Short Research Article

The Effect of L-ButhionineSulfoximine on theToxicities and Interactions of As, Cd, Hg, and Pband their composite mixture on MCF 7 Cell Line

The effect of intracellular level of GSH on the cytotoxicity and interaction of four environmentally relevant metals arsenic, cadmium, mercury and lead (As, Cd, Hg, and Pb) was investigated. L-ButhionineSulfoximine (LBSO) was used to inhibit the intracellular level of GSH in MCF 7 cells. Both individual and combined cytotoxicities of the four metals on the cells were assayed by spectrofluorometric counting of the surviving cells after 24-hour exposure. Exposure of the cells to three of the studied metals: As, Cd, and Hg resulted in the production of significantly ($p<0.5$) higher level of cellular GSH relative to the control. However, cells exposed to Pb with or without pretreatment with LBSOexhibited about 50% decrease incellular GSH.Individual metal toxicitywas higher in GSH-depleted cells relative to GSH-rich cells;however, the effect of GSH depletion was slightly metal selective as As and Hg exhibited toxicities. Cells exposed to the composite mixture of all four metals indicated additive and antagonistic interactionsin GSH depleted cells and GSH rich respectively.

Keywords: LBSO, GSH, cytotoxicity, interactions, TU, interactive index

1 Introduction

Adverse health effects are usually the consequences of human exposure to high levels of environmental contaminants. Exposure to different chemicalsoccurssimultaneously or sequentially through their natural presence in food ordue to anthropogenic activities (e.g. industrial and agricultural activities).Fortunately, in recent years, governmental agencies and various research groups have developed toxicological data and methodology for assessing chemical mixtures which are frequently encountered in the environment(Monosson 2005; Teuschler et al. 2002).Arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) are examples of some of the chemicals which humans are exposed to frequently. Toxicity studies and health concerns of these metals are important because of their consistent inclusion among the top sevencontaminants of concern in Environmental Protection Agency's (EPA) highest priority hazardous substances(ATSDR 2014).

Compared to individual chemicals, it is generally accepted that chemicals mixturesmaypose more risk depending on the combination. Unraveling the extent of the risks posed and the various forms of complications exhibited by chemical mixtures are therefore part of the goals of groups involvedin the studies of chemical mixtures and related research. Various studies suggest that mixtures of chemicals elicit toxicological interactions (antagonistic, additive and synergistic) on their target cells.(Enserink et al. 1991; Otitoloju 2003; Spehar and Fiandt 1986)For example Ishaque *et al*., 2006 showed that composite mixtures of As, Cd, Hg, and Pb,at their maximum contamination limit (MCL),demonstrated synergistic effects on *Vibrio fischeri.*Similarly, studies by Egiebor et al., 2013 revealed that the exposure of MCF 7 cells to the mixture of the combination

of thesame set ofmetals induced a synergistic effect. Several researchers have studied the cellular regulations of glutathione (GSH) and metallothionein (MT) in different cells exposed to metal ions (Barata et al., 2002; Chan and George Cherian 1992; Świergosz-Kowalewska et al., 2006; Valencia et al., 2001).In one study,researchers indicated that cellular regulations of GSH and MT were strongly linked to the cell's biochemical response when exposed to the metal ion intoxication(Bae et al. 2001).Intracellular interaction of the protective proteins with metal ions can greatly affect the cytotoxicity of these ions. Researchers have stressed that the control of the intracellular availability of the metal ions by GSH (by participating in reactions that destroy free radicals) and MT (by metal-ligand interactions) partly regulates thecytotoxicities of the metal ions in a variety of ways, thereby leading to the interactive effects observed in cells (Anderson and Reynolds 2002; Hultberg et al. 2002; Roesijadi 1994). Most importantly, exposure of cells to some metals trigger the synthesis of GSH and MT**,** whichcreates a cycle of dependence between the biochemical response and metal ions bioavailability(Roesijadi 1994).Other studies also describe the apparent increase in the intracellular concentration of MT as the GSH is depleted and how this affect bioavailability, toxicity, and interactions of cells exposed to more than one metal either sequentially or simultaneously(Hochadel and Waalkes 1997; Nakagawa et al. 1995; Roesijadi 1994).It is hoped that studying the cytotoxic effects of metals and their mixtures followingtheinhibition of cellular GSH or MT will enable scientist understand the link between the detoxifying polypeptides and the toxic effects of metals

The objective of this work was to determinethe effects of cellular GSH inhibition on the toxicity and interactions of As, Cd, Hg and Pb and their composite mixture onMCF 7 cell line.GSH was inhibited bypre-treating the cellswith LBSO.LBSO irreversibly inhibits gamma-glutamylcysteinesynthetase (the rate-limiting enzyme of GSH synthesis) thereby inhibiting cellular GSH production(Anderson and Reynolds 2002; Keogh et al., 1994).

2Materials and methods

2.1Cell culture and exposure

The four metals As 1mg/mL in 2% KOH,Cd 1mg/mL in 0.5N nitric acid, Hg 1mg/mL in 10% nitric acid and Pb 1mg/mL in 2% nitric acid, all atomic absorption standard solution, were purchased from Acros Organic (New Jersey). Dimethyl sulfoxide (DMSO), and Fluorescence Diacetate Dye (FDA) were purchased from Sigma-Aldrich Co (St. Louise, MO).MCF 7 cell lines, Trypsin-EDTA and Fetal Bovine Serum (FBS) were purchased from American Type Culture Collection (ATTC) (Manassas, VA). Minimum Essential Medium (MEM) alpha 1x, Dulbecco's Phosphate Buffered Saline (PBS), MEM without phenol, and Penicillin Streptomycin were purchased from GIBCO Invitrogen (Grand Island, NY). LBSO was purchased from Toronto Research Chemicals (North York, ONCanada). MCF 7 cells were grown in MEM alpha 1x supplemented with 10.0% FBS and 1.0% penicillin streptomycin and incubated for 24 hrs at 37^{o}C in a 5% CO_2 incubator to allow the cells to grow, and form a monolayer in the flask. Cells grown to 75-85% confluence were washed with phosphate buffer saline (PBS), trypsinized with 3 mL of 0.25% (v) trypsin, 0.3% (v) EDTA, diluted with fresh medium, and counted for experimental purposes.

To inhibit the production of cellular GSH,growth medium containing 2.5mM LBSOwas used to seed MCF 7 cells in sterile 96-well (1 x 10^4 cells/well)plates and placed in a CO_2 incubator for 24 hours. The concentration of LBSO used was pre-determined by exposing MCF 7 cells to decreasing concentration of LBSO (starting form 20mM). The concentration that could effectively inhibit GSH production with a cell survival rate of 95% was determined to be 2.5mM.Subsequentlycells were exposed to serially diluted concentrations of the individual metals and the composite mixture of the 4 metals prepared in MEM (without phenol), supplemented with 5% penicillin streptomycin. The highest concentration for the individual chemicals was 80mg/L. The mixture of the four metals was made by mixing As, Cd, Hg, and Pb stock solutions in ratio of their EPA's MCL 10:5:2:15 respectively representing a starting concentration of 20, 10, 4 and 30 mg/L respectively. The first row of each plate was used as control (medium without cell) and the second row used as negative control (cells without chemicals) andthe treated cells were incubated for 24 hours.

2.2Cell viability test by spectrofluorometric method

After carefully decanting metal-containing growth medium,cells werewashed with 100.0 µL PBS, and each of the wells was treated with 100.0µL of diluted Fluorescence Diacetatedye (FDA) solution (i.e., 50mg FDA dye in 5 mL dimethyl sulfoxide). The treated plates were placed in the incubator for 45 minutes. This allowed the surviving cells to be stained by the FDA giving them a fluorescent green color. Cell culture plates were read with Fluoroskan Ascent FL 374 (ThermoLabsystems, Finland) and the readings were converted to percent death by comparing each reading with the control (reading from the second row of each plate; 100% survival by default).Igor Pro 6.22A software, was used to generate a concentration-response for each treatment.Inbuilt sigmoid function was fitted onto the plotted data and the concentrations at various percentages responses from 20% to 80 % were estimated. To assess the interaction of the various components of the mixture, concentration addition was used to determine the toxicity of thecomposite mixture. Thus, the concentration of a mixture component was scaled for its relative toxicity generally termed Toxic Unit (TU) of that component.(Altenburger 2011; Altenburger et al. 2000)

2.3 Measurement of intracellular GSH content in MCF7 cells and LBSO pretreated MCF 7 cells.

GSH levels were analyzed in MCF7 cells using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes).(Han et al. 2008)Cells wereexposed topreviously stated concentrations of the individual metals (As, Cd, Hg, and Pb)and their composite mixture for 24 hours.Subsequently cells were exposed to CMFDA dye for 45 min. This same procedure was repeated using cells that were pretreated with LBSO for 24hr. CMFDA fluorescence intensity was determined using a FACScalibur flow cytometer (Becton Dickinson) and calculated with Cell Quest pro software. 10,000 events were collected for each sample.

2.4 Statistical Analysis

In order to determine the significance of the potential differences in the results, Student's t-test was employed. Analysis of variance (ANOVA) together with Tukey's test was applied for multiple comparisons.

3 Results and discussion

3. 1
GSH levels in MCF7 cells and LBSO pretreated MCF 7 cells.

The effect of LBSO on GSH level was studied incells pretreated with LBSO and cells without LBSO pretreatment. The GSH levels were expressed as percentages relative to the control and shown in Table 1. Compared to the control, cells without LBSO pretreatment showed increased levels of GSH after exposure to As, Cd, and Hg solutions, which is consistent with previous studies showing increased levels of GSH in cells exposed to metals.(Garcıa-Fernandez et al. 2002)The level of GSH in cells exposed to Cd and Hg increased more than 2-fold and those exposed to As increased about 1.5 fold relative to the control. Thus at $P<0.05$, the level of GSH in cells exposed to the three metals was significantly higher than the control. In cells pretreated with LBSO, the levels of GSH decreased to about half of the control for all the metals used in the treatment. Cells exposed to Hg after LBSO pretreatment showed the lowest levels of GSH. When compared to those without LBSO pretreatment, LBSO-treated cells exposed to Hg, Cd, and As showed about 14, 5, and 3-fold decreased levels of GSH respectively. The significant ($P<0.5$) decrease in GSH level in LBSO pretreated cells clearly confirmed that LBSO interfered with the cells defense mechanism.
Surprisingly, the same level of GSH was found in cells exposed to Pb, irrespective of LBSO pretreatment. Thus, cells exposed to Pb in both cases showed about 50% decrease in the levels of GSH, when compared to the control(Table 1). Although previous studies demonstrated decreased intracellular levels of GSH in cells treated with Pb(Perez et al. 2013; Wilczek et al. 2004),it was not clear why further decreased in GSH levels was not observed in LBSO-pretreated cells. A higher-than-control level of GSH at exposure to Pb would have been expected, since metals have the potential for eliciting GSH increase, as previously reported. Our result is in line with result from other studies which have shown that exposure lead canreduce cellular GSH levels.(Hunaiti and Soud 2000; Perez et al. 2013; Ullah et al. 2011)As much as 40% decrease in GSH level in human whole cells exposed to Pb was reported byHunaiti and Soud 2000

Table 1: GHS levels in cells before exposure to metals As, Cd, Hg, and Pb. The levels are shown as percentage of the control with ± standard deviation (SD) from 4 samples.

Treatment	No pretreatment	LBSO pretreatment

Control	100 ± 11	100 ± 12
As	153 ± 18	50 ± 6
Cd	247 ± 26	51 ± 6
Hg	244 ± 24	18 ± 2
Pb	53 ± 6	56 ± 6

When cells were exposed to the composite mixture ofAs, Cd, Hg, and Pb, the levels of GSH in LBSO-pretreated cells and cells without LBSO pretreatment were 14±2% 149±16% as compared to the control. Thus,LBSO markedly influenced the levels of cellular GSH when exposed to the composite mixture.It was thought thatsince the metals interact differently with GSH and potentially interfere with cellular GSH levels, varying the concentration of the metals used in the mixture will have significant effects on the GSH levels. However, no trend was observed even when different concentrations of each metal were present in the mixture.

3.2 Acute toxicity of individual metals in presence of LBSO

To assess the individual toxicity of As, Cd, Hg and Pb, two set of cells (LBSO pretreated cells and cells without LBSO pretreatment), cells wereexposed to varying concentrationsof the four individual metals. From the results (Figure 1),cells without LBSO pretreatment indicated that the four metals showed significantly ($P<0.05$) different levels of toxicity towards MCF 7 cells. Toxicity ranking of the four metals was in agreement with the EPA ranking (i.e., Hg>Cd>As>Pb).

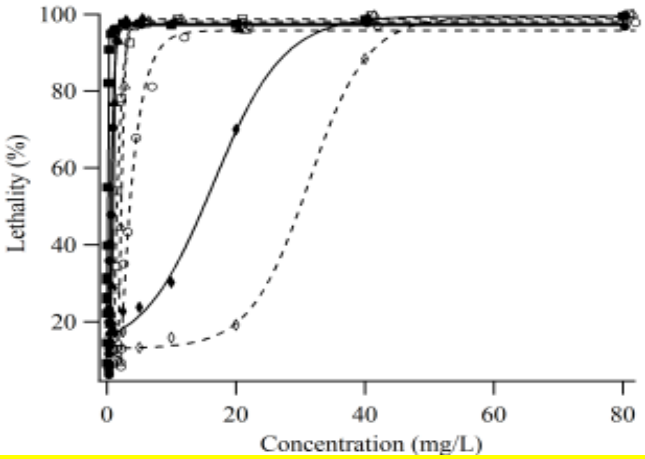

Figure 1: Acute toxicity profiles of individual metals towards LBSO (continuous line) and non-LBSO pretreated (broken line) MCF 7 cells. The symbols ●, ■, ▲, and ♦ indicate toxicity profiles As, Cd, Hg, and Pb-exposed cells respectively.

Hg was found to be the most toxic (i.e. LC_{50} 0.6 ± 0.1 mg/L) while Pb was the least toxic (i.e. LC_{50} 26 ± 2 mg/L) to MCF 7 cells based on 24 hour exposure (Figure 2).

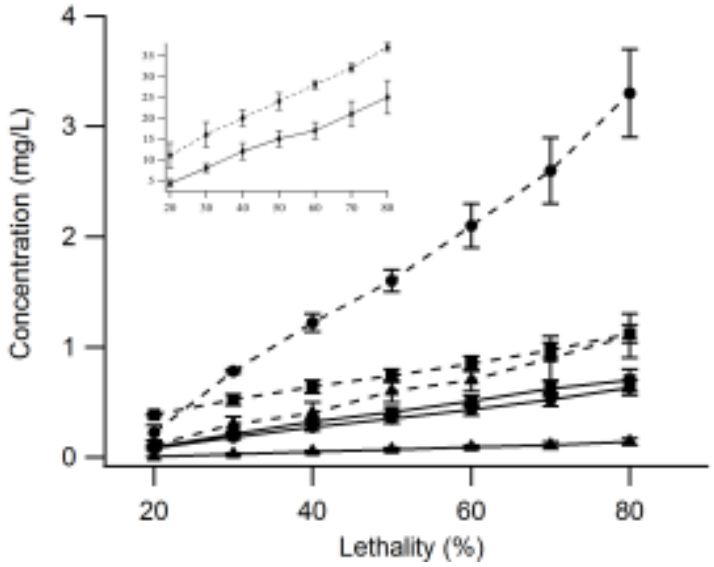

Figure 2: Mean toxicities of individual metals at various percentage responses. The markers ●, ■, ▲, and ♦ indicate As, Cd, Hg, and Pb treatments respectively, continuous line is exposure in the presence of LBSO (GSH depleting agent) broken line is the exposure in absence of LBSO, and the error bars represent standard deviation calculated from at least 9 different toxicity assays. The insert is the mean toxicities for lead.

Pretreatment of MCF 7 cells with LBSO was shown to increase the toxicity of the individual metals.Thus without GSH defense, less concentration of each metal was required to elicitcell death. In contrast to EPA ranking of the individual metal toxicities, cells pretreated with LBSO indicated that As, and Cd switched positions in the ranking. Arsenic became slightly more toxic than Cd. Nevertheless, there were no significant differences in the toxicities of the two metals, showing similar influence on GSH.
Hg and Pb remained the most and least toxic respectively among the four metals with no correlation to LBSO pretreatment (Figure 2). The LC_{50} for Hg, As, Cd, and Pbstarting form the most toxic was 0.0698, 0.346, 0.412 and 14.505 mg/L respectively. As expected, each metal demonstrated significant ($p<0.05$) increase in cells mortality with increasing concentration of the metals indicating a dose-dependent cytotoxic effect of the four metals. Pb showed the highest range between the concentration for LC_{20} and LC_{80}, this was followed by Cd, and As. The coefficient of variation(CV) ranged 6.17 to 35.64%. Mercury showed higher CV values and Cd less CV indicating highest and lowest variability respectively.
Different toxic effects of the tested metals were obtained, in the absence and presence of cellular glutathione, at 50% lethality, the toxicities of As and Hg in GSH-depleted cells were 5 and 9 times higher respectively, relative to the their toxicities in GSH-rich cells. The toxicities of Cd and Pbwere two times higher in cells pretreated with LBSO as against cell without LBSO pretreatments. Similar increases in the levels of GSH were detected in cells pretreated with LBSO before metal (As, Hg and Cd) exposure;however, Pb demonstrated significantly lower toxicity.The implication is that, when cellular GHS is depleted, the level of protective proteins like metallothioneins increases in order to keep the intracellular level of total protective proteins balanced.This assertion is supported by previous

studies that have indicatedthat decreased levels of cellular GSH induced the increased production of other protective protein (e.g., MT) (Hochadel and Waalkes 1997; Roesijadi 1994).

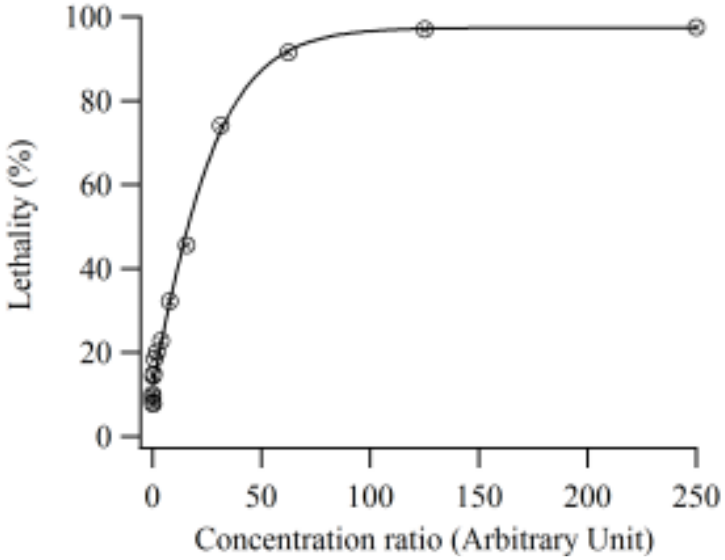

Figure 3: Concentration-response curve for the composite mixture of the four metals. Concentration ratio of 1 contains the four metals in their EPA MCL concentration (i.e., As: Cd: Hg: Pb is to 10: 5: 2: 15)

A composite mixture of the four metals was prepared based on the EPA MCL ratio. Serial dilutions of the mixture were were employed to assess concentrations at various percentage deaths of the cells. To obtain concentration-response curve (figure 3), concentration ratios were worked out from the working ratio (concentrations in the solution used) and the MCL. Lethal concentrations of each of the four metals at various percentage responses were calculated from these ratios and shown in Table 2. As expected, the ranking for the toxicity of the metals in the composite mixture in the presence of LBSO followed the EPA ranking (i.e., Hg > As > Cd >Pb). The LC_{50} for 24 hour exposure of the metals on the MCF 7 cells for the four metals Hg, Cd, As, and Pb were 0.0409, 0.102, 0.204, and 0.308 mg/L respectively (Table 2).
Toxicities for all the tested metalsproved concentration-dependent.Relying on LC50 data, it could be concluded that each of the four metals As, Cd, Hg, Pb in the mixture were respectively 2, 4, 2, and 48 more toxic than when they were present alone (Table 2).

Table 2: Toxicities (mean±SD) mg/L of the metals As, Cd, Hg, and Pb when present in a mixture in the ratio of 10:5:2:15, respectively, at various lethal concentrations, in the presence of LBSO. The mean was calculated from at least 9 different toxicity assays.

LC_x	As	Cd	Hg	Pb
LC_{20}	0.06±0.01	0.033±0.007	0.013±0.003	0.10±0.02
LC_{30}	0.12±0.02	0.06±0.01	0.024±0.004	0.18±0.03
LC_{40}	0.16±0.03	0.08±0.01	0.033±0.006	0.25±0.04

3.3 Comparison between individual and combined toxicities.

A comparative study between individual and combined toxicities, aimed at estimating toxic units for each of the metals at various LCs and the concentration of each mixture component was scaled for its relative toxicity. Toxic unit gives an estimation of the toxicity of a component in a composite mixture. The mean of the TU (toxic units) values of Hg and As were significantly higher, followed by Cd and Pb (Figure 4). It was shown that As and Cd contributed the most to the toxicity of the mixture, with equal contributions. The contribution of Hg, the second toxic metal in the mixture, was significantly lower than that of Cd and As.

The TU values of As, Cd, and Hg were slightly higher at lower response levels and leveled up at subsequent response levels. For Hg, the TU values at about 40% responses upwards. As and Cd values were reported as leveling off at 40% and 60% respectively. In the case of Pb, the TU values were the same throughout the various response levels.

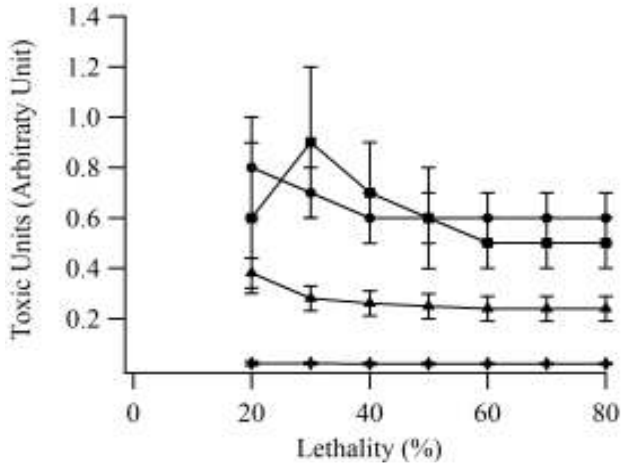

Figure 4: Toxic Units for a mixture of As, Cd, Hg, and Pb in the ratio 10:5:2:15 respectively at various lethal concentrations in the presence of LBSO. Error bars are standard deviation calculated from 9 samples. The symbols ●, ■, ▲, and ♦ indicate toxicity profiles As, Cd, Hg, and Pb-exposed cells respectively.

To evaluate the type of toxic interaction of the four metals in the composite mixture, the concentration addition model was employed. The values for the index of interaction, considered the sum of the TU values, decreased, as the percentage response increased (fig

5). The means interactive indices for all concentrations were greater than 1, pointing to a shift towards antagonistic interaction of the four metallic species.

The one-sample t-test carried out on the interactive indices at different response levels showed that the interactive indices did not significantly differ from unity (P=0.5), proving that the interactive effect of the four metals is strongly additive.The results are discussed and compared with those previously reported by researchers who demonstrated various interactive effects of metal mixtures on cells with cellular GSH(Bae et al., 2001; Ishaque et al., 2006). Their results indicated that mixtures induce synergistic to antagonistic effects. In some cells, antagonistic effect became evident as the level of protective proteins increased

The prevalent factor in the present study is the suppression of the glutathione defense system, prior to exposure of the targets to the toxicants. Consequently, the targets were less capable to exhibit effective defense against the toxicity of the various metallic species in the mixture. Nevertheless, the various mixture components possess different degrees of potency, and they behave additively in the cells deprived of glutathione defense.

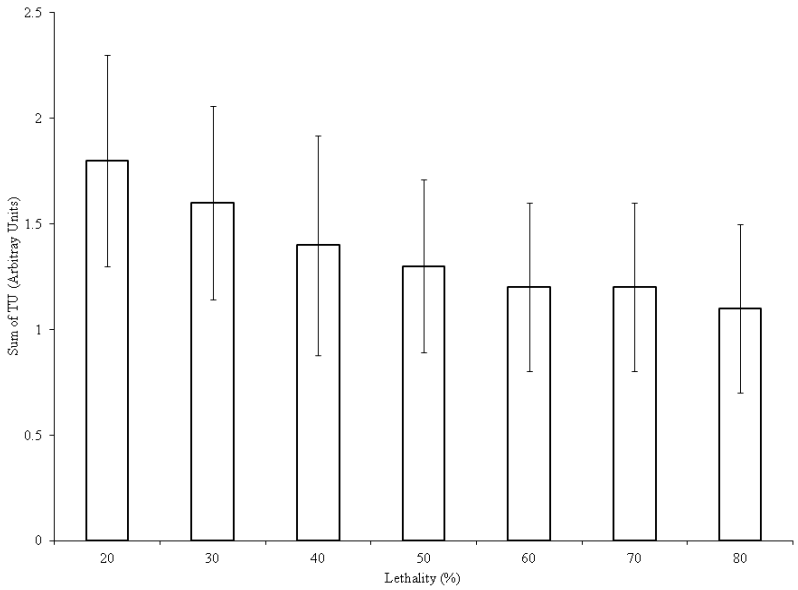

Figure 5: Interaction indices (sum of TU) for a mixture of As, Cd, Hg, and Pb in the ratio 10:5:2:15 respectively at various LCs in presence of LBSO

4Conclusions

MCF 7 cells are sensitive to the alteration in the levels of the GSH regardless of the metal used for treatment.In general, there was an increase in toxicity of the individual metals in GHS-depleted cells. However, effect of GSH depletion on the metals toxicity was metal selective, as As and Hg, appeared to be highly toxic relative to Cd, and Pb. In the composite mixture, As and Hg, contributed most and equally to the toxicity.Along the various levels of response tested, the interactive effects of the four metals acting simultaneously on GSH-depleted cellswas found to be additive.

What is not known from this study is the effect of decreasing level of GSH on the level of MT and its influence on the metal toxicity. Our next work will profile level of MT in GSH-suppressed cells and how the varying level of MT will affect the toxicity of the metals. It will also be important to understand the effect of the c elimination of the various metals on the toxicity and interactionof the remaining components in the composite mixture.

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