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# Effects of chelators on mercury, iron, and lead neurotoxicity in cortical culture

Travis Rush, Julie Hjelmhaug, Doug Lobner *

*Department of Biomedical Sciences, Marquette University, 561 N. 15th Street, Rm 464, Milwaukee, WI 53233, USA*

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ABSTRACT

Chelation therapy for the treatment of acute, high dose exposure to heavy metals is accepted medical practice. However, a much wider use of metal chelators is by alternative health practitioners for so called "chelation therapy". Given this widespread and largely unregulated use of metal chelators it is important to understand the actions of these compounds. We tested the effects of four commonly used metal chelators, calcium disodium ethylenediaminetetraacetate ($CaNa_2EDTA$), D-penicillamine (DPA), 2,3 dimercaptopropane-1-sulfonate (DMPS), and dimercaptosuccinic acid (DMSA) for their effects on heavy metal neurotoxicity in primary cortical cultures. We studied the toxicity of three forms of mercury, inorganic mercury ($HgCl_2$), methyl mercury (MeHg), and ethyl mercury (thimerosal), as well as lead ($PbCl_2$) and iron (Fe-citrate). DPA had the worst profile of effects, providing no protection while potentiating $HgCl_2$, thimerosal, and Fe-citrate toxicity. DMPS and DMSA both attenuated $HgCl_2$ toxicity and potentiated thimerosal and Fe toxicity, while DMPS also potentiated $PbCl_2$ toxicity. $CaNa_2EDTA$ attenuated $HgCl_2$ toxicity, but caused a severe potentiation of Fe-citrate toxicity. The ability of these chelators to attenuate the toxicity of various metals is quite restricted, and potentiation of toxicity is a serious concern. Specifically, protection is provided only against inorganic mercury, while it is lacking against the common form of mercury found in food, MeHg, and the form found in vaccines, thimerosal. The potentiation of Fe-citrate toxicity is of concern because of iron's role in oxidative stress in the body. Potentiation of iron toxicity could have serious health consequences when using chelation therapy.


## 1. Introduction

Environmental and occupational exposure to heavy metals such as mercury and lead are of significant concern world-wide. Such metals are known to target the mammalian central nervous system and have been implicated in the development of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Praline et al., 2007). Childhood exposure to mercury or lead is of specific concern given the deleterious effects that each of these metals impose on the developing nervous system (Ellis and Kane, 2000).

Mercury is found in multiple forms in the environment. Elemental mercury can be released from dental amalgam restorations (Patterson et al., 1985) and the mercury vapor can be readily absorbed (Hursh et al., 1976). The elemental mercury can then be converted into inorganic mercury in the body which can accumulate in the brain (Björkman et al., 2007). Methyl mercury accumulates in fish inhabiting mercury-contaminated lakes and oceans and is of specific concern because it is readily absorbed from the GI tract and is actively transported across the blood–brain barrier (Kostial et al., 2005; Kerper et al., 1992). While MeHg is thought to be cleared from the brain, it is possible that over multiple exposures significant amounts of MeHg can become demethylated to inorganic mercury and accumulate in brain tissue. Monkeys that have undergone MeHg exposure were found to have little organic mercury in their brains 6 months later, but had higher than normal inorganic mercury (Burbacher et al., 2005). Mercury has been shown to have a substantially longer half-life in the brain than in the blood stream (Rice, 1989). Thimerosal, an antiseptic containing ethyl mercury, continues to be used as a preservative of vaccines distributed and administered worldwide with its use in the US only recently decreasing (Geier et al., 2007). Direct intramuscular injection of this compound provides rapid access to the blood stream and thereby privileged access to its target organs, possibly including nervous tissue.

Lead exposure is also widespread. Commercial use of lead is quite broad, with various lead compounds serving as components in many common products such as lead-based paints found in older homes, as well as being used in the manufacture of batteries (Shukla and Singhal, 1984; Jarup, 2003).

The concerns with iron are somewhat different. While environmental exposure to iron does occur, for example, from drinking water, iron pipes, and cookware, the main concern is the

* Corresponding author. Tel.: +1 414 288 6569 fax: +1 414 288 6564.
*E-mail address:* doug.lobner@marquette.edu (D. Lobner).

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inappropriate release of iron in the body. Iron can be released from the breakdown of hemoglobin following aneurysm or blood disease. The free iron is dangerous because of its ability to generate oxygen free radicals by catalyzing the Fenton reaction (Yamazaki and Piette, 1990).

A controversial use of metal chelators has been by alternative health practitioners for the treatment of chronic health conditions. The basic idea is that chronic high levels of heavy metals are responsible for health problems such as heart disease, Alzheimer's disease, and autism, and that these diseases can be treated by chelation therapy to remove the heavy metals from the body. Statistics on how often such procedures are performed, what they are used to treat, and whether they are effective, or harmful, are limited. A published study (Barnes et al., 2004) estimated that in the year 2002, 66,000 people in the United States underwent chelation therapy. The number is likely to be increasing. Small scale clinical trials have been performed using chelation therapy with EDTA for the treatment of atherosclerotic vascular disease, none of which has showed any benefit (Guldager et al., 1993; van Rij et al., 1994; Knudtson et al., 2002). The National Institutes of Health have recently initiated a major clinical trial testing EDTA in people with coronary artery disease, the results from this trial should be available in 2010 (website: http://www.nccam.nih.gov/chelation).

At present, the primary clinical treatment of acute heavy metal poisoning is by administration of metal chelators. Chelators such as calcium disodium ethylenediaminetetraacetate ($CaNa_2EDTA$), D-penicillamine (DPA), 2,3 dimercaptopropane-1-sulfonate (DMPS), and dimercaptosuccinic acid (DMSA) have been designed to effectively mobilize and remove toxic metal molecules while limiting the disruption of homeostatic levels of essential metals such as zinc. Chelators have been shown to have varying efficacies depending on the metal to which the patient was exposed (Andersen, 2004). The purpose of the present study is to characterize the effects of each chelator on the cytotoxic effects of $HgCl_2$, MeHg, thimerosal, $PbCl_2$, and Fe-citrate. To study these interactions, we exposed murine primary cortical cultures to toxic concentrations of each metal of interest both with and without the chelators present.

## 2. Materials and methods

### 2.1. Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE, USA). Serum was from Atlanta Biologicals (Atlanta, GA, USA). All chemicals were obtained from Sigma (St. Louis, MO, USA).

### 2.2. Cortical cell cultures

Mixed cortical cell cultures containing both neuronal and glial cells were prepared from fetal (15–16 day gestation) mice as previously described (Rose et al., 1993; Lobner, 2000). Timed pregnant mice were anesthetized with isoflurane and euthanized by cervical dislocation. Dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Cultures were plated at approximately 8 hemispheres per plate. Glial cultures were prepared identical to mixed cultures except cortical cells were obtained from postnatal day 1–3 mice (McCarthy and deVellis, 1980; Choi et al., 1987). The postnatal mice were anesthetized with isoflurane and euthanized by decapitation. Glial cultures were plated at approximately 3 hemispheres per plate. Cultures were maintained in humidified 5% $CO_2$ incubators at 37 °C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

### 2.3. Induction of neuronal death

All experiments were performed on cultures 13–15 days in vitro (DIV), at this time there are approximately 150,000 neurons/well ($145{,}900 \pm 5800$; $n = 4$; data from 4 plates from 4 different dissections). Metals and/or chelators were added to the cultures in media identical to plating media except lacking serum for 24 h, at the end of which time a sample of the media was taken to perform the LDH release assay to determine the level of cell death.

### 2.4. Assay of cell death (LDH release)

Cell death was assessed in mixed or pure glial cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 h after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values, and results normalized to 100% neuronal death caused by 500 μM NMDA in mixed cultures and 100% glial death caused by 20 μM A23187 in glial cultures. Approximately 50% ($51 + 2\%$, $n = 12$) of the LDH present in mixed cultures is present in the neurons. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Gwag et al., 1995; Lobner, 2000).

### 2.5. Statistical analysis

Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Bonferroni $t$-test, with $p < 0.05$ being considered significant.

## 3. Results

### 3.1. Concentration-dependent toxicity of $HgCl_2$, MeHg, thimerosal, $PbCl_2$, and Fe-citrate

Fig. 1A–E demonstrates the concentration-dependent cytotoxicity with 24-h exposure to increasing concentrations of each of $HgCl_2$, MeHg, thimerosal, $PbCl_2$, and Fe-citrate. Cell death was assayed in each case through measuring release of the cytosolic enzyme lactate dehydrogenase. $HgCl_2$ had the most notable effects, especially at 5 μM concentrations, while causing approximately 40% cell death at a concentration of 1 μM. MeHg and thimerosal produced similar toxicity profiles, both causing approximately 40% neuronal death at 5 μM. Fe-citrate required approximately 30 μM to produce 40% neuronal death. We chose these concentrations of each compound for the following experiments to produce an intermediate level of cell death. This allowed for the observation of both increased and decreased toxicity caused by the chelators tested. $PbCl_2$ produced only 25% cell death at a concentration of 100 μM. We decided that this lower toxicity at 100 μM would be sufficient to reveal any protection or potentiation brought on by presence of a chelator and did not use a higher concentration in order to maintain physiological relevance.

### 3.2. Metal induced cell death is neuron-specific

The experiments in Fig. 1 were performed using mixed neuronal and glial cultures. To confirm which cells each metal was targeting, we exposed glial cultures to $HgCl_2$ (1 μM), MeHg (5 μM),