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Article

Effects of Lead and Cadmium on Brain Endothelial Cell Survival, Monolayer Permeability, and Crucial Oxidative Stress Markers in an *in Vitro* Model of the Blood-Brain Barrier

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Abstract: Oxidative stress, which is the loss of balance between antioxidant defense and oxidant production in the cells, is implicated in the molecular mechanism of heavy metal-induced neurotoxicity. Given the key role of lead (Pb) and cadmium (Cd) in inducing oxidative stress, we investigated their role in disrupting the integrity and function of immortalized human brain microvascular endothelial cells (hCMEC/D3). To study this, hCMEC/D3 cells were exposed to control media or to media containing different concentrations of Pb or Cd. Those exposed to Pb or Cd showed significantly higher oxidative stress than the untreated group, as indicated by cell viability, reactive oxygen species (ROS), glutathione (GSH) levels, and catalase enzyme activity. Pb also induced oxidative stress-related disruption of the hCMEC/D3 cell monolayer, as measured by trans-endothelial electrical resistance (TEER), the dextran permeability assay, and the level of tight junction protein, zona occluden protein (ZO-2). However, no significant disruption in the integrity of the endothelial monolayer was seen with cadmium at the concentrations used. Taken together, these results show that Pb and Cd induce cell death and dysfunction in hCMEC/D3 cells and, in the case of Pb, barrier disruption. This suggests blood brain barrier (BBB) dysfunction as a contributing mechanism in Pb and Cd neurotoxicities.

Keywords: blood brain barrier; reactive oxygen species; neurotoxicity; lead; cadmium

1. Introduction

Pb and Cd are two common occupational and environmental pollutants that are widely distributed. Major routes of exposure to these heavy metals include contaminated air, water, soil, and food. Pb and Cd are not physiologically or biochemically significant to humans. On the contrary, they have been implicated in a number of diseases, including central nervous system (CNS) disorders. Both Pb and Cd have been reported to be neurotoxic and to cause behavioral disorders and CNS biochemical dysfunctions [1–8].

The BBB is a potential site for neurotoxic effects of Pb and Cd. The role of the BBB is important in the development of neurological disorders seen in heavy metal poisoning because the BBB serves as the conduit by which neurotoxins enter the brain through circulation. Cd can be uptaken from the nasal mucosa or olfactory pathways into the peripheral and central neurons and cause toxicity [9,10]. Cd is also transported across the BBB by the zinc transporter and is more rapidly transported across the blood-brain and testis barriers in Cd-sensitive than in Cd-resistant strains [11,12]. Cd is reported to increase the permeability of the BBB in rats [5,13] and to penetrate and accumulate in the brain of developing and adult rats [7,14]. This leads to intracellular accumulation, cellular dysfunction, and cerebral edema. Similarly, lead is reported to cross the BBB and increase ROS production [15–17] by accumulating in the astrocytes, and impairing cell functions and perturbing glial-neuronal interactions [3,18,19].

Increasing evidence indicates that multifactorial mechanisms might be involved in metal-induced toxicity and it is suggested that one of the well-known mechanisms is production of metal-induced ROS [20–22]. As Pb and Cd are redox-inactive elements, they cannot induce ROS production directly. However, they have a high affinity for sulfhydryl groups [20] and, therefore, binding to critical sulfhydryl residues of glutathione (GSH) could alter the thiol-redox status and disrupt cellular function. They can also modulate the activity of several proteins by binding to their sulfhydryl groups, and may also inhibit expression of antioxidant enzymes such as superoxide dismutase (SOD) and catalase [23]. These metals decrease the activities of antioxidant enzymes, possibly by replacing their active site biometals and binding to their sulfhydryl groups [24]. In addition, these metals increase the free iron/copper concentration, possibly by its replacement in various proteins, which increases the cellular amount of free redox active metals, and thereby enhancing ROS production via the Fenton reaction [25]. Alternatively, Pb may inhibit δ -aminolevulinic acid dehydratase (ALAD) activity, leading to a buildup of δ -aminolevulinic acid [26]. The autooxidation of δ -aminolevulinic acid forms ROS, such as hydrogen peroxide, which may contribute to Pb-induced oxidative stress.

Neurotoxicity associated with Pb and Cd exposure may be the result of a series of small perturbations in the redox status of the brain. Imbalance of prooxidant/antioxidant ratio in the brain could oxidatively damage critical biomolecules. Involvement of oxidative damage to macromolecules in metal-induced toxicities is supported by several reports [20,27].

In the present study, the main goal was to investigate the role of oxidative stress in Pb and Cd-induced toxicity in human brain endothelial cells, hCMEC/D3. It is the brain endothelial cells that form the vascular BBB, which protects the brain from unregulated exposure to Cd and Pb. To achieve this goal, we measured ROS and intracellular GSH levels. Catalase activities were measured as they are key components of cellular defenses against hydroperoxides. Lastly, we evaluated changes in BBB permeability by measuring dextran permeability and trans-endothelial electrical resistance (TEER) in the hCMEC/D3 monolayer. We also studied the effect of lead and Cd exposure on the expression of the tight junction protein, ZO-2. The results of this study indicated that Pb and Cd are capable of inducing oxidative stress in brain endothelial cells and could be a mechanism by which they induce neurotoxicity.

2. Materials and Methods

2.1. Materials

High performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Cadmium chloride, lead (II) acetate trihydrate and all other chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Culture of Human Brain Microvascular Endothelial Cells (hCMEC/D3) and Toxicity Studies

As an *in vitro* BBB model, immortalized human brain endothelial cells, hCMEC/D3 (a gift from Dr. Pierre Courard), were seeded in 25 cm² tissue culture flasks (Sigma-Aldrich, St. Louis, MO, USA) and maintained in EBM-2 medium in humidified 5% CO₂/95% air at 37 °C. Culture medium was changed twice a week and endothelial cells at passages 28–34 were used in this study. EBM-2 medium (Lonza, Walkersville, MD, USA) was supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, and 2.5% fetal bovine serum (FBS), as recommended by the manufacturer. This fully supplemented medium was designated as Microvascular Endothelial Cell Medium-2 (EBM-2 MV, herein referred to as EBM-2 medium). For dosing cells with Pb or Cd, we used serum-free and growth-factor-free medium for all experiments instead of the fully supplemented media described above. Cells were treated with Pb and Cd for 48 h and 24 h, respectively, for all studies except for the intracellular ROS measurements. Our aim was to study toxicity of these metals under as nearly the same conditions as possible. However, differences in the relative toxicities of the metals required us to be flexible in the experimental designs, adapting to the special characteristics of each metal. Cd showed toxicity as soon as 24 h, whereas Pb did not show toxicity until 48 h. Therefore, studying the metals at a similar degree of toxicity required adapting the study times.

2.3. Determination of Cell Viability

hCMEC/D3 cells were treated with Pb or Cd, the medium was then discarded, and a Calcein AM assay kit (Biotium, Inc., Hayward, CA, USA) was used to determine cell viability relative to the control group. Briefly, the cells were seeded in a 96-well plate, at densities of approximately 10,000 cells per well. The cells were allowed to attach for 24 h, after which they were exposed to various concentrations of Pb, Cd, or serum free media for the respective period of time. PBS solution was used

to wash the cells once the designated time was reached. After two wash cycles, 100 μL of 2.0 μM Calcein AM in PBS was added to each well and then incubated at 37 °C. After 30 min, the fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using a microplate reader (FLOURstar, BMG Labtechnologies, Durham, NC, USA).

2.4. Intracellular ROS Measurement

Intracellular ROS generation was measured using a well-characterized probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA) [28]. DCFH-DA was hydrolyzed by esterases to dichlorofluorescein (DCFH), which was trapped within the cell. This nonfluorescent molecule was then oxidized to fluorescent dichlorofluorescein (DCF) by the action of cellular oxidants. A DCFH-DA stock solution (in DMSO) of 10 mM was diluted 500-fold in HBSS without serum or any other additive to yield a 20 μM working solution. Cells were washed twice with HBSS and then incubated with a DCFH-DA working solution for 1 h in a dark environment (37 °C incubator). The cells were washed twice with serum-free EBM-2 medium, and varying concentrations of Pb or Cd were added to cells and incubated for various periods of time. After this, the cells were washed twice with serum-free medium and 100 μL of serum-free media were added to each well. Then, the fluorescence was determined at 485 nm excitation and 520 nm emission, using a microplate reader.

2.5. Determination of GSH

Intracellular endothelial cell GSH content was determined by reverse phase HPLC, according to the method developed in our laboratory [29]. After treatment, hCMEC/D3 cell samples were homogenized in serine borate buffer (SBB). Twenty microliters of this homogenate were added to 230 μL of HPLC grade water and 750 μL of *N*-(1-Pyrenyl)maleimide (NPM) (1 mM in acetonitrile). The resulting solutions were incubated at room temperature for 5 min. The reaction was stopped by adding 10 μL of 2 N HCl. The samples were then filtered through a 0.45 μm filter (Advantec MFS, Inc., Dulin, CA, USA) and injected onto the HPLC system. An aliquot of 2.5 μL of the sample was injected for analysis using a fluorescence detector (Finnigan™ SpectraSYSTEM FL3000 Fluorescence Detector at $\lambda_{\text{ex}} = 330$ nm and $\lambda_{\text{em}} = 376$ nm). The HPLC column was a Reliasil ODS-1 C₁₈ column (Column Engineering, Ontario, CA, USA). The mobile phase (70% acetonitrile and 30% water) was adjusted to a pH of 2.5 through the addition of 1 mL/L of both acetic and *O*-phosphoric acids. The NPM derivatives were eluted from the column isocratically at a flow rate of 1 mL/min.

2.6. Determination of Catalase Activity

Catalase activity was measured according to the method described by Aebi [30]. Briefly the activity of catalase was measured spectrophotometrically at a wavelength of 240 nm in samples, following the exponential disappearance of H₂O₂ (10 mM). The catalase activity was calculated from the equation $A_{60} = A_{\text{initial}} - kt$, where k represents the rate constant, A_{initial} is the initial absorbance, and A_{60} is the absorbance after 60 s have passed.

2.7. Dextran Permeability Study

hCMEC/D3 cells were seeded onto collagen-coated inserts with a pore size of 0.4 μm at densities of 3×10^4 cells/well, and allowed to culture until a monolayer formed. The cell monolayer was then treated with varying concentrations of Pb or Cd. After this, the medium was removed to ensure that there were no dead cells on the membrane. It was then replaced with 200 μL of FITC labeled dextran, and the insert was transferred to a fresh plate well, containing 500 μL of serum-free medium. The plates were incubated for 30 min at room temperature, and 100 μL of the plate well solution were removed and transferred to a 96-well plate. Fluorescence was read with 485 nm excitation and 530 nm emission wavelengths using a microplate reader.

2.8. Trans-Endothelial Electric Resistance (TEER) Measurement

TEER measurement by EVOM voltohmmeter (World Precision Instrument, Sarasota, FL, USA) assessed the tightness of the hCMEC/D3 monolayer. hCMEC/D3 cells were seeded onto collagen-coated inserts with a pore size of 0.4 μm at densities of 3×10^4 cells/well, and allowed to culture until a monolayer formed (4–7 days). The cell monolayer was then treated with varying concentrations of Pb or Cd. After this, the old medium was replaced with 200 μL of fresh serum-free medium to get rid of any dead cells. The insert containing the cell monolayer was then transferred to a fresh plate containing 500 μL of fresh serum-free medium. The TEER reading was recorded immediately and TEER values were calculated as: Resistance $\times 0.32 \text{ cm}^2$ (insert surface area). Thus, resistance is proportional to the effective membrane. The final TEER value was obtained by subtracting the resistance of collagen-coated inserts from the resistance obtained in the presence of the endothelial cells.

Western Blot Analysis

Cell homogenates were prepared in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) and the protein concentration was estimated using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA). Briefly, 50 μg of cell homogenate were resolved by electrophoresis on a 4%–20% sodium dodecyl sulfate (SDS) polyacrylamide gel (120 v, 1.5 h) in a running gel buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 0.1% SDS. The samples were transferred to PVDF membranes by an iBlot[®] Gel Transfer Device (Life Technologies, Grand Island, NY, USA). Membranes were immunoblotted by using the SNAP i.d.[®] 2.0 Protein Detection System (Millipore, Billerica, MA, USA) with primary antibodies for ZO-2 and GAPDH (Cell Signaling Technology, Inc., Danvers, MA, USA) in 1:1000 dilution. Subsequently, the membrane was incubated in the respective secondary antibody (1:1000) for 10 min at room temperature. Final visualization was carried out with the enhanced chemiluminescence kit (Cell Signaling Technology, Inc., Danvers, MA, USA). The protein bands were quantitated by densitometry, where the band intensity ratio of the treated group over the untreated group, or control, was calculated.

2.9. Determination of Protein

Protein levels of the cell samples were measured by the Bradford method [31]. Bovine serum albumin was used as the protein standard.

2.10. Statistical Analysis

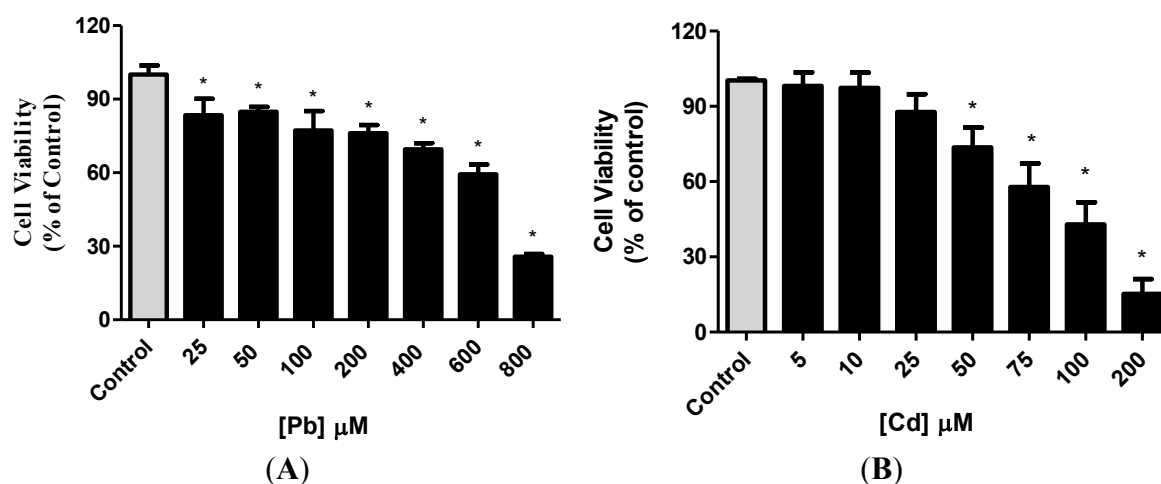
All reported values are represented as the mean \pm SD. Statistical analysis was performed using the GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistical significance was ascertained by one way analysis of variance, followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of Pb or Cd on Cell Viability

A dose-dependent decrease in cell viability was observed in hCMEC/D3 cells upon exposure to Pb or Cd (Figure 1A,B), which was confirmed using a calcein AM assay. However, Cd appears to be more cytotoxic than Pb at similar concentrations.

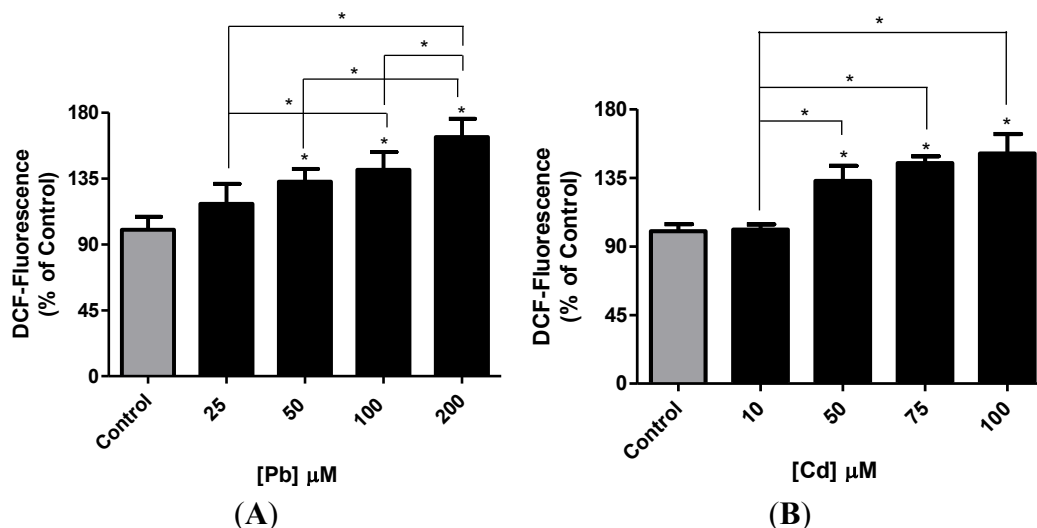
Figure 1. Cell viability of hCMEC/D3 cells. Dose-response for (A) Pb-induced cytotoxicity and (B) Cd-induced cytotoxicity. Cell viability was quantified using Calcein AM assay. Cells were exposed to different concentrations of Pb (25–200 μ M) and Cd (10–100 μ M) for 48 h and 24 h, respectively. All experiments were performed in quadruplets, and the values reported are mean \pm SD (* over the test column represents $p < 0.05$ compared to the control, and on the linking lines represents $p < 0.05$ between the two test groups).



3.2. Effect of Pb or Cd on Intracellular ROS Level

To substantiate the hypothesis that Pb or Cd were causing hCMEC/D3 cell death through oxidative stress, ROS levels were measured after the exposure of cells to Pb or Cd for 1 h. A dose-dependent increase in the production of ROS in hCMEC/D3 cells was seen with exposure to Pb or Cd (Figure 2A,B).

Figure 2. ROS levels in hCMEC/D3 cells after treatment with (A) Pb and (B) Cd. ROS levels were measured after 1 h of treatment. All experiments were performed in quadruplets, and the values reported are mean \pm SD (* over the test column represents $p < 0.05$ compared to the control, and on the linking lines represents $p < 0.05$ between the two test groups).



3.3. Effect of Pb or Cd on Intracellular GSH and Catalase Activity

To further elucidate the mechanism by which Pb or Cd induced cell death and damage, we investigated their individual effects on GSH and the critical antioxidant enzyme, catalase. GSH is one of the major intracellular thiol antioxidants in a cell. GSH levels were measured in hCMEC/D3 cells after exposure to Pb or Cd (Figure 3A,B). A lower dose of Pb at 25 μM did not increase the GSH level significantly, but treatment with higher concentrations of Pb for 48 h showed marked increases in the GSH level in comparison to control. In the case of Cd, an increase in the GSH levels was observed at all the concentrations studied. However, there was an increase followed by decrease in GSH levels. Since catalase activity is important in a cell's defense against oxidative stress, we further evaluated the role of catalase by measuring its activity in each of our treatment and control groups. The results demonstrated that exposure to Pb caused a dramatic increase in catalase activity (Figure 4A). However, there was no change in catalase activity after Cd treatment (Figure 4B).

3.4. Effect of Pb or Cd on the Integrity of the hCMEC/D3 Monolayer

Regulation of the flow of substances through intercellular spaces depends on the integrity of the BBB. Permeability studies, such as the dextran cell-permeability and TEER assays, were used in this study to assess the integrity of a monolayer of endothelial cells as they simulate the integrity of the BBB. The resistance measured across the monolayer of cells showed a decrease in resistance of approximately 6%–14% across the Pb treated groups (Figure 5A). However, for Cd treatment, an initial increase followed by a significant decrease at 100 μM Cd concentration was observed, compared to the control (Figure 5B). To further support these findings, we measured the amount of FITC labeled dextran that leaked across the monolayer, using fluorimetry after treatment. In agreement with the data from TEER, there was a 6%–60% increase in the permeability of the layer, as seen by the

increase in fluorescence across the Pb-treated groups. However, no significant changes in permeability were observed upon treatment with Cd, compared to the control. This indicated that intercellular junctions between the endothelial cells were significantly compromised with Pb treatment, but not with Cd at the concentrations studied (Figure 6A,B).

Figure 3. Intracellular GSH levels in hCMEC/D3 cells after treatment with (A) Pb and (B) Cd. Cells were exposed to different concentrations of Pb (25–200 μM) and Cd (10–100 μM) for 48 h and 24 h, respectively. All experiments were performed in quadruplets, and the values reported are mean \pm SD (* over the test column represents $p < 0.05$ compared to the control, and on the linking lines represents $p < 0.05$ between the two test groups).

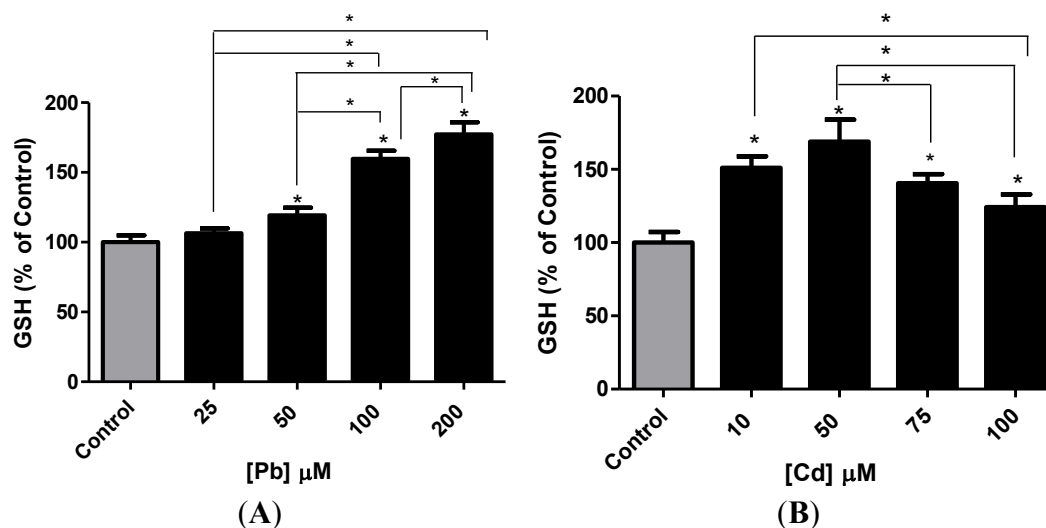


Figure 4. Catalase activity in hCMEC/D3 cells after treatment with (A) Pb and (B) Cd. Cells were exposed to different concentrations of Pb (25–200 μM) and Cd (10–100 μM) for 48 h and 24 h, respectively. All experiments were performed in quadruplets, and the values reported are mean \pm SD (* over the test column represents $p < 0.05$ compared to the control, and on the linking lines represents $p < 0.05$ between the two test groups).

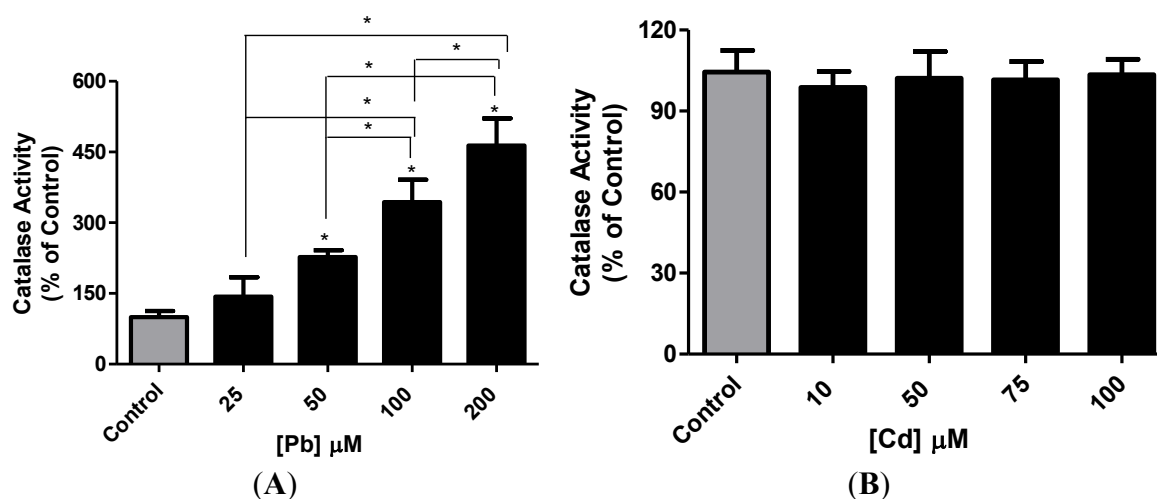


Figure 5. Effects of (A) Pb and (B) Cd on TEER in hCMEC/D3 cells. hCMEC/D3 cells were seeded onto a collagen-coated insert with a pore size of 0.4 μm at a density of 15×10^3 cells/well, and allowed to culture until a monolayer formed. The cell monolayer was then treated with different concentrations of Pb (25–200 μM) and Cd (10–100 μM) for 48 h and 24 h, respectively. Values represent mean \pm SD. All experiments were performed in triplicates, and the values reported are mean \pm SD (* over the test column represents $p < 0.05$ compared to the control, and on the linking lines represents $p < 0.05$ between the two test groups).

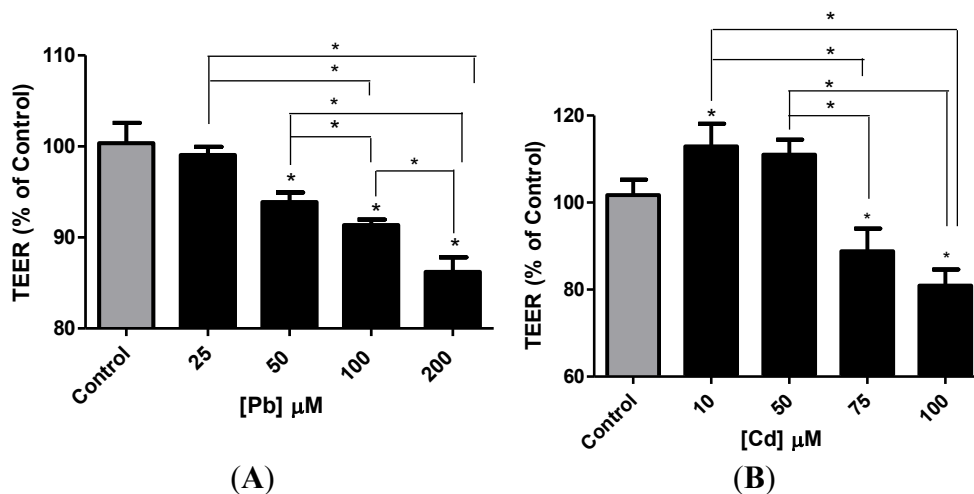
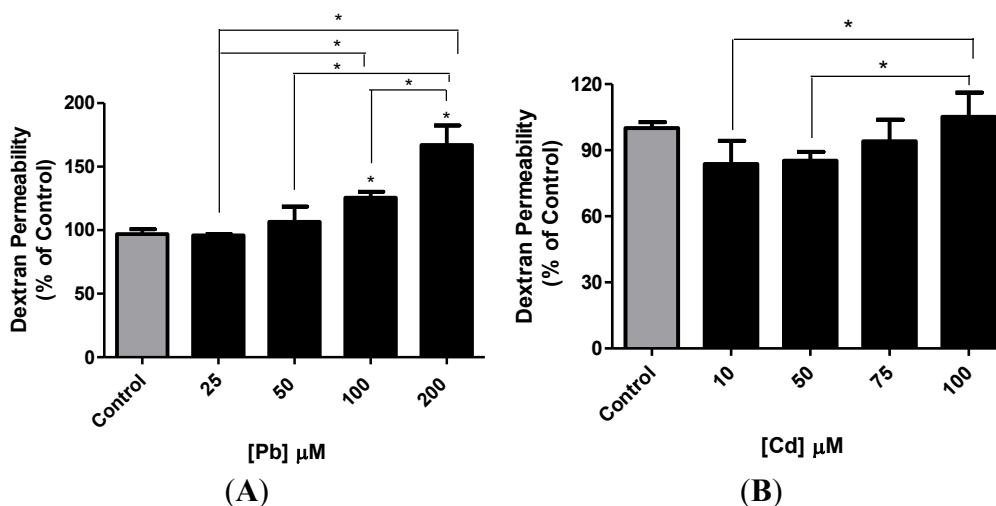


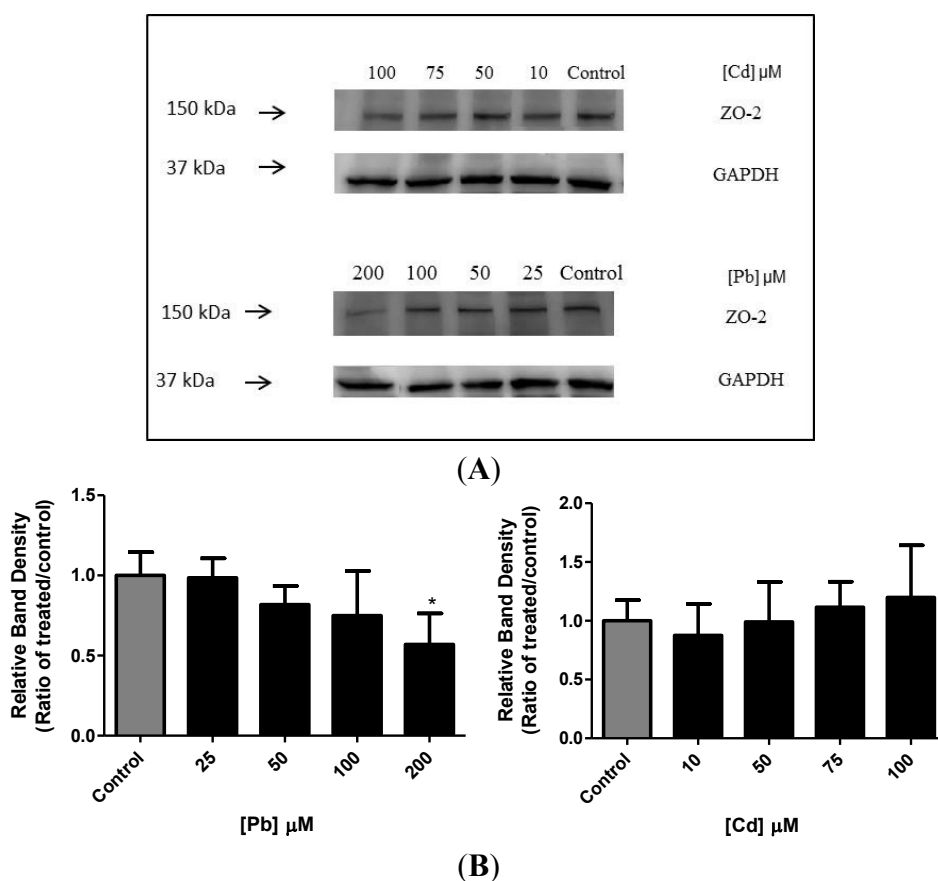
Figure 6. Effects of (A) Pb and (B) Cd on FITC-Dextran permeability in hCMEC/D3 cells. hCMEC/D3 cells were seeded onto a collagen-coated insert with a pore size of 0.4 μm at a density of 15×10^3 cells/well and allowed to grow until a monolayer was formed. The cell monolayer was then treated with different concentrations of Pb (25–200 μM) and Cd (10–100 μM) for 48 h and 24 h, respectively. Fluorescence was read with a 485 nm and 530 nm filter set. All experiments were performed in triplicates, and the values reported are mean \pm SD (* over the test column represents $p < 0.05$ compared to the control, and on the linking lines represents $p < 0.05$ between the two test groups).



3.5. Effect of Pb or Cd on the Levels of Zona Occluden Protein (ZO-2)

ZO-2 belongs to the zona occludens proteins, a family of membrane-associated guanylate kinase homologue proteins that are involved in the organization of epithelial and endothelial intercellular junctions. These proteins serve as recognition proteins for trans junction placement by linking the transmembrane strands to the actin-based cytoskeleton system. To further support the results that Pb disrupts the integrity of the BBB, the levels of ZO-2 were evaluated. A decrease ranging from 25% to 40% in the ZO-2 protein was observed upon treatment with Pb. However, no significant changes in ZO-2 levels were observed after Cd treatment (Figure 7).

Figure 7. (A) Effect of Pb and Cd on tight junction protein: ZO-2. Shown are the representative Western blots of ZO-2 in hCMEC/D3 cells treated with different concentrations of Pb (25–200 μ M) and Cd (10–100 μ M) for 48 h and 24 h, respectively. (B) The graphs represent relative densitometric analysis of treated cells over the controls. The results are representative of three individual experiments. * Values significantly different from control.



4. Discussion

Pb and Cd are neurotoxic and are implicated in the etiopathology of a growing number of neurological disorders, including Parkinson’s disease and Alzheimer’s disease [32–35]. Our previous studies [17,36,37], as well as the present one, support the hypothesis suggested by several other groups [27,38–40] that heavy metal-induced oxidative stress could be, in part, responsible for their

toxicities. In the present study, Pb and Cd each altered the oxidative stress-related parameters in hCMEC/D3 cells. Decreased cell survival in metal exposed cells was accompanied by altered antioxidant defense systems, suggesting oxidative stress as a possible mechanism for Pb- and Cd-induced damage.

Accumulating evidence indicates that heavy metal-induced neuronal toxicity is caused by the induction of ROS, which leads to oxidative stress [41,42]. Our results showed a dose-dependent increase in ROS upon treatment with Pb or Cd. These results are in agreement with previous studies that reported generation of ROS upon treatment with Pb and Cd [41,43]. This increase in ROS could be attributed to the displacement of copper and iron by Pb or Cd. The unbound free or chelated copper and iron ions then participate in oxidative stress via the Fenton reaction.

GSH is thought to be the first line of defense against metal-induced toxicity. It can act as a nonenzymatic antioxidant by direct interaction of the sulfhydryl group with ROS, or it can be involved in the enzymatic detoxification reactions for ROS, as a cofactor, or as a coenzyme [44]. Pb and Cd are reported to bind exclusively to the sulfhydryl group [45] of GSH. Our results suggest that a compensatory mechanism operates to overcome the toxicity of Pb and Cd by maintaining a high concentration of GSH in the cells. Several studies have indicated an alteration (increase or decrease) in GSH levels upon exposure to Pb or Cd. An increase [46] as well as a decrease in GSH levels has been reported upon Cd exposure [47]. Similar alterations have been observed in the case of Pb treatment. Increased GSH synthesis in Pb-poisoned rats [48] has been reported, along with a decrease in GSH levels in various organs, including the brain [17,36,39,49]. This can be explained by an initial Pb or Cd-induced GSH synthesis to counteract the stress. However, during prolonged metal exposure or at higher doses, as oxidative stress continues, GSH synthesis cannot efficiently supply the demand; therefore, GSH depletion occurs. Furthermore, the antioxidant enzymes may become inactive from direct binding of the metal to the enzyme's active sites, if the sites contain sulfhydryl groups.

Catalase is a major antioxidant enzyme, with heme as the prosthetic group, and functions by enzymatically converting hydrogen peroxide to molecular oxygen and water. Catalase activity was modulated differently by Pb and Cd exposure in our study. A significant increase in catalase activity was observed in Pb-exposed cells, whereas no change was observed in the case of Cd exposure. The literature contains contradictory evidence concerning the effects of Pb or Cd on catalase activity. Some studies have reported elevated catalase activities, indicating the involvement of metal-induced reactive oxygen intermediates, where enhancement of activity could be a compensatory mechanism to increase the detoxification process and protect cells [2,50,51]. In contrast, others observed decreased catalase activity, which may be attributed to the reduced absorption of iron or the inhibition of heme biosynthesis [39,52]. Our results with Pb exposure are consistent with previous findings, which reported significant increases in catalase activity upon Pb exposure [53]. Waisberg *et al.* [54] reported both increased and decreased catalase activities under different exposure conditions.

The BBB is essential for the normal function of the CNS. It is formed by specially modified endothelial cells that confer the BBB with low permeability and high TEER. Under physiological conditions, the integrity of the BBB is protected from oxidative stress because the BBB has high levels of antioxidant enzymes [55]. Oxidative stress is one of the important mechanisms responsible for the disruption of the BBB. This disruption allows the passage of toxic substances into the brain, leading to the development of and progression of various neurological diseases [56]. It was, therefore, crucial to

determine the potential for Pb or Cd to alter hCMEC/D3 permeability. Pb induced a dose-dependent decrease in TEER with a corresponding increase in permeability. However, for Cd, an increase followed by a decrease in TEER was observed, along with modulation in permeability. This can be explained by an initial Cd-induced increase in TEER to counteract the stress. However, at higher doses, this defense mechanism fails and therefore, decrease in TEER occurs. These results indicate the potential for Pb and Cd to disrupt cellular homeostasis and BBB integrity under severe oxidative stress conditions.

Our results with Pb are in agreement with a previous study that reported decreased TEER in a dose-dependent manner in both the RBE4-astrocyte model and the BMEC-astrocyte model upon Pb exposure [57]. Impaired BBB integrity following Pb exposure has been demonstrated by the Laterra and Bressler groups at Johns Hopkins [58,59]. Cd-induced endothelial cell alteration in the fetal brain from prenatal exposure has been previously reported [60]. In another study, no change in permeability was observed after 30 days of Cd treatment; however, a 90-day exposure increased the permeability of the BBB [61]. Exposure to either Pb or Cd has also been associated with leakage of the BBB *in vitro* [57,62] and *in vivo* [63].

Exposure to heavy metals has been linked to neurodegenerative changes, and one of the most critical factors in the development and progression of these changes is the loss of integrity of the BBB [56]. The BBB, composed primarily of the brain microvascular endothelial cells, forms a tight seal due to the presence of well-developed tight junctions (TJ) that restrict the entrance of circulating molecules and immune cells into the brain. These TJ proteins are not only involved in paracellular transport [64], but also play a role as signaling molecules involved in actin cytoskeleton reorganization [65]. TJ proteins are also highly sensitive, and respond to the changes in their microenvironment by alteration and dissociation of the occludin/ZO complex, leading to impairment of the BBB [66]. In the current study, a decrease in the levels of ZO-2 protein has been observed in cells treated with Pb, pointing to the alteration of BBB permeability in our model. However, no change in the level of ZO-2 was observed with Cd exposure in our model. Decrease in ZO-2 protein has been reported upon Pb exposure [67]. Pb is reported to increase barrier permeability by increasing intracellular calcium levels, possibly by rearrangement of scaffold proteins and their actin cytoskeleton support [68].

Apart from oxidative stress, these metals may also induce neurotoxicity by antagonizing various physiologic actions of calcium, including disruption of calcium signaling pathways and cadherins. They may modify calcium channels and prevent passage of calcium, which are important secondary messengers in the signaling pathways. They may also disrupt the function of cadherins, which are calcium-dependent cell adhesion molecules. Cadherins have typical extracellular domains and mediate adhesion via calcium-dependent interactions. Cadherins are important determinants of endothelial cell contact integrity and permeability. Cd is reported to disrupt intercellular junctions in a wide variety of epithelial cells from various tissues and species [69,70]. Displacement of calcium from these critical sites by Pb and Cd may disrupt the cell-cell interactions in nervous tissue and increase permeability. Susceptibility to endothelial injury may vary in different cell types depending on the levels and patterns of cadherin expression and may also vary with age, strain, and species of animal. The finding that metals may disrupt these barriers warrants further research in this direction.

5. Conclusions

In summary, results from the present study indicated that exposure to Pb or Cd altered the thiol redox status. This may affect the antioxidant defenses in the brain's microvasculature and lead to BBB dysfunction, resulting in more metal entering the brain. The neurotoxic effects of Pb and Cd were multi-dimensional as they were associated with both biochemical and functional changes in the endothelial cells. Our results indicate that both Pb and Cd have the potential to disrupt the integrity of the hCMEC/D3 monolayer. Toxicity induced by mechanisms other than oxidative stress may be the reason for variations in susceptibility to different metal ions. It appears that both Pb and Cd are toxic at low μM concentrations, with Cd being more toxic than Pb, as reflected by lower cell viability and a more pronounced decrease in TEER at lower concentrations compared with Pb and at a relatively shorter incubation time. Interestingly, we did not see any increase in permeability in the case of Cd treatment, which could be due to intact tight junctions and cell membranes, despite cell death after 24 h. Studies with longer treatment times would help evaluate the changes in permeability, if any. In this initial study, monocultures of hCMEC/D3 were desirable to study the direct effects of Pb and Cd on brain endothelial cells. However, to simulate the potential alterations in BBB structure and permeability, a co-culture model with pericytes and astrocytes would be more appropriate for further studies.

Although toxic metal exposure has been strictly regulated in the U.S., it is still a major concern in developing countries. This is especially true for children because of their developing brain. Measures should be taken to reduce heavy metal exposure in the general population in order to minimize the risk of adverse health effects on children as well as adults.

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Author Contributions

Shakila Tobwala designed and conducted experiments, analyzed data, and wrote the manuscript. Hsiu-Jen Wang and Joshua Warren Carey conducted experiments. Nuran Ercal and William A. Banks revised the paper critically for important intellectual content. All authors have seen and approved the manuscript.

Conflicts of Interest

The authors have no financial, consulting, or personal conflicts of interest pertaining to this work. Ercal is supported by Richard K. Vitek endowment.

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