An in vitro cytotoxic approach to assess the toxicity of heavy metals and their binary mixtures on hippocampal HT-22 cell line

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Abstract

Humans are exposed to a cocktail of heavy metal toxicants in the environment. Though heavy metals are deleterious, there is a paucity of information on the toxicity of mixtures. In this study, four common neurotoxicity heavy metals lead (Pb) cadmium (Cd), arsenic (As), and methylmercury (MeHg) were exposed individually and as mixtures to HT-22 cell line for 8 days. The study established that low dose exposures induced toxicity to the HT-22 cell line during 8 days. The results indicates potency dependent response, the toxicity of single metals on the HT-22 cells; MeHg > As > Cd > Pb. The cytotoxicity data of single metals were used to determine the mixtures interaction profile by using the dose additivity and effect additivity method. Metal mixtures showed higher toxicities compared to individual metals. Synergistic, antagonistic or additive effects of the toxicity were observed in different mixtures in low dose exposure. The interactive responses of mixtures depend on the co-exposure metal and their respective concentration. We concluded that the combined effects should be considered in the risk assessment of heavy metal co-exposure and potency. In future, comprehensive mechanistic based investigations needed for understanding the real interactive mixtures effects at molecular level.

Keywords: Mixture toxicity; Metal mixtures; Cytotoxicity; Apoptosis; Isobologram analysis.

Abbreviations: Ach E = Acetyl cholinesterase E, ANOVA = Analysis of variance, As = Arsenic, CA= concentration addition, CI= combination index, DMEM = Dulbecco's modified Eagle's medium, FBS = Fetal bovine serum, GABA = ¥-gamma-amino butyric acid, GAD = Glutamate decarboxylase, LC_{50} = Lethal concentration 50, LTP = Long-term potentiation, MeHg = Methyl mercury, MTT = 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, NMDA = N-methyl D-aspartate, OD = optical density, Pb = Lead, PI = propidium iodide, SD = Standard deviation, WHO = World Health Organization.

1. Introduction

Heavy metals are environmental pollutants of great concern because of their persistent occurrence, arising from increasing industrialisation and other anthropogenic activities (Al-Khashman and Shawabkeh, 2006; Nadal et al., 2004; Yu et al., 2011). Exposure to heavy metal compounds including lead (Pb), cadmium (Cd), arsenic (As), and methyl mercury (MeHg) has long been known to cause damage to human health (Mari et al., 2014; Morais and Garcia, 2010). The organs affected by these metals are kidney, lung, liver, gastrointestinal and haematological systems, mainly the peripheral and central nervous systems (Angelica and Fong, 2014). Because of their high degree of toxicity, these four elements rank among the priority metals that are of great public health concern (WHO, 2010). Exposure to MeHg and Pb has a significant effect on the human brain and, are well known to target the central nervous system (Aschner et al., 2007; Clarkson, 1987; Maynard et al., 2005; Sanders et al., 2009). Exposure to Cd also severely affects the function of the nervous system, leading to parkinson like symptoms, and learning disabilities (Viaene et al., 2000; Wang and Du, 2013). The exact mechanism and its neurotoxic effects, however, unresolved (Kumar et al., 1996; Mendez-Armenta and Ríos, 2007). Recently, it has been found that As is also linked to developmental neurotoxicity (Luo et al., 2009; Rodriguez et al., 2002; Tyler and Allan, 2014).

The brain is a critical target organ for Pb, MeHg mediated cognitive dysfunction effects, and Cd, As are also highly influences the brain in continuous exposure (Giasson et al., 2002). Numerous studies have been done on the toxicity of individual metals to a brain (Wu et al., 2016). In an individual metal mode of action, Pb, As and MeHg has been found as potent neurotoxicants (Johansson et al., 2007; Sadiq et al., 2012; Tyler and Allan 2014). Experimental studies proved that Cd also influences the cognitive function of the brain (Hart et al. 1989; Luo et al. 2009; Viaene et al. 2000). Generally, humans are exposed to these metals in a simultaneous manner (Stackelberg, 2013). The simultaneous exposure may exacerbate the toxic effects, most of the heavy metals are known to increase the sensitivity to cognitive dysfunction and neurodegenerative outcomes (Clarkson 1987; Snyder et al. 2005). In a recent review, we reported that the combination of metals may produce more/ less than additive due to their common binding affinity with NMDA receptor (Pb, As, MeHg), Na^{+}- K^{+} ATP ase pump (Cd, MeHg), biological Ca^{+2} (Pb, Cd, MeHg), and glutamate neurotransmitter (Pb, MeHg) (Fig 1) (Karri et al., 2016).

Chemical mixtures toxicity is effectively an infinite problem, and it is an ongoing challenge to integrate this issue into regulatory regimes (Sarigiannis and Hansen, 2012; Sharma et al., 2016). Testing of all kinds of mixtures of chemicals existing in the real world or of all possible combinations of a simple mixture of different dose levels is virtually impossible (Orton et al., 2014). Moreover, even if toxicity data of individual chemicals are available, we are still facing the immense problem of extrapolation of findings obtained at relatively high exposure concentrations in laboratory animals to a man being exposed to lower concentrations (Cassee et al., 1998). More than 95% of toxicological research studies are focused on single chemicals and almost completely neglect the mixtures (Kortenkamp et al., 2009). The available toxicity data for the mixtures of metals are very limited. Studies on exposure to heavy metal mixtures are critical since there is a lack of information on the toxicities and associated mechanisms. Some reported binary mixture data of As, Cd, and Pb on various biological endpoints are inconsistent for the same endpoints from study to study and are less relevant in terms of risk assessment (ATSDR, 2004). On the hand, prediction of mixtures effects is a great challenge because synergism or antagonism in a combination of two or more chemicals may occur and no currently available mathematical model can predict or fully solve this problem (PapeLindstrom and Lydy, 1997). Previously reported studies have established toxicity of metal mixtures on various organs and their functions: the immune system (Jadhav et al., 2007a), mortality

(Vellinger et al., 2012), neurotoxicity (Hu et al., 2013; Rai et al., 2013), bladder cancer (Feki-Tounsi et al., 2013) cytogenicity (Jadhav et al., 2006), induction of oxidative stress (Jadhav et al., 2007b), and metal mixtures interactions on essential elements (Cobbina et al., 2015). Recommendations for study design and evaluation of combined effects of metal mixtures are not clear (Tichý et al., 2002). The regulatory frameworks such as REACH in the EU are becoming more and more critical regarding the use of animal testing (Cedergreen, 2014). There are various risk assessment methods for evaluating combined exposures in practice, these methods are derived from the dose addition concept and effect addition (Scholze et al., 2014).

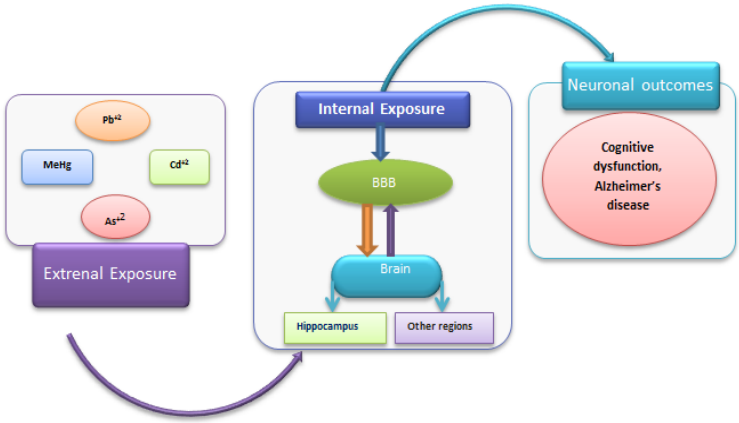

Figure 1. Conceptual diagram of metal mixtures exposure- toxicology- disease outcome scenario (hippocampus) (Karri et al., 2016)

Recent advancement in vitro techniques, with an appropriate target cell, may allow an accurate understanding of metal mixtures toxicity. However, the in vitro effects were specific to cell lines and exposure conditions. There is an ongoing discussion regarding the most appropriate method for the evaluation of mixtures interactions (Kortenkamp and Altenburger, 1998); so two methods have been employed in this study: the effect additivity model (Axelrad et al., 2002) and the alternative dose additivity model (Berenbaum, 1978). The present study explores the toxicity of individual and binary mixtures of Pb, Cd, As, and MeHg after 8 days exposure to HT-22 hippocampal cell line. 8 days exposure used in this study is considering the maximum stability of cell confluence (80-85%). For elaborating the hypothesis, we performed cytotoxicity and apoptosis of Pb, Cd, As, and MeHg alone in the mice HT-22 hippocampal cell line. Further, we extended the binary mixtures interactions study by using the response addition and dose addition whether they interact with one another when combined.

2. Materials and methods

2.1. Chemicals and media

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]), Cadmium chloride ($CdCl_2$ [CAS no: 10108-64-2]), Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), all are analytical grade and purchased from Sigma-Aldrich Química, S.L- Madrid (Spain).

2.2. Cell line and reagents

Among various research tools, neuronal cell lines are the most commonly used in vitro model for relevant mechanistic studies. With particular concerns for memory and alzheimer disease related studies, hippocampal neuronal cell lines are very limited. HT-22 is one cell line subcloned from its parent line HT4, which are immortalized mouse hippocampal neuronal precursor cells. The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies.

The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C (Niska et al., 2015). For all the experiments in 8 days duration cells were grown at 70- 80% confluence.

The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were plated at 0.57 x 10^6 cells/ mLand and grown for 24 hours before metal treatment. Duplicates wells of cells were treated with 10 exposure levels of Pb, Cd, As and, MeHg ranging from 10 to 100 µM, 0.5 to 7 µM, 0.4 to 4.2 µM, and 0.6 to 12 µM, respectively; due to the 8 days exposure medium containing given concentration was refreshed at 2 days interval for maintaining metal exposure in a long time. Metal stock solutions 100X were prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO added) and sterilized by filtration through 0.2 µm and different concentrations of a working solution of each individual metal was prepared by prior dilution of the stock solution in phosphate buffer saline (pH = 7.4) and then applying 10% working solution on DMEM culture medium.

2.3. Cytotoxicity/ MTT assay

The MTT assay was carried out using a modification of the method of Mossman (1983). The HT-22 cells were seeded in 96-well plate. After 24 h, when the cells had reached a confluence of 70–80%, they were exposed for 8 days to several concentrations of the heavy metals (Pb, Cd, As and, MeHg). After the incubation period, the medium was aspirated from well and MTT working solution at 0.5 mg/mL was added to each well. Cells were incubated at 37°C for 3 h; after this time, the MTT was removed by aspiration. Formazan crystals were dissolved in 100 µL of DMSO and placed the plates on a shaker and agitated for 5 min. The absorbance of the solubilized reduced MTT was then measured in a micro titter plate spectrophotometer reader at a wavelength of 570 nm. The measured absorbance or optical density (OD) values were converted to percent of cell viability (%) with respect to control. Cell viability (%) = Absorbance of treatment / Absorbance of Control x 100%. The cytotoxicity results were used for calculating the IC_{10} to IC_{30} of the each metal for apoptosis and mixtures interaction study.

2.4. Analysis of apoptosis by annexin V-FITC/ propidium iodide (PI)

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of the plasma membrane, Annexin V- FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, Annexin V and Propidium iodide (PI) was used to distinguish the apoptotic and necrotic cells from the alive cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to the designed doses (IC_{10}, IC_{20}, IC_{25}, and IC_{30}) of heavy metals in 12 well plates at a density of 0.56 x 10^6/mL during 8 days, control cells were made without chemical. The medium with metal concentration was refreshed every 2 days. After 8 days treatment with metals, cells were harvested by trypsinization, washed twice with ice cold PBS (p^H = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 4^0C, resuspended in 1mL of 1X binding buffer and then transfer the 100 µL of the solution to 5 mL culture tube, and added 5 µL of both annexin-V, PI to the samples.

After staining, cells were incubated for 15 min in the dark at room temperature. Cells were re-washed with 1X binding buffer 400 µL and analyzed by flow cytometry (Beckman coulter, Germany).

2.5. Assessment of interactions using the response additivity method

This method for testing interaction between chemicals has been described by Lau et al., (2006). In this model, the combined effects of two agents are thought to be equal to the sum of the effects of the single compounds. Deviations from this are either synergistic or antagonistic. In the present study, chemicals were tested at their IC_5- IC_{20} concentrations, derived from individual concentration–response curves. The mixture experiments were conducted for a total of 6 mixtures in 8 days, including Pb+Cd, Pb+As, Pb+MeHg, Cd+As, Cd+MeHg, and As+MeHg, For binary mixtures, cells were then exposed to pairs of the compounds in equal proportions. To assess the interaction between heavy metals, combined effects include: additivity, where metals are no more and no less effective in combination than they are separately; synergism, where the effectiveness of agents is increased when in combination; potentiation, where the increased effect of a toxic compound is acting concurrently with a non-toxic compound; and finally, antagonism, where the effectiveness of agents is decreased when in combination (Costa et al., 2007).

2.6. Assessment of interactions using the isobole method- dose additivity

In the current study, five different mixtures ratios of each binary pair were prepared (0:100, 25:75, 50:50, 75:25, and 100:0). Each of these mixture ratios was tested in triplicate around the mixture IC_{30} according to the method (Axelrad et al., 2002). For the data analysis of these mixtures, compusyn isobole analyses were used to assess the interaction of the metals in the mixture. The combination index (CI) - isobologram method is widely used in pharmacology to study the nature of the interaction between drugs. The interaction is analysed by using the median-effect/combination index (CI)-isobologram equation (Chou, 2010), which is based on the median-effect principle (mass-action law). This method has been applied to predict the mixture toxicity of environmental chemicals (Wang et al., 2015). The benefit of a non- constant combination toxicity study is not simply due to the property of the metals, but could also depend on the dose ratios. As the cells do not make the difference between a single metal or a combination, two metals combined at a given ratio could be considered as a third agent with its own dose effect relation. The isobole model (Berenbaum, 1978) allows the construction of graphs showing curves describing various combinations of two compounds A and B, which together produce the same, specified effect. Isoeffective doses A and B of the single compounds are connected by an additivity line, which predicts the combinations of A and B required to yield the specified effect, provided the interaction between A and B is additive (zero interaction).

This relationship is expressed by the equation: $c_A/C_A + c_B/C_B = 1$

Where c_A and c_B are the concentrations of A and B in a mixture that produce a specified effect, and C_A and C_B are the concentrations of the single agents, which on their own elicit the same effect as the mixture. Synergistic agents require lower concentrations to produce a given effect when in combination, giving concave isobole; therefore the equation is expressed as $c_A/C_A + c_B/C_B < 1$. Antagonistic combinations give convex isobole resulting in $c_A/C_A + c_B/C_B > 1$. The localization of the experimental mixture point (a, b) corresponding to the doses actually needed for a combination effect mixture with respect to the line of additivity can be translated in term of synergy, additivity, and antagonism; if experimental point below the line corresponds to a combination index (CI < 1) and indicates synergy; a point on the line corresponds to a CI = 1 and indicates simple additivity; finally a point above the line corresponds to a CI > 1 and indicates antagonism. Sometimes the CI values are >3 or much greater, especially at low effect levels (i.e., low fa level). Keep in mind that the synergy

scale is from 1 to 0 and the antagonism scale is from 1 to infinity (Chou, 2010). The limitations of the CI method are that it is highly sensitive to small changes in effect measurement at low and high concentrations and lack of statistical evaluation of synergy, additivity or antagonism (Zhao et al., 2010).

2.7. Statistical analysis of data

All experiments were performed three times (n=3) and each concentration tested in replicates. The results were given as mean ± standard deviation (SD). IC_5 to IC_{30} values calculated from dose response curve fitted by using the Graph pad prism version 5.01. Compusyn software (http://www.combosyn.com) used for non-constant ratio isobologram fitting. All data were analysed by one way ANOVA ($p^{***} < 0.05$) procedures followed by dun net's. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Cytotoxicity of individual metals (Pb, Cd, As, and MeHg) on HT-22 cell line

To characterize the effects of Pb, Cd, As, and MeHg individually on the hippocampus, we performed cytotoxicity studies in immortalized mice HT-22 hippocampal cells using the MTT assay as a measure of cell viability. As expected, all 4 metals showed a dose, dependent cytotoxic effect, expressed by decreased absorbance or optical density (OD) values of treated cells. Results for Pb, Cd, As, and MeHg dose- response curves are presented in Figure 2 and table 1. For Pb, dose- response curve clearly shows concentration dependent effect, cytotoxicity was drastically enhanced with respect to concentrations (>70 µM). The obtained results suggest that Pb induced damage in HT-22 cells is dose dependent. However, at low concentration (10-60 µM) Pb had no effect on cell viability. Exposure of HT-22 cells to different Cd concentrations (0.5–7 µM) shows a dose dependent relationship. Cells exposed to Cd concentrations from 0.5 to 2.5 µM exhibited the non-significant effect. The effect of Cd was more pronounced at 3.5 µM. We observed that the percentage of cell viability decreases with increasing Cd concentration reaching a maximum of cell death (90%) in chronic exposure. The metal As on HT-22 cells. The metal As on HT-22 cells drastically decreased the viability; the toxic effect of As was high at 1.2-4.6 µM. The effects of MeHg on HT-22 cells were high, even in the lowest concentration (0.6 µM), the cell death was statistically significant. The dose response curve inflection drastically enhanced in MeHg than other three metals. The results obtained from the MTT cytotoxicity assays indicate that there are differences between heavy metals sensitivity on hippocampus cell line. The MeHg appear to be more sensitive as indicated by LC_{50} value. This difference could be due to the different uptake mechanisms of heavy metals by the HT-22 cells this difference among the cytotoxicity results of heavy metals suggests that some underlying unique intracellular mechanism is responsible for the hippocampus damage. • The summary of cell viability data of heavy metals for different exposure times (1 day, 3 days, 8 days) are presented in the supplementary material (Annex-I) and detail study can be found in Karri et al. 2017.

Table 1: The LC_{50} values of Pb, Cd, As, and MeHg for hippocampal HT-22 cell line after different exposure times in In vitro[a]

Exposure time	Heavy metals (IC_{50})			
	Pb (µM)	Cd (µM)	As (µM)	MeHg (µM)
8 days	74.3	3.7	0.8	0.6

([a]Values of LC_{50} with 95% confidence intervals from curves shown in Figure 2. Cytotoxicity was evaluated by inhibition of MTT reduction as described in the methods).

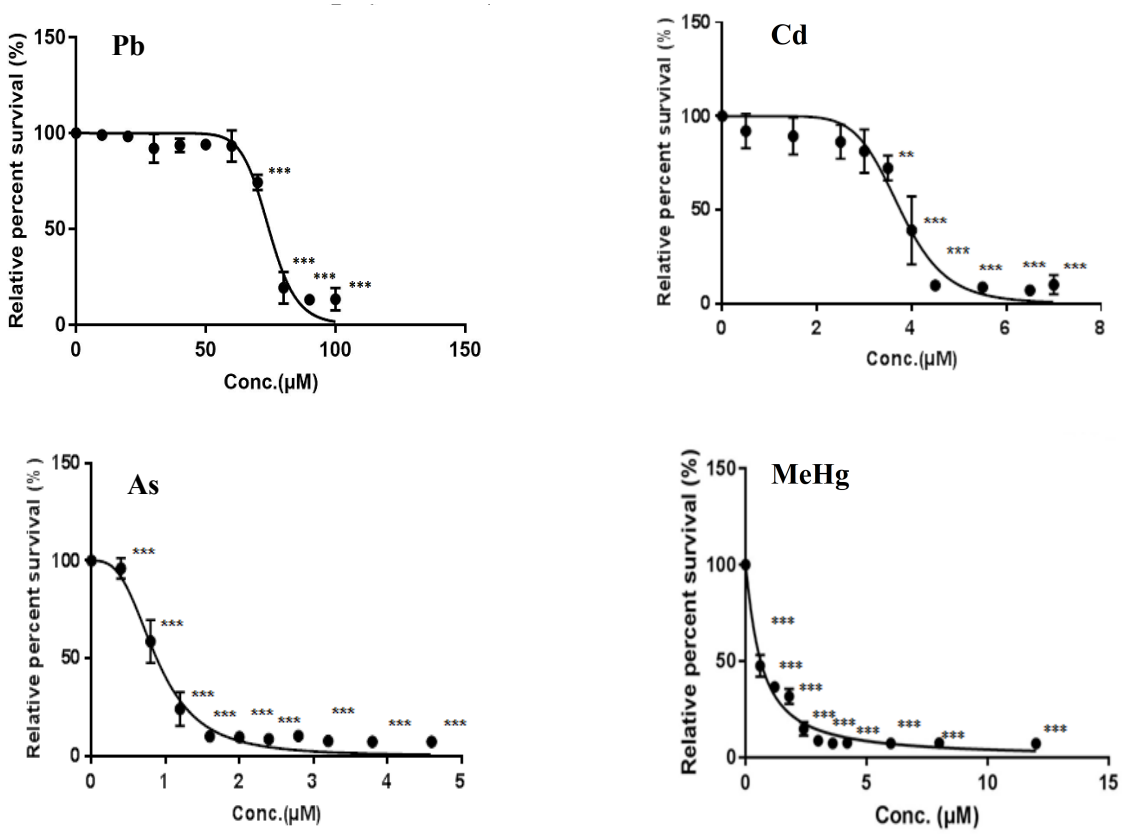

Figure 2: Dose- response curves of Pb, Cd, As, and MeHg on HT-22 cells during 8 days exposure. The one – way ANOVA followed by a dunnet's multiple comparison tests compared the control with all concentrations, asterisks indicate significantly different with control ($p^{***} < 0.05$).

3.2. Apoptosis effects of individual metals (Pb, Cd, As, and MeHg) on HT-22 cells

To gain insight into the heavy metal induced cell death in HT-22 cells, we examined the Annexin FTIC – V / Propidium iodide (PI) assay during 8 days exposure. We found that heavy metals have different potency to induce apoptosis in HT-22 cells. Firstly Pb results in Figure 3a indicates, there was a slight trend to increased apoptotic cells (%) as concentration increased, but it was not statistically significant. However, at 70 µM response was relatively significant ($p^{***} < 0.05$). Figure 3b shows that Cd apoptosis effect in HT-22 cells, the apoptosis response was similar to Pb metal; the percent of apoptotic cells was significant at 3.26, 3.37 µM ($p^{***} < 0.05$). The other two metals As and MeHg has a significant effect even at low concentration of exposure. As shows more percent of apoptotic cells at 0.60, 0.66 µM ($p^{***} < 0.05$); which indicates the potency of As was high in HT-22 cells. In MeHg the apoptosis pattern was started at 0.18 µM and reached as sudden inflection at 0.29 µM (($p^{***} < 0.05$), however, the percent of apoptotic cells are more than expected (47.6 ± 0.1). At IC_{30} (70.00, 3.37, 0.66, and 0.29 µM), the observed maximum percentage of apoptosis cells 15.4 ± 5.6, 31.8 ± 0.0, 41.9 ± 6.4, and 47.6 ± 0.1 of Pb, Cd, As, and MeHg respectively. Thus, the present study clearly shows that the apoptosis is a potency dependent as similar to cell viability. This concentration dependent pattern of apoptosis induced by heavy metals as observed in flow cytometrtic analysis of the HT-22 cells stained with PI and annexin V is summarized in Figure. 3a – 3d (dot plot).

Quantitative of total apoptotic cells (%) are shown in the Table 2A-2D. Overall flow cytometrtic analyses show that heavy metals induce apoptosis on HT- 22 cells dose dependent, and the potency of metals shows MeHg > As > Cd > Pb.

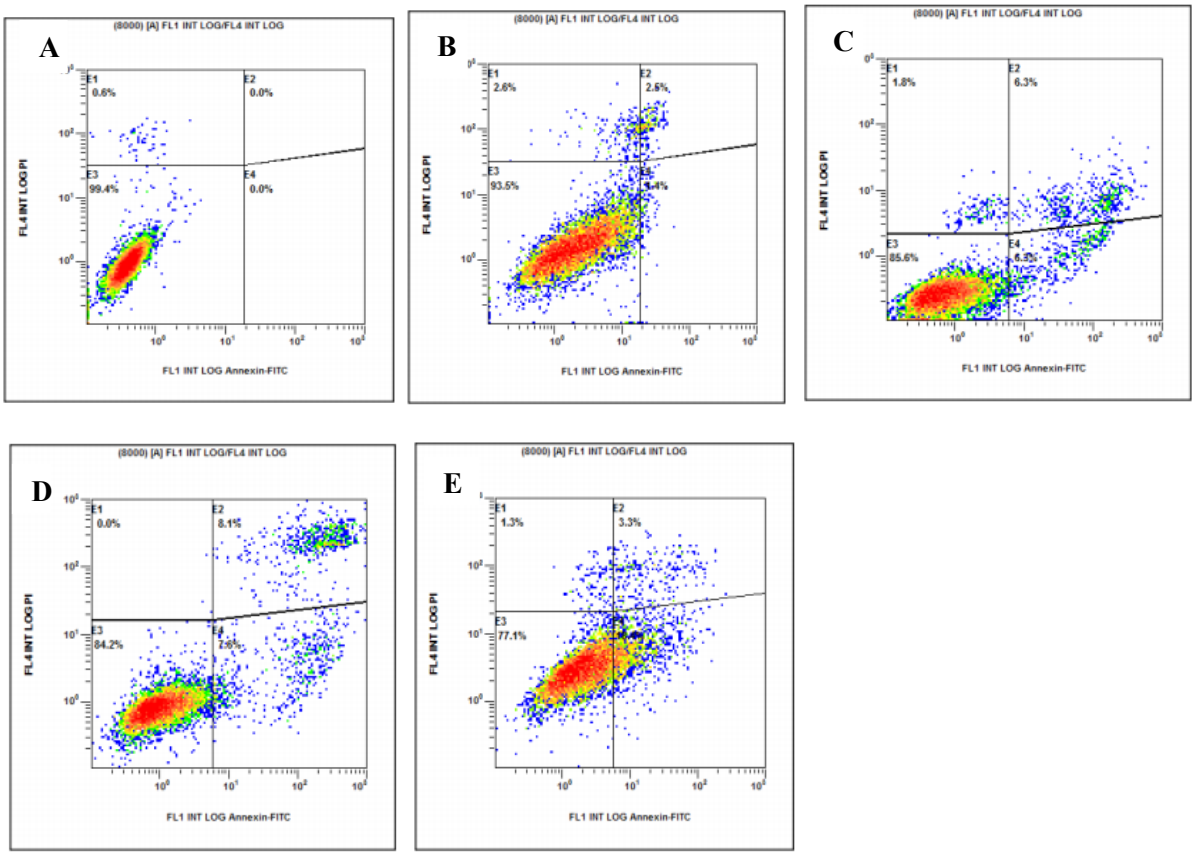

Figure 3a: Representative dot plots showing the inhibitory effect of Pb to HT-22 cells upon 8 days of exposure. A= Control, B= 63.50 µM, C= 67.34 µM, D = 68.74 µM, E= 70 µM. Lower left (LL) = Live cells (Annexin V−/ PI−), lower right (LR) = Early apoptotic cells (Annexin V +/PI−), upper left (UL) = Late apoptotic (Annexin V+/ PI+), upper right (UR) = Necrotic cells (PI+).

Table 2A: Pb Summary data of annexin – V / PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

Pb Conc. (µM)	Viable Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.8 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
63.50	94.5 ± 0.9	1.9 ± 1.7	3.6 ± 0.9
67.34	88.8 ± 2.9	9.7 ± 3.1	1.5 ± 1.0
68.74	84.6 ± 2.1	12.8 ± 2.8	2.6 ± 4.1
70	81.3 ± 6.5	15.4 ± 5.6	3.3 ± 4.6

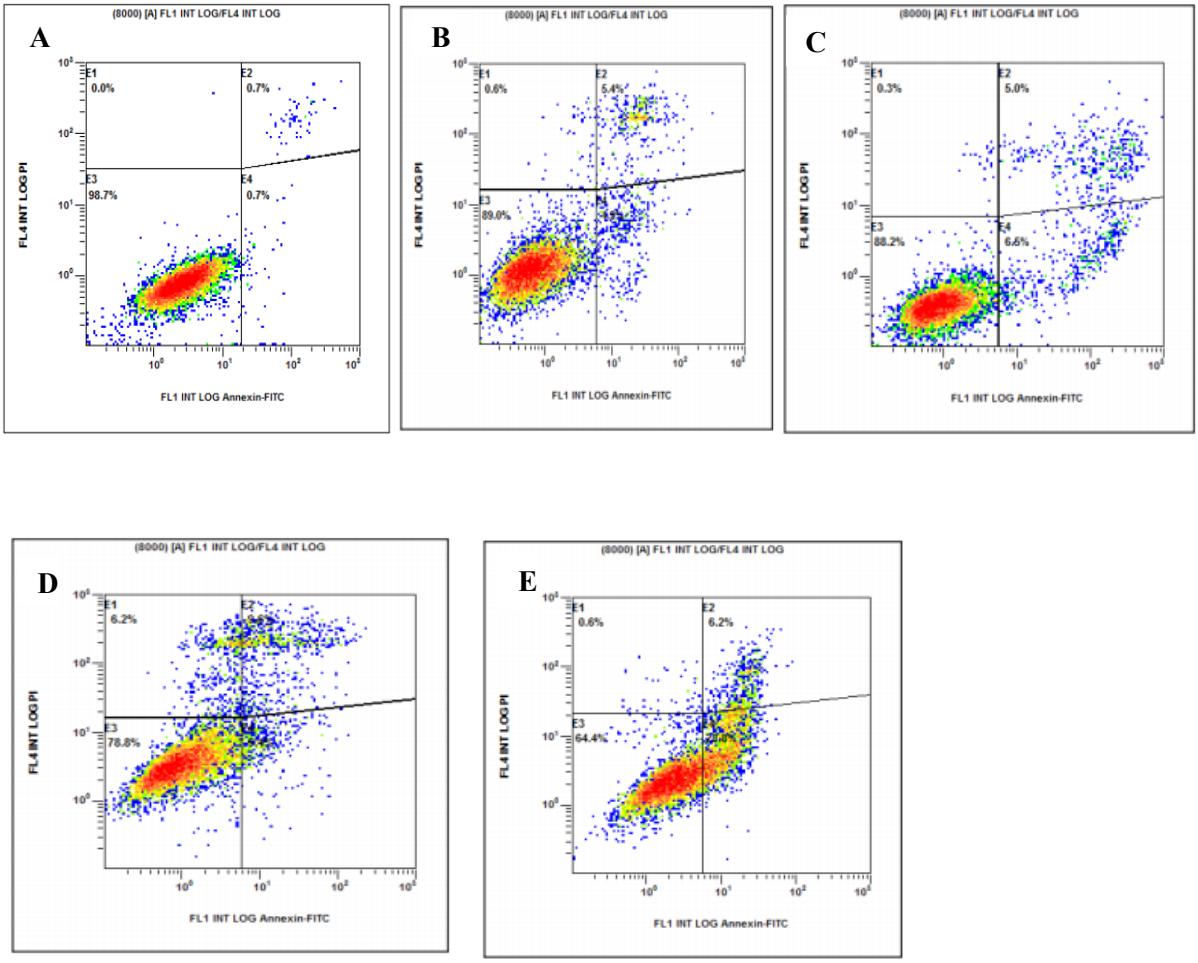

Figure 3b: Representative dot plots showing the inhibitory effect of Cd to HT-22 cells upon 8 days of exposure. A= Control, B= 2.80 µM, C= 3.14 µM, D = 3.26 µM, E= 3.37 µM. Lower left (LL)/ E3 = Live cells (Annexin V− / PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+ / PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

Table 2B: Cd Summary data of annexin – V /PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

Cd Conc. (µM)	Viable Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.9 ±0.4	0.93 ± 0.8	0.2 ± 0.3
2.80	91.5 ± 2.0	4.8 ±1.5	3.7 ± 1.0
3.14	89.0 ± 1.3	9.9 ± 0.6	0.8 ± 2.4
3.26	80.4 ± 1.8	15.7 ± 2.8	3.9 ±1.5
3.37	65 ± 1.3	31.8 ± 0.0	1.8 ± 0.0

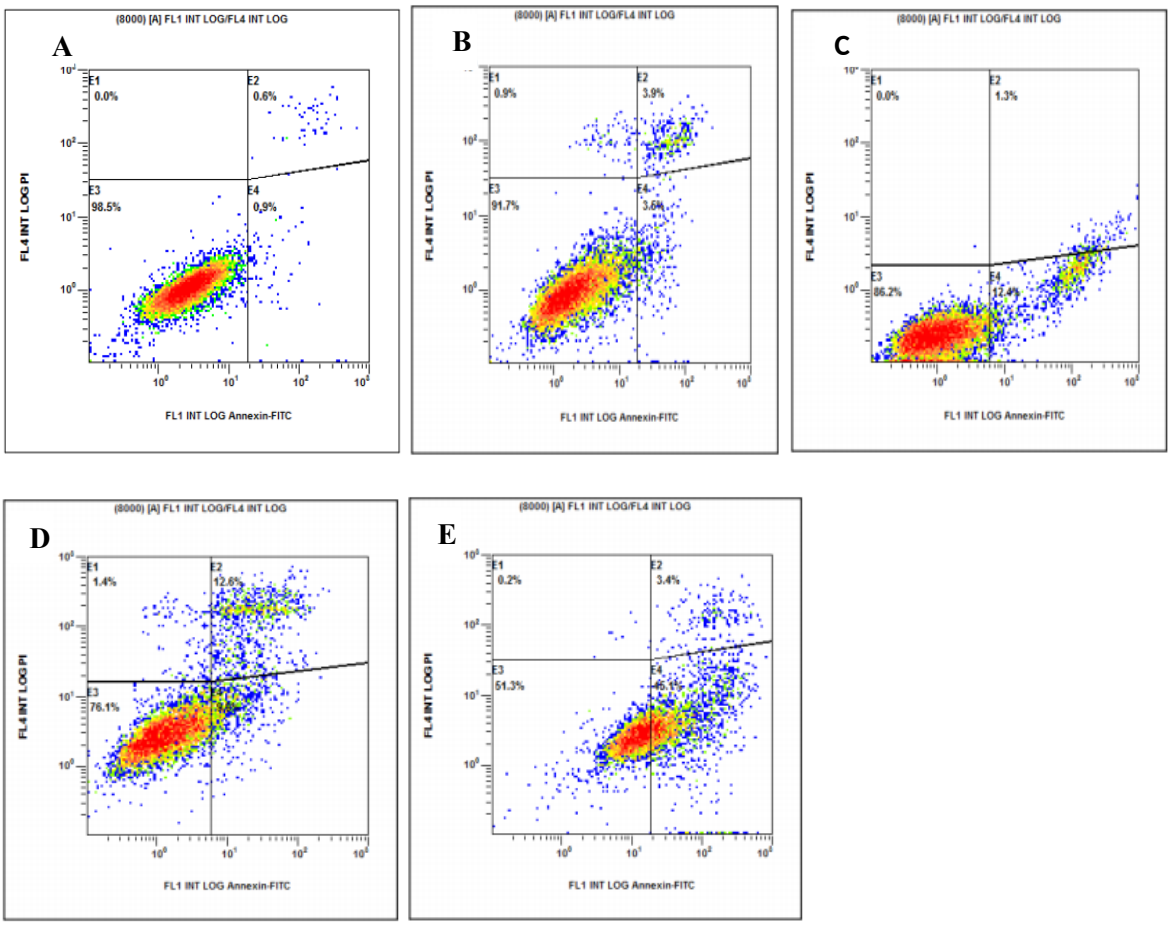

Figure 3c: Representative dot plots showing the inhibitory effect of As to HT-22 cells upon 8 days of exposure. A= Control, B= 0.42 µM, C= 0.55 µM, D = 0.60 µM, E= 0.66 µM. Lower left (LL) = Live cells (Annexin V−/PI−), Lower right (LR) = Early apoptotic cells (Annexin V + / PI−), Upper right (UR) = Late apoptotic (Annexin V+ / PI+), Upper left (UL) = Necrotic cells (PI+).

Table 2C: As Summary data of annexin – V /PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

As Conc. (µM)	Viable Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.9 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
0.42	91.7 ± 0.6	7.4 ± 0.2	1 ± 0.8
0.55	86.4 ± 1.5	13.2 ± 1.2	0.4 ± 0.5
0.60	78.9 ± 3.0	17.3 ± 4.5	3.8 ± 2.4
0.66	57.8 ± 6.4	41.9 ± 6.4	0.3 ± 0.2

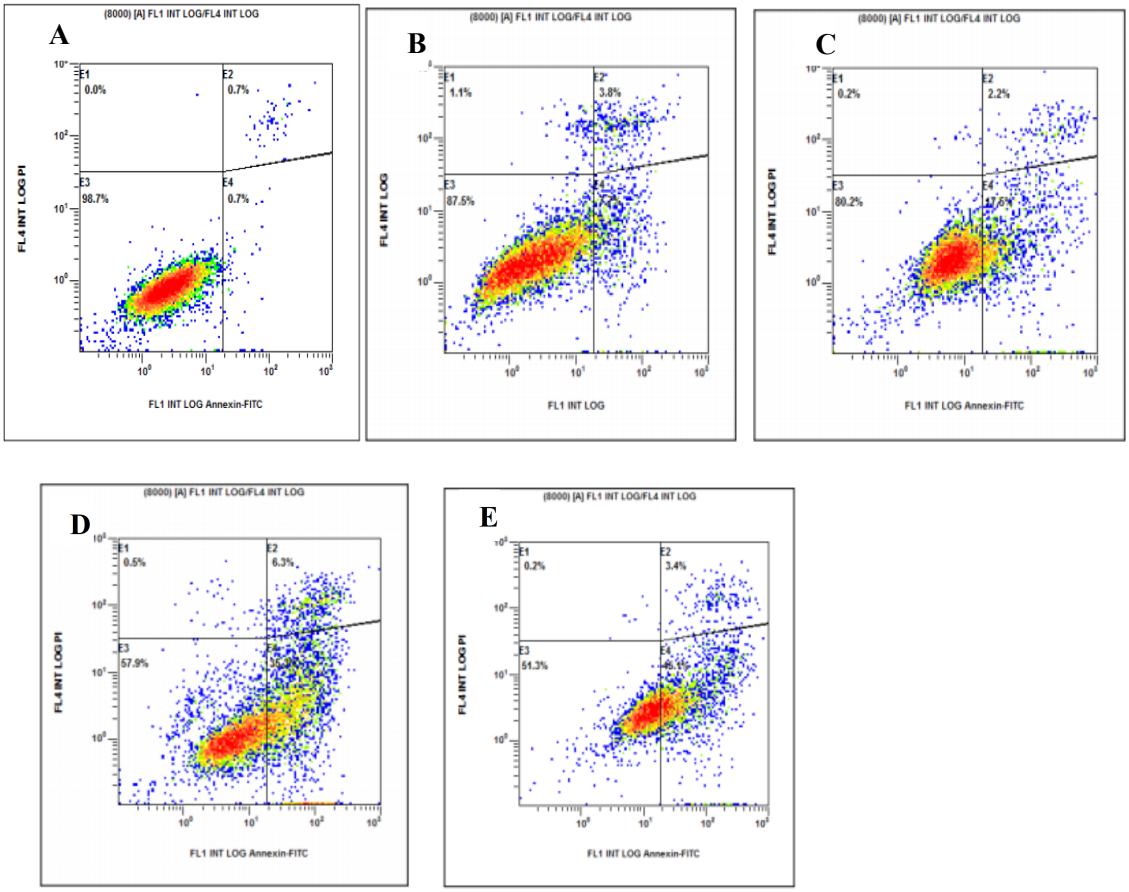

Figure 3d: Representative dot plots showing the inhibitory effect of MeHg to HT-22 cells upon 8 days of exposure. A= Control, B= 0.09 µM, C= 0.18 µM, D = 0.23 µM, E= 0.29 µM. Lower left (LL)/ E3 = Live cells (Annexin V−/ PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/ PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

Table 2D: MeHg Summary data of annexin – V /PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

Conc. (µM)	Live Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.9 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
0.09	89.8 ± 2.7	8.9 ± 1.2	1.3 ± 1.2
0.18	77.8 ± 1.5	21.2 ± 0.7	1.0 ± 0.7
0.23	68.2 ± 9.4	33.2 ± 0.3	0.7 ± 0.3
0.29	52.3 ± 7.7	47.6 ± 0.1	0.2 ± 0.1

3.4. Interactions among the Pb, Cd, As and MeHg mixtures- response additivity

The concentrations of individual metals were selected for the mixture interaction based on the MTT assay, apoptosis results. The selected concentrations were expected to cover the concentration levels from non-toxic concentrations to a toxic level. The response additivity method described by Lau et al. (2006) was utilized, in which concentrations of each compound equivalent to their IC_5, IC_{10}, IC_{15}, and IC_{20} calculated from the single metal response curve. To estimate the mixtures toxicity, we performed different combinations of metal (Pb+ Cd, Pb+ As, Pb+ MeHg, Cd+ As, Cd+ MeHg, and As+ MeHg) on mice hippocampal HT-22 cell line. Generally know that MeHg and Pb have more impact on the brain rather than Cd, As. However in mixture point of view, the obtained results suggest that some interaction mechanism is there to produce the more than expected level. Several interesting findings were observed from dose response analysis; additive results were obtained in Pb+Cd, Pb+As, As+MeHg from IC_5 to IC_{20} range of mixtures, this purely additive effect is indicative of a lack of interaction. The cytotoxicity of Cd+As mixture results was less than the additive response suggesting antagonist effect, which indicates some interaction mechanism between Cd and As inhibits the potency of toxicity on HT-22 cells. The mixtures; Pb+MeHg, Cd+MeHg showed more than the additive response, for this nature of interaction considered as a synergistic effect. The obtained results suggest that there are mainly synergistic effects in MeHg containing mixtures. The Cd role in mixtures was aberrant; in the results, Cd showed additive, antagonistic, synergistic effects, depends on the co-exposure metal. A marked reduction of cell viability was found in MeHg among Pb, Cd binary mixture. This synergistic effect can be possible due to the common mechanism and it reduces the exposure dose of individual metals in a mixture (Fig 4).

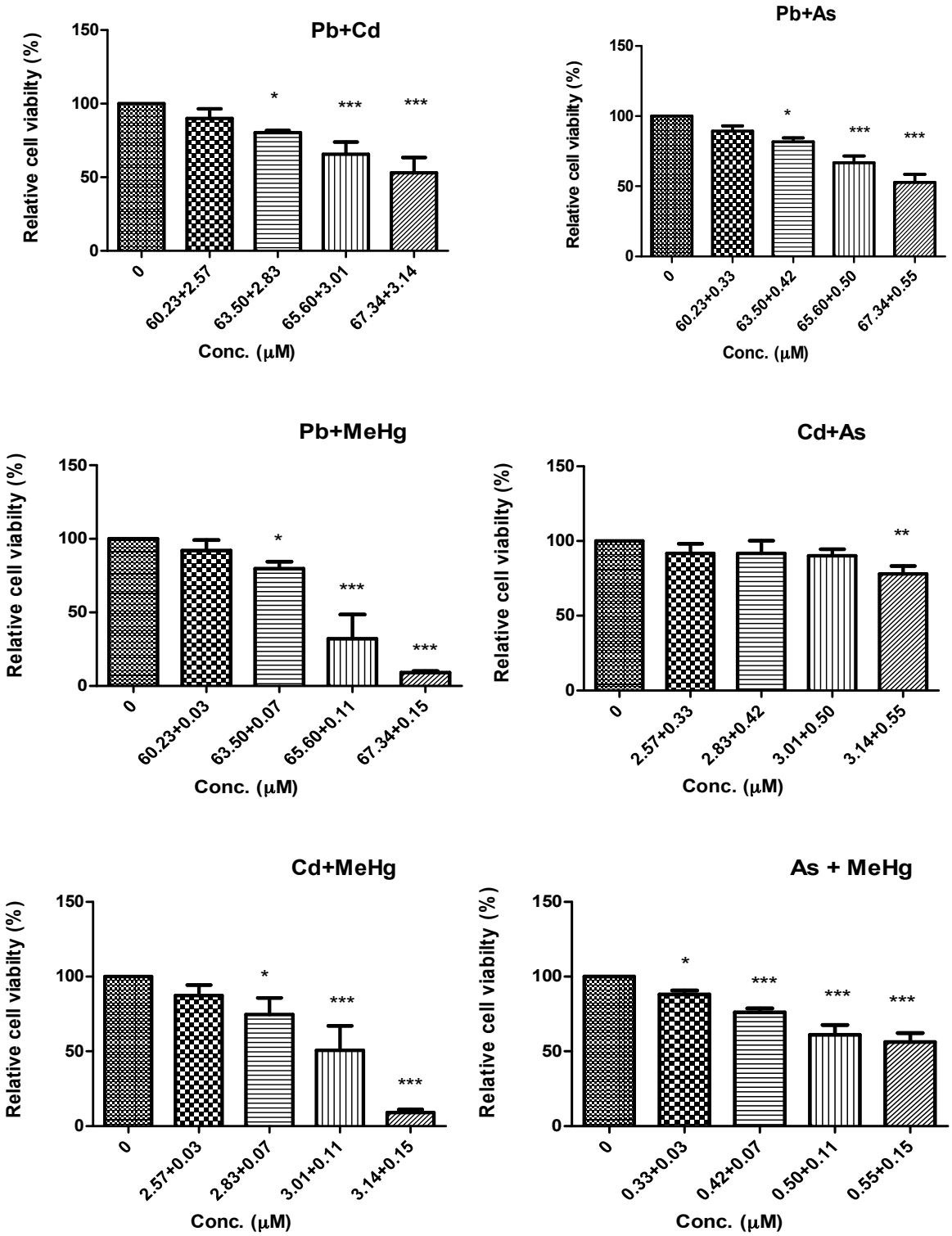

Figure 4: Effects of Pb, Cd, As, and MeHg in binary combination, on cell viability, measured by the MTT assay, in HT- 22 cell line during 8 days. In each response, concentrations of each metal corresponding to the equitoxic (in combination) were utilized. Values of IC_5, IC_{10}, IC_{15}, and IC_{20} were derived from concentration response curves reported in Figure 1. Each bar represents the mean (± SD.) of separate determinations carried out in triplicate (***$p< 0.05$).

3.5. Interactions among the Pb, Cd, As and MeHg mixtures- dose additivity

Dose additivity is an equally valid procedure for analysing interactions between metals irrespective of their mechanisms of action and aims to establish the required concentrations of individual metals within a combination that produces a specified level of effect. Individual concentration- response curves for all four metals were used to calculate the individual concentrations required to produce 30% inhibition of cell death. The effective concentration (IC_{30}) of Pb, Cd, As, and MeHg are 70.0 μM, 3.37 μM, 0.66 μM, and 0.29 μM respectively. All binary mixtures tested as non- constant ratio isobologram model and generated data points illustrated in Fig 5, the straight line between the mean single values is referred to as the additivity line and area of CI values for these two measurements the CI limits. Qualitative evaluation of curve can be in two types of descriptors; the mean value can either is within the CI belt or exist outside or inside (deviation from additivity). Mixtures interaction for Pb, Cd, As, MeHg represented in Table 3, descriptively graphic data suggest that all combinations interaction. The combination of A (X-axis) and B (Y-axis) metal using a comparison of CI values for single concentrations (100% A/ B% metal and 0% A/ 100% B, additivity line). For instance, a combination of Pb and Cd was produced CI>1 value, the significant points are shown in Fig 5, suggests that Pb and Cd were antagonistic effect (50% Pb/ 50% Cd, 75% Pb/ 25% Cd) in the mixture. The effect of Pb and As combination clearly indicates that slightly deviation from the additive line in the different ratios, the CI < 1. The isobologram of Pb+As mixture mark slightly synergistic interaction. The Pb and MeHg mixture suggests the interaction is more deviate from the expected additivity line (25% Pb/ 75% MeHg, 50% Pb/ 50% MeHg), CI value 0.7, 0.7 respectively. The combination of Cd with MeHg, and As shows the antagonistic effect, the similar results can be seen for isobole curve; however, the antagonistic potency of interaction depends on the composition of mixtures (CI values range 1.4 to 3.8). We found highest antagonistic effect in Cd+MeHg mixture (25% Cd/ 75% MeHg, 75% Cd / 25% MeHg) CI values 3.31, 2.05 respectively that indicates MeHg concentration influencing the combination effect (except MeHg+As) (Table 4).

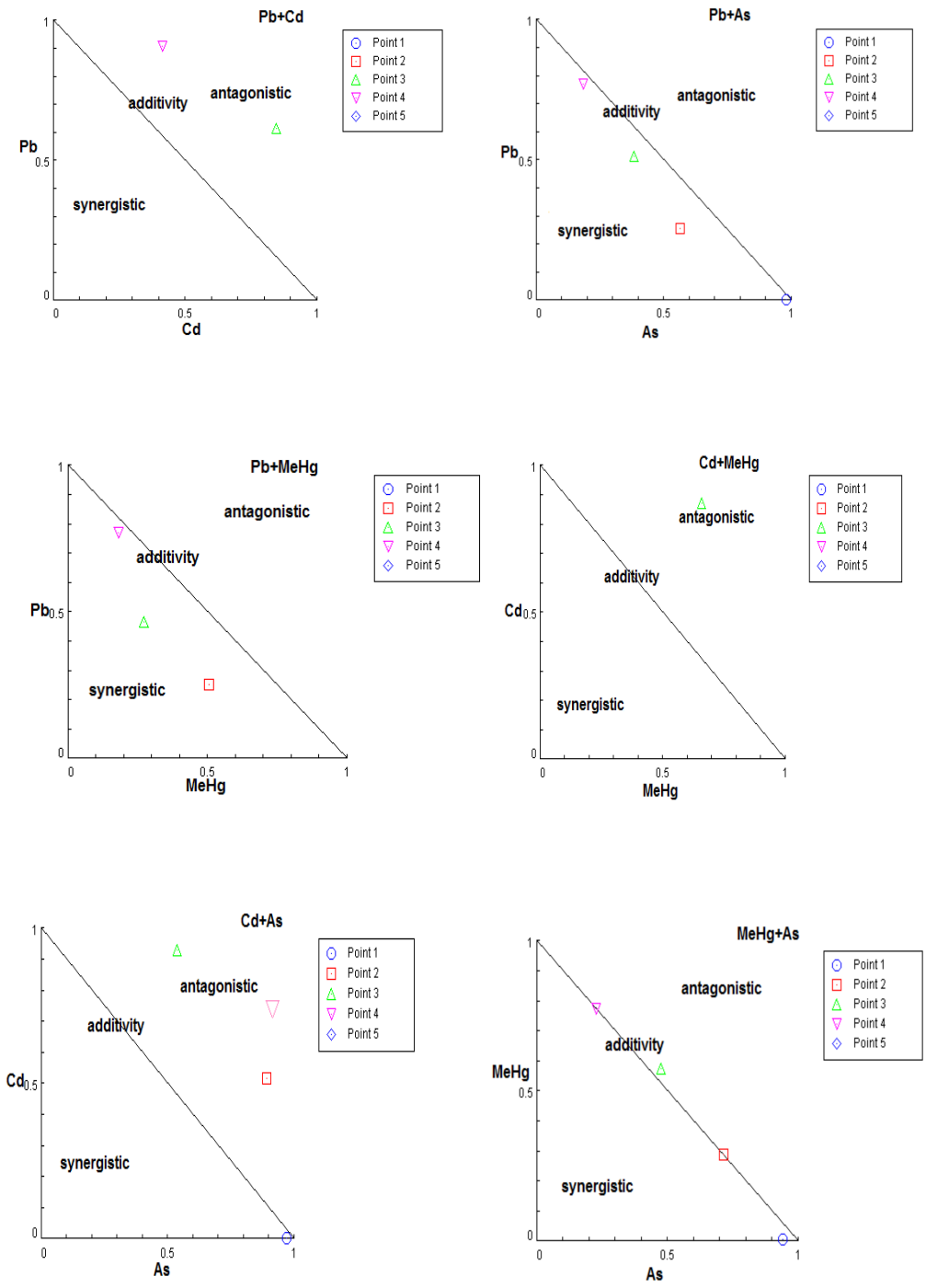

Figure 5: Normalized isobologram plot of binary mixtures of Pb, Cd, As, and MeHg on HT-22 cells. Each isobologram experiment was performed three times independently of each other; data presented for each metal combination are a mean of all three experiments. Some points are out of the limit in curve representation.

Table 3: The combination index (CI) values of each non- constant ratio of the mixture obtained from the Compusyn normalized isobole model (Note. 100% A and 100% B correspond to each metal's IC_{30} value; other metal amounts are related to a fraction of the IC_{30} as indicated).

Point	Mixture ratio		Combination Index (CI) value					
	A (%)	B (%)	Pb+Cd	Pb+As	Pb+MeHg	Cd+As	Cd+MeHg	MeHg+As
1	0	100	1.0	1.0	1.0	1.0	1.0	1.0
2	25	75	1.5	0.8	0.7	1.4	3.3	1.0
3	50	50	1.4	0.9	0.7	1.4	1.5	1.0
4	75	25	1.3	0.9	0.9	1.8	2.0	1.0
5	100	0	1.0	1.0	1.0	1.0	1.0	1.0

(*CI value =1; additive, > 1; antagonistic, <1 synergistic according to Isobologram analysis)

4. Discussion

Usually, more than one heavy metal was involved in the environmental exposure. A risk assessment of the metal mixtures would be more practical, and the combined effects are needed in future research to improve the risk evaluations. The objective of this work was to assess toxicities of heavy metals individually and as mixtures on HT-22 cell line. The results showed significant cell reduction after exposure to toxic metals. Ultimately binary mixtures induced significant cell death compared to individual metals. The individual toxicity of these four metals to different cell lines and organisms has been widely investigated; however, mixture studies are very limited relevant to hippocampal cell line. The Pb toxicity results in the HT-22 cells suggest the potency of Pb depends on the concentration. It showed that Pb could gradually decrease the viability of HT-22 cells and the result was consistent with reported studies (Xu et al., 2006). The observed neurotoxic effects of Cd in various brain cell cultures are in agreement with the current HT-22 cells cytotoxic effects (Lopez et al., 2003). The toxicity of Cd perhaps establishes the relation with Ca^{+2} ion mechanism. Orrenius and Nicotera (1994) reported that the concentration of cytoplasmic and nuclear Ca^{+2} increased in neurons as result of Cd exposure. In experimental animal models was found that As causes a potent alteration in hippocampal region (Cronican et al., 2013). In the present study As shows significant effect on HT-22 cells; the decreasing pattern of cell viability was high. Other reported studies showed that As induces cytotoxicity in human lung fibroblasts in dose dependent manner, which is consistent with current study (Vogt and Rossman, 2001). The MeHg potency is very high in HT-22 cells rather than other three metals. The obtained results are consistent with MeHg toxicity studies in glioblastoma, neuroblastoma cultures and cerebellar granule cells (CGC) (Crespo-López et al., 2007; Fonfría et al., 2005)

To further gain insight into the heavy metal toxicity; mechanistic based assay such as apoptosis might be potential to validate the MTT assay. To know the clear cytotoxic effect on HT-22 cells, we performed Annexin- V/ PI staining that allows the discrimination of viable cell, apoptotic, and necrotic cells by binding with Ca^{+2} dependent phosphatidyl serine (PS) protein in the cell membrane (Vermes et al., 1995). The obtained results confirm Pb could induce the apoptosis in HT-22 cells, however the apoptosis response was very low in tested 63.5-70 μM range; the observed results reported similarly like calcium to bind the internal metal binding site of the permeability transition pore and open it, which could initiate apoptosis in the retina (He et al., 2000). The effect of Cd was more pronounced at 3.37 μM. Reported studies have shown that Cd induces dose- dependent

apoptosis like cortical neurons (López et al., 2003). Regarding As we observed that apoptosis at a low dose (0.66 μM); these findings correlate with As induced apoptosis in neuroblastoma cells (Akao et al., 2000). The MeHg induced apoptosis in HT-22 cells was high in 0.18-0.29 μM, the similar response was observed in human SH-SY 5Y neuroblastoma cells (Ndountse and Chan, 2008). The results indicate that percentage of apoptotic cells was dependent on the metal potency like MTT assay, maximal level of apoptotic cells observed in MeHg, and potency of metals ranked MeHg > As > Cd > Pb. We are used the apoptosis to validate the MTT assay results for extending to the metal mixtures toxicity.

Based on the cytotoxicity of individual metals Pb, Cd, As, and MeHg on HT-22 cells, the response addition, dose addition methods were used to determine interaction profile of mixtures. However, there is a widespread disagreement over terminology, definitions, and models for the analysis of mixtures (Feron and Groten, 2002; Wu et al., 2016). Several methods for calculating the expected combination effect of two or more chemicals are currently in use, the majority of which can be associated with two popular basic concepts known as response additivity (Bliss 1939) and dose additivity (Loewe 1953). Response additivity focuses on measuring the effects of mixtures at only one specified concentration for each metal, thus lacking the information on concentration- response relationships. Dose additivity is an equally valid procedure for analysing interactions between agents irrespective of their mechanisms of action. However, this method requires tedious testing with a variety of concentrations for the determination of each data point on the isobologram, There is no generally accepted agreement as to which of the two concepts is more appropriate (Teuschler, 2007); therefore we have attempted to carry out this study using both models to confirm our findings. It is noteworthy that the combined toxicity of the overall metal mixtures might be different from their individual toxicities. Thus mixture studies are important to elucidate whether these interactions to such mixtures would cause deleterious effects to hippocampal cell line. We used simple effect addition and dose addition isobologram analysis concept for mixtures interactions. Response addition implies that the effects of exposure to a mixture of such chemicals are equivalent to the effects of the sum of the potency corrected doses of each component (Ince et al., 1999). The findings from Pb+Cd results consistent with experimental studies on a pregnant rat showed that combined exposure of Cd and Pb have additive effect on decreasing Na^+ / K^+ -ATP ase function, in which Cd activity is potentiated by Pb for causing failure of the Na^+/ K^+-ATPase (Antonio et al., 2003). In the current study, significant antagonism was found in Cd+As mixture; the observed antagonistic effect supported by Vellinger et al. (2012). In addition a Pb+MeHg, Cd+MeHg mixture showed similar toxic potency level which suggests the MeHg influences the equitoxic level in both mixtures however, the combination with MeHg and other metals studies are limited. The findings mainly indicate synergistic effect among MeHg and Pb, Cd in high concentration, which is an agreement with other investigators findings of synergism (FríasEspericueta et al., 2009). The other two combinations Pb+As, As+MeHg indicates the simple additive effect on HT-22 cells. Based on the single metal results, we propose that the isobologram analysis for dose additivity method, Rodea-Palomares et al., (2010) were the first to use this method in environmental risk assessment applications. In the isobologram analysis, the metal mixture impact was different from the equitoxic exposure. The combination of Cd with Pb, As MeHg indicated the antagonist effect clearly shows that co exposure of Cd metal implies the toxicity of other metal in a reduction manner. The current Cd results disagreement with reported interactions studies such as Cd with As increases the expression of stress proteins in rat and human kidney cell lines (Madden et al., 2002). The combination of Pb with As and MeHg effects found more than the additive effect according to CI value. The other combination of MeHg & As CI value on HT-22 cells suggesting a simple additive effect. These findings give significant evidence of the metal mixtures neurotoxic activity and their potential interactions depend

on the composition of elements, cell line sensitivity. The observed interactions at dose additivity, response additivity supported by (Bae et al., 2001) interactive effect of Pb, Cd, As and Cr on keratinocytes, the results showed a trend of additivity, synergism and antagonism with increasing metal mixture concentrations. However, the current mixtures toxicity is most relevant to neurotoxicity; the literature related to metal mixtures toxicity impact on hippocampus is very limited.

Table 4: Summary of metal mixtures interaction profile on HT-22 cells during 8 days exposure.

Mixture	**Interaction profile**	
	Response addition	**Dose addition**
Pb+Cd	Additive	Antagonistic
Pb+As	Additive	Slightly synergistic
Pb+MeHg	Synergistic	Synergistic
Cd+As	Antagonistic	Antagonistic
Cd+MeHg	Synergistic	Strong antagonistic
MeHg+As	Additive	Additive

5. Conclusion

From a public health point of view, it is most relevant to answer the question whether chemicals in a mixture interact in a way that results in a reduced or increased overall response when compared with the sum of the responses to the individual chemicals in the mixture, or indeed in an effect that is simply an addition of the expected effects. We investigated the toxicity of four heavy metals and their mixtures on hippocampus relevant mice HT-22 cell line for knowing their toxicity impact. The comparison between the effect addition and dose addition was useful in the evaluation of combined effects in the mixture. The nature of interaction varies according to the effect levels and the type of components in the mixtures. This binary combination of Pb, Cd, As, and MeHg displayed synergistic and antagonistic interaction at low and higher effect levels, respectively. The mixture effects should be considered in the risk assessment of co-exposure metal potency. Toxicity order of current mixtures results on HT-22 cells, Pb+MeHg> MeHg+As > Cd+As found in both dose addition and response addition. The other three mixtures (Pb+Cd, Cd+MeHg, Pb+MeHg) interaction effects are different in an each method. We concluded from the results; dose addition isobologram analysis was a more beneficial strategy for mixtures study than response additivity. We expect this approach will be of use for a wide range of metal mixtures and indeed other types of experiments for knowing the interaction profile at each point. Moreover, improved investigations on metal combined effects are needed in future studies. The outcome of this study adds to a new sense of urgency for research to examine the mechanisms associated with toxicities of metals mixtures. In future, comprehensive mechanistic based omics investigation will be useful for understanding the real interactive mixtures interactions at molecular level.

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