Original Article

Cytotoxic and Oxidative Stress Caused by Cadmium and Lead on Human Skin Fibroblast Cells

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Abstract

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Introduction: Heavy metals are important occupational and environmental pollutants that cause damage to various organs. Although there is no effective therapy for such a poisoning, metallothionein has been shown to play a key role in the detoxification of cadmium (Cd). Evidence in the literature suggests that superoxide dismutase, glutathione peroxidase, and catalase constitute important defense mechanisms against oxygen toxicity in the cells. The aim of this study was to investigate the effect of cadmium chloride and Pb-acetate on antioxidant enzymes in the human skin fibroblast cells (HF2FF).

Material and Methods: The human skin fibroblast (HF2FF) cells were incubated in serum-free medium containing 20 µM $CdCl_2$ for 18 hr three times a week. The same exposure to an equimolar dose of Pb-acetate was performed. After each exposure and after three times exposure the cells were collected and cell viability, the contents of superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), GSH and malondialdehyde (MDA) were measured.

Results: Cd caused cytotoxicity and inhibition of glutathione peroxidase (GSH-Px) and SOD activity, as well as depletion of the reduced form of glutathione (GSH) in the cell. The level of lipid peroxidation (LP) was increased, but catalase activity was not significantly altered. These defects were increased with repeated exposures. The same exposure to an equimolar dose of Pb-acetate evoked only inhibition of GSH-Px and SOD. The values of GSH, catalase and LP activity remained unchanged.

Conclusion: The inhibition of GSH-Px and SOD may be considered as an important biomarker of the toxic effect of metals.

Keywords: Cadmium, Glutathione peroxidase, Lead, Lipid peroxidation

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Introduction

Evidence in the literature suggests that superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1.), glutathione peroxidase (glutathione: hydrogen peroxide oxidoreductase, EC 1.11.1.9.) and catalase (hydrogen-peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6.) constitute important defense mechanisms against oxygen toxicity in cells (1). The cadmium (Cd) is an important occupational and environmental pollutant that causes damage to various organs (2, 3). It is a very unique mineral. It is extremely toxic and has toxic biological effects at concentrations smaller than almost any commonly found mineral. Despite this great toxicity, there is some evidence that cadmium is an essential nutrient with biological function (4).

One of the greatest effects of cadmium is depletion of selenium in the body. Indeed,

selenium is essential for cadmium removal. Selenium atoms combine with cadmium atoms and are escorted out of the body via the bile system. Therefore, there is less selenium to form glutathione peroxidase, one of the body's main antioxidants. This results in formation of greater levels of reactive oxygen species and hydrogen peroxide. Although metallothionein has been shown to play a key role in the detoxification of Cd, there is no effective therapy for Cd poisoning (5). It has already been found that a single dose of cadmium chloride caused a statistically significant increase in lipid peroxidation and depletion of the reduced form of glutathione in the liver of male mice and rats, accompanied by elevated activity of alanine aminotransferase (6). In other experiments the inhibition of CuZn-superoxide dismutase and an increase of glutathione peroxidase and catalase activity in erythrocytes of mice were found with the same single dose of cadmium chloride (7, 8). Exposure to lead has been known to adversely affect human health in urbanized communities (9). Quinlan and colleagues (10) have reported that lead (and aluminum) stimulates iron-dependent lipoperoxidation of membranes, implicating deleterious reactive oxygen species in the physiopathology of plumbism. That lead ions can directly accelerate oxidation of oxyhemoglobin (oxyHb) to methemoglobin (metHb) and inactivate several thiol enzymes have also been established (11). SOD is an enzyme used extensively as a biochemical indicator of pathological states associated with oxidative stress (12). It plays a protective role against deleterious effects triggered by superoxide radical anion. Iron ions (13), mercury poisoning (14), lead poisoning (15) and aging (16) are among the conditions with altered SOD activities. Monteiro and colleagues (17) demonstrated that the SOD activity is higher in individuals exposed to lead. The aim of this study was to investigate the effect of repeated low doses of cadmium chloride and Pb-acetate on the activity of glutathione peroxidase, SOD, and catalase in the human skin fibroblast cells (HF2FF). The influence of cadmium on lipid peroxidation and GSH content in the HF2FF was investigated as well.

Material and Methods

Chemicals and reagents

DTNB, GSH, GSSG, NADPH+H^+, ATP, LDH, PEP, pyrovate kinase, L-aminobutyrate, Pb-acetate, $CdCl_2$. 2.5 H_2O, L-Glu, fetal calf serum, antibiotics (penicillin, streptomycin), glutathione reductase, NADPH, GSH, RPMI, HBSS, thiobarbituric acid, pyridine, 1.1.3.3. tetraethoxypropane, perchloric acid, 5,5'-dithio-bis (2-nitrobenzoic acid), and trypsin were purchased from Sigma Chemical Co. (St. Louis, Mo. USA). Human skin fibroblast cell line (HF2FF) was purchased from Pasteur institute of Iran and cell culture reagents were purchased from Life Technologies (Gibco, Cergy-pontoise, France).

Cell cultures

HF2FF cell line was obtained from Pasture Institute of Iran. Cells were routinely plated at a density of 100,000 cells/plate on 6 cm coated plate and were grown in RPMI (pH 7.4), supplemented with 10% FCS and antibiotics (7 U/ml penicillin, 100 μg/ml streptomycin), and were cultured at 37°C in 5% CO_2.

Chemical treatments

Six plates of confluent monolayers were used in each assay. The medium was removed before each treatment. All treatments were carried out in serum-free media. The cells were incubated for 18 hr, 3 times a week in serum-free medium containing 20 μM $CdCl_2$. The same exposure to an equimolar dose of Pb-acetate was carried out. Untreated control cells were incubated in serum-free medium, as was done for cells exposed to $CdCl_2$ and pb-acetate.

Assay of GSH-Px activity

The activity of glutathione peroxidase (GSH-Px) was determined according to Lawrence, R.A and colleagues' protocol (18). An aliquot of 100 μl cell lysate was mixed with 800 μl reaction buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, and 1 mM GSH in PBS), and 100 μl (10 U) glutathione reductase. The mixture was vortexed and incubated at 25°C for 5 min. GSH-Px activity was monitored at 340 nm and calculated from NADPH oxidized/min/mg protein.

Assay of superoxide dismutase activity

Total SOD activity (Mn- plus CuZn-SOD) was determined as previously described by Hermes-Lima and Storey (19) under the following assay conditions: 5 mmol l^{-1} EDTA, 2.5 mmol l^{-1} $MnCl_2$, 0.25 mmol l^{-1} NADH, 4 mmol l^{-1} 2-mercaptoethanol in 50 mmol l^{-1} potassium phosphate buffer, pH 7.2. One SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation of NADH (monitored at 340 nm) by 50% (IC_{50}). Several 1-ml cuvettes were used for each sample, using increasing amounts of enzyme extract (from 0 to 150 μl); these were plotted as velocity versus amount of enzyme extract,

and an IC_{50} value was obtained. Blanks were run in the absence of 2-mercaptoetanol.

Catalase activity

Catalase activity was measured by monitoring enzyme-catalyzed decomposition of H_2O_2 (20). In summary, a solution of H_2O_2 was added to test tubes containing samples, a water blank, and an H_2O_2 standard solution (standard). After incubating for 3 min, the enzymatic reaction was terminated by addition of 1.4 ml $H_2SO_4.KMnO_4$ to each tube; they were then vortexed and the absorbance was recorded at 480 nm. One unit of catalase activity is defined as k/0.00693 (21), where k = log (S_o/S_2) x (2.3/t), S_o is absorbance of standard minus absorbance of blank, S_2 is absorbance of standard minus absorbance of sample, and the t is time interval.

Assay of malondialdehyde concentration

The peroxidation of lipids (LP) expressed as malondialdehyde production was measured by the thiobarbituric acid test (22). The lipid peroxide concentration was determined by the method measuring the amount of thiobarbituric acid (TBA) reactivity by the amount of malondialdehyde (MDA) formed during acid hydrolysis of the lipid peroxide compound. Thiobarbituric acid-reactive substances (TBARS) in HF2FF cell line was measured using modified method of Uchiyama and Mihara (22). The cells were homogenized with 1.15 mol cold KCl. Each milliliter of this homogenate was combined with 0.375 ml of 20% acetic acid and 0.375 ml of 0.6 % thiobarbituric acid solution. This mixture was heated in a boiling water bath for 60 min and then cooled in cold water for 10 minutes. Then 1.25 ml of n-butanol and pyridine (15:1) was added. The mixture was vortexed and centrifuged at 2000 rpm for 5 min to separate the phases. The absorbance of butanol phase was measured at 532 nm on spectrophotometer CECIL 1100. 1.1.3.3-tetraethoxypropane was treated in the same manner as the samples and used as external standard curve and the level of lipid peroxides was expressed as micromole MDA per milligram of protein.

Protein concentration in homogenate was measured by the method of Bradford (1976), using BSA as a standard.

Measurement of GSH

The concentration of reduced glutathione was determined according to the method of Tietze with slight modifications (23). HF2FF cells were plated at 5×10^5 cells/well in 6-well culture plates and cultured for 2 days. At the end of the incubation, the cells were washed twice with Ca^{++} and Mg^{++}-free PBS and lyzed with 0.1 ml of 3% perchloric acid for 15 min at 4°C. After centrifugation at 800 × g for 5 min, supernatants were neutralized with 0.9 ml of 0.1 M sodium phosphate/5 mM EDTA buffer, pH 7.5 (phosphate/EDTA buffer). The reaction mixture contained 20 µl of the neutralized extract, 0.96 ml of phosphate/EDTA buffer, 20 µl of 60 mM 5,5'-dithio-bis(2-nitrobenzoic acid). The increase of absorbance at 412 nm was monitored for 4 min. A standard curve of glutathione was prepared at each determination.

Cytotoxicity studies

Cellular damage induced by Cd was evaluated by trypan blue exclusion assay or by monitoring the release of lactate dehydrogenase (LDH) into the culture medium. For trypan blue assay, HF2FF cells were plated at 1×10^6 cells/dish in 60-mm culture dishes and cultured for 2 days. At the end of the incubation with $CdCl_2$, the culture medium was aspirated and reserved. The cells were detached from culture dishes by treatment with 0.25% trypsin/ 1 mM EDTA. After trypsinization, the cells were suspended in RPMI 1640 and the culture medium was returned. The mixture was centrifuged at 800 × g for 3 min to concentrate the cells. Cellular suspension and 0.4% trypan blue in Hanks' balanced salt solutions were mixed (final concentration of 0.07% trypan blue), and the number of viable cells was counted using a hemacytometer in the triplicate samples. The percentage of viable cells (cell viability) was calculated as (unstained cells)/ (stained + unstained cells) × 100.

For LDH assay, the cells were plated at 2.5×10^4 cells/well in 96-well culture plates or 5×10^5 cells/well in 6-well culture plates and cultured for 2 days. At the end of the incubation with $CdCl_2$, culture medium was removed and centrifuged at 800 × g for 10 min to obtain cell-free supernatant. Supernatant LDH activity was determined using Vassault and colleagues' method (24). The results were expressed as percentage of the maximum amount of LDH released from samples that had been treated with 1% Triton X-100 (percentage release) (Fig. 1).

Statistical analysis

The data was presented (mean ±SD) for each treatment group. For each experimental endpoint, each group was compared with the control by one-way ANOVA. Two-way ANOVA was used to describe statistical differences ($p<0.05$) across treatments and time points.

Table 1: Activity of GSH-Px and SOD in the HF2FF cell line after treatment with Cd-chloride and Pb-acetate. For other notes see *Fig. 1*.

Enzyme	Control	Cd			Pb		
		1st 20uM	2nd 20uM	3rd 20uM	1st 20uM	2nd 20uM	3rd 20uM
Catalase (i.u/mg)	12.3±2.1	12.0±3.3	11.9±4.2	12.4±3.6	12.5±2.8	11.7±1.8	13.4±3.3
SOD (i.u./mg)	56.8±11.9	66.1±16.4*	68.8±17.6*	78.4±20.7**	57.9±18.4	64.2±4.4*	65.0±4.9*
GSH-px (m.iu/mg)	8.57±1.4	8.09±0.6	7.56±1.1*	6.17±0.7**	8.14±1.4	8.62±1.2*	9.31±1.2**

* Significantly different from corresponding values in control, p<0.05
** Significantly different from corresponding values in control, p<0.01

Results

In this study oxidative stress effects of $CdCl_2$ and Pb-acetate on the HF2FF cells were examined. The oxidative damage or free radical production was observed following treatment with $CdCl_2$ and Pb-acetate. A significant decrease in cell viability and an increase in LDH activity were observed in cells exposed to $CdCl_2$ and Pb-acetate, compared to the control (unexposed) cells (Fig 1).

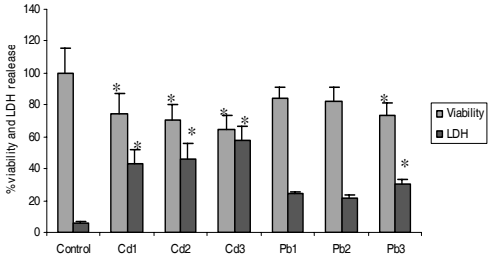

Fig. 1: Cytolethality of $CdCl_2$ and Pb-acetate on the HF2FF cell line. The cells were exposed to 20 μM $CdCl_2$ for 18 hr, and repeated doses in 1 week. The same equimolar Pb-acetate was added to cells. Cell viability and LDH leakage were determined after the end of exposure to $CdCl_2$ and Pb-acetate. Each value (mean ± S.D.) of cell viability (n = 6) and LDH leakage (n = 6) represents the percentage of viability in control and the percentage of LDH release, respectively.
* p<0.01 was considered as significant.

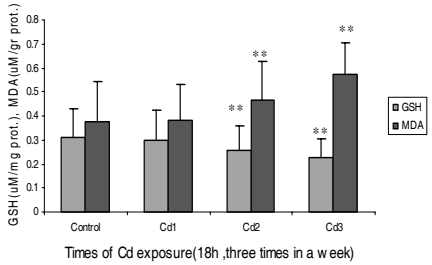

Fig. 2: The level of lipid peroxidation and the content of GSH in the HF2FF cells after 24 hr treatment with 20 μM of $CdCl_2$. The cells were exposed to Cd-chloride for 18 hours, three times a week. Data represent mean ±95% confidence limits from 6 wells of cell culture.
** p<0.01 vs. the control value.

Cellular levels of antioxidants, GSH, MDA, SOD, catalase, and GSH-Px were determined. Following exposure to repeated doses of 20 decrease in GSH was observed at 18 hr (75–91% of control) and after 1 week (49–68% of control) (Fig 2).

A significant decrease in GSH-Px and a significant increase in SOD activities were observed in cadmium treated cells for 18 hr (83-91% and 116% compared to control, respectively), and after 1 week (61–72% and 138% compared to control, respectively), but the change of catalase activity was not statistically significant (Table 1).

In contrast to GSH, SOD, and GSH-Px, Cd treatment led to a significant increase in MDA level in HF2FF cells (151% of control cells) (Fig. 2).

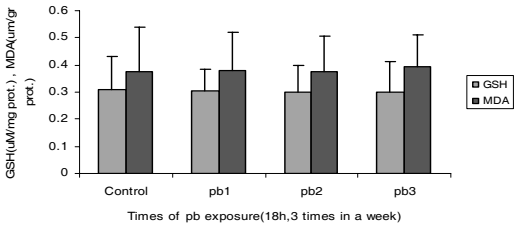

Fig. 3: The level of lipid peroxidation and the content of GSH in the HF2FF cell line after the treatment of 20 μM of Pb-acetate. The cells were exposed to Pb-acetate for 18 hours, three times a week. For other notes see Fig 1.

Alteration of SOD and glutathione peroxidase in inhibition of GSH-Px was not significant in Pb-acetate treated cells for 18 h (102% and 89-95% reduction over control, respectively). However after 1 week, both SOD and GSH-Px activity increased significantly (108-114.5% and 101.5-109% compared to controls, respectively) (Table 1).

Discussion

The mechanisms responsible for the toxicity of cadmium are not well understood. Alteration of the activity of antioxidant enzymes is not only associated with natural factors, but also with inorganic and organic contaminants. Concurrent with the increase of oxidative radicals, the activity antioxidant enzymes in organisms exposed to metals may vary (25, 26). Lipid peroxidation can be one of the consequences of oxidative stress, a situation

which occurs when the production of ROS exceeds that of the antioxidant defense systems (26). The ROS can be inactivated through the action of antioxidant enzymes, such as superoxide dismutase and catalase. The process of lipid peroxidation determines the alteration in the structure of cell membrane (27). In this study, we did not find any changes in lipid peroxidation products after acute exposure to sub-lethal cadmium concentration, indicating efficient response of cellular protection mechanisms against cadmium toxicity.

In this context, Cd increased MDA in the HF2FF cells at any concentration and time examined. In contrast, Pb-acetate failed to increase MDA in the HF2FF cells at any concentration or time examined. GSH, an intracellular peptide is found in nearly all cell types (28), acts as an antioxidant to detoxify a variety of endogenous and exogenous free radicals. Decreased intracellular GSH levels have been implicated in the pathogenesis of a number of degenerative conditions and diseases including cancer (29). Depletion of GSH in cells therefore renders them more susceptible to oxidative damage.

The results of the 18 hr experiment with Pb-acetate in HF2FF cells suggest that the inhibition of glutathione peroxidase activity is not a sufficient stimulus for induction of lipid peroxidation. The increase in lipid peroxidation was only observed when glutathione peroxidase activity was inhibited simultaneously with glutathione depletion. This condition occurred using Cd chloride (Fig 1). The selenium-containing enzyme glutathione peroxidase can catalyze practically the reduction of all hydroperoxides, including organic hydroperoxides and hydrogen peroxide, at the expense of the donor substrate-GSH. Current knowledge about the redox status of selenium during the catalytic cycle of glutathione peroxidase has been reviewed elsewhere (30). The significant decrease in glutathione peroxidase activity in the experiments with Cd-chloride (Table 1) is probably due to interaction of this metal with selenium. The selenium concentration is then insufficient to maintain both the optimal glutathione peroxidase activity and the detoxification of the metal. The interaction of cadmium and silver with sodium selenite and the influence of selenite on the survival ratio and the retention and distribution of these metals have been studied in previous experiments on mice (31, 32). The selenium detoxification reaction with heavy metals depends probably on the metabolic reduction of selenium to hydrogen selenide (33, 34) which may react with metal to give inert metal selenide (34). The influence of silver on the activity of glutathione peroxidase which is due to its interaction with selenium has been reported in experiments *in vivo* (35). *In vitro* inhibition of this enzyme system by heavy metals has also been reported (36). Several experiments demonstrate that cadmium and lead are also able to influence significantly the activity of the important seleno-enzyme – GSH-Px *in vivo*. This inhibition of GSH-Px by metals might be considered as one of the most important biomarkers of environmental pollution. Monteiro and colleagues (17) demonstrated that individuals exposed to lead have increased antioxidant defenses (SOD and GSPx) and found a nonlinear relationship between lead and SOD. In current study, we obtained a linear correlation between these variables with repeated low doses of lead. There was, however, no significant difference in catalase activity. The biochemical basis for explaining the correlation between lead and SOD is not evident. However, the increase of the antioxidant defenses in response to lead exposure may reflect a protective response to the deleterious effects of oxyradicals. The involvement of reactive oxygen species in lead poisoning has also been addressed recently by Ercal and colleagues (36), who demonstrated a decrease in the concentrations of reduced glutathione (GSH) and an increase in the concentrations of oxidized glutathione (GSSG) and malondialdehyde in lead acetate-treated mice.

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