



Effects of lead intoxication on intercellular junctions and biochemical alterations of the renal proximal tubule cells

L.G. Navarro-Moreno^a, M.A. Quintanar-Escorza^c, S. González^b, R. Mondragón^a, J. Cerbón-Solorzáno^a, J. Valdés^a, J.V. Calderón-Salinas^{a,*}

^a Department of Biochemistry, Center for Research and Advanced Studies Center (CINVESTAV) IPN, México City, Mexico

^b Electron Microscopy Unit, Center for Research and Advanced Studies Center (CINVESTAV) IPN, México City, Mexico

^c Departamento de Bioquímica, Facultad de Medicina, Universidad Juárez del Estado de Durango (UJED), Durango, Dgo., Mexico

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ABSTRACT

Lead intoxication is a worldwide health problem which frequently affects the kidney. In this work, we studied the effects of chronic lead intoxication (500 ppm of Pb in drinking water during seven months) on the structure, function and biochemical properties of rat proximal tubule cells. Lead-exposed animals showed increased lead concentration in kidney, reduction of calcium and amino acids uptake, oxidative damage and glucosuria, proteinuria, hematuria and reduced urinary pH. These biochemical and physiological alterations were related to striking morphological modifications in the structure of tubule epithelial cells and in the morphology of their mitochondria, nuclei, lysosomes, basal and apical membranes. Interestingly, in addition to the nuclei, inclusion bodies were found in the cytoplasm and in mitochondria. The epithelial cell structure modifications included an early loss of the apical microvillae, followed by a decrement of the luminal space and the respective apposition and proximity of apical membranes, resulting in the formation of atypical intercellular contacts and adhesion structures. Similar but less marked alterations were observed in subacute lead intoxication as well. Our work contributes in the understanding of the physiopathology of lead intoxication on the structure of renal tubular epithelial cell–cell contacts in vivo.

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1. Introduction

Lead intoxication is one of the leading occupational health problems. This metal causes a broad range of biochemical, physiological and behavioral dysfunctions. Many authors propose that the formation of free radicals is the most important molecular mechanism of the lead toxicity. As a consequence, enhanced lipid-peroxidation, DNA damage, and altered calcium and sulfhydryl homeostasis may occur. Exposure to lead can result in significant alterations in multiple organs (Rendón et al., 2007). Lead toxic effects are mainly manifested in kidney as renal tubular damage and renal failure. Renal failure is associated with a reduced glomerular filtration, interstitial fibrosis and epithelial damage (Hong et al., 1980). Only one hour after intestinal absorption, lead accumulates in bone, kidney, liver and other organs (Yu, 1983; Maldonado-Vega et al., 1996, 2002) and lead is excreted via glomerular filtration and tubular secretion (Vander et al., 1979). Lead enters as a divalent cation to renal proximal tubule cells across the

apical and basement membranes, similar to calcium in other cells (Vander et al., 1979; Calderón-Salinas et al., 1999a,b).

Exposure of rats to lead in drinking water or by intraperitoneal injection induces kidney histopathological lesions, principally in proximal tubule cells (cytomegaly, karyomegaly, necrosis, vacuolization, mitochondrial swelling, lysosomal pleomorphism and nuclear inclusion bodies) (Fowler et al., 1980).

Renal function depends on cell integrity. Intercellular junctions maintain the epithelial cell polarity and the intercellular sealing between baso-lateral membranes in order to keep the asymmetrical distribution of molecules in both sides of the epithelium necessary for transepithelial transport (González-Mariscal et al., 2000; Mitic et al., 2000).

Heavy metals as cadmium affect intercellular junctions, with abnormal topological distribution of tight junction proteins such as claudin-2, claudin-3 and claudin-5 (Prozialeck et al., 2002). Lead exposure selectively alters the cellular level of claudin-1, which, in turn, reduces the tightness and augments the permeability of tight blood cerebrospinal fluid (CSF) barrier. The immature barrier appears to be more vulnerable to lead toxicity than the mature, well-developed, brain barrier, the fact possibly contributing to lead-induced neurotoxicity among young children (Shi and Zheng, 2007).

* Corresponding author. Tel.: +52 55 5747 3955; fax: +52 55 5747 3391.
E-mail address: jcalder@cinvestav.mx (J.V. Calderón-Salinas).

In this report, we provide evidence upon novel structural modifications of the intercellular junctions of the renal proximal tubule epithelial cells of chronically lead-exposed rats, and their correlations with molecular and physiological alterations. In addition, nuclear and cytoplasmic inclusion bodies were found in the renal cells of subacute lead-exposed rats.

2. Methods

2.1. Animals and intoxication schedules

Wistar male rats (90–110 g) were maintained on Diet Science (Picolab Rodent Diet 20–5058) and randomly divided into three groups of 15 rats each. The first group was non-exposed (control group); this group drank water without lead for seven months. The second group was daily intoxicated with lead acetate to obtain 500 ppm of lead as in drinking water *ad libitum* during seven months (chronic intoxication). The third group was treated with lead acetate to obtain 50 mg (0.25 mmol) of lead per kg of body weight by intraperitoneal administration every 48 h during 14 days (subacute intoxication). Previous studies indicated that these doses were the most convenient for this model, i.e. high lead concentrations induced severe damage and death (data not shown).

2.2. Determination of pH, glucose, blood and protein in urine

Rats were placed in individual metabolic cages without food and water for 12 h in the light prior to collection of urine samples over a 4 h period. Urine-testing strips (Multistix, Bayer Co.) were used for examination of hematuria, glucose, pH and protein (Keller et al., 2000; Shibata et al., 1986). Urine containing a trace or more of protein was tested quantitatively for protein by the Lowry method, with bovine seric albumin as standard (Lowry et al., 1951).

Table 1

Lead concentration in blood and kidney in rats exposed and non exposed to lead (500 ppm). Results are presented as the mean \pm the standard deviation.

Lead concentration/Group	Non exposed ($n = 20$)	Exposed ($n = 20$)
In blood ($\mu\text{g}/\text{dl}$)	4.3 ± 0.8	$43.0 \pm 7.6^*$
In kidney ($\mu\text{g}/\text{mg}$ of tissue)	0.2 ± 0.002	$7.2 \pm 1.2^*$

* $P < 0.001$ student's t -test.

Table 2

Protein, glucose and blood in urine and urinary pH in rats exposed and non exposed to lead (500 ppm). Results are presented as the mean \pm the standard deviation; the range appears in parenthesis. ($n = 20/\text{group}$).

Group	Urinary pH	Protein in urine (mg/dl)	Glucose in urine (mg/dl)	Blood in urine (erythrocytes/ml)
Non exposed	7.25 ± 0.8 (7.0–7.5)	15 ± 0.2 (0–30)	n.d	n.d
Exposed	$8.0 \pm 0.9^*$ (7.5–8.5)	$1300 \pm 200^*$ (100–2000)	50 ± 6.1 (0–100)	15 ± 2.1 (0–25)

n.d: Not detected.

* $P < 0.001$ student's t -test.

Table 3

Calcium, proline and serine incorporation in kidney proximal tubule cells in rats exposed and non exposed to lead (500 ppm). Results are presented as the mean \pm the standard deviation. ($n = 20/\text{group}$).

Group	Calcium incorporation (nmol/mg/5 min)	Proline incorporation (nmol/mg/5 min)	Serine incorporation (nmol/mg/5 min)
Non exposed	0.058 ± 0.010	0.065 ± 0.00	0.08 ± 0.005
Exposed	$0.045 \pm 0.005^*$	n.d	n.d

n.d: Not detected.

* $P < 0.001$ student's t -test.

2.3. Determination of lead in blood and kidney

Rats were sacrificed by cervical dislocation, blood was obtained by cardiac puncture and lead concentration ($\mu\text{g Pb}/\text{dl}$ of blood) was measured by voltametry in a lead-analyzer/3010B (ESA Inc.) (Matson et al., 1974; Maldonado-Vega et al., 1996). Both kidneys were removed and renal cortex was dissected and homogenized; the homogenate (1 mg protein/ml) was digested with nitric acid and suspended with Metex[®] (ESA Inc.). To minimize the matrix effect on the absorption peak, standard curves were calculated using a recovery test of 5 lead concentrations between 10 to 50 μg (recovery of 85–113%). Precision of analyses were 87–104% (using ESA high and low lead calibrators) and detection limit was 1 $\mu\text{g}/\text{dl}$ (Maldonado-Vega et al., 1996; Cortina-Ramírez et al., 2006).

2.4. Calcium, proline and serine uptake

Renal cortex was disaggregated with 1% collagenase and renal proximal tubule cells were separated in Percoll density gradients and centrifuged at 2000g (González-Mariscal et al., 2000). A sample of proximal tubule cells (1 mg protein/ml) was incubated (37 °C) in PBS plus 1 mM $^{45}\text{Ca}^{2+}$ (18 $\mu\text{Ci}/\text{ml}$), 1 mM ^3H -Proline (10 $\mu\text{Ci}/\text{ml}$) or 1 mM ^3H -Serine (10 $\mu\text{Ci}/\text{ml}$); at 1, 2, 3, 4, 5 min aliquots of 100 μl were collected and placed in tubes containing mineral oil and centrifuged 30 s at 12,000g in a Beckman centrifuge. Cell pellets were collected and suspended in scintillation liquid and the radioactivity was determined in a Beckman LS 6500 scintillation counter. Incorporation initial velocity was calculated; by slope of linearization of 1–4 min adjust as previously described (Calderón and Cerbón, 1992; Calderón-Salinas et al., 1999a,b).

2.5. Oxidative damage evaluation

Oxidative damage in renal proximal tubules was evaluated by lipid peroxidation by the thiobarbituric acid reactive species assay (TBARS). Renal proximal tubules (1 mg/ml) were washed two times in isotonic solution. 10 ml of this sample was suspended in 400 μl PBS, 25 μl butylated hydroxytoluene (BHT; added to stop additional oxidation) and 25 μl of 30% trichloroacetic acid (TCA). This solution was incubated at 4 °C for 2.5 h and centrifuged at 500g in a Beckman centrifuge. The supernatant was collected and incubated at 100 °C in 75 μl of 0.1 M EDTA and 250 μl of 1% thiobarbituric acid (TBA). The absorbance was measured in a Beckman DU-650 spectrophotometer at 520 and 600 nm, thus eliminating

non specific absorbance at 600 nm. Results are expressed as pmol of malondialdehyde equivalents per mg of protein (Trigueros-Gaisman et al., 1998). The equivalents of malondialdehyde were

calculated using a calibration curve in a matrix of renal proximal tubules and considering a molar extinction coefficient of 1.56×10^5 M/cm.

2.6. Transmission electron microscopy

Lead exposed and non-exposed rats were killed by cervical dislocation at 0 and 7 months of treatment. Kidneys were rapidly removed, immersed in dissection solution (250 mM sucrose, 2.3 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 25 mM KCl, 0.1 mM PMSF and 20 mM NaN₃) at 4 °C, and 2 mm slices of kidney cortex were dissected from the isolated kidneys. Slices were washed in PBS phosphate-buffered saline (PBS, in mM: 138 NaCl, 2.7 KCl, 8.1 Na₂HPO₄,

Table 4

Lipid peroxidation in kidney cortex in rats exposed and non exposed to lead (500 ppm). Results are presented as the mean \pm the standard deviation. (n=20/Group).

Group	TBARS concentration (pmol/mg)
Non exposed	170 \pm 10
Exposed	455 \pm 66*

* $P < 0.001$ student's *t*-test.

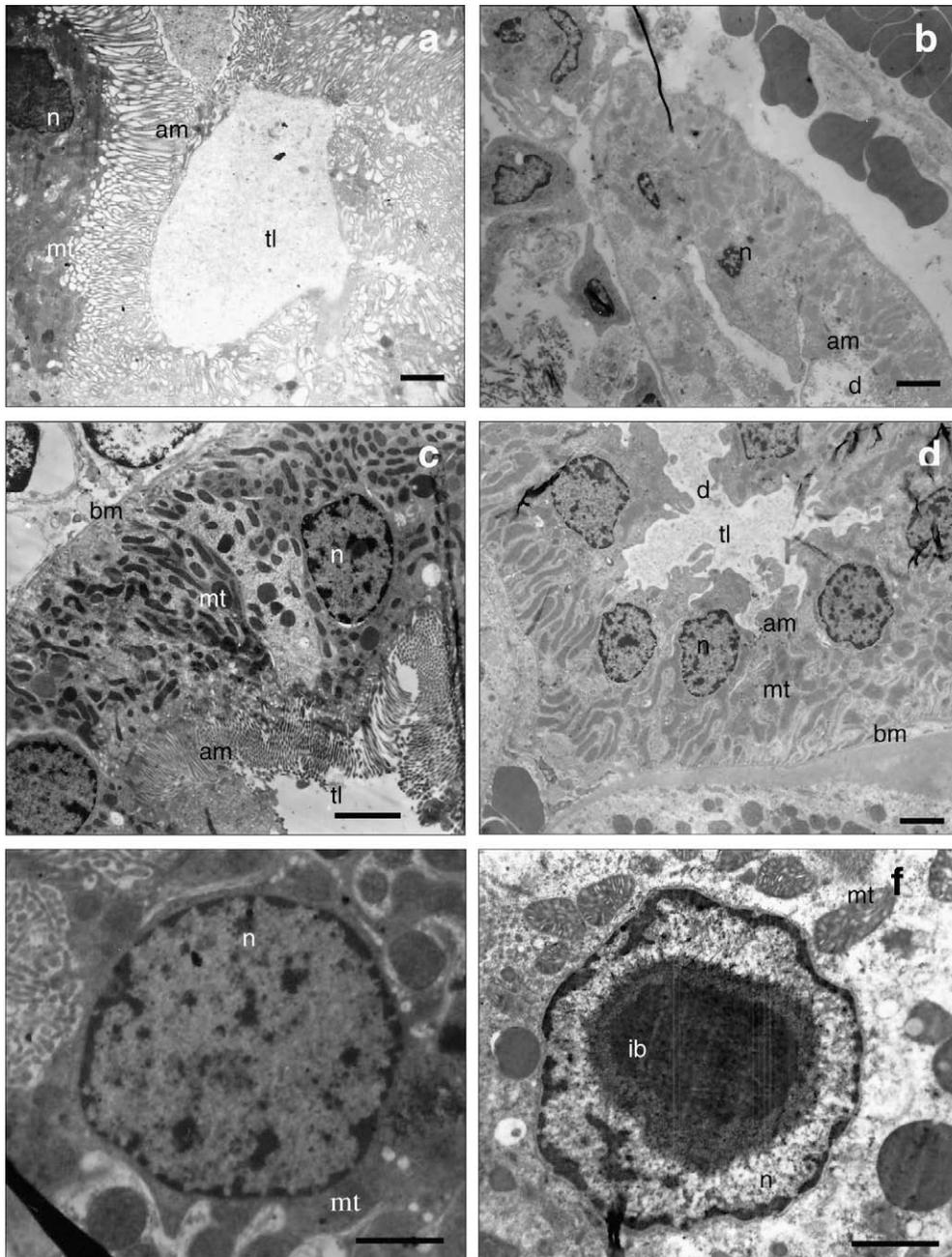


Fig. 1. Electron micrographs of proximal tubules from non-lead exposed (a, c and e) and lead-exposed rats (chronic intoxication b, d and f). Micrographs show reduction of lumen, microvillus and lost of the brush border of renal proximal tubules (a vs b), alteration in the morphology of cellular organelles (mitochondrial swelling) (c vs. d) and nuclei with intranuclear inclusion bodies (e vs. f). Tubular lumen (tl); microvillus (m); apical membrane (am); basal membrane (bm); mitochondria (mt), nuclei (n), detritus (d) and inclusion bodies (ib). Scale bar = 2 μ m.

1.1 KH₂PO₄, pH 7.0), fixed with 2.5% glutaraldehyde in PBS for 1 h at room temperature, and processed as previously described (Mondragón and Frixione, 1996); in brief, slices were fixed in 1% osmium tetroxide, dehydrated with ethanol and embedded in Spurr's resin, thin sections were obtained in a Leica ULTRACUT R (Leica Microsystems, Austria) and stained with Reynolds solution. Sections were viewed in a JEOL 2000 EX transmission electron microscope.

2.7. Statistical significance

The difference between means was compared using student's *t*-test, statistical SPSS-w version 14. The levels of significance were $P < 0.001$.

3. Results

3.1. Physiological and biochemical renal alterations in lead-exposed rats

The effect of lead intoxication on the rat kidneys was verified as increased lead concentrations in blood (10-fold) and renal cortex (36-fold) were observed in lead-exposed rats when compared to non-exposed rats (Table 1).

Also, some parameters of urinary function were altered. The urinary pH was increased (0.75 pH units) and the presence of glucose and blood were evident in urine. Protein concentration in urine was 87-fold higher in lead-exposed rats whereas only traces were found in non-exposed rats (Table 2).

Important biochemical alterations were found in proximal tubule cells of lead-exposed group. Calcium uptake was diminished 23%, and notably, proline and serine incorporation was not detected (Table 3). The oxidative damage, measured as TBARS concentration, was 2.6-fold higher in renal proximal tubules of lead-exposed animals (Table 4).

3.2. Morphological alterations on kidney proximal tubules in lead-exposed rats

Several morphological alterations in architecture, shape and cellular and subcellular organization of renal proximal tubules were observed in lead-exposed animals (Fig. 1b, d and f). In contrast to the characteristic tubular shape and the presence of typical brush border oriented toward the tubule lumen in the non-exposed rats (Fig. 1a, c and e), loss of the brush border on the apical membrane of epithelial cells and tubules with a collapsed lumen was found in the lead-exposed (Fig. 1b and d).

Characteristic structures, morphologically similar to intranuclear inclusion bodies (~5 μm structures formed by an electrodense core with a peripheral layer of fibrillar material), lysosome-like electrodense bodies distributed in all cytoplasm and notorious morphological alterations were found in cells of lead-exposed animals (Fig. 1f). In addition, severe structural damages in organelles and membranes, including the formation of membrane whirls at the basal membrane and disruption of the apical brush border membranes (Fig. 1b), mitochondrial swelling were frequently observed (Fig. 1d).

In rats exposed to subacute lead intoxication, we found small (~1 μm) lead inclusion bodies (Fig. 2a) within the nuclei, mitochondria and cytoplasm of proximal tubule cells (Fig. 2b). In contrast, inclusion bodies were not observed in mitochondria and cytoplasm in chronically-exposed rats.

3.3. Morphological alterations on intercellular junctions in proximal tubules

The main morphological alterations of cell junctions in kidney proximal tubules originated by after chronic intoxication appear in Fig. 3, and are interpreted as a possible sequence of events: (a) alterations in apical membrane with disappearance of the brush border; (b) collapse of tubule lumen; (c) rearrangement of intercellular junctions, numerous desmosome-like junctions that appear as electrodense patches located toward the apical membrane face of the epithelial cells; (d) a close interaction between apposed epithelial cells were formed as apical-apical junctions; and (e) the presence of apical-apical junctions increasing the sealing of the collapsing tubule.

4. Discussion

In the present study we exposed rats to 500 ppm of lead during 7 months and this chronic exposition induced significant alterations in renal function. The intense proteinuria and hematuria observed, indicated a glomerular failure; whereas the notably glucosuria and urinary pH alteration indicated a tubular damage, principally in the proximal tubule where reabsorption takes place.

The above mentioned physiological alterations are related with an increased lipid peroxidation in renal proximal tubule membranes of lead-exposed (Table 4).

The effect of chronic intoxication on renal proximal tubule cell membranes leads to a decrement in calcium incorporation and a total inability of proline and serine uptake, indicating that the physiology of these cells is severely affected in agreement with the magnitude of the structural changes observed. Nephropathy and renal failure in workers exposed to lead has been reported

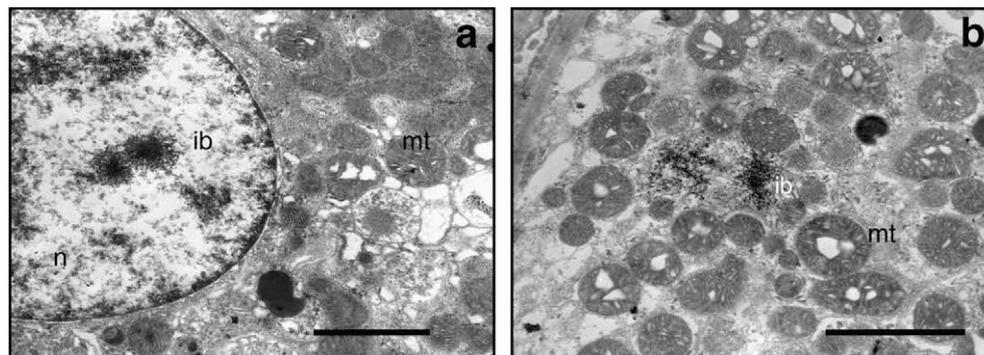


Fig. 2. Electron micrographs of inclusion bodies in nucleus (a), mitochondria and cytoplasm (b) of proximal tubule cells of rats with intraperitoneal administration of lead (subacute intoxication). Mitochondria (mt); nuclei (n); inclusion bodies (ib). Scale bar = 2 μm.

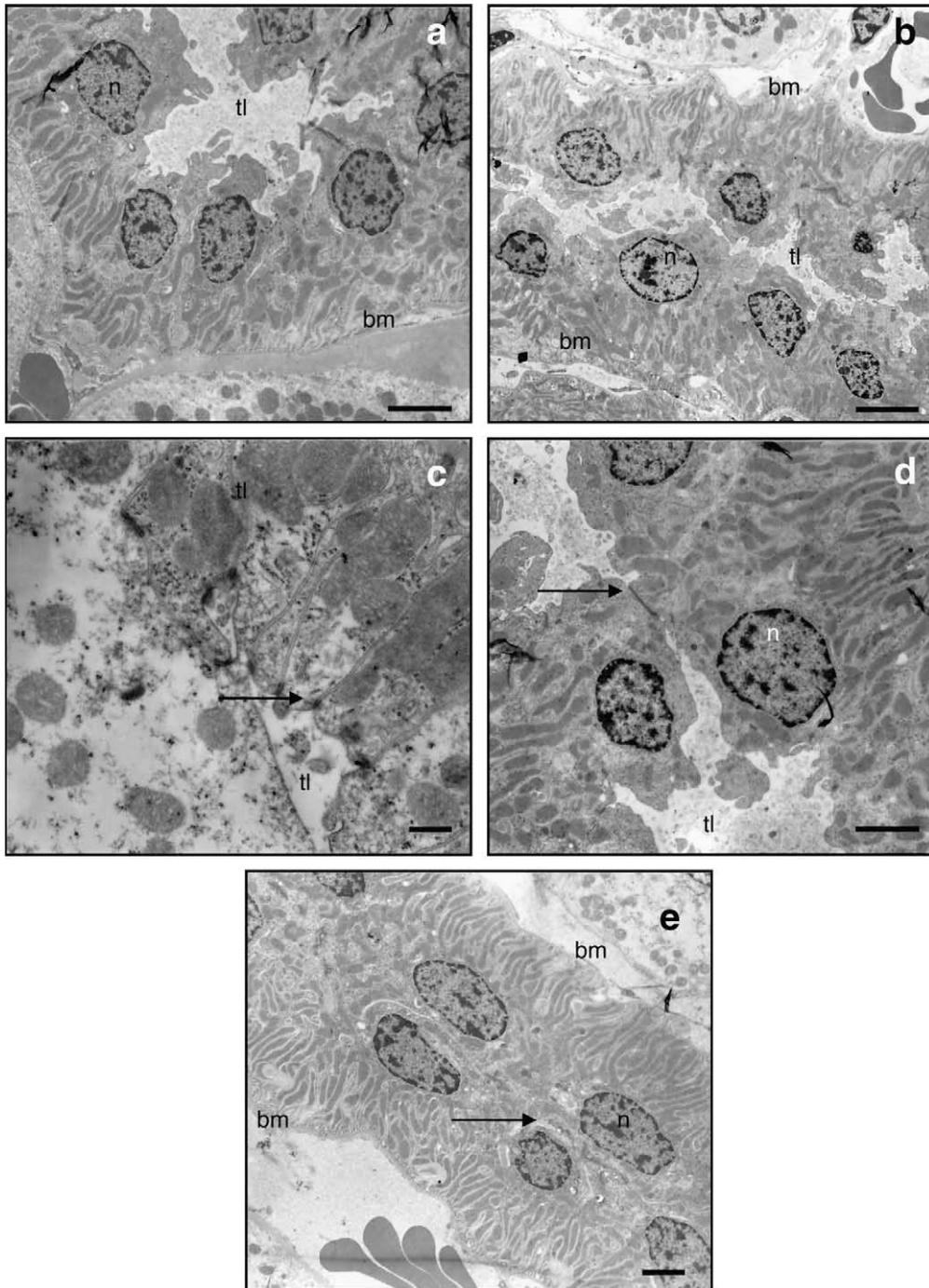


Fig. 3. Electron micrographs of intercellular junctions of proximal tubule cells of lead-exposed rats (chronic intoxication). (a) Lost of apical microvillus and changes in membrane integrity. (b) Collapse of tubule structure with intercellular apposition. (c) Formation of intercellular adhesion junctions between apposed cells. (d) Abnormal junction apical-apical membrane with formation of intercellular junctions. (e) Lost of lumen and sealing of apical membranes. Tubular lumen (tl); basal membrane (bm); nuclei (n). Arrows indicate the cell adhesion structures in abnormal junctions. Scale bar = 2 μ m.

(Hong et al., 1980) and alterations in the nucleus, mitochondria and membranes of proximal tubule cells have been observed in lead-exposed individuals (Goyer, 1968; Fowler, 1993; Bernard and Becker, 1998). The lead induces membrane damage by stereospecific-interaction protein-binding (i.e. binding to divalent cation domains), electrostatic interaction (i.e. interacting with negative domains), by interaction with sulfhydryl groups (mercaptides). It also induces oxidative damage to the membranes by accumulation of oxidant metabolites (such as aminolevulinic acid, free protoporphyrins, heme and iron ions) and by direct or indirect inhibition of antioxidant enzymes, reducing the total antioxidant protection of

the cell, affecting membrane structure and function and altering physiological processes of organs and tissues (Rendón et al., 2007). These damages are reflected in cellular structural changes and explain the close relationship between the morphological changes found in the kidneys of lead exposed animals with the molecular and physiological changes showed in this study.

In addition to the morphological damages, structural and morphological changes have been concomitant with cellular lead resistance or with a lower sensitivity to lead-induced kidney damage (Shelton and Egle, 1982; Shelton et al., 1986; Fowler, 1993). Accordingly, the presence of intranuclear inclusion bodies has been

reported in acute lead exposed rats (Oskarsson and Johansson, 1987) and in lead treated epithelial cells cultures (McLachlin et al., 1980).

Interestingly, we found small inclusion bodies (~1 µm diameter) in the nuclei, mitochondria and cytoplasm of lead-exposed animals (0.25 mmol of lead per kg of body weight by intraperitoneal administration every 48 h during 14 days, subacute intoxication). Considering that the intranuclear inclusion bodies of chronically lead-exposed rats are larger (up to 5 µm), the presence of smaller inclusion bodies in other parts of the cell suggests their initial formation, probably via cytoplasmic protein synthesis, association with lead and polymerization prior to preferential mobilization to the nucleus where they terminate their polymerization and growth and consolidate its association with lead as suggested by Shelton and Egle (1982), Shelton et al. (1986), and Fowler (1993).

These findings and the proteins able to bind lead are novel and interesting systems to study lead-protein interaction (e.g. homo- or hetero- polymerization).

Consistent with an apical-apical membrane junction structure of kidney tubule cells, an atypical orientation of intercellular junction not described previously in the literature was found in lead-exposed rats. This novel atypical cell–cell junction structure may be formed after microvillae loss and reduction or disappearance of renal proximal tubule lumen. These morphological alterations were concomitant with the biochemical changes observed and together contribute to the understanding of the physiopathological events that occur in kidney during chronic lead intoxication. The structural changes in cells and in cell–cell junctions might alter severely, polarity and vectorial transport (reabsorption and permeability) of epithelial cells (McNeill et al., 1990; Calderón et al., 1998). Changes in the distribution of specific proteins in tight junctions have been described in kidney and epithelial cells cultures when exposed to cadmium (Prozialeck et al., 2002; Pérez et al., 2004; Arreola-Mendoza et al., 2006).

The presence of lead could mimic or compete with physiological cations such as calcium. Lead can interact with proteins such as calmodulin and it can be structured and combined with salts in the bone by replacing calcium of the apatite and hydroxyapatite crystals (Dowd et al., 2008). In this way, once the lead has induced cell damage, either directly or through oxidative processes, altering the structure of tubular renal tissue, its continuous presence may establish altered lead-calcium associations on sites that require calcium for stable interactions. For instance, lead might compete with calcium in the establishment and maintenance of several intercellular junctions, including tight junctions and desmosomes (Cerejido et al., 2008). Somehow lead could modify the tight junctions, altering their role as a barrier to the lateral diffusion of membrane proteins and lipids, modifying their asymmetrical distribution. These could induce changes on intermolecular interactions of calcium dependent cadherines involved in the maintenance of desmosomes. The disanchorage of cadherines may allow their lateral membrane diffusion reaching even the apical plasma membrane. Collapse of the luminal space may facilitate the approaching of opposite cells with the formation of atypical intercellular apical-apical junctions. Further research is necessary to confirm or discard these possibilities. In order to strengthen the effect observed by microscopy, studies on intercellular junctions biomarkers (immuno-histochemistry) are being carried out in our laboratory, as well as experiments to disclose different alterations in other cells of lead exposed rats.

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