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STRUCTURAL CHANGES AND RETINAL CELL LAYERS DAMAGES INDUCED BY CDCL2 TREATMENT IN RATS

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ABSTRACT Cadmium (Cd) is a highly toxic environmental heavy metal. Thus, the effects of Cd in human and animal eyes are still under investigations. Adult male rats were divided into two groups: the first group received 200 µg Cd/L in their drinking water and 3.10^{-5} µg/kg of Cd in their food for five weeks; the second group (a control group) received regular water and standard rat chow in an identical manner.After five weeks, Cd levels were measured in retina using graphite-furnace-spectrophotometry, with values normalized to protein levels. Immunohistochemistry and western blot analysis were performed to assess the structural effects of Cd on all retinal layers. Higher Cd levels were found in the neural retina of cadmium treated animals compared to controls. Cd induced statistically significant decreases in retinal cell layers thickness and density. Cone photoreceptors were particularly affected, with reduced expression of cone opsins. Apoptotic nuclei were observed in all retinal layers of Cd treated animals. The retinal exposure to Cd caused structural changes and apoptotic effect in rat retinal layers after 5 weeks of CdCl2 treatment.

KEYWORDS : Cadmium; retina; cell damages; apoptosis.

INTRODUCTION

Cadmium (Cd) is an environmental contaminant and because of its non-decomposable character, it can be harmful in natural environment. Occupational and environmental pollution with Cd results mainly from mining, metallurgy industry and manufacture of nickel–cadmium batteries, agriculture industry, pigments and plastic stabilizers. Smoking, as well as food, water and air contaminations are also important sources of Cd intoxication in humans (Waalkes, 2003; Satarug et al., 2010; Jia et al., 2010). This pollutant causes important ecological repercussions and significant health damages to humans, animals and plants. Accumulation of cadmium into humans' organs elicits the need for public health and research organisms to develop biomarker for Cd detection into these organs (Fernandez et al., 2010; Ghnaya et al., 2007; Sharma et al., 2010). Cadmium also accumulates in various ocular tissues such as lens, retina, ciliary body, and vitreous (Erie et al., 2005; Grubb et al., 1985; Wills et al., 2008a, 2008b, 2009). Cadmium accumulation in eye tissues is associated with gender, age, smoking as well as pathological status. Elevated cadmium levels have been reported in cataractous lenses compared to clear human lenses (Ramakrishnan et al., 1995; Cekic, 1998a, 1998b). Moreover, upon cadmium exposure through intravenous injection in rats, there was a significant increase in pathological changes such as lipid peroxidation in the lens (Mosad et al., 2010). Thus, the existence of cadmium in eye lenses of human and animal models have been established but the mechanisms of cadmium-induced pathological alterations are not known yet. However, it was shown that cadmium exposure caused changes in nucleus and mitochondria of retinal photoreceptors and ganglion cells during organogenesis in Cd2+ treated mouse embryo (Yargiçoglu et al., 1999).

In our study we hypothesize that cadmium, a major smoke constituent, could cause photoreceptors changes and retinal cell layer damages. We investigate the possible mechanisms of Cd toxicity and if Cd could cause toxicity in retinal cells through induction of apoptosis leading to cell death.

MATERIALS AND METHODS

Animals and housing conditions

Twenty four 10-week-old Wistar rats weighing 222 ± 16 g were randomly divided into two experimental groups (n = 12 in each group). The control group of animals was not treated with Cd and the remaining experimental group received 200 µg Cd/L (as CdCl2) in their drinking water for 5 weeks. Exposure duration, Cd dose, as well as the administration method were chosen on the basis of the previous reports (Messaoudi et al., 2009, 2010). Animals were housed, according to the EEC 609/86 Directives regulating the welfare of the experimental animals, in individual stainless steel cages at 24 ± 1°C at a relative humidity of 45%±10%, under cyclic lighting conditions (12hrs light ± 300 lux/12hrs dark). Both groups had access to food and drinking water *ad libitum*. Concentration of Cd in the diet was 3.10-5 µg/kg dry weight. To evaluate the daily intakes of Cd in experimental group and express this as ppm/kg b.w., the 24-h consumption of drinking water and body weight were monitored during the whole experiment. Drinking water consumption and daily Cd intake were investigated according to the method described by Brzoska et al. 2008. The last day of experimentation, final body weight of each animal was recorded and rats were sacrificed.

Tissue preparation

Animals were sacrificed by C02 inhalation followed by decapitation. Whole eyes were harvested and either fixed in 4% paraformaldehyde in PBS (0,01M, pH=7.4) for 12hrs at 4°C, or the retinas were dissected out under a binocular microscope and snap frozen in liquid nitrogen and then cryosectioned using Leica CM 3050 S cryostat.

Immunohistochemistry

For immunohistochemical study, the data were analyzed after five weeks of CdCL2 treatment. The enucleated rats eyes used for immunohistochemistry were immersion fixed in 4% paraformaldehyde for 12 hrs. Eye cups were generated by removing the anterior segment. Eye cups were cryoprotected with 30% sucrose overnight, then embedded in Tissue-Tek OCT (Sakura Finetek, USA, Torrance, CA, USA). 10 µm frozen sections were cut in the sagittal (vertical) plane through the optic nerve head (Chen et al., 2003). Sections were permeabilized with Triton X-100 (0.1% in PBS for 5 min) and then saturated with PBS containing 0.3% bovine serum albumin, 0.3% Tween-20 and 0.1% sodium azide for 30 min. Sections were incubated overnight at 4°C with the following primary antibodies diluted in the same buffer used for saturation. Antibodies used were: monoclonal anti-rhodopsin antibody Rho-4D2 (Hicks and Molday, 1986), polyclonal anti-mouse MW-cone opsin (From Dr Cheryl Craft, Doheny Eye Institute, university of Southern California, Los Angeles, USA) used at 1 µg⁄mL final concentration. Secondary antibody incubation was performed at room temperature (22°C) for 2h with Alexa goat anti-rabbit or anti-mouse IgG-conjugated antibodies (Molecular Probes Ltd, Eugene, OR, USA) (5 µg⁄mL). Cells nuclei were stained with 4,6-diamino-phenyl-indol-amine (DAPI, Sigma). Slides were washed thoroughly, mounted in 1:2 ratio of PBS:glycerol and observed as described above.

Immunoblot analysis

After sacrifice of animals, neurosensory retinas were isolated and immediately frozen at -80°C. The method for western blot was performed as described before Saidi et al., 2011. Membranes were incubated with the following primary antibodies: monoclonal anti-Rhodopsin (Hicks and Molday, 1986), and anti-mouse MW-cone opsin (generous gifts from Dr Cheryl Craft, Doheny Eye Institute, university of Southern California, Los Angeles, USA). Primary antibodies were diluted into PBS buffer to a final concentration of 0.0004 µg⁄mL and incubated with the

membranes overnight at 4°C with agitation. The membranes were washed thoroughly and then incubated with goat anti-rabbit IgG-horseradish peroxidase secondary antibody diluted to a final concentration of 0.08µg⁄mL (1:10000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactive bands were visualized with chemiluminescence (Immobilon (P)Western, Millipore, Bedford, MA, USA), according to the manufacturer's instructions. Apparent molecular masses were estimated by comparison to pre-stained molecular size markers (Invitrogen-Life Technologies, Gaithersburg, MD, USA). The membranes were then incubated with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, IL, USA) for 20 min at room temperature, washed thoroughly and the same procedure as described above was performed with monoclonal anti-tubulin antibody (sc-20012; Santa Cruz Biotechnology Inc.) diluted to a final concentration of 50µg / mL. The secondary antibody was a goat anti-mouse IgG-horseradish peroxidase (0.05 µg/mL; 1:15000). Immunoreactive bands were detected by chemiluminescence (Immunobilon TM western, Millipore, Bedford, MA, USA). The relative abundance of individual proteins was evaluated by scanning densitometry (Image J, National Institute of Health, Bethesda, MD, USA).

Dosage of Cadmium

Methods for analysis of cadmium in animal ocular tissues followed protocols for measurements in human tissues that have been previously developed in the trace metal facility at UTMB (Alcock, 1987; Ramanujam et al., 1999). Digestion of tissue samples were carried out using 0.5-1.0 ml of 30% hydrogen peroxide (GFS Chemicals; Powell, OH) at 70-80 °C in a drying oven for 48-72h to a white colored ash followed by 0.1 ml of Ultra-pure nitric acid (GFS Chemicals) until completely ashed. Concentrations of Cd in the digested samples were determined by Graphite furnace atomic absorption spectrophotometry (GF-AAS). GF-AAS was performed using a Perkin-Elmer Model-5100 atomic absorption spectrophotometer equipped with a Perkin-Elmer HGA-600 graphite furnace with a Zeeman-5100 deuterium arc correction and a Perkin-Elmer-60 auto-sampler attached to the graphite furnace. These analytical methods have been used previously for analyses of metals in eye tissues (Grubb et al., 1985). Heavy metal concentrations from two eyes of the same animal were averaged and treated as one observation (total number of observations equals 12). Cadmium concentrations in control and Cd-treated animals were compared using ANOVA test. The level of significance was p<0,05.

Microscopic Evaluation and Measurement Procedures

Microscopic evaluation of retinas included scanning tissue sections for evidence of gross disease followed by morphometric analysis, which included measurements of the thickness of total retina, outer nuclear layer, inner nuclear layer and ganglion cell layer. Thickness measurements were made in the posterior retina at eight points, four on either side of the optic nerve that were approximately 200 to 300 µm apart. These measurements were then averaged to yield a measurement for that particular section. For each animal analyzed, four separate eye sections were measured. All measurements were obtained with a microscope and digital camera (Zeiss LSM10).

In Situ Detection of DNA Fragmentation by TUNEL Assay

The TUNEL assay was performed using the *in situ* apoptosis detection kit with fluorescein (Cell Death Detection Kit; Roche Diagnostics, Basel, Switzerland), according to method published (Boudard et al., 2009). Tissues were observed with epifluorescence by using standard fluorescence excitation and emission filters. Each section was scanned systematically from the temporal to the nasal ora-serrata for fluorescent cells indicative of apoptosis. To distinguish between structures that autofluoresced versus those that were TUNEL positive, all slides were examined first with the rhodamine filter and then with the FITC filter. Autofluorescent structures were visible under both filters, whereas TUNEL-positive cells were detectable only with the FITC filter. Positively labeled cells were counted in the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL).

Cell counting

Density of cell nuclei was determined for each condition (three fields of each of three individuals per group) by light microscope examination of retinal sections. Images were obtained within the posterior retina, within 1 µm of the optic nerve head (Hughes, 1991). Cell layer thickness and number of nuclei per layer were calculated within a standard rectangle placed across captured images, equivalent to an area of 0.07 mm^2.

Statistical analysis

Data are shown as the mean standard error of the mean. All results were compared with control animals as well as to the Cd-exposed animals (Cd). For all our experiments, a two-way ANOVA was used to analyze the differences between two groups, followed by a Dunnett's test with a threshold of significance of p<0.05 and p<0.01 to detect specific differences between the treated and control eyes, using a statistical software package (GraphPad Prism5).

RESULTS

Comparison of Cadmium content in control and Cd-treated eyes

Cadmium is known to accumulate in the lung, liver, and kidney and to produce cytotoxic effects in these organs. Our results confirm that cadmium is present in rat neural retina following Cd-treatment (Table 1). These results support those of Erie et al., 2005 who reported that cadmium levels in the RPE-choroid were two-fold greater than the cadmium concentrations found in the neural retina. However, our measurements of Cd in neural retina were comparable to their values (82 to 60 pmol/mg wet weight, compared to 65 to 8 pmol/mg wet weight for the present study).

A summary of individual protein measurements and mean values for control, Cd-treated eyes is presented in Table 1. The data were next sorted after five weeks treatment for further analysis. Total protein levels in the neural retina were 10% lower in Cd treated eyes than in control eyes.

Table 1: Total retinal proteins and cadmium concentration in adult rat retina exposed or not to Cd during 5 weeks, n = 12.

Cell layer	ONL	INL	CGL
Controls	0 ± 0	0 ± 0	0 ± 0
Cd+ group	72,35 ± 0,43*	75,27 ±1,38*	80,14 ± 4,57*

Data are expressed as means ± SE of animals.
* Statistically significant differences : p < 0,001 in comparison with controls.

The Cd content in the neural retina was measured using GF-AAS. In the subsequent analyses, all data were normalized to protein concentration following previously published methods because of the low dry weights of the samples (Newsome et al., 1996).

Normalized cadmium means and *standard error* of the *means* (SEMs) values for cadmium in different neural retina are compared in Table 1. Mean levels of Cd was highest in the Cd-treated retinas in comparison to control. Control retina's Cd averaged 23,67 ± 0,64 picomoles/µL protein, whereas Cd-treated retina levels for this metal was 59,63 ± 1,5 picomles/µL protein. Mean values for Cd-treated retinas were statistically significant (*p<0,05 compared to control eyes).

Structural changes in retinal cell layer

Quantitative measurement of retinal layer thickness and cell density showed significant decreases in layers thicknesses (ONL, INL, CGL) in Cd-treated retina (39,14; 27,81; 11,04 µm, respectively) compared with control layers (43,24; 44,98; 13,15 µm, respectively) (Fig. 1a). The ONL cell number shown in (Fig. 1b) was not different between Cd-treated eyes (136,83) and control animals (138).

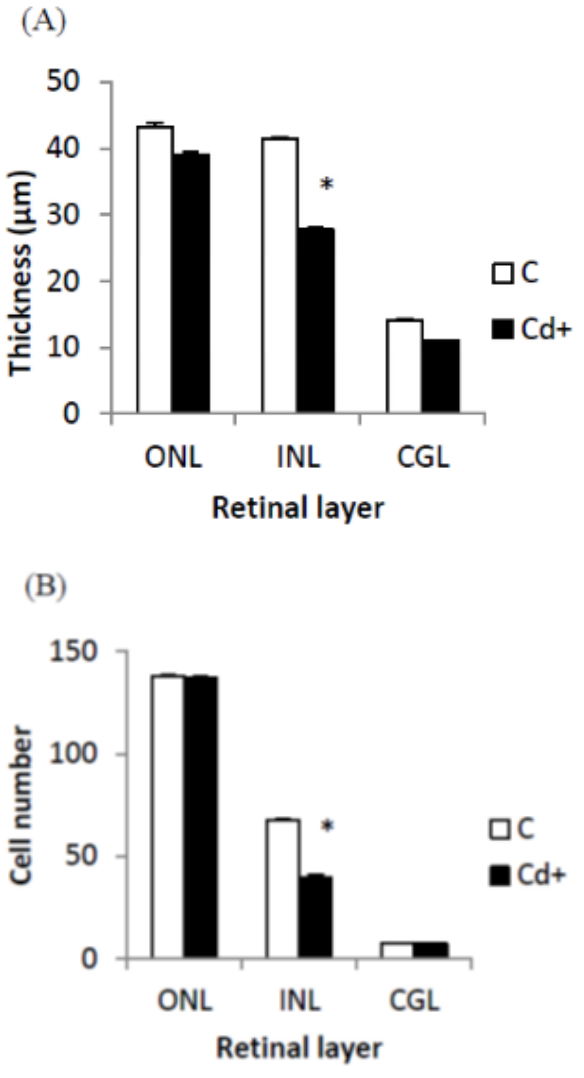

Figure 1: Quantitative measurements of retinal layers thickness (A) and cell density (B) in control (C), Cd^{+} animals. There were significant decreases in INL thicknesses and CGL cell numbers in Cd^{+} compared with C animals (*P<0.05; ANOVA).

Regarding the INL, Cd-treated eyes have a significant decrease in cell number in comparison with control eyes. No significant difference was shown in GCL cell number between control and Cd-treated eyes.

Cd decreases opsins immunostaining ofp-hot-ore-ceptors

Imm-unohistochemistry was used to study structural changes of photoreceptors (cones and rod). Photoreceptors cells of retina in control group showed normal neural cells structure. We observed a decrease of photoreceptors opsins immunostaining (MW-opsin for cones, Rho-4D2 for rod) in Cd-treated retina (Fig. 2) while the rhodopsin immunostaining seems fairly normal.

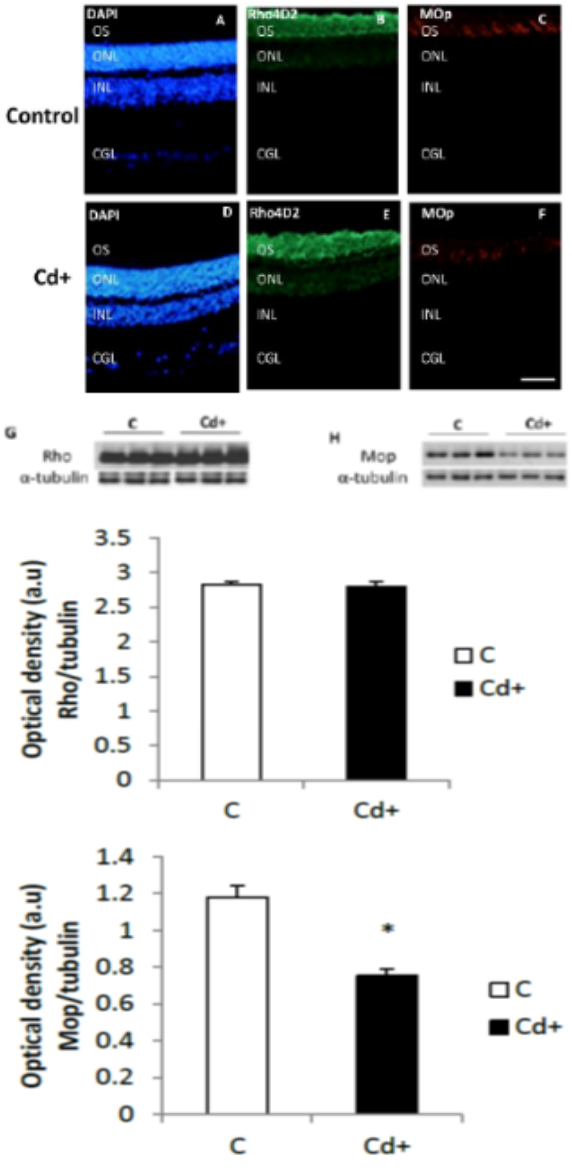

Figure 2: Rod and cone photoreceptor opsins in rat retina. Strong rhodopsine (Rho) immunolabeling was observed in rod outer segments (OS, green) in control (B) and Cd+ retina (E). Staining of cone mid wave length opsin (Mop) was localized to numerous cone outer segments (OS, red) in Control (C) in comparaison with Cd+ retina (F). Western blot analysis and quantification of Rho and Mop expression (G-H). Rho and tubulin (Tub) immunoreactivity showed no difference in intensity between C and Cd+ samples (G). Triplicate samples are shown above corresponding columns. Means and SEM significantly values were obtained in the neural retina in Cd-treated retinas compared to controls (H) (*p<0,05 compared to control eyes). Scale bar: 50µm.

We performed comparisons between control and Cd-treated whole retinal extracts by western blot analysis. As shown in (Fig. 2) MW-cone opsin immunoreactivity observed at ~40 kDa

presented a fainter band in the Cd-treated retinas compared to the control (Fig. 2g,h). Rod opsin immunoblots were performed using heated samples (Fig. 2g). Cd treatment does not seem to affect the integrity of rods as shown by the immunohistochemistry and western blot analysis (Fig. 2).

Percentage of in situ Cell Death Detection (TUNEL Analysis)

In order to investigate the effects of Cd on cell viability, control and Cd-treated retinas were stained with TUNEL immunolabeling (Fig. 3).

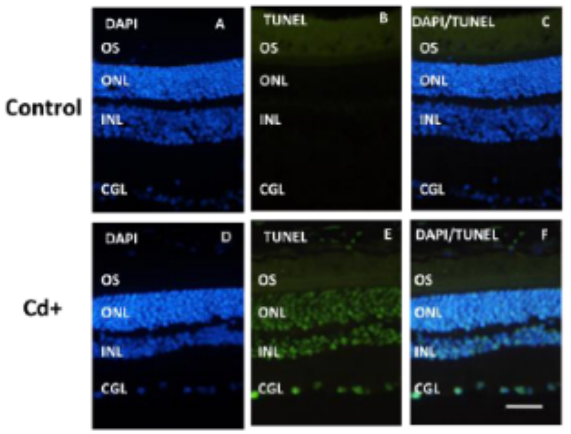

Figure 3: Localization of apoptotic cells in the retina after 5 weeks of cadmium treatment. In the Cd-treated rat retina (D-F), the entire remaining ONL, INL and CGL was filled with apoptotic cells. Scale bar: 50 µm.

Cd induced apoptosis in all retinal cells layers. The Cd-induced apoptosis was 72,35%, 75,27% and 80,14% respectively in outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) significantly different from control eyes (P<0,001) (Table 2).

Table 2: Total number of TUNEL positive nuclei in control and Cd-treated animals during 5 weeks, n = 6 animals.

Cell layer	ONL	INL	CGL
Controls	0 ± 0	0 ± 0	0 ± 0
Cd+ group	72,35±0,43 *	75,27 ±1,38*	80,14 ±4,57*

Data are expressed as means ± SE of animals.
* Statistically significant differences: p < 0,001 in comparison with controls.

DISCUSSION

Recent reports have demonstrated Cd accumulation in human retina and implicated Cd in development as well as progression of smoking-related age-related macular degeneration (AMD) (Erie et al., 2005; Wills et al., 2008a, 2008b, 2009), where the reported Cd levels in the retinal pigment epithelium (RPE)/choroid tissues are approximately double (4000 mg/g tissue) in smokers compared to non-smokers (1650 mg/g tissue) (Erie et al., 2005). Higher Cd levels have been found in the neural retina and RPE of AMD patients (90 and 220 pmol/mg protein, respectively) compared to patients without AMD (40 and 120 pmol/mg protein, respectively) (Wills et al., 2009). In our study we have demonstrated that the rat retina accumulate Cd during five weeks of Cd exposure. These results are similar than the study of Wills et al., 2009.

In order to be toxic, cadmium must enter the cell and be available as a free inorganic ion intracellularly (Thévenod, 2003). Uptake of free cadmium into the cytoplasm is not well known (Shaikh et al., 1995; Endo et al., 2002), however, previous work in hepatocytes suggests that entry of this metal into the cell may involve L-type calcium channels or use transport pathways for metals such as zinc or copper. The mechanism of action of heavy metals on the photoreceptors is not clear yet. A known difference between rods and cones is the outer segment morphology (Sjöstrand, 1953). Rod outer segments contain saccules or disks, which are enclosed by the plasma membrane but isolated from that membrane; these may function in the generation of the rod receptor potential (Hagins and Yoshikami, 1974). Cones usually have no such disks and their lamellae, which are analogous to rod disks and are continuous with the extracellular fluid (Sjöstrand, 1953). This morphological difference may somehow account for the fact that Pb2+, Hg2+, and Cd2+, depress the rod receptor potential amplitude but leave the cones unaffected. Our results show a decrease of cone photoreceptors opsins immunostaining in Cd-treated retina (Fox and Sillman, 1979).

Cd2+ promotes apoptosis, manifested by typical morphological changes and inter-nucleosomal DNA fragmentation (Fox and Sillman, 1979). In our study the percentage of apoptotic cells in retinal tissues after cadmium treatment was dramatically higher than control retinas. Even if the exact mechanisms by which Cd2+ exerts its apoptotic effects are not completely understood, Cd2+ has been demonstrated to disturb Ca2+ homeostasis in embryonic cells (Ishido et al., 1998; Biagioli et al., 2001). Calcium is a ubiquitous intracellular signal responsible for controlling numerous cellular processes including cell proliferation, differentiation, and survival/death (Clapham, 2007).Studies have shown that Cd disrupts intracellular free calcium ([Ca2+]i) homeostasis, leading to apoptosis in a variety of cells, such as skin epidermal cells, hepatic cells, lymphoblastoid cells, mesangial cells, renal tubular cells, astrocytes, NIH 3T3 cells, thyroid cancer cells, and thymocytes (Yeh et al., 2009; Wills et al., 2009; Son et al., 2010; Xie et al., 2010). As a second messenger, Ca2+ mediates physiological responses of neurons to neurotransmitters and neurotrophic factors.
Cd2+ may affect the cell membrane permeability to Ca+2 ions and disturb Ca2+ channel dynamic and intracellular Ca2+ transport. Cd2+ and Ca2+ are competitive ions. Once Cd2+ is accumulated, the Ca2+ uptake efficiency decreases leading to further deterioration of subsequent Ca2+ uptake. In a Ca2+ free system, DNA fragmentation is increased by Cd2+ (Lohmann and Beyersmann, 1993; Meinelt et al., 2001; Roccheri et al., 2004).

CONCLUSION

Cd is an environmental contaminant and because of its non-decomposable character, it can be toxic for the nature. In this study, rat retinas were used in order to assess the ultrastructural and molecular effects of Cd on retinal layers. Apoptic effects in ONL, INL and GCL were observed in rat retinas following CdCl2 treatment during five weeks.

The mechanism of action of heavy metals on the photoreceptors is not clear yet. The toxic effects of Cd on rat retinas cell indicate that environmental heavy metals such as Cd could be important potential factors in retinal neurons death associated with retinal diseases particularly related to smoking.
A growing interest has been focused on factors that can protect against Cd toxicity. It has been mainly noted that Zinc (Zn), an essential transition metal for cellular growth factor and differentiation (Smart et al., 2004), can protect several tissues from the harmful effects of Cd in several organs such as kidney (Brzoska and Moniuszko-Jakoniuk., 2001), liver, brain and bone (Brzoska et al., 2008; Kumar et al., 1996). In RPE, supplementation with high-dose of zinc (80 mg) provides some protection (Satarug et al., 2008), but the mechanism(s) underlying such protection has not been fully elucidated. Thus, the roles of Zinc on Cadmium retinal toxicity warrant further investigation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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