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Full Length Article

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.

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 et al., 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 Rios, 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

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; NMDAN, -methyl-D-aspartate; OD, optical density; Pb, lead; PI, propidium iodide; SD, standard deviation; WHO, World Health Organization

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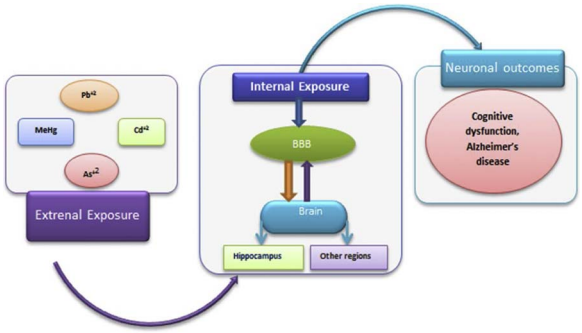

Fig. 1. Conceptual diagram of metal mixtures exposure- toxicology- disease outcome scenario (hippocampus) (Karri et al., 2016).

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; Karri et al., 2016; 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 (Pape-Lindstrom 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 (Tichy 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).

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 °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 0.57×10^6 cells/mL and grown for 24 h 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 100× 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 × 100%. The cytotoxicity results were used for calculating the