Summer 2018

Hexavalent Chromium Toxicity on Human Epithelial Cells and Protection by Ascorbic Acid and Epigallocatechin Gallate

Timmy Mayotte
Olivet Nazarene University, Tpmayotte01@yahoo.com

Hexavalent Chromium Toxicity on Human Epithelial Cells and Protection by Ascorbic Acid and Epigallocatechin Gallate

Timmy Mayotte and Ryan Himes

Olivet Nazarene University Pence-Boyce Research Program

Summer 2018

Abstract

Hexavalent chromium or Cr(VI) is a known carcinogen in human beings, though the exact mechanism of carcinogenicity is still unknown. The same chemical compound is also found at varying levels in the water sources of more than 200 million Americans. While the government currently regulates total chromium levels, they have yet to determine a permissible exposure limit for Cr(VI). There is currently no method of preventative treatment for the chemical. The focus of this study was to determine the mechanism of carcinogenicity of Cr(VI) as well as confirm the viability of antioxidants as a preventative treatment. Because of Cr(VI)'s strong oxidative power, we hypothesized that the chemical causes DNA mutation and cell death via oxidation and that antioxidants could prevent this from occurring. To test this theory, we exposed human cell culture to Cr(VI) and samples of Cr(VI) cotreated with either vitamin C or epigallocatechin gallate (EGCG). An Ames test was also performed to determine the mutagenicity of Cr(VI) as well as cotreatments of the toxicants with antioxidant.

It was found that Cr(VI) is significantly toxic to cell culture at concentrations of 200 ppb (parts per billion) or more. Both vitamin C and EGCG blocked this effect at 10 ppm (parts per million) and 15 ppm, respectively. Neither antioxidant was observed to be cytotoxic when treated alone. Cr (VI) was also found to be significantly mutagenic at 20 ppb and up. This mutagenicity was significantly reduced by cotreatment with 20 ppm vitamin C at 200 and 2000 ppb Cr. Vitamin C was not found to be mutagenic when treated individually. With this combined data, we can conclude that hexavalent chromium is both cytotoxic and mutagenic via an oxidative mechanism and these effects can be abrogated by antioxidants. Though continued study is merited, this information further validates the protective potential of antioxidants.

Introduction

Hexavalent Chromium

The toxicological effects of hexavalent chromium (Cr(VI)) compounds have been widely studied over the years. Specifically, data have been collected to assess its carcinogenic effects on humans through case study (Yu, 2013), its toxicological effects on rats (Geetha et al., 2003), and on a number of cellular cultures as well (Majone et al., 2002). While there exist 3 different oxidative states for chromium, the hexavalent form has been found to be much more toxic than the quadrivalent form or the trivalent form, which in fact is an essential element for humans (Sun et al., 2015). Unfortunately, the toxicant can be found in hexavalent form in the tap water of nearly two thirds of the United States' drinking supply (Andrews & Walker, 2016). While the environmental protection agency (EPA) monitors total chromium levels in our water, hexavalent levels are not monitored, and the toxicant is ingested daily. The EPA's current maximum contaminant level (MCL) for total chromium is 100 ppb, however Cr(VI) at 100 ppb could have detrimental effects on a population. In humans, hexavalent chromium toxicity through ingestion has been known to cause cardiovascular, gastrointestinal, hematological, hepatic, renal, and neurological damage, and in severe cases, causes cancer or death (Yu, 2013). Furthermore, roughly ten percent of inorganic Cr(VI) is absorbed through the intestinal tract (Yu, 2013). Excretion of absorbed chromium occurs primarily via urine. In humans, the kidney excretes about 60% of an absorbed Cr(VI) dose in the form of Cr(III) within 8 hours of ingestion. Approximately 10% of an absorbed dose is eliminated by biliary excretion, with smaller amounts excreted in hair, nails, milk, and sweat (Kiilunen & Kivisto, 1983). Clearance from

plasma is generally rapid (within hours), whereas elimination from tissues is slower, with a half-life of several days (ATSDR, 2012).

Chromium is a naturally occurring element and often found in both the hexavalent and trivalent states in natural watersheds (Loyaux-Lawniczak et al, 2001). However, chromium compounds are often used for chromium plating and other industrial uses as well (Kamerud et al., 2013). Disposal of chromium containing commercial products and coal ash from electric utilities and other industries are major sources of chromium releases into the soil (Barceloux 1999). Solid waste and slag produced during chromate manufacturing processes can be potential sources of chromium exposure as well (Barceloux 1999). Improper disposal and maintenance of the chemical at these facilities can cause environmental contamination and drinking water pollution (Cone, 2009). A recent study done on Illinois water showed that hexavalent chromium concentrations were actually higher in treated water than those in untreated water. Cr(VI) levels on surface water were found to be 0.3 ppb, and levels in bedrock aquifers at 1.1 ppb, whereas those in treated water supplies were 2.4 ppb, indicating that water treatment practices may ironically play a role in increasing concentrations (Mills & Cobb, 2015).

Mechanism of Toxicity

The mechanism of toxicity of Cr(VI) has been found to be induction of oxidative stress which further leads to cell toxicity and cell death (Bagchi et al., 2002; Chiu et al., 2010). Oxidative stress results when reactive oxygen species (ROS), either produced endogenously as a consequence of normal cellular functions or derived from external sources, cause damage that exceeds the cell's ability to resist oxidation (Martindale & Holbrook, 2002). When ROS originate

from exogenous sources, they are either taken up directly by cells from the extracellular matrix or produced as a consequence of the cell's exposure to some environmental antagonist (Martindale & Holbrook, 2002). Transient fluctuations in ROS serve important regulatory functions such as in aerobic respiration, but when present in high levels, ROS can cause severe damage to DNA, protein, and lipids (Martindale & Holbrook, 2002). A number of cellular defense mechanisms have evolved to combat the accumulation of ROS. These include various non-enzymatic molecules such as glutathione, and vitamins A, C, and E, as well as enzymatic scavengers of ROS like superoxide dismutase and catalase (Martindale & Holbrook, 2002). Unfortunately, these systems of defense are not always adequate to counteract the production of ROS, resulting in what is termed a state of oxidative stress. Because of its 6+ oxidation state, Cr(VI) is a very powerful oxidizer.

Ames Assay

Previous study shows that not only are hexavalent chromium compounds carcinogenic, but they are also directly mutagenic towards deoxyribonucleic acid (DNA) (Petrilli & Deflora, 1976). In this procedure, a genetically engineered strain of *Salmonella typhimurium* was exposed to differing solutions to test mutagenic potential. When exposed to a mutagen, this specific strain of bacteria will revert from a state of auxotrophy (inability to produce the essential amino acid histidine), to a state of prototrophy (ability to produce histidine). This genetic reversion allows the bacteria to survive and replicate whereas a lack of mutation will result in death. Bacterial survival, observed by colorimetric determination of its growth media, is therefore a direct indicator of DNA mutagenesis. This same study showed that the mutagenic

effects were caused directly by the Cr(VI) and not due to a metabolic byproduct within the bacteria (Petrilli & Deflora, 1976).

Reduction Potential

One study showed that the use of certain microorganisms as a biological filter could be used to lower chromium levels (Thatoi et al., 2014), and several others have shown that antioxidants have chromium reduction potential against chromium as well (Chrysochoou & Reeves, 2016; Geetha et al., 2003). Antioxidants lower the oxidation state of chromium from 6+ to the lower, less harmful state of 3+. One study showed that epigallocatechin gallate (EGCG) directly reduces Cr(VI) in solution (Chrysochoou & Reeves, 2013), while another showed that Vitamin C also reduces Cr(VI) in solution and in the past has been used as a topical treatment against Cr(VI) skin exposure (Yu, 2013). A third study tested the effect of antioxidants extracted from the plant *Hippophae rhamnoides* on albino rats when co-fed with hexavalent chromium. Results suggested that rats which had been fed antioxidants along with the chromium compounds exhibited declines in tumor growth both in size and frequency than those which had been fed chromium compounds without any antioxidant present (Geetha et al., 2003). While this study demonstrates that a plant extract with antioxidant properties prevents Cr(VI)-induced toxicity at the organismal level, little is known about the effects of specific antioxidants on cellular culture. To date, there have been no studies of Cr(VI) and antioxidant cotreatment upon human cell culture, and while antioxidants are known to reduce Cr(VI), there is still uncertainty in regard to which antioxidants are most effective. Additionally, the extent to which antioxidants are protective has yet to be elucidated; whether they protect against cytotoxicity

upon the cell membrane and within the cytoplasm, or if they protect against mutagenesis within the nucleus as well.

Methods

Cellular culture, compounds, and storage

Human intestinal epithelial (HInEpi) cells were obtained from the American Type Culture Collection (ATCC) and were sustained on the cell line's respective ATCC media. Human embryonic kidney (HEK) cells were obtained from a secondary passage in nitrogen storage in house at Reed Hall of Science, though the original passage was obtained from Loyola University. These cells were sustained on Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, and 1% L-Glutamine, all of which were sourced from Sigma Aldrich. Cells were incubated at 37°C and 5% CO_2 and passaged once confluent using trypsin-EDTA. Both L-Ascorbic Acid and Epigallocatechin Gallate were obtained from Sigma Aldrich. The Cr(VI) (hexavalent chromium) compound used for experimentation was potassium chromate, also obtained from Sigma Aldrich.

Determination of treatment concentration

Experimental Cr(VI) concentrations were tested in magnitudes of 10 ranging from 2 ppb (parts per billion) to 20,000 ppb to simulate environmental conditions as well as coincide with previous literature. These concentrations were used for both cell proliferation and Ames procedures. Antioxidant concentrations were determined through experimentation, starting at

a 1:1 ratio of antioxidant to Cr(VI) and adjusted accordingly based on response until effects were seen.

Solution Preparation

Stock solutions of Cr(VI), EGCG and ascorbic acid were prepared by dissolving solute in double-distilled water at a concentration of 2000 ppm and then filter sterilizing under a filtrated hood. Once treatment concentrations had been determined as described above, smaller aliquots of stock solution were mixed with the appropriate cell media to bring the final mixture to the desired experimental concentration. Experimental solution containing both Cr(VI) and an antioxidant for cotreatment were prepared by bringing both a Cr(VI) solution and an antioxidant solution to twice their desired final concentration in media and then adding the two solutions together to dilute the sample down to its experimental concentration. Stock solutions were stored at 4°C and were remade several times throughout experimentation to avoid expiration. Experimental solutions were made within 24 hours of use.

Cellular proliferation assay

All cell culture work was done under a sterile hood. Both the HInEpi and HEK cells were passaged in T-75 flasks in their untreated, respective media until confluency was reached. These cells were then passaged onto a 24-well plate and again cultured within their untreated media until wells reached confluency. Treatment groups were then run in quadruplicate, allowing for 6 sample groups per plate. Cells were exposed to solution for 72 hours. To depict qualitative results, photos were taken of wells at points of interest along the way using a Nikon TXI.

After the 72-hour incubation period, media was aspirated and then cells were trypsinized and suspended in solution. 10 µl of solution was taken from each well and then mixed with equal parts Trypan blue. After at least a minute to allow for cell staining to occur, 10 µl of cell suspension/trypan stain solution was drawn off and dispensed over a hemocytometer for counting of viable cells to determine number of cells per treatment group.

Ames assay

Ames test kits were purchased from Environmental Bio-detection Products Inc. (EBPI) and the assay was carried out according to manufacture instructions. The lyophilized bacterial culture was suspended in a liquid media (Reagent G) 12-16 hours prior to experimentation. Once suspended, the bacteria were placed in a shaking incubator at 37°C to replicate and grow overnight. The following day, experimental samples were prepared by diluting the Cr(VI) stock solution in sterile water to desired concentrations. Turbidity within the bacterial culture verified growth and the OD600 of the solution was measured by spectrophotometry. This OD (optical density) value was then used to bring the bacterial suspension to a desired concentration through a series of calculations. 3 samples of each treatment group were placed in a 24-well plate for the bacterial exposure period. Positive, negative and sterility controls were also done on the exposure plate. Added into each well was the treatment solution, exposure media, and the bacterial suspension, and the plate was incubated at 37°C for 100 minutes. During incubation period, a reversion media was prepared for the 96-well plates. After the bacteria had been incubated for 100 minutes, the plate was removed and solution from each well was pipetted into a tube containing the premade reversion media. Using loading boats and a multichannel pipette, each sample was pipetted into 48 wells of a 96-well plate. The 96-well

plates were then placed in an incubator at 37°C for 3 days to allow for revertant bacteria to grow. After the 3 day incubation period, plates were scored by colorimetric determination with yellow and partial-yellow wells indicating genetic reversion.

Statistical analysis

The statistical analysis for both the cell proliferation data and the Ames assay data were done by a two-tailed t-test data with p-values < 0.05 determined to be statistically significant.

Results

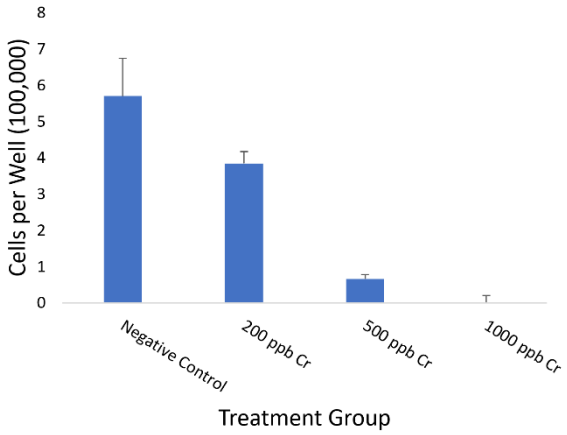

Figure 1: Dose response to Cr(VI) Human embryonic kidney cells were exposed to different doses of Cr(VI) ranging from 200 ppb - 1000 ppb. A negative dose response to Cr(VI) was observed.

Cr(VI) was shown to exhibit a negative dose response when exposed to HEK cells. Figure 1 shows that the negative control group of untreated media resulted in 570,000 cells per well after a three-day exposure period. 200 ppb Cr(VI) solution resulted in 383,750 cells per well, and 500 ppb Cr(VI) resulted in 66,250 cells per well while 1000 ppb completely eradicated all cells in the treatment group. These data made way for the addition of antioxidants to Cr(VI) solution.

Figure 2 shows that 500 ppb chromium was kept constant while varying concentrations of vitamin C were added as cotreatment. 50 ppm vitamin C was also run independently as a

control group and yielded similar cell counts as the negative control. 500 ppb Cr(VI) cotreated with 5 ppm VC increased cells per well from 500 ppb Cr(VI) alone. These counts were 186,250 and 66,250 respectively. Wells cotreated with Cr(VI) and 10 ppm VC yielded 467,500 cells per well and those cotreated with VC concentrations at 25 ppm yielded 590,000 cells per well. There is no statistically significant difference between the negative control and 50 ppm VC. There is also no significant difference between 500 ppb Cr(VI) cotreated with 25 ppm VC and the negative control.

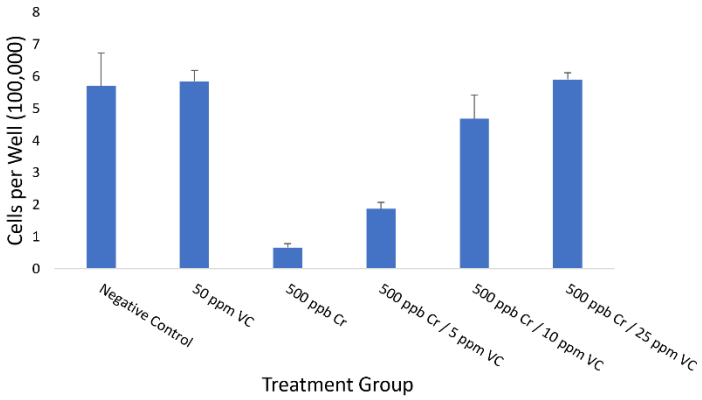

Figure 2: Dose response to Cr(VI) and vitamin C cotreatment. Human embryonic kidney cells were exposed to cotreated samples of 500 ppb Cr(VI) and differing concentrations of vitamin C. Likelihood of survival increased with the addition of vitamin C. Cr(VI) was completely mitigated by 25ppm vitamin C.

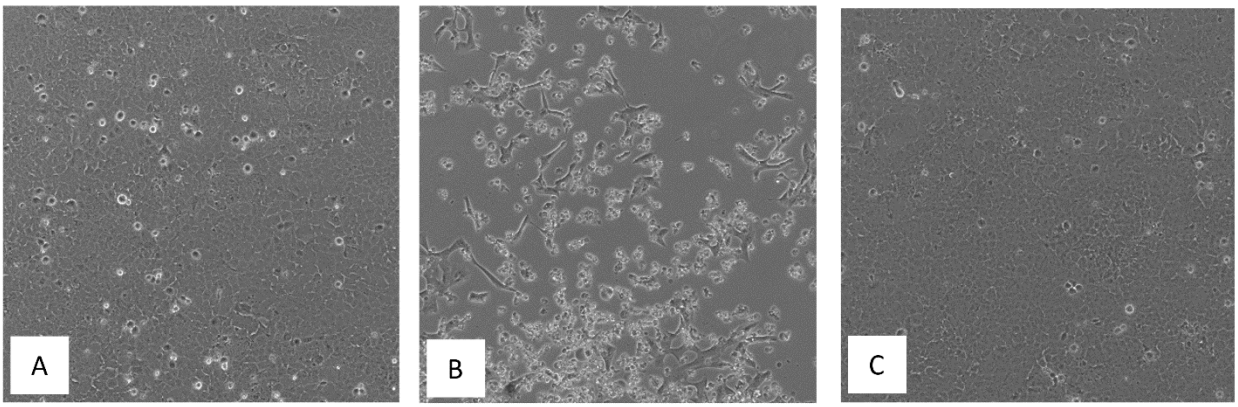

Figure 3: Human intestinal epithelial cells exposed to Cr(VI) and vitamin C. Cells were exposed to untreated cell media (panel A), as well as 500 ppb Cr(VI) (panel B) and 500 ppb Cr(VI) cotreated with 25 ppm vitamin C (panel C). Cotreatment mitigated nearly all observed effects of Cr(VI). This result was replicated on human embryonic kidney cells. These images were taken 3 days after exposure.

These results were replicated qualitatively upon a second cell line. Human Intestinal epithelial cells were exposed to the same treatment groups as shown in Figures 1 and 2. Due to

difficulties in the cell counting procedure for this particular line of cells, representative pictures of treatment groups were taken to serve as alternative results to quantification. Panel B in Figure 3 depicts 500 ppb Cr(VI) exposure whereas panel C shows cotreatment with 25 ppm VC. Panels A and C look relatively similar, while panel B shows undeniable cytotoxicity and cell necrosis.

HEK cells were also exposed to Cr(VI) solutions cotreated with EGCG. Figure 4 depicts exposure to 500 ppb Cr(VI) and solutions cotreated with EGCG ranging from 5 ppm to 50 ppm. In this trial, 500 ppb Cr(VI) resulted in 94,500 cells per well. 500 ppb Cr(VI) cotreated with 5 ppm EGCG also resulted in 94,500 cells per well. Upon further addition of EGCG, cell survival increased up to the final treatment group of 500 ppb Cr(VI) with 50 ppm EGCG which yielded a cell count of 460,250. Negative control in this exposure trial yielded 796,250 cells per well and 50 ppm EGCG, 759,500.

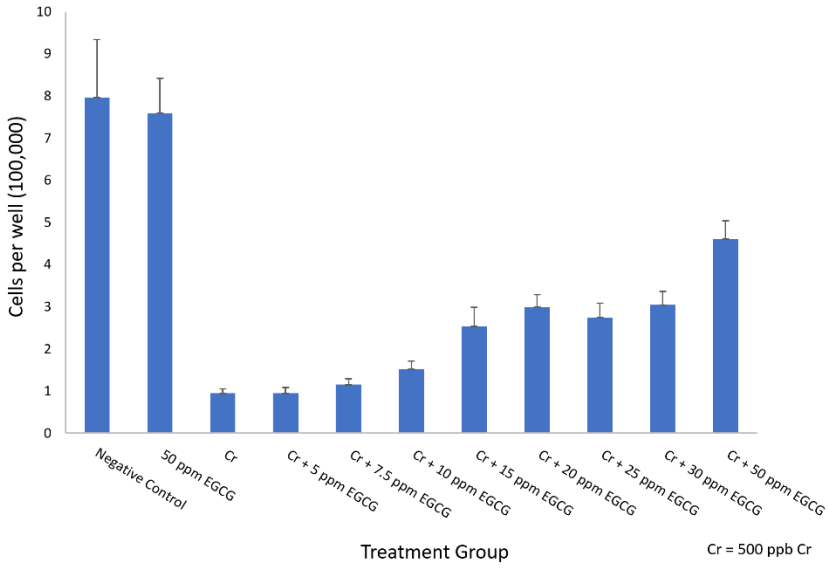

Figure 4: Dose response to Cr(VI) and EGCG cotreatment. Human embryonic kidney cells were exposed to solutions of 500 ppb Cr(VI) cotreated with varying concentrations of EGCG. As EGCG concentrations increased, cell survival also increased.

Figure 5 depicts the results of the Ames assay, which was performed identically five separate times throughout the course of experimentation. As Cr(VI) concentrations increased from 2 ppb to 2000 ppb, percent mutagenicity increased. Treatment groups of 20, 200 and 2000 ppb were significantly more mutagenic than the negative control. 200 ppb and 2000 ppb

Cr(VI) cotreated with 20 ppm vitamin C were significantly less mutagenic than their Cr(VI)-only treated counterparts. 20 ppm vitamin C was not found to be mutagenic itself.

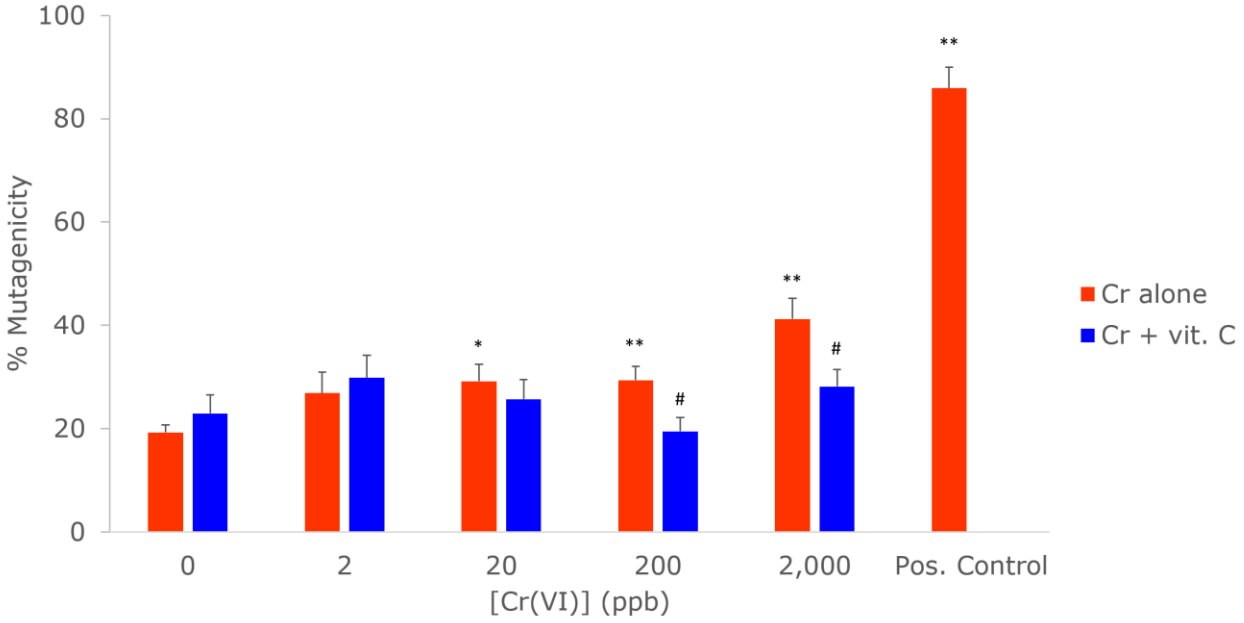

Figure 5: Bacteria exposed to Cr(VI) and vitamin C to test mutagenic potential. Bacterial cultures exposed to solution containing higher levels of Cr(VI) were more likely to mutate DNA. Those exposed to high levels of Cr(VI) as well as vitamin C were less likely to mutate. * denotes p-value < 0.05 when compared to negative control, ** denotes p-value < 0.005 when compared to negative control, # denotes p-value < 0.05 compared to that concentration of Cr(VI) alone.

Discussion

These data suggest that Cr(VI) is cytotoxic via an oxidative mechanism. After exposing human embryonic kidney cells to a range of Cr(VI) concentrations, we can see that the contaminant has a direct influence on cell death, with higher concentrations of chromium leading to a lower likelihood of cell survival (Figure 1). These same results were also observed qualitatively on Human Intestinal Epithelial cells, with higher concentrations of Cr(VI) leading to greater cell death. Furthermore, these effects were mitigated by the addition of antioxidants

within cell solution. Both ascorbic acid and epigallocatechin gallate prevented cytotoxicity and necrosis (Figures 2 and 4). This evidence suggests that Cr(VI) is indeed cytotoxic via an oxidative mechanism as the presence of an antioxidant reduced cytotoxicity.

Ascorbic acid was found to be a much more potent protective chemical than epigallocatechin gallate (Figures 2 and 4). This could be due to the vast difference in the size of the two molecules as ascorbic acid is much smaller than its counterpart. It's unclear where the reduction of Cr(VI) into Cr(III) is occurring, whether inside the cell in the cytoplasm or outside the cell within the culture media, though it is probable that the majority is occurring in the media before the toxicant enters the cell.

Data from the Ames assay continues to suggest that Cr(VI) is mutagenic by way of oxidation. As bacterial strains were exposed to increasing concentrations of the toxicant, percent mutation increased as well. When exposed to solution cotreated with antioxidant however, percent mutation was essentially nullified as levels were brought back down to those observed within the negative control group, regardless of the concentration of Cr(VI). That is, any group treated with vitamin C exhibited no significant change from the negative control, even at the highest treated concentration of Cr(VI) (2000 ppb).

Furthermore, neither antioxidant tested was found to be cytotoxic or mutagenic in-and-of-itself. This serves as crucial information as the ultimate purpose that they may serve as is a preventative protectant, pre-treated within a drinking water supply. We cannot pre-treat the water with a level of protectant so high that it itself becomes a harmful contaminant. This

concern is needless as both vitamin C and EGCG showed great protective potential at levels at which they themselves were harmless.

It appears that Cr(VI) is more mutagenic towards bacterial DNA at lower concentrations (20 ppb) than it is cytotoxic towards mammalian cells (200 ppb). This result indicates that bacterial cells are either more sensitive to Cr(VI), or that mutagenicity is also occurring in the mammalian cells at concentrations like that in the bacteria, but that the mutations occurring are nonlethal or nonharmful. In either case, the information merits further research into Cr(VI) mutagenicity in a mammalian cell line.

Additionally, this study focused on the cotreatment of antioxidants with Cr(VI). Specifically, both bacterial cells in the Ames test and human cells in the cell proliferation assay were exposed to both the toxicant and the protectant simultaneously. In both assays, the cotreated solution was prepared 1-10 hours before cell exposure. Because of this, the chemical interaction taking place between the two compounds likely occurs in solution. Another interesting continuation of this study would be examining the differences between a cotreatment with antioxidant (as performed here in this study) and a pre-treatment or post-treatment with antioxidant.

These data come to the scene at a point in time where millions of Americans are exposed to hexavalent chromium in their daily water supply (Andrews & Walker, 2016). The protective potential of antioxidants is promising, and applications of this research are numerous. For example, just as many European countries began adding fluoride to public water supplies in the early 90's to prevent dental caries, antioxidant may be added to water supplies

known to have higher concentrations of Cr(VI), or areas at higher risk of Cr(VI) contamination. This reduction potential may also be applied to mitigate other powerful oxidizers. Though continued study is merited, this information further validates the protective potential of antioxidants and will be helpful for government agencies and organizations in determining safe levels of water chromium.

References

Ames, B., Durston, W., Yamasaki, E., & Lee, F. (1973). *Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection*. Proceedings of the National Academy of Sciences USA, 70(8), 2281-2285.

Andrews, D., & Walker, B. (2016). *'Erin Brockovich' Carcinogen in Tap Water of more than 200 Million Americans.* Environmental Working Group.

ATSDR, Agency for Toxic Substances and Disease Registry. (2012). *Toxicological Profile for Chromium.*

Bagchi, D., Stohs, S.J., Downs, B.W., Bagchi, M., & Preuss, H.G. (2002). *Cytotoxicity and Oxidative Mechanisms of Different forms of Chromium*. Toxicology, 180, 5-22.

Barceloux, D. G. (1999). *Chromium*. Toxicology, 37(2), 173-94.

Casarett, L. J., Klaassen, C. D., Amdur, M. O., & Doull, J. (1996). *Casarett and Doull's toxicology: The basic science of poisons*. New York: McGraw-Hill, Health Professions Division.

Chiu, A., Shi, J., Hill, R., Katz, A., (2010). *Review of Chromium (VI) Apoptosis, Cell-Cycle-Arrest, and Carcinogenesis.* J Environ Sci Health C Environ Carcinog Ecotoxicol Rev., 28(3), 188–230

Chrysochoou, M., & Reeves, K. (2016). *Reduction of Hexavalent Chromium by Green Tea Polyphenols and Green Tea Nano Zero-Valent Iron*. Bulletin of Environmental Contamination and Toxicology, 98(3), 353-358.

Cone, M. (2009). *Chromium in Drinking Water Causes Cancer*. Environmental Health News, (February, 20).

Dalcin, M.G., Pirete, M.M., Lemos, D.A., Ribeiro, E.J., Cardoso, V.L., & Resende, M.M. (2011). *Evaluation of Hexavalent Chromium Removal in a Continuous Biological Filter with the use of Central Composite Design*. Journal of Environmental Management, 92(4), 1165-1173.

Division of Public Water Supplies. (2011). *Assessment of Total Chromium in Illinois Community Water Supplies*. Illinois Environmental Protection Agency.

Geetha, S., Sai Ram, M., Mongia, S., Singh, V., Ilavazhagan, G., & Sawhney, R. (2003). *Evaluation of Antioxidant Activity of Leaf Extract of Seabuckthorn (Hippophae rhamnoides) on Chromium(VI) Induced Oxidative Stress in Albino Rats*. Journal of Ethnopharmacology, 87(2-3), 247-251.

Kamerud, K.L., Hobbie, K.A., & Anderson, K.A. (2013). *Stainless Steel Leaches Nickel and Chromium into Foods during Cooking*. Journal of Agricultural and Food Chemistry, 61, 9495-9501.

Kiilunen, M., & H. Kivisto, (1983). *Exceptional pharmacokinetics of trivalent chromium during occupational exposure to chromium lignosulfonate dust*. Scandinavian Journal of Work, Environment & Health 9(3), 265-71.

Levis, A.G., Bianchi, V., Tamino, G., & Pegoraro, B. (1978). *Cytotoxic Effects of Hexavalent and Trivalent Chromium on Mammalian Cells in Vitro.* Br. J. Cancer, 37, 386-396.

Loyaux- Lawniczak, S., Lecomte, P., & Ehrhardt, J.J. (2001). *Behavior of Hexavalent Chromium in a Polluted Groundwater: Redox Processes and Immobilization in Soils*. Environmental Science and Technology, 35(7), 1350-1357.

Majone, F., Bianchi, V., Dal Toso, R., Debetto, P., Levis, A.G., Luciani, S., & Tamino, G. (2002). *Mechanisms of Chromium Toxicity in Mammalian Cell Cultures.* Toxicology, 17(2), 219-224.

Martindale, J. L., & Holbroook, N. J. (2002). *Cellular response to oxidative stress: Signaling for suicide and survival*. Journal of Cellular Physiology, 192(1), 1-15.

Mills, P.C., & Cobb, R.P. (2015). *Hexavalent and Total Chromium at Low Reporting Concentrations in Source-Water Aquifers and Surface Waters Used for Public Supply in Illinois*. U.S. Geological Survey Scientific Investigations Report 2015–5020, 58.

Oze, C., Bird, D.K., & Fendorf, S. (2007). *Genesis of Hexavalent Chromium from Natural Sources in Soil and Groundwater*. Proceedings of the National Academy of the Sciences, 104(16), 6544-6549.

Petrilli, F., & DeFlora, S. (1976). *Toxicity and Mutagenicity of Hexavalent Chromium on Salmonella typhimurium*. Applied and Environmental Microbiology, 33(4), 805-809.

Strober, W. (2001). *Trypan Blue Exclusion Test of Cell Viability*. National Institute of Allergy and Infectious Diseases, 3, 10.1002.

Sun, H., Brocato, J., & Costa, M. (2015). *Oral Chromium Exposure and Toxicity*. Curr Environmental Health Report, 2, 295–303.

Thatoi, H., Das, S., Mishra, J., Rath, B.P., & Das, N. (2014). *Bacterial Chromate Reductase, a Potential Enzyme for Bioremediation of Hexavalent Chromium: A Review.* Journal of Environment Management, 146, 383-399.

Von Burg, R. (1993). *Chromium and Hexavalent Chromium.* Journal of Applied Toxicology, 13(3), 225-230.

Yu, D. (2013). *Chromium Toxicity*. Agency for Toxic Substances and Disease Registry.