The Effects of Copper, Manganese, and Vanadate Mixtures on Caco-2 Cell Cultures: A Case for the Precautionary Principle

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Abstract

This paper is a commentary on the usefulness of the Precautionary Principle as a risk management tool in the protection of public health when concrete scientific evidence is absent. The presence of metals in drinking water is known to affect human health, even in small amounts. While, certain metals are regulated, others are not due to a lack of data to support the concern that metal mixtures in trace amounts may pose a risk to human health. In this preliminary study, we assessed the viability of the Caco-2 (HTB-37) human adenocarcinoma cell line after a series of exposure experiments with mixtures of Cu, Mn, and V. Concentrations ranges of 0.25 mM, and 30 mM of Cu, Mn, and V mixtures were employed, and resazurin and XTT assays were used to evaluate mixture effects on cell viability. Cell proliferation was observed with V at low concentrations (>1 mM) and Cu at high concentrations (< 1 mM) in tests of single-metal treatment. Results were summarized and evaluated from the perspective of the Precautionary Principle.

1. Introduction

Environmental pollutants are most often composed of unidentified chemical mixtures, which in turn nullify the idea that a person is exposed to only a single chemical agent at any given time (Bliss, 1939). Hence, the predictive potential of toxicological data based on single chemicals is inadequate for chemical mixture-based risk assessments (Malich et al., 1998).

Limits set by the United States Environmental Protection Agency (U.S. EPA) for some metals, such as copper (Cu) and manganese (Mn), can still result in toxic effects at the cellular level prior to manifesting at the macroscopic level. Additionally, there are certain substances such as vanadium (V) that are not currently regulated by the EPA, which current research indicates may produce deleterious effects after long-term exposure. Taking into consideration these complexities, regulators can prevent irreparable harm to human health by applying preventative measures (Raffensperger and Tickner, 1999).

Therefore, this exposure study attempted to identify the effects of metal mixtures (at levels actually consumed in drinking water by some Houston-area residents) on Caco-2 cells. Bear in mind that the term “exposure” is usually interpreted as an internal dose of a drug given to a patient to produce an effect in pharmacology; we define exposure as treatment of cells and sub-cellular organelles in vitro to solutions of metal compounds in cell biology. As a result, the assessment of metal exposure in this study encompasses the contact between humans and metals through drinking water without measuring the actual amounts of the metals retained by the cell culture (Seiler et al., 1994).

2. Role of the Precautionary Principle

Environmental standards for drinking water contaminants have been put in place by regulatory agencies for individual compounds.

However, as mentioned previously, drinking water may contain a complex mixture of chemical contaminants, such as trihalomethanes, pesticides, and heavy metals. Consequently, the likelihood of one or more of these contaminants to affect the toxicity or pharmacokinetic behavior of another compound is possible and of great interest. This is the challenging aspect when setting drinking water standards that are protective of human health should such interactions occur. There are guidelines put forward by the EPA and other agencies for conducting risk assessments for chemical mixtures, but there is insufficient data to indicate or measure the extent to which the health effect of one compound is changed in any way by co-exposure to another (Brown et al., 1994).

Conventional risk regulation is usually concerned with the establishment of hazard levels below which a resultant harm is acceptable or of no consequence with respect to the law (e.g., Maximum Contaminant Levels). Setting these types of limits can pose a problem for regulators; therefore, now there is a reverse in the burden of proof. There is no requirement for a completely defendable risk assessment, which could be perceived as precautionary-based (Rogers, 2003).

Generally speaking, scientific disciplines, like epidemiology, can ascertain compelling links between a disease state and a particular contaminant/pollutant; however, scientific proof is often lacking. Many studies have employed animal models and show evidence of disease but making the connection with human subjects is often unclear. Thus, because this type of proof is difficult to obtain, some governments have already taken a *weight-of-evidence* approach and applied the PP in order to protect public health (Hileman, 2009).

3. Methods

Inductively Coupled Plasma-Mass Spectrometry analyses of Houston-area municipal drinking water samples were carried out to assess metals of interest. Water samples were collected during several sampling trips and analyzed using U.S. EPA Method 200.8 at Texas Southern University's NASA-URC. Cu, Mn, and V were selected, and subsequent toxicity testing employing several assays followed. First, stock solutions of the metals of interest were prepared using sulfates of Cu, Mn, and sodium orthovanadate obtained from Sigma-Aldrich. The concentrations of primary focus were carefully weighed and used to treat tumor cells. These cell grow more readily in low serum media, hence the serum free treatment was include for comparison. Serum can adsorb treatment metals and reduce the concentrations available to the cells (Johns Hopkins Technical Report, 1992).

Fetal bovine serum (FBS) is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors (Sigma Aldrich, 2013). So all experiments labeled serum-free should be interpreted as media with no FBS and those labeled complete, did contain FBS as part of the culture process. Salts were dissolved in serum-free media and stored 1–2 days prior to treatment. The solutions were later used in toxicity tests on the Caco-2 cell line obtained from ATCC (Manassas, VA). Metals used in all experiments were in the form of sulfates because of some concern for the preferential absorption of oxyions in the cell culture. This was not an issue for the form of vanadium used in these experiments because sulfates would not be taken up preferentially in place of vanadate as an oxyion.

Cell culture experiments were performed using various chemical mixture treatments, and cell viability was measured using the resazurin and XTT biological assays. According to the manufacturer's instructions, all absorbances must be adjusted for a true value to be obtained. It should be noted that absorbance values obtained were read directly but were unadjusted as recommended by the manufacturer. Only one wavelength was used in each experiment even though the manufacturer suggested a second wavelength be used to eliminate error. This study was a preliminary, range-finding investigation into continued cell viability after each treatment. Error bars were calculated at the 5% level and viability results were compared to control results.

4. Results and Discussion

Significant information concerning trace metal toxicity in human intestinal cells was revealed. The results indicated that at both the 1.0-ppm and 30-mM concentrations, a correlation was found between increased viability in the Cu-only treatment and increased viability with the mixture. The opposite was true for V in this same test as viability decreased with the single-metal treatment at both the 1.0-ppm and 30-mM levels. These results are shown in both experiments 1 and 2 below.

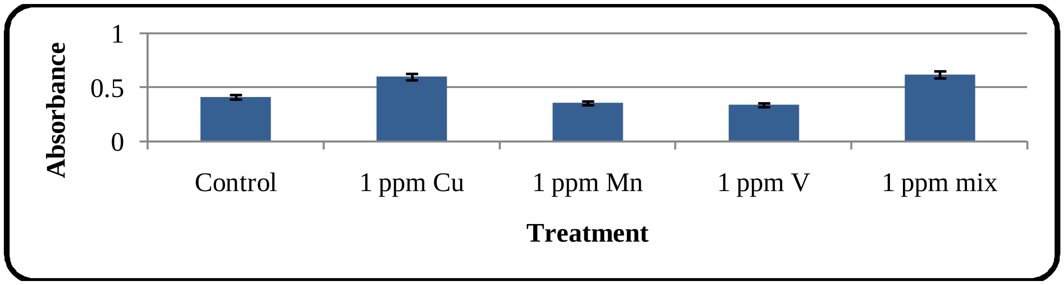

Figure 1 Resazurin Results for 1ppm Metal Treatments on Caco-2 Cell Cultures after 24 hr exposure

Table 1 Resazurin Assay Data for 1ppm Metal Treatments on Caco-2 Cell Cultures after 24hrs exposure

TREATMENT					
	Serum-free	Cu	Mn	V	Mixture
Avg Abs	0.42±0.13	0.60±0.06	0.36±0.009	0.34±0.06	0.62±0.19

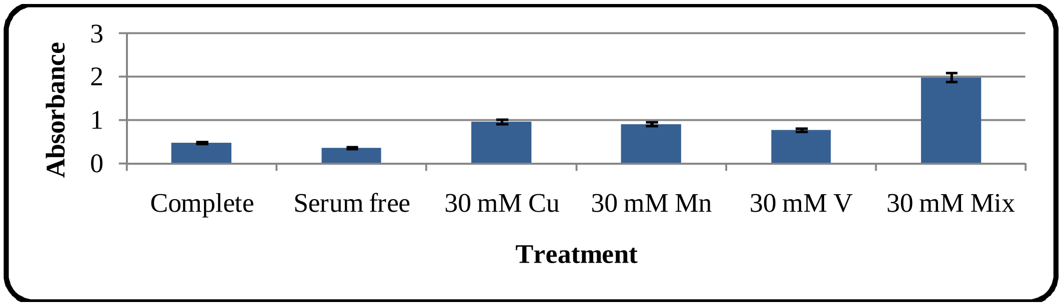

Figure 2 Resazurin Data for the Effects of 30mM Metal Treatments on Caco-2 Cell Cultures after 24 hr exposure

Table 2 Resazurin Assay Data for 30mM Metal Treatments on Caco-2 Cell Cultures

TREATMENT						
	Complete	Serum-free	Cu	Mn	V	Mixture
Avg Abs	0.47±0.08	0.35±0.03	0.96±0.11	0.91±0.06	0.77±0.13	1.99±0.22

Preliminary analysis of the data from another range-finding mixture experiment indicated the Caco-2 cell line showed increased absorbance compared with controls after exposure to a 0.004-mM Cu, 0.0059-mM Mn, and 0.0054-nM V mixture (~1 ppm in all cases). Researchers have conducted studies using similar concentration ranges (0.2-0.5 ppm vanadate) and found no toxic effects after single element exposure (Bishayee and Chatterjee, 1995). This is very interesting, and future longer-term exposure may induce a different result at the 1-ppm concentration. In this experiment, the results obtained showed similar trends with respect to the absorbances obtained for the mixture and Cu. The mixture takes on the character of the Cu in increasing the proliferation rate of the culture. These absorbances were obtained at 24.2 ºC, and the control was serum-free. This concentration was used as a high-test value because there already exists, data in the literature concerning the behavior of these metals and results were corroborated.

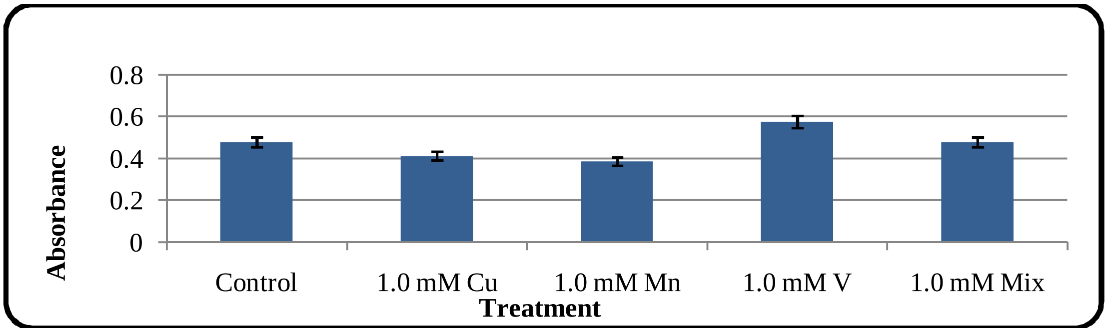

Figure 3 Resazurin Data for the Effects of 1.0mM Metal Treatments on Caco-2 Cell Cultures after 24 hr Exposure

Table 3 Resazurin Assay Data for 1.0mM Metal Treatments on Caco-2 Cell Cultures

TREATMENT					
	Serum-free	Cu	Mn	V	Mixture
Avg Abs	0.48±0.05	0.41±0.04	0.39±0.04	0.58±0.04	0.48±0.05

Conversely, when viability increased in the V-only treatment, the effects of the mixture, and Cu- and Mn-only treatments on viability appeared to be negative (i.e., decreased at both the 0.25-mM and 1.0-mM levels). Therefore, it seemed that the V in the mixture countered the effects of Cu and Mn in the same mixture at lower concentrations. Equally, the effects of Cu were the opposite at those same lower concentrations.

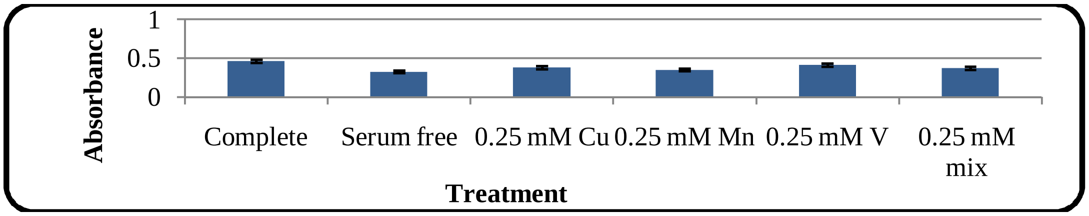

Figure 4 Effects of both 0.25mM Single Metals and Mixture on Caco-2 Cell Cultures after 24 hr exposure

Table 4 XTT Data for Various Metal Mixture Treatments on Caco-2 Cells after 24h Culture

TREATMENT									
	Serum	Serum-free	0.25 mM Cu	0.25 mM Mn	0.25 mM V	0.25 mM Mix	1.0 mM Mix	30 mM Mix	0.5g Mix
Avg Abs	1.18 ±0.23	1.08 ±0.22	0.47 ±0.06	0.53 ±0.08	0.55 ±0.09	0.93 ±0.36	0.73 ±0.27	2.68 ±0.42	1.56 ±0.31

The 0.25-mM concentration was selected as the concentration of focus because the entire investigation was based on low-level metal exposure. The mixture at 0.25 mM had an absorbance that fell between the serum-free result and that of the normal culture, indicating that the combination of these particular metals at this concentration does not cause the degree of viability decrease as the individual metals at the same levels. Again, these results were taken with only 24 hours of exposure. At lower concentrations, some loss of viability occurred. The experiment using 0.5 g of a single metal and the metal mixture was corroborated with the results of the XTT assay. All relevant values were recorded in Table 4.

When cells were grown in a V-only solution, the cultures were consistent with the results in the literature (Fargašová and Beinrohr, 1998). On the other hand, when Cu increases cell viability, the mixture also seemed to mimic this same pattern. Therefore, there is an indication of competition among the metals in the mixture. When the viability of V-treated cells increased, the opposite was seen in the mixture and Cu treatments. The role of Mn seems unclear as its behavior varied among all the tests. It is obvious that more testing needs to be done to clarify the conflicting Mn data. There is definitely an interaction between the metals, which varies with concentration, and length of exposure.

Results showed cell proliferation with V at low concentrations (>1 mM) and with Cu at high concentrations (< 1 mM). The mixtures also mimicked the pattern of the single metals at each respective concentration. There also appeared to be some synergistic interactions between the metals that were evident in the behavior of the mixtures, which has been confirmed in the literature (Crans et al, 1989). This synergism is suspected to occur between Cu and V, and concentration is believed to be a definite factor in the behavior of the mixtures. There is still some uncertainty as to the role played by Mn because its behavior was inconsistent over the 0.25-mM–30-mM concentration range.

5. Conclusions and Future Studies

Advances in exposure assessment have made it possible to accurately measure the types, concentrations, durations, and locations of human exposure to environmental pollutants. (Steinemann, 2004). In the case of trace-metal risk assessment, the EPA has carried out numerous risk assessments that deal with types of toxicity and has developed references doses (Rfds) for a large number of chemicals, including essential trace metals (Goldhaber, 2003). However, there are metals that are not currently being scrutinized, such as V, which, in combination with other metals, may have underlying effects on other metals in a mixture.

As seen in this study, there are still many unanswered toxicological questions that remain. What is clear is the fact that mixtures behave differently from the individual metals, depending upon the environment. For individuals who already suffer with preexisting conditions like colon cancer, they may be at even a higher risk. The presence of metals like vanadium especially in finished drinking water has been under investigation by several research groups but there is very limited data as it relates to human toxicity. The drive to do these studies is often hindered by the perception of safety because of the absence of a regulatory driving force. In other words, vanadium is very rarely monitored by public or private water systems, and its presence in finished water is often overlooked by policymakers (Gerke et al., 2009).

Therefore, in the interest of public health and economic sustainability, it is prudent that policy makers continue to improve upon existing laws and enforcement procedures. Some of the unanswered questions alluded to in the section above can be accomplished by the following additional studies:

1. Interactions and effects of multiple toxicant exposures based on the toxicological nature of the mixtures.
2. A more detailed analysis of trace metal mixture effects in other colon cancer cell lines is also needed.
3. Epidemiological implications of exposures to these mixtures. More specifically, there is a need to better understand how this long-term exposure affects patients with compromised health issues, as in the case of cancer patients and diabetics.
4. There is a need for the development of more efficient assays as tools for testing environmental health risks and analysis of biological impact of these mixtures on human health (SETAC, 1987).
5. Applications of the PP in environmental policy-related decisions should be promoted, especially in poor and disadvantaged communities around the globe.

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