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Cover Page Footnote

I would like to thank Dr. Ryan Himes for his help throughout this project. His guidance, encouragement, and friendship were truly a blessing that I'll remember for years to come. This work was made possible by funding from the Olivet Honors Program, the Pence- Boyce research grant, and the Hippenhammer grant. I also owe thanks to Drs. Long, Rosenberger, and Sharda for their insight in work with cell culture, statistical analysis, and the writing of this document. Though I've been able to take part in great opportunities like this research project during my undergraduate experience, perhaps nothing is greater than the overall ability to study at a place like Olivet. I cannot express how crucial it is to learn science in a place where you can develop your thoughts freely without opinions forced upon you. To be presented with evidence and allowed the chance to independently understand it, all the while surrounded by Christian faculty who encourage you to walk in God's will and listen to His Spirit is a great gift. I would not be the person I am today without the community at this university. I also owe a tremendous thank you to my friends and family for their interest in and support of this project.



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ABSTRACT

Hexavalent chromium, or Cr(VI), is a potent oxidizer and known carcinogen that is found at varying levels in the water sources of more than 200 million Americans. However, the exact mechanism of carcinogenicity remains unknown, and though the government currently regulates total chromium levels they have yet to determine a permissible exposure limit for Cr(VI). Moreover, there is currently no preventative treatment for Cr(VI). Because of Cr(VI)'s strong oxidative power, we hypothesized that it causes DNA mutation and cell death via oxidation and that antioxidants could prevent this from occurring. To test this, we first assessed the viability of human cell culture exposed to Cr(VI) with or without either of the antioxidants vitamin C or epigallocatechin gallate (EGCG). Further, an Ames test was performed to determine the mutagenicity of Cr(VI) with and without either antioxidant.

We 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, while neither antioxidant was observed to be cytotoxic when treated alone. Cr(VI) was also found to be significantly mutagenic at 20 ppb and greater. This mutagenicity was significantly reduced by cotreatment with 20 ppm vitamin C at 200 and 2000 ppb Cr(VI), while vitamin C was not found to be mutagenic when tested individually. With these combined data, we conclude that Cr(VI) 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 against toxicants like Cr(VI).

Keywords: Hexavalent chromium, epigallocatechin gallate, ascorbic acid, cytotoxicity, mutagenicity, 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), and its toxicological effects on rats (Geetha et al., 2003) and cell cultures (Majone et al., 2002). Though there exist three 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 is in fact 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). Though the environmental protection agency (EPA) monitors total chromium levels in drinking water, hexavalent levels are not monitored, and the toxicant is ingested daily. The EPA's current maximum contaminant level for total chromium is 100 ppb, yet it is unclear if 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).

Chromium is a naturally occurring element and often found at low levels 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 and can elevate these low levels (Kamerud et al., 2013). Disposal of chromium containing commercial products and coal ash from electric utilities are major sources of chromium releases into the soil. 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 Cr(VI) at these facilities can cause environmental contamination and drinking water pollution (Cone, 2009). A recent study done of Illinois water showed that hexavalent chromium concentrations were actually higher in treated water than those in untreated water. Indeed, 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). Though these levels are well below the EPA-regulated limit, it is still unknown whether these levels are detrimental to human health.

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). In their study, Martindale and Holbrook found that 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). They went on to find that 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 an environmental antagonist, such as Cr(VI). 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. 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 potent oxidizer that can challenge the limited capacity of natural antioxidative systems.

Ames assay

Previous research shows that not only are hexavalent chromium compounds carcinogenic, but they are also directly mutagenic towards deoxyribonucleic acid (DNA) (Petrilli & Deflora, 1976). In this study, 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 Cr(VI) and not due to a metabolic byproduct within the bacteria (Petrilli & DeFlora, 1976). This information therefore indicated that not only is Cr(VI) toxic to organisms but is also directly mutagenic toward cellular DNA, with a preventative treatment yet to be elucidated.

Reduction potential

Different approaches have been utilized to reduce the oxidizing potential of Cr(VI). For example, one study showed that the use of certain microorganisms as biological filters could be used to lower chromium levels (Thatoi et al., 2014), while several others have shown that antioxidants are effective (Chrysochoou & Reeves, 2016; Geetha et al., 2003). Antioxidants lower the oxidation state of chromium from hexavalency to the lower, less harmful state of trivalency. One study showed that epigallocatechin gallate (EGCG) directly reduces Cr(VI) in solution (Chrysochoou & Reeves, 2013), whereas 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 that had been fed antioxidants along with the chromium compounds exhibited declines in tumor growth both in size and frequency compared to those without antioxidants (Geetha et al., 2003). Though 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 at the cellular level. To date, there have been no studies of Cr(VI) and antioxidant cotreatment on human cell culture, and though 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. We hypothesized that when co-treated with an antioxidant, hexavalent chromium would exhibit less cytotoxicity on a human cell culture. We also hypothesized that when co-treated with an antioxidant, hexavalent chromium would exhibit less mutagenicity towards bacterial DNA.

METHODS

Cell 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 Dr. Seth Robia (Loyola University Chicago). 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% CO₂ and passaged once confluent using trypsin-EDTA. Both L-ascorbic acid and epigallocatechin gallate were obtained from Sigma Aldrich. The Cr(VI) 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 ten 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. 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 twenty-four hours of use.

Cellular proliferation assay

Both the HInEpi and HEK cells were passaged in T-75 flasks in respective media until confluency was reached. These cells were then passaged onto a 24-well plate and again cultured until wells reached confluence. Treatment groups were then run in quadruplicate, allowing for six sample groups per plate that 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 inverted microscope with phase contrast.

After the 72-hour incubation period, media was aspirated and cells were trypsinized and suspended in solution. 10 µl of solution were taken from each well and 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 were 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 manufacturer's instructions. The lyophilized bacterial culture was suspended in a liquid medium (Reagent G) 12 to 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 (Appendix 1). Three samples of each treatment group were placed in a 24-well plate for the bacterial exposure period. Positive, negative, and

sterility controls were also included 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 the incubation period, a reversion medium 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 medium. 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 three days to allow for revertant bacteria to grow. After the three 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. The error bars depicted in the figures below show standard error of the mean.

RESULTS

To determine if antioxidants could prevent Cr(VI)-induced cytotoxicity, we first had to establish Cr(VI) toxicity without cotreatment with antioxidants. This also helped us determine a good concentration of Cr(VI) to use for cotreatment. HEK cells exposed to increasing concentrations of Cr(VI) displayed a marked reduction of cell viability. **Figure 1** shows that Cr(VI) levels of 1000 ppb completely eradicated all cells in the treatment group. These data indicate not only the severity of Cr(VI) exposure but also provide a basis for a range of exposure doses and cellular susceptibility. For this study, we chose to use 500 ppb Cr(VI) as an intermediate dose to elucidate the potency of the antioxidants vitamin C and EGCG.

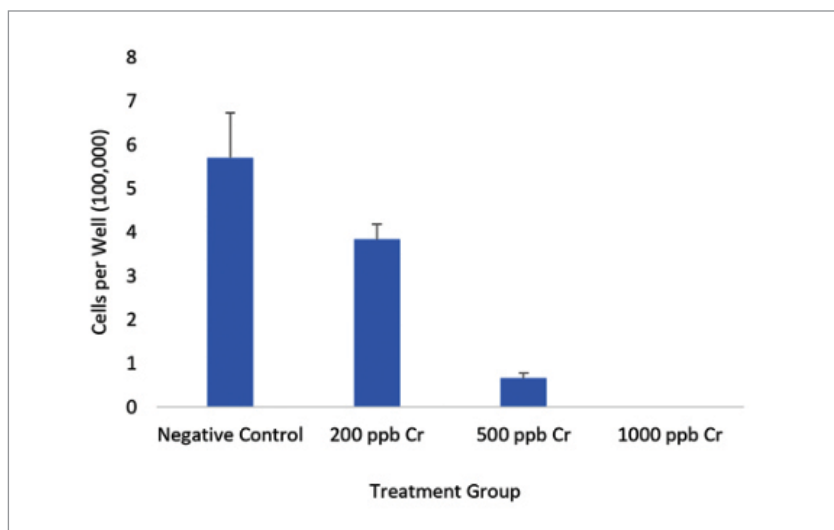


Figure 1: Human embryonic kidney cells display reduced viability upon increasing exposure to Cr(VI). HEK cells were exposed to different doses of Cr(VI) ranging from 200 ppb to 1000 ppb. A negative dose response to Cr(VI) was observed.

Next, we wanted to determine if the presence of vitamin C could prevent Cr(VI)-induced cytotoxicity. To test for this, chromium concentrations were kept constant at 500 ppb 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, indicating a lack of cellular toxicity of vitamin C alone. Figure 2 shows that 500 ppb Cr(VI) cotreated with as little as 5 ppm vitamin C more than doubled the cells per well from 500 ppb Cr(VI) alone. Furthermore, responses were observed in a dose dependent manner in wells cotreated with 10 and 25 ppm vitamin C. Importantly, there was no significant difference observed between 500 ppb Cr(VI) cotreated with 25 ppm vitamin C and the negative control.

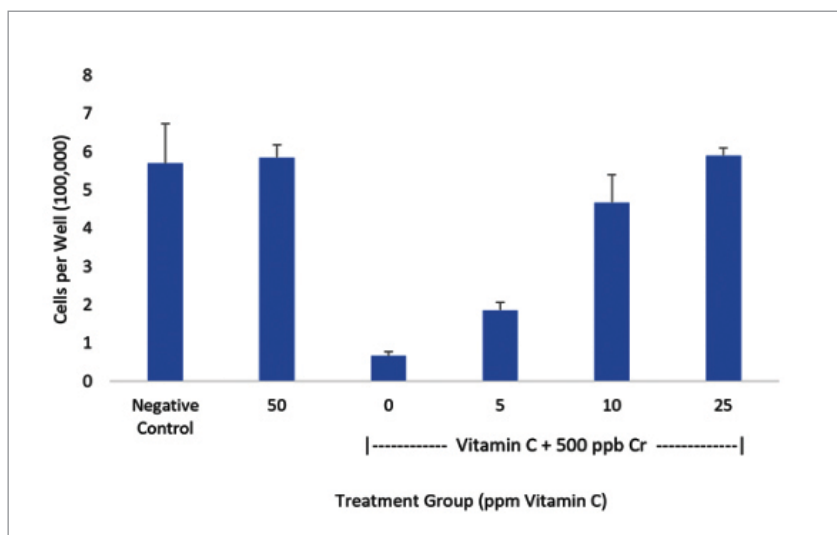


Figure 2: The antioxidant vitamin C can prevent toxicity of Cr(VI). Human embryonic kidney cells were exposed to cotreated samples of 500 ppb Cr(VI) and differing concentrations of vitamin C. Likelihood of survival increased with the addition of vitamin C. Cr(VI) was completely mitigated by 25 ppm vitamin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

These results were also replicated qualitatively upon a second cell line. Human intestinal epithelial cells were exposed to the same treatment groups as the above mentioned HEK cells. 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. As shown in **Figure 3**, cotreatment with 25 ppm vitamin C markedly improved cytotoxic effects of 500 ppb Cr(VI). Together, these data suggest that Cr(VI) toxicity and the protective effect of vitamin C can be broadly applied across different cell types.

Not only were we interested in the protective potential of vitamin C, but antioxidants at large. To test this theory, we exposed HEK cells to solutions cotreated with 500 ppb Cr(VI) and varying concentrations of EGCG to compare the efficacy of the two antioxidants (**Figure 4**). As in the case of vitamin C, EGCG demonstrated protective effects in a dose dependent manner starting at 7.5 ppb EGCG. However, unlike the complete protection from Cr(VI) toxicity that was observed with vitamin C at a lower dose, even the highest dose of EGCG was only able to protect approximately 60% of the HEK cells.

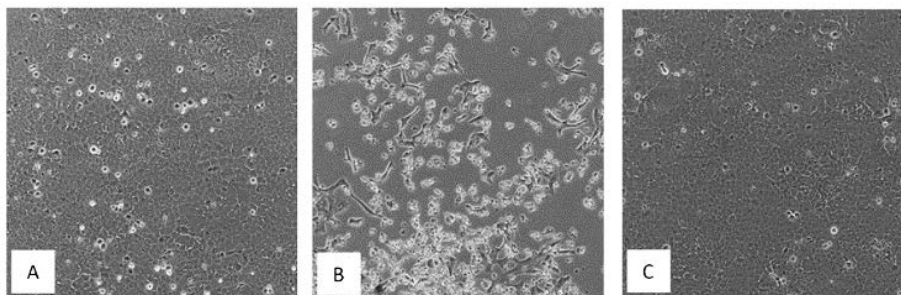


Figure 3: Vitamin C protects human intestinal epithelial cells from Cr(VI)-induced cytotoxicity. Cells were exposed to untreated cell media (panel A), as well as 500 ppb Cr(VI) (panel B) and 500 ppb Cr(VI) cotreated with 25 ppm vitamin C (panel C). Cotreatment mitigated nearly all observed effects of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

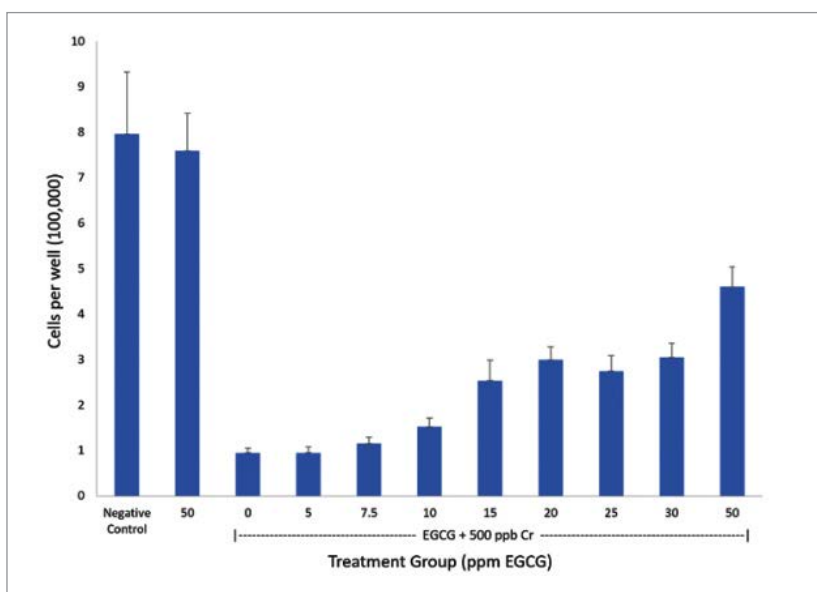


Figure 4: The antioxidant EGCG can prevent toxicity of Cr(VI). Human embryonic kidney cells were exposed to solutions of 500 ppb Cr(VI) cotreated with varying concentrations of EGCG. As EGCG concentrations increased, cell survival also increased.

We next wanted to test the protective potential of antioxidants against Cr(VI)-induced mutagenicity. To test this, bacteria were exposed to Cr(VI) as well as Cr(VI) cotreated with vitamin C in an Ames assay. **Figure 5** shows that as Cr(VI) concentrations increased from 20 ppb to 2000 ppb, percent mutagenicity increased. Treatment groups of 20, 200, and 2000 ppb were significantly more mutagenic than the negative control. Cotreatment with 20 ppm vitamin C significantly reduced the mutagenicity of 200 ppb and 2000 ppb Cr(VI). Moreover, 20 ppm vitamin C was not found to be mutagenic itself.

DISCUSSION

After exposing human cell cultures to a range of Cr(VI) concentrations, we observed that increasing concentrations of Cr(VI) was associated with cell death of both HEK

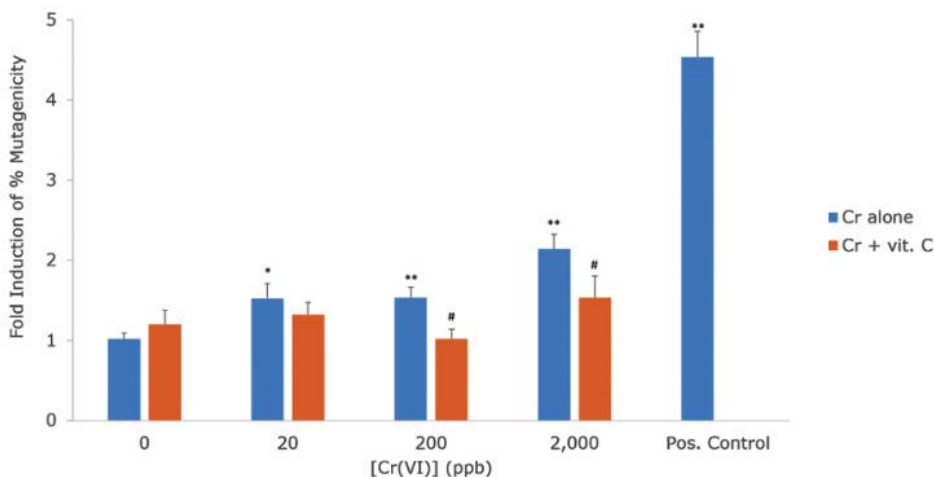


Figure 5: Bacterial cells cotreated with vitamin C and Cr(VI) exhibit less mutagenesis than those without the antioxidant. Bacterial cultures exposed to solution containing higher levels of Cr(VI) were more likely to mutate DNA. Those exposed to high levels of Cr(VI) as well as vitamin C were less likely 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. The positive control is a 12.5 ppb sodium azide solution.

and HIE cells (Figures 1 and 3). However, we found that the detrimental effects of Cr(VI) were mitigated by the addition of the antioxidants vitamin C or EGCG in a dose dependent manner (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.

Vitamin C was found to be a much more potent protective chemical than EGCG (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. This is because the Cr(VI) was exposed to the antioxidant in solution hours before being dispensed over the cells. Indeed, metabolic clearance of Cr(VI) may likewise occur prior to cellular interaction. 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 eight 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). Therefore, a majority of the reduction interactions occurring between antioxidants and Cr(VI) likely take place before the two components enter the cell. However, while clearance from plasma is generally rapid (within hours), whereas elimination from tissues is slower, with a half-life of several days (ATSDR, 2012).

Data from the Ames assay further suggests that Cr(VI) is indeed mutagenic by way of oxidation. Though previous studies have shown that Cr(VI) is mutagenic (Petrilli

& DeFlora, 1976), the novel cotreatment with antioxidants performed in this study show that chemical reduction decreases mutagenicity, indicating that the oxidative mechanism of Cr(VI) plays a critical role in mutagenesis. 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 concentration of Cr(VI) (2000 ppb).

Furthermore, neither antioxidant tested was found to be cytotoxic or mutagenic in-and-of-itself. Thus, they may serve as a preventative protectant as pre-treatment 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 in this case 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 may suggest two things. One reason for this discrepancy may be that bacterial cells are more sensitive to Cr(VI); this is probably a result of the vast differences between prokaryotic and eukaryotic cells. Eukaryotes have several other cellular components that Cr(VI) may interact with before reaching the DNA within the nucleus. The second conclusion may be that mutagenicity is also occurring in the mammalian cells at concentrations similar to that in the bacteria, but that the mutations occurring are nonlethal or nonharmful. This would be a reasonable assumption, as a mammalian eukaryotic cell has far more DNA than a prokaryotic bacterium (Alberts et al., 2015). Additionally, a prokaryotic genome has fewer regions of noncoding DNA (20%) than that of a eukaryote (98%) (Alberts et al., 2015). Therefore, there is a greater likelihood that a mutagen would affect a critical region in the bacterial genome, while eukaryotic cells are afforded a degree of insulation by vast noncoding stretches of DNA that can absorb mutagens such as Cr(VI). In either case, this merits further research into Cr(VI) mutagenicity in a mammalian cell line.

This study focused only on the cotreatment of antioxidants with Cr(VI) as opposed to pre-treatment or post-treatment. 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 one to ten hours before cell exposure. Because of this, the chemical interaction taking place between the two compounds likely occurs in solution. An interesting continuation of this study would be examining the differences between a cotreatment with antioxidant (as performed in this study) and a pre or post-treatment with antioxidant. This would provide information as to whether antioxidants could protect against impending Cr(VI) exposure or help cells recover from prior Cr(VI) exposure. These assays would also shed more light as to where the majority of the chemical reduction of the chromium is occurring, either in solution or within the cells themselves.

These data come at a time when hundreds of 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 1990s to prevent dental caries, antioxidants may be useful additions to water supplies known to have higher concentrations of Cr(VI) or areas at higher risk of Cr(VI) contamination. Moreover, these findings suggest that antioxidants as reducing agents may also be applied in mitigation of other harmful 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.

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APPENDIX

$X = OD_{600}$ measurement for overnight bacteria, $Y =$ Working concentration
(For this specific strain, TA100, the working concentration is 0.05 at OD_{600}),
 $Final\ volume = 6\ mL$.

Volume of overnight bacteria (mL) required for dilution = $\frac{Y}{X} \times Final\ volume$

Example: TA100 was grown overnight and recorded an $OD_{600} = 0.65$

Volume of TA100 required for dilution = $\frac{(0.05)}{(0.65)} \times 6 = 0.46\ mL$

Therefore, 0.46 mL of overnight bacterial solution will be added to 5.54 ml of Reagent N to bring the final volume to 6 mL.