Dysfunctions in Alzheimer's Dementia Hallmarks with Pyrethroids and Piperonyl Butoxide Pesticide Synergy

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ABSTRACT: There are over 5 million Americans with Alzheimer's dementia (AD). A positive correlation between chronic pesticide exposure and AD prevalence. This study's goal is to elucidate the toxicity of synergistic pesticides pyrethroid (PY) and piperonyl butoxide (PBO) on the induction of cytotoxicity, tau phosphorylation, and amyloid-ß (Aß). An MTT assay measured cell viability and cytotoxicity. A lactate dehydrogenase (LDH) assay determined extracellular LDH and an ELISA determined Aß and tau protein expression. Exposure to PY and PBO synergistically reduced cell viability greater than PY and PBO individually. Exposure to synergistic PY and PBO increased LDH cytotoxicity in a time-dependent manner. PY and PBO synergistically increased AB and tau protein expression, indicating low concentrations and long-term treatment were capable of inducing AB and tau protein expression. PY and PBO synergistically induced multiple hallmarks of AD, suggesting chronic low concentration exposure to pesticides is likely correlated with AD development. Future investigations should focus on the pathophysiological mechanisms of PY and PBO's induction on AD hallmark protein expression. Researching these effects will improve the safety and health of pesticide users.

KEYWORDS: Neurodegenerative Diseases; Alzheimer's Disease; Pyrethroid; Piperonyl Butoxide; Amyloid-Neurodegenerative Diseases; Alzheimer's Disease; Pyrethroid; Piperonyl Butoxide; Amyloid-ß;;

Introduction. There are currently 5.8 million Americans with Alzheimer's dementia (AD). By 2050, over 14 million Americans will be affected by AD, according to the Alzheimer's Association1. It is estimated that 95% of Alzheimer's patients suffer from late-onset Alzheimer's dementia (LOAD). LOAD is characterized by developing AD at age 65 or older. While LOAD is the most common form of AD, there are no known causes2. Risk factors for LOAD include both genetic and environmental factors; the main genetic factor identified is the Apolipoprotein E gene[3]. This study aims to research pesticides as a main environmental factor in the development of AD.

Annually, 5.6 billion pounds of pesticides are used worldwide, with 1 billion pounds used in the United States alone[4]. One popular agricultural pesticide is pyrethroid (PY), a synthetic neurotoxic insecticide developed from pyrethrin found in chrysanthemums[6]. PYs are considered nontoxic to large mammals but recent studies have shown it is high toxic to mice and human cell lines[7,8]. Meta-analysis data has demonstrated a positive association between chronic pesticide exposure and AD prevalence[9]. PY pesticides are known to induce oxidative stress, acetylcholinesterase, apoptosis, and mitochondrial dysfunction, all which mirror hallmarks of AD. Thus, pyrethroid presence in neurons is questioned as a risk factor of AD[10,11]. PY disrupts proper voltage gated sodium channel (VGSC) kinetics and prolongs depolarization of the channel[12]. PY can also act upon a voltage gated calcium channel to change its kinetics channel and cause an influx of calcium ions[13]. This imbalance of calcium ions in mitochondria can cause reactive oxidative species and cell death associated with AD neurological dysfunctions. Since PY can be metabolized quickly by esterases and cytochrome p450, PY is applied with piperonyl butoxide (PBO), a chemical synergist. PBO inhibits cytochrome p450 function to increase PY toxicity[5]. In this study, a combination of PY and PBO were used synergistically to mimic the common application of PY pesticides[5,14.]

The purpose of this study is to examine the cytotoxicity of PY and PBO on dysfunctions to AD hallmark proteins. Aß and tau phosphorylation were the two main hallmarks studied. Previous studies have shown both Aß and tau-phosphorylation to be associated with the glycogen synthase kinase pathway can be activated with PY[15].

Results and Discussion. Cytotoxicity of PY and PBO.

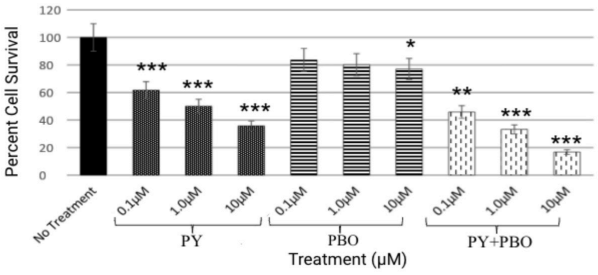

Figure [1]. The effect of PY and PBO on human neuroblastoma cell survival. MTT assay was performed [72] hours after initial treatment of PY, PBO, or a combination of PY and PBO. * = $p<^{0.05}$; ** = $p<^{0.005}$; *** = $p<^{0.001}$. Data is presented as mean $^{+}$_ SEM from eight samples.

DOI: 10.36838/v2i2.10

In eight replicative experiments, SK-N-SH human neuroblastoma cells were treated with increasing concentrations of PY, PBO, or in combination at 0.1 μM, 1.0μM, and 10μM for 72 hours prior to performing an MTT assay to determine cell viability.

The synergistic relationship between PY and PBO was present, indicating their synergistic ability functions in human neuroblastoma cells at low concentrations of 0.1μM treatment of PY and PBO. Cell survival percent at 0.1 μM of PY+PBO was 35.39%; individual treatments of PY and PBO had cell survival rates of 47.80% and 79.16%, respectively (Figure 1; $p<0.05$). Even at low concentrations of 0.1μM, PY+PBO still produced significant results, indicating its synergistic neurotoxicity could have implications in AD neurological hallmarks. All the concentrations applied to the cells demonstrated that PBO+PY reduced cell survival compared to individual treatments. The addition of PBO to PY increased the cytotoxic ability of the two chemicals thus causing less cell viability.

LDH Assay. In eight replicate experiments, SK-N-SH human neuroblastoma cells were treated with increasing concentrations of PY, PBO, or in combination at 0.1 μM, 1.0μM, and 10μM for 72 hours before an LDH assay was performed to determine cytotoxicity.

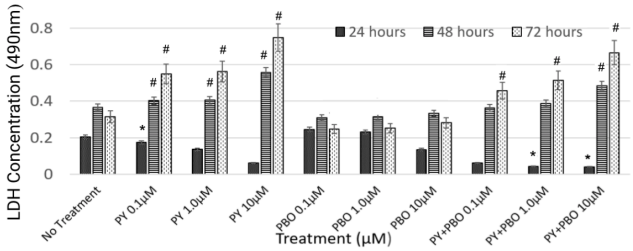

Figure 2. Cytotoxicity of PY and PBO in a Time Dependent Manner. Human neuroblastoma cells were treated with various concentrations of PY and PBO. Statistical analysis was performed in comparison to no treatment groups. * = $p<0.05$; # = $p<0.005$. Data is presented as mean ± SEM from eight samples.

A time- and dose-dependent relationship was established between PY and PY+PBO. The synergistic ability of PY+PBO is not shown by the LDH assay, but rather in the PY which produced significantly greater cytotoxicity than PY+PBO. This suggests that PBO may have a neuroprotective role in mitochondrial related pathways. This is supported by the individual PBO treatment at 48 and 72 hours where the LDH concentrations released by the neuroblastoma cells was less than the overall LDH released by neuroblastoma cells receiving no treatment (Fig 2; $p<0.05$). The lack of PBO effect on LDH concentrations may be due to PBO's unique characteristic to act as both an inhibitor and inducer of cytochrome p450. The result of this is that PBO inhibits cytochrome p450 function thus maintaining the LDH concentration. Further experiments into PBO reactions is needed to determine the significant role of PBO in combination with PY. Nevertheless, individual PY produced significant cytotoxicity at low concentrations of 0.1μM, indicating its toxicity has great consequences in mitochondrial function associated with AD mitochondrial dysfunction.

Neuronal Death. A Trypan Blue Exclusion Assay was performed 72 post treatment of human neuroblastoma cells with 0.1μM, 1.0μM, and 10.0μM of PY and/or PBO to determine neuronal death count.

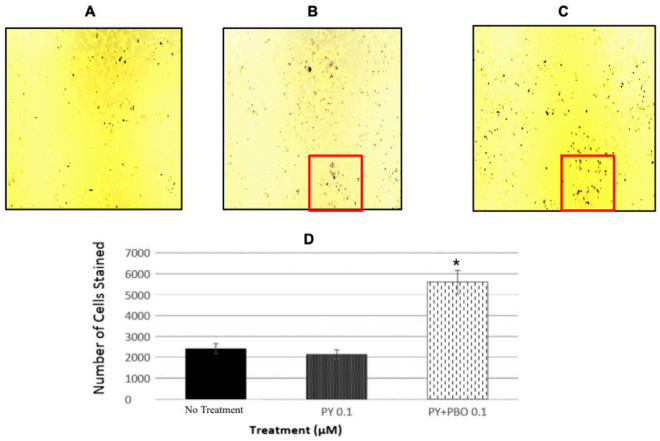

Figure 3. Neuronal death caused by long–term PY and PBO treatment. A) Control. B) PY; 0.1μM. C) PY and PBO; 0.1μM. D) Quantified human neuroblastoma cells 72 hours post Trypan Blue Exclusion Assay to determine cellular death via staining with Trypan Blue. * = $p<0.005$. Data is presented as mean ± SEM from twelve samples.

Neuronal death was greater with PY+PBO than the individual treatment of PY. The synergy of PY+PBO is cytotoxic and most likely causes oxidative stress to produce cell death. This could indicate PY+PBO induces apoptosis via oxidative stress. The neuroblastoma cells without PY and/or PBO treatment had cell death that could be from the naturally occurring death of cells. Additionally, neuronal death (a less specific hallmark of AD) caused by PY+PBO could be associated with AD cellular hallmark development. PBO was not tested in this experiment because of previous results that determined PBO has no significant effects on cell viability at 0.1μM and 1.0 μM.

Aß ELISA. An ELISA was performed 72 hours after human neuroblastoma cell treatment with 0.1μM, 1.0μM, or 10μM of PY and/or PBO to determine dose-dependent Aß concentration with the synergy of PY+ PBO and Aß induction level on neuroblastoma cells.

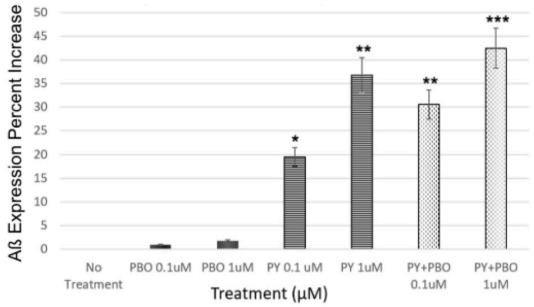

Figure 4. ELISA of PY and PBO on Aß protein concentration. * = $p<0.05$; ** = $p<.005$; *** = $p<0.001$. Data is presented as mean ± SEM from four repeated experiments.

Individual treatment with 0.1μM and 1.0μM PBO induced little to no Aß expression. However, PY individual treatment induced a 19.89% and 36.72% increase in Aß expression, respectively. The synergistic effect of PBO and PY significantly increased Aß expression to 30.47% and 42.31% at 0.1μM and 1μM, respectively, supporting the hypothesis that pesticides are able to induce Aß production (Fig. 4; $p<0.05$). Low concentration and chronic synergistic pyrethroid exposure are demonstrated as a contributing factor to the development of AD hallmarks. Furthermore, the increase in Aß expression could be caused by increased oxidative stress that PY and PBO induce, or PY+PBO stimulation of key enzymes in Aß production such as ß-secretase.

Tau ELISA. An ELISA was performed 72 hours after human neuroblastoma cell treatment with 0.1μM, 1.0μM, or 10μM of PY and/or PBO to determine dose-dependent tau protein concentration in treatments with a combination PY and PBO on neuroblastoma cells.

Individual treatment with 1.0μM PY and PBO resulted in an 18.62% and 10.47% respective increase compared to the no treatment group, indicating that long-term low concentration exposure to neuroblastoma cells elevates tau protein levels. PBO's influence on tau protein expression is significant as it suggests PBO cytotoxicity could cause tau expression. Since PBO does not significantly change cell viability but does significantly increase tau protein levels it is possible that, like tau protein in AD, PBO does not result in immediate neuron death but a gradual decline in neurons. The synergistic ability of PY and PBO caused significantly more tau protein expression compared to both the control group and the cells with treated individually with PY or PBO. At 1.0μM, tau protein expression increased by 27.81%, suggesting that at chronic low concentration exposure to PY+ PBO causes dysfunctions in the tau protein pathology (Fig. 5; $p<0.05$).

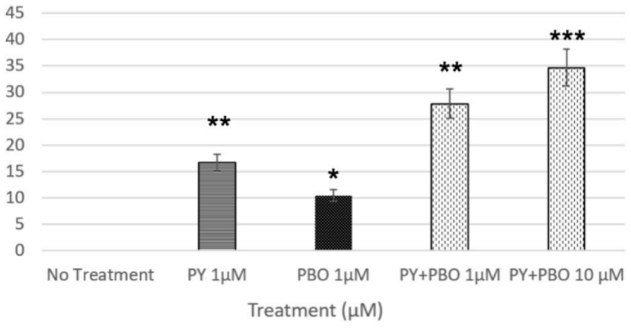

Figure 5. ELISA of PY and PBO on tau protein expression. Human neuroblastoma cells were treated with various concentration of PY and PBO for 72 hours before an ELISA was performed to determine tau phosphorylated protein concentration. * = $p<0.05$; ** = $p<.005$; *** = $p<0.001$. Data is presented as mean +_SEM from eight samples.

Conclusion. PY and PBO both demonstrated consistent cytotoxic synergistic effects on human neuroblastoma cells. Their combines neurotoxic effects indicate their potential as contributing factors to AD development. Aß protein expression, tau protein expression, and cellular toxicity all increased after a 72-hour treatment of PY and PBO. This reveals PY and PBO's potential influence on AD neurological hallmark development as well as the exposure to low concentrations of 0.1μM of PY+PBO can cause cytotoxicity associated with neuronal death and decline. The increase in Aß and tau protein demonstrates that PBO and PY have the potential to contribute to the pathophysiological formation of AD. Aß increase can be induced by oxidative stress and increases in reactive oxidative species like superoxides or hydrogen peroxides16. Pyrethroid exposure to mitochondria reduces superoxide dismutase (SOD), a key antioxidant enzyme that reduces superoxides into oxygens. A reduction in SOD leads to a buildup of unmetabolized toxic superoxide molecules that increase oxidative stress17. Cytotoxicity and oxidative stress could result from the prolonged depolarization period caused by PY targeting VGSC. Cytotoxicity in low dosages of these pesticides may also lead to neuronal loss which can be contributed to other neurodegenerative diseases. Calculating the average amount of pesticides that an individual is exposed to is difficult, but the concentrations would be similar to the low concentrations used in the experiments.

Methods.Chemicals and Reagent Preparation. Pyrethroid extract, 2-(2-Butoxyethoxy) ethyl (6-propylpiperonyl) ether (PBO), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Sigma-Aldrich. Pyrethroid solutions were created using serial dilutions of 10μM, 1.0μM, and 0.1μM using ethanol concentrations. PBO solutions were created using serial dilutions of 10μM, 1.0μM, and 0.1μM using Human Tau ELISA kit and Amyloid-ß 42 Human ELISA Kit purchased from Invitrogen. All chemicals purchased were of analytical or technical grade.

Cell Culture. SK-N-SH HTB-11 human neuroblastoma cells were obtained from the American Type Culture Collection. Neuroblastoma cells were cultured in a humidified 5% CO2 atmosphere at 37 ˚C in MEM media.

MTT Assay. An MTT assay was conducted to measure the induction of PY and PBO on SK-N-SH human neuroblastoma cell. The cell cultures were treated with 0.1mM, 1.0 μM, or 10.0 μM of either individual PY or PBO or synergistically treated with PY and PBO. Samples were incubated for 72 hours to mimic the chronic low concentration pesticide exposure in agricultural settings. After incubation, excess MTT was removed and 50μL of DMSO was pipetted into each well to solubilize the cells. Absorbance was measured at 490 nm using a BioRad iMark™ Microplate Absorbance Reader.

Lactate Dehydrogenase Assay. Lactate dehydrogenase (LDH) measured LDH concentration based on a coupled enzyme reaction. LDH assay kit was purchased from ThermoFisher Scientific. LDH catalyzes the reaction of lactate to pyruvate by reducing NAD+ to NADH. NADH is used by diaphorase to reduce tetrazolium salt to a red formazan product which can be measured spectrophotomically and is directly proportional to LDH concentration. Cells were treated with 0.1μM, 1.0μM, and 10.0μM, of PY, PBO, or PY+PBO for 72 hours before LDH assay was performed. Red formazan product was measured with a BioRad iMark™ Microplate Absorbance Reader at 490nm and 680nm.

Trypan Blue Exclusion Assay.Cell viability and cellular death were measured using 0.4% Trypan Blue Solution. After PY and/

DOI: 10.36838/v2i2.10

or PBO treatment for 72 hours, SK-N-SH human neuroblastoma cells were treated with Trypan Blue. Viable cells have membranes that are impermeable to the Trypan Blue. Images of each well were taken immediately after straining using a light microscope. ICTN plugin within ImageJ was used to calculate the number of viable cells.

Enzyme-linked immunosorbent assay (ELISA) .Aß protein expression was measured using ELISA conducted according to the manufacturer's manual. After ELISA was performed, the Bio-Rad iMark Microplate Absorbance Reader was set to 450 nm. Tau protein expression was also measured using an ELISA conducted according to the manual. All ELISA procedures were performed 72 hours after human neuron models were treated with 0.1μM, 1.0μM, and 10.0μM, of PY, PBO, or PY+PBO.

Data Analysis. One-way ANOVA was used to determine statistical significance where all experiments were repeated three times with $p<0.05$ as the statistical significance. The statistical significance of the data was in comparison with cells that received no treatment.

Acknowledgements. I want to thank my mentor, Dr. Wei Zhu, for his support and guidance during this research process, especially his continued encouragement to find opportunities to present my research. I would also like to thank my teacher, Dr. Serena McCalla, for being a constant source of inspiration and motivation.

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