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# Toxicologic evidence of developmental neurotoxicity of Type II pyrethroids cyfluthrin and alpha-cypermethrin in SH-SY5Y cells

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ABSTRACT

We attempted to identify cellular mechanisms as an approach to screen chemicals for the potential to cause developmental neurotoxicity. We examine, in SH-SY5Y cells, whether apoptosis and oxidative stress via reactive oxygen species (ROS) generation, caspase 3/7 activation, gene expression (Bax, Bcl-2, Casp-3, BNIP3, p53 and Nrf2) alterations and necrosis by release of cytosolic adenylate kinase (AK), underlie direct effects of the pyrethroids cyfluthrin and alpha-cypermethrin. We also determined transcriptional alterations of genes (TUBB3, NEFL, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A, SYN1 and PIK3C3) linked to neuronal development and maturation. Our results indicate that cyfluthrin and alpha-cypermethrin have the ability to elicit concentration-dependent increases in AK release, cellular ROS production, caspase 3/7 activity and gene expression of apoptosis and oxidative stress mediators. Both pyrethroids caused changes in mRNA expression of key target genes linked to neuronal development. These changes might reflect in a subsequent neuronal dysfunction. Our study shows that SH-SY5Y cell line is a valuable *in vitro* model for predicting development neurotoxicity. Our research provides evidence that cyfluthrin and alpha-cypermethrin have the potential to act as developmental neurotoxic compounds. Additional information is needed to improve the utility of this *in vitro* model and/or better understand its predictive capability.

## 1. Introduction

Developmental neurotoxicity is any effect of a toxicant on the developing nervous system before or after birth that interferes with normal nervous system structure or function. A number of existing chemicals will probably meet the criteria for requiring developmental neurotoxicity testing, however, limitations in the available data for a number of chemicals indicate that the triggering schema may not be sufficient to elicit testing of all chemicals that may be developmental neurotoxicants. This causes concern as there is a regulatory need to identify chemicals that may induce neurotoxicity during development. Developmental neurotoxicity of pesticides is being of growing interest in recent years due to the increasing reports of neuropsychiatric and neurodegenerative disorders. A number of epidemiologic studies have linked pesticide exposure to developmental effects. Exposure to these substances during early development may lead to neurotoxic effects manifested at a later phase of life. Insecticides such as organochlorides, organophosphates, and carbamates are becoming discontinued in over-the-counter products due to their toxicity and persistence in the environment. These pesticides are now being replaced by synthetic pyrethroids, in part due to their greater selective toxicity to insects, lower toxicity to mammals and limited persistence in the environment. However, there is relatively little known about the developmental neurotoxicity of pyrethroids (Shafer et al., 2005).

The pyrethroid class of insecticides was derived from natural compounds (the pyrethrins) isolated from the *Chrysanthemum* genus of plants (Casida, 1980). All pyrethroids contain several common features: an acid moiety, a central ester bond, and an alcohol moiety (Fig. 1). The acute mammalian toxicity of pyrethroids has been well characterized (Soderlund et al., 2002; Verschoyle and Aldridge, 1980). Based on toxic clinical signs in the rat, pyrethroids have been divided into two types: a) compounds that produce a syndrome consisting of aggressive sparring, increased sensitivity to external stimuli, and fine tremor progressing to whole-body tremor and prostration (Type I, or T syndrome); and b) compounds that produce a syndrome consisting of pawing and burrowing, profuse salivation, and coarse tremor progressing to choreoathetosis and clonic seizures (Type II, or CS syndrome). Structurally, a key difference between Type I and Type II pyrethroids is the absence

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**Abbreviations**

| | |
|---|---|
| AK | adenylate kinase |
| AKDR | adenylate kinase detection reagent |
| BSA | bovine serum albumin |
| CAMK2A | calcium/calmodulin dependent protein kinase II alpha |
| CAMK2B | calcium/calmodulin dependent protein kinase II beta |
| Bax | Bcl-2-associated X protein |
| Bcl2 | B-cell lymphoma 2 |
| BNIP3 | Bcl-2 interacting protein 3 |
| Casp-3 | caspase 3, apoptosis-related cysteine peptidase |
| DCFH | 2′,7′-dichlorofluorescin diacetate |
| DEVD | aspartic acid-glutamic acid-valine-aspartic acid |
| DMEM F12 | Dulbecco's Modified Eagle Medium/Nutrient Mixture |
| DMSO | dimethyl sulfoxide |
| DNT | developmental neurotoxicity |
| DPBS | Dulbecco's phosphate buffered saline |
| EC30 | concentrations that produce a 30% decrease in cell viability |
| EC50 | concentrations that produce a 50% decrease in cell viability |
| FAD | flavin adenine dinucleotide |
| FBS | fetal bovine serum |
| GAP43 | growth associated protein 43 |
| HEPES | N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| LOAEL | low observed adverse effect level |
| MTT | 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide |
| NADPH | β-nicotinamide adenine dinucleotide phosphate |
| NEFH | neurofilament triplet L protein |
| NEFL | neurofilament triplet L protein |
| NOAEL | no observed adverse effect level |
| Nrf2 | nuclear factor-erythroid-2-related factor-2 |
| PIK3C3 | phosphatidylinositol 3-kinase catalytic subunit type 3 |
| p53 | tumor protein 53 |
| ROS | reactive oxygen species |
| SH-SY5Y | human neuroblastoma cell line |
| SYN1 | synapsin 1 |
| TUBB3 | tubulin beta-3 |
| WNT3A | wnt family member 3A |
| WNT5A | wnt family member 5A |
| WNT7A | wnt family member 7A |

or presence, respectively, of a cyano group at the α carbon of the 3-phenoxybenzyl alcohol moiety of the compound. Thus, the Type I/II or T/CS nomenclatures are useful as general classification schemes (Soderlund et al., 2002). Cyfluthrin and alpha-cypermethrin used in this study are Type II pyrethroids (Fig. 1). The primary mode of pyrethroid action in both insects and mammals is disruption of voltage-sensitive sodium channels. In general, Type I compounds prolong channel opening only long enough to cause repetitive firing of action potentials (repetitive discharge), whereas Type II compounds hold open the channels for such long periods of time that the membrane potential ultimately becomes depolarized to the point at which generation of action potentials is not possible. These differences in prolongation of channel open times are hypothesized to contribute to the differences in the CS and T syndromes after exposure to Type II and I pyrethroids, respectively (Ray, 2001). Although sodium channels are important targets for the neurotoxic effects of pyrethroids in mammals, other

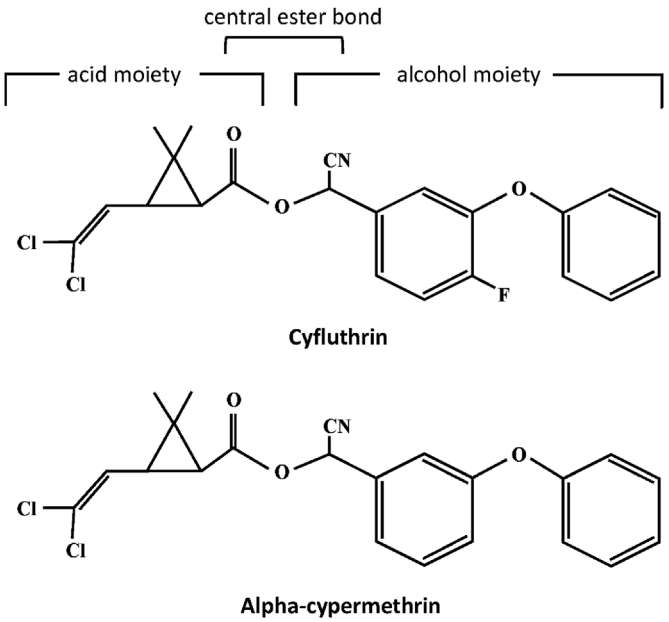


**Fig. 1.** Chemical structures of cyfluthrin and alpha-cypermethrin.

targets, particularly voltage-gated calcium and chloride channels, have also been implicated as alternative or secondary sites of action for a subset of pyrethroids (Soderlund, 2012).

Pyrethroid insecticides are one of the most commonly used residential and agricultural insecticides. Altogether, epidemiological and experimental findings indicate that this class of insecticides is classically considered to be safer to the human health than other classes, based on the increased use of pyrethroids and studies showing that pregnant women and children are exposed to pyrethroids (Berkowitz et al., 2003; Morgan et al., 2007; Lu et al., 2006, 2009), there are concerns over the potential for developmental neurotoxicity. Recent studies evaluated the effects of prenatal pyrethroid exposure on children's neurodevelopment and identified evidence of deleterious outcomes. Xue et al. (2013) quantified several pyrethroid levels in the urine of pregnant women and reported an inverse association between prenatal pyrethroid exposure and measures of motor function, social adaptation and intelligence at 1 year of age in Chinese infants. Shelton et al. (2014) found a positive association between residential proximity to Type II pyrethroids preconception and during the third trimester of gestation and both autism spectrum disorders and developmental delay (delayed cognitive or adaptive development). The increasing demand for developmental neurotoxicity data in regulatory decisions and the growing desire to employ human scientific approaches that consider animal welfare as part of the experimental design underscore the need for alternative developmental neurotoxicity protocols that can quickly and efficiently yield data geared towards public health decision-making. Combining *in vivo* data sets with *in vitro* approaches are important for regulatory decisions. In this context, it has been suggested that *in vitro* models including SH-SY5Y cells could be used to screen for chemical effects on critical cellular events of neurodevelopment, including neurite outgrowth (Radio et al., 2008). SH-SY5Y cell line is an established model in experimental neurological studies (Pahlman, 1990; Krishna et al., 2014). Keeping in view the present usage scenario of pyrethroids worldwide and reports on their potential as developmental neurotoxicants, this study was undertaken to evaluate potential neurotoxic effects of the Type II pyrethroids cyfluthrin and alpha-cypermethrin, compounds that are extensively applied to control pests in residential and agricultural settings, to treat head lice and scabies in humans and fleas in pets, for public health vector control, and for disinfection of commercial aircrafts (Anadón et al., 2009, 2013a; USEPA, 2011, 2017a,b). Despite beneficial roles as insecticides,

cyfluthrin and alpha-cypermethrin enter the brain, accumulate in significant quantity and exert neurotoxicity in the non-target organisms (Mun et al., 2005; Malkiewicz et al., 2006; Nasuti et al., 2007; Singh et al., 2011a,b; 2012a,b; Anadón et al., 2013b; Rodriguez et al., 2016, 2018). In the present study, using SH-SY5Y cells, we attempt to elucidate cellular mechanisms by which the Type II pyrethroids cyfluthrin and alpha-cypermethrin might affect neuronal development. We investigated if the pyrethroids cyfluthrin and alpha-cypermethrin evoke adenylate kinase (AK) release, reactive oxygen species (ROS) production, caspase 3/7 activation, as well as elicit alterations in apoptosis and oxidative stress signaling pathways related to crucial roles on neuronal processes. In this context, we also determined the expression of genes linked to neuro-(developmental) toxicity, neurite growth and regeneration such as TUBB3, NEFL, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A, SYN1 and PIK3C3 transcripts.

## 2. Material and methods

### 2.1. Chemicals and reagents

The test substances were the Type II pyrethroids cyfluthrin and alpha-cypermethrin. The test substance cyfluthrin [cyano (4-fluoro-3-phenoxyphenyl) methyl 3- (2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate], a defined mixture of the 4 diastereoisomeric enantiomer pairs [diastereomer I (cis) (23.6%), diastereomer II (cis) (17.9%), diastereomer III (trans) (34.6%) and diastereomer IV (trans) (21.4%)], (CAS No.: 68359-37-5), ≥97.5% purity, molecular weight 434.3 g/mol, was provided by Bayer AG (Wuppertal-Elberfeld, Germany). The test substance alpha-cypermethrin, a 1:1 mixture of the pair of enantiomers (*R*)-α-cyano-3-phenoxybenzyl(1*S*,3*S*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and (*S*)-α-cyano-3-phenoxybenzyl(1*R*,3R)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate (CAS No.: 67375-30-8), ≥99% purity, molecular weight 416.3 g/mol, was provided by BASF Española S.L. (Barcelona, Spain).

The compounds 2′,7′-dichlorofluorescin diacetate (DCFH), 3-[4,5 dimethylthiazol- 2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (DPBS, D8537), fetal bovine serum (FBS), flavin adenine dinucleotide (FAD), bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid (HEPES), and β-nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma-Aldrich, St Louis, MO 63103, USA. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F12) was obtained from Biowhitaker Lonza (Walkersville, MD, USA). Penicillin and streptomycin were obtained from Invitrogen (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory available purity.

### 2.2. Cell line and culture condition

Human dopaminergic neuroblastoma SH-SY5Y cell line was obtained from European Collection of Authenticated Cell Cultures (ECACC 94030304), Sigma-Aldrich. SH-SY5Y cells were maintained in DMEM-F12 medium supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. For assays, SH-SY5Y cells were sub-cultured in 96-well plates at a seeding density of $5 \times 10^4$ cells per well. Cells were treated with the drugs before confluence in DMEM-F12 with 1% FBS. A vehicle group containing 0.1% DMSO was employed in parallel for each experiment. All SH-SY5Y cells used in this study were used at a low passage number (< 13).

### 2.3. Cell viability (MTT assay)

Cell viability, virtually the mitochondrial activity of living cells, was measured by quantitative colorimetric assay with MTT, as described previously (Denizot and Lang, 1986). Briefly, 50 μL of the MTT labeling reagent, at a final concentration of 0.5 mg/ml, was added to each well at the end of the incubation period and the plate was placed in a humidified incubator at 37 °C with 5% $CO_2$ and 95% air (v/v) for an additional 2 h period. Metabolically active cells convert the yellow MTT tetrazolium compound to a purple formazan product. The insoluble formazan was dissolved with DMSO; colorimetric determination of MTT reduction was measured spectrophotometrically at 540 nm, using a microplate reader (Spectrostar Nano, BMG LABTECH GmbH, Ortenberg, Germany). Control cells treated with DMEM-F12 were taken as 100% viability.

### 2.4. Adenylate kinase measurement

The bioluminescent ToxiLight TM bioassay (Lonza, Walkersville, Inc. USA) is a nondestructive cytotoxicity highly sensitive assay designed to measure cell membrane damage in mammalian cells and cell lines in culture. It quantitatively measured the release of cytosolic AK from the membranes of damaged cells (Crouch et al., 1993). The ToxiLight TM bioassay utilizes AK enzyme activity, which phosphorylates ADP to form ATP. Before the assay, $10^4$ cells per well in 96-well plates were treated with different dilutions of cyfluthrin or alpha-cypermethrin. After 24 h of the different treatments [cyfluthrin (0.01–100 μM) or with alpha-cypermethrin (0.01–100 μM)], 100 μL of cell supernatants were deposited in a 96-well white plate (White, Flat Bottom, Corning, USA). Then 100 μL of AK Detection Reagent (AKDR) were added to each well, and luminescence was measured after 5 min using a luminometer (FLx800, BioTek, Winooski, VT, USA) and expressed as AK release (%).

### 2.5. Reactive oxygen species (ROS) measurement

ROS formation was measured following the Wang and Joseph procedure (Wang and Joseph, 1999) by the DCFH assay using a microplate reader. After being oxidized by intracellular oxidants, DCFH becomes dichlorofluorescein and emits fluorescence. By quantifying fluorescence, a fair estimation of the overall oxygen species generated under the different conditions was obtained. For the assay of direct effect of pyrethroids, cells were seeded in 96-well plates at a rate of $2 \times 10^5$ cells per well and changed to FBS-free medium and the different cyfluthrin or alpha-cypermethrin concentrations the day after. After 24 h, 10 μM DCFH was added to the wells for 30 min at 37 °C. Then, cells were washed twice with DPBS. Multiwell plates were immediately measured in a fluorescent microplate reader (FLx800 Fluorimeter, BioTek, Winooski, VT, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. By quantifying fluorescence over a period of 60 min, a reliable estimation of the overall oxygen species generated under the different conditions was obtained.

### 2.6. Caspase 3/7 activity determination

The caspase-Glo 3/7 assay is a homogenous, luminescent assay that measures caspase-3 and -7 activities (Liu et al., 2005). The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD (aspartic acid-glutamic acid-valine-aspartic acid), in a reagent optimized for caspase activity, luciferase activity, and cell lysis. The addition of the Caspase-Glo reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase present.

The Kit Caspase-Glo 3/7 assay (Promega) provides the Caspase-Glo 3/7 buffer and lyophilized Caspase-Glo 3/7 substrate. These were equilibrated at room temperature prior to use. The contents of the buffer solution were transferred into the bottle containing the substrate. The content was dissolved by mixing to form the Caspase-Glo 3/7

reagent. This reagent can be stored at 4 °C for up to 1 week. The reagent can be frozen at −20 °C for longer storage.

SH-SY5Y cells (15 × $10^3$ cells per well) were grown in a 96-well white plate (White, Flat Bottom, Corning, USA) and exposed to cyfluthrin (0.01–100 μM) and alpha-cypermethrin (0.01–1000 μM) for 24 h. After 30 min at room temperature, 50 μL of Caspase-Glo 3/7 reagent was added to 50 μL of culture medium containing the cells previously treated in each well. After shaking the plate at 350 rpm during 30 s, an incubation period of 60 min at room temperature in the dark was needed to stabilize the signal before luminescence measurement with the luminometer. Luminescence was measured using the plate reader (FLx800, BioTek, Winooski, VT, USA).

### 2.7. RNA isolation and cDNA synthesis

Neuroblastoma SH-SY5Y cells were co-incubated with cyfluthrin (5 μM) or with alpha-cypermethrin (42 μM) for 24 h. Total RNA was extracted using the Trizol Reagent method (Invitrogen) and purified using RNeasy MinElute Cleanup Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) according to the manufacturer protocol. The final RNA concentration and purity was determined using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Madrid, Spain), obtaining A260/A280 ratios between 1.9 and 2.1 in all the samples. First-strand cDNA was synthesized from 5 μg total RNA by reverse transcription using RT2 First Strand kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, starting with a genomic DNA elimination step. At the end, cDNA was diluted 1:10 in nuclease-free water and stored at −80 °C for further analysis.

### 2.8. Measurement of mRNA gene expressions linked to apoptosis, oxidative stress and neurodevelopmental toxicity by Real-Time PCR

Quantitative Real-Time PCR assays for Bax, Bcl-2, Casp-3, BNIP3, p53 and Nrf2, genes linked to apoptosis and oxidative stress, and for TUBB3, NEFL, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A, SYN1 and PIK3C3, genes that may be associated to neurodevelopmental toxicity, were performed to analyze mRNA gene expressions (Table 1). Reactions were run on a Real-Time PCR system, BioRad CFX96, using $RT^2$ SYBR Green qPCR master mix (Qiagen, Hilden, Germany) according to manufacturer's protocol. Concentration of each primer was 400 nM and thermal protocol was as follows: 95 °C for 10 min, followed by 40 cycles composed of 15 s at 95 °C and 1 min at 60 °C. Sequences of primers are presented in Table 1. Relative changes in gene expression were calculated according to Pfaffl (2001), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as housekeeping gene (with tested no differences between groups) and extracting efficiencies from raw data using LinRegPCR free software (Ramakers et al., 2003).

### 2.9. Statistical analysis

Six replicates for each experimental condition were performed, and the presented results were representative of these replicates. Data are represented as mean values ± standard error of the mean (SEM). Comparisons between experimental and control among groups were performed by one-way ANOVA followed by the Tukey's *post-hoc* test, using GraphPad Prism 6. Statistical difference was accepted when $P < 0.05$.

For cell viability (MTT assay), cytotoxic concentrations of cyfluthrin and alpha-cypermethrin EC30 and EC50 values (concentrations that produce a 30% and 50% decrease in cell viability) were calculated by concentration-response curve (Sigmoidal fitting) with Origin-Pro 9 Software.

For quantitative Real-Time PCR assays, data are represented as mean value ± SEM. Statistical analysis was carried out using the Student's *t*-test. Statistical significance was defined at $P < 0.05$.

Concentrations of cyfluthrin and alpha-cypermethrin corresponding to EC30 values were used for determine the effects of these insecticides on the expression of genes linked to apoptosis, oxidative stress and neuro-(developmental) toxicity.

## 3. Results and discussion

There is increased need for reliable and efficient screening tools to evaluate, identify, and prioritize chemicals for their potential to induce developmental neurotoxicity. *In vitro* test systems offer the possibility of providing an early screen for a large number of chemicals, and could be useful in characterizing the mechanism of action or the developmental processes that are particularly affected by the test chemical. A significant obstacle with the current *in vivo* methods for the identification of developmental neurotoxicants is the lack of explicit regulatory guidance on how to quantitate the risks of developmental neurotoxicity [either for low observed adverse effect level (LOAEL) or no observed adverse effect level (NOAEL), or for benchmarks]. Moreover, it is difficult to interpret the methods in terms of their predictive value for human health. However, our findings together demonstrate that *in vitro* test systems may be not only a convenient but also an appropriate model for the toxicological study of pyrethroid insecticides.

We have studied for the first time the mechanisms of cellular action linked with neurodevelopmental toxicity of the Type II pyrethroids, cyfluthrin and alpha-cypermethrin, on human dopaminergic neuroblastoma SH-SY5Y cells.

### 3.1. Effects of cyfluthrin and alpha-cypermethrin on SH-SY5Y cell viability

This measure was based on the cleavage of MTT by the mitochondrial enzyme succinate dehydrogenase; it was used to evaluate human cell viability. The results show that the two insecticides cause cellular death for human SH-SY5Y cells. Concentrations, 0.01, 0.1 and 1 μM of cyfluthrin and concentrations 0.01, 0.1, 1, 2.5, 5, 7.5, 10 and 25 μM of alpha-cypermethrin did not affect neuronal survival (Fig. 2). The EC30 and EC50 for cyfluthrin were calculated to be 5.05 ± 1.51 and 19.11 ± 9.40 μM, respectively, equivalent values to those previously calculated (Martínez et al., 2019). The EC30 and EC50 values for alpha-cypermethrin were 42.47 ± 1.17 and 78.57 ± 2.34 μM, respectively. The EC50 for alpha-cypermethrin was also equivalent to that previously reported (Romero et al., 2017).

**Table 1**
Sequences of forward and reverse primers for apoptosis, oxidative stress and neurodevelopment related genes.

| Genes | Primer forward sequence | Primer reverse sequence |
|---|---|---|
| *Housekeeping gene* | | |
| GAPDH | GAGAAGGCTGGGGCTCATTT | AGTGATGGCATGGACTGTGG |
| *Apoptosis and oxidative stress related genes* | | |
| Bax | CCCCCGAGAGGTCTTTTTCC | CCTTGAGCACCAGTTTGCTG |
| Bcl-2 | TCTCATGCCAAGGGGGAAAC | TCCCGGTTATCGTACCCTGT |
| Casp-3 | GTGGAGGCCGACTTCTTGTA | TTTCAGCATGGCACAAAGCG |
| BNIP3 | CCTCAGCATGAGGAACACGA | GCCACCCCAGGATCTAACAG |
| p53 | GAACAAGTTGGCCTGCACTG | GAAGTGGGCCCCTACCTAGA |
| Nrf2 | CTGGTCATCGGAAAACCCCA | TCTGCAATTCTGAGCAGCCA |
| *Neurodevelopment related genes* | | |
| TUBB3 | CCGAAGCCAGCAGTGTCTAA | AGGCCTGGAGCTGCAATAAG |
| NEFL | CTGGAAATCGAAGCATGCCG | TGATCGTGTCCTGCATAGCG |
| NEFH | CTGGAGGCACTGAAAAGCAC | CTGGTAGGAGGCAATGTCGG |
| GAP43 | AGGGAGAAGGCACCACTACT | GGAGGACGGCGAGTTATCAG |
| CAMK2A | CATGGTTTGGGTTTGCAGGG | CCGGCTTTGATCTGCTGGTA |
| CAMK2B | GAGGACGGAGCGAGCAGAT | GACGCACGATGTTGGAATGC |
| WNT3A | TCTACGACGTGCACACCTG | CCTGCCTTCAGGTAGGAGTT |
| WNT5A | AGCAGACGTTTCGGCTACAG | TGCCCCCAGTTCATTCACAC |
| WNT7A | GCGTCTCGCACACTTGCAC | CCGCGCTTTCCGGTTCATAG |
| SYN1 | TACAACGTACCCCGTGGTTG | TTTGGCATCGATGAAGGGCT |
| PIK3C3 | GCTGTCCTGGAAGACCCAAT | TTCTCACTGGCAAGGCCAAA |

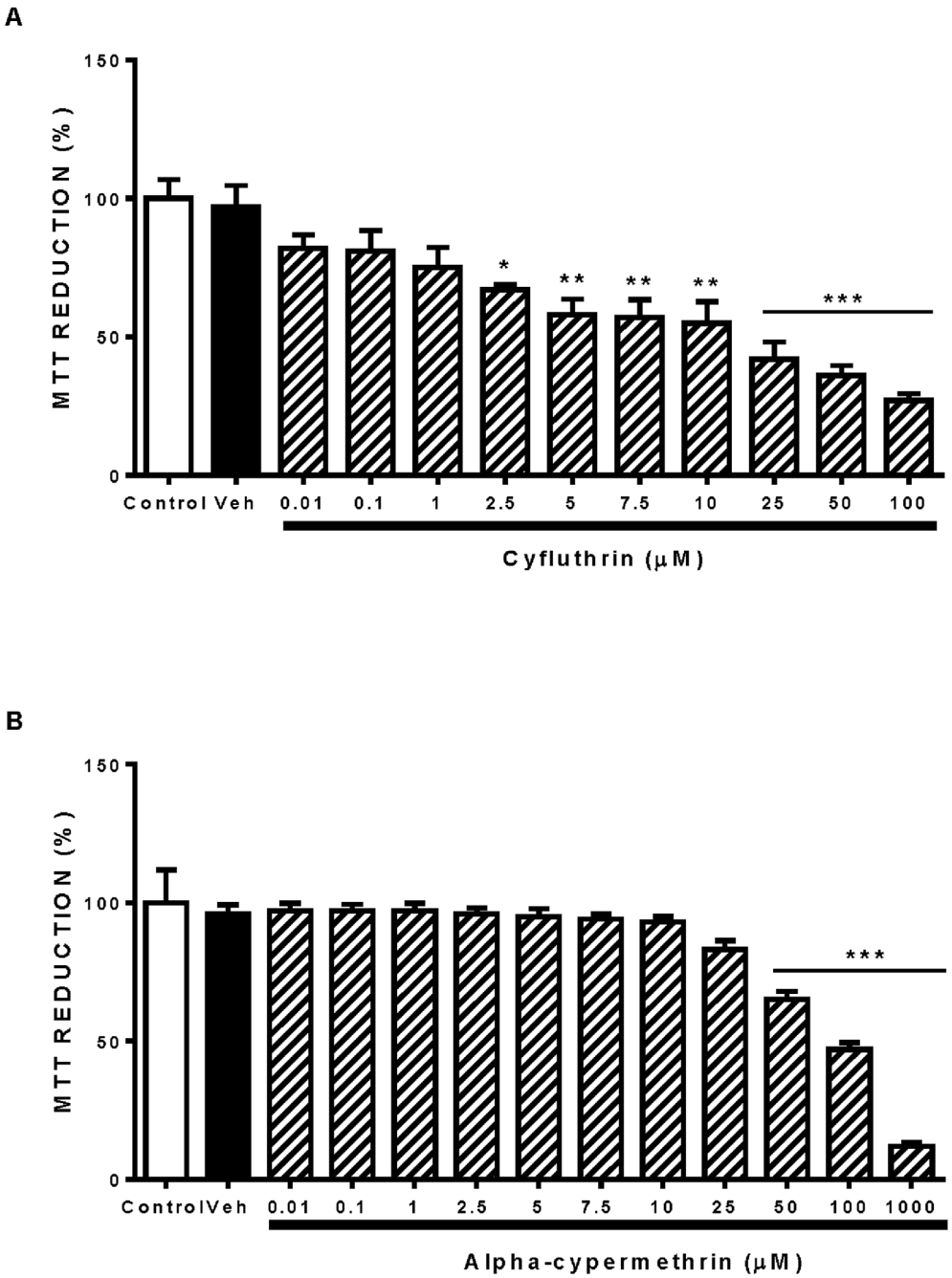


**Fig. 2.** Cytotoxicity induced by cyfluthrin and alpha-cypermethrin on SH-SY5Y cell viability after 24 h incubation period. Cell viability was measured as MTT reduction **(A** and **B)**. Data were normalized as % control (white column). Cells treated with DMSO (0.1%) were the negative control [vehicle (Veh), black column]. Data represent the mean ± SEM of six independent experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ compared to vehicle.

### *3.2. Effects of cyfluthrin and alpha-cypermethrin on cell membrane integrity*

ToxiLight TM bioassay is a nondestructive cytotoxicity assay designed to quantitatively measure the release of AK from damaged cells. It utilizes the AK enzyme activity, which phosphorylates ADP to form ATP. The amount of resultant ATP is then analyzed by measuring light intensity produced from bioluminescent firefly luciferase reaction. AK is a robust protein present in all eukaryotic cells, which is released into the culture medium when cells are damaged (the membrane integrity is disrupted during necrosis or secondary necrosis that occurs as a result of apoptosis). We measured AK activity after its release in the medium revealing cytoplasmic membrane rupture, corresponding to a necrosis and/or a secondary necrosis at the end of apoptosis (Taatjes et al., 2008). Our study demonstrates for the first time that both insecticides provoke an increase in AK release on human SH-SY5Y cells. Cells treated with 2.5, 5, 7.5, 10, 25, 50 and 100 µM of cyfluthrin showed an increase in AK release of 43%, 48%, 52%, 102%, 119%, 178% and 253%, respectively, compared to control cells (Fig. 3A), whereas alpha-cypermethrin significantly induced a significant increase in AK release of 40%, 176% and 289%, respectively, only at higher concentrations of 25, 50 and 100 µM (Fig. 3B). The pyrethroid alpha-cypermethrin induces cell membrane damage (by AK release) at 10 times higher concentrations than cyfluthrin (Fig. 3B). We demonstrate that cyfluthrin is more toxic in human SH-SY5Y cells than alpha-cypermethrin, especially on cell membrane. We suggest that cellular death may be mostly due to apoptosis overall after adenylate kinase release.

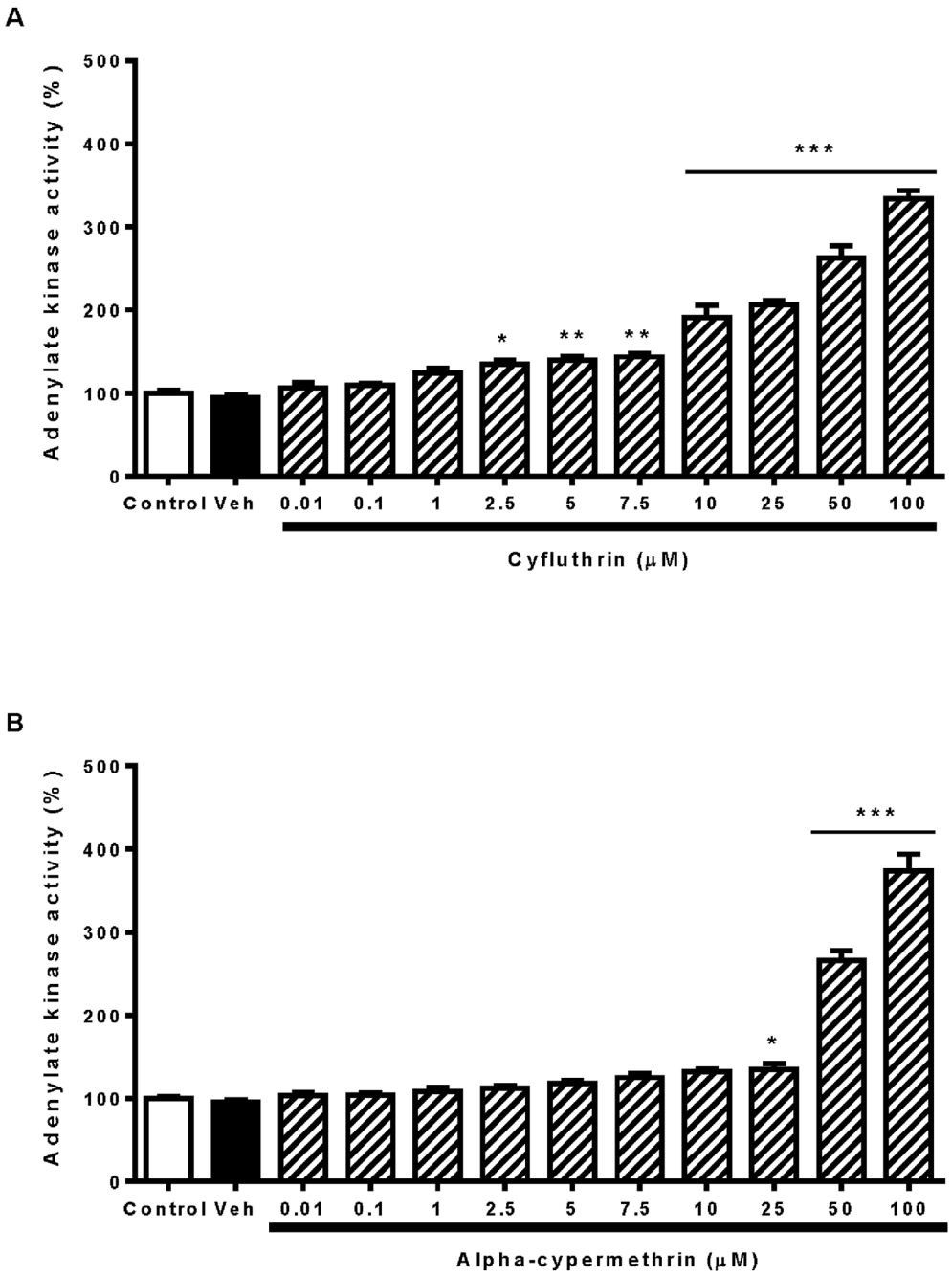


**Fig. 3.** Adenylate kinase activity induced by cyfluthrin **(A)** and alpha-cypermethrin **(B)** on SH-SY5Y cells after 24 h incubation period. Data were normalized as % control (white column). Cells treated with DMSO (0.1%) were the negative control [vehicle (Veh), black column]. Data represent the mean ± SEM of six independent experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ compared to vehicle.

### *3.3. Effects of cyfluthrin and alpha-cypermethrin on oxidative stress induction and programed cell death (apoptosis)*

Reactive oxygen species (ROS) are produced by cellular metabolic reactions, and have been implicated in the pathogenesis of several diseases, including atherosclerosis, cancer, and Alzheimer's disease (Dreher and Junod, 1996; Westhuyzen, 1997; Knight, 1997). ROS are harmful because they react with and modify all classes of cellular macromolecules and critical cellular targets that cause behavioral abnormalities, cytotoxicity, and mutagenic damage. Moreover, it has been postulated that dopaminergic neurons are more susceptible to the damage caused by ROS because of the potential neurotoxicity of dopamine itself. In fact, a recent work in our laboratory (Martínez et al., 2019) demonstrated that 5 μM cyfluthrin caused 86% increase in ROS generation in SH-SY5Y cells. In the present study, treatment of SH-SY5Y cells for 24 h with the pyrethroids cyfluthrin and alpha-cypermethrin produced a significant concentration-dependent increase in the cellular ROS generation (Fig. 4). As expected, cyfluthrin enhanced the response of ROS formation in comparison with alpha-cypermethrin. Cyfluthrin at concentrations of 2.5, 5, 7.5, 10, 25, 50 and 100 μM significantly induced an increase of 22%, 94%, 97%, 102%, 104%, 121% and 135%, respectively (Fig. 4A), whereas alpha-cypermethrin at concentrations of 5, 7.5, 10, 25, 50 and 100 μM significantly induced an increase of 24%, 30%, 32%, 58%, 71%, 89% and 89%, respectively (Fig. 4B). Nevertheless, alpha-cypermethrin exposure of SH-SY5Y cells evokes an increase in the generation of ROS at concentrations below than those

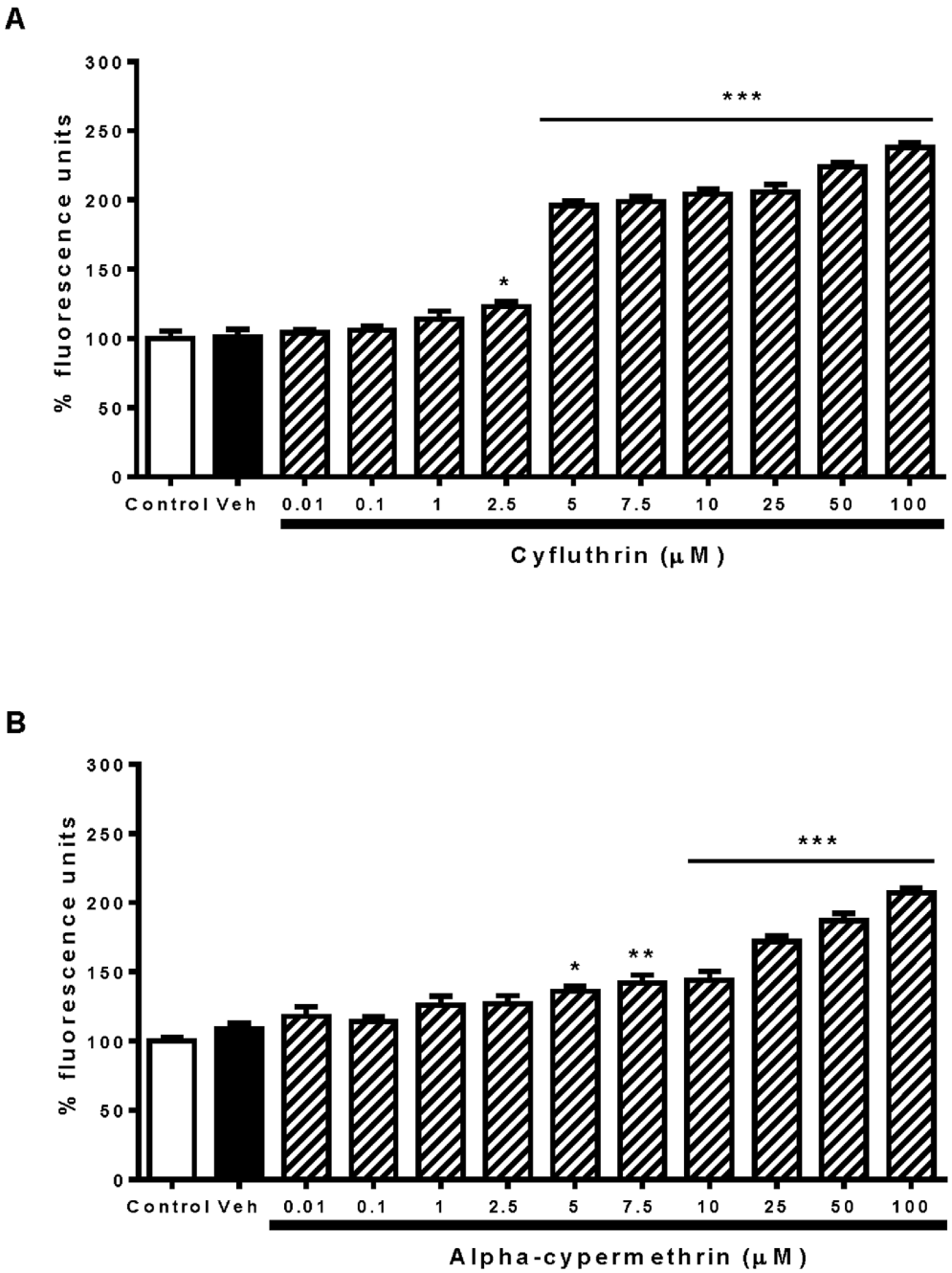


**Fig. 4.** ROS production by cyfluthrin **(A)** and alpha-cypermethrin **(B)** on SH-SY5Y cells after 24 h incubation period. Data were normalized as % control (white column). Cells treated with DMSO (0.1%) were the negative control [vehicle (Veh), black column]. Data represent the mean ± SEM of six independent experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ compared to vehicle.

necessary for cytotoxicity. There is evidence that melatonin and *N*-acetylcysteine (NAC), both powerful scavengers of reactive oxygen species (Reiter et al., 2001; Shahripour et al., 2014) significantly attenuated oxidative stress markers including the nitric oxide (NO) production induced by alpha-cypermethrin (Romero et al., 2017) and the NO and intracellular malondialdehyde (MDA) productions induced by cyfluthrin (Martinez et al., 2019). The important point is that our results indicate the need to assess oxidative injury after fetal or neonatal exposures to the pyrethroids cyfluthrin and alpha-cypermethrin *in vivo*. Enhanced ROS production may represent one of the mechanisms by which pyrethroids injure the immature brain.

Because of ROS may serve as common mediators in programmed cell death in response to many toxicants and pathological conditions (Galluzzi et al., 2007), we have also elucidated that cyfluthrin- and alpha-cypermethrin-induced cell death can be due, at least in part, to apoptosis via increased activity of caspase-3/7 (Fig. 5). The caspases are activated by both pyrethroids, but cyfluthrin is more toxic than alpha-cypermethrin. Cyfluthrin concentrations of 2.5, 5, 7.5, 10, 25, 50 and 100 μM cause an increase in caspase 3/7activity of 24%, 25%, 26%, 27%, 43%, 49% and 66%, respectively (Fig. 5A). This apoptotic pathway was also activated for alpha-cypermethrin at levels 20 times higher (Fig. 5B). Concentrations of alpha-cypermethrin of 25, 50 and 100 μM significantly induced a caspase 3/7activity increase of 22%, 40% and 66%, respectively (Fig. 5B).

Respect to effects of cyfluthrin and alpha-cypermethrin on apoptosis and oxidative stress related gene transcriptions in SH-SY5Y cells, in our laboratory, we previously demonstrated that mRNA expression of Bax, Bcl-2, Casp-3, BNIP3, p53 and Nrf2 significantly increased after

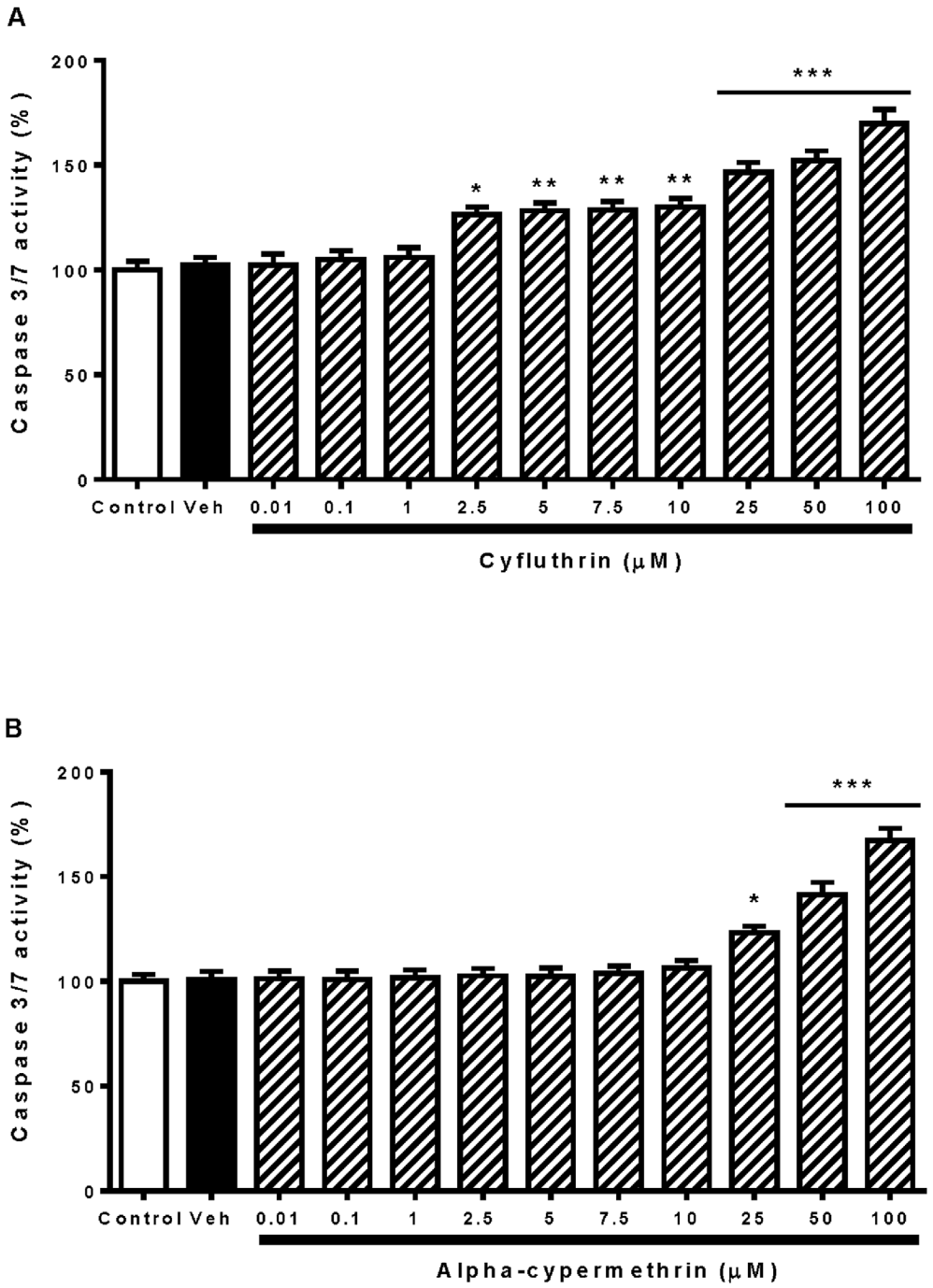


**Fig. 5.** Caspase-3/7 activity induced by cyfluthrin **(A)** and alpha-cypermethrin **(B)** on SH-SY5Y cells after 24 h incubation period. Data were normalized as % control (white column). Cells treated with DMSO (0.1%) were the negative control [vehicle (Veh), black column]. Data represent the mean ± SEM of six independent experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ compared to vehicle.

cyfluthrin exposure [5 μM, concentration equivalent to EC30 value on cell viability (MTT assay)]. In the most genes, the mRNA levels induced by cyfluthrin were partially reduced by melatonin (Martinez et al., 2019). In the present study, we revealed by Real-Time PCR assays that alpha-cypermethrin exposure [(42 μM, concentration equivalent to EC30 value on cell viability (MTT assay)] caused also an increase of these gene expressions (Fig. 6). Alpha-cypermethrin provided a significant increase of Bax, Bcl-2, Casp-3, BNIP3 and p53 (2-fold, $P < 0.05$) and Nrf2 (3-fold, $P < 0.01$) mRNA expression compared to control group (Fig. 6). These data suggest that apoptosis and oxidative stress mechanisms are the main target biological alterations related to the toxicity induced by pyrethroids.

### *3.4. Effects of cyfluthrin and alpha-cypermethrin on transcriptional pathways linked to neuro-(developmental) toxicity in SH-SY5Y cells*

Considerable evidence has been accumulated supporting a role for $H_20_2$ and other ROS as activators of signaling pathways and transcription factors. Reactive oxygen interacts with receptors, second messengers and transcription factors, altering gene expression and influencing cell growth and survival (Palmer and Paulson, 1997). The exact nature of the relationship between oxidative stress and transcriptional activation of important genes linked to developmental neurotoxicity activity is unclear. Developing nervous system is expected to be highly sensitive and may act as a preferential target for chemicals

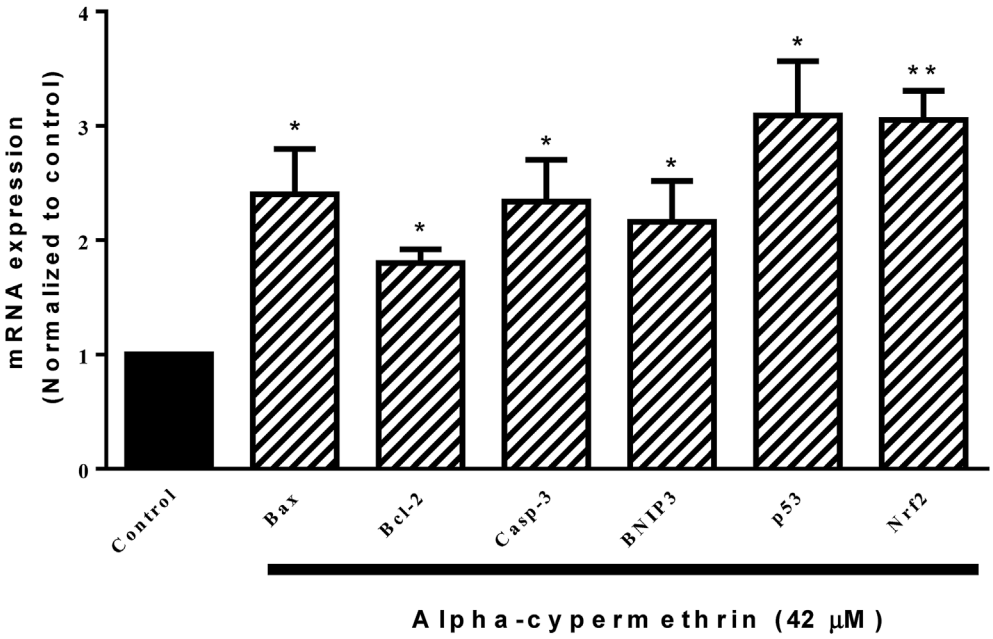


**Fig. 6.** Effect of alpha-cypermethrin (42 μM) on gene expressions linked to apoptosis and oxidative stress pathways (Bax, Bcl-2, Casp-3, BNIP3, p53 and Nrf2) in SH-SY5Y cells. Cells treated with DMSO (0.1%) were the negative control (black column). Data represent the mean ± SEM of six independent experiments. *$P < 0.05$ and **$P < 0.01$ compared to control.

because the development of dopaminergic neurons mainly occurs during postnatal periods. We evaluated by RT-PCR assays whether the insecticides, cyfluthrin and alpha-cypermethrin, affected the expression of selected target genes linked to neuro-(developmental) toxicity, neurite growth and regeneration. Concentrations of cyfluthrin (5 μM equivalent to 2 μg/mL) and alpha-cypermethrin (42 μM equivalent to 17 μg/mL), corresponding to EC30 values on cell viability (MTT assay), were used to determine the effects of these insecticides on the mRNA expressions of genes involved in normal neural cell development and cell growth: TUBB3, NEFL, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A, SYN1 and PIK3C3. We decided to focus on these "realistic" concentrations because it has been documented that these concentrations are within the range of the derived NOAEL values from oral acute and subchronic neurotoxicity studies performed in rats for cyfluthrin and alpha-cypermethrin according to EU Regulation No 528/2012 from 2012 to 2016.

The Fig. 7AB presents responsiveness of eleven genes to insecticide treatment of SH-SY5Y cells. Cyfluthrin (5 μM) significantly reduced the gene expression of NEFL (−15%) and SYN1 (−35%) compared to control group (Fig. 7A) and increased the gen expressions of TUBB3, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A and PIK3C3 (206%, 112%, 98%, 172%, 185%, 135%, 91%, 153% and 76%, respectively) compared to control group (Fig. 7A). Also, the insecticide alpha-cypermethrin (42 μM) significantly reduced the gene expressions of NEFL (−11%) and SYN1 (−19%) compared to control group (Fig. 7B) and increased the gen expressions of TUBB3, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A and PIK3C3 (69%, 121%, 83%, 63%, 60%, 134%, 109%, 116% and 59%, respectively) compared to control group (Fig. 7B).

The strongest transcriptional alterations occurred for Type II pyrethroid cyfluthrin (Fig. 7A) with (i) significant induction of the expression of TUBB3 mRNA (206%), strictly related to neurite outgrowth and widely used as a neuronal marker in developmental studies (Katsetos et al., 2003); (ii) significant induction of the expression of the two CAMK2 isoforms (CAMK2A mRNA, 172%; CAMK2B mRNA, 185%) which can lead to neuronal apoptosis (Chen et al., 2011); (iii) significant induction of the expressions of NEFH (112%), the basic function of NEFH is supporting axonal structure (Liu et al., 2004), and GAP43 (98%), GAP43 plays a pivotal role not only during development but also in axonal remodeling in the adult brain (Grasselli and Strata, 2013) and (iv) significant induction of the WNT signaling pathways (WNT3A mRNA, 135%; WNT5A mRNA 91%; WNT7A mRNA 153%) with crucial roles in neuronal development and maturation. WNTs are involved in diverse cellular functions that include neuronal migration, neuronal polarization, axon guidance, dendrite development, and synapse formation (Inestrosa and Arenas, 2010; Rosso and Inestrosa, 2013), all of which are essential steps in the formation of functional neural connections. In relation to the Type II pyrethroid alpha-cypermethrin also particularly increased the mRNA expressions of NEFH (121%), GAP43 (83%), and WNT signaling pathways (WNT3A, 134%; WNT5A, 109%; WNT7A, 116%) (Fig. 7B). This clearly confirm that these insecticides available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from treated crops pyrethroids.

In conclusion, our study shows that the SH-SY5Y cell line is a valuable model in the initial identification of compounds with developmental neurotoxicity, and that associated transcriptional alterations may support the identification of developmental neurotoxicity. The application of *in vitro* data directly to risk assessment will require a more complete understanding of the mechanisms underlying the expression of toxicity *in vivo*, as well as the ability to extrapolate from effective concentrations obtained *in vitro* to likely target tissue levels *in vivo*. The present study focused on developmental neurotoxicity of the Type II pyrethroids, cyfluthrin and alpha-cypermethrin. We show up data demonstrating our approach to correlating effects on mechanisms of cellular action that include increases in AK release, generation of ROS, and caspase 3 and 7 activation with the up-regulation of TUBB3, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A transcripts linked to neuronal development and maturation, which is an important finding. The analysis of expressional changes of these transcripts would be used as biomarkers for detection and initial evaluation of potential developmental neurotoxicity of pyrethroids, using SH-SY5Y cells. Additional information using this *in vitro* model is necessary to understand its utility in screening approaches for developmental neurotoxicity.

## CRediT authorship contribution statement

**María-Aránzazu Martínez:** Conceptualization, Methodology, Supervision. **Bernardo Lopez-Torres:** Investigation, Formal analysis, Resources. **José-Luis Rodríguez:** Investigation, Formal analysis. **Marta Martínez:** Investigation, Visualization, Writing - original draft, Writing

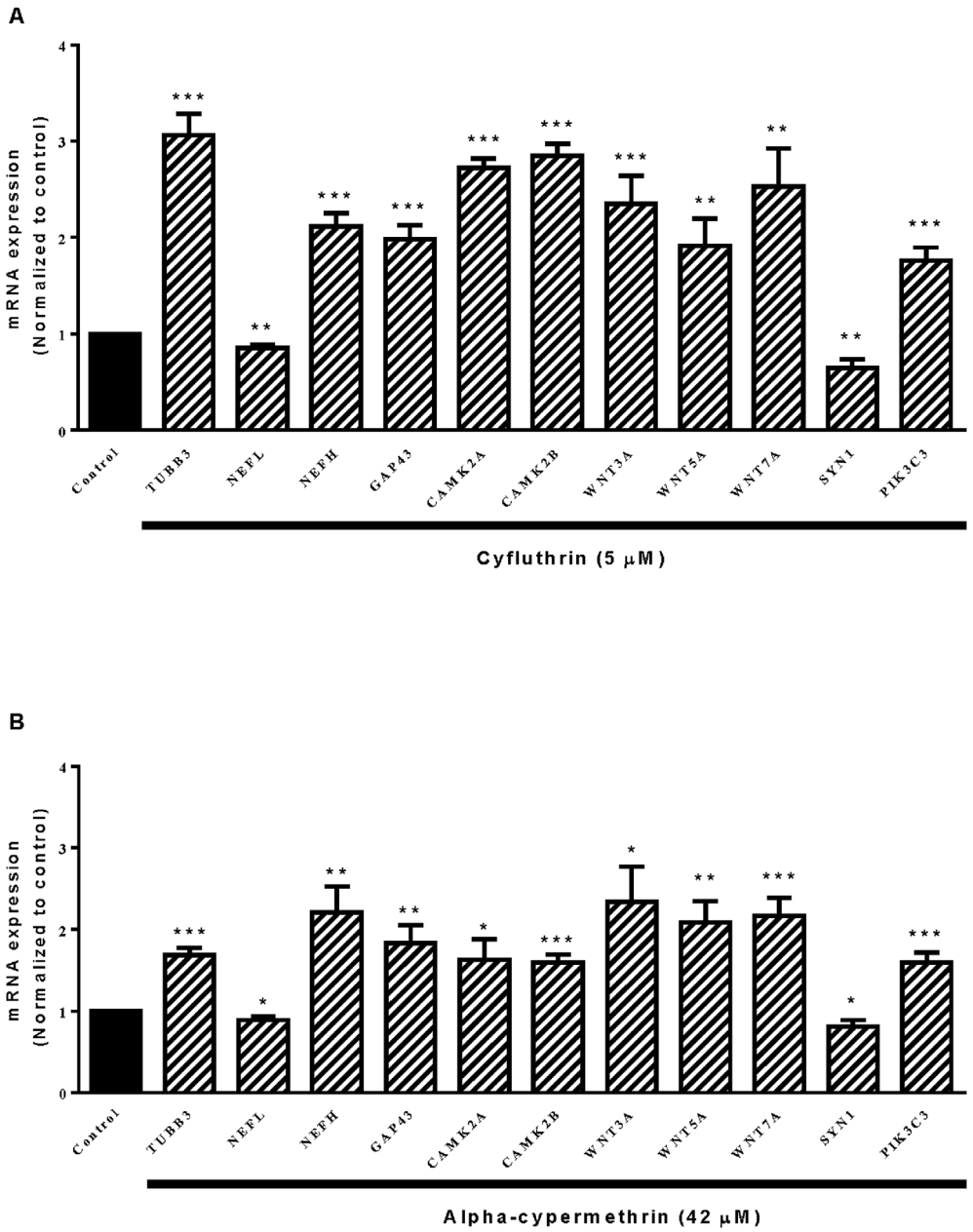


**Fig. 7.** Effect of cyfluthrin (5 μM) **(A)** and alpha-cypermethrin (42 μM) **(B)** on gene expressions linked to neuronal development toxicity (TUBB3, NEFL, NEFH, GAP43, CAMK2A, CAMK2B, WNT3A, WNT5A, WNT7A, SYNI and PIK3C3) in SH-SY5Y cells. Cells treated with DMSO (0.1%) were the negative control (black column). Data represent the mean ± SEM of six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control.

- review & editing. **Jorge-Enrique Maximiliano:** Investigation, Resources. **María-Rosa Martínez-Larrañaga:** Conceptualization, Methodology, Investigation. **Arturo Anadón:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Irma Ares:** Investigation, Validation, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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