Mutat Res Gen Tox En 826 (2018) 25–32

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

journal homepage: www.elsevier.com/locate/gentox

Cypermethrin: Oxidative stress and genotoxicity in retinal cells of the adult zebrafish

E.V. Paravani[a], M.F. Simoniello[b], G.L. Poletta[b,c], F.R. Zolessi[d,e], V.H. Casco[a,*]

[a] Laboratorio de Microscopia Aplicada a Estudios Moleculares y Celulares, Facultad de Ingeniería, Universidad Nacional de Entre Ríos (UNER), Ruta 11 Km 10, IBB-CONICET-UNER, Oro Verde, 3101, Entre Ríos, Argentina
[b] Cátedra de Toxicología, Farmacología y Bioquímica Legal, FBCB-UNL, Ciudad Universitaria, CC242 Paraje El Pozo S/N, 3000, Santa Fe, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917 (C1033AAJ), CABA, Argentina
[d] Sección Biología Celular, Facultad de Ciencias, Universidad de la República, Uruguay
[e] Cell Biology of Neural Development Laboratory, Institut Pasteur de Montevideo, Uruguay

ARTICLE INFO

Keywords:
DNA damage
Apoptosis

ABSTRACT

Cypermethrin (CM), widely used for control of indoor and field pests, is one of the most common contaminants in freshwater aquatic systems. We evaluated CM genotoxicity and the activities of superoxide dismutase (SOD) and catalase (CAT) in retinal cells of adult zebrafish. Histological and immunofluorescence techniques show the presence of apoptotic cells in the zebrafish retina after 9 d of treatment with 0.6 μg/L CM. Histone γ-H2AX, a marker of DNA damage, was detected in both outer and inner nuclear layers; caspase-3, an apoptotic marker, was detected in the outer nuclear layer. In the comet assay, the cells were sensitive to hydrogen peroxide-induced DNA damage, showing a dose-dependent response. We observed a positive comet assay response to CM that was dose- and time-dependent. Following exposure to CM, SOD and CAT enzyme activities, and *sod* and *cat* mRNA levels, increased. These results indicate that CM causes DNA damage and oxidative stress and can induce apoptosis in retinal cells.

1. Introduction

Pyrethroids are toxic to fish under laboratory conditions [1]. In the environment, their effects might be different, due to interactions with suspended matter, bottom sediments, and aquatic plants [2] and depending on field use conditions. Most risk assessments are based on data from experiments in freshwater model ecosystems, rather than descriptive hydrobiological research. Cypermethrin ([cyano-(3-phenoxy-phenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate, CM) is a widely-used type II pyrethroid pesticide. Because of its high toxicity to target organisms, such as insects, and low mammalian toxicity, CM is widely used for agricultural and domestic applications. Consequently, CM is one of the most common contaminants in freshwater aquatic systems [3,4]. CM residues have been detected in water and sediment samples from streams and rivers draining major agricultural districts [5]. According to previous reports, the concentration of CM in surface water is < 1 μg/L, in most cases, but it can be as high as 2.8 μg/L [6]. Chemical exposure may cause oxidative stress and reactive oxygen species (ROS) generation in fish [7,8]. Under normal physiological conditions, ROS are detoxified by antioxidant enzymes such as SOD and CAT [8,9]. However, CM may induce oxidative stress in fish. Uner et al. [10] found that SOD and CAT levels were significantly increased in the livers and kidneys of the freshwater fish species *Oreochromis niloticus* and *Cyprinus carpio* after 10-d exposure to 3.0 μg/L CM. Oxidative stress can also cause DNA damage and apoptosis. Jin et al. [11] reported that CM exposure induces hepatic DNA damage and alters the transcription of oxidative stress and apoptosis genes in adult female zebrafish. We demonstrated that amphibian tadpoles subjected to sublethal and acute doses of CM suffer apoptosis in several brain regions [12–14]. To analyze CM effects in a system simpler than the amphibian brain, we have evaluated morphological, biochemical, and molecular changes in the zebrafish retina.

The multilayered vertebrate retina, like other regions of the central nervous system (CNS), develops from a relatively homogeneous, single-layered sheet of neuroepithelial cells. The retina is a highly-conserved structure, having almost identical anatomical and physiological characteristics throughout vertebrate taxa [15]. The teleost retina is an excellent model for studying xenobiotic effects on neurogenesis. It is arranged in discrete layers, facilitating observations of morphological changes and toxic effects on neuronal cells and their synaptic connections. Here, we have analyzed histological alterations, DNA damage, enzyme activities, and the expression of genes related to oxidative stress

* Corresponding author.
E-mail address: vcasco@ingenieria.uner.edu.ar (V.H. Casco).

https://doi.org/10.1016/j.mrgentox.2017.12.010
Received 25 July 2017; Received in revised form 19 December 2017; Accepted 24 December 2017
Available online 27 December 2017

and apoptosis in the retinas of adult zebrafish exposed to environmentally relevant concentrations of CM.

2. Materials and methods

2.1. Chemicals

Cypermethrin, commercial formulation SHERPA®, 25% CM active ingredient, was used. The stock and test solutions were prepared by dissolving the material in 0.1% acetone (Cicarelli, BA, Argentina). Stock solutions were stored in the dark at 4 °C. H_2O_2 was purchased from Cicarelli. RPMI-1640 medium was purchased from HyClone (Thermo Scientific, Logan, UT). Dimethyl sulphoxide (DMSO), low-melting-point agarose (LMPA), acridine orange, and other reagents for the comet assay (CA) as well as general laboratory chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Zebrafish is an excellent vertebrate model system for toxicology studies [16]. We used 8-month-old fish from a laboratory stock produced by brood fish. The fish were acclimatized for 48 h in glass tanks with dechlorinated tap water at 26.5 ± 1 °C, pH = 6.8–7.5, hardness 50–100 mg/L $CaCO_3$, conductivity 410–440 μS/cm^2, dissolved oxygen concentration 5.5–6.2 mg/L, photoperiod 14 h light/10 h dark [17]. They were fed twice daily with flake food (TetraMin®, Germany) and once daily with brine shrimp. Fish were sacrificed by cooling in ice and retinas were extracted and processed as described below, for comet assay studies, histological analysis, and oxidative stress determination.

The use of animals in this study was approved by the Institutional Animal Care and Use Committee at the National University of Entre Rios and Italian University Institute of Rosario (Rosario, Argentina; protocol N°028/12).

2.3. In vivo CM bioassay

In vivo experiments were carried out in triplicate treatments, with five fish per treatment. Each treatment included a no-CM control. Fish were maintained in dechlorinated tap water with 0.1% acetone. Groups were exposed for 3, 6, 9, or 12 d, at 0.3 or 0.6 μg/L CM concentrations, with daily changes of the solution. CM stock solution (in acetone) was diluted with water. The CM concentrations used were based on reported concentrations in aquatic environmental systems [6,18]. Under normal environmental temperatures and pH, CM is stable to hydrolysis, with half-life > 50 d, and to photodegradation, with half-life > 100 d [19].

2.4. Retinal cell extraction

The eyes of each specimen were extracted under a microscope, connective tissue was removed, extra-ocular muscles and optic nerves were cut, and the eyes were finally extracted with forceps. Lenses were removed by performing a small corneal incision with watchmaker tweezers, applying slight pressure on the eye. All cells obtained from the complete retina were used for analysis.

2.5. Histological analysis

Eyes of control and exposed animals were fixed in 4% paraformaldehyde (PFA) in PBS 1X, pH = 7.2 for 4 h at room temperature and then processed according to Casco et al. [14]. Cured blocks were cross-sectioned at 0.5 μm thickness with an Ultracut-S ultramicrotome Reichert (Leica, Austria), and semi-thin sections were stained with toluidine blue (Merck Millipore, Billerica, MA) and recorded with a CCD camera coupled to an Olympus BX50 microscope at 40X and 100X.

2.6. Immunofluorescence

Eyes were fixed in 4% PFA, OCT-embedded and cryo-sections were made at 10 μm thickness. Non-specific antibody binding was blocked with 1% bovine serum albumin, 0.2% Triton X-100 in PBS for 30 min at 20–23 °C. Primary and secondary antibodies were each incubated for 1 h at room temperature. Antibodies were diluted in the blocking solution as follows: anti-γ-H2AX antibody, 1:300 (Abcam, Cambridge, UK, ab11174); anti-active caspasa-3 antibody, 1:300 (Abcam, ab2302). The secondary antibody was goat anti-rabbit IgG, Alexa 488-conjugated, 1:1000; Molecular Probes, Eugene, OR. (now Abcam ab150077). Nuclei were counterstained with methyl green zinc chloride salt (Abcam, ab146287). Photomicrography was performed with a laser confocal system, LSM800 (Carl Zeiss AG). Acquisitions of z-stacks were performed with Fiji software.

2.7. Comet assay

Isolated retinas were washed with PBS, 25 μl, and gently disrupted with a micropipette tip. Retinal cell suspensions were diluted with RPMI-1640, 1:19 (v/v) for *in vitro* exposure or used directly for the comet assay (CA) in the *in vivo* CM exposure studies.

Cell viability was determined with fluorescent DNA-binding dyes. Cell suspension was mixed with a working solution of acridine orange and ethidium bromide (EB), each 100 μg/ml, prepared in Ca^{2+}, Mg^{2+}-free PBS, and then examined under a fluorescence microscope (40X). 100 cells per sample were counted and the percentage of viable cells was determined [20]. Retinal cells obtained after *in vitro* and *in vivo* exposures were used for CA as follows: cell suspension, 50 μl, was mixed with 1% LMPA, 200 μl, and slides were prepared as follows. Slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM trizma base, 1% Triton X-100, 10% DMSO; pH = 10) for 24 h, incubated in freshly made alkaline solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13) for 10 min, electrophoresed 10 min at 300 mA and 25 V (0.70 V/cm) and then neutralized (0.4 M trizma base, pH = 7.5). Finally, samples were dehydrated in ethanol and left to dry [21]. Lysis, unwinding, and electrophoresis were conducted at 4 °C and the preparations were kept in the dark. All samples were coded for 'blind' analysis, stained with EB (2 μg/ml), and comet images of 100 randomly selected cells (50 cells from each of two replicated slides) were scored from each sample under a fluorescence microscope. Comet images were analyzed and registered using a monochromatic, refrigerated Apogee CCD camera (Andor, Belfast, UK), coupled to an epifluorescence Olympus BX50 microscope (Olympus, Japan). Images of 100 randomly selected nucleoids were scored from each animal. Cells were classified into five classes according to their tail size and intensity (from no damage: class 0, to maximum damage: class 4) (Fig. 4A–D), resulting in a single DNA damage score (Damage Index, DI) for each animal. Comet cell profiles with a small or nonexistent head and an extremely prominent and diffuse tail were not scored due to the possibility that they were apoptotic or necrotic cells [22]. The DI was calculated as follows:

DI = n1 + 2n2 + 3n3 + 4n4, where: n1, n2, n3, and n4 are the numbers of cells in damage classes 1, 2, 3, and 4 [23,24]. Slide analysis was carried out double-blind.

Twenty zebrafish were used to determine basal values of DNA damage and to standardize the assay conditions, using H_2O_2, a known genotoxic agent. Immediately after the acclimation period, fish were sacrificed by a thermal shock in ice water, and retinal cells obtained as previously described. Half of the samples were used for basal value determinations and the remaining samples for technique standardization, as follows: cell suspensions were made to 1:19 (v/v) with RPMI and then exposed to H_2O_2 (2.5, 5.0, or 10.0 μM) for 10 min at 25 °C. Control experiments were carried out in water. Tubes were centrifuged at 400 × g for 10 min and cells used for the CA. The H_2O_2 concentrations were based on previous studies [21,25].

2.8. Enzyme activity assay

Retinas were homogenized with a teflon microcentrifuge sample pestle (Thomas Scientific) in PBS, 50 µl, and then centrifuged. The supernatants were collected and used to determine SOD and CAT activities. SOD activity was measured in a spectrophotometer at 550 nm as the inhibition of reduction of nitro blue tetrazolium chloride by superoxide anion radical generated during oxidation of xanthine by xanthine oxidase [26]. The reaction mixture contained 50 mM sodium carbonate dissolved in 50 mM K, Na-phosphate buffer (pH = 7.8, 25 °C), 0.1 mM EDTA, 0.1 mM xanthine, and 25 µM nitro blue tetrazolium chloride. The activity of SOD was calculated according to a standard curve and expressed as U/mg protein [27]. CAT activity was determined by recording the absorbance of the generated stable chromophore at 405 nm due to H_2O_2 consumption, according to the Aebi method [28].

2.9. Analysis of gene expression

Total RNA of retinas of zebrafish treated with CM was isolated using GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. The ratio of absorbance at 260 *vs.* 280 nm was used to verify the quality of RNA in each sample. Subsequently, RNA was denatured at 65 °C for 15 min. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used. RT products were used directly for polymerase chain reaction (PCR), in an Ivema T-18 thermocycler (Llavallol, B. A., Argentina) using the following program: denaturation, 10 min, 95 °C; 30 cycles of 1 min at 95 °C, 90 s at 50 °C, and 90 s at 72 °C. Oligonucleotide primers were used to detect the expression of β-actin, superoxide dismutase (AY N° 195857), and catalase (AF N°170069); see Table 1. β-Actin expression was used for normalization.

2.10. Statistical analysis

Statistical analysis was performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Results are given as means ± standard error (SE). Variables were tested for normality with the Kolmogorov-Smirnov test and homogeneity of variance between groups was verified by Levene's test. We used one-way ANOVA followed by Dunnett's test for the comparison of the DI between each group and its control, for both *in vitro* and *in vivo* exposures. A linear regression was conducted for the *in vivo* exposure to test a concentration-response relationship between DI and CM. $p < 0.05$ was considered significant.

3. Results

3.1. Histological analysis

Fish exposed to CM, 0.3 µg/L, showed no significant morphological changes at any treatment times compared to the controls (Fig. 1A and B). for fish exposed to 0.6 µg/L CM, this was also true for the first 9 d (Fig. 2A and B), but, after 12 d, apoptotic figures were seen in the outer (photoreceptors) and inner (amacrine, bipolar, and horizontal cells) nuclear layers (Fig. 2C and D).

Table 1
Sequences of primer pairs used in the PCR reactions.

Target gene	Accession no.	Primer sequences (from 5′ to 3′)	Product length (bp)
sod	AY195857	F: 5′-CCGGACTATGTTAAGGCCATCT-3′ R: 5′-ACACTCGGTTGCTCTCTTTTCTCT-3′	123
cat	AF170069	F: 5′-AGGGCAACTGGGATCTTACA-3′ R: 5′-TTTATGGGACCAGACCTTGG-3′	499
β-actin	AF057040	F: 5′-ATGGATGAGGAAATCGCTGCC-3′ R: 5′-CTCCCTGATGTCTGGGTCGTC-3′	106

3.2. Immunofluorescence

γ-H2AX: Retinas of fish treated with 0.6 µg/L CM for 3 d did not stain for γ-H2AX in any of the nuclear retinal layers (data not shown), but at 12 d, γ-H2AX staining was seen in the outer nuclear layer, corresponding to the rod and cone cells (Fig. 3A), and in the inner nuclear layer (Fig. 3B). Caspase-3: Retinas of fish exposed to 0.6 µg/L CM for 3 d were negative (data not shown), but at 12 d, a nuclear dotted pattern of immuno-labeling was seen in the photoreceptor cells (Fig. 3C); no signal was observed in the control retinas (Fig. 3D).

3.3. In vitro assay

A significant DI increase was seen in retinal cells exposed *in vitro* to H_2O_2, at all concentrations tested, and the increase was dose-dependent; $R^2 = 0.9574$; $p < 0.05$ (Fig. 4).

3.4. In vivo assay

No mortality occurred in the *in vivo* treatments. Retinal cell DI values of fish exposed to CM, 0.3 µg/L, increased significantly with exposure time, compared to controls. At 0.6 µg/L CM, a statistically significant increase in DI of retinal cells was seen as early as 3 d (Fig. 5A, Table 2).

3.5. Effects of CM on antioxidant enzyme activities

Oxidative stress may be one of the mechanisms of pyrethroid neurotoxicity [29]. For fish exposed to CM, 0.3 µg/L, SOD activities in retinas increased, but not significantly, compared to controls (Fig. 6A, Table 3). However, at 0.6 µg/L CM, SOD activities showed statistically significant increases beyond 3 d treatment (Fig. 6B, Table 3). CAT activities in retinas of zebrafish exposed to CM showed significant increases (Fig. 6C and D, Table 3), especially at 0.6 µg/L.

3.6. Effects of CM on sod and cat gene expression

Expression levels (mRNA) of the *sod* and *cat* genes were determined by semi-quantitative RT-PCR (RT-*sq*PCR). For the fish exposed to CM, 0.6 µg/L for 3 d, retina *cat* mRNA levels increased compared to the control group, but *sod* mRNA did not. After 12 d, mRNA levels of both genes were significantly increased (Fig. 7).

4. Discussion

The possible adverse effects of pyrethroids on aquatic environments, including fish, have become an important issue [30]. Fish are often used as sentinel organisms because they have prominent roles in the trophic chain, react to low concentrations of mutagens, and may accumulate toxic substances, both directly, from contaminated water, and indirectly, by eating contaminated aquatic organisms [31]. Pollutant effects on vertebrate retinal cells have been reported by several groups [32–37]. We chose to study the effect of CM on the zebrafish retina, a system derived from the CNS with a simpler organization than the vertebrate brain.

Retinal cells of zebrafish exposed to CM showed significant changes, including apoptotic bodies, in the photoreceptor cell layer and, to a lesser extent, in the inner nuclear layer. In contrast to the amphibian larvae brain [12–14], these changes occurred at CM concentrations much lower than those found in surface water or runoff from agricultural practices in Argentina [3], and were already seen after relatively short exposures. To test

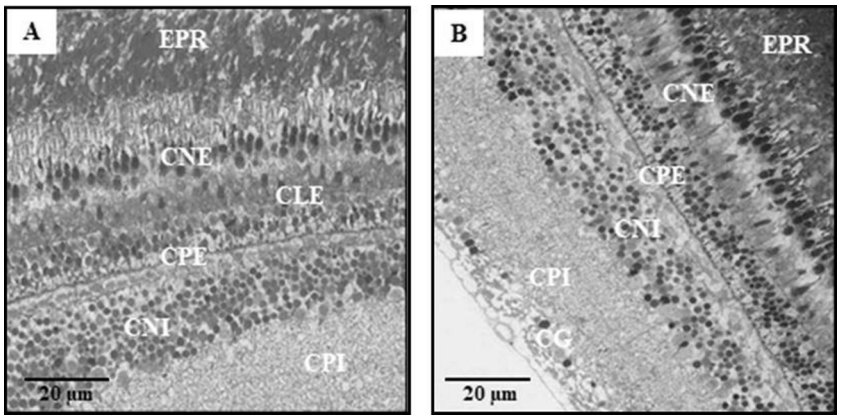

Fig. 1. Retinal histological sections of zebrafish exposed to CM, 0.3 µg/L. Morphological changes observed in the retinas of zebrafish at 12 d (A) compared to controls (B). Toluidine blue stain. ONL: Outer Nuclear Layer; OLM: External Limiting Membrane; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Internal Plexiform Layer; GCL: Ganglion Cell Layer.

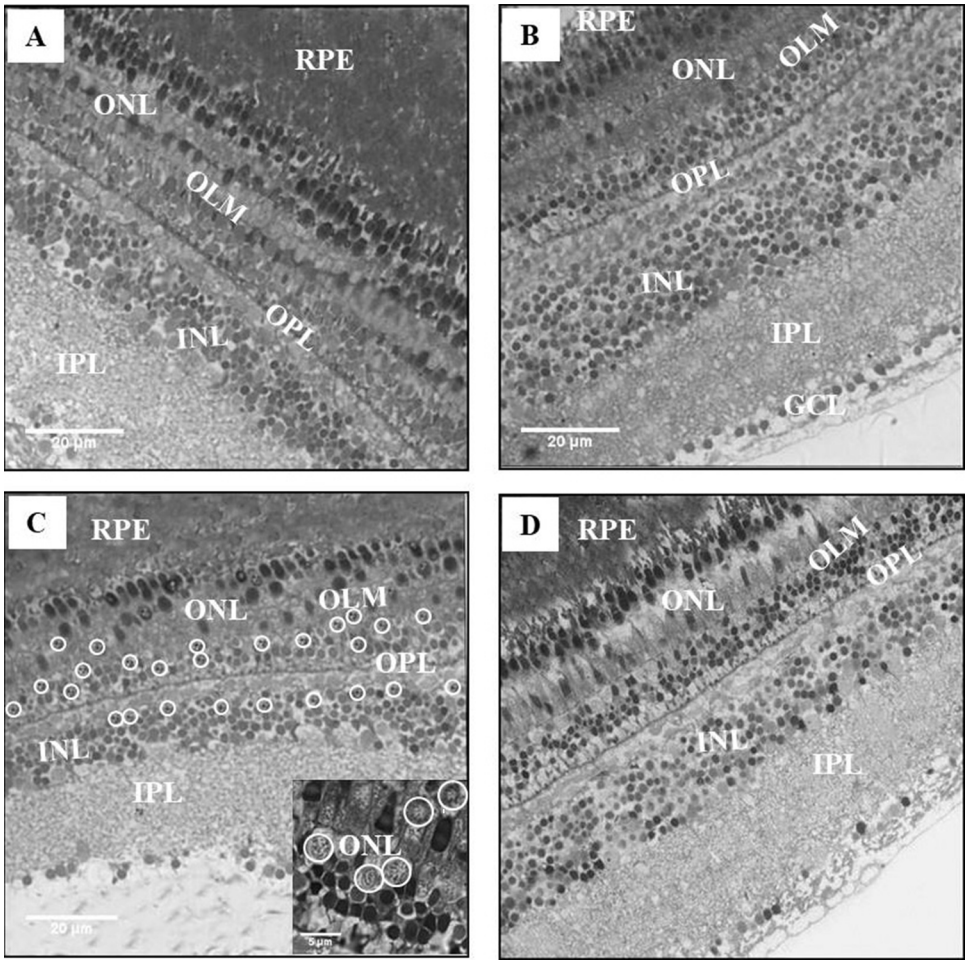

Fig. 2. Retinal histological sections of zebrafish exposed to CM, 0.6 µg/L. No significant morphological changes were observed during the first 9 d of exposure (A) relative to the control zebrafish (B). After 12 d exposure, the retinas showed apoptotic nuclei in the photoreceptor layers (C); controls are shown in (D); the red outlines show apoptotic figures. The inset shows the morphology of the nuclei. Toluidine blue stain. ONL: Outer Nuclear Layer; OLM: External Limiting Membrane; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Internal Plexiform Layer; GCL: Ganglion Cell Layer.

whether the morphological changes were indeed due to apoptosis, we used immunofluorescence with antibodies to γ-H2AX [38] and caspase-3 [39]. After 12 d exposure, γ-H2AX immunoreactivity was observed on both the outer and inner nuclear layer cells, while caspase-3 immunoreactivity was observed only in the outer layer. These results are consistent with the work of Rogakou et al. [38], who showed that γ-H2AX is seen as an early response to DNA fragmentation, prior to activation of caspase-3. Our studies are consistent with the view that double-strand DNA breaks are an early event in the apoptotic pathway [40] and may explain the differential responses of retinal cells seen in our experiments. Shi et al. [41] showed that, in 4-h-post-fertilization zebrafish embryos exposed to CM, caspase-3 activity increases, *p53* gene expression increases, and *ogg1* gene expression decreases. The studies of Jin et al. [18] demonstrated that mRNA levels of some apoptosis genes, such as *p53, puma, bax, apaf1, caspase-9* and *caspase-3*, were significantly up-regulated after exposure to 3 or 10 µg/L CM. In the same study, the activities of caspase-3 and caspase-9 also increased significantly after CM exposure. These findings suggest that CM induces DNA damage in the photoreceptor cells (possibly by a specifically neurotoxic mechanism) and that this damage cannot be adequately repaired. Cells in the inner nuclear layer may avoid apoptosis by more effective DNA repair.

The comet assay measures DNA damage in single cells [42]. BDI values for retinal cells reported here are somewhat higher than those found by Poletta et al. [43] in gill cells of *Prochilodus lineatus*. The difference may be either cell type-related or species-related; this remains to be determined.

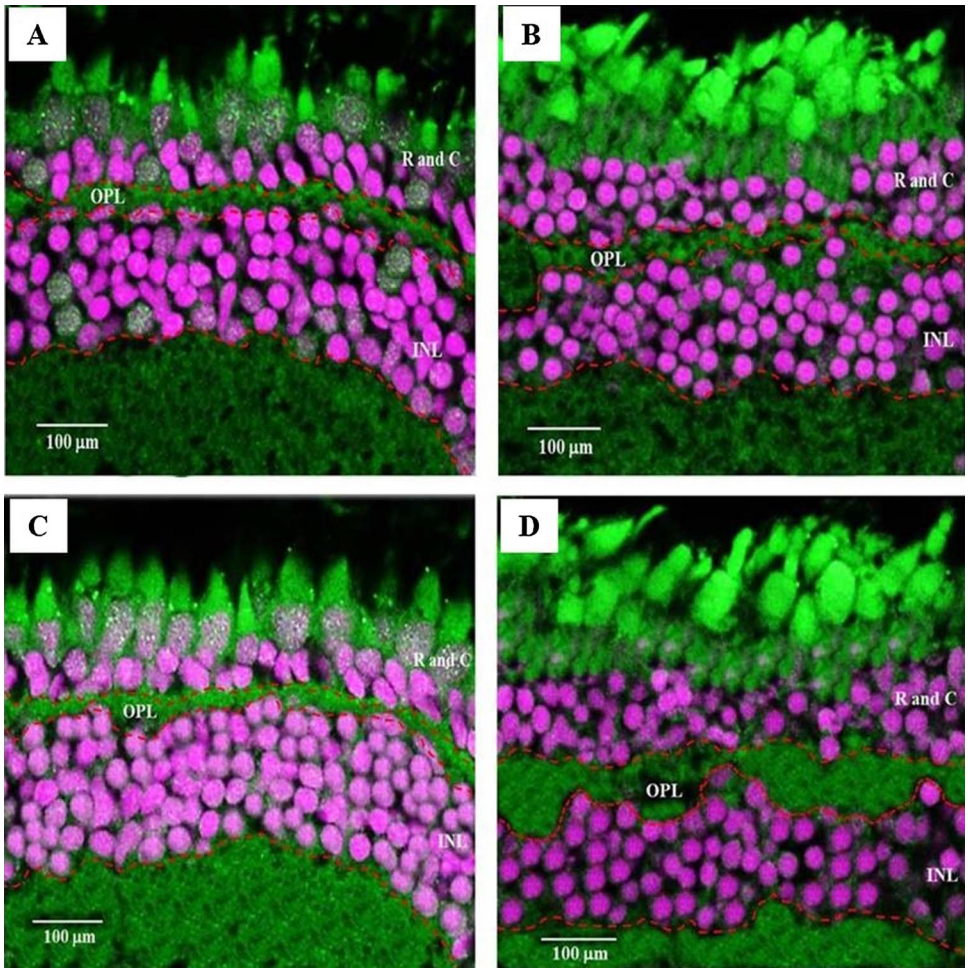

Fig. 3. Immunofluorescence analysis of zebrafish retinal cells exposed to CM, 0.6 µg/L, for 12 d; antibodies to γ-H2AX and caspase-3, respectively. (A) Retinas of exposed fish ($n = 5$) showed immunoreactivity at the level of outer nuclear layer (rods and cones) and the inner nuclear layer with the anti γ-H2AX antibody. (B) Retinal cells of the control animals are devoid of immunoreactivity for γ-H2AX. (C) Retinas of exposed fish ($n = 5$) showed immunoreactivity at the level of outer nuclear layer (rods and cones) with the caspase-3 antibody. (D) Retinal cells of the control animals are devoid of immunoreactivity for caspase-3. The green color observed on the external segments of the photoreceptors is the autofluorescence of the visual pigments. R and C: Rods and Cones; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

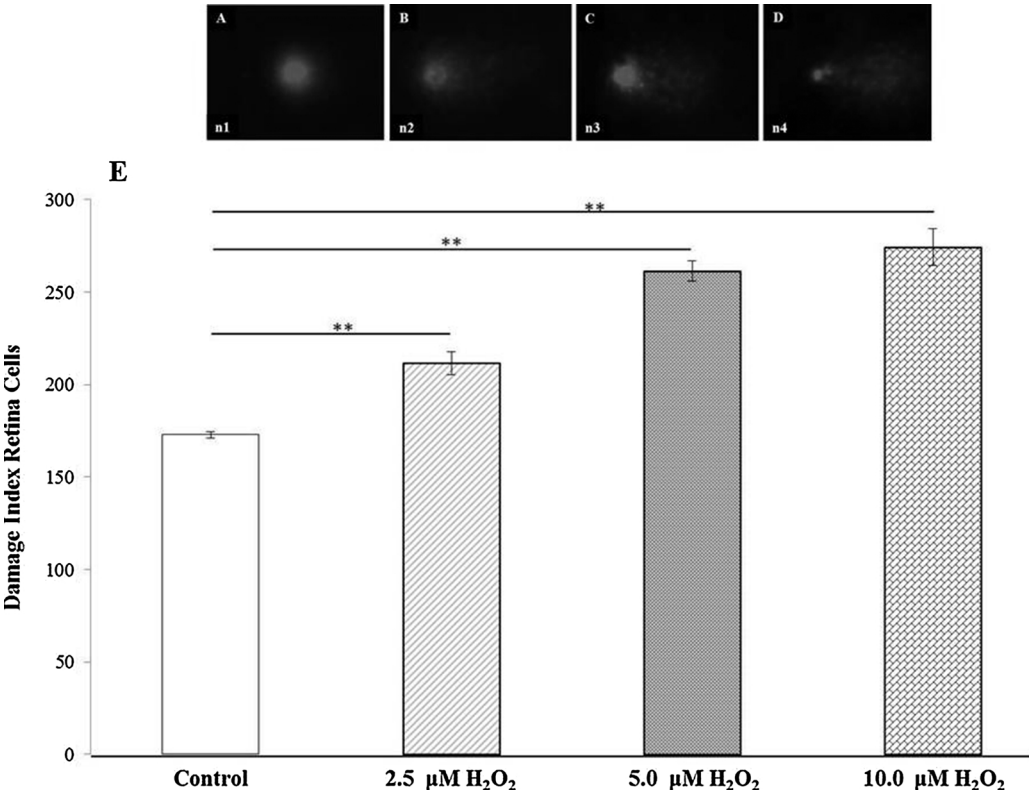

Fig. 4. Comet assay. (A) Class 0 (undamaged); (B) Class 1 (slightly damaged); (C) Class 2 (medium damage); (D) Class 3 (highly damaged). (E) Damage index (DI) values in retina cells of zebrafish after *in vitro* exposure to H_2O_2 ($n = 5$). Control; 2.5 µM H_2O_2; 5.0 µM H_2O_2; 10.0 µM H_2O_2. Values significantly different from the control are indicated by asterisks (**$p < 0.01$).

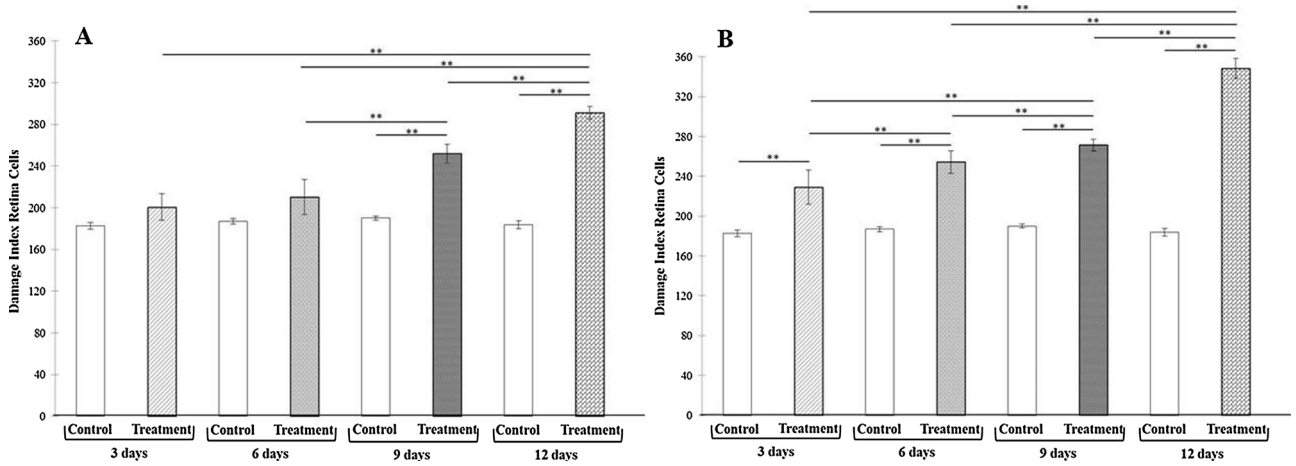

Fig. 5. Damage index (DI) values of DNA in retinal cells of zebrafish exposed to CM at 0.3 (A) (n = 5) and 0.6 μg/L (B) (n = 5). Statistically significant differences are indicated by asterisks (**p < 0.01).

Table 2
Damage index in retinal cells of zebrafish adults exposed *in vivo* to CM.

	CM (μg/L)	Damage Index (mean ± SE)			
		3 d	6 d	9 d	12 d
Controls	0	182.6 ± 3.2	187.0 ± 2.5	190.0 ± 1.9	183.8 ± 3.7
Treatment (low)	0.3	200.6 ± 12.7	210.2 ± 16.8	252.0 ± 8.9	290.8 ± 6.3
Treatment (high)	0.6	229.2 ± 17.3	254.4 ± 11.1	271.4 ± 5.77	348.6 ± 9.8

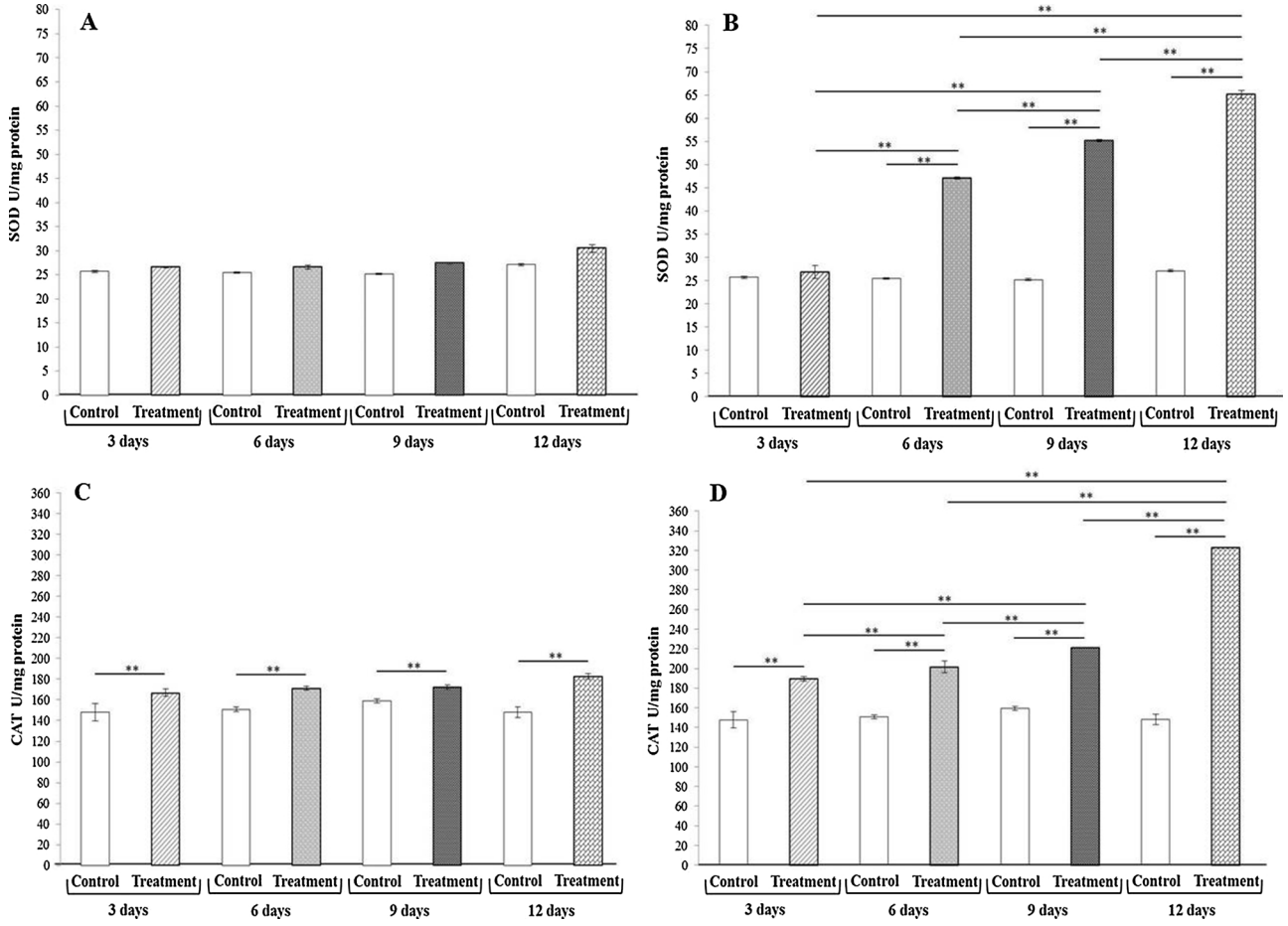

Fig. 6. Activities of SOD (A and B) and CAT (C and D) in the retinal cells of zebrafish exposed to CM, 0.3 and 0.6 μg/L, respectively, for 3, 6, 9, and 12 d (n = 5, each). Statistically significant differences are indicated by asterisks (**p < 0.01).

Table 3
Activities of superoxide dismutase (SOD) and catalase (CAT) in zebrafish retina exposed to CM.

		Enzyme activity (U/mg protein)				
		CM (μg/L)	3 d	6 d	9 d	12 d
SOD	Control	0	25.7 ± 0.2	25.5 ± 0.2	25.2 ± 0.2	27.1 ± 0.2
	Treatment (low)	0.3	26.6 ± 0.2	26.6 ± 0.6	27.4 ± 0.2	30.5 ± 0.7
	Treatment (high)	0.6	26.9 ± 1.4	47.1 ± 0.2	55.2 ± 0.3	65.1 ± 0.9
CAT	Control	0	147.8 ± 8.4	150.8 ± 2.3	159.2 ± 1.9	135.1 ± 0.2
	Treatment (low)	0.3	167.0 ± 3.4	171.2 ± 1.9	172.4 ± 2.1	182.8 ± 2.5
	Treatment (high)	0.6	189.4 ± 2.3	201.7 ± 6.1	221.2 ± 0.2	322.8 ± 0.2

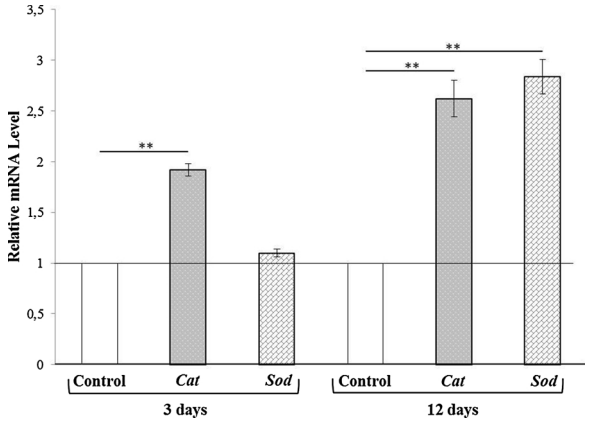

Fig. 7. Gene expression levels of *cat* and *sod* in the retina of zebrafish exposed to 0.6 μg/L CM ($n = 6$). Statistically significant differences are indicated by asterisks (**$p < 0.01$).

We tested H_2O_2, an oxidative damaging agent [25], and observed DNA damage in retinal cells after 10 min *in vitro* exposure. Poletta et al. [43] observed H_2O_2-induced DNA damage in gill cells of *P. lineatus*. Gills are an important tissue for uptake of contaminants by fish; gills are directly exposed to the environment and gill cells are constantly dividing. Indeed, gills are a standard organ for study of water pollution effects [31]. Our work indicates that retinal cells may be even more sensitive than gill cells for such studies.

Positive comet assay results for CM have been found in other species. Patel et al. [44] reported that the compound causes DNA damage in mouse brain, liver, and kidney. Poletta et al. [43] observed DNA damage in epithelial gill cells of *P. lineatus* after acute *in vivo* exposures.

The teleost retina has a pool of multipotent progenitor cells within the circumferential germinal zone, which are continuously renewed, forming more neurons. If retinal cells are damaged at early stages, these progenitor cells can regenerate all retinal cell types [45]. Over time, this pool of cells may be lost, or they may lose their differentiation potential, and therefore damage sensitivity increases. The teleost retina may provide an excellent model for studying neuronal regeneration after damage caused by neurotoxic substances. One possible factor accounting for the genotoxic effects of CM is that, because of its small size and hydrophobicity, CM can easily cross cell membranes, reaching and interacting with DNA through its acid moieties [46]. The binding of CM to DNA may lead to destabilization of the DNA structure and duplex unwinding, inducing chromosomal damage.

Alternatively, oxidative stress [47] may cause the retinal damage. Analysis of the expression of the genes encoding SOD and CAT has been used to detect toxicity and monitor the effects of chemical pollutants [48,49]. Oxidative stress and DNA damage may be closely related [50]. ROS can induce oxidative DNA damage, including strand breaks and base and nucleotide modifications [51]. Differential expression of the genes encoding these antioxidant proteins has been used to detect biological toxicity and/or to monitor the impact of chemical pollutants [52]. Increased activity of ROS-scavenging enzymes such as SOD and CAT may protect cells against the effects of CM. Many genes related to the oxidative stress response have been identified in zebrafish [53,54]. Jin et al. [18] argued that CM produces oxidative stress in hepatocytes of adult zebrafish, altering mRNA levels of the antioxidant enzymes SOD and CAT.

5. Conclusion

CM causes detrimental effects in retinal cells of zebrafish within a short time of exposure and at concentrations much lower than those that may be found in runoff and surface waters in natural environments. The effects include apoptosis, DNA damage, increased expression of caspase-3 and γ-H2AX genes, alterations in antioxidant enzyme activities, and increased expression of their respective genes. Our results provide important information about the mechanisms of CM-induced neurotoxicity and also suggest that zebrafish can serve as an excellent model for studying the developmental toxicity of environmental contaminants.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was partly funded by the PICTO ANPCYT-UNER-2009-209 to V.H. Casco and PID-SCYT-UNER N° 6119-2010 to E.V. Paravani. We are very thankful to Mr. Sebastian Graziati for fish husbandry and technical help and to Prof. Diana R. Kohan for her contributions in the statistical analysis. The anti-γ-H2AX antibody was kindly provided by Dr. Daniel Prieto.

References

[1] K. Haya, Toxicity of pyrethroid insecticide to fish, Environ. Toxicol. Chem. 8 (1989) 381–391.
[2] I.R. Hill, Aquatic organisms and pyrethroids, Pestic. Sci. 27 (1989) 429–457.
[3] D. Marino, A. Ronco, Cypermethrin and chlorpyrifos concentration levels in surface water bodies of the Pampa Ondulada Argentina, Bull. Environ. Contam. Toxicol. 75 (2005) 820–826.
[4] P. Carriquiriborde, J. Díaz, H. Mugni, C. Bonetto, A.E. Ronco, Impact of cypermethrin on stream fish populations under field-use in biotech-soybean production, Chemosphere 68 (2007) 613–621.
[5] S.A. Burr, D.E. Ray, Structure-activity and interaction effects of 14 different pyrethroids on voltage-gated chloride ion channels, Toxicol. Sci. 77 (2004) 341–346.
[6] S. Jergentz, H. Mugni, C. Bonetto, R. Schulz, Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina, Chemosphere 61 (2005) 817–826.
[7] Y. Jin, X. Zhang, L. Shu, L. Chen, L. Sun, H. Qian, W. Liu, Z. Fu, Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*), Chemosphere 78 (2010) 846–852.
[8] A. Valavanidis, T. Vlahogianni, M. Dassenakis, M. Scoullos, Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants, Ecotoxicol. Environ. Saf. 64 (2006) 178–189.
[9] X. Zhang, P. Xie, D. Li, R. Tang, H. Lei, Y. Zhao, Time-dependent oxidative stress responses of crucian carp (*Carassius auratus*) to intraperitoneal injection of extracted microcystins, Bull. Environ. Contam. Toxicol. 82 (2009) 574–578.
[10] N. Uner, E.O. Oruç, M. Canli, Y. Sevgiler, Effects of cypermethrin on antioxidant

enzyme activities and lipid peroxidation in liver and kidney of the freshwater fish, *Oreochromis niloticus* and *Cyprinus carpio* (L.), Bull. Environ. Contam. Toxicol. 67 (2001) 657–664.
[11] Y. Jin, S. Zheng, Z. Fu, Embryonic exposure to cypermethrin induces apoptosis and immunotoxicity in zebrafish (*Danio rerio*), Fish Shellfish Immunol. 30 (2011) 1049–1054.
[12] M.F. Izaguirre, R.C. Lajmanovich, P.M. Peltzer, A.P. Soler, V.H. Casco, Cypermethrin-induced apoptosis in the telencephalon of *Physalaemus biligonigerus* tadpoles (*Anura: Leptodactylidae*), Bull. Environ. Contam. Toxicol. 65 (2000) 501–507.
[13] M.F. Izaguirre, M.N. Vergara, V.H. Casco, CAS role in the brain apoptosis of *Bufo arenarum* induced by cypermethrin, Biocell 30 (2006) 309–320.
[14] V.H. Casco, M.F. Izaguirre, L. Marín, M.N. Vergara, R.C. Lajmanovich, P. Peltzer, A.P. Soler, Apoptotic cell death in the central nervous system of *Bufo arenarum* tadpoles induced by cypermethrin, Cell Biol. Toxicol. 22 (2006) 199–211.
[15] B.J. Melloni, How the retina works, Am. Fam. Phys. 4 (1971) 81.
[16] Y.J. Dai, Y.F. Jia, N. Chen, W.P. Bian, Q.K. Li, Y.B. Ma, Y.L. Chen, D.S. Pei, Zebrafish as a model system to study toxicology, Environ. Toxicol. Chem. 33 (2014) 11–17.
[17] M. Westerfield, The Zebrafish Book, A Guide for the Laboratory Use of Zebrafish (*Danio Rerio*), 4th ed., University of Oregon, Eugene, OR, 2000.
[18] Y. Jin, S. Zheng, Y. Pu, L. Shu, L. Sun, W. Liu, Z. Fu, Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (*Danio rerio*), Chemosphere 82 (2011) 398–404.
[19] EXTOXNET, The Extension Toxicology Network, Pesticide Information Profiles (PIPs), (2017) (Accessed 26 October 2017) Available at: http://extoxnet.orst.edu/pips/ghindex.htmlS.
[20] S. Mercille, B. Massie, Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells, Biotechnol. Bioeng. 44 (1994) 1140–1154.
[21] M.F. Simoniello, F. Gigena, G. Poletta, A. Loteste, E. Kleinsorge, M. Campana, J. Scagnetti, M.J. Parma, Alkaline comet assay for genotoxic effect detection in neotropical fish *Prochilodus lineatus* (Pisces, Curimatidae), Bull. Environ. Contam. Toxicol. 83 (2009) 155–158.
[22] D.W. Fairbairn, P.L. Olive, K.L. O'Neill, The comet assay: a comprehensive review, Mutat. Res. 339 (1995) 37–59.
[23] C.M. Gedik, S.W. Ewen, A.R. Collins, Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells, Int. J. Radiat. Biol. 62 (1992) 313–320.
[24] M. Dusinska, A.R. Collins, The comet assay in human biomonitoring: gene-environment interactions, Mutagenesis 23 (2008) 191–205.
[25] A.M. de Miranda Cabral Gontijo, R.E. Barreto, G. Speit, V.A.V. Reyes, G.L. Volpato, G.M.F. Salvadori, Anesthesia of fish with benzocaine does not interfere with comet assay results, Mutat. Res. 534 (2003) 165–172.
[26] H.P. Misra, I. Fridovich, The oxidation of phenylhydrazine: superoxide and mechanism, Biochemistry 15 (1976) 681–687.
[27] Y.T. Zhang, Q.S. Zheng, J. Pan, R.L. Zheng, Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants, Basic Clin. Pharmacol. Toxicol. 95 (2004) 53–58.
[28] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[29] C. Nasuti, R. Gabbianelli, M.L. Falcioni, A.D. Stefano, P. Sozio, F. Cantalamessa, Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids, Toxicology 229 (2007) 194–205.
[30] Y.F. Sasaki, F. Izumiyama, E. Nishidate, S. Ishibashi, S. Tsuda, N. Matsusaka, N. Asano, N. Saotome, T. Sofuni, M. Hayashi, Detection of genotoxicity of polluted sea water using shellfish and the alkaline single-cell gel electrophoresis (SCE) assay: a preliminary study, Mutat. Res. 393 (1997) 133–139.
[31] T. Cavas, S. Ergene-Gözükara, Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents, Aquat. Toxicol. 74 (2005) 264–271.
[32] P. Simoniello, F. Trinchella, S. Filosa, R. Scudiero, D. Magnani, T. Theil, C.M. Motta, Cadmium contaminated soil affects retinogenesis in lizard embryos, Exp. Zool. A Ecol. Genet. Physiol. 321 (2014) 207–219.
[33] M. Korbas, B. Lai, S. Vogt, S.C. Gleber, C. Karunakaran, I.J. Pickering, P.H. Krone, G.N. George, Methylmercury targets photoreceptor outer segments, ACS Chem. Biol. 18 (2013) 2256–2263.
[34] L. Huang, C. Wang, Y. Zhang, M. Wu, Z. Zuo, Phenanthrene causes ocular developmental toxicity in zebrafish embryos and the possible mechanisms involved, J. Hazard. Mater. 15 (2013) 172–180.
[35] X. Zhang, D. Jones, F. Gonzalez-Lima, Neurodegeneration produced by rotenone in the mouse retina: a potential model to investigate environmental pesticide contributions to neurodegenerative diseases, J. Toxicol. Environ. Health A 69 (2006) 1681–1697.
[36] W. Dong, W. Muramoto, Y. Nagai, K. Takehana, J.J. Stegeman, H. Teraoka, T. Hiraga, Retinal neuronal cell is a toxicological target of tributyltin in developing zebrafish, J. Vet. Med. Sci. 8 (2006) 573–579.
[37] F. Yu, Z. Wang, B. Ju, Y. Wang, J. Wang, D. Bai, Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E, Exp. Toxicol. Pathol. 59 (2008) 415–423.
[38] E.P. Rogakou, W. Nieves-Neira, C. Boon, Y. Pommier, W.M. Bonner, Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139, J. Biol. Chem. 275 (2000) 9390–9395.
[39] G.M. Cohen, Caspases: the executioners of apoptosis, Biochemistry 326 (1997) 1–16.
[40] A. Rebbaa, X. Zheng, F. Chu, B.L. Mirkin, The role of histone acetylation versus DNA damage in drug-induced senescence and apoptosis, Cell Death Differ. 13 (2006) 1960–1967.
[41] X. Shi, A. Gu, G. Ji, Y. Li, J. Di, J. Jin, F. Hu, Y. Long, Y. Xia, C. Lu, L. Song, S. Wang, X. Wang, Developmental toxicity of cypermethrin in embryo-larval stages of zebrafish, Chemosphere 85 (2011) 1010–1016.
[42] G. Frenzilli, M. Nigro, B.P. Lyons, The comet assay for the evaluation of genotoxic impact in aquatic environments, Mutat. Res. 681 (2009) 80–92.
[43] G.L. Poletta, F. Gigena, A. Loteste, M.J. Parma, E.C. Kleinsorge, M.F. Simoniello, Comet assay in gill cells of *Prochilodus lineatus* exposed in vivo to cypermethrin, Pestic. Biochem. Physiol. 107 (2013) 385–390.
[44] S. Patel, A.K. Pandey, M. Bajpayee, D. Parmar, A. Dhawan, Cypermethrin-induced DNA damage in organs and tissues of the mouse: evidence from the comet assay, Mutat. Res. 607 (2006) 176–183.
[45] R.C. Marcus, C.L. Delaney, S.S. Easter, Neurogenesis in the visual system of embryonic and adult zebrafish (*Danio rerio*), Vis. Neurosci. 16 (1999) 417–424.
[46] P.N. Saxena, L.K.S. Chauhan, S.K. Gupta, Cytogenetic effects of commercial formulation of cypermethrin in root meristem cells of *Allium sativum*: spectroscopic basis of chromosome damage, Toxicology 216 (2005) 244–252.
[47] A. Barzilai, K.I. Yamamoto, DNA damage responses to oxidative stress, DNA Repair 3 (2004) 1109–1115.
[48] D.L. Sheader, T.D. Williams, B.P. Lyons, J.K. Chipman, Oxidative stress response of European flounder (*Platichthys flesus*) to cadmium determined by a custom DNA microarray, Mar. Environ. Res. 62 (2006) 33–44.
[49] S. Woo, S. Yum, D.W. Kim, H.S. Park, Transcripts level responses in a marine medaka (*Oryzias javanicus*) exposed to organophosphorus pesticide, Comp. Biochem. Phys. C Toxicol. Pharmacol. 149 (2009) 427–432.
[50] Y.M. Chung, Y.S. Bae, S.Y. Lee, Molecular ordering of ROS production mitochondrial changes, and caspase activation during sodium salicylate-induced apoptosis, Free Radic. Biol. Med. 34 (2003) 434–442.
[51] M.R. Bennett, Reactive oxygen species and death: oxidative DNA damage in atherosclerosis, Circ. Res. 88 (2001) 648–650.
[52] D.L. Sheader, T.D. Williams, B.P. Lyons, J.K. Chipman, Oxidative stress response of European flounder (*Platichthys flesus*) to cadmium determined by a custom DNA microarray, Mar. Environ. Res. 62 (2006) 33–44.
[53] P.M. Craig, C.M. Wood, G.B. Mc Clelland, Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*), Am. J. Physiol. Regul. Integr. Comp. Physiol. 293 (2007) 1882–1892.
[54] Y. Liu, J.S. Wang, Y.H. Wei, H.X. Zhang, M.Q. Xu, J.Y. Dai, Induction of time-dependent oxidative stress and related transcriptional effects of perfluorododecanoic acid in zebrafish liver, Aquat. Toxicol. 89 (2008) 242–250.