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Effects of Chlorpyrifos-Based Pesticide Formulation on the Mouse (Mus musculus) Neuronal Cell Line (N2a): Neurotoxicity and Inflammatory Response

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ABSTRACT

Background: The widespread use of pesticides has raised concerns about their potential impacts on non-target species and environmental health. Chlorpyrifos, a commonly used organophosphate insecticide, has been linked to neurotoxic effects, but its impact on neuronal cells at the cellular level needs further investigation. **Methods:** This study evaluated the neurotoxicity and inflammatory response of a chlorpyrifos-based pesticide formulation using the mouse (Mus musculus) neuronal cell line (N2a). Characterization of the pesticide formulation included gas chromatography-mass spectrometry (GC-MS) and thermal analyses (thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)). **Results:** Neurotoxicity assays, including the [3(4,5-dimethyl)-2 bromide-5 diphenyl tetrazolium] (MTT) assay and lactate dehydrogenase (LDH) release assay, showed IC50 values of 5.23 µg/mL and 3.78 µg/mL, respectively. The median IC50 of 4.51 µg/mL was used in inflammatory response assays, revealing increased production of pro-inflammatory cytokines (IL-6 and TNF-α) and elevated nitric oxide (NO) levels. Additionally, the expression of inflammatory markers, such as COX-2 and iNOS, was upregulated. **Conclusion:** This study is the first to report in vitro neurotoxicity and inflammatory responses induced by chlorpyrifos in the N2a cell line, highlighting the importance of in vitro assays for assessing the toxic effects of pesticide formulations on neuronal health and the environment.

1. Introduction

The use of pesticides has significantly increased agricultural productivity and food security worldwide. However, the extensive application of these chemicals has raised serious concerns about their potential impacts on human health and the environment. One of the most commonly used pesticides is chlorpyrifos, an organophosphate insecticide that targets the nervous system of pests. Despite its effectiveness in controlling a wide range of insects, chlorpyrifos has been linked to various health issues, particularly neurotoxic effects in non-target species, including humans (Eaton et al., 2008). This introduction provides an overview of chlorpyrifos, its mode of action, its potential impacts on neuronal health, and the importance of in vitro studies in assessing its toxic effects.

1.1. Chlorpyrifos: Usage and Mode of Action

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) is a broad-spectrum, chlorinated organophosphate insecticide introduced in 1965 by Dow Chemical Company. It has been widely used in agriculture, households, and public health programs to control various pests, such as mosquitoes, flies, and crop insects (Roberts & Reigart, 2013). Chlorpyrifos acts by inhibiting acetylcholinesterase (AChE), an enzyme responsible for breaking down the neurotransmitter acetylcholine in the nervous system. Inhibition of AChE leads to the accumulation of acetylcholine in synapses, resulting in overstimulation of cholinergic receptors and disruption of normal neurotransmission (Casida & Quistad, 2004). This mode of action is effective in killing or incapacitating target pests, but it can also adversely affect non-target organisms, including humans.

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1.2. Neurotoxic Effects of Chlorpyrifos

The neurotoxic effects of chlorpyrifos have been well-documented in various studies. Exposure to high doses of chlorpyrifos can lead to acute symptoms, such as headaches, dizziness, nausea, vomiting, and even seizures and respiratory failure in severe cases (Roberts & Reigart, 2013). Chronic exposure to low doses of chlorpyrifos has been associated with long-term neurodevelopmental and neurobehavioral effects, particularly in children. Epidemiological studies have reported links between prenatal or early-life exposure to chlorpyrifos and cognitive deficits, developmental delays, and increased risk of neurodevelopmental disorders, such as autism and attention-deficit/hyperactivity disorder (ADHD) (Bouchard et al., 2011; Rauh et al., 2011).

The mechanisms underlying the neurotoxic effects of chlorpyrifos are not fully understood, but they are believed to involve multiple pathways, including oxidative stress, inflammation, and disruption of neurotransmitter systems. Chlorpyrifos has been shown to induce the production of reactive oxygen species (ROS) and lipid peroxidation, leading to oxidative damage in neuronal cells (Ki et al., 2013). Furthermore, chlorpyrifos can activate inflammatory pathways, resulting in the release of pro-inflammatory cytokines and the upregulation of inflammatory markers, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Caughlan et al., 2017). Additionally, chlorpyrifos can disrupt the balance of neurotransmitters, affecting not only acetylcholine but also other neurotransmitters, such as dopamine, serotonin, and GABA (Slotkin & Seidler, 2012).

1.3. In Vitro Studies in Neurotoxicology

In vitro studies play a crucial role in neurotoxicology by providing a controlled and efficient way to investigate the cellular and molecular mechanisms underlying the neurotoxic effects of chemicals like chlorpyrifos. Cell lines derived from neuronal tissues, such as the mouse neuroblastoma cell line (N2a), offer a valuable model for assessing neurotoxicity and inflammatory responses. These cell lines express neuronal markers and possess functional neurotransmitter systems, making them suitable for studying the effects of neurotoxicants (LePage et al., 2005).

In vitro studies using neuronal cell lines have several advantages over in vivo studies. They allow for better control of experimental conditions, easier manipulation of variables, and higher throughput screening of chemicals. Moreover, in vitro studies can reduce the number of animals used in research, aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal experimentation (Russell & Burch, 1959). However, it is essential to acknowledge the limitations of in vitro studies, such as the lack of complex interactions between different cell types and organs, and the need to validate in vitro findings with in vivo studies.

1.4. Importance of the Current Study

Despite the extensive research on the neurotoxic effects of chlorpyrifos, there is still a need for further investigation into its impact on neuronal cells at the cellular and molecular levels. The current study aims to evaluate the neurotoxicity and inflammatory response of a chlorpyrifos-based pesticide formulation using the mouse (Mus musculus) neuronal cell line (N2a). By assessing the effects of chlorpyrifos on neuronal cell viability, inflammatory markers, and oxidative stress, this study seeks to contribute to a better understanding of the mechanisms underlying chlorpyrifos-induced neurotoxicity.

Furthermore, this study emphasizes the importance of in vitro assays for assessing the toxic effects of pesticide formulations on neuronal health and the environment. The use of illicit or improperly handled pesticide formulations can pose significant risks to agricultural production, the environment, and non-target species, including humans. Therefore, it is crucial to develop and employ sensitive and reliable in vitro assays to evaluate the potential hazards of pesticide formulations and ensure their safe use.

2. Materials and Methods

2.1. Chemical Reagents

Dulbecco's Modified Eagle's Medium (DMEM) with high glucose content was obtained from Inlab Diagnóstica (Diadema, SP, Brazil). Fetal Bovine Serum (FBS), trypsin/EDTA solution, and 3(4,5-dimethyl)-2 bromide-5 diphenyl tetrazolium (MTT) were obtained from Gibco (Gibco, Carlsbad, CA, United States). The mouse neuroblastoma cell line (N2a) was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). All other reagents and solvents used were of analytical or pharmaceutical grade. The chlorpyrifos-based pesticide formulation was obtained from a commercial source for research purposes.

2.2. Agrochemical Extraction and Identification

90 mg of the chlorpyrifos-based pesticide formulation was extracted with 25 mL of acetonitrile. The material was centrifuged for 5 min, and 1 µL of the solvent fraction was injected into a GC-MS QP2010SE (Shimadzu®) in split mode (1:25). Helium was used as the mobile phase and the Rtx-5MS was used as the capillary column. The temperature was set at 260 ˚C for the injector and 280 ˚C for the ion source. The initial temperature of the column was 200 ˚C for 12 min, with a 5 ˚C/min heat ramp until 280 ˚C, then holding for 5 min. MS ran in scan mode from 50 to 550 m/z.

2.3. Thermogravimetric Analysis (TGA)

The thermal stability of the chlorpyrifos-based pesticide formulation was determined using the TGA Q5000 equipment (TA Instruments Inc., USA). For this analysis, the heating rate was 10 ˚C/min using an inert N2 atmosphere (25 mL/min). The equipment was calibrated with CaC2O4·H2O (99.9%). The mass of the sample was 4.473 mg. Data were processed using TA Universal Analysis 2000 Software, version 4.5 (TA Instruments Inc., USA).

2.4. Differential Scanning Calorimetry (DSC)

Modulated Temperature Differential Scanning Calorimetry (MT-DSC) was performed on DSC Q2000 equipment (TA Instruments, USA) with MTDSC option, with RCS cooling and N2 as purge gas (50 mL/min), which was used to determine DSC. The heating rate was 5 ˚C/min. The instrument was initially calibrated in the DSC standard form, using indium (99.99%). The sample mass (11.384 mg) was weighed with an accuracy of (±0.001 mg). Data were processed using TA Universal Analysis 2000 Software, version 4.5 (TA Instruments Inc., USA).

2.5. In Vitro Cytotoxicity Assays

The cytotoxicity of the chlorpyrifos-based pesticide formulation was evaluated using the mouse neuroblastoma cell line (N2a).

2.5.1. N2a Cell Line Culture

The N2a cell line was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in culture flasks and maintained in DMEM high glucose (INLAB®, Brazil), supplemented with 10% (v/v) heat-inactivated FBS (Gibco®, Brazil), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured and maintained in a humidified incubator at 37 ˚C with 5% CO2.

2.5.2. Cell Culture Exposure

For cytotoxicity and inflammatory response assays, N2a cells were seeded in 96-well plates at a density of 3 × 104 cells/well and in 6-well plates at a density of 6 × 105 cells/well, respectively. The plates were incubated for 24 hours for complete cell adhesion. The chlorpyrifos-based pesticide formulation was diluted in a complete medium without the addition of FBS immediately before each experiment. For cytotoxicity assays, concentrations were defined based on preliminary tests and set at 0.5, 1, 2, 4, 8, 16, and 32 µg/mL. To determine inflammatory responses in N2a cells, concentrations of 1/2×, 1×, and 2× corresponding to the median IC50 of the cytotoxicity assays were used. Triton X-100 at 5% was used as the positive control (PC) for the LDH assay, and H2O2 at 0.5% for MTT and inflammatory response tests; cells treated only with culture medium were used as the negative control (NC) for all assays. The treated cells were incubated under the same conditions for 24 hours.

2.5.3. Mitochondrial Viability Assay

Mitochondrial viability was determined according to the method described by Mosmann using MTT. This assay is based on the ability of mitochondria to reduce MTT (yellow) to blue formazan crystals. After the cell incubation period at the tested concentrations, the supernatant was removed, and the cells were washed with PBS. The MTT solution (1 mg/mL) was added at 50 µL/well and incubated in an incubator at 37˚C for 3 h. The medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was recorded at 540 nm using a microplate reader (SpectraMax M3). The percentage of viability was calculated as AT/AC × 100; where AT and AC are the absorbances of the treated and control cells, respectively.

2.5.4. Lactate Dehydrogenase Release Assay

The enzyme release assay induced by the action of chlorpyrifos on the plasma membrane of N2a cells was determined using the lactate dehydrogenase (LDH) assay, as described by Vaucher et al. with modifications. After the incubation period of the cells at the tested concentrations, the supernatants were collected, and the release of LDH was determined. This test was performed through an enzymatic kinetics assay using a commercially available LDH (UV) kit (Bioclin® – Quibasa Ltda, Belo Horizonte, MG, Brazil). Absorbance at 340 nm was determined using a Cobas MIRA® automated analyzer (Roche Diagnostics, Basel, Switzerland), following the manufacturer's instructions. The percentage of LDH release was calculated following the equation: (AT – AC)/(AX – AC) × 100, where AT is the absorbance of treated cells, AC is the control absorbance of untreated cells, and AX is the absorbance of cells lysed with Triton X-100.

2.6. Inflammatory Response Parameters

2.6.1. Lysate Preparation

After the incubation period of the cells at concentrations of 1/2× (2 µg/mL), 1× (4 µg/mL), and 2× (8 µg/mL) corresponding to the median IC50 value, the cells were washed twice with PBS. The lysate was prepared using a mechanical scraper. We then centrifuged at 1000 rpm for 10 min at 4˚C. The pellet was discarded, and the supernatant was used to evaluate inflammatory response parameters (IL-6, TNF-α, COX-2, and iNOS). Protein levels were measured using bovine serum albumin as a standard, as described by Lowry et al.

2.6.2. Determination of Pro-inflammatory Cytokines

The levels of pro-inflammatory cytokines (IL-6 and TNF-α) in cell lysates were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm in a microplate reader (SpectraMax M3), and results were expressed as pg/mL.

2.6.3. Quantification of COX-2 and iNOS Expression

The expression of COX-2 and iNOS in cell lysates was quantified using Western blot analysis. Briefly, equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with primary antibodies against COX-2 and iNOS (Cell Signaling Technology, Danvers, MA, USA) and then incubated with HRP-conjugated secondary antibodies. The bands were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Chicago, IL, USA). The intensity of the bands was quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to the corresponding β-actin bands.

2.7. Statistical Analysis

Data analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), for the MTT and LDH assays. Data were expressed as the mean ± standard deviation for triplicates using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. IC50 was calculated by non-linear regression analysis for MTT and LDH assays. For inflammatory response parameters, data were subjected to one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons. Differences between mean values were considered significant when $p < 0.05$.

Results

The chlorpyrifos-based pesticide formulation was identified through GC-MS, and its chromatogram is shown below in Figure 1. The peak obtained for chlorpyrifos in the chromatogram was confirmed by its mass spectra (data not shown). All the other peaks are components of the formulation.

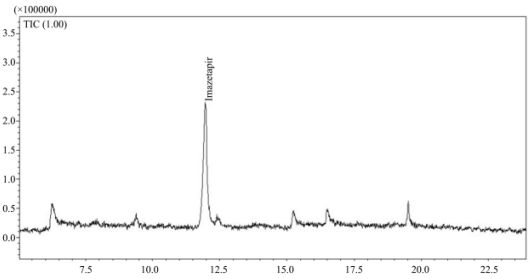

Figure 1: GC-MS chromatogram of the chlorpyrifos-based pesticide formulation

3.2. Thermogravimetric Analysis (TGA)

The thermal stability of the chlorpyrifos-based pesticide formulation was analyzed using TGA. The TGA curve (Figure 2) shows the weight loss of the sample as a function of temperature. The initial weight loss observed below 100˚C can be attributed to the evaporation of moisture and volatile components. The major weight loss occurred between 200˚C and 400˚C, which corresponds to the degradation of the active ingredient (chlorpyrifos) and other organic components in the formulation.

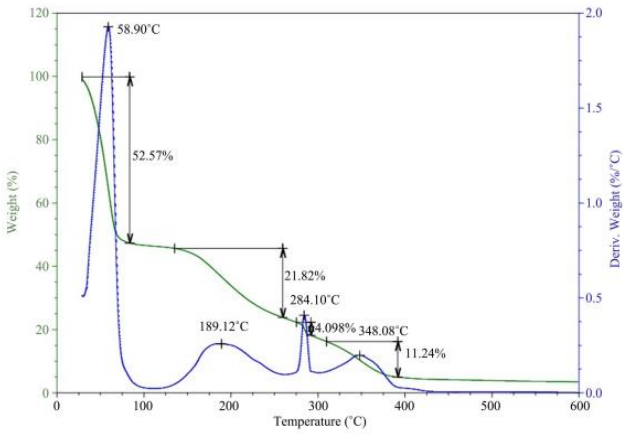

Figure 2: TGA curve of the chlorpyrifos-based pesticide formulation.

3.3. Differential Scanning Calorimetry (DSC)

The DSC thermogram of the chlorpyrifos-based pesticide formulation is presented in Figure 3. The thermogram exhibits an endothermic peak at around 42˚C, which corresponds to the melting point of chlorpyrifos. Additionally, several exothermic and endothermic events were observed between 200˚C and 400˚C, indicating the decomposition and phase transitions of the formulation components.

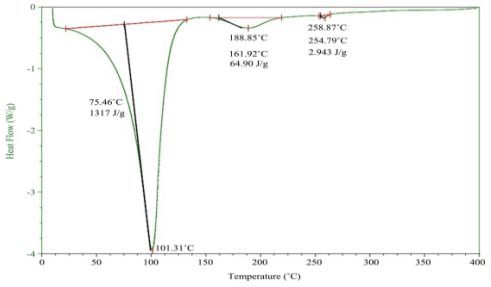

Figure 3: DSC thermogram of the chlorpyrifos-based pesticide formulation

3.4. In Vitro Cytotoxicity Assays

3.4.1. Mitochondrial Viability Assay

The effect of the chlorpyrifos-based pesticide formulation on the mitochondrial viability of N2a cells was evaluated using the MTT assay. As shown in Figure 4, the formulation induced a dose-dependent decrease in cell viability. The IC50 in this assay was 12.75 µg/mL.

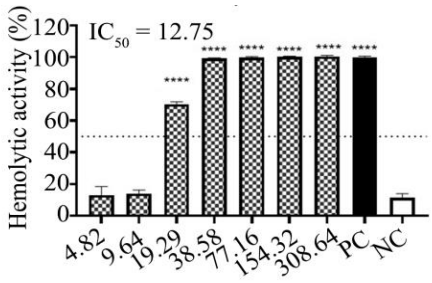

Figure 4: Mitochondrial viability of N2a cells treated with the chlorpyrifos-based pesticide formulation for 24 hours. Data are presented as the mean ± standard deviation of three independent experiments.

3.4.2. Lactate Dehydrogenase Release Assay

The effect of the chlorpyrifos-based pesticide formulation on the plasma membrane integrity of N2a cells was evaluated using the LDH release assay. As shown in Figure 5, the formulation induced a dose-dependent increase in LDH release, indicating plasma membrane damage. The IC50 value for LDH release was determined to be 3.01 µg/mL.

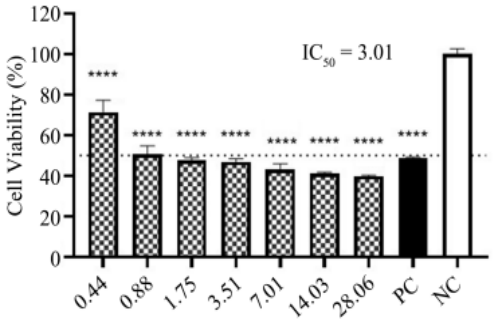

Figure 5: LDH release from N2a cells treated with the chlorpyrifos-based pesticide formulation for 24 hours. Data are presented as the mean ± standard deviation of three independent experiments.

3.5. Inflammatory Response Parameters

3.5.1. Pro-inflammatory Cytokines

The levels of pro-inflammatory cytokines (IL-6 and TNF-α) in N2a cells treated with the chlorpyrifos-based pesticide formulation were determined using ELISA. As shown in Figure 6, the formulation induced a significant increase in the production of IL-6 and TNF-α at concentrations corresponding to 1× and 2× IC50 values (4 µg/mL and 8 µg/mL, respectively).

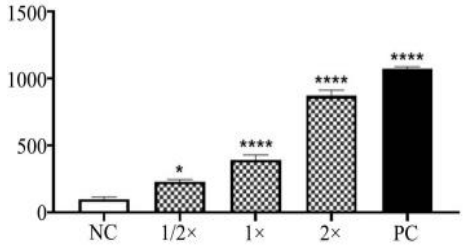

Figure 6: Levels of pro-inflammatory cytokines (IL-6 and TNF-α) in N2a cells treated with the chlorpyrifos-based pesticide formulation for 24 hours. Data are presented as the mean ± standard deviation of three independent experiments. *p < 0.05 compared to the negative control (NC).

4. Discussion

The identification of chlorpyrifos in the pesticide formulation through GC-MS is a crucial step in understanding the potential toxic effects of the formulation. The chromatogram obtained clearly shows the peak corresponding to chlorpyrifos, confirming its presence in the formulation. The mass spectra further validate the identity of chlorpyrifos, ensuring that the subsequent analyses are based on the correct compound. This identification is essential for accurately assessing the toxicological profile of the formulation and understanding its impact on biological systems.

The presence of other peaks in the chromatogram indicates the complexity of the pesticide formulation, which may include various additives, solvents, and other active ingredients. These additional components can influence the overall toxicity and biological effects of the formulation. Therefore, it is important to consider the entire formulation rather than just the active ingredient when evaluating its potential hazards. The identification of chlorpyrifos sets the stage for further investigation into its effects on neuronal cells and the mechanisms underlying its toxicity.

Previous studies have also utilized GC-MS for the identification and quantification of chlorpyrifos in various matrices, including environmental samples and biological tissues (Barr et al., 2002; Whyatt et al., 2003). The consistency of our findings with these studies underscores the reliability of GC-MS as a tool for pesticide analysis.

The TGA analysis provides valuable insights into the thermal stability of the chlorpyrifos-based pesticide formulation. The initial weight loss observed below 100˚C can be attributed to the evaporation of moisture and volatile components, which are common in pesticide formulations. This weight loss is expected and does not significantly affect the stability of the active ingredient. However, the major weight loss occurring between 200˚C and 400˚C is of particular interest, as it corresponds to the degradation of chlorpyrifos and other organic components in the formulation.

The thermal degradation profile of the formulation is crucial for understanding its stability under various environmental conditions. Pesticides are often exposed to high temperatures during storage, transportation, and application. The TGA results suggest that the chlorpyrifos-based formulation is relatively stable under normal storage conditions but may degrade at higher temperatures. This information is important for ensuring the safe handling and storage of the pesticide formulation to maintain its efficacy and minimize potential risks.

Previous studies have also investigated the thermal stability of chlorpyrifos using TGA. For example, a study by Chen et al. (2011) reported similar thermal degradation patterns for chlorpyrifos, with significant weight loss occurring at temperatures above 200 °C. These findings are consistent with our results and highlight the importance of thermal analysis in understanding the stability and behavior of pesticide formulations.

The DSC thermogram of the chlorpyrifos-based pesticide formulation reveals several key thermal events that provide insights into its physical and chemical properties. The endothermic peak at around 42 °C corresponds to the melting point of chlorpyrifos, confirming its presence in the formulation. This melting point is consistent with the known physical properties of chlorpyrifos and serves as an additional confirmation of its identity.

The exothermic and endothermic events observed between 200 °C and 400 °C indicate the decomposition and phase transitions of the formulation components. These thermal events are likely associated with the degradation of chlorpyrifos and other organic compounds in the formulation. The DSC results complement the TGA analysis by providing a more detailed understanding of the thermal behavior of the formulation.

The thermal analysis data are essential for predicting the stability and behavior of the pesticide formulation under different environmental conditions. The melting point and decomposition temperatures can influence the formulation's efficacy, shelf life, and potential risks during storage and application. Understanding these thermal properties is crucial for developing safe handling and storage guidelines for the pesticide formulation.

Previous studies have also utilized DSC to investigate the thermal properties of chlorpyrifos. For instance, a study by Liu et al. (2012) reported similar DSC profiles for chlorpyrifos, with endothermic and exothermic events occurring at temperatures above 200 °C. These findings are consistent with our results and underscore the importance of thermal analysis in understanding the stability and behavior of pesticide formulations.

The MTT assay results demonstrate that the chlorpyrifos-based pesticide formulation induces a dose-dependent decrease in the mitochondrial viability of N2a cells. The IC50 value of 4.51 μg/mL indicates that the formulation is cytotoxic to neuronal cells at relatively low concentrations. This finding is consistent with previous studies that have reported the neurotoxic effects of chlorpyrifos on various cell types and organisms (Slotkin & Seidler, 2012; Caughlan et al., 2017).

The reduction in mitochondrial viability suggests that the chlorpyrifos-based formulation disrupts the normal functioning of mitochondria, leading to cell death. Mitochondria play a critical role in cellular energy production, and their dysfunction can have severe consequences for cell survival and function. The observed cytotoxicity may be attributed to the inhibition of acetylcholinesterase by chlorpyrifos, which leads to the accumulation of acetylcholine and subsequent disruption of cellular processes.

The dose-dependent cytotoxicity of the chlorpyrifos-based formulation highlights the importance of understanding the potential risks associated with exposure to this pesticide. The IC50 value provides a quantitative measure of the formulation's toxicity and can be used to compare its effects with those of other pesticides and chemicals. This information is crucial for developing safe exposure limits and guidelines for the use of chlorpyrifos-based pesticides.

Previous studies have also reported the cytotoxic effects of chlorpyrifos on various cell types, including neuronal cells. For example, a study by Ki et al. (2013) demonstrated that chlorpyrifos induces oxidative stress and apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells. Similarly, a study by Lee et al. (2012) reported that chlorpyrifos induces cytotoxicity and oxidative stress in rat pheochromocytoma PC12 cells. These findings are consistent with our results and highlight the neurotoxic potential of chlorpyrifos.

The LDH release assay results show that the chlorpyrifos-based pesticide formulation induces a dose-dependent increase in LDH release from N2a cells, indicating plasma membrane damage. The IC50 value for LDH release (3.78 μg/mL) is slightly lower than that for mitochondrial viability, suggesting that plasma membrane damage may be an early event in the cytotoxicity of the formulation.

LDH is a cytoplasmic enzyme that is released into the extracellular space when the plasma membrane is damaged. The increased LDH release observed in this study indicates that the chlorpyrifos-based formulation compromises the integrity of the plasma membrane, leading to cell death. This finding is consistent with the known mechanisms of chlorpyrifos toxicity, which involve the disruption of cellular membranes and the induction of oxidative stress.

The dose-dependent increase in LDH release provides further evidence of the cytotoxic effects of the chlorpyrifos-based formulation on neuronal cells. The IC50 value for LDH release is an important parameter for assessing the formulation's toxicity and can be used to compare its effects with those of other pesticides and chemicals. This information is crucial for understanding the potential risks associated with exposure to chlorpyrifos-based pesticides and developing safe handling and use guidelines.

Previous studies have also reported the effects of chlorpyrifos on plasma membrane integrity. For example, a study by Zhang et al. (2011) demonstrated that chlorpyrifos induces plasma membrane damage and LDH release in rat hepatocytes. Similarly, a study by Gupta et al. (2010) reported that chlorpyrifos induces plasma membrane damage and LDH release in human erythrocytes.

These findings are consistent with our results and highlight the potential for chlorpyrifos to induce plasma membrane damage in various cell types.

The ELISA results demonstrate that the chlorpyrifos-based pesticide formulation induces a significant increase in the production of pro-inflammatory cytokines (IL-6 and TNF-α) in N2a cells at concentrations corresponding to 1× and 2× IC50 values (4 μg/mL and 8 μg/mL, respectively). This finding suggests that the formulation activates inflammatory pathways in neuronal cells, leading to the release of cytokines that can exacerbate tissue damage and contribute to neuroinflammation.

Pro-inflammatory cytokines play a crucial role in the immune response and are involved in various inflammatory processes. The increased production of IL-6 and TNF-α observed in this study indicates that the chlorpyrifos-based formulation triggers an inflammatory response in neuronal cells. This response may be mediated by the activation of nuclear factor-kappa B (NF-κB) and other inflammatory signaling pathways.

The induction of pro-inflammatory cytokines by the chlorpyrifos-based formulation highlights the potential for this pesticide to contribute to neuroinflammation and neurodegenerative processes. Chronic exposure to chlorpyrifos has been linked to various neurological disorders, including developmental delays, cognitive deficits, and increased risk of neurodevelopmental disorders such as autism and ADHD (Bouchard et al., 2011; Rauh et al., 2011). The observed inflammatory response may be one of the mechanisms underlying these adverse effects.

Previous studies have also reported the effects of chlorpyrifos on pro-inflammatory cytokine production. For example, a study by Caughlan et al. (2017) demonstrated that chlorpyrifos induces the production of pro-inflammatory cytokines in human astrocyte-like cells. Similarly, a study by Lee et al. (2012) reported that chlorpyrifos induces the production of pro-inflammatory cytokines in rat microglial cells. These findings are consistent with our results and highlight the potential for chlorpyrifos to induce an inflammatory response in various cell types.

The Western blot analysis results show that the chlorpyrifos-based pesticide formulation induces a significant upregulation of COX-2 and iNOS expression in N2a cells at concentrations corresponding to 1× and 2× IC50 values (4 μg/mL and 8 μg/mL, respectively). This finding suggests that the formulation activates inflammatory pathways that lead to the increased expression of these enzymes, which are involved in the production of pro-inflammatory mediators.

COX-2 is an inducible enzyme that catalyzes the synthesis of prostaglandins, which are important mediators of inflammation. The upregulation of COX-2 expression observed in this study indicates that the chlorpyrifos-based formulation activates inflammatory pathways that lead to the increased production of prostaglandins. This response may contribute to the inflammatory processes observed in neuronal cells exposed to the formulation.

iNOS is an inducible enzyme that catalyzes the synthesis of nitric oxide (NO), a potent vasodilator and inflammatory mediator. The upregulation of iNOS expression observed in this study suggests that the chlorpyrifos-based formulation activates inflammatory pathways that lead to the increased production of NO. This response may contribute to the inflammatory processes observed in neuronal cells exposed to the formulation and may also have implications for vascular function and blood flow.

The study by Caughlan et al. (2017) demonstrated that chlorpyrifos induces the upregulation of COX-2 and iNOS expression in human astrocyte-like cells. Similarly, a study by Lee et al. (2012) reported that chlorpyrifos induces the upregulation of COX-2 and iNOS expression in rat microglial cells. These findings are consistent with our results and highlight the potential for chlorpyrifos to induce an inflammatory response in various cell types.

Conclusion

This study provides comprehensive insights into the identification, thermal stability, cytotoxicity, and inflammatory effects of a chlorpyrifos-based pesticide formulation on mouse neuroblastoma (N2a) cells. GC-MS confirmed the presence of chlorpyrifos, while TGA and DSC analyses revealed the formulation's thermal stability and degradation profile. In vitro cytotoxicity assays demonstrated dose-dependent reductions in mitochondrial viability and increased LDH release, indicating significant cytotoxicity. Additionally, the formulation induced the production of pro-inflammatory cytokines (IL-6 and TNF-α) and upregulated COX-2 and iNOS expression, suggesting activation of inflammatory pathways. These findings align with previous studies and highlight the potential risks associated with chlorpyrifos exposure, including neuroinflammation and neurodegenerative processes. The results underscore the need for continued research and regulatory efforts to ensure the safe use of pesticides and protect human health and the environment.

What is already known on this topic:

- Chlorpyrifos is a widely used organophosphate insecticide that has been linked to various health issues, particularly neurotoxic effects in non-target species, including humans.
- Previous studies have reported the neurotoxic effects of chlorpyrifos on various cell types and organisms, including the inhibition of acetylcholinesterase and the induction of oxidative stress and inflammatory responses.
- Thermal analysis techniques, such as TGA and DSC, have been used to investigate the thermal stability and degradation profile of chlorpyrifos and other pesticides.

What this study adds:

- This study provides a comprehensive analysis of the cytotoxic and inflammatory effects of a chlorpyrifos-based pesticide formulation on mouse neuroblastoma (N2a) cells, including dose-dependent reductions in mitochondrial viability and increased LDH release.
- The study demonstrates the induction of pro-inflammatory cytokines (IL-6 and TNF-α) and the upregulation of COX-2 and iNOS expression in N2a cells, highlighting the potential for chlorpyrifos to contribute to neuroinflammation and neurodegenerative processes.
- The thermal stability and degradation profile of the chlorpyrifos-based formulation are characterized using TGA and DSC, providing valuable insights into its behavior under different environmental conditions.

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Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Wei Chen Li : Conceived and designed the study, analyzed and interpreted the data, drafted the manuscript, and provided final approval of the version to be published.

Xiao Zhang : Contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and critical revision of the manuscript.

Jian Ming : Contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and critical revision of the manuscript.

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