

Investigating the Role of Protein Kinase C Expression as an Indicator of Apoptotic Activity after Deltamethrin Exposure

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Introduction: Apoptosis, or programmed cell death, is a mechanism by which a cell can effectively kill itself when it becomes damaged or poses a threat to an organism's health. While it is a tool that cells use to prevent irreparable damage to whole tissues, uncontrolled apoptosis has been linked to neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease. The apoptotic process occurs in a series of steps, but its exact mechanism is unclear (Hossain and Richardson, 2011).

A commonly used pyrethroid pesticide, deltamethrin, has been found to play a role in inducing apoptosis. Deltamethrin is a Type II pyrethroid that targets nervous system function in insects by delaying the closing of sodium ion channels within a neuron (Johnson *et al.*, 2010). This leads to accumulation of calcium in the neuron, which activates the ER stress pathway, in turn, denaturing proteins. The denaturation of these proteins likely contributes to neurodegenerative diseases, as protein unfolding phenomena have been observed as part of multiple cases of neurodegeneration (Hossain and Richardson, 2011).

There have been no chronic health effects attributed to deltamethrin in humans. It is less toxic to mammals than to insects, due to larger size, higher core body temperatures, and decreased ion channel sensitivity. As a Type II pyrethroid, deltamethrin is speculated to cause long-term effects on ion channels other than the sodium channel (Johnson *et al.*, 2010).

Caspases are a family of proteins that have various roles in the apoptosis cascade, with caspase-3 being the most widely studied. Caspase-3 is a major player in apoptosis, acting as a final effector protein in the apoptosis-inducing signaling cascade (Hossain and Richardson, 2011). Other proteins involved in apoptosis include Bcl-2, an anti-apoptotic protein, and protein kinase C (PKC), the different isoforms of which play different roles in cell death (Day *et al.*, 2009; Hardin *et al.*, 2016). In the apoptosis-inducing cascade, PKC lies upstream of the effector caspase-3. The isoforms of PKC associated with inducing apoptosis are PKC- α and PKC- β while isoforms PKC- ζ and PKC- δ are linked to anti-apoptotic processes (Knox *et al.*, 1993; Newton, 1995; Pongracz *et al.*, 2002).

Previous studies have identified deltamethrin-concentration-dependent increases in caspases associated with apoptotic processes (Hossain and Richardson, 2011). It is unknown how caspases are activated in the apoptosis-inducing pathway after deltamethrin exposure, but it may be due to activation of an upstream protein.

This study was conducted to determine if PKC plays a role in activating caspases after deltamethrin exposure. To do so, SH-SY5Y cells were exposed to different concentrations of deltamethrin for 24 and 48 hours, and the concentration of PKC- α , β , and γ were determined. The hypothesis stated that PKC concentrations would increase with time and with concentration of deltamethrin.

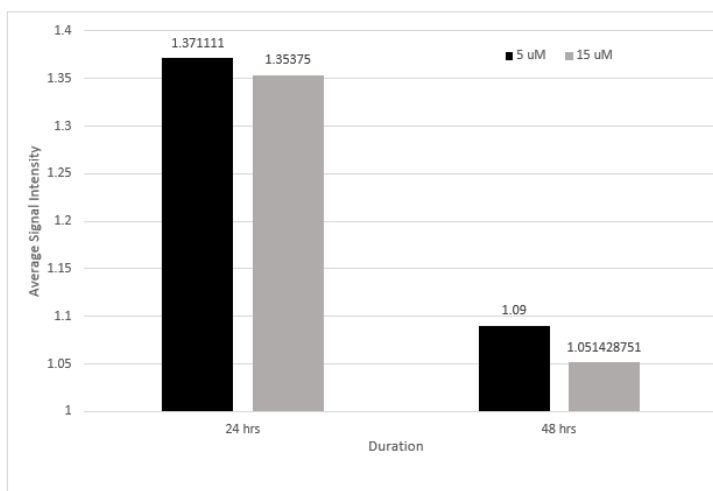
Materials & Methods: Neuroblastoma cells (SH-SY5Y, passages 15-19) were grown in HAM's F12 media with 10% fetal bovine serum (FBS) and incubated at 37 °C with 5% CO₂. Media was replaced every three days until cells reached 80% confluency, when they were differentiated using 10⁻⁷ M retinoic acid. After three days, cells were treated with 5 μ M or 15 μ M deltamethrin or exposed to 0.02% ethanol in media as a control. Cells were incubated for 24 and 48 hours, then lifted with 0.25% trypsin-EDTA, and lysed with lysis buffer (50 μ L 100 mM NaF, 25 μ L 200 mM Na₃VO₃, and 25 μ L 1:200 Protease Inhibitor Cocktail III to 5 mL RIPA Lysis Buffer). The lysate was frozen at -20 °C. A total of three replicates was conducted.

To determine the total concentration of protein in each lysate sample, a standard BCA protein assay was generated. The lysate samples were then diluted 1:1 using sample buffer, boiled for 5 minutes, then placed on ice. A total of 20 μ g of protein was loaded in each well for gel electrophoresis in 12% SDS-polyacrylamide gels. The gel, a nitrocellulose membrane, and filter papers were equilibrated in transfer buffer for 10 minutes, and proteins were transferred from the gel to the membranes. The nitrocellulose membranes were rinsed in 1X TBS for two minutes on a rocker at room temperature. The TBS was discarded, and the membranes were blocked in TBS Odyssey Blocking Buffer on a rocker for one hour at room temperature. The membranes were incubated overnight at 4°C with an anti-

PKC antibody (1:1000, Abcam) in blocking buffer. Membranes were brought to room temperature, then rinsed three times in 0.1% TBST for five minutes. The membranes were then incubated with light-sensitive secondary antibody (1:10,000, Abcam) in Blocking Buffer at room temperature on a rocker for one hour. The membranes were washed in 0.1% TBST three times for five minutes each, and finally, rinsed for five minutes in 1X TBS. Two pieces of filter paper were used to cover the light sensitive membranes as they dried. They were placed in the Fc Odyssey (LiCor) machine, which detected the protein signal. A two-way ANOVA was used to analyze the data (Jamovi).

Results: The BCA assay detected protein in all samples. The average signal intensities of PKC after 24 hours were 1.371 (± 0.551) for 5 μ M deltamethrin and 1.354 \pm (0.762) for 15 μ M deltamethrin. After 48 hours, the average signal intensities were 1.09 \pm (0.116) for 5 μ M and 1.051 \pm (0.344) for 15 μ M. While there was a trend of decreased PKC concentration from 24 to 48 hours, and the 15 μ M deltamethrin treatment had a consistently lower signal compared to the 5 μ M deltamethrin treatment, the statistical analysis of the data showed no significant difference in treatment ($p=0.28$) or duration of treatment ($p=0.13$). There was no interaction between these values ($p = 0.55$) (Figure 1).

Figure 1.



Discussion: The hypothesis of a time- and dose- dependent increase of PKC concentration in response to deltamethrin exposure in SH-SY5Y cells was rejected. The data indicate that the changes in PKC concentration observed between treatments and time were not significant. This implies that PKC itself is not an effector in the deltamethrin-induced apoptosis cascade, thereby eliminating it as a subject from similar experiments in future.

Although PKC is not an option for further study, there are other proteins involved in apoptosis that could be

investigated to determine the mechanism for induced cell death due to deltamethrin exposure. According to Shi (2004), only the expression of caspase-9 has been studied. The activation of caspases necessitates the cleavage of their inactive zymogen forms. Caspase-9 has catalytic activity that is moderate in its zymogen form, and only somewhat more active in its cleaved form (Shi, 2004).

Cleavage of caspase-9 occurs when it binds an apoptotic protease activating factor, Apaf-1. In the presence of ATP/dATP, Apaf-1 recruits caspase-9 to form a complex known as the apoptosome. Once bound to Apaf-1, Caspase-9 can become active and then activates caspase-3, one of the effector caspases in the apoptotic cascade (Danial and Korsmeyer, 2004). Therefore, Apaf-1 is a potential protein target for future studies with deltamethrin, as it plays an essential role in the ultimate activation of caspase-3 (Brentnall *et al.*, 2013).

Not only could future studies focus on other proteins such as Apaf-1, but they may also include different pesticides. Deltamethrin is a Type II pyrethroid used as an insecticide that targets the nervous system (Chi *et al.*, 2013). Other classes of pyrethroids include the Type I pyrethroid Metofluthrin, a newer insecticide, which may have carcinogenic effects due to genotoxicity (Deguchi *et al.*, 2009). Although Type I and Type II pyrethroids have different mechanisms of action, they are both pyrethroids. Future studies could examine the effects on concentration of Apaf-1, PKC, or various caspases after treatment with Metofluthrin, a representative Type I pyrethroid.

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