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Lead and cadmium induced cytotoxic and genotoxic effects on HL-60 and Jurkat cell lines

Esmeralda Dautović[1], Maida Šljivić Husejnović[1], Martina Bergant[2], Damir Sabitović[3], Nahida Srabović[1], Aida Smajlović[1], Lejla Begić[1], Adaleta Softić[1*]

[1]*University of Tuzla, Faculty of Pharmacy, Tuzla, Bosnia and Herzegovina*
[2]*University of Nova Gorica, Laboratory for Environmental and Life Sciences, Nova Gorica, Slovenia*
[3]*University Clinical Center Tuzla, Tuzla, Bosnia and Herzegovina*

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Abstract

Humans are exposed to a mixture of toxic heavy metals in the environment. Because of the lack of information on the toxicity of their mixtures, in this study, two common heavy metals, lead and cadmium, were introduced individually and as mixtures in HL-60 and JURKAT cell lines for 24 hours. Our experimental results have shown that these two heavy metals induced cytotoxic and genotoxic effects in both cell lines. Also, cadmium exhibited a higher cytotoxic and genotoxic potential than lead. The cytotoxicity data of single metals were used to determine the mixtures interaction profile by using the effect additivity method. Metal mixtures showed synergistic effect in HL-60 cells and antagonistic effect in JURKAT cells, compared to individual metals. The combined effects should be considered in the risk assessment of heavy metal co-exposure and potency.

***Correspondence**
E-mail: adaleta.mulaomerovic@untz.ba

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Introduction

Heavy metals are released by natural events and human activities into the environment. They are used in multiple technological, domestic, medical and agricultural applications, which led to their wide distribution, raising concerns over their potential effects on human health. In developed countries, human exposure to toxic metals has lately been decreasing, but in other parts of the world, it is increasing (Jarup, 2003). Heavy metals are systemic toxicants that induce multiple organ damage, even at lower levels of exposure (Duffus, 2002). In general, lead (Pb), cadmium (Cd), methylmercury (MeHg) and arsenic (As) are some of the most toxic metals that humans are exposed to. They target essential organs like brain, liver and kidney, causing neurotoxicity, hepatotoxicity and nephrotoxicity

(WHO, 2007). These four elements rank among the priority metals that World Health Organization has marked as great public health concerns (WHO, 2010). Lead has unique physical and chemical properties that make it suitable for great variety of applications. Because of its numerous industrial applications, lead is a common environmental and occupational contaminant widely distributed around the world. Even though the toxic effects of lead and its compounds have been extensively investigated in a variety of systems the existent data regarding its mutagenic and carcinogenic properties are contradictory. Lead has been classified as possible human carcinogen (group 2B) (IARC, 1987) and its inorganic compounds as probable human carcinogens (group 2A) (IARC, 2006). According to Agency for Toxic Substances and Disease Registry (ATSDR, 1999), one of the major mechanisms of lead toxicity is through biochemical processes that include lead's ability to inhibit or mimic the actions of calcium and to interact with proteins. Acute and chronic exposure to lead may influence immune response, which may lead to an increase in the incidence of allergies, infectious diseases, autoimmune processes or cancer (Dietert et al., 2004; Hsiao et al., 2011). Numerous experimental studies have shown that lead can influence the levels of immunoglobulins, numbers of lymphocytes, peripheral blood mononuclear cells and macrophages (Başaran & Undeğer, 2000; Mishra et al., 2003). Also, it may cause impaired responses to mitogens and depression of neutrophil functions (Undeğer & Başaran, 1998). Due to its prolonged biological half-life, low rate of excretion and high accumulation capacity in soft tissues, cadmium is one of the most toxic heavy metals. It is a widespread toxicant because environmental levels have steadily risen with an increase in the production of cadmium for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings, and alloys (Lalor, 2008; Daud et al., 2013). Humans are exposed to cadmium through occupational (industries) and non-occupational activities (cigarette smoking and consumption of contaminated foods and water; Lalor, 2008; Daud et al., 2009; Daud et al., 2013). The toxic effects of cadmium have been extensively studied in in vivo and *in vitro* systems. It has been classified as a group I carcinogen (IARC, 1993) and as a probable human carcinogen (group B1) by Environmental Protection Agency (EPA; Merrill et al., 2001). Cadmium affects metabolic processes including membrane transport, energy metabolism and protein synthesis. It may also act on DNA, directly or indirectly, by interfering with gene control and repair mechanisms (Beyersmann & Hechtenberg, 1997; Beyersmann & Hartwig, 2008). Numerous studies have shown that cadmium damages mammalian organs including the kidneys, testes, lungs, and the hematopoietic, cardiovascular, and nervous systems (Siu et al., 2009; Sabolić et al., 2010). It can induce apoptosis via the mitochondrial pathway in cell lines (Long et al., 2008; Szuster-Ciesielska et al., 2000; Jiang et al., 2014). Cadmium causes apoptosis in cell culture systems at low and moderate concentrations (e.g., 0.1-10 μmol/L). At higher concentrations (>50 μmol/L), necrosis becomes evident (Templeton & Liu, 2010). 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 (Karri et al., 2018). Although many studies offer evidence that lead and cadmium are multi-target toxicants, little is known about the effect of these two metals in mixture, especially of their joint effect on the immune cells (Yedjou et al., 2003; García-Lestón et al., 2010; Bernhoft, 2013; Rukhsanda et al., 2014) . Hence, in the present study human leukemia HL-60 cells and Jurkat T cells were used as models to explore the single and combined cytotoxic effect of lead and cadmium on immune cells. Also, the genotoxic potential of each individual metal was analyzed.

Materials and methods

Chemicals, reagents and antibodies

All analyses that include heavy metals were performed using analytical grade chemicals, reagents and standards. For all solution preparations and dilutions only double-distilled deionized water was used. Primary antibody anti-phospho-histone H2A.X was obtained from Merck Millipore. Secondary antibody conjugated with Alexa Fluor 488 and PrestoBlue™ cell viability reagent was obtained from Thermo Fisher Scientific.

In vitro culture of the cell lines

Promyeloblastic HL-60 cell line was cultured in RPMI 1640 medium (Sigma) supplemented with 20% heat inactivated (HI) FBS (Sigma) and 1% penicillin/streptomicin antibiotics (Sigma), in humidified atmosphere containing 5% CO_2 at 37°C. Jurkat human T lymphocyte cell line was cultured in RPMI 1640 medium (Sigma) containing 10% FBS (Sigma) and 1% penicillin/streptomycin antibiotics (Sigma). For each experiment cells were grown to 80% confluence in 75cm2 culture flasks.

Analysis of cell viability/cytotoxicity by Presto blue

For each experiment HL-60 and Jurkat cells were seeded (3 X 104 cells/well) in 96 well plates and grown for additional 24 h after exposure to a metal. Triplicate wells were treated with different levels of Pb (10-100 μM), Cd (2.5-10 μM) or their mixtures. Pb and Cd standards were prepared with reagent-grade chemicals. Untreated cells were used as negative control while cells treated with 30% dimethyl sulfoxide (DMSO) in RPMI nutrient medium provided positive control. The cells were cultivated in RPMI culture medium supplemented with 1/10 volume of Pb or Cd metal solutions to achieve final concentrations of Pb (10-100 μM) or Cd (2.5-10 μM) in culture medium. Pb and Cd stock solutions were prepared in double-distilled deionized water and solutions were sterilized by filtration through 0.2 μm sterile syringe filters. The Presto blue assay was carried out according to the manufacturer's instructions (Thermo Fisher Scientific). After 24 h treatment with RPMI culture medium supplemented with 1/10 volume of Pb or Cd metal solutions, cells were incubated with Presto blue reagent. The absorbance was measured after 2 h of incubation at 570 nm wavelength using microplate reader. The measured absorbance values were converted to cell viability percentage with negative control as a reference.

Assessment of interaction using the effect method "effect additivity"

The concentrations of Pb and Cd for the analysis of mixture interaction were selected from their individual cytotoxic effects. The effect additivity method, described by Lau et al. (2006) was utilized, in which concentration of each compound equivalent to its IC20 (concentration that induced 20% cytotoxic effect) was calculated from single metal response curve. In order to assess the effect of metals mixture, the cells were treated with either a single metal at a concentration of IC20 or with a mixture calculated to produce the same effect of 20% inhibition if their effects were simply additive (i.e., zero interaction). The cells were exposed to pairs of the metals in the following proportions: 100% Pb: 0% Cd, 75% Pb: 25% Cd, 50% Pb: 50% Cd, 25% Pb: 75% Cd, and 0% Pb: 100% Cd. Each experiment was performed in independent triplicates. In this model of synergy (Axelrad et al., 2002), the combined effect of two agents is considered to be equal to the sum of the effects of single compound; thus the cytotoxic effect, produced by any mixture, can be predicted. Deviations from this expected constant inhibition (zero interaction) produced by the calculated IC20 are either synergistic (above expected zero effect), or antagonistic (below expected zero effect). The statistical analysis was performed according to Student's two-tailed t-test; p values below 0.05 were considered to be significant.

Genotoxicity analysis

Indirect immunofluorescence method with primary anti-phospho hystone H2A.X antibody was used to determine genotoxicity of Pb and Cd in cell lines. For the assessment of genotoxicity, HL-60 and Jurkat cells were seeded in 24 well plates and treated for 24 h with different levels of Pb or Cd water standards. Cells were treated with RPMI culture medium supplemented with 1/10 volume of Pb or Cd metal solutions Cells were treated with 10% metal solutions and 90% RPMI culture medium. Untreated cells were used as negative control. After 24 h of metal treatment, HL-60 and Jurkat cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). The cells were permeabilized by 0.5% Triton X-100 in PBS, pH 7.4 for 5 minutes at RT and nonspecific staining was blocked with 5% BSA (Sigma) in PBS, pH 7.4 for 30 minutes. The cells were labeled with primary mouse monoclonal anti γH2A.X antibody (Merck Millipore) in 0.5% BSA in PBS for 1 hour at 37°C. The cells were then washed and incubated with secondary goat anti-

mouse antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific) for 1 hour at RT. After final wash, the cells were mounted on slides with ProLongTM Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). The images were taken using fluorescence microscope Olympus IX81 with Prior ProScan11 (Olympus Q Color 5 imaging system, Slide Book 5.0 software) at a 400x magnification. The genotoxic effects were evaluated by the chi square test; p values below 0.05 were considered to be significant.

Results and Discussion

To characterize the individual effects of Pb and Cd on HL-60 and Jurkat cells, Presto blue viability assay was performed. As expected, these metals caused cytotoxic effects in both cell lines (Figure 1).

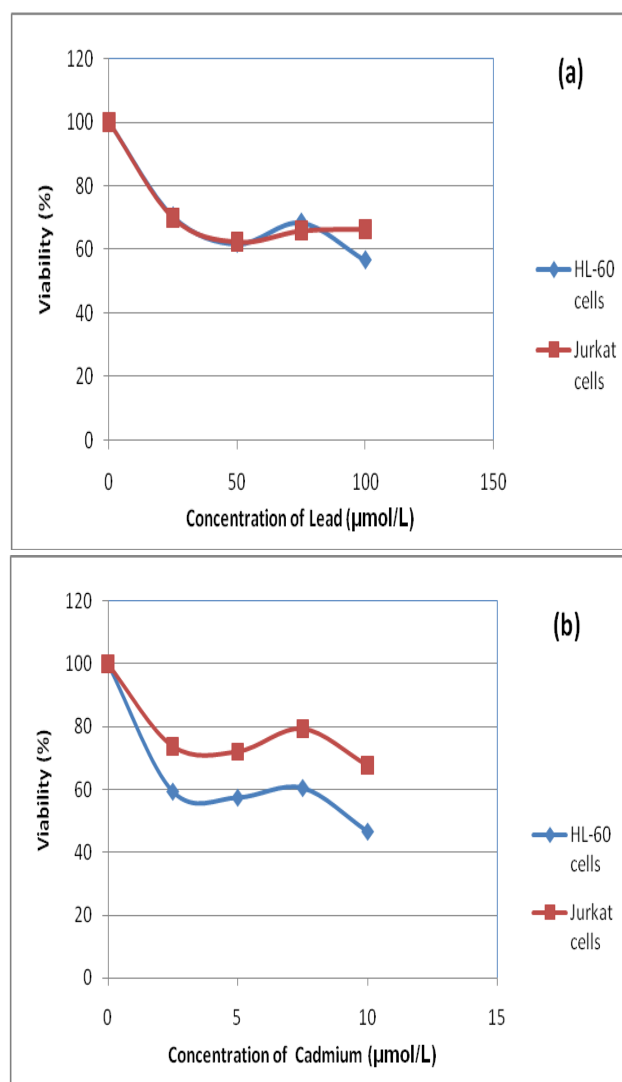

Figure 1. Viability of HL-60 and Jurkat cells after 24 hours exposure to increasing concentrations of Pb (a) and Cd (b)

Our results are in accordance with the results of Yedjou et al. (2003), who found that lead induced cytotoxicity in HL-60 cells, and with the results of Nemmiche et al. (2011) who found that cadmium significantly decreases viability of Jurkat cells. Cadmium exhibited higher cytotoxic potential, which means that the same percent of cytotoxicity was observed at significantly lower concentration of cadmium compared to lead. It was interesting that in both cell lines the most pronounced cytotoxic effect was observed at the lowest concentrations of metals (2.5 µmol/L for cadmium and 25 µmol/L for lead). Further increase in metal concentration did not lead to proportional decline in cell viability, in both lines, respectively. The studies on metal mixtures toxicity have revealed that the effects of mixtures are hard to predict as all possible outcomes have been observed (Norwood et al., 2003; Vijver et al., 2011). These interactions can be conflicting across various experiments (Norwood et al., 2003), or can be concentration dependent (Sharma et al., 1999; Liu et al., 2015). The concentrations of Pb and Cd for the analysis of their interaction were selected from their individual cytotoxic effects. In the absence of interactions, two compounds in combinations at fixed concentrations have the same effect as the individual compounds at those same concentrations; this was taken as the "theoretical expected values"." Significant synergistic effect was observed in HL-60 cells for Pb and Cd in the 50:50 mixture; the cytotoxic effect (28.05%) of mixture was significantly different ($p=0.0471$) from the expected value (Figure 2a). On the contrary, in Jurkat cells, the antagonistic effect was observed, the 50:50 mixture did not cause cytotoxicity ($P<0.0001$) (Figure 2b). These results could be explained by the fact that two examined cell lines originate from different progenitor cells and thus have different patterns of protein expression, some of which could be in interaction with heavy metals and ultimately result in counter-effects. Damage of genetic material after exposure to Pb and Cd was analyzed with indirect immunofluorescence. For the assessment of genotoxicity of lead, both cell lines were exposed for 24 hours to increasing concentrations of lead nitrate water standards, ranging from 25 µmol/L to 4000 µmol/L. For the assessment of genotoxicity of cadmium, both cell lines were exposed to increasing

concentrations of cadmium nitrate water standards, ranging from 10 µmol/L to 160 µmol/L.

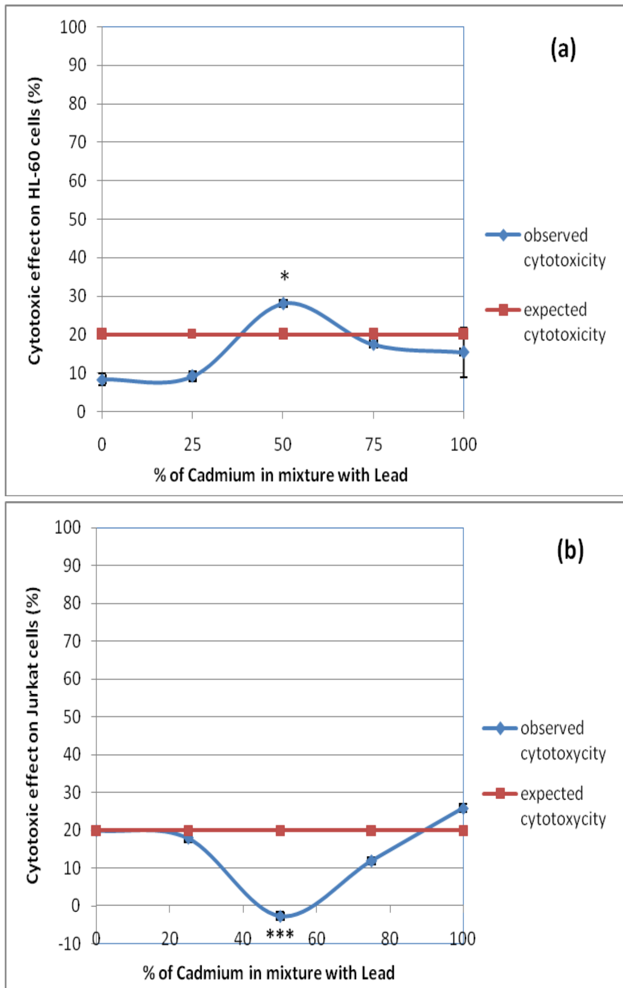

Figure 2. The relationship between cytotoxic effect and the percentage of Pb and Cd in a mixture in HL-60 cells (a) and Jurkat cells (b). Error bars represent standard error of mean (n=3). The 50:50 mixture of Pb and Cd produced significantly higher cytotoxic effect than expected in HL-60 cells and no cytotoxic effect in Jurkat cells, *p<0.05 and ***p<0.0001.

After the exposure, the cells were labeled with anti-phospho- histone H2A.X antibody which binds to the sites of DNA double strand breaks and DNA damage was observed by fluorescence microscopy. Based on the intensity of green fluorescence, positive cells with fractures in the DNA strands (strong intensity) and negative cells (low intensity, auto fluorescence) were distinguished. For each sample, approximately 200 cells were analyzed and the percentage of positive cells (those with damaged genetic material) was calculated.

Representative images of genotoxic effects are shown in Figure 3. In general, lead exhibited fewer genotoxic effects relative to cadmium. Significant genotoxic activity of lead in HL-60 cells was observed at a concentration of 4000 µmol/L (7.11% of cells were labeled) compared to untreated cells (3.45% labeled cells due to various stress factors, cell division), calculated by chi square test ($\chi2=8.48$, $p<0.05$).

Genotoxic activity of lead in Jurkat cells was observed at a concentration of 1000 µmol/L (9.69% of cells were labeled). When compared to untreated cells (2.82% labeled cells), this percent of positive cells was significantly higher ($\chi2=6.65$, $p<0.05$). Our results are consistent with the results of other numerous studies that have demonstrated the genotoxic potential of lead in different cell cultures (Garcia-Leston et al., 2010).

However, the results obtained using different detection methods, different cell lines as experimental models and different lead salts, were diverse with respect to the concentration of lead that causes genotoxic effects.

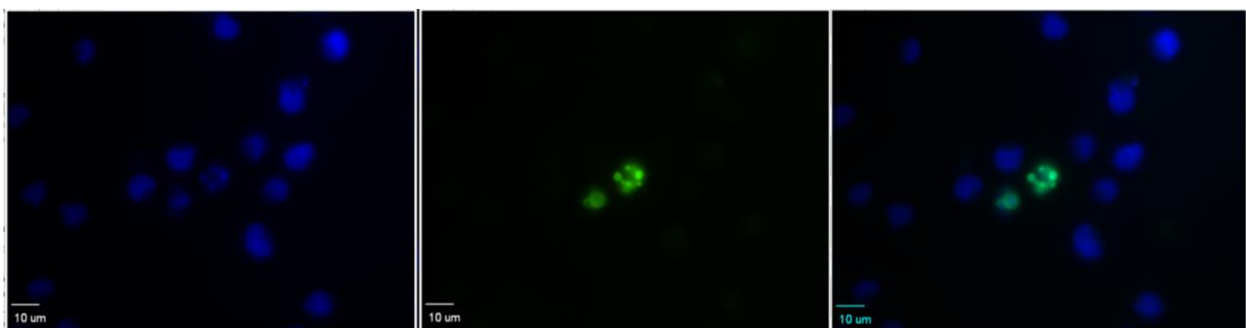

Figure 3. Genotoxic effects of 10µmol/L Cd on HL-60 cells after 24 hours exposure. Cells stained with DAPI are visible in violet-blue color and show the total number of all cells in the vision field of the microscope (left). Positive cells or cells with DNA damage that are stained with anti-phospho-histone H2A.X antibody and secondary antibody conjugated with Alexa Fluor 488 are visible in green (center). Merge (right) obtained by a ImageJ software. Each dot in the cell nucleus that emits a strong green fluorescence represents DNA strand break.

After extensive research of available publications, we concluded that the genotoxic activity of lead has not been investigated earlier by detecting the double strand breaks with anti-phospho-histone H2A.X antibody. On the other hand, cadmium exerted greater genotoxic activity, which showed dose dependence in both cell lines (Figure 4).

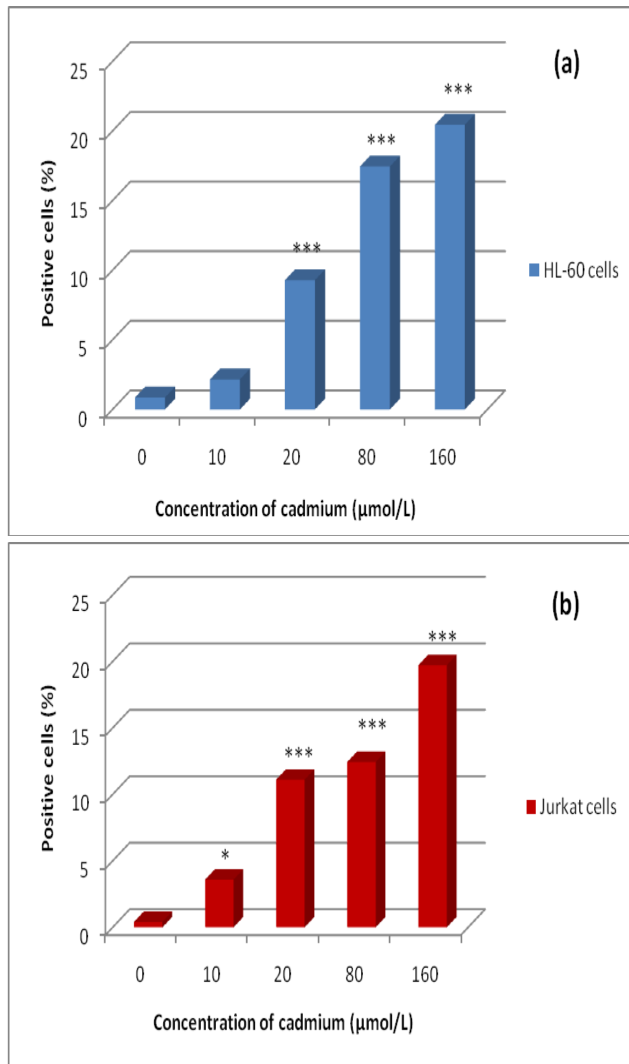

Figure 4. Genotoxic effects of cadmium on HL-60 cells (a) and Jurkat cells (b). *p<0.05 and ***p<0.0001.

The number of stained (positive) cells and unstained (negative) cells (in both cell lines) after the exposure to cadmium concentration gradient are presented in Table 1. Statistical significance between the frequencies of stained cells after exposure to certain Cd concentration and negative control (untreated cells) was tested by a Chi-square test whose values are also presented in the Table.

Although the toxic effects of lead and cadmium compounds have been extensively studied, inconsistent results have been obtained about their mutagenic, clastogenic and carcinogenic properties. Valverde et al., (2001) suggested that these metals cause genotoxicity and carcinogenicity not by direct interaction with DNA, but by indirect interactions, such as oxidative stress. The authors found an induction of lipid peroxidation and an increase in free radical levels in the different organs of mice after inhalation of lead acetate or cadmium chloride, suggesting the induction of genotoxicity and carcinogenicity by indirect interactions with DNA.

Conclusions

Heavy metals are ubiquitous and occur as heterogeneous mixtures in the environment. Our results indicate that mixtures of Pb and Cd cause synergistic cytotoxic effects in HL-60 cells. In general, the combined effects should be considered in the risk assessment of heavy metal co-exposure and potency. In order to reduce the environmental and public health effects of heavy metal pollution, governments and health agencies need to pay additional attention to the environment and anthropogenic activities.

Table 1. Cadmium induced genotoxic effects

	Number of HL-60 cells			Number of Jurkat cells		
Cd (µM)	positive	negative	χ^2	positive	negative	χ^2
0	4	242		1	245	
10	5	226	0.66	8	215	6.29*
20	21	195	14.73***	23	184	25.67***
80	41	194	35.47***	28	197	29.47***
160	45	175	43.76***	43	175	50.25***

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Conflict of interest

Authors declare that they have no conflict of interests.

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