the hypothesis that toxicants, such as arsenic, accelerate the program of senescence. Human (IMR-90) primary diploid fibroblasts were chronically exposed to arsenic at nanomolar concentrations and the impact on the expression of senescent-associated mRNAs was evaluated. IMR-90 cells were exposed either to untreated media or exposed to 130nM and 330nM AsCl₃ supplemented media. RNA samples were extracted weekly from IMR-90 cells and RTPCR was conducted. Arsenic-exposed IMR-90 cells displayed increases in all senescence-associated transcripts monitored, those include Interleukin-6, Interleukin-8, Interleukin-alpha and the cyclin-dependent kinase inhibitor protein P16. At day 40, post AsCl₃ exposure, a near 25-100 fold increase in senescent transcript expression was observed. Thus, there appears to be a direct relationship between the duration of toxicant stress, and the increased transcription level of senescent transcripts. Our studies suggest that low dose AsCl₃ exposure promotes senescent transcript expression and very likely accelerates the senescent program that has the potential of creating a cellular niche that is permissive to disease progression.

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Introduction:

Arsenic is a naturally occurring element that is found in several components of earth's atmosphere. It is chemically classified as a metalloid, meaning that it behaves as both a metal and nonmetal. When referring to arsenic exposure, it is classified as either organic or inorganic arsenic, which refers to the bonding of Carbon and Hydrogen. Inorganic arsenic is commonly found in soil and minerals such as copper and lead, and is the primary concern of public health officials. Due to its widespread natural occurrence, arsenic cannot be easily filtered from the environment. It becomes toxic when, "reacting with oxygen or other molecules present in air, water, soil, or by the action of bacteria that live in soil or sediment" (11). This leads into the metabolic pathway of how toxicants are metabolized and are affected by reactive oxygen species (ROS).

There are several ways in which people are exposed to arsenic every day, some of which include the foods we eat, drinking water, and the air we breathe. According to the Center of Disease Control, the average inorganic arsenic consumption is about 3.5 micrograms per day. Governmental officials have approved exposure in drinking water up to 10 parts per billion or 130 nanomolar (11). Upon consumption, arsenic is processed and passed through the body and is excreted through urine within several days of ingestion. Animal testing has shown that chronic exposure to arsenic's methyl and dimethyl compounds results in damage to the urinary bladder and the kidneys (11). Several medical conditions linked to chronic arsenic exposure include, renal and bladder cancer, diabetes mellitus, poor healing skin lesions, immune dysfunction and cardiovascular disease (3). These conditions are also presented in individuals with chronic inflammation and or mitochondrial disorders.

Mitochondrial disorders are characterized as, "genetic defects of the oxidative phosphorylation pathway which can affect different organs and tissues" (10). One of the many functions of mitochondria are to generate the signaling oxidant H_2O_2 . Several environmental stressors are commonly known to alter mitochondrial function, leading to an increase in the production of the reactive oxygen species in the form of H_2O_2 . Studies have been done performing acute arsenic exposure on fibroblast cells. Results showed a

significant increase in oxidative stress along with increased levels of ROS (12). Conclusive studies have been done to establish that the signaling network of mitochondrial derived H_2O_2 are constitutively engaged in senescent cells (10). The phase of senescence can be both protective and destructive towards cells.

Senescence is a protective phase that restricts cell-cycle progression, preventing the spread of damage to the next cell generation (2). When cells are exposed to cellular stress early on in life, the beneficial effects include tumor suppression and tissue repair. However, as senescent cells accumulate with age, the senescence-associated secretory phenotype (SASP) can disrupt tissues and contribute to age-related pathologies, including cancer (6). Cells exposed to intercellular or extracellular stress such as oncongenic reagents will transition cells into senescence. Studies using other environmental toxicants have successfully induced premature cellular senescence by promoting intracellular ROS production (13). In all cases cellular senescence is accompanied by an increase in several key senescence markers. We mainly focus on the soluble signaling factors, interleukins and chemokines. Interleukin-6 is the most prominent cytokine of the Senescence-Associated Secretory Phenotype (SASP). IL-6 is associated with DNA damage and oncogenic stress induced senescence in both human and mouse fibroblasts (2) (4). Due to the fact that IL-6 is directly controlled by persistent DNA damage signaling, it makes it a strong, useful target for our experiment. Interleukin-8 is also recognized for its role in the detection of cellular inflammation. IL-8 mainly functions as a chemoattractant for neutrophils at sites of inflammation (5). Research has been done on the increased production of IL-6 and IL-8 in the presence of oncogenic stress (5) (9). IL-1 α is a key senescence-associated (SA) proinflammatory cytokine that acts as a critical upstream regulator of the SA secretory phenotype, involved in surface cell signaling (9). When under cellular stress IL-1 α is produced by monocytes and macrophages as a proprotein. These proproteins are released in response to cell injury (2) (4) (7). Additionally, P16 is primarily known for being a tumor suppressor gene, and is induced by oncogenic stressors (5) (6). A recent study was done using mice to test the importance of the P16 gene and its role in cellular senescence during the process of aging.

In this study, genetically engineered mice designed to express premature aging underwent additional genome manipulation. A promoter for the gene P16Ink4A was attached to an apoptotic protein, which was designed to only be turned on in the presence of a drug. After genomes were manipulated the mice were split into two groups. The mice that did not receive the apoptotic protein inducing drug experienced regular side effects of aging, such as muscle atrophy and scoliosis. However, the mice that received the drug remained essentially ageless. In this case, every time the P16 tumor suppressing gene was turned on, such as in the program of senescence. The apoptotic protein would then kill the cell, leading to a clearing of senescent cells (1). This study confirms the importance of the gene P16 and how upregulation of this gene results in health conditions associated with aging. Throughout this experiment, IL-6, IL-8, IL-1 α , and P16 will be used to test for upregulation in senescent associated secretory phenotypes.

As previously mentioned, senescence can be used as a protective factor towards cells. However, due to the senescence-associated secretory phenotype (SASP), inflammation can also be detrimental towards cells. Chronic inflammation, is associated with many age-related pathophysiologic processes and

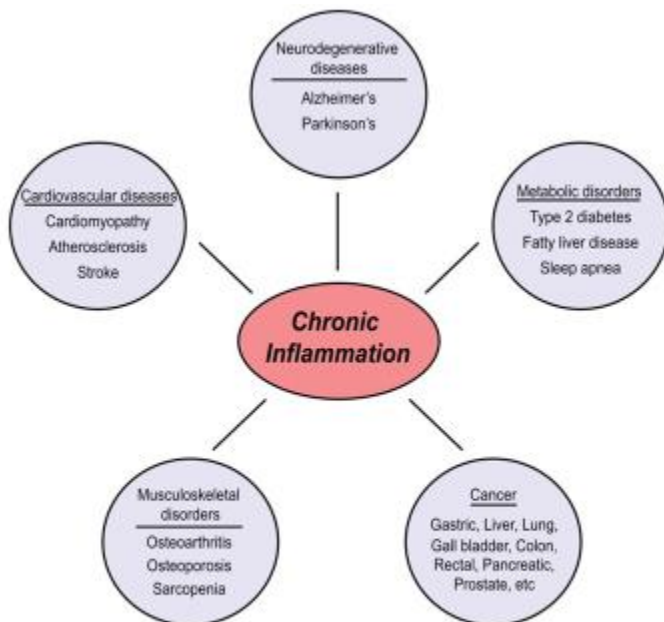


Figure I. Diagram represents different diseases that evolve from chronic inflammation. In addition, these diseases often are associated with increasing age.

diseases, “including Alzheimer’s disease, diabetes, atherosclerosis, osteoarthritis and cancer, among others. In addition, chronic inflammation is also associated with normal aging” (4). Studies show that in normal healthy aging individuals, there is a 2–4 fold increase in the levels of pro-inflammatory mediators such as IL-6, IL-8 and tumor necrosis factor (TNF) α in individuals 50 years of age or older (4).

This fold increase is compared to younger

healthy individuals. As individuals age, the duration in which they are exposed to several environmental toxicants inevitably increases. Part of the human body's response to stress factors, is the initiation of inflammation, as a way to become more equipped to fight the stressor. However, chronic inflammation leads to a list of age-related diseases. Interestingly enough, several age-related diseases associated with chronic inflammation also correlate with diseases associated with chronic arsenic exposure. This raises an important question as to whether-or not aging itself drives chronic inflammation, or if there is something else causing chronic inflammation, which in turn drives aging. By studying the inflammatory senescent factors listed above, we hope to gain a better understanding of the pathway that connect environmental toxicants, chronic inflammation, and cellular senescence in which leads to several age-related diseases.

Whether arsenic specifically promotes senescence is not known, and our studies will determine whether this toxicant has the potential to exacerbate degenerative disease progression by accelerating the senescent program. Our studies indicate that low doses of arsenic can prematurely increase the levels of many senescent-associated transcripts that have the potential to exacerbate age-associated chronic inflammation.

Materials and Methods:

Materials. Minimum essential medium with Earle's salts and L-glutamine (MEM), Phosphate buffered saline solution (PBS), Penicillin, Streptomycin, TRIzol, Quant-iT OliGreen cDNA quantification kit, Platinum SYBR Green, Eva Green, and qPCR Supermix-UDG and GAPDH. Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine serum (FBS). Maxima H Minus First Strand cDNA Synthesis Kit manufactured by Thermo Science Molecular Biology. Real time RT-PCR primers were purchased from Integrated DNA Technology. All other chemicals were from Fisher Scientific and were of Molecular grade or higher in quality.

Homo Sapiens (IMR-90) fetal lung diploid fibroblasts Growth conditions for IMR-90 cells are briefly described. They are grown on 10mL sterile gamma irradiated tissue cell culture plates. MEM media contains, L-glutamine, 10% FBS, and are stored at 37 °C in a 21% Oxygen incubator. For the arsenic supplemented media, concentrations of 130nM AsCl₃ and 330nM AsCl₃ were used. This media was replaced every two days, and cells were split and samples extracted every four to five days (once a week). When performing media change, the plate was washed with 4mL of PBS, and fresh media was added. When cells were split, they were always split in a 1:4 fashion. This treatment was administered for eight weeks.

Real-Time RT-PCR The IMR-90 cells were grown for four to five days in their arsenic-free or supplemented (130nM, 300nM) media. RNA was isolated from confluent plates using TRIzol reagent according to the manufacturing protocol and quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific). cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis kit according to the manufacture's protocol. cDNA was quantified using qPCR Supermix quantification kit according to the manufacture protocol. Total cDNA, 500ng, per reaction was amplified with Platinum SYBR Green and Eva Green qPCR SuperMix-UDG kit according to manufacture. A applied Biosystems 7500 Real Time PCR System was used to test for the primers IL-6, IL-8, IL-1 α , and cyclin-dependent kinase

inhibitor protein P16. These human gene-specific primer pairs were designed using IDT-DNA Primer Quest, Primer Bank.

Results

IMR-90 fibroblast cells were exposed to chronic low dose arsenic in the form of AsCl_3 for forty days. The arsenic-supplemented media had an arsenic concentration of 130nM, (equivalent to 10ppb) and 330nM (equivalent to 25ppb). Once a week cell samples were taken from culture and a PCR was conducted testing for the following senescent expressing cytokines; IL-6, IL-8, IL-1 α , and cyclin-dependent kinase inhibitor protein P16 protein. Figure 2. represents the first set of RNAs that were extracted after five days of treatment. First, analyzing the upregulation of IL-6, we saw a 3.47 fold change in the 130nM treated cells compared to the non-treatment cells, and a 6.21 fold change in the 330nM cells. While no other significant differences were observed in the levels of the other senescent transcripts monitored.

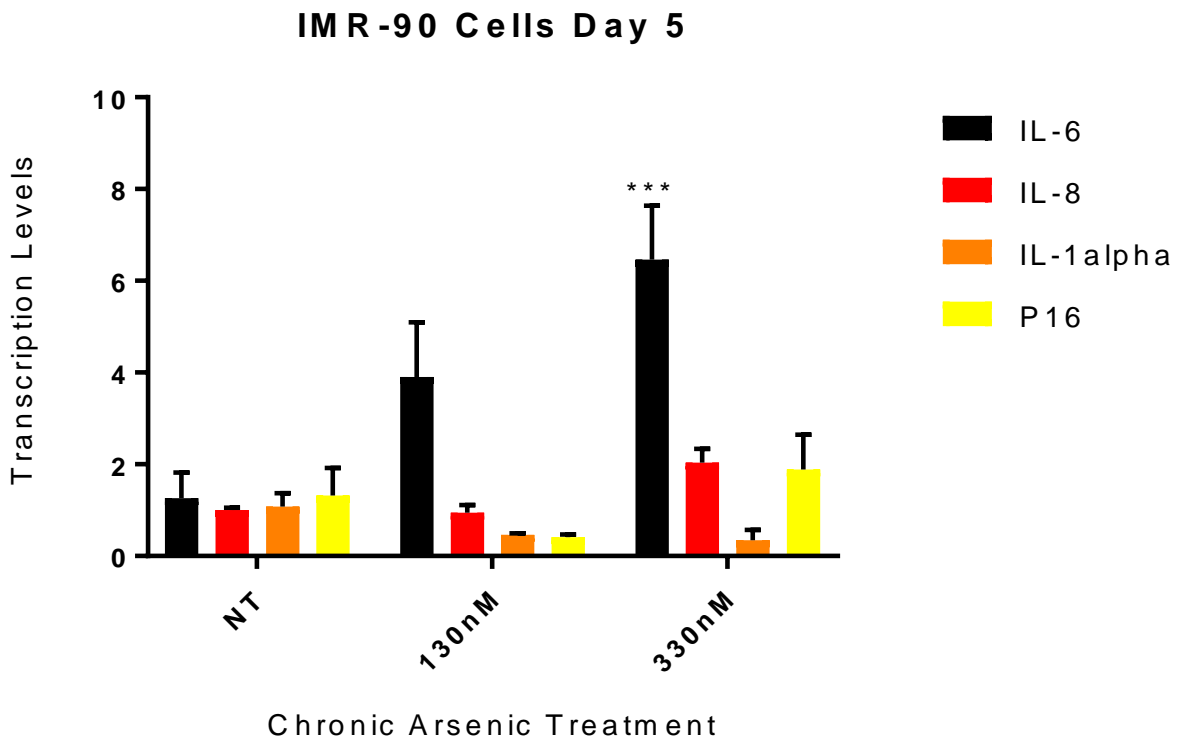


Figure 2. Fibroblastic IMR-90 cells after five days of chronic low dose arsenic exposure. The X-axis represents the different treatments; non-treatment cells, cells exposed to 130nM AsCl₃ concentration, and cells exposed to 330nM AsCl₃ concentration. The following cytokines were chosen for transcription level analysis; IL-6, IL-8, IL-1 α , and senescent protein P16. Two way ANOVA was used to test for statistical significance between the control cells and experimental cells. *** represents statistical significance between the control and the IL-6 expressed in the 330nM cells. A P value \leq 0.001 between the two samples was recorded.

Carrying forward with the exposure, we continued to see an increase in the senescent phenotypes being produced. Figure 3. displays upregulation in senescence cytokines after twenty-one days of chronic low dose arsenic treatment. As presented in the bar graph below, the levels of IL-8 and P16 were most affected. 21-days of AsCl₃ exposure increased IL-8 expression by 2.67 and 4.88 fold in response to 130nM and 330nM doses, respectively. The cell cycle inhibitor P16 showed a significant 8.54 and 17.25 increase in transcript levels in response to 130nM and the 330nM AsCl₃, respectively. No differences were observed in IL-1 α expression at day 21.

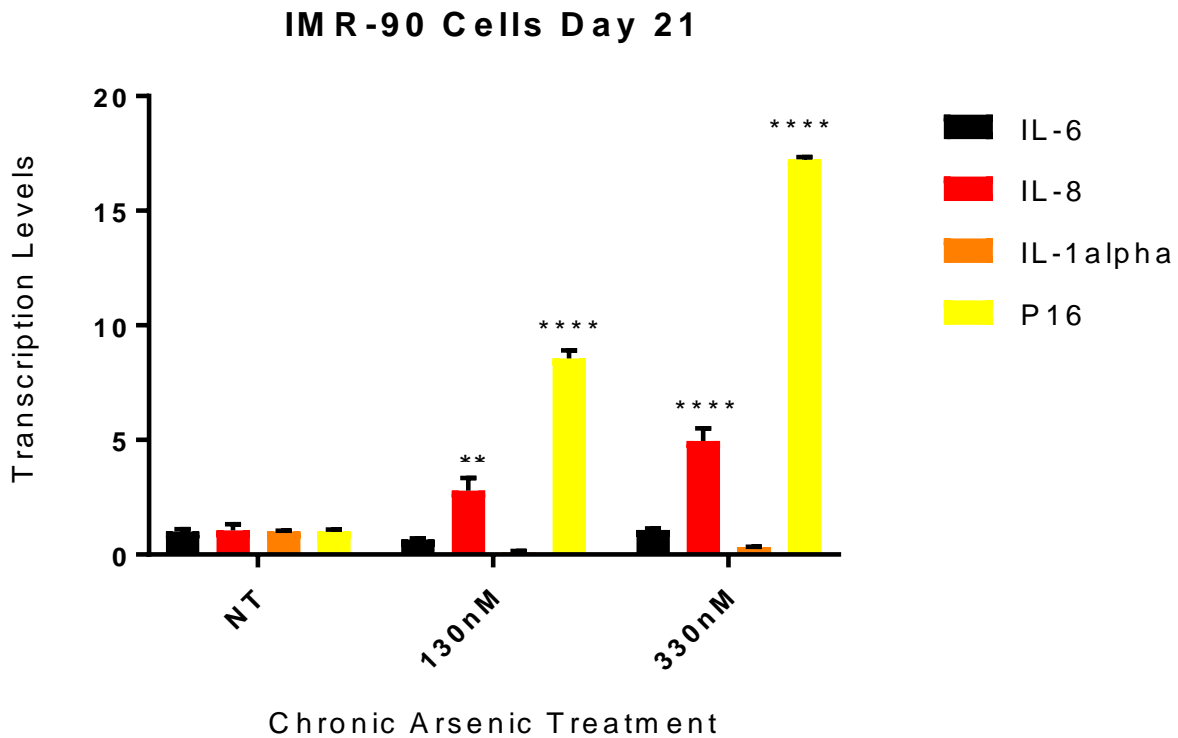


Figure 3. IMR-90 cells after twenty one days of chronic arsenic exposure. Compared to the non-treatment control group, small fold changes were observed for the IL-6 and IL-1 α cytokines. IL-8 and P16 protein were most affected. Statistical significance using two way ANOVA was seen in the increased transcription levels of both, IL-8 and protein P16. Two stars (**) represents a P value ≤ 0.01 . For the three cases where four stars are represented (****) a P value ≤ 0.0001 was calculated.

Weekly RNA samples were continuously extracted, testing for a constant increase in our selected senescent cytokines. Day forty represents the last day of the chronic low-dose arsenic exposure. RNA samples were again extracted and our five selected cytokines were tested for an increase in cellular transcription levels using PCR. Figure 4. represents the upregulation in SASP molecules observed here. Starting with IL-6, the fold change compared to the non-treatment group was 28.83 for the 130nM cells, and 19.97 for the 330nM cells. As for IL-8 we continue to see upregulation of 15.36, and 33.35

respectively for the 130nM and 330nM. With day forty we see a dramatic increase in IL-1 α transcript levels. The 130nM and 330nM exposure caused an 88.76 and 76.07 fold induction of IL-1 α , respectively. Lastly, we looked at the P16 protein which also expressed significant upregulation. The 130nM cells experienced a 37.83 fold change, and the 330nM experienced a 32.31 fold increase.

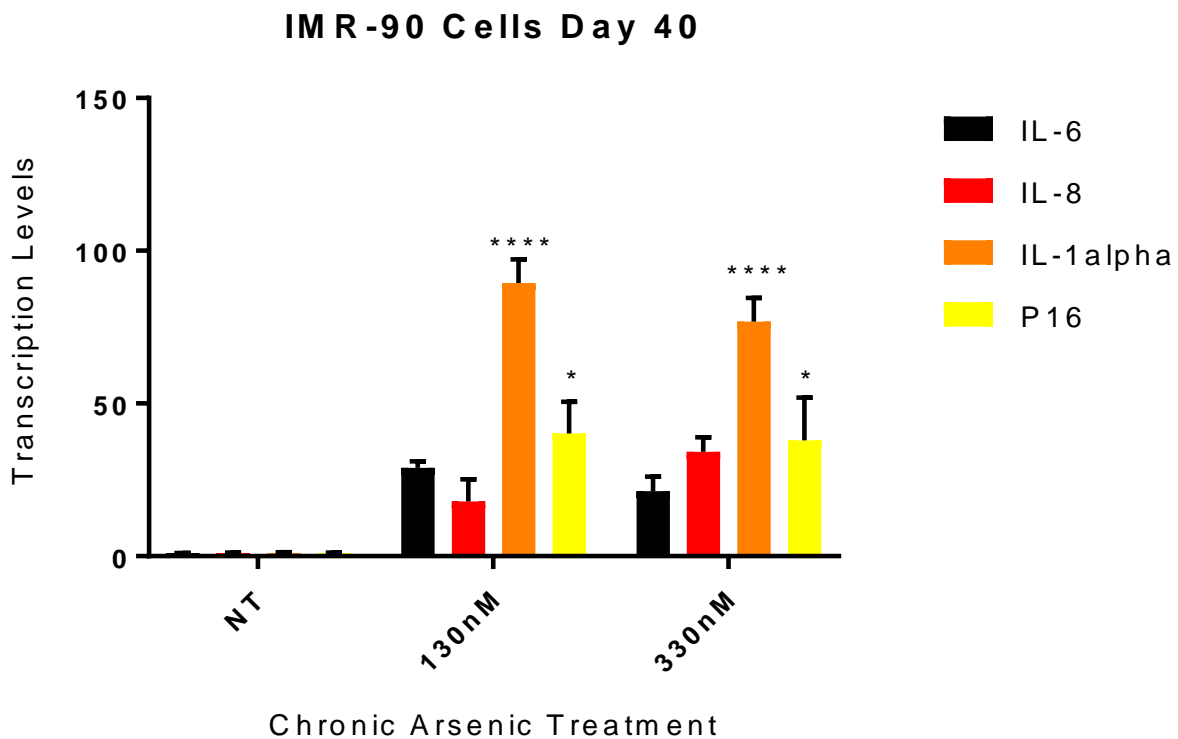


Figure 4. IMR-90 cells, after forty days of chronic arsenic induced exposure. There was significant upregulation in the transcription of our selected cytokines. However, the transcription levels for IL-1 α specifically is reaching an outstanding fold change of almost 100 fold, compared to our controlled non-treatment cells. Significant increase was observed in both the P16 protein, and IL-1 α cytokines. Regarding the P16 protein, a one star (*) significance was seen between the control and the 130nM cells, with a P value ≤ 0.05 . The significance between the control and the 330nM cells was represented by a P value ≤ 0.05 . In regard to the IL-1 α , there was a significant increase in transcription seen for both the

130nM and 330nM cells. Four stars (****) are used for both to represent a P value ≤ 0.0001 . It is evident that toxicant cellular stress induced upon these cells is resulting up increased senescent proteins.

Over the 40 day chronic low dose exposure, we observed an increasing trend in all four senescence marking cytokines. Cells exposed to both 130nM AsCl₃ and 330nM AsCl₃, expressed a dramatic increase in SASP molecules produced. Figures 5. displays the increasing trend of all four of our senescence marking molecules produced by cells exposed to 130nM AsCl₃. Figure 6. represents a similar concept, except figure 6, represents cells exposed to 330nM AsCl₃.

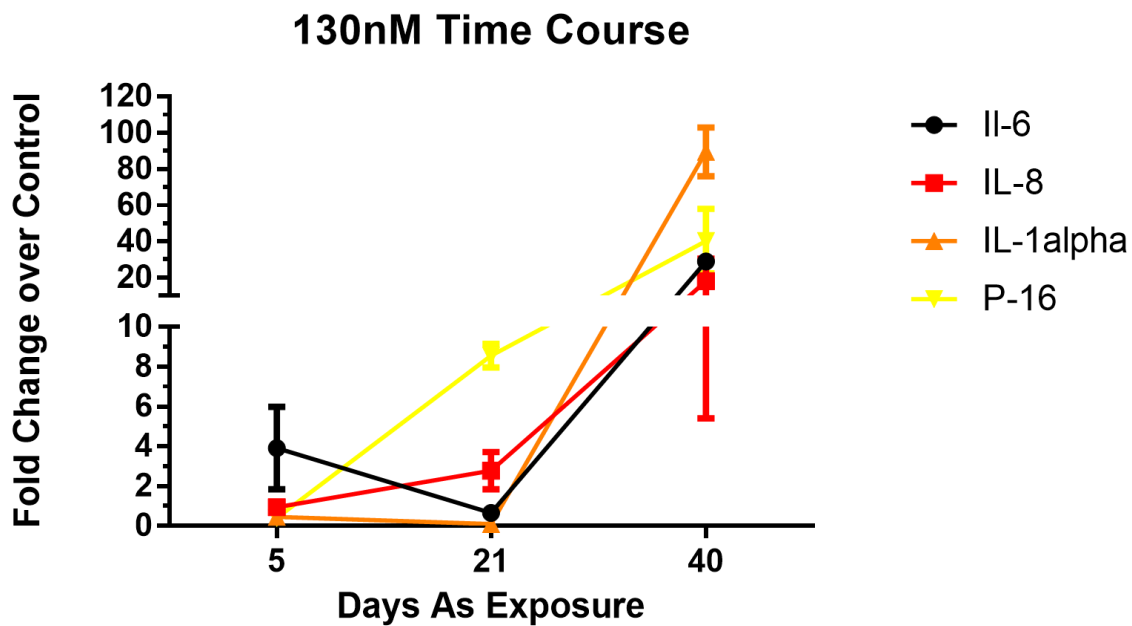


Figure 5. IMR-90 cells exposed to 130nM AsCl₃ for 40 days. This graph represent the increase in each of our senescent marking cytokines through the duration of our exposure. The Y-axis the fold change of our cytokines in experimental cells over control cells. The X-axis represents the three days in which PCR tested for upregulated cytokines. Here, n=3 and the error bars are represented by SEM.

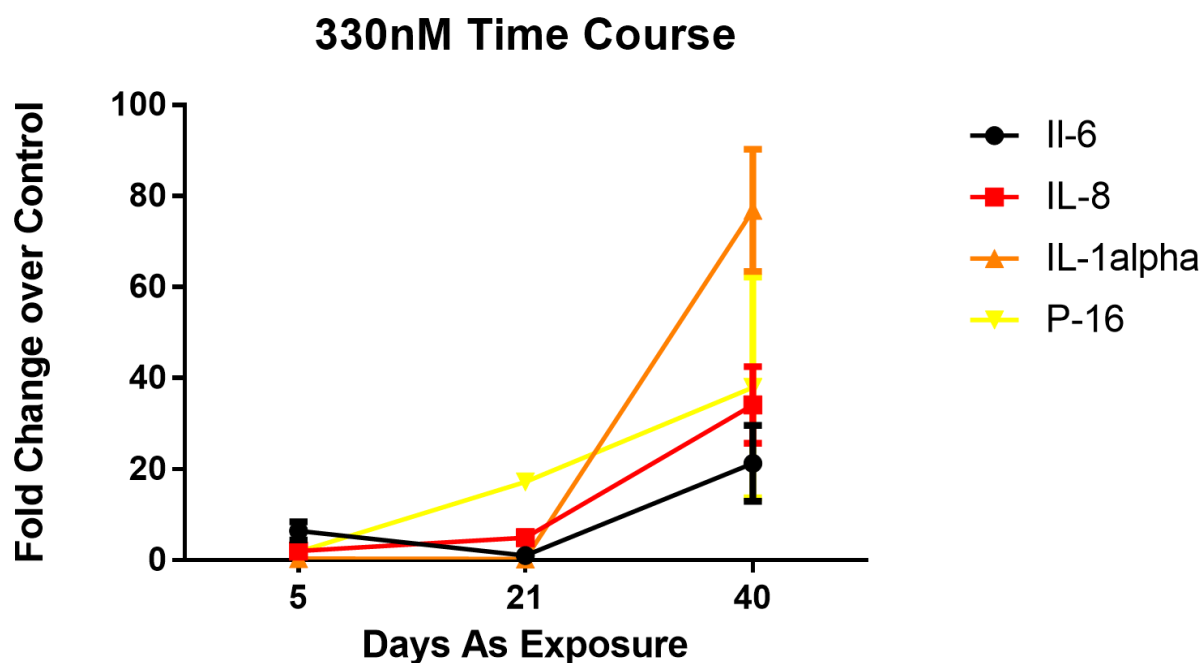


Figure 6. IMR-90 cells exposed to 330nM AsCl_3 for 40 days. This graph represent the increase in each of our senescent marking cytokines through the duration of our exposure. The Y-axis the fold change of our cytokines in experimental cells over control cells. The X-axis represents the three days in which PCR tested for upregulated cytokines. Here, $n=3$ and the error bars are represented by SEM.

The selected cytokines IL-6, IL-8, IL-1 α , and cyclin-dependent kinase inhibitor protein P16 were chosen to track the rate and quantity in which cells were becoming senescent. By day five of the treatment, a threefold change was observed. As the arsenic exposure continued, the transcription levels of senescent proteins also continued to increase. On day twenty one, certain cytokines had reached a fold change as high as seventeen. Furthermore, after forty days, a near 100 fold change in transcript levels was observed. Thus, there is a direct relationship between the quantity of senescent cytokines expressed throughout a cell, and the duration in which they are exposed to the toxicant.

In addition, the same treatment was performed using *Mus musculus* fibroblast cells. When performing this experiment, two additional variables were implemented. Two MEF cell lines were used, one wild type line, and one knock out line regarding the gene *Alkbh8*. This gene is known to have an effect on the way in which cells can adapt to cellular stress. The second variable of interest was, percentage of environmental oxygen during incubation. Due to the strong correlation between ROS and cell damage, we wanted to see if decreasing the percentage of available oxygen would decrease the amount of ROS produced. Thus, hypothesizing that these cells may have a better adaptive response to the arsenic treatment. However, the PCR results for these cells were not conclusive.

Discussion

The purpose of this project was to see how prominent senescence-associated cytokines are effected by chronic low-dose arsenic exposure. Further understanding this process and the associated metabolic pathways, holds the key to understanding age related diseases. If we are able to deduce the molecules that are activating senescent pathways, researchers and health professionals can further educate the public which external toxicants to more specifically stay away from. If we are able to control our exposure to certain destructive cellular toxicants, we may become closer to understanding the secrets behind several age-related diseases. The end all goal is to achieve prolonged life without diseases associated with age, such as, renal failure, cancer, rheumatoid arthritis, dementia, and several other diseases previously mentioned.

Due to its wide spread natural occurrence, arsenic was selected as our toxicant of choice. Both the Environmental Protection Agency and the Food and Drug Administration approves arsenic standards in drinking water up to 10 parts per billion or 130 nanomolar (11). In this study, over the course of forty days, IMR-90 fetal lung fibroblasts were exposed to chronic low-dose arsenic at concentrations of 130nM and 330nM. Results show, that in just forty days there was an increase in cellular senescence and SASP factors were produced. After five days of arsenic exposure we saw that the only cytokine expressing an upregulation was IL-6. Studies using melanoma cells showed that IL-6 inhibits proliferation of early cell damage but not advanced-stages (5). Thus, we may be experiencing the same effect here. IL-6 may be the initial cytokine indicating cellular inflammation, but as the arsenic exposure continued, the other cytokines became more prominent. This same study notes P16 showing an upregulation later on in the progression of melanoma due to its predominant role in tumor suppression (5). In addition, results from an experiment to test the importance of P16 as a tumor suppressor gene and its role in senescence, concluded that the program of senescence activates the P16 gene. This agrees with the data we collected at day 21 and day 40 of our exposure. As the arsenic exposure continued and cells became senescent, we saw an increasing expression in the P16 protein. Lastly, IL-1 α ability to act as a surface receptor, may

help explain why at day 40 we saw such a dramatic upregulation. As cells become senescent and SASP molecules are released, they signal neighboring cells, further promoting inflammation (6). At day 40, PCR displayed a dramatic increase in all four of our senescence marking cytokines. Thus, this leads us to think that there were several cells sending out signals to neighboring cell. These signals may have been detected and expressed by the upregulation in IL-1 α . With that in mind, we question how chronic low dose arsenic exposure, (as seen in drinking water) affects the progression of cellular senescence in mammals. Throughout the duration of this experiment, a change in senescent phenotypes was observed. There was a clear association between quantity of senescent cytokines produced and the duration of arsenic exposure.

Although our results suggest that arsenic induces the senescence program, there is still much work to be done. The rate of cell proliferation was not recorded throughout this experiment. The IMR-90 cells entered this experiment at passage number eight and were cultured up until passage seventeen. Under the microscope, visual observations were made noticing the reduced rate of proliferation around week three of exposure. The protocol called for cells to be split 1:4 once a week to maintain optimal growth under these conditions. For the first three weeks the non-treatment, 130nM and 330nM cells visually looked as if they were proliferating at the same rate. After four days of incubation, all three 10mL plates containing the different media concentrations had reached one hundred percent confluency and were ready to be split. From this point forward the non-treatment cells continued to proliferate the fastest, while the treated cells experienced a reduced growth rate. Before each RNA extraction, the cells should have been plated allowing for the rate of proliferation to be calculated. Although (P16) is a strong indicator of the qualitative rate at which cells are becoming senescent, unless its level of regulation is expressed relative to cell number, we cannot determine the actual number of cells becoming senescent. Thus, further testing must be done, carefully recording the rate of cell proliferation in order to obtain quantitative data as to the number of senescent cells increasing from week to week.

In addition to how the cells in culture were analyzed, changes could also be made to the techniques used to run PCR. When RNA samples were tested using PCR, our Actin control, plus our four selected cytokines were plated simultaneously using one plate. Therefore, each plate represented an older sample of cells. Due to the variability that exists between each plate of PCR reactions. It is hard to accurately say that the change in the cytokine transcription levels is solely due to the exposure of chronic low- dose arsenic. One way to help control these confounding variables would be to run every PCR plate at the same time every day (or on the same day). In addition, instead of plating cells by their age. It would be beneficial to organize the plate by primers. Meaning, for one plate we would load eight weeks of RNA sample on one plate, and just test one primer at a time. This way we can control some of the inevitable variation, and have a more accurate way to quantify the week to week changes that are taking place within the cells.

Overall, our results demonstrate that under these conditions that cellular proliferation decreases and that rate of cells becoming senescent is more rapid when cells *in vitro* are exposed to arsenic as an external toxicant. It would be interesting to see if the same results are observed when conducted *in vivo*, using *Mus musculus* as our experimental species. Due to the level of senescent proteins and other tumor suppressor genes present throughout different diseases, we would expect for *Mus musculus* to develop certain age-related diseases if exposed to this same arsenic treatment. A potential effect for mice being exposed to arsenic contaminated drinking water, is renal sensitivity and or renal cancer (3). Arsenic is detoxified and passed through the kidneys before it is excreted through the bladder (3). However, with chronic exposure, a buildup of arsenic and dangerous levels of reactive oxygen species promotes nuclear reprogramming (8) (9). Pending discoveries may change the standards in which the Environmental Protection Agency and the Food and Drug Administration approve arsenic contaminated drinking water. Additional topics of interest include ways in which cellular senescence may be reversed, and or ways in which the rate of senescence progression can be delayed.

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