Research Paper Biological Science

# Protective Effect of Zinc on Cdcl2 Toxicity in Muller Glia and Synaptic Rat Retinal Cells Layers

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**ABSTRACT**

Cadmium (Cd) is regarded as one of the most toxic heavy metal elements for the environment. In this study, we report the effects of co-exposure of Cd and Zn on rat retinal cell layer. Forty two adult males rats, were divided into four groups: the first group received 200µg Cd/L in their drinking water for five weeks, the second group received 200 µg Zn /L, the third group received Cd and Zinc (200µg Cd+ 200µZn/L), finally a control group received tap water in identical manner.
After five weeks, Cd and Zn 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. Cd induced statistically significant decreases in retinal cell layers thickness and density accompanied by profound alterations in glial cells (down-regulation of glutamine synthetase, up-regulation of glial fibrillar acidic protein), decrease of synaptophysin expression (SVP38) and modification of protein kinase alpha (PKCα) expression. Percentage of apoptotic nuclei were reduced in all retinal layers of Cd-Zn treated animals in comparison with Cd-treated retina only. This fact may be explained by the role of Zn in metallothionein (Mt) synthesis, a Cd detoxification agent. The retinal co-exposure of Cd+Zn showed decrease of several changes observed after five weeks of CdCl2 treatment, and revealed the protective effect of Zn on Cd toxicity.

**KEYWORDS** Cadmium; Zn; retinal cells; GCL, synaptic cells.

## INTRODUCTION

The heavy metal cadmium (Cd) is known to have important ecological repercussions and causes significant health damages to humans, animals and plants. Cd accumulates in various ocular tissues such as lens, retina, ciliary body and vitreous [1-5]. 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 [6] can protect several tissues from the harmful effects of Cd on the antioxidant system in several organs such as kidney [7], liver, brain and bone [8-10].

Zn is known to be necessary for the visual cycle and photoreceptor survival [11]. This metal act as co-factor for the antioxidant enzyme Cu, Zn-superoxide dismutase which catalyzes the conversion of superoxide to oxygen and hydrogen peroxide (H2O2) [4]. Zn also stimulates protective cellular stress-signaling pathways and stabilizes proteins, making them less vulnerable to oxidation. Zn levels in the retina are high compared to other tissues and a large number of Zn binding proteins are present in the retina [12,13]. Zn deficiencies are associated with night blindness and macular degeneration [14-16].

The precise mechanisms for Zn transport in the retina are still unclear. Zn binds to metallothionein (Mt), a key regulator of the storage and transport of this metal [17]. The toxic metal cadmium interferes with Zn metabolism, partly due to its shared binding site with Zn on Mt and because it competes for binding with zinc and other metals on transport proteins [17].

In our study we hypothesize that Cd, a major smoke constituent, could cause several cell layer damages in rat retina. We investigate the glial mechanism of Cd toxicity through induction of apoptosis leading to cell death and synaptic changes and the protective role of Zn co-exposure.

## METHODS

Animals and housing conditions: Forty two10-weeks-old Wistar rats weighing 222 ± 16 g were randomly divided into three experimental groups (n = 12 in each group). The first group received 200 µg Cd/L (as CdCl2)in their drinking water for 5 weeks, the second group received 200 µg Zn /L, and the third remaining experimental group received 200µg Cd/L + 200µg Zn/L in their drinking water, the control group of animals was not treated with Cd. Exposure duration, Cd and Zn doses, as well as the administration method were chosen on the basis of the previous reports Messaoudi et al. [18,19]. 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). The three groups had access to food and drinking water ad libitum. Concentrations of Cd and Zn in the diet are, respectively, 3 and 5 (10-5 µg/kg dry weight). To evaluate the daily intakes of Cd and Zn in experimental group and express this as ppm/kg b.w., the 24h 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 and Moniuszko-Jakoniuk [20]. 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. For immunohistochemical study, the data were analyzed after five weeks of Cd and Cd+Zn treatments. The enucleated rats eyes used for immunohistochemistry were fixed in 4% paraformaldehyde for12 hrs. Eye cups were generated by removing the anterior segment and 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 [21] using Leica CM 3050 S cryostat.

Immunohistochemistry: Cryosections 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. The primary antibodies used were: monoclonal anti-Metallothionein (Mt) antibody (1:100 dilution, UC1MT ab 12228, Abcam)used at a final concentration of 10 mg/ml, polyclonal anti-Glial Fibrillary Acidic Protein (GFAP) (1:500 dilution, Z0334, DAKO), monoclonal anti-synaptophysin (SVP38) (1:100 dilution, Santa Cruz, sc12737), mouse monoclonal protein kinase alpha (PKCα) (Santa Cruz Biotechnology,(H-7) sc-8393), monoclonal anti-glutamine synthetase (GS) (1:500 dilution, Santa Cruz, sc-74430)antibodies. 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 and then mounted in 1:2 ratio of PBS:glycerol.

Immunoblot analysis: The method for western blot was performed as described before Saidi et al. [22]. Membranes were incubated with the following primary antibodies: anti-GFAP (polyclonal GFAP, Z0334, DAKO), anti-mouse monoclonal anti-metallothionein (Mt) (UC1MT ab 12228, Abcam), monoclonal anti-synaptophysin (SVP38) (Santa Cruz, sc12737), mouse monoclonal protein kinase alpha (PKCα) (Santa Cruz Biotechnology, (H-7) sc-8393), monoclonal anti-glutamine synthetase (GS) (Santa Cruz, sc-74430). 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 Immuno Research 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 (0.05µg / 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 [23,24]. 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 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 [25].

Heavy metal concentrations from two eyes of the same animal were averaged and treated as one observation (total number of observations equals 12). Cadmium and Zinc concentrations in control, Zn+ and Cd-treated animals (Cd, Cd+Zn) were compared using ANOVA test. Level of significance was considered when 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, and inner nuclear 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 [26]. Tissues were observed with epifluorescence microscope, 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 1µm of the optic nerve head [27]. Cell layer thickness and number of nuclei per layer were calculated within a standard rectangle placed across captured images, equivalent to an area of 0.07mm2.

Statistical analysis: Data are shown as the mean standard error of the mean. 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 (Graph Pad Prism5).

**RESULTS**

Comparison of Cd and Zn content in control, Cd-treated and Cd+Zn-treated eyes: The Zn and Cd content in the neural retina were measured using GF-AAS (Table 1).

In the subsequent analyses, all data were normalized to proteins concentration following previously published methods because of the low dry weights of the samples [16].

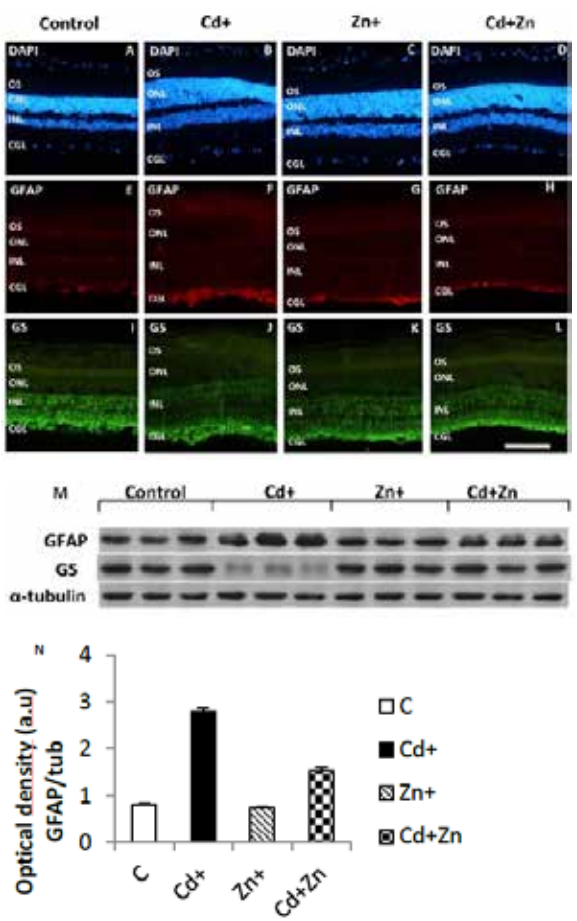
Table 1. Comparison of Cd and Zn content in control, Cd+, Zn+ and Cd+Zn-treated eyes during 5 weeks, n = 12.

| | Cd retina (picomoles/ mg protein) | Zn retina (nanomoles/µL) |
|---|---|---|
| Controls | 9,66 ± 0,63 | 6,35 ± 0,29 |
| Cd+ group | 58,68 ± 1,72* | 7,85 ± 0,33 |
| Zn+ group | 9,79 ± 0,44 | 11,99 ± 0,22 |
| Cd+ Zn group | 57,73 ± 1,04 * | 11,83 ± 0,24* |

Data are expressed as means ± SE of animals.
* Statistically significant differences : p < 0,05, in comparison with controls, Zn+ group and Cd+Zn group.

Normalized cadmium means and ***standard error*** of the ***means*** (SEMs) values for cadmium and zinc in the neural retina are compared in (Table 1). Mean levels of both metals were highest in the Cd-treated and Cd+Zn-treated retinas in comparison to control and Zn-treated animals. Control retina's Cd and Zinc averaged 9,66 ± 0,63 picomoles/mg proteins and 6,35 ± 0,29 nanomoles/mg proteins, whereas Cd-treated retina levels for these metals were 58,68 ± 1,72 picomoles/mg proteins and 7,85 nanomoles/mg proteins± 0,33, respectively. Zn-treated animals have have 9,79 ± 0,44 picomles/mg proteins and 11,99 ± 0,22 nanomoles/mg proteins. The cadmium content of the Cd+Zn-treated retina was 57,73 ± 1,04 picomles/mg protein (equivalent to Cd values in the Cd-treated retina), and the zinc content 11,83 ± 0,24 nanomoles/mg protein.

Cd-caused glial expression disorders: Glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) are the best markers among others reflecting altered glial reactivity in the retina [28,29]. We studied the effect of Cd on the GFAP levels in the rat retina. We observed an intense immunostaining of GFAP in Cd-treated animals compared to the control and Zn-treated animals (Figure 1 (F,E, G)).



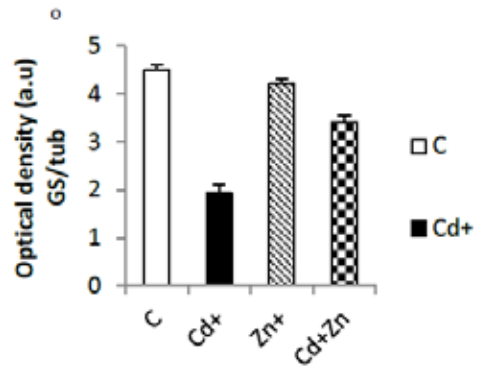
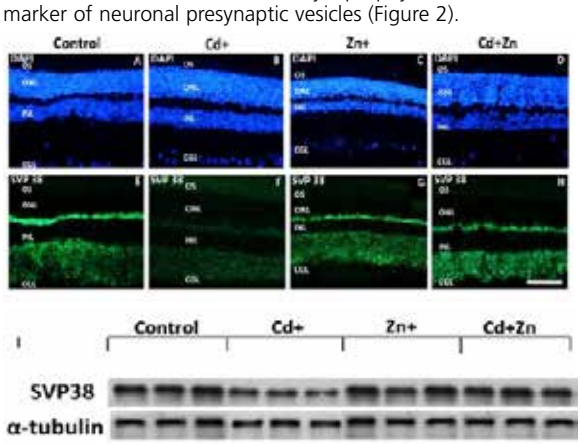




Figure 1: Glial alterations in rat retinas. Immunohistochemical and Western blot analyses of glial fibrillar acidic protein (GFAP) was strong in Cd+ (F,M) and Cd+Zn (H,M) treated retinas compared to the control (E,M) and Zn+ (G,M) animals. Western blots for GFAP, GS and α-tubulin are shown for three different animals of each group (M). Increased GFAP expression in Cd+ and Cd+Zn animals was confirmed by densitometric scanning of Western blots, expressed in arbitrary units (a.u.) normalized to α-tubulin (n=3 retinas per point) (N). Glutamine synthetase (GS) immunoreactivity was robust across the entire control retina and reduced the Cd+ retinas (J). Quantitative Western blots analysis confirmed the decrease of GS expression in the Cd+ group in comparison to control, Zn+ and Cd+Zn animals (O). Scale bar: 50 µm (A–L). (*P<0.01, ANOVA).

In the Cd+Zn treated retina (Figure 1(H)) the GFAP staining was at an apparent lower level compared to the Cd-only treated retina but appeared higher to the control. This suggests that the increase of GFAP expression was primarily caused by Cd treatment. We examined also the immunostaining of glutamine synthetase (GS). GS immunoreactivity was robust across the entire retina in Control (C) but seemed reduced in Cd-treated animals (Figure 1 (F)). Cd-treated animals (Figure 1 (J)) have an lower apparent GS rate in comparison to control, Zn+ and Cd+Zn-treated animals (Figure 1(I, K, L)).

We further investigated whether Cd had toxic effect on GFAP expression in neural rat retina at the molecular level. We determined the level of the GFAP in control, Cd+ and Cd+Zn retinas by western blot analysis (Figure 1 (M, N)). Increased GFAP expression in Cd+ and Cd+Zn animals was confirmed by densitometric scanning of Western blots. Upon Cd+Zn treatment, the GFAP level was significantly increased compared to the control and Zn+ (1,52 a.uvs. 0,8 a.u and 0,74 a.u, respectively) while the GFAP level was almost two times higher in the Cd+retinas compared to the Cd+Zn retinas(2,78 a.u vs.1,52 a.u) (Figure 1 (N)).Quantitative Western blots analysis confirmed the decrease of GS expression in the Cd+ group in comparison to control, Zn+ and Cd+Zn animals (O).

Role of Zn on Cd-induced synaptic malfunction: To test our hypothesis that cadmium may attenuate synaptic function, we stained retinal sections with Synaptophysine 38 (SVP38), a marker of neuronal presynaptic vesicles (Figure 2).



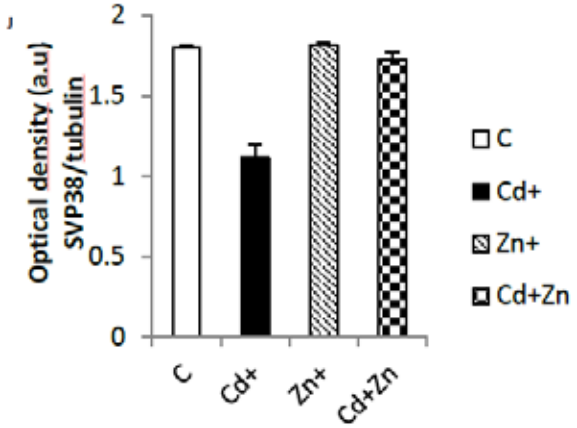
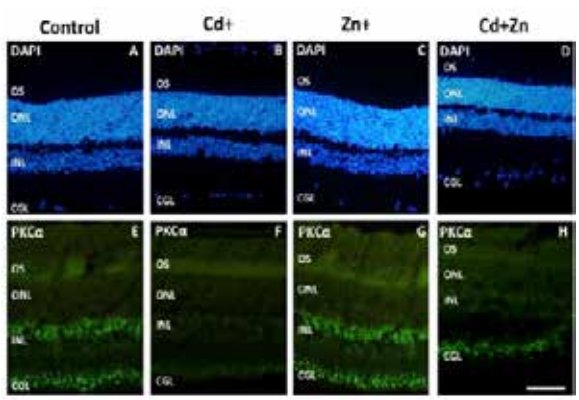


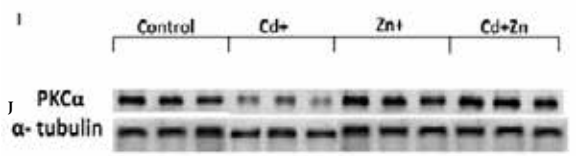

Figure 2: Strong synaptophysin (SVP38) immunostaining in neural plexiform layers of control (E), Zn+ (G) and Cd+Zn (H) retinas compared to the Cd+ animals (F). Western blot analysis and optical density quantification of SVP38 expression showed a significant increase in the retinas co-exposed to Cd and Zn compared to Cd only (K). (*P<0.05, ANOVA compared to control eyes). Scale bar: 50 µm.

Immunodetection of SVP38 was intense in control rat retina compared to Cd, Zn+ and Cd+Zn treated animals. The western blot confirmed the data shown by immunohistochemistry (Figure 2 (I,J)), where SVP38 was reduced in Cd+ compared to control, Zn+ and Cd+Zn retinas, however this reduction was more significantly increased in Cd+Zn retinas (Figure 2 (I,J)) compared to Cd+ animals.

Cd may attenuate also the synaptic function by decreasing SVP38 expression in Cd-treated animals versus control group. This alteration can be explained by several mechanisms related to Ca2+ion exchange.

In the retina, PKC isoforms are found in bipolar [30], ganglion [31,32], amacrine [33], pigment epithelial and cones cells [34]. PKCα is mainly expressed by rod bipolar cells and, to a lesser extent, by amacrine cells and cones [33,35-39]. In our study, bipolar cells were stained with antibodies against PKCα, restricted to this cell type within the neural retina. In control retina, PKCα antibody showed clear staining in cell bodies within the inner nuclear layer (INL), as well as their axons and synaptic ending in the inner plexiform layer (IPL), corresponding to rod and cone ON bipolar cells (Figure 3).



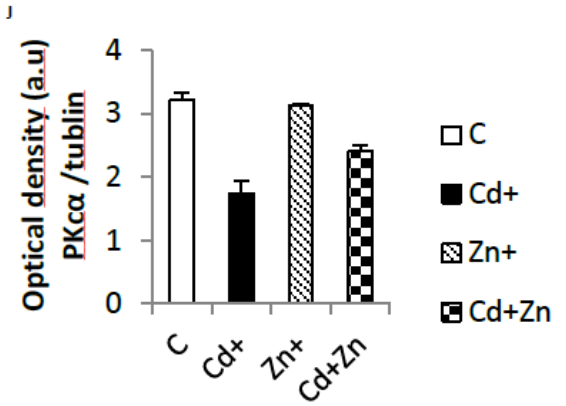




Figure 3: Protein kinase C alpha (PKCα) immunostaining was intense in neural retinal layers in Control (E) and Zn+ (G) versus Cd+ (F) and Cd+Zn rats. Western blot analysis and optical density quantification of PKCα showed it significantly decreased in Cd+ retinas in comparison to control (I,J). (*P<0.05, ANOVA). Scale bar: 50 µm.

Cd+ and Cd+Zn treated retinas showed almost complete disappearance or reduction of PKCα staining respectively (Figure 3 (F,H)), which was confirmed by quantitative western blot analysis as described earlier (triplicate lanes for control, Cd+, Zn+ and Cd+Zn treated retinas) (Figure 3 (I,J)).

Role of Zn on Cd-induced apoptosis in retinal cell layer: In order to investigate the effects of Cd on cell viability, control and Cd-treated retinas were stained with TUNEL immunolabeling (Figure 4).

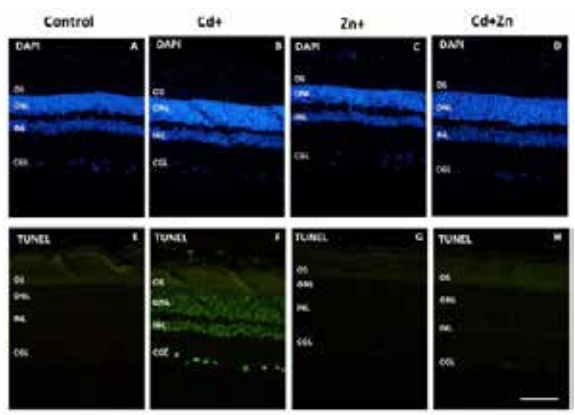



Figure 4: Apoptosis induced by cadmium in rat retinal cell layer. In Cd-treated animals, the entire remaining ONL, INL and CGL was filled with apoptotic cells (B,F) in comparison with control (A,E), Zn+ (C,G) and Cd+Zn treated animals (D,H). In Cd+Zn treated animals (D,H), only the innermost cell rows of the INL, corresponding to the position of amacrines cells contained some TUNEL-positive cells. Scale bar, 50 µm.

Cd induced apoptosis in different retinal cells layers. An increase in apoptosis was observed in outer nuclear layer (ONL), inner nuclear layer (INL) and ganglionic cell layer (GCL) when we treated with Cd (200µg/L) (Figure 4 (F)). The Cd induced apoptosis was 72,35%, 75,27% and 80,14% in ONL, INL and GCL, respectively, significantly different from control eyes (p<0,001) (Table 2). We showed absence of apoptotic cells in the ONL but a low percentage of apoptotic cells in the INL (3,81%) and GCL (12,34%) in Cd+Zn (200µg Cd/L + 200µg Zn/L) treated animals (Table 2).

We showed also absence of Tunel-positive neuclei in control and Zn-treated animals. We conclude that zinc has a preventive effect in all retinal layers in comparison to Cd+ group, but

the neuroprotective process remains to be studied further.

Protective effect of Zn on Cd-induced cell death in retinal cells: Metallothionein (Mt) expression was assessed in rat retina exposed to Cd+, Zn+ or Cd+Zn by ***immunoblotting analysis*** using monoclonal anti-Mt antibody (Figure 5).

Table 2. Total number of TUNEL-positive neuclei in control , Cd+, Zn+ and Cd+Zn treated animals , 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 * |
| Zn+ group | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Cd+Zn group | 0 ± 0 | 3,81 ± 0,18 | 12,34 ± 0,10 |

Data are expressed as means ± SE of animals.
* Statistically significant differences : p < 0,05, in comparison with controls, Zn+ group and Cd+Zn group.

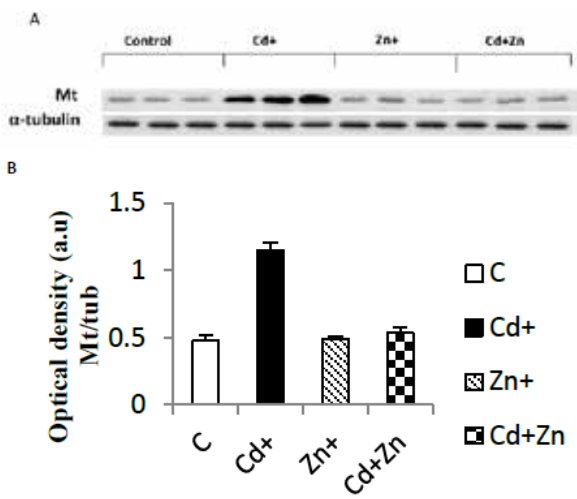



Figure 5: Western blot analysis and quantification of Metallothionein (Mt) optical density (a.u) showed it significantly increased in Cd+ retinas compared to the control, Zn+ and Cd+Zn animals (A,B) (*P<0.05, ANOVA).

In our study Mt Western blot analysis showed intense immunoreactive bands at 45kDa of molecular weight in Cd-treated animals in comparison to control, Zn+ and Cd+Zn treated animals. Quantification of Mt optical density (a.u) showed it significantly increased in Cd+ retinas (1,15 ± 0,05) (G,H) compared to the control (0,47 ± 0,04), Zn+ (0,49 ± 0,02) and Cd+Zn animals (0,53± 0,04) (p<0.05, ANOVA). Zn may play a protective role against Cd toxicity by reducing Mt expression in the retina, but the biochemical process remains unknown.

**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) [1,3-5]. In this study we have demonstrated that rat retina accumulates Cd during 5 week of treatment. This result is similar to the study of Wills et al. [5]. Higher Cd levels have been found in the neural retina of AMD patient eyes (90 pmol/mg protein) compared to non-AMD eyes (40 pmol/mg protein) [5].

However, our measurements of Cd in neural retina were comparable to their values (82 to 60 pmol/mg wet weight, compared to 60 to 25 pmol/mg wet weight for the present study). In other studies, the authors have compared the distribution of cadmium to other trace metals (zinc and copper) in the same samples [3,4]. Again, distinct differences in the distribution of cadmium, zinc and copper in retinal tissues were obtained. One possible reason for this apparent lack of leakage might be the binding of cadmium and other metals to cellular proteins. In the present study, zinc levels in retinal tissues from Cd+Zn treated rat were approximately 2 to 3 times higher than levels in control animals.

In order to be toxic, cadmium must enter the cell and be available as a free inorganic ion intracellularly [40]. However, previous work in hepatocytes suggests that entry may involve L-type calcium channels or use transport pathways for metals such as zinc or copper.

Cadmium is a well known blocker of voltage-gated Ca2+ channels and causes a decrease in depolarization-evoked exocytotic release of neurotransmitters from nerve terminals [41-44].

Namely, cadmium caused a decrease in glutamate accumulation by synaptic vesicles inside of nerve terminals in the preparation of digtonin permeabilized synaptosomes; lowered the the proton gradient of synaptic vesicles in synaptosomes; and in isolated synaptic vesicles. It was shown that high concentrations of Cd can cause astrocytic cell death because of mitochondrial impairment through pathways dependent on Ca2+ and reactive oxygen species [45-47].

Taking into account the above facts, we propose that synaptic malfunction caused by the metals may results from: a decrease in exocytotic release of neurotransmitters that is associated not only with the blockage of voltage-gated Ca2+ channels but also with incomplete filling of synaptic vesicles; a decrease in glutamate uptake that is due to not only direct influence of the metals on glutamate transporters and also due to partial dissipation of the proton gradient of synaptic vesicles. Cadmium related abnormal release of glutamate by regulated exocytosis indicates alterations in stimulated neurotransmission. Whereas malfunction of Na2+-dependent glutamate transporters may provoke an increase in the extracellular level of glutamate, and thus significant changes in spontaneous brain activity [48]. Borisova et al. showed that Cadmium in concentration of 50μM immediately caused significant dissipation of the proton gradient of isolated synaptic vesicles [49].

Glutamine synthetase (GS) is the key enzyme in ammonia assimilation and catalyses the ATP-dependent condensation of NH4+ with glutamate to produce glutamine [50]. It has been shown that during exposure to Cd, a decrease in GS activity takes place in maize [51], pea [52], bean [53] and rice [54]. More recently, it was demonstrated that Cd decreased GS activity in soybean nodules [55]. We show similar results in this report, that the decrease and increase of GS and GFAP respectively, caused by CdCl2 were evident five weeks after Cd-treatment.

To the best of our knowledge, this is the first report of Cd-induced decreases in protein kinase C expression in bipolar cells and may be related to perturbed inner retinal function seen in Cd-treated animals, such as alterations in cell bodies within the INL, as well as their axons and synaptic ending in the IPL, corresponding to rod and cone ON bipolar cells.

In our study the percentage of apoptotic cells in retinal tissues after cadmium treatment was dramatically higher than that of the control retinas. These results confirmed previous findings that cadmium induces cell apoptosis and indicate indirectly that cadmium could activate aspartic-acid-specific cystein proteases[ (caspases) in neural retina [56,57].

Numerous studies show that increased Zn supply may reduce Cd absorption and accumulation, and prevents or reduces the adverse actions of Cd [58], whereas Zn deficiency can intensify Cd accumulation and toxicity. Zn is required for the optimum activity of more than 200 enzymes, including those involved in the synthesis and repair of DNA and RNA and thus the related protein synthesis and tissues repair responses [59]. Mutual interactions between Cd and Zn may occur at various stages of both metal's metabolism, e.g. absorption, distribution in the organism and excretion, as well as at the stage of Zn biological functions. We have shown in the present study that

co-exposure of Cd with Zn at concentration (200µg/L both) reduced the intracellular effect of Cd in rat retina, most likely through inhibition of Cd2+ uptake. This explanation remains to be substantiated in future studies with direct measurements of Cd uptake or knockdown of the metal transporter ZIP8, which transports cadmium with high affinity and greatly sensitizes cells to cadmium-induced cell death [60]. Our data suggest that large molar quantities of Zn would be required for effective inhibition of Cd uptake, raising concern about its toxicity or side effects. It can be argued, however, that Zn is an inducer of the metal binding protein metallothionein (Mt) that protects against increased oxidative stress induced by Cd [61, 62].

Mt are low-molecular-weight, cysteine-rich, metal-binding proteins. Mt genes are readily induced by various physiologic and toxicologic stimuli. Because the cysteines in Mt are absolutely conserved across species, it was suspected that the cysteines are necessary for function and Mt is essential for life.

Enhanced consumption of Zn may decrease Cd absorption from digestive tract and its accumulation in the organism and induce the synthesis of Mt, a cadmium-binding protein, which is widely implicated in the sequestration of this metal, and as a result it may prevent the toxic effects of Cd, including the retina damage [63-65]. This fact is related, in a large part, to the Mts which are low molecular–weight proteins with the cysteine content able to sequester excessive metals such as Cd, Cu and Zn.

The expression of Mt can be induced by metals, hormones and various stressors. However, metals, especially Cd and Zn, are the most potent inducers of the protein [66].

Cadmium is known as a strong inducer of Mt expression [63]. Mt is known to be made in the retina, but its function and the cells that make this protein remain unclear [16, 67-70]. Lu et al. (2002) shown that Mts are made in a variety of cells of the human retina [61]. Using RPE cells that express Mt-1 under an inducible promoter, it is shown that this protein may be involved in protection against oxidative stress and apoptosis in the eye.

Mt has been shown the high capacity to bind zinc ions in mammalian cells [54]. Huang et al. (2006) demonstrated that cadmium significantly induce Mt expression in E. coli by western blot analysis [71]. Since the zinc ions are believed to stabilize the three-dimensional structure of SOD protein and to confer the enzyme activity of SOD, Huang et al. (2006) suggest that high level of apo-Mt induced by cadmium may sequester zinc form the Cu, Zn-SOD enzyme to decrease the SOD activity [71].

The induction of Mt by Cd and the subsequent sequestration of Cd by Mt protect tissues from Cd toxicity. The protective role of Mt against Cd toxicity has been unequivocally established, not only for acute Cd poisoning, but also for chronic Cd toxicity, and Cd carcinogenesis. The cellular defense mechanisms of Mt, heat shock proteins and other compensatory mechanisms modulated toxicity. However, the effectiveness of cellular defenses diminished with prolonged exposure and increasing mixture complexity.

## CONCLUSION

In conclusion, our results suggest that Cd caused structural and molecular changes in all retinal layers in rat retina following Cd and Cd+ Zn treatment during 5 weeks.

It has been mainly noted that Zn is an essential transition metal for cellular growth factor and differentiation, and can protect several tissues from harmful effects of Cd on the antioxidant system in several organs.

We showed that the decrease in the activity of GS and the increase of GFAP expression caused by Cd treatment were evident five weeks after treatment. Cd may attenuate also the synaptic function by decrease of SVP38 expression in Cd+ and Cd+Zn-treated animals versus control group. This alteration can be explained by several mechanisms related to ion exchange Ca2+. Cadmium related abnormal release of glutamate by regulated exocytosis indicates alterations in simulated neurotransmission. PKCα expression was altered after 5 weeks of Cd-treatment.

We have shown in the present study that co-exposure of Cd with Zn at concentration (200µg/L both) reduced the intracellular effect of Cd in rat retina, most likely through inhibition of Cd uptake. In our study Mt immunostaining was shown in all the retinal layers of all groups, but was significantly increased in Cd-treated animals in comparison with both control and Cd+Zn treated animals suggesting that Zn may play a protective role against Cd toxicity in the retina through Mt inhibition. Zinc treatment may contribute to reduce Mt expression, but the biochemical process remains unknown.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

## ACKNOWLEDGMENTS

The authors thank Cristina Sandu (Département de Neurobiologie des Rythmes, CNRS UPR 3212, INCI, Strasbourg, France) for helpful discussion and Domitille Boudard for expert technical assistance. This work was supported by the Fritz Tobler Foundation.

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