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Early lead exposure increases the leakage of the blood–cerebrospinal fluid barrier, *in vitro*

Lewis Zhichang Shi and **Wei Zheng***
School of Health Sciences, Purdue University, West Lafayette, IN 47907, USA

Abstract

The cell type blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier (BCB) is entirely different, ie, endothelia in BBB and epithelia in BCB. Nonetheless, both barriers share a common character – the tight junctions (TJ) between adjacent cells. This study investigated the consequence of lead (Pb) exposure on the tightness of BCB. In an *in vitro* BCB transwell model, using immortalized choroidal epithelial Z310 cells, we found that early exposure to Pb (prior to the formation of tight barrier) at 5 and 10 μM, significantly reduced the tightness of BCB, as evidenced by a 20% reduction in transepithelial electrical resistance (TEER) values ($P < 0.05$), and > 20% increase in the paracellular permeability of [^{14}C]sucrose ($P < 0.05$). Exposure to Pb after the formation of tight barrier, however, did not cause any detectable barrier dysfunction. RT-PCR and Western blot analyses on typical TJ proteins revealed that Pb exposure decreased both the mRNA and protein levels of claudin-1, with the membrane-bound claudin-1 more profoundly affected than cytosolic claudin-1. Pb exposure, however, had no significant effect on ZO1 and occludin. These data suggest that Pb exposure selectively alters the cellular level of claudin-1, which, in turn, reduces the tightness and augments the permeability of tight blood–CSF barrier. The immature barrier appears to be more vulnerable to Pb toxicity than the mature, well-developed, brain barrier, the fact possibly contributing to Pb-induced neurotoxicity among young children.

Keywords

blood–brain barrier; blood–CSF barrier; claudin-1; lead (Pb); permeability

Introduction

Lead (Pb)-induced toxicity remains a major public health issue, not only in developing countries, but also in many developed countries.[1,2] The metal is still used as a gasoline additive, present in certain types of batteries, as a unique chemical additive in the production of tableware, and applied in some ammunitions and hair dye products.[3,4] Pb toxicity has been associated with altered liver, kidney, lung, cardiovascular, and/or immune functions.[5,6] Yet, the primary target for Pb toxicity is the central nervous system, particularly in children with clinical manifestations of impaired neurobehavioral development, cognitive deficiency, and low intelligence.[7-9]

For Pb to enter the brain, the metal must first pass across brain barriers that safe-guard the homeostasis of the central nervous system, ie, the blood–brain barrier (BBB), between the blood and brain interstitial fluid, and the blood–cerebrospinal fluid barrier (BCB), between

*Correspondence: Wei Zheng, PhD, Professor of Health Sciences, Purdue University School of Health Sciences, 550 Stadium Mall Drive, CIVL-1163D, West Lafayette, IN 47907-2051, USA wzheng@purdue.edu.

the blood and cerebrospinal fluid (CSF). Previous studies have shown that brain barriers are the targets of Pb toxicity. At high concentrations, Pb disrupts the cerebral capillary endothelial cells, as evidenced by extensive cerebral hemorrhage and extravascular distribution of albumin, which is normally excluded from the brain parenchyma because of its large molecular weight.[10-12] When Pb was administered to mice subcutaneously (2.5 and 5 μg/g), the treatment reportedly interfered with normal BBB function.[13] The toxic effect of Pb on brain barriers has been known to be age-dependent, with much higher vulnerability associated with infants and toddlers than adults.[14,15] In addition, Pb exposure has been associated with a significant accumulation in the choroid plexus in humans, as well as animal models.[16-18] The sequestration of Pb in the choroid plexus is believed to cause an altered production of transthyretin, leading to a distorted transport of thyroid hormone by the BCB.[18-21] However, the direct effect of Pb on the tightness or permeability of the BCB has never been investigated.

A common characteristic between BBB and BCB is the presence of tight junctions (TJs) between adjacent barrier cells. TJs between the endothelia of BBB or epithelia of BCB are the intricate combination of transmembrane and cytoplasmic proteins linked to an actin-based cytoskeleton system, which allows the TJ to form a seal while remaining capable of rapid modulation and regulation of substance exchange between the blood and brain fluid compartments. Typical TJ proteins include claudin-1, occludin, and ZO-1.[22-25] Claudins constitute the backbone of TJ strands, by forming dimmers and binding homotypically to claudins on adjacent cells to produce the primary seal of the TJ. Occludin functions as a dynamic regulatory protein, whose presence in the membrane is correlated with increased electrical resistance across the membrane and decreased paracellular permeability.[26,27] The TJ also consists of several accessory proteins necessary to form structural support for the TJ, such as the zonula occludens proteins (ZO1, ZO2, ZO3), which belong to the family of membrane-associated guanylate kinase-like proteins (MAGUKs). These proteins serve as recognition proteins for TJ placement by linking the transmembrane strands to the actin-based cytoskeleton system, and by serving as a supporting structure for signal transduction.[28,29] To our knowledge, whether Pb exposure alters the production and assembly of TJs at brain barriers has not been studied.

This study, using an *in vitro* BCB model based on newly immortalized choroidal Z310 cells,[30,31] aimed to investigate if and how Pb exposure affected the tightness of the BCB. To examine the sensitivity of the BCB to Pb exposure, two exposure paradigms were used: (1) early Pb exposure prior to the formation of TJs; and (2) late Pb exposure post the formation of TJs. The functional integrity of *in vitro* BCB model was assessed by examining the transepithelial electrical resistance (TEER) and the [^{14}C]sucrose permeability. The effect of Pb exposure on the expression of typical TJ proteins was analysed by conventional RT-PCR and Western blot, in order to reveal the mechanism of Pb toxicity on brain barriers.

Materials and methods

Materials

Chemicals and assay kits were obtained from the following sources: Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, gentamycin from Gibco (Grand Island, NY); methylthiazolyldiphenyl-tetrazolium bromide (MTT), epidermal growth factor (EGF), collagen solution (Type I), rabbit anti-mouse actin antibody from Sigma (St Louis, MO); dexamethasone from Calbiochem (San Diego, CA); [^{14}C]sucrose (specific activity: 55 mCi/mmol) from Moravek Biochemicals (Brea, CA); RNeasy mini kit and RNase-free DNase set from Qiagen (Valencia, CA); $MgCl_2$ solution, PCR buffer, dNTP, Oligo dT and MuLV reverse transcriptase from Applied Biosystems (Foster City, CA); polyclonal rabbit anti-human claudin-1, anti-human occludin, and anti-

human ZO-1 from Zymed (San Francisco, CA). The ECL detection system was from Amersham Biosciences (Piscataway, NJ). Western blot stripping buffer was from Pierce (Rockford, IL). Transwell-COL culture wells were purchased from Costar (Cambridge, MA). The Eco-lite cocktail was from MP Biomedicals (Irvine, CA).

In vitro BCB transwell model and Pb treatment

The characteristics of immortal rat choroidal epithelial Z310 cells have been described in a previous publication.[31] For normal growth of Z310 cells, the cells were maintained in a growth medium containing DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL of gentamycin, in a humidified incubator with 95% air–5% CO_2 at 37°C. The cells were passaged twice a week.

Transwell devices with an insert (12 mm in inner diameter with and polyester permeable membrane) were used for trans-barrier study. The permeable membrane attached to the bottom of the insert, which was designated as the inner chamber, was pre-coated with 0.1% collagen for 4–5 hours in a sterile hood. An aliquot of 2.0×10^5 Z310 cells suspended in 0.9 mL of the normal growth medium, supplemented with 1 μM dexamethasone, was added to the inner chamber, which was inserted into the outer chamber, containing 1.3 mL of the same medium. The culture continued for 48 hours and the medium was changed every other day thereafter.

For the early Pb exposure study, 24 hours after seeding Z310 cells in the inner chamber, the above described normal growth medium was replaced with normal growth media plus 5 and 10 μM of Pb acetate as the exposed groups, or 10 μM of Na acetate as the control, in both inner and outer chambers. For the late Pb exposure study, Z310 cells were cultured in the normal growth medium until the formation of cell monolayer (~6 days). The cells were then rinsed with 1 × PBS (pH = 7.4) and serum free medium (SFM) once, and continued to culture in the SFM containing 5 and 10 μM of Pb acetate as the exposed groups, or 10 μM of Na acetate as the control, in both inner and outer chambers, for the remainder of the experiment. SFM was composed of DMEM medium plus 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin, 100 μg/mL gentamycin, 10 ng/mL epidermal growth factor (EGF), 5 μg/mL of insulin and transferrin, 5 ng/mL of sodium selenite and fibroblast growth factor (FGF), and 50 ng/mL prostaglandin E1.[20]

Determination of barrier permeability

The formation of the monolayer barrier was judged by three criteria: (1) the cell grew to a confluent monolayer without visible spaces between cell clusters under an inverted light microscope; (2) the height of the culture medium in the inner chamber had to be at least 2 mm higher than that in the outer chamber for at least 24 hours; and (3) a constant TEER value across the cell monolayer was obtained. The TEER value was measured using an epithelial volt-ohmmeter (EVOM; World Precision Instruments, Sarasota, FL) after cells were cultured in the transwell chambers for at least 2 days. The net value was calculated by subtracting the background value, which was measured on a collagen-coated, cell-free chamber (blank), from the value of a cell-seeded chamber.

The confluent cultures, evidenced by the aforementioned three criteria, were used for the [^{14}C]sucrose permeability study, to investigate the paracellular diffusion across the cell monolayer. All the transport studies were performed in SFM at 37°C.[20] An aliquot (6.4 μL) of [^{14}C]sucrose stock solution (in 2% ethanol, specific activity: 495 mCi/mmol; concentration: 0.1 mCi/mL; Moravek Biochemicals, Inc., Brea, CA) was added into the outer chamber to a final concentration of 1.0 μM. An aliquot (20 μL) of SFM was immediately removed from the outer chamber and designated as C_D. At each time point (3,

5, 15, 30, and 45 min, 1 hour and 2 hours), aliquots (20 μL) of SFM were removed from the inner chamber (ie, the receiver chamber) and replaced with an equal volume of freshly prewarmed SFM, in order to keep the hydrostatic pressure. All the samples were mixed with Eco-lite cocktail and counted with a Packard Tri-Carb 2900 TR liquid scintillation analyser (counting efficiency for ^{14}C: 95%). The permeability coefficients (P_E) of [^{14}C]sucrose across the Z310 cell monolayer were calculated as follows. The slopes ($\Delta C_R/\Delta t$, mg/mL per minute) from the linear range (< 60 min) of time course were obtained by linear regression analyses. Each data set was used to calculate the total and blank permeability coefficients by Equation 1:

$$P_T \text{ or } P_B = \frac{V_R}{A * C_D} \Delta C_R / \Delta t$$

where P_T represents the total permeability coefficient (cell monolayer+membrane+coating, cm/min); P_B, the blank permeability coefficient (membrane+coating, cm/min); V_R, volume of the receiver (0.9 mL); A, surface area of transport membrane (1.1 cm^2); C_D, sucrose concentration in donor chamber (mg/mL); C_R, sucrose concentration in receiver chamber (mg/mL); and t, time (min). The permeability coefficient of epithelial barrier (P_E) is then obtained from Equation 2:[32,33]

$$\frac{1}{P_e} = \frac{1}{P_T} - \frac{1}{P_B}$$

Assessment of cell viability following Pb exposure

At the end of the transport study, each transwell was rinsed three times with ice-cold 1 × PBS (pH = 7.4). An aliquot (200 μL) of methylthiazolyldiphenyltetrazolium bromide (MTT) stock solution (2 mg/mL in PBS) was added to each insert and incubated at 37°C for 4 hours. After removal of MTT solution and rinsing the inserts with 1 × PBS three times, the membranes in the inserts were peeled off and placed in Eppendorf tubes. An aliquot of acidic isopropanol (0.1 MHCl in absolute isopropanol) was added to the samples. The absorbance was measured at 570 nm with DU 530 UV/Vis spectrophotometer (Beckman Coulter, Fullerton, CA).

Reverse transcriptase (RT)-PCR of claudin-1, ZO1, and occludin

Z310 cells were cultured in the normal growth medium to about 70% confluence (observations under an Olympus inverted light microscope) on 35-mm tissue culture dishes, before exposure to Pb acetate (0.1, 1.0, and 10 μM), or Na acetate (10 μM) for 24 hours. Total RNAs were isolated from cells using the RNeasy mini kit and RNase-free DNase set from Qiagen, and reverse-transcribed with MuLV reverse transcriptase (Applied Biosystems) and amplified by PCR reactions using the synthesized primers, for claudin-1, 5′-GCA TGC CTT AAC ACA GAG AAA CGG-3′ (forward), 5′-AAA CCC TGC TCC CGC ACG TAA CTT-3′ (reverse), which yielded a 339 bp product; for ZO1, 5′-CAG CCA AGG CCT GCA TAG CCA TGG-3′ (forward), 5′-ACG CAG GAG CCT CGC CCC GCA GCT GCA-3′ (reverse), which yielded a 312 bp product; for occludin, 5′-AGG AAC ACA TTT ATG ATG AAC AGC-3′ (forward), 5′-ACG GAC AAG GTC AGA GGA-3′ (reverse), which yielded a 293 bp product; and for housekeeping gene GAPDH, 5′-TTC CGC AAG TTC ACC TAC C-3′ (forward), 5′-CGG GCC GGC CAT GCT TTA CG-3′ (reverse), which yielded a 361 bp product. Each PCR cycle was composed of denaturing (94°C, 30 s), annealing (30 s), and extension (72°C, 45 s). Annealing temperatures of PCR setting depended on the specific requirement of used primers. Minus RT and minus template

controls were included to check the contamination of genomic DNA in the RNA preparations and in the PCR reagents. The RT-PCR products were electrophoresed on 2% agarose gel and photographed with a Polaroid camera. The band density was analysed with UN-SCAN-IT software (Silk Scientific, Inc. Orem, Utah), and the values were normalized to those of GAPDH in the same samples. The percentage decreases or increases in Pb-exposed samples were estimated based on the values of controls.

Western blot analyses of claudin-1, ZO1, and occludin

Z310 cells with about 70% confluence in cultures were treated with either 10 μM Na acetate (as the control group) or 5 and 10 μM Pb acetate (as the treatment groups) for 24 hours. Total proteins were isolated by harvesting cells in 1 mL homogenization buffer containing 5 μM pepstatin A, 10 μM leupeptin, 7.5 μM *trans*-epoxysuccinyl-l-leucylamido(4-guanidino) butane (E-64), 25 μM bestatin, 0.4 μM aprotinin, 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium vanadate, 50 mM NaF, 1 mM beta-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM 2-mercaptoethanol (ME), 1.0% Triton X-100, 150 mM NaCl, and 0.1% SDS in 20 mM Tris–HCl buffer, pH 7.5. The preparation was rocked on ice for 30 min to solubilize the membrane-bound fraction of TJ proteins, followed by sonication using Sonifer Model 250 at duty cycle 20% and output four to six for 25 pulses. After centrifugation at 10 000 × *g* for 20 min at 4°C, the supernatant was collected and stored, as the total protein preparation, in −70°C until use.

Protein concentration was determined by the Bio-Rad method. Equal amounts of proteins were loaded onto 10–20% Tris–HCl SDS-PAGE gel (Bio-Rad) for claudin-1 (22 kDa) and occludin (65 kDa) studies, and onto 4–20% Tris–HCl SDS-PAGE gel (Bio-Rad) for ZO1 (225 kDa) studies. After electrophoresis, the gels were immunoblotted with the primary antibodies for targeted TJ proteins (rabbit anti-human claudin-1, -occludin, and -ZO-1; Zymed). The membranes were further treated with secondary antibodies (goat anti-rabbit IgG) and visualized using an ECL detection kit (Amersham Biosciences). For determination of internal standard β-actin (42 kDa), the members were striped with Western blot stripping buffer (Pierce), and immunoblotted with rabbit anti-mouse actin antibody. The bands were analysed using densitometry with UN-SCAN-IT software. The values of TJ proteins were normalized by those of actin obtained from the same gel. The percentage of decrease/increase in Pb-treated groups was estimated based on the control values.

For preparation of membrane-bound or non-membrane-bound fractions for claudin-1 study, the method of Jou *et al*.,[34] was followed with minor modifications. Sub-confluent Z310 cells (about 70% confluence) were treated with 10 μM Na acetate in the control group, or 1 and 5 μM Pb acetate in the Pb-treated groups for 24 hours, and scraped in 1 mL Triton X-100 extraction buffer containing 5 μM pepstatin A, 10 μM leupeptin, 7.5 μM E-64, 25 μM bestatin, 0.4 μM aprotinin, 500 μM AEBSF, 1 mM EGTA, 1 mM EDTA, 1 mM sodium vanadate, 50 mM NaF, 1 mM beta-glycerolphosphate, 1 mM PMDF, 15 mM 2-ME, 150 mM NaCl and 1.0% Triton X-100 in 20 mM Tris–HCl, pH 7.5 and incubated on ice for 20 min. After centrifugation at 10 000 × *g* for 20 min, the supernatant was collected and designated as the Triton X-100/detergent-soluble (or cytosolic) fraction. For the insoluble fraction, the pellet from the above preparation was dissolved by pipetting in 100 μL SDS buffer (replace 1% Triton X-100 with 1% SDS in the above recipe), followed by sonication with Sonifer Model 250 at duty cycle 20% and output four to six 25 pulses, and diluted with 900 μL Triton X-100 extraction buffer. The resulting preparation was further incubated at 4°C for 20 min, followed by centrifugation at 10 000 × *g* for 20 min; the supernatant was designated the Triton X-100/detergent-insoluble (or membrane bound) fraction.

Statistic analysis

Statistical comparisons were performed using oneway ANOVA (Prism 3.0, GraphPad Software Inc., San Diego, CA) or Student's *t*-test, as appropriate. A probability of $P < 0.05$ was considered statistically significant. Linear regression analyses were performed using KaleidaGraph (version 3.6, Synergy software, Reading, PA). The quality of fit was determined by evaluating the coefficient of determination (γ^2). Data were reported as mean ± SD.

Results

Exposure to Pb prior to the formation of tight barrier

Under the normal culture condition, the tight barrier in Z310 cell-based transwell model was formed between 6 and 8 days with TEER values about 90 ohm-cm^2. The TEER reached the maximum (106.59 ± 6.5 ohm-cm^2) on day 10. These values are comparable to or even better than those obtained from the transwell model based on rat primary choroidal epithelial cells (Figure 1).

For the early Pb exposure study, Z310 cells were exposed to Pb acetate or Na acetate (as the control) in the normal growth medium, 24 hours after cell seeding in the inner chamber. The exposure continued until the end of the experiment. The TEER values measured on day 12 after the initial cell seeding were significantly lower in Pb-treated groups than in controls (Figure 2). There was about 20% reduction compared to controls ($P < 0.05$).

After the TEER measurements, the same transwell preparations were used for the [^{14}C]sucrose permeability study. Consistent with the TEER measurement, Pb exposure significantly increased the [^{14}C]sucrose permeability coefficients by 20–22% compared to the control group ($P < 0.05$) (Figure 3). Noticeably, MTT assay did not show any significant colorimetric changes in Pb-treated cells compared to the controls, suggesting that Pb exposure at the current dose regimen did not cause significant cellular injury, at least not to cellular metabolic and mitochondrial functions. Hence, the alterations in the tightness of the BCB barrier appear to be associated with functional changes in the barrier rather than the reduced overall health of Z310 cells, as affected by Pb toxicity.

Exposure to Pb after formation of tight barrier

As shown in Figure 1, Z310 cells normally formed the tight barrier between 6 and 8 days in the inner chamber. Thus, the cells cultured in the inner chamber were exposed to Pb on day 6, and assessed for barrier tightness on days 8, 10 and 12 post-initial cell seeding, to assess the Pb effect on barrier function after the barrier was formed. In all tested time points, no significant changes of TEER values were observed between Pb-exposed groups and controls (Figure 2). The [^{14}C]sucrose permeability study further confirmed that Pb exposure did not affect the paracellular leakage of [^{14}C]sucrose across the Z310 barrier (Figure 3). In addition, the MTT assay at the end of Pb-treatment did not detect any significant changes in absorption between Pb-treated and control groups.

Selective inhibition by Pb of claudin-1 expression

The expression of TJ mRNAs, as they were affected by Pb exposure, was investigated by RT-PCR. Pb exposure reduced the density of bands corresponding to claudin-1, but had no effect on occludin or ZO-1 (Figure 4). By densitometry analyses, Pb exposure caused a 27% reduction of claudin-1 mRNAs (Table 1). It appears that Pb treatment selectively affected the TJ proteins.

To verify the Pb inhibitory effect on claudin-1, Western blot analyses were performed to examine the protein level of claudin-1, occludin, and ZO-1, between Pb-treated and control cells. By autoradiograph, there was an apparent decline of claudin-1 bands in Pb-treated cells, but no visible changes in occludin and ZO-1 bands (Figure 5). Analyses by densitometry using β-actin to normalize the targeted protein bands demonstrated that the total amount of claudin-1 was significantly lower in Pb-treated groups than in the controls, about 38% reduction ($P < 0.01$). No reduction of occludin and ZO-1 proteins was seen in Pb-treated cells (Table 2).

Since claudin-1 exists both in cytosol and on membrane,[30] we further separated membrane-bound fractions from cytosolic fractions to investigate Pb effect on cellular claudin-1 protein levels. As shown in Figure 6, the membrane-bound claudin-1 was significantly reduced even at a low level (1 μM) of Pb exposure ($P < 0.01$), while the cytosolic claudin-1 was not affected. At the higher exposure dose (5 μM), both cytosolic and membrane-bound claudin-1 proteins were significantly reduced, with the membrane-bound more so than cytosolic claudin-1.

Discussion

Using an *in vitro* BCB transwell model based on choroidal Z310 epithelial cells, we found that early exposure to Pb prior to the formation of intercellular TJs significantly decreased the tightness of the barrier structure. Pb toxicity to the barrier likely pertained to its selective inhibition of the expression of claudin-1, an essential TJ protein. In contrast, late exposure to Pb, ie, after the TJs were formed in the cell monolayer, did not affect the tightness of the choroidal epithelial barrier. Western blot studies further demonstrated that the membrane-bound claudin-1 was more abundantly reduced than cytosolic claudin-1 following Pb exposure.

During the early developmental stage, the intactness of brain barrier systems, via the formation of TJs between barrier cells, is critical for brain development, differentiation, and maturation.[35,36] The microvasculature of the developing brain is particularly vulnerable to Pb insult. Studies have shown that Pb exposure induces acute encephalopathy among children,[14,37] as characterized by cerebellar hemorrhage, increased blood–brain barrier permeability, and manifested edema. The Laterra and Bressler groups at Johns Hopkins also demonstrated an impaired blood–brain barrier integrity following Pb exposure.[38-40] While Pb has been known to accumulate in the choroid plexus,[41] the direct evidence of Pb action on the TJ structure in the choroid plexus has never been sought. The data presented in this report not only confirm the detrimental effect of Pb on the tight barriers, but also demonstrate that Pb exposure in choroidal epithelial cells appears to result in a decrease in the tightness and augmentation of the permeability of the blood–CSF barrier, *in vitro*.

The structure of brain barriers during the early stage of brain development is immature and rather ‘leaky’. While the formation is nearly complete in most brain areas at birth, the brain barriers remain functionally immature and permeable, and in some species, it remains in such a state long after birth.[42,43] An age-dependent maturation also occurs in the BCB.[44] During early development, the leaky structure of brain barriers is physiologically necessary to accommodate the high demand of blood-borne nutrients by the growing brain. However, this relative openness of the barriers in early life renders the brain highly susceptible to insults from exposure to toxic substances, typified by Pb neurotoxicity in young children. The results in this study demonstrate that the structure of the immature BCB is more vulnerable to Pb toxicity than well-developed, mature barrier structure. Hence, our data suggest that, in spite of the ready access of Pb to the brain at a young age, the metal, en route to brain compartments, may disrupt the barrier structure, ultimately leading to neurotoxicity.

How Pb toxicity causes the destruction of the barrier intactness is not entirely known. Possible mechanisms include the activation of protein kinase C (PKC) by Pb, which subsequently increases the transcription of immediate early response genes (c-fos and c-jun), leading to an overexpression of vascular endothelial growth factor (VEGF) and dysfunction of the BBB.[38,39,45,46] Activation of PKC by Pb has also been demonstrated in the choroidal epithelial cells,[47] although the relationship between PKC activation and tightness of the BCB has not been established. Our data clearly demonstrate a selective inhibition by Pb of the expression of claudin-1 in choroidal epithelial cells, as supported by both RT-PCR on claudin-1 mRNA expression and Western blot on the protein level. Since claudins provide the primary seals of TJs between adjacent cells,[48] the reduced claudin levels would be expected to weaken the tightness of the barrier structure. This postulated mechanism is further strengthened by the observation that the membrane-bound claudin-1 was more profoundly affected by Pb treatment. The questions remain, though, as to how Pb exposure suppresses claudin-1, and whether PKC activation or Pb effect on immediate, early response genes, is associated with claudin-1 expression/production. More in-depth studies are thus needed to understand Pb toxicity on brain barriers.

In summary, exposure to Pb prior to the formation of TJs significantly decreases the tightness of the Z310 cell-based *in vitro* barrier. The mechanism appears to be associated with a selective inhibition by Pb of the expression of claudin-1. Pb-induced damage to immature barrier may contribute to the vulnerability of children to environmental Pb.

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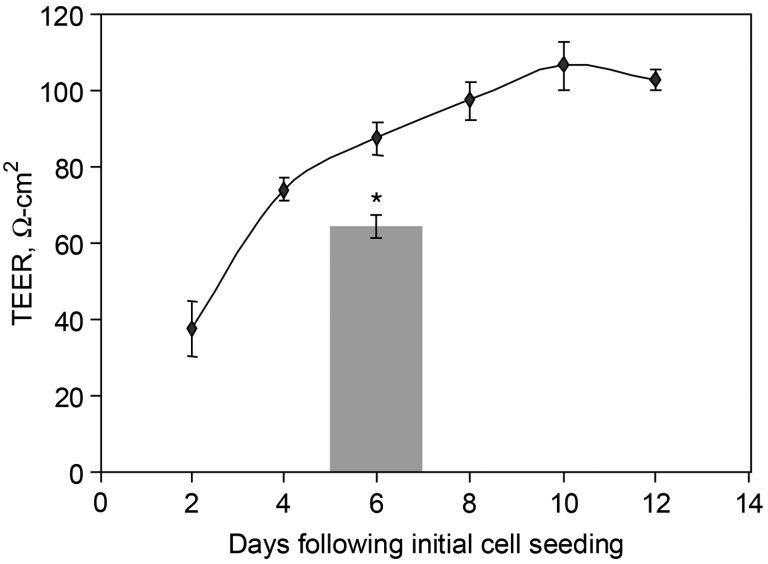

Figure 1.

Time dependence of TEER values in Z310 cell-based *in vitro* transwell model. Cells were seeded on day '0' and TEER values determined at the time indicated. The curve represents the experiments performed on Z310 cells. The inserted bar represents the value obtained from the BCB transwell model based on primary culture of rat choroidal epithelial cells. Data represent mean ± SD, $n = 3–4$. *$P < 0.05$ compared to the data in Z310 model on Day 6.

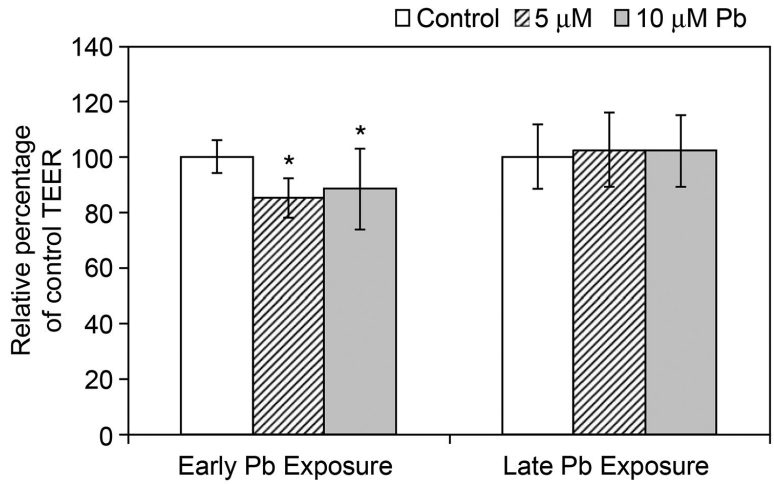

Figure 2.

Effect of Pb exposure on the TEER values of *in vitro* BCB model. For early Pb exposure study, cells were exposed to Pb acetate (5 or 10 μM) or Na acetate (10 μM, as the control) in the normal growth medium 24 hours after initial cell seeding. For late Pb exposure study, the cells were treated with Pb in the serum free medium on day 6. Media were changed every other day until the end of the experiment. TEER values were measured on day 12 after initial cell seeding. Data represent mean ± SD, $n = 3–4$. *$P < 0.05$ compared to controls.

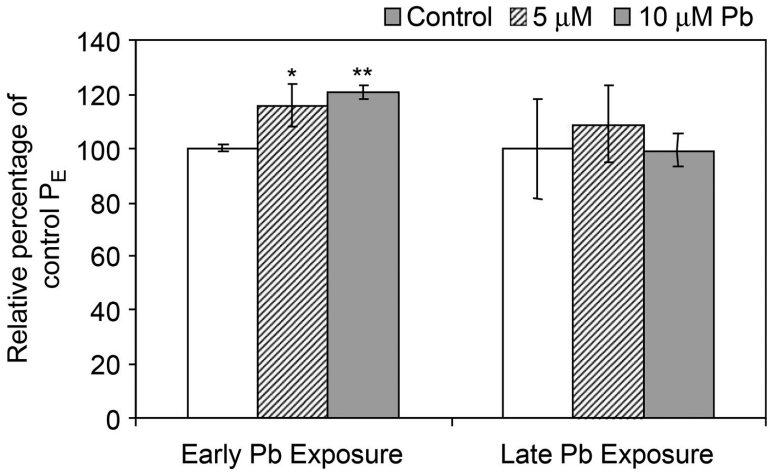

Figure 3.

Effect of Pb exposure on [^{14}C]sucrose permeability coefficient across the *in vitro* BCB model. Pb exposure paradigm (ie, early or late exposure) has been described in the legend to Figure 2. Following TEER measurements, [^{14}C]sucrose permeability was determined on day 12 for the early and late exposure group, respectively. Data represent mean ± SD, n = 3–4. *P < 0.05, **P < 0.01 compared to controls.

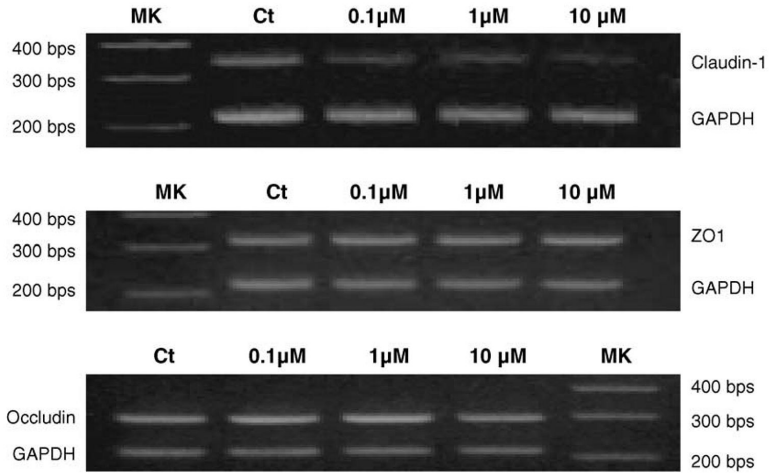

Figure 4.

Pb exposure selectively inhibited the levels of claudin-1 mRNA in Z310 cells. Sub-confluent Z310 cells (~ 70%) were exposed to Pb acetate (0.1, 1.0 and 10 μM), or Na acetate (10 μM as control) for 24 hours. The mRNA levels of claudin-1 (339 bp), ZO1 (312 bp), occludin (293 bp) and GAPDH (361 bp) were quantified by RT-PCR. Ct, control; MK, base pair marker.

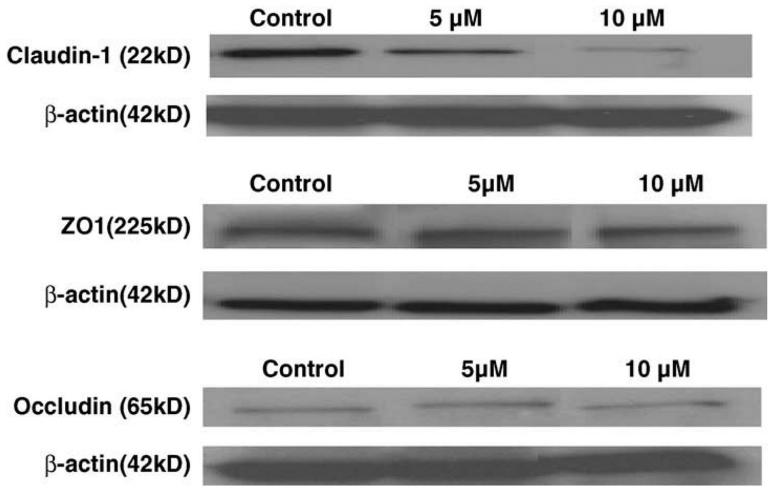

Figure 5.
Pb exposure selectively inhibited the protein level of claudin-1 in Z310 cells. Cells were exposed to Pb acetate (5.0 and 10 μM), or Na acetate (10 μM as control) for 24 hours. The protein levels of claudin-1, ZO1, occludin, and β-actin were quantified by Western blot analyses.

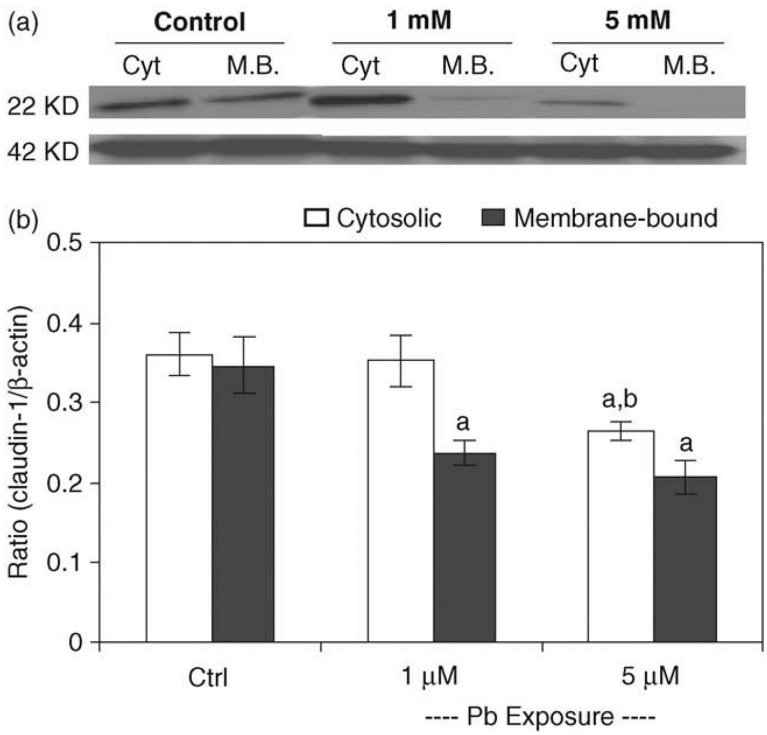

Figure 6.

Pb exposure had more profound effect on membrane-bound claudin-1 than cytosolic claudin-1. Cells were exposed to Pb acetate (1.0 and 5.0 μM), or Na acetate (10 μM as control) for 24 hours. The cytosolic and membrane-bound fractions were separated using Triton X-100, as described in the methods section. (A) A representative Western blot gel shows that bands at 22 and 42 kDa correspond to claudin-1 and β-actin, respectively. Cyt, cytosolic fraction; MB, membrane-bound fraction. (B) The protein levels of claudin-1 and β-actin were quantified by densitometry. Data represent mean ± SD, $n = 3–4$. (a) $P < 0.05$ compared to values in controls. (b) $P < 0.05$ compared to values in 1 μM Pb-treated group.

Table 1

Effect of Pb exposure on the levels of mRNAs encoding TJ proteins in Z310 cells

	Control	*Pb-exposed*		
		0.1 μM	*1 μM*	*10 μM*
Claudin-1	0.67 ± 0.03	0.51 ± 0.06*	0.51 ± 0.03*	0.49 ± 0.08*
Occludin	1.26 ± 0.18	1.16 ± 0.46	1.25 ± 0.36	1.02 ± 0.37
ZO-1	1.02 ± 0.20	1.16 ± 0.20	1.15 ± 0.19	1.14 ± 0.18

The levels of mRNA were determined by RT-PCR. The densitometry values of mRNA were normalized by those of GAPDH in the same samples and presented in the table. Data represent mean ± SD, $n = 3-4$.

*$P < 0.05$ compared to controls.

Table 2

Effect of Pb exposure on the levels of TJ proteins in Z310 cells

	Control	*Pb-exposed*	
		5 μM	*10 μM*
Claudin-1	0.37 ± 0.01	0.27 ± 0.03*	0.23 ± 0.02**
Occludin	0.22 ± 0.03	0.24 ± 0.05	0.20 ± 0.03
ZO-1	0.42 ± 0.02	0.45 ± 0.02	0.43 ± 0.04

The levels of TJ proteins were determined by Western blot. The amounts of proteins were normalized by those of β-actin in the same samples and presented in the table. Data represent mean ± SD, $n = 3-4$.

* $P < 0.01$ compared to controls.

** $P < 0.001$ compared to controls.