Efficacy of oral DMSA and intravenous EDTA in chelation of toxic metals and improvement of the number of stem/progenitor cells in circulation

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Abstract

Metal toxicity represents a significant public health concern and contributes to many toxic metal-exposure related diseases. In particular, toxic metals promote the oxidative stress in stem cells and endothelial progenitor cells (EPCs). EPCs that are presented in peripheral blood function to replenish aging damaged endothelial cells and play important role in the maintenance of vasculature and neovascularization. Given the links between oxidative injury, endothelial cell dysfunction and vascular disease, we focused our investigation on the response of CD34-positive cells to chelation by DMSA.

In addition, we compared the effectiveness of DMSA and EDTA in the chelation of toxic metals and the excretion of essential metals.
The data were compared for 160 subjects after oral DMSA (500mg) chelation and 250 subjects after intravenous EDTA (1g-3g) chelation.
Our in vitro experiments were designed to compare the intrinsic toxicity of two antidotes and their effectiveness in lead chelation.
One of the important results of our study shows that the removal of toxic metals improves the number of stem/progenitor cells in circulation. After a series of five DMSA treatments, the number of CD34+ cells in circulation was increased on 50%-160% (p<0.05).
Our in vitro experiments demonstrated that toxic metals (lead and mercury) inhibit mesenchymal stem cell metabolism and proliferation, and that stem cells are more sensitive to these metals than differentiated cells.
In comparison with EDTA, DMSA was more effective in the extraction of lead, but treatment by this antidote resulted in a very high excretion of copper (55 folds). Clearance of essential metals during chelation by 1g-3g of EDTA was increased over twenty-fold for zinc and manganese.
In summary, we suggest that chelation therapy shows promise in repairing metal toxicity damage and restoring circulating stem cells.

Keywords: chelation, DMSA, EDTA, stem/progenitor cells, toxic metals.

Introduction

Toxic metals such as lead, mercury, aluminum, and arsenic represent a significant public health concern [1-3]. They can induce behavioral dysfunction, damage central and peripheral nervous systems, compromise kidney and liver function, and damage haemopoietic and cardiovascular systems [4-9]. The major mechanisms in causing heavy metal toxicity include alteration of cellular pro-oxidant anti-oxidant homeostasis and binding of metal ions to key cellular proteins [10, 11]. Lead, for instance, increases the generation or reactive oxygen species (ROS), which in turn causes nucleic acid damage and lipid peroxidation in cell membranes. This damage is directly related to vasoconstriction and hypertension [12,13]. Lead also alters gene expression and cell differentiation due to its substitution for zinc or calcium in key regulatory proteins [14, 15].

Generation of ROS such as superoxide and hydrogen peroxide can have a detrimental effect on endothelial cells (ECs), endothelial progenitor cells (EPCs), and vascular smooth muscle

cells [16]. EPCs, bone marrow derived CD34-positive stem cells that mature into endothelial cells, replenish aging or damaged ECs that line blood vessels [17-19]. Decreasing EPC numbers are associated with coronary diseases (including atherosclerosis, diabetes, and hypertension) and increased cardiovascular risk [20-23]. The effects of toxic metals on the cellular oxidation-reduction balance can damage progenitor cells. For example, lead is not only cytotoxic toward stem cells but also interferes with their differentiation [24-25]. This suggests a mechanism in which toxic metals can harm the vascular system by inducing generation of ROS, which in turn hinder the replenishment of vessel walls by endothelial progenitor cells.

Chelation therapy has been proposed for clearing toxic heavy metals from the body [26-29], as it not only removes contaminating metals but also decreases free radical production [30]. The two most commonly used chelation agents to date are ethylenediaminetetraacetic acid (EDTA) and dimercaptosuccinic acid (DMSA). Safety and efficacy for these two agents have been studied [29, 31]. In the present study, we focused on how toxic metal exposure, and subsequent chelation therapy, affects circulating CD34 positive cell numbers. We also present results concerning the toxicity of lead and mercury to stem cells (mesenchymal stem cells, MSCs), endothelial progenitor cells and differentiated cells (endothelial cells and fibroblasts).

Materials and Methods

Study population

Data were obtained with full IRB approval and consent from over four-hundred subjects given chelation therapy for long-term non-occupational toxic metal exposure. The breakdown of specific treatments is as follows: oral DMSA (500 μg) for 160 subjects; intravenous low dose Na-EDTA (1 g) for 50 subjects; and intravenous high dose EDTA (Ca-EDTA or Na-EDTA) (3 g) for 200 subjects. Prior to treatment, each subject was screened for adequate kidney functioning by assaying fasting serum creatinine (levels below 1.7 mg/dL were considered acceptable). Urine samples were collected pre and post treatment for toxic metal excretion analysis.

For five subjects, selected for long-term exposure and at least three amalgam fillings, we conducted a more detailed analysis: these subjects were given a series of five DMSA doses and blood samples were collected at the beginning and end of their treatments. In these subjects, we measured complete blood count (CBC), circulating CD34-positive cell levels, and urine metals.

Measurements of samples from subjects

Body burdens and excretion rates of toxic and essential metals were determined through urine analysis. For patients treated by EDTA, aluminum, lead, cadmium and manganese levels in urine were measured by the Perkin-Elmer 4100ZL atomic absorption spectrometer with graphite furnace by standard protocols. Calcium, magnesium, iron, copper and zinc were also analyzed on a Perkin-Elmer Inductively Coupled Plasma atomic emission spectrometer (Model 3300 DV). For patients treated by DMSA, heavy metal analyses of urine were undertaken by the King James Laboratory (Cleveland, OH). All measurements of metals were performed using Inductively Coupled Plasma -Atomic Emission spectroscopy (ICP-AES).

The procedure of stem/progenitor cells measurements was based on gating strategy defined by the ISHAGE guidelines [32]. Cells were selected that expressed bright CD34 antigen and dim CD45 antigen along with low side-angle light scatter characteristics. In our case, we considered circulating CD34 positive cells as precursor of EPCs. Our focus on the CD34 marker is work [33], in which blood–derived cells from which endothelial cells in culture were developed were described as cells expressing this antigen. Moreover, recent studies demonstrate that CD34+ cells not expressing leukocyte antigen (CD45-) form endothelial colony-forming units and those expressing CD45 demonstrate hematopoietic properties.

PBMCs were separated from whole blood, re-suspended in 100 μl (0.5M cells per 100 μl) of a buffer (PBS+0.5% BSA), and stained by 20 μl of antibodies (Stem cells kit, Beckman Coulter) during 15 min. Flow cytometry was performed on "Cell Lab Quant SC" (Beckman Coulter) equipped with 22 mW argon laser tuned at 488 nm. Quantification of CD34+ cells was performed on separated PBMCs by two–color immunostaining using anti-CD45-FITC and anti-CD34-PE. The percentage of CD34 positive cells was calculated based on the measured number of leukocytes (CD45-positive cells) and the number of circulating CD34-positive cells per micro liter was recalculated by normalization on the concentration of leukocytes determined by the Complete blood count (CBC) test.

In Vitro studies

We conducted in vitro experiments using human endothelial cells (HUVEC, Cascade Biologics), lung fibroblasts (CCD 18lu, ATCC), mesenchymal stem cells (BioE Co) and EPCs developed in our laboratory by long-term culture from PBMCs. Endothelial cells and EPCs were grown in medium M-200 (Cascade Biologics) supplemented with 2% fetal bovine serum (FBS), hydrocortisone, human epidermal factor, fibroblast growth factor, and heparin. CCD 18lu cells and MSCs were grown in DMEM medium (ATCC) and MSCGM (Lonza) with 10% FBS. Chelating agent concentrations for in vitro studies were based on clinical dosages used for chelation by EDTA-Ca and DMSA.

Cellular lead levels were determined as described previously [34]. Cells seeded in the 24–well plates were exposed to different concentrations of lead and antidotes in the medium with a different concentration of serum (including serum free medium) for 24h to 48h. After exposure, cells were harvested by rinsing with 2 mM EDTA in PBS and then detached from the wells by trypsin. Cells were counted with hemacytometer

and, after centrifugation, dried several hours in an oven at 100 ºC. Once dried, cells were digested overnight using 200 μL concentrated nitric acid. The digested cells were diluted with 1.8 ml of matrix modifiers. Matrix modifier was prepared by adding in 500 ml volumetric flask 2.5 ml of 10% TX-100, 1 g ammonium phosphate monobasic, and 1 ml concentrated nitric acid. The total level of lead was then measured by flame atomic absorption spectroscopy (AAS).

Cellular ATP levels were determined using The CellTiter-Glo Luminescent cell Viability Assay kit (Promega). Cell proliferation was measured by BrdU Flow kit (BD Pharminogen).

Statistics

Statistical analysis was performed by Systat (release 13) and Kaleidagraph. For non-normal distributions, pre- and post-treatment parameters were compared using the non-parametric Wilcoxon test with significance at 5%. The paired t-test was used to compare differences before and after treatment.

Results

Oral administration of 500 mg DMSA (single dose) led to measurable urinary excretions of lead, arsenic, mercury and aluminum, as shown in **Figure 1**. The excretion exceeded the pretreatment levels and the excreted values varied between (IQR) 0.5-3.0 ug/L (mean 1.48 ug/L) for lead, 0.16-0.9 ug/L (mean 0.4 ug/L) for mercury, 3.6-10.6 ug/L (mean 6.5 ug/L) for aluminum, 0.2-13ug/L (mean 2.67 ug/L) for arsenic.
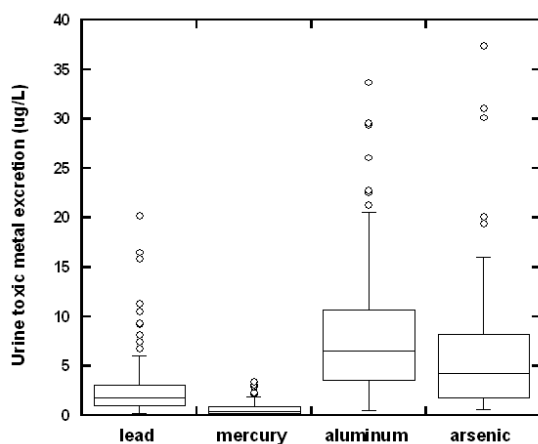

FIGURE 1. Urinary excretion of lead (Pb), mercury (Hg), aluminum (Al), and arsenic (As) in six-hours following chelation therapy with 500 mg DMSA. Boxes represent upper and lower quartiles, with the center line indicating the median.
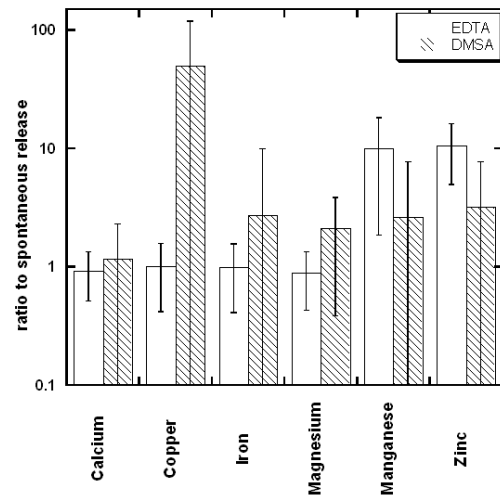

FIGURE 2. The urine excretion ratios (excretion relative to that occurring spontaneously) of essential metals calcium, copper, iron, magnesium, manganese, and zinc after treatment by the equimolar concentrations of EDTA and DMSA (1 g and 500 mg). DMSA chelation of essential minerals was the most dramatically for copper, Clearance of essential metals during EDTA chelation was significantly increased for zinc and manganese.

Figure 2 shows the ratio of post-chelation urinary essential mineral excretion to spontaneous essential mineral excretion after treatment with the equimolar concentration of EDTA and DMSA (1g and 500 mg). Copper is excreted most dramatically (55-fold above normal) by DMSA, while the other minerals see roughly two-fold increases in excretion after chelation. Similar trends were previously observed with EDTA [31]. Clearance of essential metals during chelation by 1g-3g of EDTA was increased over twenty-fold for zinc and manganese, and for other essential metals such as iron and calcium, the increase in excretion was two-four folds.

Average ratios of post chelation values to spontaneous release values for toxic metals after single treatment by different dosages of intravenous EDTA (1 gram and 3 grams) and oral 500 mg dosage of DMSA are shown in **Figure 3**. For toxic metals, DMSA was most efficient in removing lead and arsenic, was comparable to EDTA in removing aluminum, and was least efficient in regard to cadmium and mercury. Data of chelating mercury and arsenic by EDTA were not obtained, but published results show that EDTA does not cause excretion of these metals [35]. In comparison to Na-EDTA and Ca-EDTA, chelation with DMSA was more effective in removing lead.

In five subjects who were given several rounds of DMSA chelation, spontaneous release (pre-chelation level) is
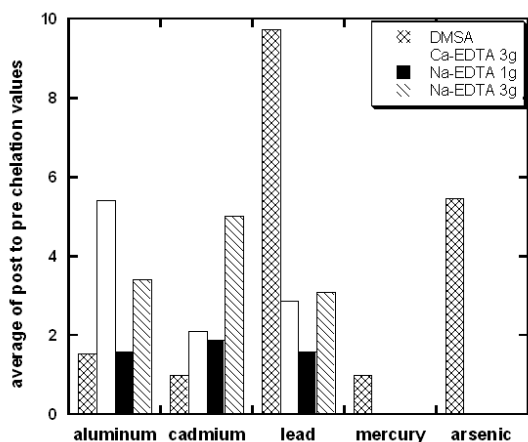
FIGURE 3. The urine excretion ratios of toxins aluminum, cadmium, lead, mercury and arsenic after 500 mg oral DMSA chelation and intravenous EDTA chelation (1 g and 3g). Data demonstrate that DMSA was more effective than EDTA in removing lead. DMSA was effective in removing arsenic, and high concentrations of EDTA were effective in chelation of aluminum and cadmium.

TABLE 1. Spontaneous urine excretions of toxic metals prior to chelation therapy (pre) are compared to maximum urine excretions attained (max) over several rounds of DMSA chelation for five subjects.

	Pb (µg/L)		Mg (µg/L)		As (µg/L)		Al (µg/L)	
Subject	Pre	Max	Pre	Max	Pre	Max	Pre	Max
I	1.1	1.9	0.060	0.062	1.1	1.1	11.6	24.4
II	0.12	1.7	0.11	0.21	0.12	1.1	2.4	7.3
III	0.14	0.28	0.53	0.76	0.14	0.94	8.0	13.5
IV	0.16	1.3	0.056	0.056	0.16	2.8	7.2	17.0
V	0.13	1.7	0.044	0.046	0.13	1.4	0.33	6.5

compared to maximum post-chelation extraction levels in **Table 1**. For most of the subjects, the maximum release of lead, mercury and arsenic was measured after first chelation. Aluminum excretion remained elevated over several rounds of chelation therapy, so that peak values generally occurred later. **Figure 4** shows how lead and mercury excretion levels varied over time. Several treatments were required to return toxic mineral levels to background. Moreover, excretion levels for zinc (with EDTA treatment) and copper (with DMSA treatment) remained high over the course of several chelation treatments, suggesting that supplements for these nutrients should be provided during treatment [36].

Using five subjects exposed to multiple rounds of DMSA therapy, we examined the effect of chelation on circulating CD34-positive cells. Quantification of circulating CD34-positive

cells by fluorescence–activated cell sorter analysis is shown in **Figure 5**. Freshly prepared peripheral blood mononuclear cells were stained by anti-CD34–PE and anti-CD45–fluorescein isothiocyanate (FITC). The initial gate on histogram EV versus SS (Figure 5A) creates a region to include mononuclear cells and exclude debris. The second gate on Figure 5B (FITC staining versus SS) creates a rectangular region that includes all CD45+ leukocytes and eliminates platelets and red blood cells. The third gate on histogram PE versus SS (Figure 5C) separates from all leukocytes the cells that express high level of CD34 fluorescence. The last histogram FITC verses SS (Figure 5D) was adjusted to include cells that have high level of CD34 expression and low level of CD45 expression. In addition, this region is set to include events with small side scattering, which is characteristic of stem/progenitor cells.

Base on this protocol, the number of CD34+/CD45⁻ cells was determined and compared for five subjects before and after DMSA chelation.

While CBC did not indicate any statistically significant changes in red cell counts, white cell counts, platelets, or hemoglobin levels, the number of circulating CD34-positive cells increased from an average of 2.3 ± 0.7 cells/µL before treatment to an average of 3.5 ± 1.2 cells/µL after treatment (p<0.05). Four of five subjects saw an increase in their circulating progenitor (CD34+) cell numbers, with increases ranging from 50% to 160% (**Figure 6**). The increase in CD34+ cell numbers correlated with the total (area-under-the curve) five-day mercury excretions.

We examined the effects of mercury (methyl mercury, MeHg, and mercury (II) chloride, $HgCl_2$) and lead (lead acetate, PbAc,
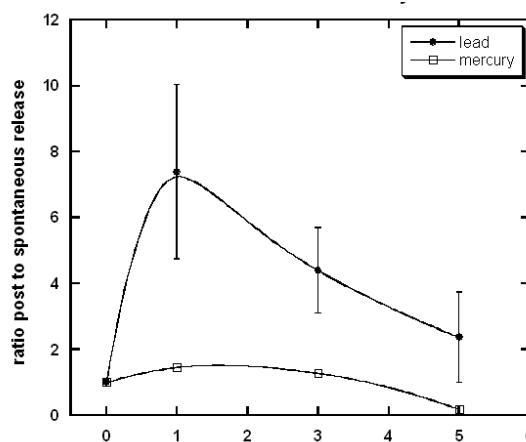

FIGURE 4. Urine lead and mercury levels over the course of five chelation treatments. Values represent averages for five subjects. According to these data, several treatments are required to return level of the metals to background level.
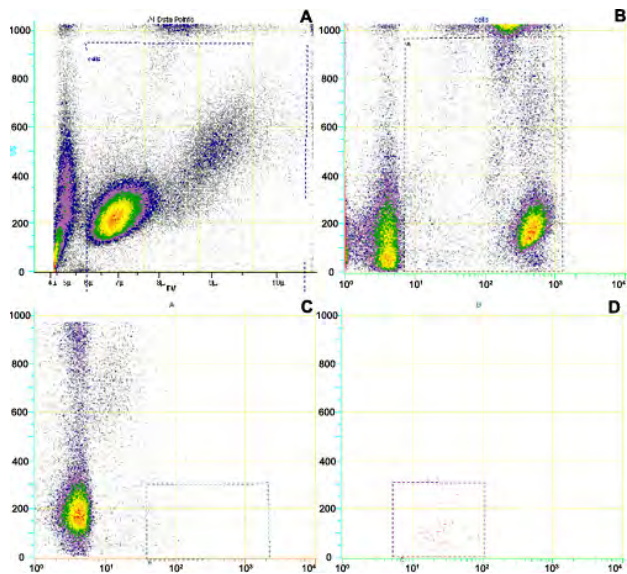
FIGURE 5. The method of CD34-positive cell selection is presented in histograms A-D. Histogram A (EV verses SS) creates a region to include mononuclear cells and exclude debris. Histogram B (FITC verses SS) creates a rectangular region to include all CD45+ leukocytes and eliminate platelets and red blood cells. Histogram C (PE verses SS) creates a region to separate CD34+ cells in population of leukocytes. Histogram D (FITC verses SS) creates the region to include all events with CD45 low/dim staining from events with CD34 bright staining.
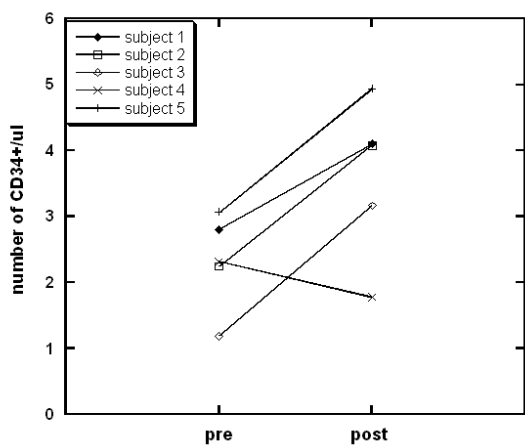

FIGURE 6. Circulating CD34-positive cells in five subjects given five rounds of DMSA therapy.
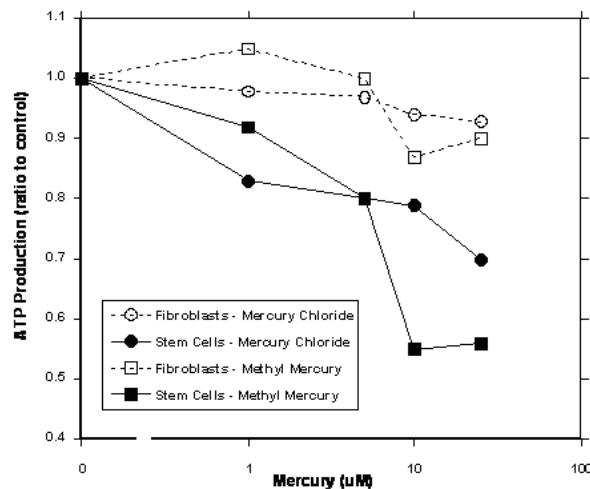

FIGURE 7. ATP production rate after treatment with mercury chloride or methyl mercury, expressed as ratio of ATP production after treatment to that of untreated controls. Treatments were carried out in vitro with mesenchymal stem cells and lung fibroblasts. The level of ATP in cells was measured after 24 hours of mercury exposure.

and lead (II) chloride, $PbCl_2$) on the energy metabolism (ATP production) of mesenchymal stem cells and CCD18lu normal lung fibroblasts. Results are shown in **Figure 7**. Two trends are observed by inspection: mercury reduces ATP production rates in a concentration dependent fashion, and MSC are more sensitive to this effect than the CCD18lu fibroblast line. It also appears that the methyl mercury form may have a greater effect than the mercury chloride form, particularly for the stem cells. This was observed morphologically under the microscope: cultured MSCs demonstrated higher level of cell damage with organic Hg in comparison to inorganic Hg. The metabolic effect of mercury is serum dependent: when serum concentrations were increased from 2% to 10%, the stem cells were more protected. The methyl mercury concentration required for 50% inhibition of ATP production was roughly 20 μM in 2% serum, but rose to 40 μM at 10% serum.

ATP production data for lead, either in the form of lead acetate or lead chloride, demonstrated that as in the case of mercury, lead reduces the ATP production rate in a concentration dependent fashion, and it has a stronger effect on MSC than on the normal cell line. Unlike mercury, the inorganic form of lead seems to have a stronger effect on the metabolism than the organic form.

It should also be noted that, comparing lead to mercury, the latter reduces the metabolism at lower concentrations than the former. For example, 10 μM methyl mercury reduced ATP production in MSC by 45%. At that concentration, lead chloride reduced MSC ATP production by 25%.
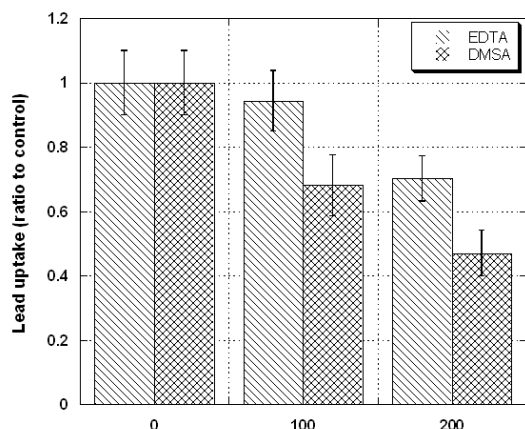
FIGURE 8. Cellular lead uptake rate in fibroblasts after exposure to the antidotes Ca-EDTA and DMSA. Different concentration of the chelating agents and lead were added in cell culture with serum free medium. After 12 h of incubation, cells were washed by PBS, detached by trypsinization, counted and processed for their lead content by AAS. The controls are the cells that were exposed to lead and not treated by antidotes. Error bars indicate standard errors.

To compare the intrinsic toxicity of antidotes we examined how the chelation agents EDTA and DMSA affected ATP production rates in cells (endothelial cells and lung fibroblast) after exposure to different concentrations of these antidotes. For DMSA, there was no effect at concentrations of 100 μM or 200 μM, while concentrations of 400 μM decreased the ATP production rate by roughly seven percent. These cells were more sensitive to EDTA, but the effect was still slight. EDTA at 400 μM decreased the ATP production of fibroblasts by twelve percent, and a six percent reduction was seen at 200 μM. Overall, these data suggest that the agents are essentially non-toxic. It should also be noted that what little toxicity to cells we observed only occurred in a serum free growth medium, and serum provided complete protection from the metabolic effects.

Finally, we compared the effects of DMSA and EDTA on lead uptake by normal differentiated fibroblasts in vitro. All experiments were performed in a serum free medium, as serum reduces cellular lead uptake by two orders of magnitude (data not shown). The protective effect of serum on lead uptake can be explained by the lead binding to albumen and other proteins. In addition, molecules having phosphates and carbonates can neutralize the damage to cells caused by ionic lead. Results of the in vitro lead uptake assay are shown in **Figure 8**. While both chelation agents reduce cellular lead uptake in a concentration dependent fashion, DMSA appears to have the larger effect. For example, 200 μM DMSA reduced cellular lead uptake by fifty percent, while the same molar concentration of Ca-EDTA reduced lead uptake by thirty percent.

Discussion

Based on the results above, the following conclusions can be drawn:

- The toxic metals lead and mercury inhibit the mesenchymal stem cell metabolism and proliferation and adult differentiated cell metabolism in vitro. In fact, MSCs are more sensitive to these metals than differentiated cells such as lung fibroblasts and HUVEC.
- The chelating agent DMSA increases circulating CD34-positive cell numbers in vivo.
- In comparison to EDTA, DMSA chelation by eqiumolar concentration is better at extracting lead and arsenic, comparable in extracting aluminum, and less efficient in extracting cadmium.
- In the extraction of essential minerals during chelation, DMSA is highly 'efficient' in removing copper, and is in general more likely than EDTA to cause extraction of essential minerals (with the exception of manganese and zinc, which are extracted more in EDTA chelation).
- DMSA shows some ability to extract mercury, but this requires several treatments.
- In vitro lead uptake assay showed that while both antidotes reduce cellular lead uptake in a concentration dependent fashion, DMSA appears to have the larger effect.

This is, to our knowledge, the first study demonstrating in a clinical setting that DMSA chelation increases circulating Cd34-positive cell numbers. Detrimental effects of mercury [37, 38] and lead [39] on stem cell proliferation and differentiation have been reported, and it has been demonstrated that the oxidative stress induced apoptosis in MSCs, embryonic stem cells and endothelial progenitor cells [40-42]. This leads to the idea that toxic metal exposure could be particularly harmful to stem cells in vivo, and in turn could be detrimental to systems that rely on stem cell replenishment, such as the cardiovascular system. Published correlations between EPC numbers and cardiovascular risk factors bear this out [20]. The ability of chelation therapy to replenish CD34-positive and EPC numbers could provide an important justification for using chelation to repair cardiovascular system damage caused by toxic metal exposure.

There have been some reports in literature that toxic metal exposure can damage peripheral blood mononuclear cells [43-45], primarily by interfering with the electron transport chain in mitochondria. In particular, arsenic and cadmium can induce lymphoid cell apoptosis [43], while environmentally relevant concentrations of mercury, cadmium, and lead in vivo are associated with reduced viability and activity of white blood cells, including T-cells, B-cells, macrophages, and natural killer cells [46-48]. In five subjects receiving multiple DMSA chelation treatments, we attempted to see if white blood cell numbers changed during the course of treatment. We saw no statistically significant change in red cell or white cell numbers, although this may be due to our small sample size. However, the mean

granulocyte counts decreased from 5.1 ± 1.2 K/μL to 4.2 ± 0.5 K/μL post chelation, number of monocytes was increased on average 23%±14%, number of lymphocytes was increased on 5%-33%, but these differences were not statistically significant. Perhaps a larger study would uncover an effect.

The metal excretion data for DMSA in this work, along with data for EDTA presented here and published previously [31], provide some guidelines for the clinical use of these chelation agents. Our previous work suggested that, for several reasons including potential cellular toxicity and non-linear dose dependence of extraction, multiple low doses of Na-EDTA or Ca-EDTA were preferable to high doses, and that Ca-EDTA was the better of the two antidotes for aluminum removal. Our data show that DMSA represents an improvement over EDTA for lead and arsenic chelation, but with a cost of higher extraction of essential minerals. The fifty-five-fold increase in copper extraction is particularly dramatic, suggesting that copper monitoring and supplementation may be necessary during chelation therapy.

In summary, we think chelation therapy shows promise in repairing metal toxicity damage and restoring circulating stem cell populations. We hope to embark on a larger scale study to gain more data on changes in white cell and progenitor cell numbers before and after chelation therapy.

Acknowledgment

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