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# ***Inhibition of Neurite Outgrowth Upon Acute Exposure to Chlorpyrifos***

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Biology

## **Abstract**

Chlorpyrifos is an organophosphate insecticide used on agricultural crops to control different pests. Chlorpyrifos (CPF) has toxic effects on the human nervous system that can result from exposure orally, topically and through inhalation. Effects of low dose exposures can range from nausea and dizziness to muscle twitching, tremors, and loss of coordination. More severe exposures can result in convulsions and even paralysis. It was hypothesized that if neurons in culture were exposed to 2  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 20  $\mu\text{M}$  CPF, then neurite outgrowth would decrease with increasing concentration. In order to test this hypothesis, the cells were exposed to three different concentrations of CPF (2  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 20  $\mu\text{M}$ ) and a control. After 24 hours of exposure, the cells were fixed and microscopic images of neurites were captured. The data indicate that increased concentrations of CPF result in a significantly decreased neurite outgrowth. It has been suggested that CPF exposure contributes to Parkinson's disease and decreased neurodevelopment. Decreased neurite outgrowth due to CPF exposure could contribute to the development of these conditions.

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## **Introduction**

Chlorpyrifos (CPF) is a well-known organophosphate insecticide used to control many different pests. By inhibiting cholinesterase, CPF can cause toxicity in humans and animals (Raszewki *et al*, 2014). Oral or dermal exposure and inhalation of CPF can cause a range of symptoms. Low doses of exposure to CPF can cause nausea, dizziness, muscle twitching, tremors, and even loss of coordination. Exposures to higher concentrations can lead to convulsions and paralysis.

It is suggested that exposure to CPF contributes to the development of Parkinson's disease (PD) (Dhillon *et al*, 2008) and adversely affects neurodevelopment *in utero* (Betancourt *et al.*, 2007). Parkinson's disease is neurodegenerative disease that is caused by the loss of dopaminergic neurons and the concomitant loss of dopamine, which is an inhibitory neurotransmitter. The lack of dopamine being transmitted between neighboring neurons causes the tremors associated with PD (Parkinson's Disease and Environmental Factors, 2014).

For normal cell-cell communication to occur in the nervous system, the neurites that extend from neighboring neurons and release neurotransmitters must be in proximity to one another. Decreased neurite growth contributes to neurological disorders, such as autism and epilepsy, as neurons cannot communicate effectively (Bicknell *et al*, 2018). Exposure to CPF can result in symptoms similar to these neurological disorders, perhaps due to a lack of communication between neurons. It was hypothesized that exposure of SH-SY5Y cells to increasing concentrations of CPF would result in decreased neurite outgrowth. To test the hypothesis, the SH-SY5Y cell line was used because it is a reliable model to study toxic effects on the nervous system due to pesticide exposures (Raszewki *et al*, 2014).

## **Methods**

SH-SY5Y cells (passages 13 -22) were maintained at 37°C in a humidified incubator. The media was replaced every two to four days depending on confluency of the cells. Using the trypan blue exclusion method, cell viability was determined and cells were plated at 50,000 cells/ml in 0.5 ml media on a 24-well clean bottom, black walled, tissue culture treated plate (Eppendorf).

## Differentiating and Treating Cells

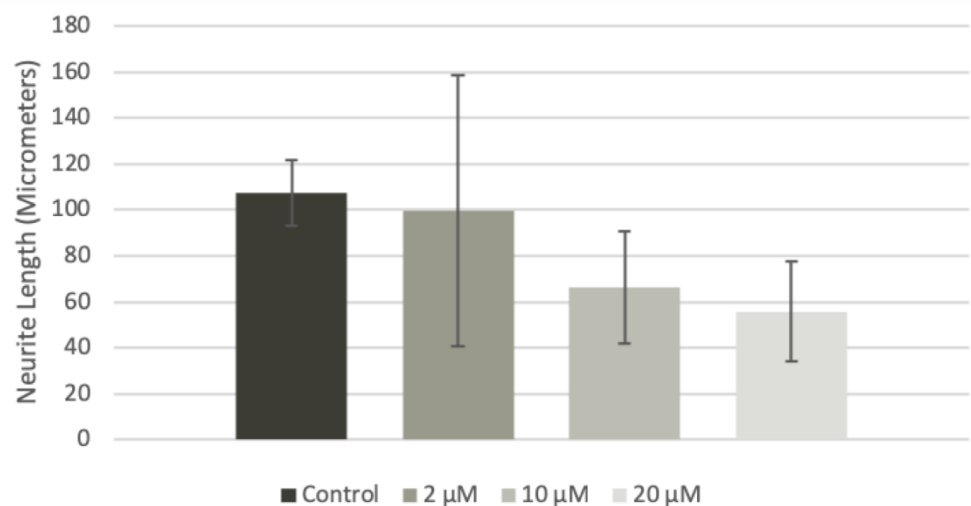
Cells were differentiated to a normal phenotype with  $10^{-7}$ M retinoic acid in media and incubated for three days. Chlorpyrifos (ChemService) was diluted in media for concentrations of 2  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M. The negative control was 0.01% ethanol in media. A volume of 0.5 ml of each treatment was added to the appropriate well in the 24-well plate. Cells were placed in  $37^{\circ}\text{C}$  in a humidified incubator for 24 hours.

## Imaging Cells and Measuring Neurites

After 24 hours, CPF was aspirated off of each well using either vacuum suction or a sterile DPTP. Each well was washed 2 times with sterile 1X PBS, then fixed with 4% paraformaldehyde for 10 minutes. After removing the paraformaldehyde, each well was washed three times with sterile PBS, then permeabilized with 0.1% Triton X for 10 minutes. The Triton X was removed and each well was washed 3 times with sterile 1X PBS, and blocked with 1% BSA at room temperature for 1 hour. After aspirating the BSA off of each well, beta-3 tubulin mouse antibody (1:200, Cell Signaling Technology) was added to each well and incubated for 3 hours at room temperature. The antibody was removed and cells were incubated with a goat anti-mouse secondary antibody (1:2000, CST) for 45 minutes at room temperature. Cells were imaged with a ZOE cell imager (BioRad). This procedure was repeated two more times, for a total of three replicates. Neurite outgrowth was measured with ImageJ imaging software. Neurites longer than the cell body were evaluated (n = 70 neurites for control; n= 59 neurites for 2  $\mu$ M and for 10  $\mu$ M; and 42 neurites for 20  $\mu$ M). A one-way ANOVA with pairwise comparisons was used to analyze the data.

## Results

No significant difference was found between the control and 10  $\mu$ M (p = 0.18). There was a significant decrease in neurite outgrowth between the control and 2  $\mu$ M (p < 0.001), and between the control and 20  $\mu$ M CPF (p < 0.001). A significant decrease in neurite outgrowth was also found between 2  $\mu$ M CPF and 10  $\mu$ M CPF (p = 0.004) and between 2  $\mu$ M CPF and 20  $\mu$ M CPF (p < 0.001). There was a significant decrease in neurite outgrowth when CPF concentration increased from 10  $\mu$ M CPF and 20  $\mu$ M CPF (p = 0.02) (Figure 1).



## Discussion

It was expected that there would be a decrease in the length of neurite outgrowth with increasing concentrations of CPF. The hypothesis was supported by the data. There was no significant difference in neurite length between the control and 2  $\mu$ M CPF. The concentration of 2  $\mu$ M CPF did not reduce the growth of neurites. However, there was a significant decrease in neurite outgrowth if the CPF concentration reached 10  $\mu$ M compared to the control and 20  $\mu$ M concentration. A decrease in neurite outgrowth was also observed as the CPF concentration increased from 2  $\mu$ M CPF to 10  $\mu$ M CPF, and from 2  $\mu$ M CPF to 20  $\mu$ M CPF. The results indicate that exposure to increasing CPF concentrations will cause a significant decrease in neurite outgrowth.

The decrease in neurite outgrowth of dopaminergic neurons will result in decreased communication between neurons. Neurons will not receive dopamine from their neighbors and so may not be inhibited from excitation. This can cause symptoms that are seen in conditions such as Parkinson's disease and epilepsy. Furthermore, when neurons do not communicate normally *in utero*, there will be an adverse effect on neurodevelopment (Betancourt *et al.*, 2007).

Agricultural workers are chronically exposed to CPF since it is the most widely used insecticide in the United States. Consumers of fruits and vegetables and residential users are also exposed to this insecticide. Users of CPF may experience nausea, dizziness, muscle twitching, and even loss of coordination if they do not follow manufacturer's instructions. The results of this study emphasize the importance of educating consumers and residential users, and of protecting agricultural workers from chronic exposure to pesticides, particularly CPF.

## References

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