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Genotoxicity Induced by Cypermethrin in the Zebrafish Retina

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Abstract

Cypermethrin (Cyp), is one of the most common contaminants in freshwater aquatic systems. We evaluated its possible genotoxic effect and oxidative stress in retinal cells of adult zebrafish exposed to 0.3 $\mu\text{g/L}$ and 0.6 $\mu\text{g/L}$ Cyp. Both the histological and immunofluorescence (IF) techniques showed the presence of apoptotic cells in the zebrafish retina after 9 days of treatment with 0.6 $\mu\text{g/L}$ Cyp. Thus, histone $\gamma\text{-H2AX}$, a double-stranded DNA damage marker, was immunodetected in both the outer and inner nuclear layer after exposure to 0.6 $\mu\text{g/L}$ Cyp for 12 days, while the anti-caspase-3 apoptotic antibody was detected in the outer nuclear layer. Compared with the morphological evidence, the damage index (DI) showed significant differences with 0.3 $\mu\text{g/L}$ from day 9, while with 0.6 $\mu\text{g/L}$ all the stages evaluated showed very significant differences. According to these results, it was verified that the activities of superoxide dismutase (SOD) and catalase (CAT) increased significantly after exposure to 0.6 $\mu\text{g/L}$ Cyp. The same treatment caused a significant positive regulation of the mRNA levels of both genes. These results indicate that Cyp causes DNA damage and oxidative stress. This pyrethroid also has the potential to induce apoptosis in the cells of the retina.

Keywords: genotoxicity, cypermethrin, zebrafish, retina

1. Introduction

Pyrethroid insecticides are potent neurotoxicants for various insect and vertebrate groups. Pyrethroids exhibit a typical toxic action pattern of a strongly excitatory effect on the nervous system [1]. The main target sites for pyrethroids are the voltage-dependent sodium

channels of neuronal membranes [2–4]. Based on different behavioral, neurophysiological, and biochemical profiles, two classes of pyrethroids have been identified. The type I are associated with hyperexcitation and weak tremors, while type II, who have a cyano group, are associated with a more complex syndrome, including clonic seizures [1, 5]. Type II pyrethroids are extensively applied to control pests in residential and agricultural environments [6].

Cypermethrin, ((RS)- α -cyano-3-phenoxybenzyl (1RS) cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate), is a type II synthetic pyrethroid insecticide that has high insecticidal activity, relatively low toxicity in birds and mammals, and adequate air and light stability [7, 8]. This compound has a wide range of uses in numerous crops such as cotton, cereals, vegetables, and fruit trees, where it controls many insects including lepidopterans, coleopterans, and hemipterans. It is also used in food storage sites, to control flies and fleas in domestic farms and extensively for the control of cockroaches, mosquitoes, and lice [9].

It presents a moderate persistence in the environment and its residues are not usually accumulated in a significant way. In soil, it has a half-life between 4 and 56 days, being more persistent in anaerobic environments. Under aerobic conditions, it is moderately sensitive to light and undergoes microbial degradation. In water, it is stable in neutral or acidic conditions, with a half-life that exceeds 50 days and a photolysis resistance greater than 100 days. In surface water bodies, the Cyp concentration decreases rapidly by adsorption to the sediment, suspended particles, and plants.

Despite the beneficial roles, Cyp enters the brain, accumulates in a significant amount, and exerts neurotoxicity on non-target organisms [10]. In rodents, Cyp induces nigrostriatal dopaminergic neurotoxicity and, if co-administered, increases the neurodegenerative potential of other toxic chemical substances [11, 12]. Pyrethroids also induce neurotoxic chronic effects in humans [13]. In aquatic organisms, previous studies by our group allowed to determine the effect of Cyp on the tadpole's brain of two South American anurans species [14–16]. In these studies, developed under laboratory conditions using sublethal and acute Cyp doses, both their survival rate and altered brain morphology were analyzed. In the studies using prometamorphic *Bufo arenarum* larvae, high mortality rates (~65–70%) were observed in animals exposed to 39 and 156 $\mu\text{g/L}$ Cyp per 96 h [16]. In this species, the LC_{50} at 96 h was 110 $\mu\text{g/L}$, so the sensitivity of this species coincides with that previously reported for *Physalaemus biligonigerus* [14]. At the histological and ultrastructural levels, the most affected telencephalon regions were marginal and intermediate layers, surrounding the brain ventricles. TUNEL and DNA fragmentation assays confirmed the time and dose-dependent increase in the number of apoptotic telencephalic cells, as well as the DNA damage of the immature brain cells of *B. arenarum* tadpoles. However, the CNS of vertebrates is immensely complex and we were not able to determine the possible apoptotic mechanisms triggered by Cyp. For this reason, many studies focused to understand the processes triggered by drugs and neurotoxicant substances that can act on the CNS are conducted by studying a relatively simple and

accessible region of the CNS, such as the retina. In the present study, we investigate, in an *in vivo* model such as adult zebrafish, the possible link between oxidative stress and apoptosis in the relatively simple neuronal system of zebrafish retina.

Zebrafish is one of the most widely used vertebrates as a model organism for genetic studies [17, 18] and environmental toxicology [19–22]. In addition, it has been used in studies of ecological monitoring of the environment and during evaluations of multiple pollutants such as organic, endocrine disruptors, and heavy metals [23–25].

Environment pollution, especially aquatic, is a serious problem all over the world. Not only does it affect the survival and reproduction of aquatic organisms but it has a negative impact on human health, fundamentally via bioconcentration processes. Sensitivity to different pollutants makes zebrafish an ideal model organism for environmental monitoring. Characteristic changes in morphology, gene expression, behavior, and physiology can be observed as biological indicators. The International Organization for Standardization first published the zebrafish toxicity test in 1984. Thereafter, several countries promulgated their own toxicity testing standards through their use. Many environmental pollutants, including pyrethroids, interfere with the functions of the endocrine system, affect development, produce DNA damage, and induce oxidative stress in zebrafish [26–29].

This *in vivo* study was performed to characterize Cyp dose- and time-dependent toxicity using histology, immunofluorescence (IF) and comet assay (CA) studies, superoxide dismutase (SOD) and catalase (CAT) enzyme activity as well as *sod* and *cat* gene expression (Figure 1).

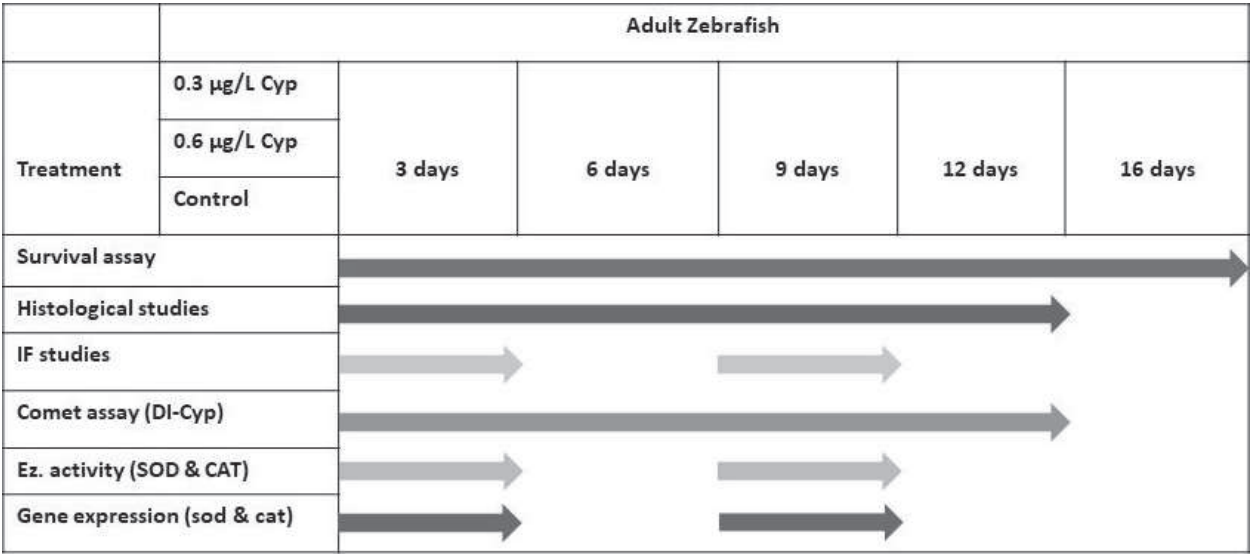


Figure 1. Summary of experimental procedures and techniques used during this work.

2. *In vivo* cypermethrin bioassay

Based on reported concentration of Cyp in agroecological aquatic systems [27, 30], adult fish kept in aquarium according to [31] were treated with 0.3 and 0.6 $\mu\text{g/L}$ Cyp solutions. Each treatment was carried out by triplicate, with their respective control, for 3, 6, 9, 12, and 16 days, with daily changes of the solution. All fish exposed to 0.3 $\mu\text{g/L}$ Cyp survived for the first 12 days, while the survival rate was 80% at the end of the bioassay. Regarding the fish group exposed to 0.6 $\mu\text{g/L}$ Cyp, the survival rate at the 12 days of exposure was 100%; from day 13, the percentage was 80%, decreasing to 50% on day 15 to finally reach 30% at the end of the bioassay. Based on the survival rate with both Cyp concentrations, it was decided to perform all the remaining *in vivo* studies with both concentrations, using 12 days as maximum exposure time.

3. Histological and immunofluorescence analysis

Eyes of control and *in vivo* exposed animals were processed according to Casco et al. [16]. The cured blocks were cross-sectioned at 0.5 μm of thickness using an ultramicrotome. Semi-thin sections were toluidine blue stained and recorded by the charge-coupled device (CCD) camera, coupled to an Olympus BX50 microscope at 40 and 100 \times . In the histological study, just after 9 days of treatment, zebrafish exposed to 0.6 $\mu\text{g/L}$ Cyp show significant changes in retinal cell layers. Apoptotic-like cells could be viewed mostly in the photoreceptor cell layer and, to a lesser extent, in the inner nuclear layer (**Figure 2**).

Unlike what was reported for the amphibian larvae brain [14–16], in the present work the major changes in the retinal cell layers were triggered with Cyp concentrations much lower than those found in surface water or runoff from agricultural practices in Argentina [32] and during relatively short exposure periods.

In addition to the possible effect on alterations in nerve impulse transmission by the modification of voltage-dependent sodium channel behavior, more recently the studies by Mun et al. [11] determined that Cyp causes oxidative stress neurotoxicity in rats. These effects are associated with an increased production of reactive oxygen species (ROS). As has been proven in numerous studies, oxidative damage of DNA is an inevitable consequence of cellular metabolism, but toxic insults increase the propensity to raise the levels of ROS. These interact with DNA, leading to serious consequences for cells [33]. Based on this background, it was hypothesized that retinas of zebrafish exposed for 12 days at 0.6 $\mu\text{g/L}$ Cyp undergo a series of alterations that together lead to modifications in the synaptic connections of their neurons and in the formation of apoptotic figures in the inner and outer nuclear layer cells. To corroborate the histological observations about the increase of apoptotic bodies on the fish retinal cells layers submitted for prolonged periods (12 days) to the highest concentration of Cyp (0.6 $\mu\text{g/L}$), an IF study was carried out using microscopy confocal laser techniques.

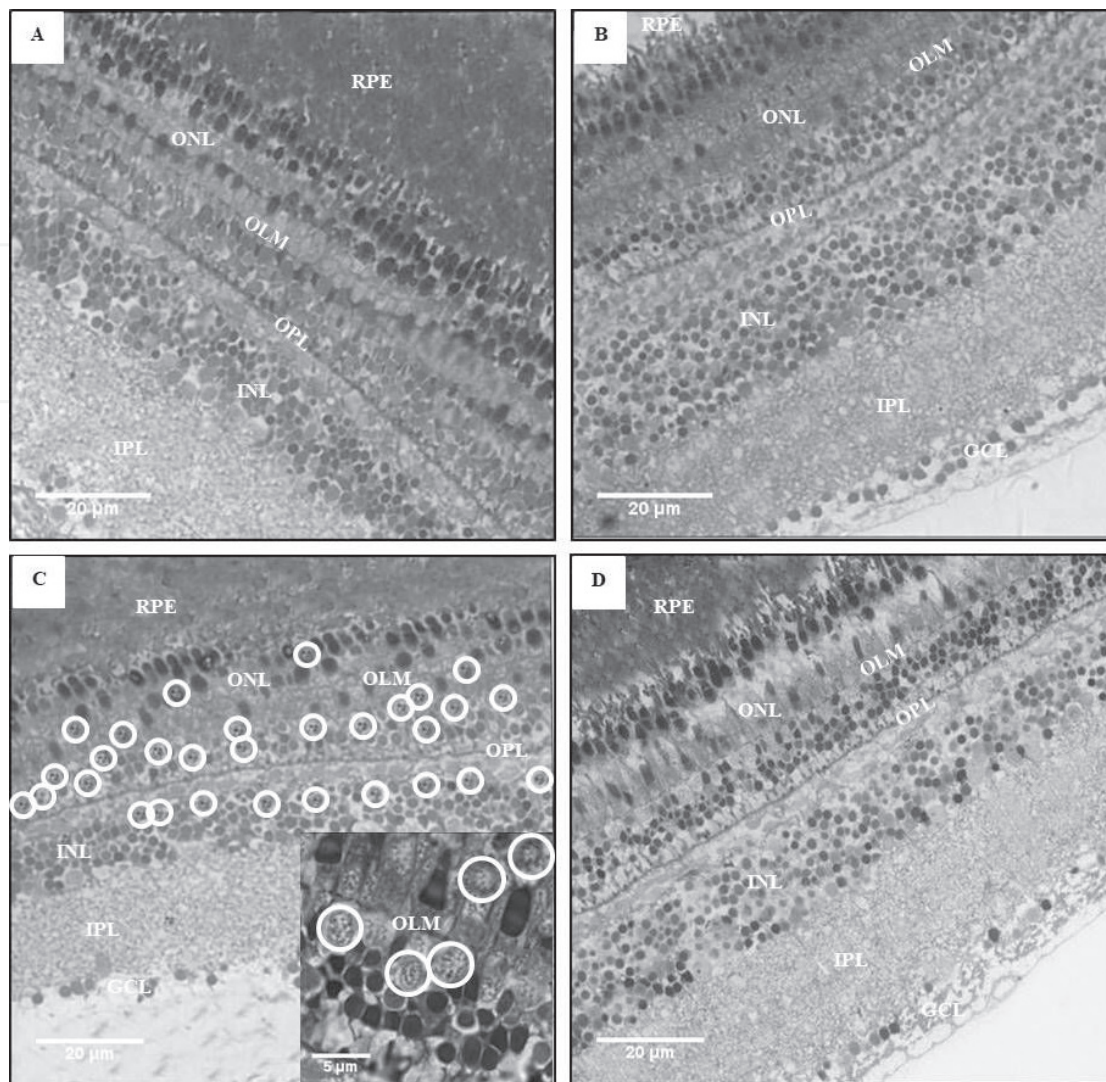


Figure 2. Retinal histological sections of zebrafish exposed to 0.6 $\mu\text{g/L}$ Cyp. No significant morphological changes were observed during the first 9 days of exposure (A) in relation to the control of the zebrafish (B). After 12 days of exposure, the retinas of zebrafish showed apoptotic nuclei of the photoreceptor layers (C), in comparison with the control (D), the white's circle show apoptotic figures. Inset shows more precisely the nuclei morphology. ONL: external nuclear layer; OLM: external limiting membrane; OPL: external plexiform layer; INL: internal nuclear layer; IPL: internal plexiform layer; GCL: Ganglion Cell Layer.

The immunofluorescence (IF) reactions were done by using the anti- $\gamma\text{-H2AX}$ antibody to identify cells with double strand DNA damage [34] and simultaneously the anti-caspase-3 antibody, since caspase-3 has been identified as a key executor and one of the most important downstream players in apoptosis pathways [35]. In this case, adult fish eyes were fixed in 4% paraformaldehyde, optimal cutting temperature (OCT)-embedded and cryo-sectioned at 10- μm thickness. The primary antibodies used were anti- $\gamma\text{-H2AX}$ (1/300) (Abcam, Cambridge, Ab11174) and anti-caspase-3 (Abcam, Cambridge, UK, ab2302) (1/300) and proceeded according to Izaguirre et al. [14]. Photomicrography was performed with a laser confocal system Zeiss, LSM800 (Carl Zeiss AG). Acquisitions of z-stacks were performed with

Fiji software. In this study, coinciding with the histological evidences, after 12 days of exposure, the anti- γ -H2AX immunoreactivity was found on both the outer and the inner nuclear layer cells (**Figure 3A and B**), while the caspase-3 immunoreactivity was only detected at the outer nuclear layer (**Figure 4A and B**).

These results are consistent with the Rogakou et al. [34] works, who showed that the phosphorylation of histone H2AX is an early response to DNA fragmentation, prior to activation of the protease caspase-3. According to our studies, the rapid activation of γ -H2AX compared

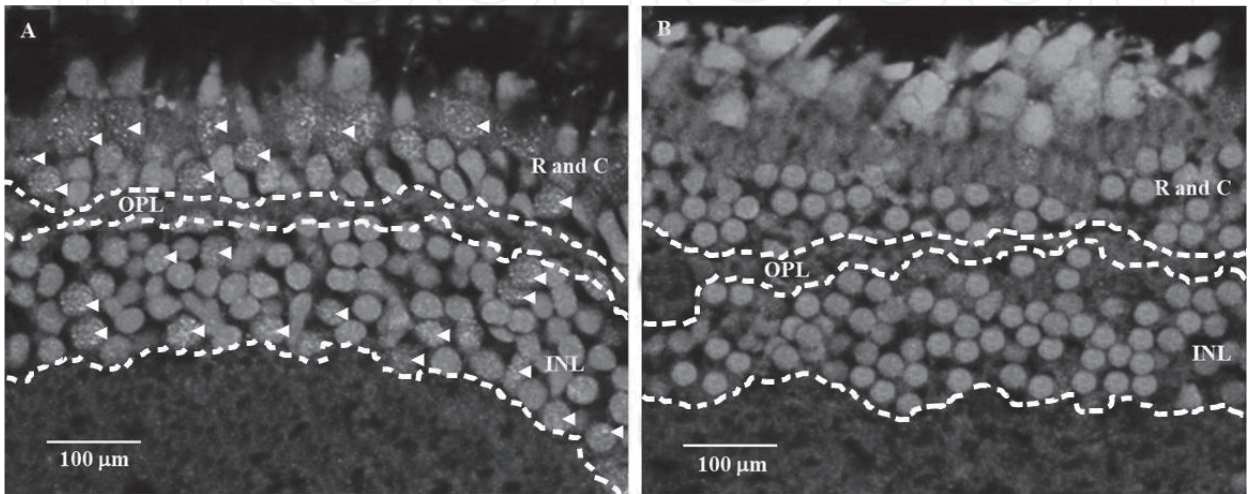


Figure 3. Immunofluorescence labeling of zebrafish retinal cells exposed to 0.6 μg/L Cyp for 12 days using anti γ -H2AX antibody. (A) Retinas of exposed fish showed immunoreactivity at the level of outer nuclear layer and the inner nuclear layer (arrowheads). (B) Retinal cells of the control animals are devoid of immunoreactivity for γ -H2AX. R and C: Rods and Cones; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer.

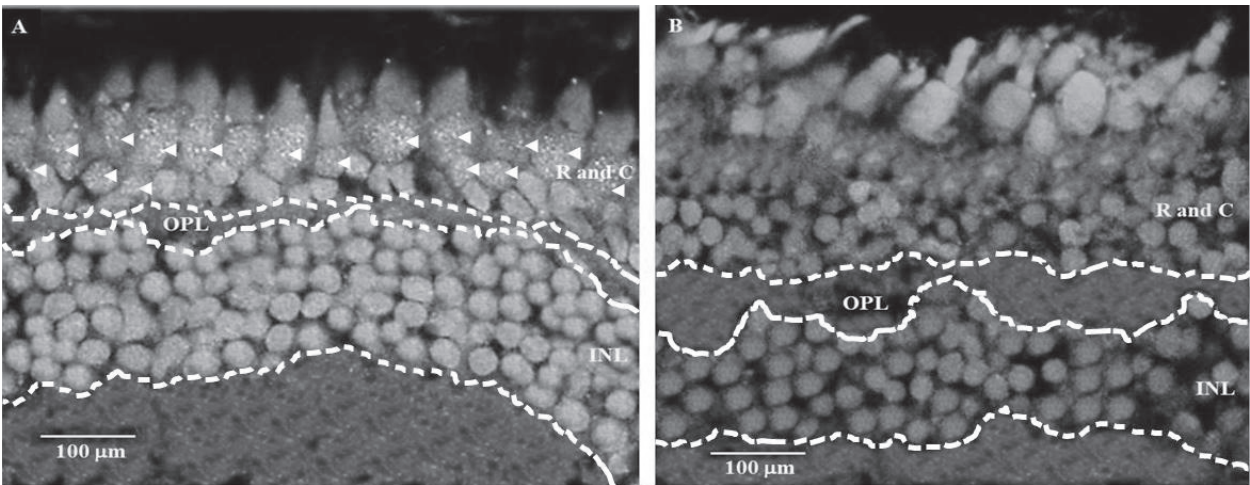


Figure 4. Immunofluorescence labeling of zebrafish retinal cells exposed to 0.6 μg/L Cyp for 12 days using anti caspase-3 antibody. (A) Retinas of exposed fish showed immunoreactivity at the level of outer nuclear layer (arrowhead). (B) Retinal cells of the control animals are devoid of immunoreactivity for caspase-3. R and C: Rods and Cones; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer.

to caspase-3 suggests that the double-stranded DNA breakdown may represent an early event in the apoptotic pathway if the DNA molecule is not repaired [36] and could explain the differential response of the retinal cells found in our experiments.

Similar results were also found in other species. For example, Patel et al. [37] reported that Cyp induced DNA damage in vital mice organs such as the brain, liver, and kidney, of animals intraperitoneally injected with 12.5, 25, 50, 100, and 200 mg/kg body weight of Cyp, for 5 consecutive days. Mukhopadhyay et al. [38] have found similar results, revealing a significant dose-dependent increase in DNA damage in the cells of brain ganglia and anterior midgut of *Drosophila melanogaster* exposed to low concentrations of Cyp (0.0004, 0.0008, 0.002, 0.2, and 0.5 ppm). In fish, Poletta et al. [39] found a significant increase in DNA damage of epithelial gill cells of *P. lineatus* after *in vivo* acute exposure (96 h) to 0.15 and 0.3 µg/L Cyp compared to controls.

Therefore, cells of the zebrafish retina also prove to be sensitive to DNA damage produced by 0.3 and 0.6 µg/L Cyp with similar behavior. In addition, we can see that, from the 9 days of exposure, the increase in DNA damage is very significant compared with the control and with the other exposure groups. Such high level of damage detected indicates that it is very sensitive to pyrethroids for long-term exposure.

In several studies, it was verified that histone γ -H2AX is an indicator sensitive to the breakdown of double-stranded DNA induced by chemical agents [40, 41]. Recently, using combination of IF, flow cytometry, and Western blot, Huang et al. [42] demonstrated that the expression of γ -H2AX in a murine macrophages cell line, exposed for 48 h at different Cyp concentrations, is significantly induced following a dose-dependent model. In these studies, Cyp was found to reduce cell viability and induce apoptotic processes. In the same study, it was shown that Cyp also increases the production of reactive oxygen species (ROS) and causes DNA damage in a dose-dependent manner. On the other hand, arrest in the G1 phase of the cell cycle induced by this pyrethroid is associated with increased expression of wild-type p21 and p53 as well as the down-regulation of cyclins D1 and E as well as protein kinase CDK4. Additionally, these studies demonstrated that Cyp treatment activates MAPK-signaling pathways by inducing the c-Jun (JNK) N-terminal kinases, the phosphorylation of extracellularly regulated protein kinases, ERK 1/2, and increased levels of the cleaved poly ADP-ribose polymerase (PARP). In the same study, pretreatment with the antioxidant N-acetylcysteine (NAC) effectively abolishes both Cyp-induced cell cytotoxicity, such as G1 cell cycle arrest, DNA damage, PARP activity, JNK activation, and ERK 1/2. In addition, specific inhibitors of JNK (SP600125) and ERK 1/2 (PD98059) reverse the phosphorylation of both molecules and attenuate apoptosis. Taken together, these data suggest that Cyp causes immune cell death through the induction of cell cycle arrest and JNK/ERK-mediated ROS-regulated apoptosis.

Of great interest is the recent finding that both in rat retinal ganglion cell enriched cultures and in ischemia/reperfusion mouse visual axis retinal injury models, JNK inhibitors (SP600125 and TAT-JNK-III) both *in vivo* and *in vitro* caused dose-dependent and significant protection in both models [43]. In this study, it was shown that in cell cultures both JNK inhibitors provoke significant and dose-dependent protection against glutamate excitotoxicity and death of retinal ganglion cells induced by the removal of trophic factors from the culture medium. In

addition, I/R injury showed both the thinning of the entire retina, as well as the internal plexiform and nuclear laminae, as well as a significant decrease in the number of ganglion cells. As indicated earlier, similar results were obtained in our *in vivo* assays of the animals treated with 0.6 µg/L Cyp for 12 days.

Regarding the immunopositive signal of caspase-3 observed in photoreceptors of zebrafish, it suggests that these cells have entered an irreversible pathway of cellular apoptosis due to the increase in the ROS production, leading to DNA rupture and therefore the expression of proteins involved in the cellular apoptosis pathway is increased. The results obtained here are consistent with previous reports showing the effect of Cyp on different cell types, which exhibit alterations in different molecules involved in the cellular apoptosis pathway. Thus, Jin et al. [44] show that exposure of zebrafish embryos to 3 and 10 µg/L Cyp for 3 days induces apoptosis and immunotoxicity, confirming an increase in the activity of caspase-3 and -9 after exposure. This same group, by analyzing messenger RNA levels of different genes related to programmed cell death (p53, Apaf-1 and caspase-3), reported that Cyp induces oxidative stress, DNA damage, and apoptosis, showing that they are significantly increased, whereas the ratio between Bcl-2/Bax genes decreases significantly after exposure to 1 and 3 µg/L Cyp for 8 days. More recently, Raszewski et al. [45, 46] demonstrate that Cyp exposure induces dose- and time-dependent apoptosis in the SH-SY5Y undifferentiated human brain cell line. This work demonstrates that Cyp causes increases in caspase-3 activity, while a decrease in Bcl-2 and Bax concomitantly occurs. However, unlike Kim et al. [43] studies, in the experiments performed with the SH-SY5Y cell line, the application of signal transduction inhibitors SP600125 (from JNK), PD98059 (from ERK 1.2), SL-327 (from MEK1 and MEK2), and SB202190 (from p38 MAPK) failed to attenuate the effect of the pesticide in the cultures of this neural cell type.

From the analysis of the results of the *in vivo* model described here, the existence of at least two groups of retinal cells with a differential sensitivity to Cyp exposure could be postulated. Thus, while photoreceptors would appear to be prone to oxidative DNA damage by excessive ROS generation and would respond by increasing the expression of proteins that lead to cell death by apoptosis (immunopositive for γ-H2AX and caspase-3), the horizontal, bipolar, and amacrine cells would be sensitive to DNA damage by oxidative stress (immunopositive for γ-H2AX) but, at the time of the test, it is not verified that they enter the apoptosis pathway (immunonegative for caspase-3).

A possible alternative explanation (although not exclusive) for the differential behavior of outer and inner nuclear layer cells, to the cytotoxic effect of Cyp, could be due to the tissular regeneration processes in the retina of bone fish [47]. It is known that these are due to the process of proliferation of the ring of embryonic neuroepithelial cells, known as the germinal peripheral zone. Bernardos et al. [48] studies allowed to demonstrate that Müller cells (MC) are responsible ones for these processes. This cell population is located mainly in the inner nuclear layer. Its proliferation rate increases in response to lesions, maintaining the pool of undifferentiated cells and undergoing differentiation processes, to form neuronal progenitor cells [49].

Based on the regenerative capacity of these cells in teleost fish, and given the possible molecular mechanisms involved in the genotoxicity induced by Cyp, the results would suggest that

although this pyrethroid causes a generalized DNA damage in all neural cells of the retina, it could be as much a selective mechanism of activation of the apoptotic route, as a differential process of regeneration in the different cellular plates. Thus, because the MCs are in the inner nuclear layer, the replacement of the horizontal, amacrine, and bipolar cells could be faster than the rest of the cell types.

4. Comet assay

Based on the results obtained in the histological and IF studies, and since the most solid hypothesis suggests that the effect of Cyp on retinal cells could be mediated by the generation of oxidative stress, the studies were deepened, combining the CA techniques, to determine the damage index DNA, and activity of the antioxidant enzymes SOD and CAT as a measure of the oxidative stress caused by exposure to Cyp. These studies were completed with the analysis of *sod* and *cat* gene expression.

Cell viability was determined before the application of the CA by fluorescent DNA-binding dyes. A total of 100 cells were counted per sample and the percentage of viable cells was determined [50]. The CA was performed as described for Singh et al. [51] with modifications required by retinal cells of zebrafish: DNA unwinding during 10 min and electrophoresis at 300 mA, 0.7 V/cm, during 10 min too. Cells were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (damage index, $DI = n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4$), where n_1 , n_2 , n_3 , and n_4 are the number of cells in each class of damage, respectively [52].

In the CA of retinal cells, with the concentration of 0.3 µg/L Cyp, it is verified that until the 9 day of the bioassay, there are no significant differences in relation to their controls; from day 9 onwards, there are statistically very significant differences ($p < 0.01$) regarding both the controls and the previous stages (**Figure 5**).

The bioassays performed with 0.6 µg/L Cyp showed DI with statistically very significant differences ($p < 0.01$) both with respect to their controls and between the different stages evaluated in the bioassay (**Figure 6**).

As previously stated, there are many reports postulating that pyrethroids in general and Cyp in particular are oxidative stress triggers; however, direct genotoxic mechanisms cannot be ruled out. Thus, spectral studies of calf thymus DNA demonstrated a bathochromic shift in UV absorption spectra, revealing that Cyp could bind with DNA. According to these studies, the recognition and reaction of Cyp with DNA is attributed to the vibratory modes of the active site and it is postulated that the possible mechanism of this interaction would be responsible for the chromosomal aberrations verified in the bioassay [52]. The molecular mechanisms responsible for the genotoxicity of pyrethroids are not clearly understood yet; however, chromosomal aberrations, sister chromatid exchange, and micronuclei formation observed in plants and animals clearly indicate that these compounds interact with DNA and induce their damage.

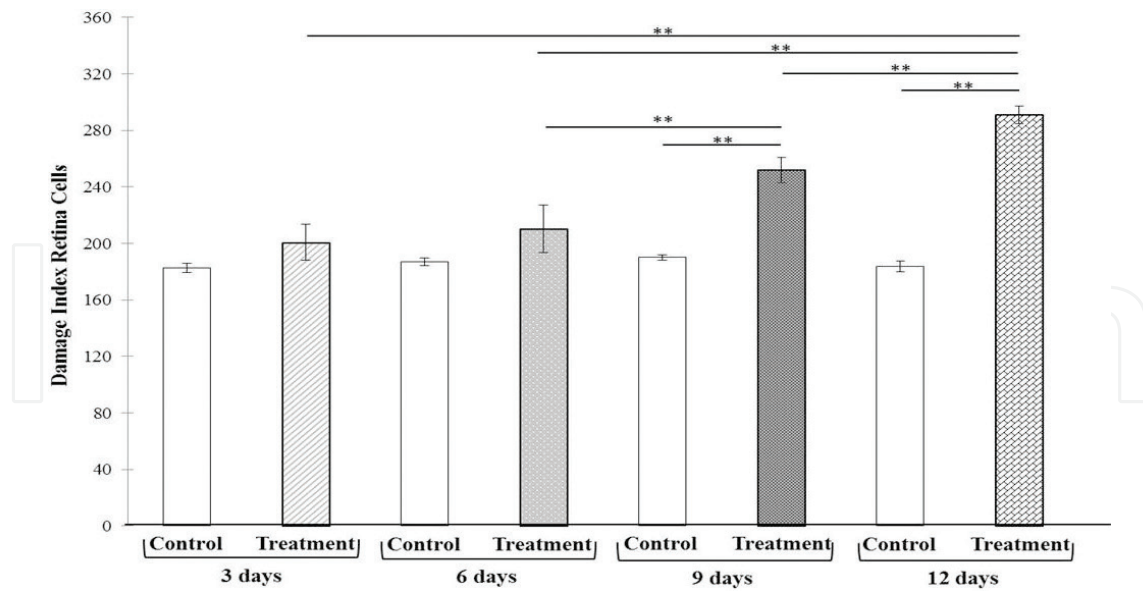


Figure 5. Bar graphic showing damage index values of DNA in retinal cells of zebrafish exposed to 0.3 µg/L of Cyp. Statistically significant differences are indicated by asterisks (** $p < 0.01$).

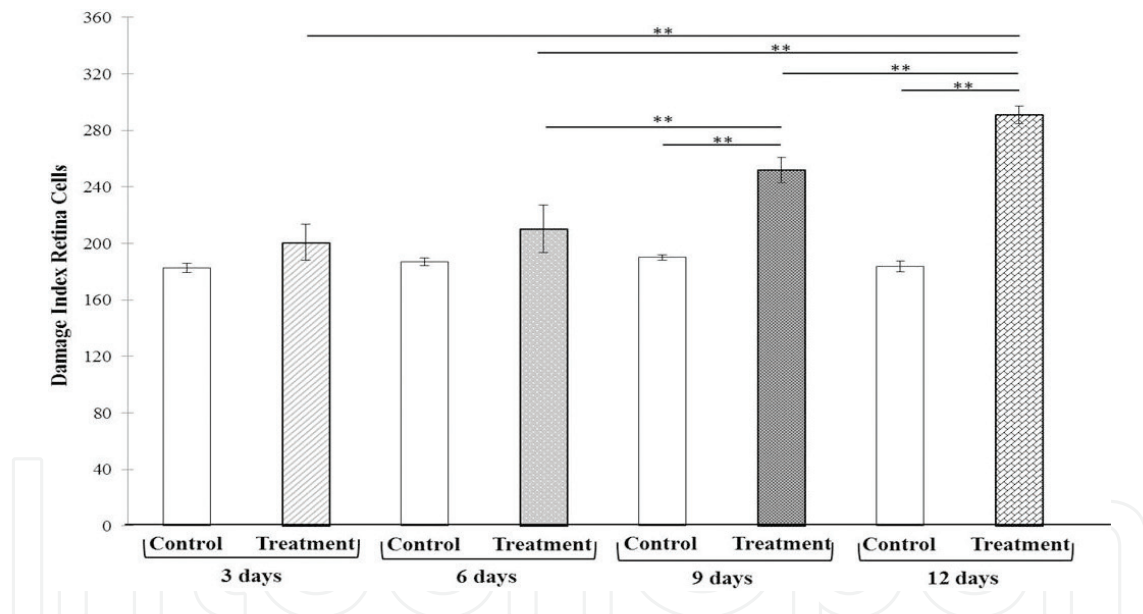


Figure 6. Bar graphic showing damage index values of DNA in retinal cells of zebrafish exposed to 0.6 µg/L of Cyp. Statistically significant differences are indicated by asterisks (** $p < 0.01$).

In addition to the direct interaction damages, those caused by the generation of free radicals as determined by the Giray et al. [53] works could be added in which rats treated with Cyp showed increases in lipid peroxidation at the cerebral and hepatic levels. Because of its small size ($V = 536.40 \text{ \AA}^3$) and hydrophobicity, the molecule of Cyp can easily cross cell membranes, reach, and interact with DNA through its acid moieties. To understand this interaction, the molecule can be divided into two parts (**Figure 7**). Region 1 would constitute the “active site” of the molecule. It contains highly electronegative atoms such as chlorine, active ester, and dimethyl groups. The crystallographic data demonstrate that this portion offers a relatively

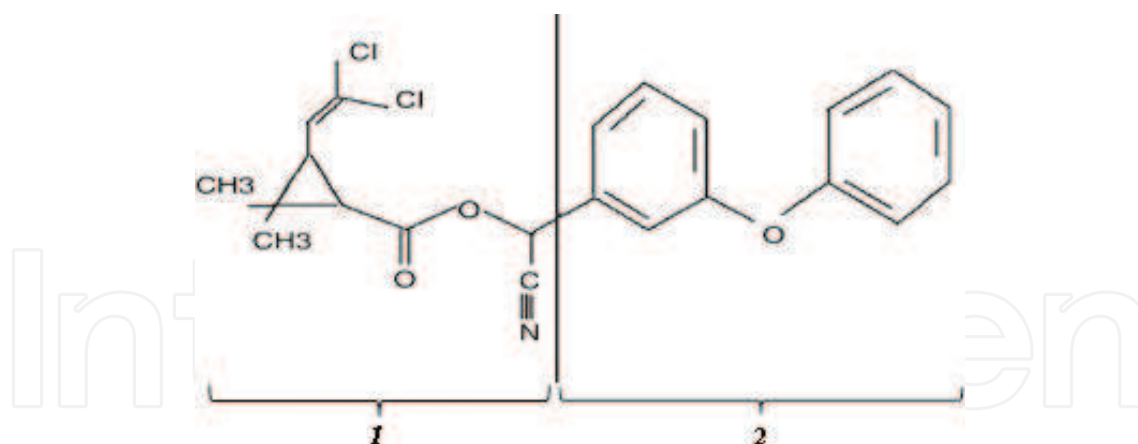


Figure 7. Cypermethrin structure showing regions (1) postulated as responsible for the interaction with DNA and (2) stabilizing support region. Adapted from [54].

flexible structure [52] which would allow the cyano, carbonyl, methyl, and chloride groups to be located nearby and interact with the DNA. On the other hand, region 2 constitutes the comparatively rigid part of the molecule providing support for the stabilization of such interaction (**Figure 7**). The molecular vibration of the atoms that involve the active region 1 would polarize the DNA molecule, promoting the binding. The binding of Cyp to DNA through polarization can lead to the destabilization of the DNA structure and the duplex unwinding, inducing chromosomal damage. Such binding was demonstrated in UV absorption studies.

Thus, the results of the CA studies as well as the morphological and IF studies allow to postulate that Cyp (or possibly its metabolites) could directly interact with the DNA molecule causing the duplex rupture.

5. Enzyme activity and gene expression of SOD and CAT

For these determinations, retinas were homogenized and then centrifuged. SOD activity was spectrophotometrically measured at 550 nm [55]. The activity of SOD was calculated according to the standard curve of SOD and expressed as U/mg protein [56]. On the other hand, CAT activity was determined by recording the absorbance of the generated stable chromophore at 405 nm due to H_2O_2 consumption according to Aebi's method [57]. In these studies, we found that the activity of SOD (**Figure 8**) and CAT (**Figure 9**) increases when retinal cells were exposed to 0.6 $\mu\text{g/L}$ Cyp. Like other organisms, fish can neutralize the elevated ROS levels in their systems, with protective ROS-scavenging enzymes such as SOD and CAT. Thus, it is possible that an increase in the activity of these enzymes contributes to the elimination from the cells of ROS induced by Cyp exposure.

Then, we examined the representative genes that encode proteins that are used to combat oxidative stress (such as SOD and CAT) to determine whether these might serve as molecular endpoints for Cyp exposure in zebrafish. The total RNAs of retinas from adult fish treated with Cyp 0.6 $\mu\text{g/L}$ for 3 and 12 days were isolated and processed according to Jin et al. [26]. Reverse transcriptase (RT) products were used directly for the polymerase chain reaction

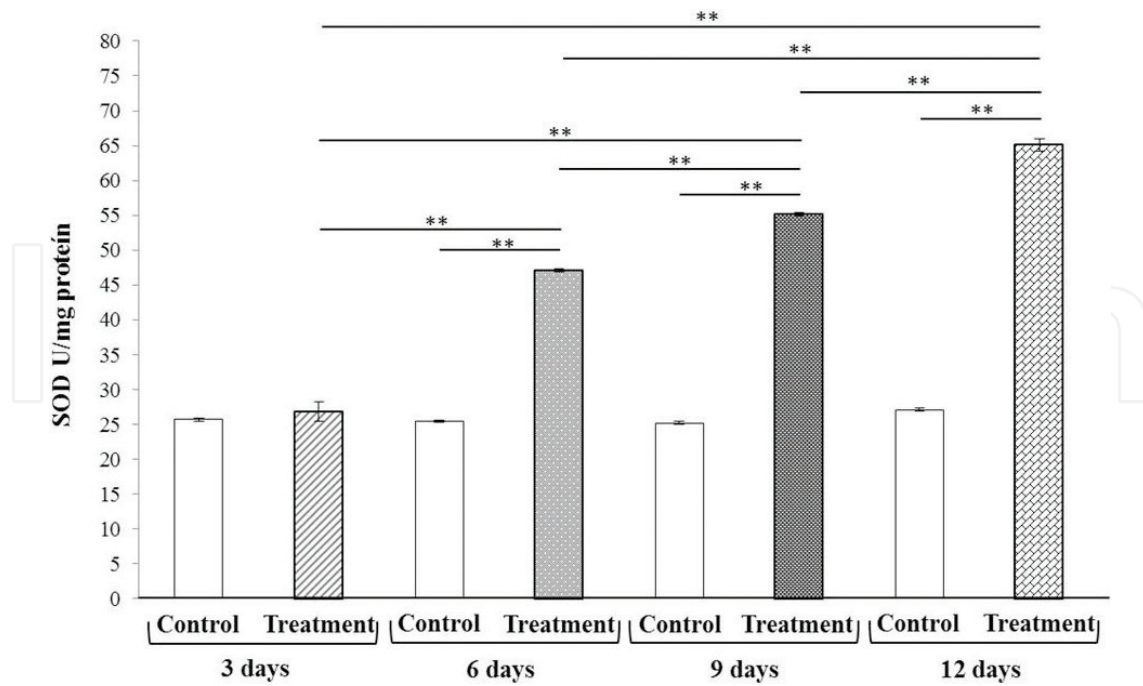


Figure 8. Bar graphic showing the values of SOD activity in the retinal cells of the zebrafish exposed to 0.6 µg/L Cyp. Statistically significant differences are indicated by asterisks (** $p < 0.01$).

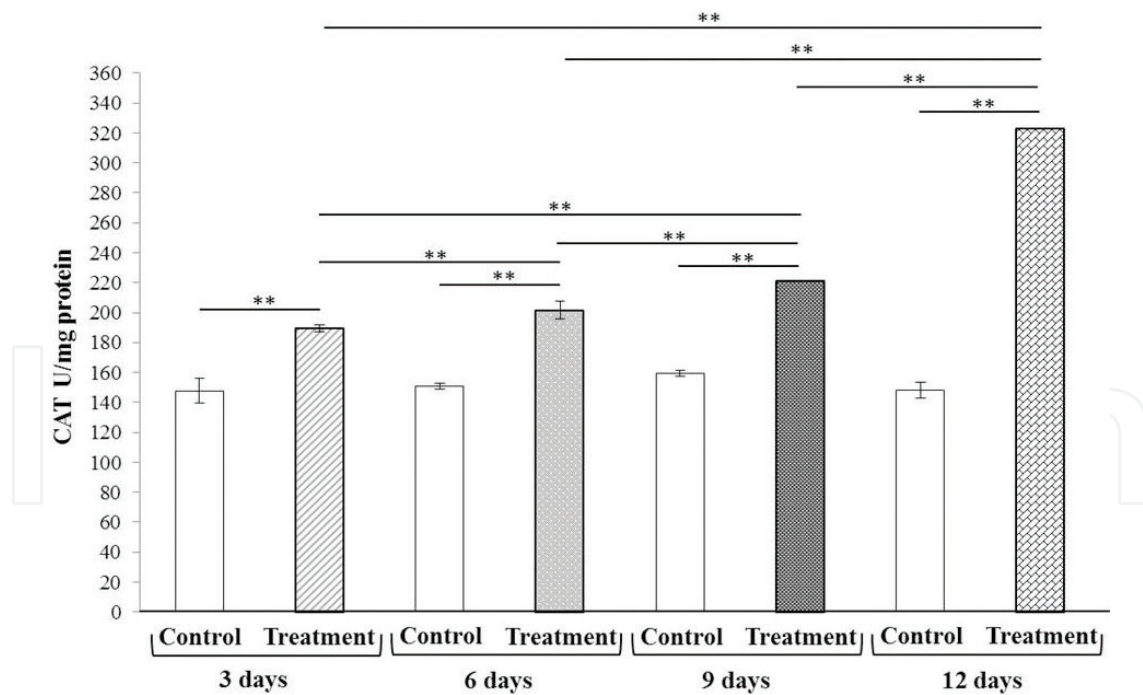


Figure 9. Bar graphic showing the values of CAT activity in the retinal cells of the zebrafish exposed to 0.6 µg/L Cyp. Statistically significant differences are indicated by asterisks (** $p < 0.01$).

(PCR). These were performed using an Ivema T-18 thermo-cycler (Llavallol, BA, Argentina) using the following program: denaturation for 10 min at 95°C, followed by 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 gene

expression of β -actin, SOD (AY N° 195,857), and CAT (AF N° 170,069). As housekeeping gene, β -actin transcripts were used for data standardization. We found that the expression levels of the mRNA of SOD and CAT enzymes had a significant increase in the retinal cells of the zebrafish after 12 days of exposure to the highest concentration of Cyp (0.6 μ g/L) (**Figure 10**), which is consistent with the findings in the evaluation of enzyme activity. Therefore, we suggest that the increased levels of mRNA and its close relationship with the increase in enzyme activity are related to the removal mechanisms of ROS.

The toxicity of pesticides in fish is related to an increased production of ROS, leading to oxidative damage [58]. Fish, like many other vertebrates, try to reduce the damage caused by oxidative stress by using an antioxidant defense system. The first line of defense consists of antioxidant molecules, such as glutathione, vitamin C and E, and carotenoids [59]. Antioxidant enzymes include another defense mechanism, including SOD, CAT, glutathione peroxidase (GPx), and glutathione S-transferase (GST) [60–62]. Several studies have shown that synthetic pyrethroids are extremely toxic to fish and aquatic invertebrates [63–65]. The antioxidant defense system of living organisms can be analyzed by the activity and gene expression of enzymatic antioxidants, such as SOD, CAT, and GPx [66].

Our results demonstrate that, in zebrafish retinas, the CAT enzyme is more sensitive and would respond more rapidly than SOD as a defense mechanism to the increase in the production of ROS to avoid the possible oxidative effect caused by Cyp.

Accordingly, several studies have shown that Cyp produces an imbalance in the production of ROS and consequently an alteration in the activity of the antioxidant enzymes SOD and CAT in various species and organs as a defense mechanism to oxidative stress. Kale et al. [66] reported that erythrocytes of rats exposed to 2500 mg/kg of Cyp showed an increase in the

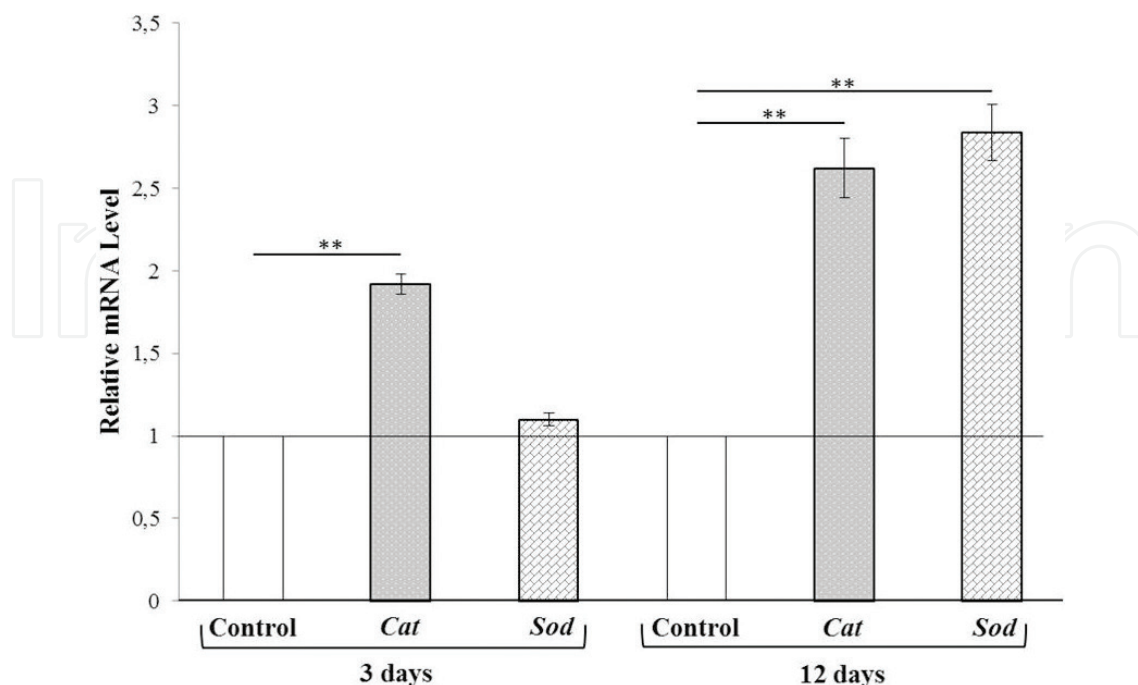


Figure 10. Bar graphic depicting the gene expression levels of *cat* and *sod* in the retina of the zebrafish exposed to 0.6 μ g/L Cyp. Statistically significant differences are indicated by asterisks (** $p < 0.01$).

activity of the antioxidant enzymes SOD and CAT because of the increase in ROS production. In addition, they demonstrate that the gradual decrease in the activity of both enzymes, after a period, is due to a decrease in the production of formed ROS. More recently, studies by Yonar et al. [58] indicate that Cyp induces an increase in the enzymatic activity of SOD and CAT in blood, liver, kidney, and gills of *Cyprinus carpio*, showing that pesticides can induce oxidative stress, leading to the generation of free radicals and causing peroxidation of lipids. Increased lipid peroxidation and ROS production may affect the activity of antioxidant enzymes, which are sensitive indicators of increased oxidative stress.

In addition, in the present study, genes encoding stress-sensitive SOD and CAT enzymes were used as molecular biomarkers of the bioassay for stress evaluation by Cyp and to complement the morphological, immunofluorescence, and EC studies. Similar studies have been performed to evaluate the behavior of other species using real-time PCR techniques as well as RNA microarrays [67–69]. At present, there are few studies evaluating the gene expression of SOD and CAT in zebrafish retina in response to agents that promote oxidative stress [70] and no studies have been reported relating stress oxidative stress caused by Cyp, and the gene expression of antioxidant enzymes in the retina. Induction of CAT mRNA, SOD, and GPx expression was recently demonstrated by Jin et al. [27] (4 days) or low concentrations (1 µg/L) for prolonged exposures (8 days), the use of high concentrations of Cyp (3 µg/L) for moderate exposures (4 days) or low concentrations (1 µg/L). In this work, it is proposed that the levels of expression promoted by the pyrethroid are the product of increased oxidative stress. In the present study, doses are much lower than those used by Jin et al. [27], obtaining greater discrimination of the behavior of two of the enzymes of response to oxidative stress. Thus, mRNA levels of the SOD enzyme in retinas of zebrafish treated with 0.6 µg/L for 3 days did not cause significant increases, whereas those corresponding to CAT resulted in very significant levels of expression. When the transcript levels of both genes were evaluated toward the end of the bioassay (12 days), both genes responded with very significant levels of expression.

In conclusion, the RT-*sq*PCR assay adopted in this work is an effective technique for the rapid determination of the oxidative effect in zebu fish exposed to Cyp. It is suggested that the significant increase in the levels of gene expression and enzymatic activity of SOD and CAT would be closely related to the mechanisms that would be put in place to decrease or eliminate the oxidative stress caused by the high production of ROS and thus avoid oxidative damage from pyrethroid exposure.

6. Conclusions

This study demonstrates that Cyp generates a variety of detrimental effects in retinal cells of zebrafish over a relatively short time of exposure and at concentrations much lower than those found in runoff and surface water in natural environments. Effects observed include apoptosis, DNA damage, increased expression of γ -H2AX and caspase-3 genes, and alterations in antioxidant enzyme activities as well as in the expression of their respective genes. Our results not only provide important information to fully understand the potential mechanisms

of Cyp-induced neurotoxicity but also suggest that zebrafish can serve as an ideal model for studying developmental toxicity of environmental contaminants.

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References

- [1] Aldridge WN. An assessment of the toxicological properties of pyrethroids and their neurotoxicity. *Critical Reviews in Toxicology*. 1990;**21**:89-104. DOI: 10.3109/10408449009089874
- [2] Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. *Annual Review of Entomology*. 1989;**34**:77-96. DOI: 10.1146/annurev.en.34.010189.000453
- [3] Vijverberg HPM, van den Bercken J. Neurotoxicological effects and the mode of action of pyrethroid insecticides. *Critical Reviews in Toxicology*. 1990;**21**:105-126. DOI: 10.3109/10408449009089875
- [4] Narahashi T. Neuronal ion channels as the target sites of insecticides. *Pharmacology & Toxicology*. 1996;**78**:1-14. DOI: 10.1111/j.1600-0773.1996.tb00234.x
- [5] Verschoyle RD, Aldridge WN. Structure-activity relationships of some pyrethroids in rats. *Archives of Toxicology*. 1980;**45**:325-329. DOI: 10.1007/BF00293813
- [6] USEPA. Pyrethroids and Pyrethrins. 2013. Available from: <http://www.epa.gov/oppsrrd1/reevaluation/pyrethroids-pyrethrins.html> [Accessed:10-12-2016]
- [7] Kaufman D, Russell B, Helling C. Movement of cypermethrin, decamethrin, permethrin, and their degradation products in soil. *Journal of Agricultural and Food Chemistry*. 1981;**29**:239-245. DOI: 10.1021/jf00104a008
- [8] Bradberry SM, Cage SA, Proudfoot AT, Vale JA. Poisoning due to pyrethroids. *Toxicological Reviews*. 2005;**24**:93-106. DOI: 10.2165/00139709-200524020-00003
- [9] DeMicco A, Cooper KR, Richardson JR, White LA. Developmental neurotoxicity of pyrethroid insecticides in zebrafish embryos. *Toxicological Sciences*. 2009;**113**:177-186. DOI: 10.1093/toxsci/kfp258

- [10] Malkiewicz K, Koteras M, Folkesson R, Brzezinski J, Winblad B, Szutowski M, Benedikz E. Cypermethrin alters glial fibrillary acidic protein levels in the rat brain. *Environmental Toxicology and Pharmacology*. 2016;**21**:51-55. DOI: 10.1016/j.etap.2005.06.005
- [11] Mun JY, Lee WY, Han SS. Effects of cypermethrin on the dopaminergic neurons in the progressive hemiparkinsonian rats. *Toxicology Mechanisms and Methods*. 2005;**15**:399-404. DOI: 10.1080/15376520500194742
- [12] Nasuti C, Gabbianelli R, Falcioni ML, Di Stefano A, Sozio P, Cantalamessa F. Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. *Toxicology*. 2007;**229**:194-205. DOI: 10.1016/j.tox.2006.10.015
- [13] Power LE, Sudakin DL. Pyrethrin and pyrethroid exposures in the United States: A longitudinal analysis of incidents reported to poison centers. *Journal of Medical Toxicology*. 2007;**3**:94-99. DOI: 10.1007/BF03160917
- [14] Izaguirre MF, Lajmanovich RC, Peltzer PM, Soler AP, Casco VH. Cypermethrin-induced apoptosis in the telencephalon of *Physalaemus biligonigerus* tadpoles (Anura: Leptodactylidae). *Bulletin of Environmental Contamination and Toxicology*. 2000;**65**:501-507. DOI: 10.1007/s001280000152
- [15] Izaguirre MF, Vergara MN, Casco VH. CAS role in the brain apoptosis of *Bufo arenarum* induced by cypermethrin. *Biocell*. 2006;**30**:309-320
- [16] Casco VH, Izaguirre MF, Marín L, Vergara MN, Lajmanovich RC, Peltzer P, Soler AP. Apoptotic cell death in the central nervous system of *Bufo arenarum* tadpoles induced by cypermethrin. *Cell Biology and Toxicology*. 2006;**22**:199-211. DOI: 10.1007/s10565-006-0174-1
- [17] Dai YJ, Jia YF, Chen N, Bian WP, Li QK, Ma YB, Chen YL, Pei DS. Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry*. 2014;**33**:11-17. DOI: 10.1002/etc.2406
- [18] Norton W, Bally-Cuif L. Adult zebrafish as a model organism for behavioral genetics. *Neuroscience*. 2010;**11**:90. DOI: 10.1186/1471-2202-11-90
- [19] Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences*. 2005;**86**:6-19. DOI: 10.1093/toxsci/kfi110
- [20] Nagel R. DarT: The embryo test with the Zebrafish *Danio rerio* a general model in ecotoxicology and toxicology. *ALTEX*. 2002;**19**:38-48. DOI: 10.1371/journal.pone.0091874
- [21] Sipes NS, Padilla S, Knudsen TB. Zebrafish as an integrative model for twenty-first century toxicity testing. *Birth Defects Research. Part C, Embryo Today*. 2011;**93**:256-267. DOI: 10.1002/bdrc.20214
- [22] Yang L, Ho NY, Alshut R, Legradi J, Weiss C, Reischl M, Mikut R, Liebel U, Muller F, Strahle U. Zebrafish embryos as models for embryo toxic and teratological effects of chemicals. *Reproductive Toxicology*. 2009;**28**:245-253. DOI: 10.1016/j.reprotox.2009.04.013

- [23] Murk AJ, Legler J, Denison MS, Giesy JP, van de Guchte C, Brouwer A. Chemical-activated luciferase gene expression (CALUX): A novel *in vitro* bioassay for Ah receptor active compounds in sediments and pore water. *Fundamental and Applied Toxicology*. 1996;**33**:149-160. DOI: 10.1006/faat.1996.0152
- [24] Mizell M, Romig ES. The aquatic vertebrate embryo as a sentinel for toxins: Zebrafish embryo dechoriation and perivitelline space microinjection. *The International Journal of Developmental Biology*. 1997;**41**:411-423
- [25] Goolish EM, Okutake K, Johnson P. The behavioral response of zebrafish to hypergravity conditions. *Journal of Gravitational Physiology*. 2000;**7**:99-100
- [26] Jin Y, Zhang X, Shu L, Chen L, Sun L, Qian H, Liu W, Fu Z. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). *Chemosphere*. 2010;**78**:846-852. DOI: 10.1016/j.chemosphere.2009.11.044
- [27] Jin Y, Zheng S, Pu Y, Shu L, Sun L, Liu W, Fu Z. Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (*Danio rerio*). *Chemosphere*. 2011;**82**:398-404. DOI: 10.1016/j.chemosphere.2010.09.072
- [28] Shao B, Zhu L, Dong M, Wang J, Xie H, Zhang Q, Du Z, Zhu S. DNA damage and oxidative stress induced by endosulfan exposure in zebrafish (*Danio rerio*). *Ecotoxicology*. 2012;**21**:1533-1540. DOI: 10.1007/s10646-012-0907-2
- [29] Ma Y, Han J, Guo Y, Lam PK, Wu RS, Giesy JP, Zhang X, Zhou B. Disruption of endocrine function in *in vitro* H295R cell-based and in *in vivo* assay in zebrafish by 2,4-dichlorophenol. *Aquatic Toxicology*. 2012;**107**:173-181. DOI: 10.1016/j.aquatox.2011.11.006
- [30] Jergentz S, Mugni H, Bonetto C, Schulz R. Runoff-related endosulfan contamination and aquatic macroinvertebrate response in rural basins near Buenos Aires, Argentina. *Archives of Environmental Contamination and Toxicology*. 2005;**46**:345-352. DOI: 10.1007/s00244-003-2169-8
- [31] Westerfield M. The zebrafish book. In: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). 5th ed. Eugene: University of Oregon Press; 2007. Paperback
- [32] Marino D, Ronco A. Cypermethrin and chlorpyrifos concentration levels in surface water bodies of the *Pampa Ondulada*, Argentina. *Bulletin of Environmental Contamination and Toxicology*. 2005;**75**:820-826. DOI: 10.1007/s00128-005-0824-7
- [33] Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: Mechanisms, mutation, and disease. *The FASEB Journal*. 2003;**17**:1195-1214. DOI: 10.1096/fj.02-0752rev
- [34] Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *The Journal of Biological Chemistry*. 1998;**273**:5858-5868. DOI: 10.1074/jbc.273.10.5858
- [35] Elmore S. Apoptosis: A review of programmed cell death. *Toxicologic Pathology*. 2007;**35**:495-516. DOI: 10.1080/01926230701320337

- [36] Rebbaa A, Zheng X, Chou PM, Mirkin BL. Caspase inhibition switches doxorubicin-induced apoptosis to senescence. *Oncogene*. 2003;**22**:2805-2811. DOI: 10.1038/sj.onc.1206366
- [37] Patel S, Pandey A, Bajpayee M, Parmar D, Dhawan A. Cypermethrin-induced DNA damage in organs and tissues of the mouse: Evidence from the comet assay. *Mutation Research*. 2006;**607**:176-183. DOI: 10.1016/j.mrgentox.2006.04.010
- [38] Mukhopadhyay I, Chowdhuri DK, Bajpayee M, Dhawan A. Evaluation of *in vivo* genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline comet assay. *Mutagenesis*. 2004;**19**:85-90. DOI: 10.1093/mutage/geh007
- [39] Poletta GL, Gigena F, Loteste A, Parma MJ, Kleinsorge EC, Simoniello MF. Comet assay in gill cells of *Prochilodus lineatus* exposed *in vivo* to cypermethrin. *Pesticide Biochemistry and Physiology*. 2013;**107**:385-390. DOI: 10.1016/j.pestbp.2013.10.007
- [40] Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A. H2AX: The histone guardian of the genome. *DNA Repair (Amst)*. 2004;**3**:959-967. DOI: 10.1016/j.dnarep.2004.03.024
- [41] Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. Histone H2A variants H2AX and H2AZ. *Current Opinion in Genetics & Development*. 2002;**12**:162-169. DOI: 10.1016/S0959-437X(02)00282-4
- [42] Huang F, Liu Q, Xie S, Xu J, Huang B, Wu Y, Xia D. Cypermethrin Induces Macrophages Death through Cell Cycle Arrest and Oxidative Stress-Mediated JNK/ERK Signaling Regulated Apoptosis. *Int. J. Mol. Sci*. 2016;**17**:885. DOI: 10.3390/ijms17060885
- [43] Kim BJ, Silverman SM, Liu Y, Wordinger RJ, Pang IH, Clark AF. *In vitro* and *in vivo* neuroprotective effects of cJun N-terminal kinase inhibitors on retinal ganglion cells. *Molecular Neurodegeneration*. 2016;**11**:30. DOI: 10.1186/s13024-016-0093-4
- [44] Jin Y, Zheng S, Fu Z. Embryonic exposure to cypermethrin induces apoptosis and immunotoxicity in zebrafish (*Danio rerio*). *Fish & Shellfish Immunology*. 2011b;**30**:1049-1054. DOI: 10.1016/j.fsi.2011.02.001
- [45] Raszewski G, Lemieszek MK, Łukawski K, Juszczak M, Rzeski W. Chlorpyrifos and cypermethrin induce apoptosis in human neuroblastoma cell line SH-SY5Y. *Basic & Clinical Pharmacology & Toxicology*. 2015;**116**:158-167. DOI: 10.1111/bcpt.12285
- [46] Raszewski G, Lemieszek MK, Łukawski K. Cytotoxicity induced by cypermethrin in human neuroblastoma cell line SH-SY5Y. *Annals of Agricultural and Environmental Medicine*. 2016;**23**:106-110. DOI: 10.5604/12321966.1196863
- [47] Hitchcock PF, Raymond PA. The teleost retina as a model for developmental and regeneration biology. *Zebrafish*. 2004;**1**:257-271. DOI: 10.1089/zeb.2004.1.257
- [48] Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. *The Journal of Neuroscience*. 2007;**27**:7028-7040. DOI: 10.1523/JNEUROSCI.1624-07.2007

- [49] Thummel R, Kassen SC, Enright JM, Nelson CM, Montgomery JE, Hyde DR. Characterization of Müller glia and neuronal progenitors during adult zebrafish retinal regeneration. *Experimental Eye Research*. 2008;**87**:433-444. DOI: 10.1016/j.exer.2008.07.009
- [50] Mercille S, Massie B. Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells. *Biotechnology and Bioengineering*. 1994;**44**:1140-1154. DOI: 10.1002/bit.260440916
- [51] Singh NP, Mc Coy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*. 1988;**175**:1841-1891. DOI: 10.1016/0014-4827(88)90265-0
- [52] Simoniello MF, Gigena F, Poletta G, Loteste A, Kleinsorge E, Campana M, Scagnetti J, Parma MJ. Alkaline comet assay for genotoxic effect detection in neotropical fish *Prochilodus lineatus* (Pisces, Curimatidae). *Bulletin of Environmental Contamination and Toxicology*. 2009;**83**:155-158. DOI: 10.1007/s00128-009-9771-z
- [53] Giray B, Gürbay A, Hincal F. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. *Toxicology Letters*. 2001;**118**:139-146 DOI: S0378427400002770 [pii]
- [54] Saxena PN, Chauhan LKS, Gupta SK. Cytogenetic effects of commercial formulation of cypermethrin in root meristem cells of *Allium sativum*: spectroscopic basis of chromosome damage. *Toxicology*. 2005;**216**:244-252. DOI: 10.1016/j.tox.2005.08.008
- [55] Misra H, Fridovich I. Superoxide dismutase and the oxygen enhancement of radiation lethality. *Arch. Biochem. Biophys.* 1976;**176**:577-581. DOI: 10.1016/0003-9861(76)90201-0
- [56] Zhang YT, Zheng QS, Pan J, Zheng RL. Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants. *Basic Clin Pharmacol Toxicol* 2004;**95**:53-8. DOI: 10.1111/j.17427843.2004.950202.x
- [57] Aebi's H. Catalase *in vitro*. In: *Meth. Enzymol.* 1984;**105**:121-127. DOI: 10.1016/S0076-6879(84)05016-3
- [58] Yonar ME. Protective effect of lycopene on oxidative stress and antioxidant status in *Cyprinus carpio* during cypermethrin exposure. *Environ. Toxicol.* 2013;**28**:609-616. DOI: 10.1002/tox.20757
- [59] Alvarez RM, Morales AE, Sanz A. Antioxidant defences in fish: Biotic and abiotic factors. *Rev. Fish Biol. Fisher.* 2005;**15**:75-88. DOI: 10.1007/s11160-005-7846-4
- [60] Storey KB. Oxidative stress: Animal adaptations in nature. *Braz J. Med. Biol. Res.* 1996;**29**:1715-1733
- [61] Droge W. Free radicals in the physiological control of cell function. *Physiol. Rev.* 2002;**82**:47-95
- [62] Valavanidis A, Vlahogianni T, Dassenakis M, Scoullas M. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.* 2006;**64**:178-189. DOI: 10.1016/j.ecoenv.2005.03.013

- [63] Casida JE, Gammon DW, Glickman AH, Lawrence LJ. Mechanism of pyrethroid insecticides. *Annu. Rev. Pharmacol. Toxicol.* 1983;**23**:413-418. DOI: 10.1146/annurev.pa.23.040183.002213
- [64] Smith TM, Stratton GW. Effects of synthetic pyrethroid insecticides on nontarget organisms. *Residue Rev.* 1986;**97**:93-120
- [65] Polat H, Erkoç FU, Viran R, Kocak O. Investigation of acute toxicity of beta-cypermethrin on guppies *Poecilia reticulata*. *Chemosphere.* 2002;**49**:39-44. DOI: 10.1016/S0045-6535(02)00171-6
- [66] de Zwart LL, Meerman JH, Commandeur JN, Vermeulen NP. Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic. Biol. Med.* 1999;**26**:202-226. DOI: 10.1016/S0891-5849(98)00196-8
- [67] Kale M, Rathore N, John S, Bhatnagar D. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. *Toxicol. Lett.* 1999;**105**:197-205. DOI: 10.1016/S0378-4274(98)00399-3
- [68] Sheader DL, Williams TD, Lyons BP, Chipman JK. Oxidative stress response of European flounder (*Platichthys flesus*) to cadmium determined by a custom cDNA microarray. *Marine Environmental Research.* 2006;**62**:33-44. DOI: 10.1016/j.marenvres.2006.03.001
- [69] Woo S, Yum S, Kim DW, Park HS. Transcripts level responses in a marine medaka (*Oryzias javanicus*) exposed to organophosphorus pesticide. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology.* 2009;**149**:427-432. DOI: 10.1016/j.cbpc.2008.10.100
- [70] Nellore J, Pauline PC, Mohanan SP, Ravikumar R, Pillai RC. Vinca rosea normalizes oxidative stress and inhibits hyperglycemia induced increase in VEGF in zebrafish retina. *Research Journal of Pharmaceutical, Biological and Chemical Sciences.* 2013;**4**:927-936