# Comparative In vitro Toxicity Evaluation of Heavy metals (Lead, Cadmium, Arsenic, and Methylmercury) on HT -22 Hippocampal Cell line

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**Abstract:** Heavy metals are considered some of the most toxic environmental pollutants. Exposure to heavy metals including lead (Pb), cadmium (Cd), arsenic (As), and methyl mercury (MeHg) have long been known to cause damage to human health. Many recent studies have supported the hippocampus as the major target for these four metals for inflicting cognitive dysfunction. In the present study, we proposed hippocampal relevant in vitro toxicity of Pb, Cd, As, and MeHg in HT- 22 cell line. This study reports, initially cytotoxic effects in acute, subchronic, chronic exposures. We further investigated the mechanistic potency of DNA damage and apoptosis damage with the observed cytotoxicity. The genotoxicity and apoptosis were measured by using the comet assay, annexin-V FTIC / propidium iodide (PI) assay respectively. The results of cytotoxicity assay clearly demonstrated significant concentration and time dependent effects on HT-22 cell line. The genotoxic and apoptosis effects also concentration dependent fashion with respect to their potency in the range of $IC_{10}$ –$IC_{30}$, maximal level of damage observed in MeHg. In conclusion, the obtained result suggests concentration and potency dependent response; the maximal level of toxicity was observed in MeHg. These novel findings support that Pb, Cd, As, and MeHg induces cytotoxic, genotoxic, and apoptotic effects on HT-22 cells in potency dependent manner; MeHg> As> Cd> Pb. Therefore, the toxicity of Pb, Cd, As, and MeHg could be useful for knowing the common underlying molecular mechanism, and also for estimating the mixture impacts on HT-22 cell line.

**Key words:** Heavy metals in vitro toxicity; Dose response; HT-22 cell line; DNA damage; Apoptosis.

**Abbreviations:** Ach E = Acetyl cholinesterase E, ANOVA = Analysis of variance, As = Arsenic, DAPI = 4′, 6-diamidino-2-phenylindole , DMEM = Dulbecco's modified Eagle's medium, FBS = Fetal bovine serum, GABA = γ-gamma-amino butyric acid, GAD = Glutamate decarboxylase, $IC_{50}$ = Inhibitory concentration 50, LTP = Long- term potentiation, MeHg = Methyl mercury, MMS = Monomethyl sulfonate, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NMDA = N-methyl-D-aspartate, OD = Optical density, Pb = Lead, PI = Propidium iodide, PS = Phosphatidylserine, 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 [1–3]. Exposure to heavy metal including lead (Pb), cadmium (Cd), arsenic (As), and mercury including methyl mercury (MeHg) has long been known to be hazardous to human health [4, 5]. Lead (Pb) exposure is widespread with broad use as major components in many commercial products such as lead-based paint batteries [6–8]. Cadmium (Cd) is a widespread industrial and environmental pollutant. Fumes generated during industrial operations [9, 10] and cigarette smoking containing Cd represents a significant source of exposure which is mainly absorbed via inhalation route [11]. However, a low amount of Cd in dietary food and its gastrointestinal absorption is found to be very low [12]. Contaminated drinking water is the main source of human exposure to arsenic (As); other less common sources of As exposure include coal combustion and food particularly in U.S [13–15]. Mercury is released into the environment primarily through the burning of fossil fuels, such as coal. The released mercury contaminates the surface waters, and enters the aquatic ecosystem as MeHg. MeHg is formed from inorganic mercury by the action of microbes that live in aquatic systems and with longer half-life biomagnified in aquatic food chains [17]. Consumption of contaminated fish and other aquatic seafood is the primary source of MeHg exposure to humans [16]. MeHg has a specific concern in human because it readily absorbed from the gastrointestinal tract (GI), and is actively transported across the blood brain barrier (BBB) [17, 18]. The affected organ systems by these four metals are renal, pulmonary, hepatic, gastrointestinal and haematological systems along with peripheral and central nervous systems [17]. Our previous study showed that Pb, Cd, As, and MeHg disrupts the hippocampus by inflicting the cognitive dysfunction in both children and adults [18] .

Exposure to MeHg and Pb has a significant effect on the human brain and are well known to targeting the central nervous system [19–22]. A specific population such as children are more susceptible concerning their development phase of the nervous system [23–25]. Exposure to Cd also severely affects the function of the nervous system, leading to Parkinson disease and learning disabilities [26, 27]. However, the exact mechanism and its neurotoxic effects are still ambiguous [28, 29]. The adverse effects of inorganic As on health are well known and include cancer, skin lesions, and lung diseases [30–32]. Many previous studies have been made that links As exposure with developmental neurotoxicity disorders [33–35]. The toxicity level of Pb, Cd, As, and MeHg are high in humans thus, these four elements rank among the priority metals that are of great public health significance [36]. Recently, Karri et al. reported that Pb, Cd, As, and MeHg exposure has the potency to damage the hippocampus region of brain by interacting with different molecular mechanisms. For instance; Pb interacts with N-methyl D-aspartate (NMDA) receptor [37], MeHg indirectly affects NMDA receptors functioning via inhibiting glutamate decarboxylase (GAD) and glutamate (Glu) transporter [38]. Cd inhibits the $Na^{+}$ / $K^{+}$ - ATP ase [39]. Arsenic suppresses the NMDA receptors activity in the hippocampus, where these receptors play a pivotal role in synaptic plasticity, learning and memory [34, 40].

The main objective of this study was to find the individual metal (Pb, Cd, As, and MeHg) toxicity on HT-22 hippocampal cell line in a comparative manner. In this study, we considered a well-established mammalian HT-22 hippocampal cell line to be more relevant for the model of hippocampal toxicity. For elaborating the hippocampal toxicity hypothesis, initially we performed cell viability via MTT assay of Pb, Cd, As, and MeHg in mice HT-22 hippocampal cell line to different exposure scenarios such as acute (1 day), subchronic (3 days) and chronic (8 days). Further, we extended to genotoxicity and apoptosis studies by using low toxic concentrations. These two assays were helpful for

elucidating genotoxicity and apoptosis mechanism of heavy metals in HT-22 cells during chronic exposure.

## 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 of the cell line sub cloned from its parent line HT4, which are immortalized mouse hippocampal neuronal precursor cells [41].

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 (Pan-Biotech- Germany), and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% $CO_2$ in air at $37^0$ C. For all the experiments cells were grown at 70-80% confluence. We have checked the cell confluence in different time intervals (1 day, 3 days, and 8days); confluence was stable until 8 days thus considered as chronic exposure.

The cells were cultured in 75 $cm^2$ cell culture flasks. For experimental purpose, cells were seeded at 1 x $10^6$ cells/ mL (acute), 0.57 x $10^6$ cells/mL (subchronic & chronic) in 96 well plates 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 4.6 µM, 0.6 to 12 µM, respectively, for acute (1 day), subchronic (3 days) and chronic (8 days) assays. In subchronic and chronic exposure, the medium containing given metal concentration was refreshed at every 2 days interval for maintaining metal exposure. 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 (PBS, pH = 7.4). The applying solution contains 10 % metal solution and 90% DMEM culture medium.

### 2.3. Analysis of Cell viability by MTT assay

The MTT assay was carried out using a modification of the method of Mossman (1983). MTT reagent is taken up into cells and reduced in mitochondria dependent reaction to yield a formazan product. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. MTT was dissolved at 5 mg/mL in phosphate buffered saline. This stock solution was filtered through a 0.2 µm filter and stored at -80°C. 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 hours; after this time, the MTT was removed by aspiration. Formazan

products 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.

**2.4. Analysis of DNA damage by Comet assay**

To measure the DNA damage induced by Pb, Cd, As, and MeHg at low concentrations ($IC_{10}$ to $IC_{30}$), the alkaline comet assay was conducted according to the OECD guidelines with some modification. To determine the genotoxic potential of Pb, Cd, As, and MeHg in HT-22 cells, cells were seeded in 96 well plate (1 x $10^6$) and incubated at 37 °C in 5% $CO_2$ for 24 hours for cells attachment. To knowing the genotoxicity effect, we applied the low / chronic $IC_{10}$ - $IC_{30}$ concentration of Pb, Cd, As, and MeHg to the cells during 24 hours, also Monomethyl sulfonate (MMS) 400 µM was used as positive control. After incubation, each well of cells was washed with 50 µL PBS (pH = 7.4), then added 50 µL trypsin and double the amount of DMEM (100 µL). The collected cells were centrifuged at 3000 rpm for 3 min at 4 °C. The supernatant was discarded and the cell pellets were collected. Next, 160 mL of low melting point agarose (LMP – 0.5%) was added to the tube and mixed with the cells. This mixture was deposited on pre-gelatinized slides (normal melting point agarose, 1.5%) and then slides were placed in lysis solution for at least 1 hour for electrophoresis. After denaturation (20 min) and alkaline electrophoresis (25 V, 300 mA, 20 min); the slides were neutralized, fixed and kept refrigerated until the time of analysis. The slides were stained with DAPI (4′, 6-diamidino-2-phenylindole), inspected visually and analyzed using the Nikon epifluorescence microscope, comet analysis scoring system (Comet assay - IV) software (Perceptive instrument, UK). In DAPI staining comets showed different levels of DNA damage and found only a slight decrease in total fluorescence with an increasing fraction of DNA in the tail. The most commonly used parameters for measuring the genotoxicity are tail length, the relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in the tail), and tail moment [42]. Collins et al. reported that tail intensity is a most rational parameter for measuring the DNA damage even in low dose exposure, and analysis of 50 comets per slide is recommended [43]. The experiment was repeated three times.

Cell viability was determined along with the comet assay for each treatment. After the treatment, the cells were harvested by trypsinization, and 20 µL of the cell suspension was mixed with 20 µL of trypan blue (Gibco) in 1.5 mL micro tube. The cells were counted in a neuabauer chamber using a light microscope. The viability found was more than 80% in each exposure.

**2.5. Analysis of apoptosis by Annexin V-FITC/Propidium iodide (PI) staining**

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of plasma membrane, Annexin V- FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, Annexin V and Propidium iodide (PI) were used to distinguish the apoptotic and necrotic 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 for 8 days and control cells were made without chemical. The medium with metal concentration was refreshed every 2 days. After chronic 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 $4^0$C, resuspended in 1mL 1X binding buffer and then transfer the 100 µL of the solution to 5 mL culture tube, and was added 5 µL of both Annexin-V and PI to the samples. After staining, the cells were incubated for 15 minutes in the dark at room temperature. Cells were re-washed with 1X binding buffer 400 µL and analyzed by flow cytometry (Beckman coulter, Germany).

**2.6. Statistical analysis**

All experiments were performed three times (n=3) and each concentration was tested in a duplicate manner. The results were given as mean ± standard deviation (SD). $IC_{10}$ to $IC_{30}$ and $IC_{50}$ values were calculated from dose response curve fitted by using the Graph pad prism version 5.01. The results of MTT assay were analysed by two way ANOVA and Tukey's test; P**** values < 0.0001 were considered as statistically significant. The results of genotoxicity and apoptosis were analysed using one - way ANOVA and Dunnett's test; P*** values ≤ 0.05 were considered as statistically significant.

## 3. Results and Discussion

### 3.1. Dose response relationship of heavy metals (Pb, Cd, As, and MeHg) on HT-22 cell line

To characterize the effects of Pb, Cd, As, and MeHg individually on hippocampus during different exposures, we performed cell viability studies in mice HT-22 hippocampal cells by using the MTT assay. As expected, all 4 metals showed a concentration, time - dependent cytotoxic effect, expressed by decreased absorbance or optical density (OD). Results for Pb, Cd, As, and MeHg concentration response experiments at acute (1 day), subchronic (3 days), and chronic (8 days) exposures are presented in Figure 1 (A, B, C and D). The results showed significant concentration and time depended effects. Two-way ANOVA test showed a statistically significant effect of time and concentration in Pb, Cd, As, and MeHg toxicity ($p^{****} < 0.0001$). The mean $IC_{50}$ values are presented in Table 1 which is defined as the concentration required for inhibiting the level of MTT by 50% compared to the non-exposed control.

Firstly Pb concentration - response curves appeared to be similar for the acute and subchronic exposure scenario and cell death was observed very low even at high concentrations. However, during chronic exposure, cell death was significantly higher at concentrations > 70 µM, indicating that sensitivity of Pb towards hippocampal cells is time depended. It can be seen from Table 1, that Pb toxicity in acute exposure was very low for which we were unable to accurately determine the $IC_{50}$ concentration in experiment. In subchronic and chronic exposure the $IC_{50}$ was determined to be 117.6 µM and 74.3 µM respectively. The obtained results suggest that Pb induced damage in HT - 22 cells at low conc. (10-60 µM) has no effect on MTT reduction / cells at any time point (Figure 1, A). It showed that Pb could gradually decrease the viability of HT-22 cells and this result was consistent with reported studies [44]. In acute exposure, the maximum cell death 30% was observed at 100 µM, which is in agreement with Pb induced cell death in cortical cell cultures [45]. In case of cytotoxicity of Cd in HT-22 cells, a decrease in the viability of cells with increasing exposure time and concentration (Figure 1, B) can be observed. Cells exposed to Cd concentrations from 4.5 to 7 µM exhibited statistically significant ($p^{****} < 0.0001$) decreases in the cell viability after acute exposure. Increasing incubation time to subchronic and chronic exposures, toxicity being observed between 3.5 to 7 µM is high. However, in acute exposure Cd did not affect the HT- 22 cells at 0.5 to 3.5 µM. At concentrations lower than 0.5 µM, Cd showed no effect at any of the time point. The chronic effect of Cd was more pronounced at 4 µM compared to the acute and subchronic exposures. We observed that the percentage of cell viability decreases with increasing Cd concentration reaching maximum cell death (90%) in chronic exposure than acute and subchronic. The toxic effects of Cd on HT-22 cells at different exposure times were further compared in terms of $IC_{50}$ value at each time point as shown in in Table 1. Previously observed neurotoxic effects of Cd in various brain cell cultures are in agreement with the current HT-22 cells cytotoxic effects [46]. Cd shows inflection at 3.5 µM. ($IC_{50}$ = 3.7 µM) for chronic exposure. The response is clearly dose and time dependent. These results are similar to the reported dose dependent Cd cytotoxicity in neuronal glia cultures [47].

Concerning the cytotoxic effects of As on HT-22 cells, we can observe a linear concentration - response relationship (Figure 1, C). A gradual decrease in the cell viability with increasing a concentration in the acute and subchronic exposure can be observed. Increasing incubation time to chronic exposure resulted in increasing toxicity being observed between 0.8-1.2 μM. Moreover, for acute and subchronic assays the response was very low. In acute exposure, the response elevated at a concentration of 2.8 μM. Previous studies reported that As showed cytotoxicity even at a micromolar concentration [48] and similar results were observed in HT-22 cells. Other cell line studies showed that As induces cytotoxicity in human lung fibroblasts in dose dependent manner, which is consistent with current study [49]. The impact of MeHg in HT - 22 cells is provided in Figure. 1, D. In acute exposure from 0.6 to 1.8 μM cell death was very low, but for the same range of concentration in subchronic exposure, significantly greater cell death was observed. Two - way ANOVA test indicated statistically significant effect for time and concentration in MeHg toxicity (p**** $< 0.0001$). In chronic exposure even in the lowest concentration (0.6 μM) the cell death was significant. However, in acute exposure MeHg showed significant effects at a concentration of 2.4 μM. The obtained MeHg concentration- response curves differed with respect to their exposure duration; response curve has a similar slope in subchronic and chronic exposures than acute exposure. The curve inflection also drastically enhanced in chronic exposure when compared to the acute and sub-chronic exposures. However, the differences in the cell viability for acute and subchronic exposures are relatively narrow indicating that high potency of MeHg in hippocampal cells (HT-22). The obtained results consistent with MeHg toxicity studies in glioblastoma, neuroblastoma cultures [50] and cerebellar granule cells (CGC) [51].

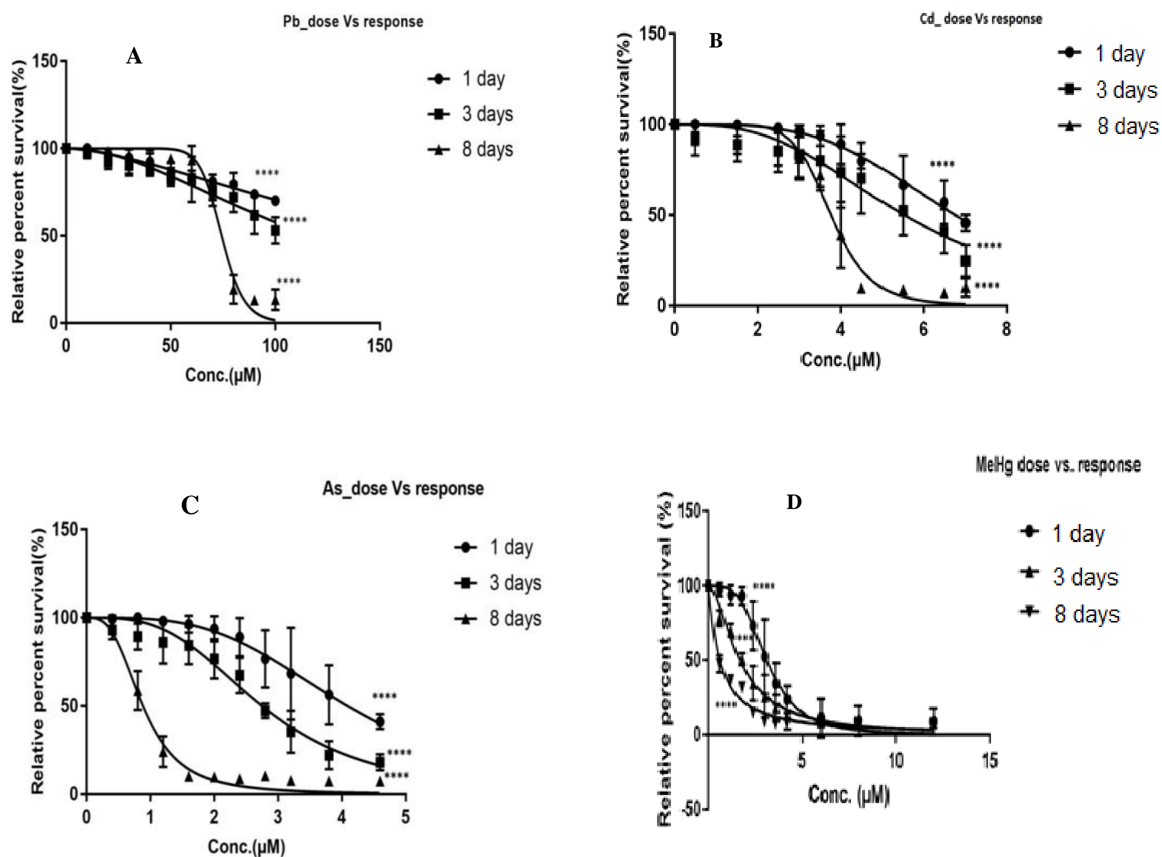

**Figure 1:** Cytotoxicity concentration response curve for Pb (A), Cd (B), As (C), and MeHg (D) after acute, subchronic, and chronic exposure. The two – way ANOVA followed by a Tukey's multiple comparison tests compared the control with all concentrations. Asterisks indicate significantly different in each exposure (p**** < 0.0001).

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

| Exposure time | Heavy metals ($IC_{50}$) | | | |
|---|---|---|---|---|
| | Pb (µM) | Cd (µM) | MeHg (µM) | As (µM) |
| Acute (1 day) | 172.2 | 6.7 | 3.1 | 4.0 |
| Subchronic (3 days) | 117.6 | 5.5 | 1.7 | 2.7 |
| Chronic (8 days) | 74.3 | 3.7 | 0.6 | 0.8 |

([a]Values of $IC_{50}$ with 95% confidence intervals from curves shown in Figure 1, Cytotoxicity was evaluated by inhibition of MTT reduction as described in the methods).

The obtained MTT assay results showed that the dose response curves for Cd, As, and MeHg are different from Pb. This difference could be due to the different uptake mechanisms of heavy metals by the HT-22 cells. Our results suggest that the sensitivity of metals is similar in all exposure scenarios, which indicate that the cell up taking concentration always depends on the type of metal. For the Pb, Cd, As metals we observed that at time dependent effect at low concentration, however at a high concentration a time independent effect. The toxicity mechanism of Pb in HT-22 cells is entirely different from Cd, As, MeHg and showing unique in dose response curve shape. In Pb, duration of exposure is a critical factor for reaching the toxic or lethal state concentration in cells. The studied four metals showed clear variation in acute and chronic exposure rather than subchronic exposure; eventually the environmental exposure of heavy metals to humans has been decisive in continuous / chronic exposure. The obtained data showed that MeHg was the most toxic. However, the high toxicity of MeHg is due to its ability to be readily absorbed into the cells and potency [52]. Taking into account the above HT-22 cell line based dose response toxicity results and the literature of in vivo molecular pieces of evidence support that the heavy metals Pb, Cd, As and MeHg have strong affinity to damage the hippocampus [24, 26, 53–55]. The obtained chronic exposure MTT results were useful to extend the further mechanistic based assays such as DNA damage and apoptosis. So that cells were treated with a range of $IC_{10}$ to $IC_{30}$ concentration (µM), this concentration was calculated from the chronic dose response curve.

### 3.2. Genotoxic effects of Pb, Cd, As, and MeHg on HT-22 cells

For knowing the clear genotoxicity relation of heavy metals on HT-22 cells, we determined whether the heavy metal toxicity is associated with DNA damage in HT-22 cells by using comet assay. Comet assay is a rapid, simple, and sensitive technique used as a quantitative assessment of toxic effects on DNA damage. In general, effectively in vitro comet assay works after short term exposure due to the fact that cells have DNA repair mechanism used to correct DNA damages and moreover they can double themselves during long time exposure [56, 57]. To evaluate the heavy metals induced genotoxic / DNA damage in HT-22 cells; cells were treated with Pb, Cd, As, and MeHg in the range of $IC_{10}$ to $IC_{30}$ for 24 hours. The degree of DNA damage was quantified by the percentage of tail intensity (%). The representative comet assay images of controls and heavy metals treated HT-22 cells are presented in Figure 2. This Figure shows a significant increase in the percentage of DNA damage with respect to negative control. There was a concentration dependent increase in percentage of DNA damage. The mean percentage of DNA damage was 0.58 ± 0.02 in the negative control cells without metal/chemical treatment. For Pb, Cd, As, and MeHg at $IC_{25}$ concentration (68.74, 3.26, 0.60, and

0.23 μM) the percentages of the DNA damage were 7.17 ± 0.05%, 6.24 ± 0.8%, 10.5 ± 1.2%, and 11.11 ± 2.5%, respectively. At $IC_{30}$ concentration (70, 3.37, 0.66, 0.29 μM) treatment, the percentages were 11.11 ± 2.5%, 12.80 ± 1.4%, 13.46 ± 2.72 %, and 20.3 ± 2.6 for Pb, Cd, As, and MeHg, respectively. Overall, the DNA damage was significantly different ($p^{***} < 0.05$) from the control as compared to the treatment groups (Figure 3). The effect of MeHg was more pronounced than in the other three metals. The results generated from the comet assay indicated; heavy metals were able to induce the DNA fragmentation to HT-22 cell in low concentration during the 24 hrs. Quantitative histograms of the comet tail intensity (%) were represented in Figure 3; these data suggest that upon increased exposure concentration to HT-22 cells, the cellular DNA became more damaged. We found that these metals are able to produce genotoxicity during 24 hours exposure in a concentration dependent manner. Heavy metals have shown a strong comparable genotoxic potential and are able to cause DNA damage in the hippocampal cells in the range of $IC_{10}$ - $IC_{30}$. Pb genotoxicity response depends on the type of cell line [58], Pb has the lowest potency to induce the DNA damage in the range of 63.5 - 70 μM on HT-22 cells. Sanders et al. also reported that Pb induced DNA damge was very low in PC-22 cells [59]. Other studies have also indicated that Pb might cause DNA damage in vivo and in vitro and that the increased levels of DNA damage observed depends duration of exposure [60]. Robbiano et al. reported significant dose dependent increases in DNA damage in primary rat and human kidney cells by Pb[61]. The possible mechanism for the occurrence of DNA damage by the Pb might be increase the rate of free radical formation leading to DNA damage [62]. The other metal Cd has similar to Pb in 2.8 - 3.26 μM range, the significant DNA damage found at 3.25 μM. In reported studies Cd showed that DNA damage was a concentration - dependent manner in human liver carcinoma cells [63]. Other reports have shown evidence that reactive oxygen species (ROS) are involved in DNA damage induced by Cd [64]. As induced DNA damage in HT- 22 cells results showed significant increase in DNA damage which was consistent with previous studies [65]. Kumar et al. reported As induces DNA fragmentation in HL-60 cells in a dose dependent manner, similarly we propose that HT-22 cells exposed to As undergo DNA damage [66]. There is evidence that low - dose exposure (0.5 μM) to MeHg may lead to DNA damage in fibroblasts and Chinese hamster ovary cells [67]. Several studies have also observed mercury inhibiting the DNA repair system [68]. Grotto et al. demonstrated the genotoxic effects of MeHg in rats during chronic exposure [69]. We found that MeHg has high potency in HT-22; the dose dependent response was linear in range of 0.07-0.26 μM. Our results demonstrated that heavy metal induces DNA damage to HT-22 cells in a dose dependent fashion with respect to their potency, suggestive clear evidence that MeHg may be a potent DNA damaging agent against hippocampal cell line when used at even low dose. We are expecting several possible mechanisms might be involved in the induction of the DNA damages other than apoptosis due to the initiation of cell DNA damage during the 24 hrs. instead of 8 days [70]. Hartwig reported that reactive oxygen species (ROS) generation induced by heavy metals could be reason to genotoxic mechanisms in mammalian cells [71].

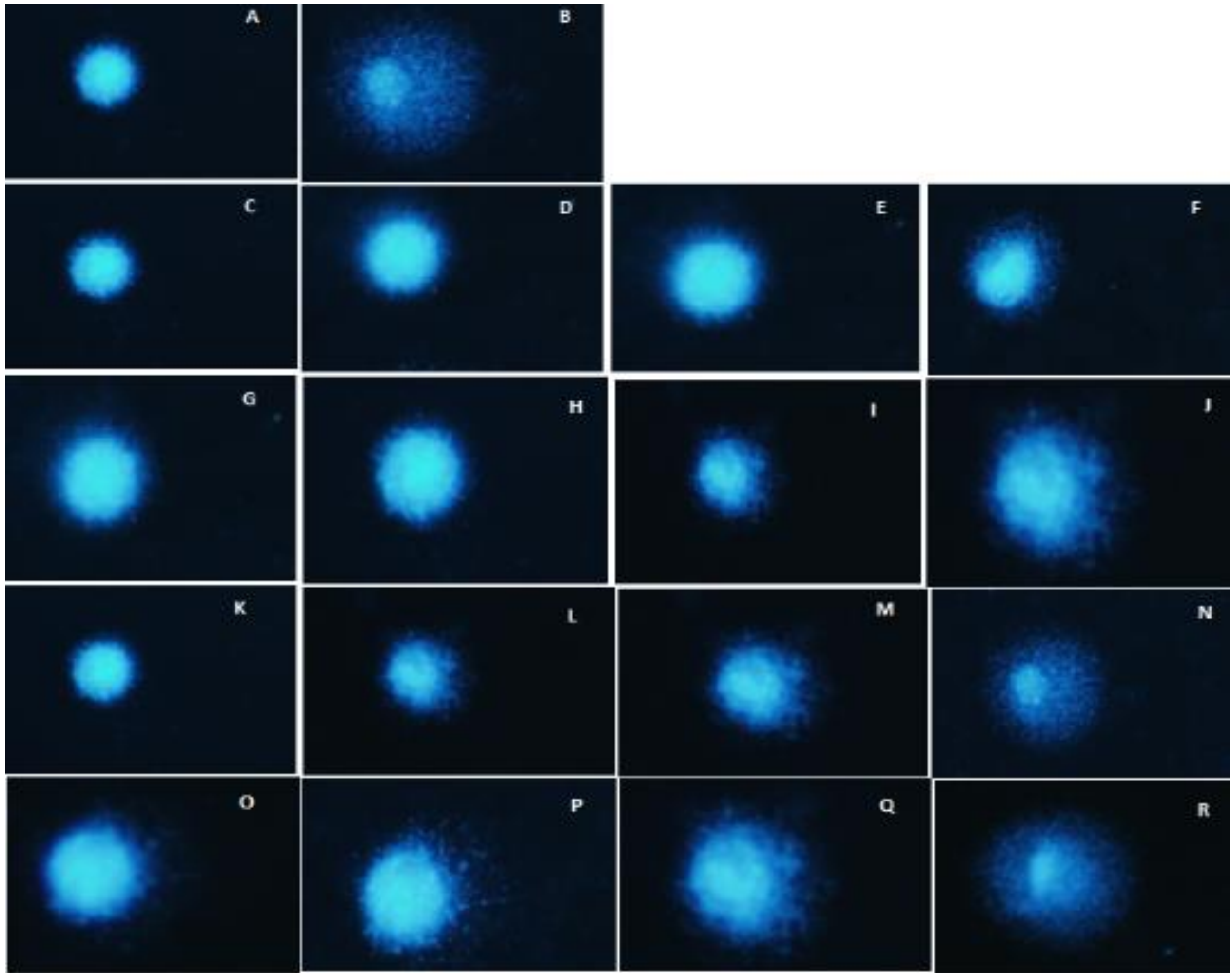


Figure 2: Representative Comet assay images of HT-22 cells. Untreated control (A), Positive control (MMS; B); Treated with: Pb (C to F) at 63.50, 67.34, 68.74, 70.00 µM; Cd (G to J) 2.80, 3.14, 3.26, 3.37 µM; As (K to N) at 0.42, 0.50, 0.60, 0.66 µM; MeHg (O to R) at 0.09, 0.18, 0.23, 0.29 µM.

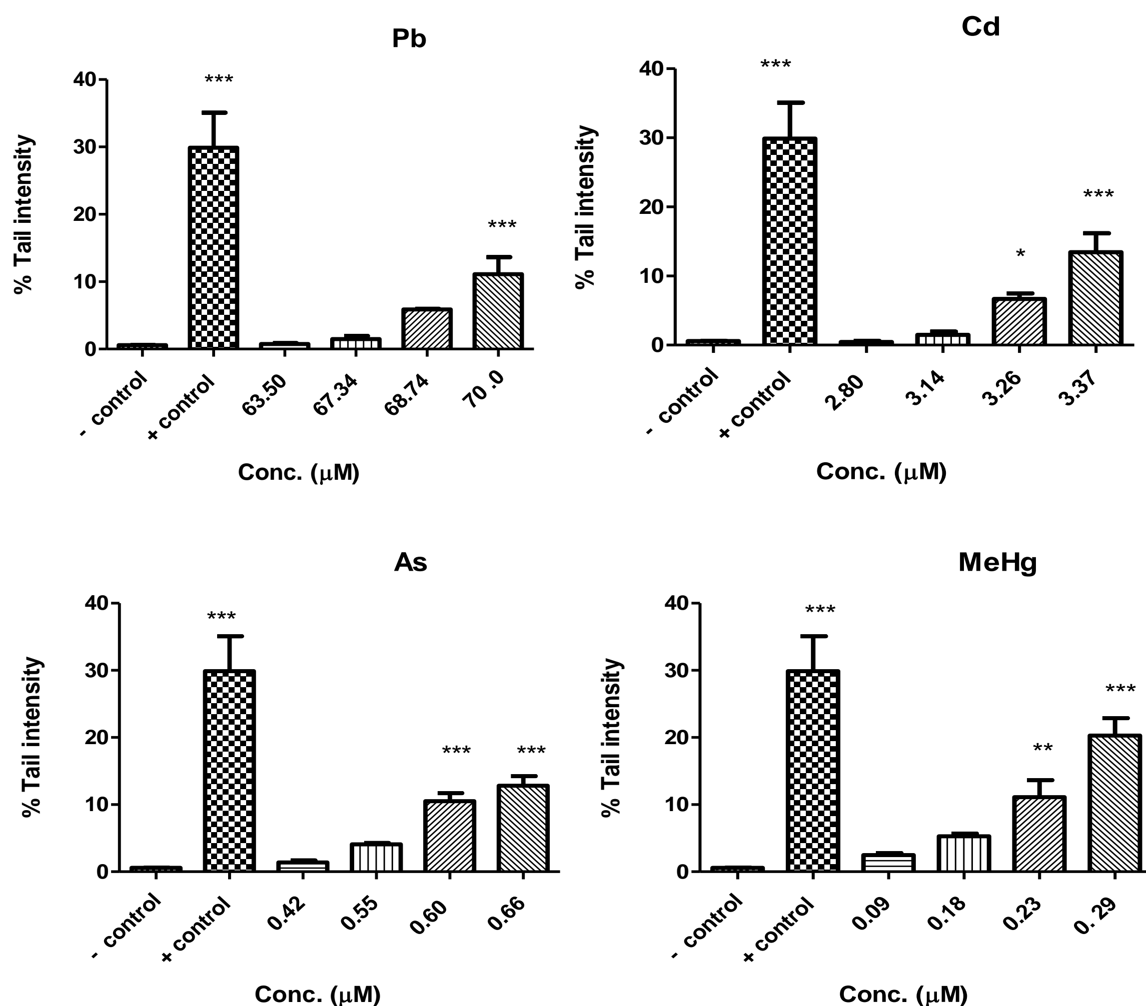


Figure 3: Effect of Pb, Cd, As, and MeHg on DNA in HT-22 cells as assessed with comet assay. Each value represents the tail intensity (% DNA damage), Mean ± SD of three independent experiments. Significantly different from the negative control ($p^{***} < 0.05$) according to the Dunnett's test.

### 3.3. Apoptosis effects of Pb, Cd, As, and MeHg on HT-22 cells:

We carried out Pb, Cd, As, and MeHg; induced apoptosis in HT- 22 cells during chronic exposure. To accomplish this objective, we performed Annexin- V/ Propidium iodide (PI) staining that allows the discrimination of viable cell, apoptotic, and necrotic cells by binding with $Ca^{+2}$ dependent phosphatidyl serine (PS) protein in cell membrane [72]. Apoptosis assay is helpful to gain insight into the heavy metal induced cell death mechanism in HT-22 cells. We found that heavy metals have different potency to induce apoptosis in HT-22 cells. Firstly Pb results in Figure 4.1 indicates, there was a slight trend to increased apoptotic cells (%) as concentration increases, but it was not statistically significant. However, at 70 µM response was relatively significant ($p^{***} < 0.05$). The present study confirms the Pb could induce the apoptosis in HT-22 cells, however the apoptosis response was very low in tested 63.5-70 µM range; it confirms that Pb shown low potency and need more concentration or exposure time for raising the response. Pb is 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 [73]. Figure 4.2 shows that the Cd apoptosis effect in HT-22 cells, the apoptosis response was similar to Pb metal; the percent of apoptotic cells was a significant at 3.26, 3.37 µM ($p^{***} < 0.05$). Cd showed concentration dependent manner in HT-22 cells, effect of Cd was

more pronounced at 3.37 μM. Hart et al. (1999) reported Cd induced apoptosis in rat lung epithelial cells is a time and dose dependent manner. Other reports have shown that Cd induces dose dependent apoptosis in cortical neurons [46]. Cd has also been evidenced in U-937 cells from human lymphoma [74], in rat kidney epithelial cells [75], and in rat fibroblasts [76] as a dose dependent manner apoptosis. Several studies demonstrated that Cd induced apoptosis might be associated with a ROS production [77]. The other two metals As and MeHg have the significant effect even at a low concentration of exposure. As showed more percent of apoptotic cells at 0.60, 0.66 μM ($p^{***} < 0.05$); which indicates that the potency of As was high in HT-22 cells; these findings are relevant with As induced apoptosis in neuroblastoma cells [78]. The sensitivity of As in different cell lines might be due to some underlying molecular mechanism [79]. Miller et al. reported that As induces apoptosis in cell lines due to the generation of ROS [80], which enhances DNA damage [81] and subsequently the brain cell death [82] . 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); similar response was observed in human SH-SY 5Y neuroblastoma cells [83]. The reported MeHg studies in rat cerebellar granule cells (CGC) indicates apoptosis is cell line specific effect [84]. In cerebellar neurons treated with MeHg up to 0.3 μM showed morphological changes characteristic to apoptosis depending the concentration [85]. At $IC_{30}$ (70, 3.37, 0.66, and 0.29 μM), the observed maximum percentage of apoptosis cells 15.4 ± 5.6, 31.8 ± 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 mechanism is potency dependent as similar to cell viability and genotoxicity. 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. 4.1 - 4.4 (dot plot). Quantitative histograms of total apoptotic cells (%) are shown in the Figure 5, these data suggest that the apoptotic cells percent was increased with respect to concentration. Among the four metals, the Pb showed very low toxicity, and MeHg showed high toxicity potency.

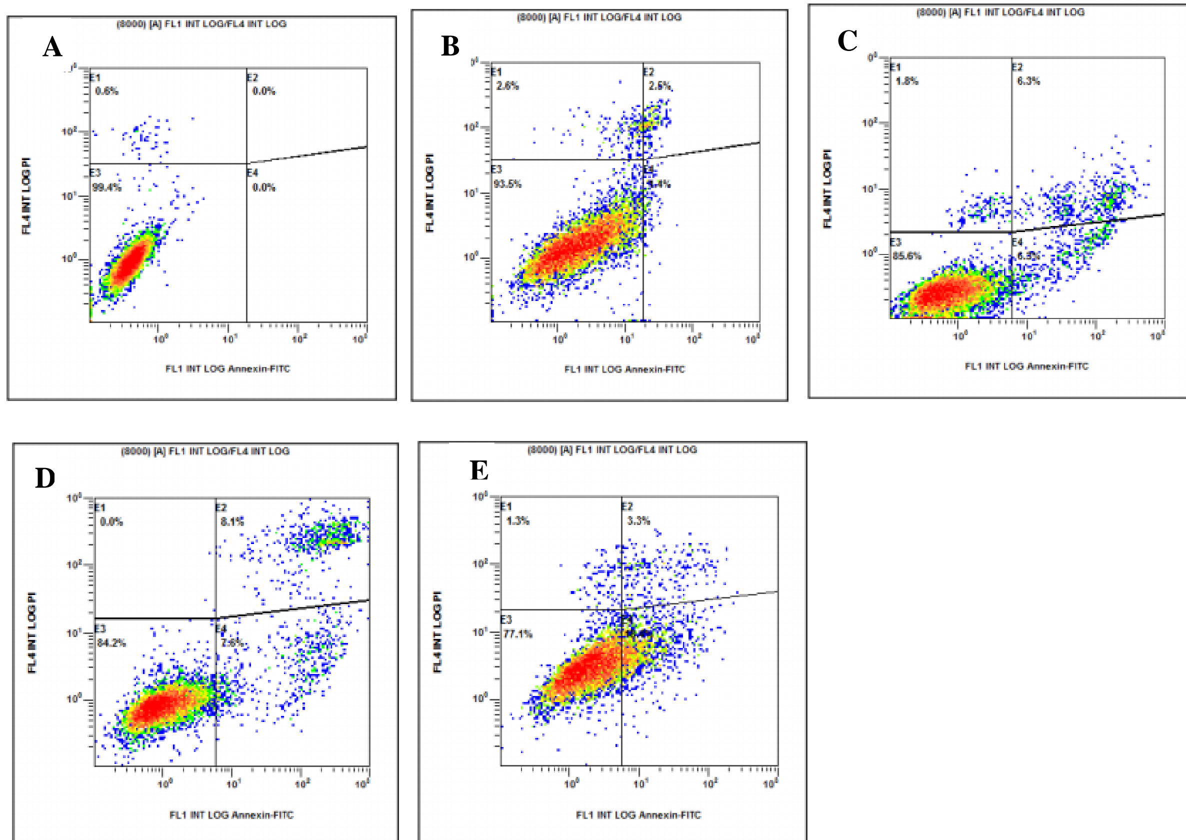

Figure 4.1: Representative dot plots showing the inhibitory effect of Pb to HT-22 cells upon chronic (8 days) of exposure. A= Control, B= 63.50 µM, C= 67.34 µM D = 68.74 µM, E= 70.0 µM. Lower left (LL)/ E3 = Live cells (Annexin V− / PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+ / PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

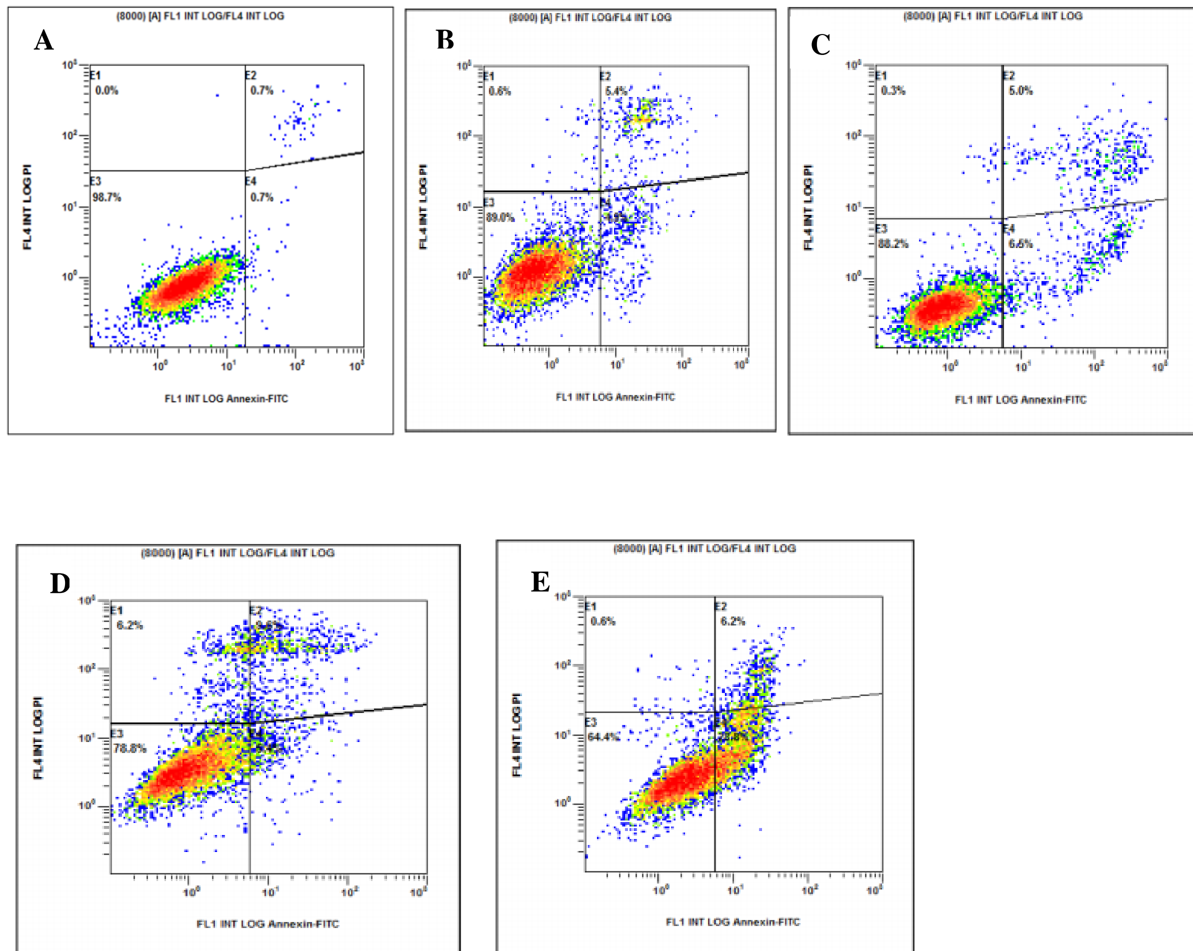


Figure 4.2: Representative dot plots showing the inhibitory effect of Cd to HT-22 cells upon 8 days of exposure. A= Control, B= 2.80 µM, C= 3.14 µM, D = 3.26 µM, E= 3.37 µM. Lower left (LL)/ E3 = Live cells (Annexin V− / PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+ / PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

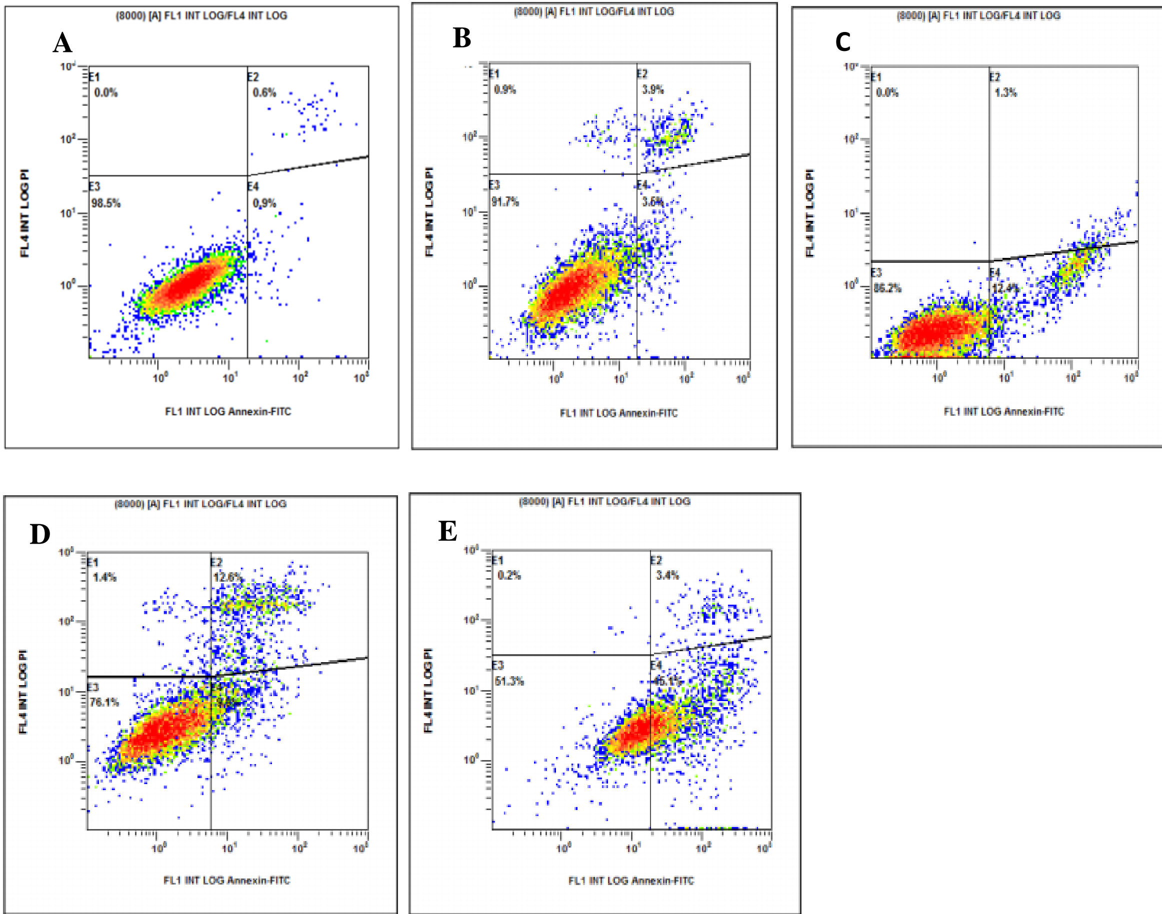


Figure 4.3: Representative dot plots showing the inhibitory effect of As to HT-22 cells upon 8 days of exposure. A= Control, B= 0.42 µM, C= 0.55 µM, D = 0.60 µM, E= 0.66 µM. Lower left (LL) = Live cells (Annexin V−/PI−), Lower right (LR) = Early apoptotic cells (Annexin V + / PI−), Upper right (UR) = Late apoptotic (Annexin V+ / PI+), Upper left (UL) = Necrotic cells (PI+).

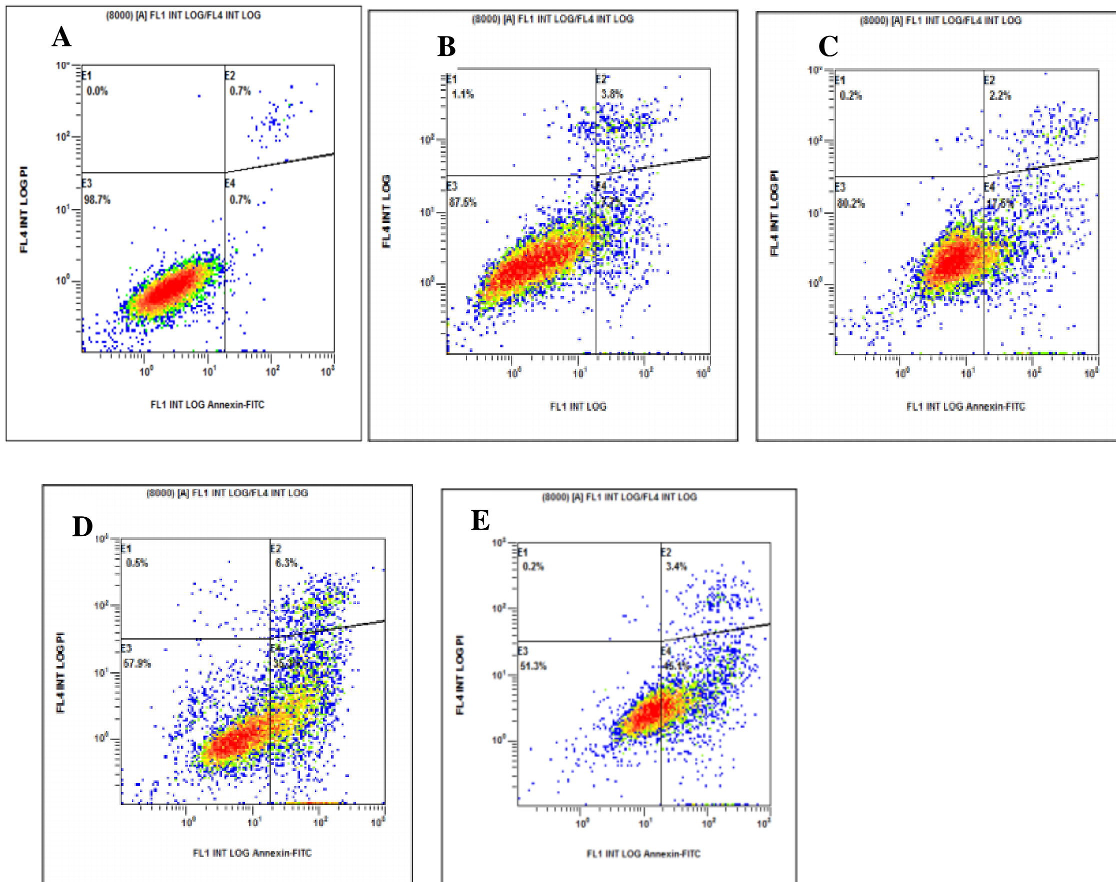


Figure 4.4: Representative dot plots showing the inhibitory effect of MeHg to HT-22 cells upon 8 days of exposure. A= Control, B= 0.09 µM, C= 0.18 µM, D = 0.23 µM, E= 0.29 µM. Lower left (LL)/ E3 = Live cells (Annexin V−/PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

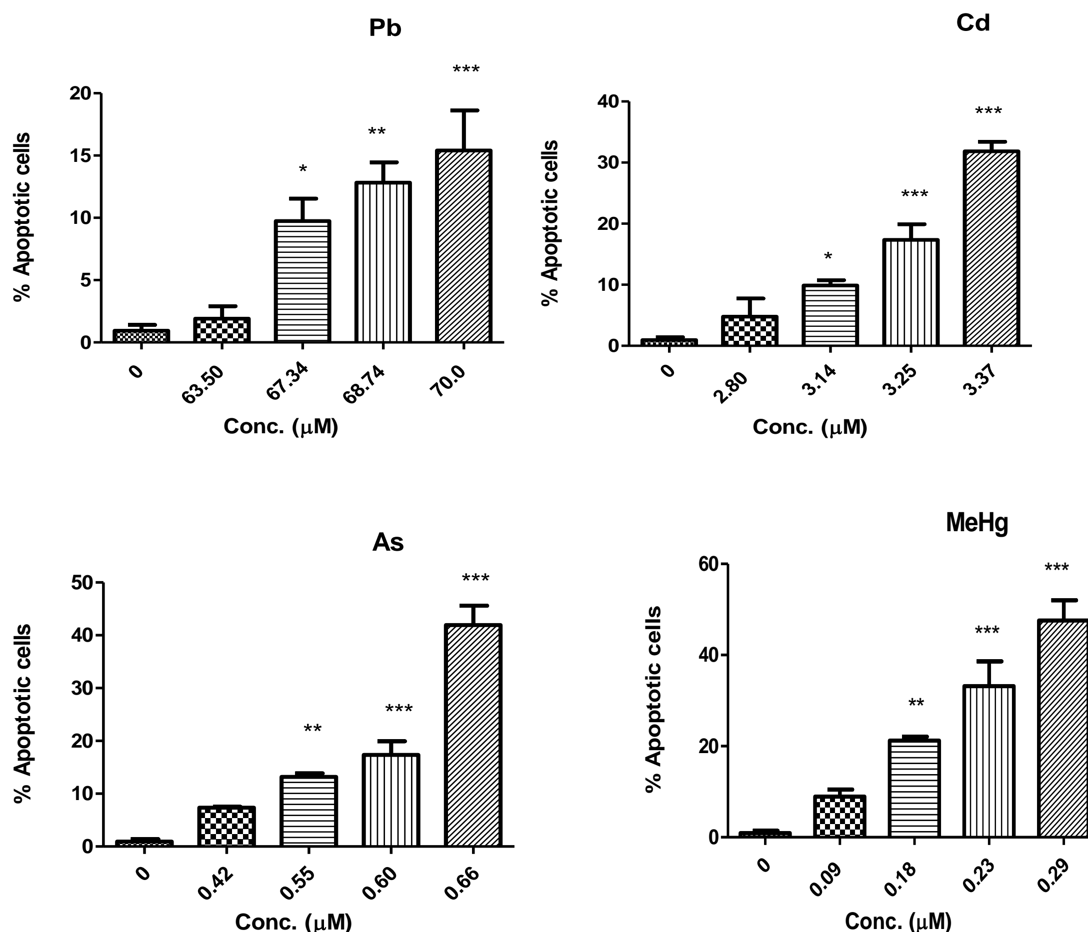


Figure 5: Quantification by flow cytometry of Pb, Cd, As, and MeHg induced apoptosis cells (%). Each point represents a mean ± SD of 3 experiments. Results are expressed as percentages of apoptosis with regard to the total cells ***significantly different ($p^{***} < 0.05$) from the control, according to the Dunnett's test.

## 4. Conclusion

In recent years, environmental exposures to hazardous metals, such as Pb, Cd, As, and MeHg have been significant toxicological concerns in the hippocampus. We assessed the cytotoxicity of four metals in hippocampal based HT-22 cells during 1 day, 3 days, 8 days; considered as acute, subchronic, chronic exposure. For more elaborating basic toxic mechanism, chronic cytotoxicity studies were further extended to a DNA damage and apoptosis in the range of low concentration ($IC_{10}$ – $IC_{30}$). Cytotoxicity, genotoxicity, and apoptosis findings are almost identical in potency. Moreover, current study demonstrated that Pb has weak effect and MeHg has high potency in HT-22 cells. The obtained apoptotic assay results indicates heavy metal potency MeHg > As > Cd > Pb as similar to MTT assay and genotoxicity. The current study demonstrated that heavy metals differential toxicity on HT-22 cells. The parameterization of the dose response curve supporting the heavy metals toxicity was comparable among all exposure conditions. The limitation of the current study is not exploring the specific mechanism on HT-22 cell during chronic exposure. The findings from apoptosis and genotoxicity results are helpful for extending further to oxidative stress based studies to know the underlying molecular mechanism related to neurodengerative diseases. In future, the common cytotoxicity of Pb, Cd, As, and MeHg on HT-22 cell line could be useful for designing the metal mixtures (binary, ternary) interaction studies and omics based studies.

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