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Investigation of Paraxon-induced PC12 Cells Viability, Cytotoxicity, and Apoptosis in the Presence of Recombinant Butyrylcholinesterase (BCHE)

Morteza Mirzaei[1,2], Hamid Tebyanian[3,4], Saeed Izadpanah[2], Rezvan Seid Moradi[2], Mona Rastegar Shariat Panahi[2], Mehrdad Moosazadeh Moghaddam[2], Ali Mohammad Latifi[2*]

[1] Department of Biology, Faculty of Sciences, University of Guilan, University Campus 2, Rasht, Iran
[2] Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[3] School of Dentistry, Baqiyatallah University of Medical Sciences, Tehran, Iran
[4] Research Center for Prevention of Oral and Dental Diseases, Baqiyatallah University of Medical Sciences, Tehran, Iran

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Abstract

Today, the use of pesticides is an essential issue for health control. Organophosphorus insecticides are considered the most widely used pesticides in the world and as they have the potential to eliminate pests, they can endanger human health by inhibiting acetylcholinesterase family enzymes. In this research, we aimed to study the effects of paraxon, the most important metabolite of organophosphorus insecticide partition, on the viability, cytotoxicity, and apoptosis occurrence in the presence of butyrylcholinesterase (BCHE). In this study, the effects of the viability, cytotoxicity, and apoptosis of paraxon-induced PC12 cells were investigated in the presence of butyrylcholinesterase (BCHE). The cell viability was assessed using the MTT test. The cell cytotoxicity was calculated through Lactate Dehydrogenase (LDH) test. The caspase activity was used to measurement of caspase-3 activity. Mitochondrial membrane potential (MMP) was studied by rhodamine123 and also, TUNEL staining was performed to quantification of apoptosis index. Paraxon induced cell cytotoxicity and decrease cell viability of PC12 cells and BCHE inhibited the paraxon function in a dose-dependent manner. Paraxon disrupted the MMP and increased the function of caspase but BCHE suppressed paraxon-induced apoptosis through inhibition of paraxon function. Treatment of PC12 cells by paraxon increased cell death index. BCHE can use in the treatment of poisoning with organophosphorus insecticides because of its potential to suppress paraxon-induced cell death. BCHE inhibits the paraxon function in a dose-dependent manner.

Keywords: PC12 cells, Viability, Cytotoxicity, Apoptosis, Butyrylcholinesterase

1 Introduction

Pesticides are chemical compounds or biological agents that are commonly used in agriculture and other modern industries to remove the pests such as insects, plants, bacteria, and fungus from human life (1-3). Insecticides have a straight effect on human health and inhibit the growth of disease-causing agents, but to be honest, the dangerous effects of these insecticides cannot be ignored (4, 5). A group of insecticides called organophosphorus is among the pesticides that have harmful effects on human health and the environment. Therefore, the use of these pesticides should be consistent with previous knowledge of their toxicity, function, and optimal use conditions. Choline esterases are organophosphorus pesticide targets (6, 7). Organophosphorus suppress the function of choline esterase which leads to aggregation of unhydrolyzed acetylcholine in the human body and results in toxicity and poisoning (8, 9). These compounds are lipophilic and store in adipose tissues. The release of these compounds leads to the induction of toxicity in patients (10, 11). Malathion, Demeton, Diazinon, Dichlorvos, Gusathion, and Parathion are the most important organophosphorus insecticides (12, 13). There is sufficient evidence that reveals parathion have a carcinogenic effect on animals but Carcinogenicity of parathion in human is unclear (14).

Parathion, as a cholinesterase inhibitor, absorbs through the skin, mucous membrane, and moth and interrupts the nervous system. Paraxone is the principal metabolite of parathion in the body and may lead to many symptoms such as convulsions, abdominal pain, diarrhea, lung edema, and respiratory arrest (15). Therefore, paraxone is a reliable choice for scientists to study the toxicity of organophosphates (16, 17). In this study, we aim to investigate the power of paraxon in the inhibition of Butyrylcholinesterase (BCHE). Therefore, we treated PC12 cells, neuron-like cell line, with BCHE and exposed these cells to different concentrations of paraxon.

***Corresponding author:** Ali Mohammad Latifi, Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran, E-Mail: amlatifi290@gmail.com

2 Materials and Methods

2.1 Cell Culture

In this study, PC12 cells were selected because of their stimulatory to nerve cells. We were grown the PC12cells in DMEM culture media (Gibco) which exposed to below supplementary compounds: fetal bovine serum (FBS, Gibco) 10%, non-essential amino acid (NEAA, Sigma) 1%, L-glutamine (Sigma) 2mM, penicillin (Sigma) 100IU/ml, and streptomycin (Sigma) 100µg/ml and kept these materials in T-25 cm2 tissue culture flasks. Then, we incubated the cells at 37 ºC in 5% CO2 and trypsinated the cells by trypsin-EDTA 0.25% (Sigma) at the time of 70 to 80% of confluency of the cell cultures. Finally, the cells were sub-cultured at a density of 1×104 cells/well in 24-well culture plates.

2.2 Cell Treatment

For cell treatment, 12h after plating, we cultured the cells in 96-well media by the concentration of 1* 104 cell/ml. Then, we washed the cells by PBS and treated the cells three groups. 1: control group without BCHE and paraxon. 2: cell cultured treated by different concentrations of paraxon (1,10,20,50,100,200,500 and 1000µm) 3: cell cultured treated by different concentrations of paraxon (1,10,20,50,100,200,500 and 1000µm) and BCHE (1,10,20,50,100,200,500 and 1000µm).

2.3 Cell Viability (%) (MTT assay)

In this study, the viability of the cells by MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. In this regard, we loaded 10×103 cells into a 96-well plate. Then 200µL of DMEM media was added to the wells. DMEA contained 0.2% BSA. 200µL of treatments were added to the wells after 24h and incubated for 24h. So, after removing the wells supernatants, the cells were incubated in 50µL (5 mg/mL) of MTT solution for 3h. For formazan crystals to dissolve, 100µL of dimethyl sulfoxide was added to each well after removing the supernatant. 30m later formazan crystals were dissolves and we measured the optical density of the wells by an enzyme-linked immunosorbent assay (ELISA) reader. The optical density was observed at 570 and 630 nm. The following formula calculated the viability of the cells:

Cell viability (%) = (A570, 630(sample)/A (control)) × 100

2.4 Cell Cytotoxicity

At first, 104 cells were cultured in 24 well cultures for 12h. In this study, lactate dehydrogenase (LDH) release, which indicates the cell disruption or damage, was the basis of the quantification of cell cytotoxicity. Cytotoxicity of the cells was measured using the LDH Cytotoxicity Detection Kit (Roche, Germany) based on the protocol of the company. The absorbance of samples at 490 or 492nm revealed the activity of LDH. In this regard, an ELISA Reader (EL800; USA) was used by more than 600nm reference wavelength. We replicated the experiments at least three times and repeated each condition four times. The following formula calculated the cells cytotoxicity:

Cell cytotoxicity (%) = (A490, (sample)/A (control)) × 100

2.5 Caspase-3 Assay

The caspase activity colorimetric assay kit (Bio-techne) was used to measurement of the caspase-3 activity of treated cells. In this regard, we used a plate reader through the manufacturer's protocol. We repeated the experiments at least twice.

2.6 Detection of mitochondrial membrane potential (MMP)

PC12 cells, 3×104 cells/well, were cultured in different treatments for 24h and washed by PBS. Then, the cells were incubated in darkness by 1 µM of rhodamine 123, a cell-permeable cationic fluorescence probe, for 30min at 37°C. An ELISA Reader, 488nm excitation and 525nm emission, measured, the mitochondrial membrane potential by reference wavelength of more than 630nm. All experiments were replicated three times and all conditions four times.

2.7 Quantification of apoptosis incidence

In this study, we fixed the cell in PBS which contained 4% w/v paraformaldehyde at pH =7.4 and room temperature for 10 min. Terminal Uridine deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) staining was performed to quantification of apoptosis index. In this regard, an in situ cell death detection kit (Roche) was used based on the manufacturer's protocol. Eight fields were selected randomly under a fluorescent microscope (Olympus AX-70) for counting the TUNEL positive cells. The apoptotic incidence (index) was calculated by dividing the number of apoptotic cells by total cells.

2.8 Data analysis

Data and results were analyzed by horse software. Post hoc Tukey test was used to compare differences between treatment and control groups. The one-way ANOVA analysis was used to compare the differences between groups. Charts were plotted by the excel software.

3 Results

3.1 Assessment of BCHE activity

Investigation of BCHE activity in paraxon-treated PC12 cells revealed that paraxon inhibited the BCHE activity in competitive models. As the concentration of paraxon increases activity of BCHE decreases significantly. A high concentration of paraxone completely inhibits the activity of BCHE and high concentrations of BCHE need higher concentrations of paraxon for complete inhibition (Figure 1).

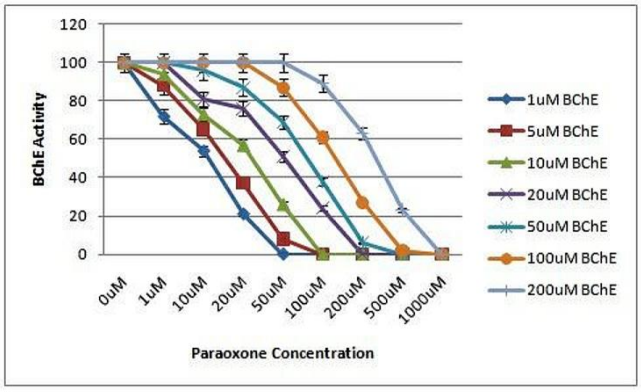

Figure 1: Activity of different concentrations of BCHE in the presence of different concentrations of paraoxon

3.2 Cell viability

MTT test showed us that an increase of paraxon concentration significantly leads to a decrease of cell viability in PC12 cells which were treated by different concentrations of paraxon (Figure 2A). MTT test showed the viability of the cells in the presence of different concentrations of paraxon and BCHE enzyme. The viability of control cells, which were not treated by paraxon, was close to 100%. But, as the paraxon was added to the cells, cell viability significantly decreased. An increase of paraxon resulted in a powerful decrease in PC12 cell viability. A high concentration of paraxon leads to 0% cell viability (Pv≤0.05). There was a significant difference between the cell viability of control and paraxon-treated PC12 cells (Figure 2B).

3.3 Cell cytotoxicity

Cell cytotoxicity of control cells, which were not treated by paraxon, was about 100% but cytotoxicity of PC12 cells was significantly increased through the increase of paraxon concentration. It showed us that there is a significant difference between cytotoxicity of control and paraxon-treated cells. It means that paraxon induces cytotoxicity in PC12 cells (Pv≤0.05) (Figure 3A) LDH test revealed that the BCHE enzyme significantly suppresses the paraxon-induced cytotoxicity. Paraxon induces cytotoxicity in PC12 cells. An increase in paraxon leads to a significant increase in PC12 cell cytotoxicity. BCHE enzyme in all concentrations inhibited the paraxon-induced cell cytotoxicity. An increase of BCHE enzyme leads to high-performance cell cytotoxicity inhibition (Pv≤0.05). Because of competitive inhibition, a high concentration of paraxon leads to uninhabitable cell cytotoxicity in PC12 cells (Figure 3B).

3.4 Cell death index

Comparison of paraxon-treated PC12 cells and control PC12 cells, which contained no paraxon, showed us that paraxon induced cell death significantly. The results of the TUNEL test revealed that an increase of paraxon leads to enhancement of cell death induction (Pv≤0.05). There is a significant difference between the cell death index of the control group and paraxon-treated PC12 cells (Figure 4A). BCHE enzyme, in all studied concentrations, suppressed the paraxon-induced cell death significantly. An increase of BCHE enzyme led to a decrease in cell death in paraxon-treated PC12 cells (Pv≤0.05). BCHE enzyme cannot suppress cell death in high concentrations of paraxon because of competitive inhibition (Figure 4B).

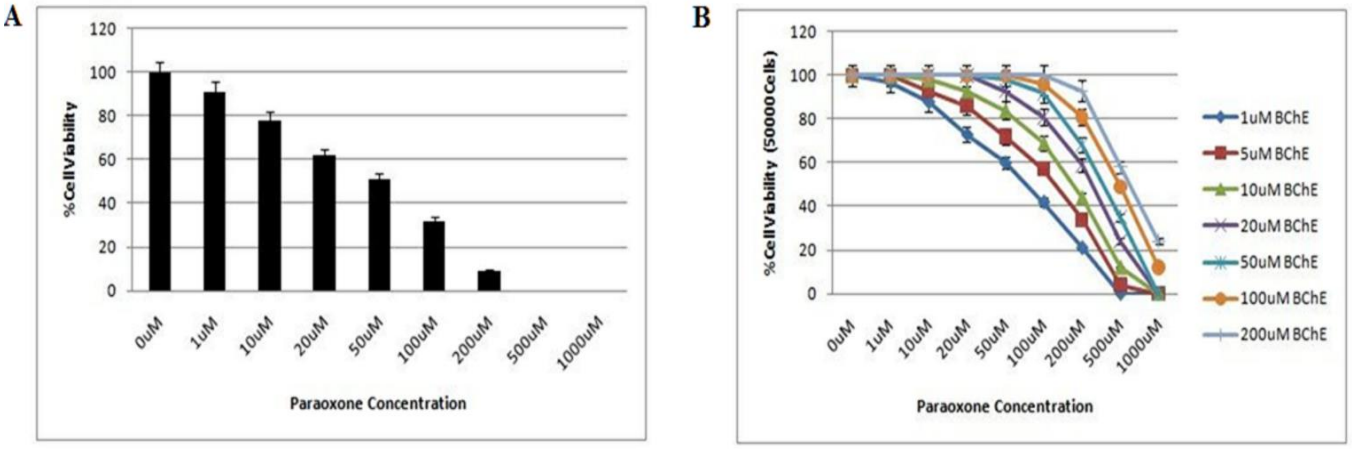

Figure 2: MTT test. The cell viability of PC12 cells in different treatments of paraxon (A); MTT test. Viability of the PC12 cells in the presence of different concentrations of paraxon and BCHE (B)

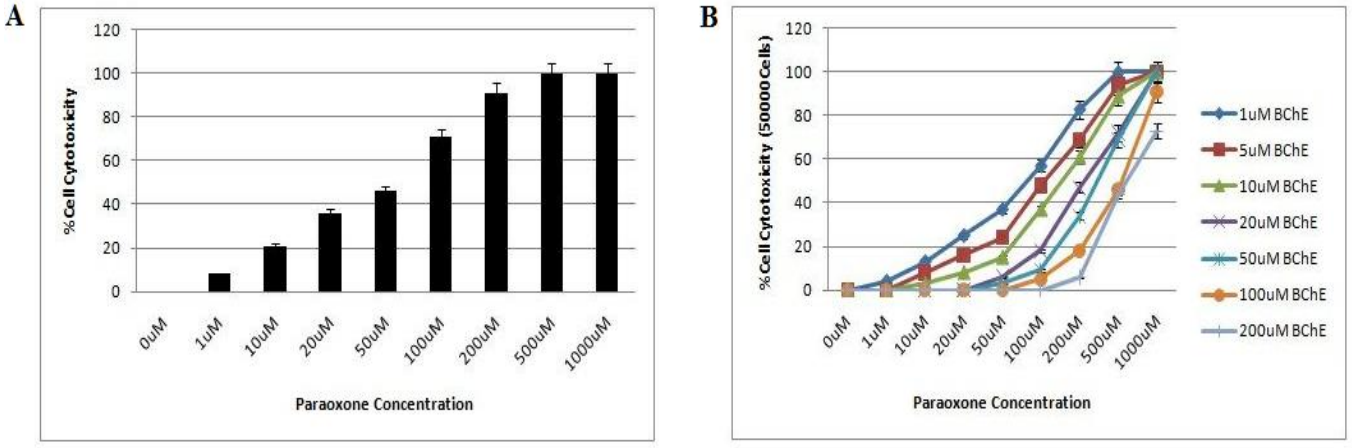

Figure 3: LDH test. Cytotoxicity induced by different concentrations of paraxon in PC12cells (A); LDH test. Cytotoxicity of the PC12 cells in the presence of different concentrations of paraxon and BCHE (B)

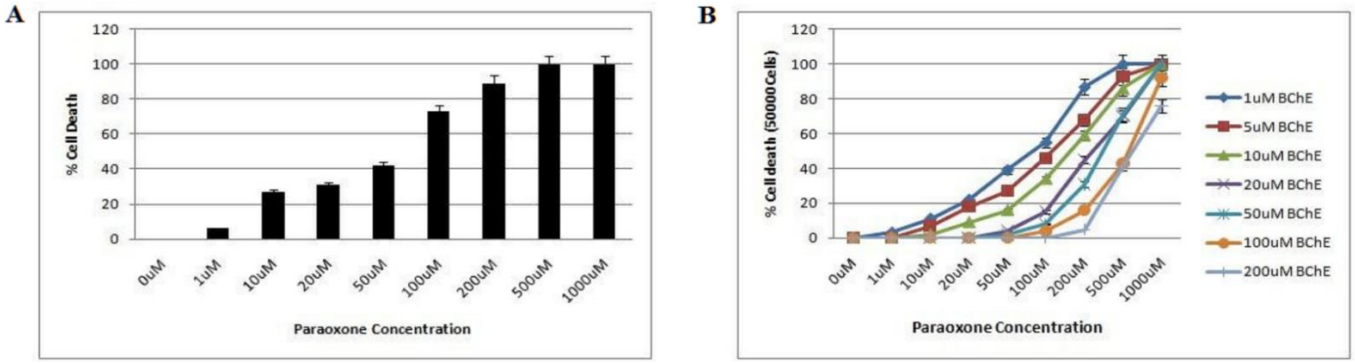

Figure 4: TUNEL test. The cell death index of PC12 cells in different treatments of paraxon (A); TUNEL test. Cell death index of the PC12 cells in the presence of different concentrations of paraxon and BCHE (B)

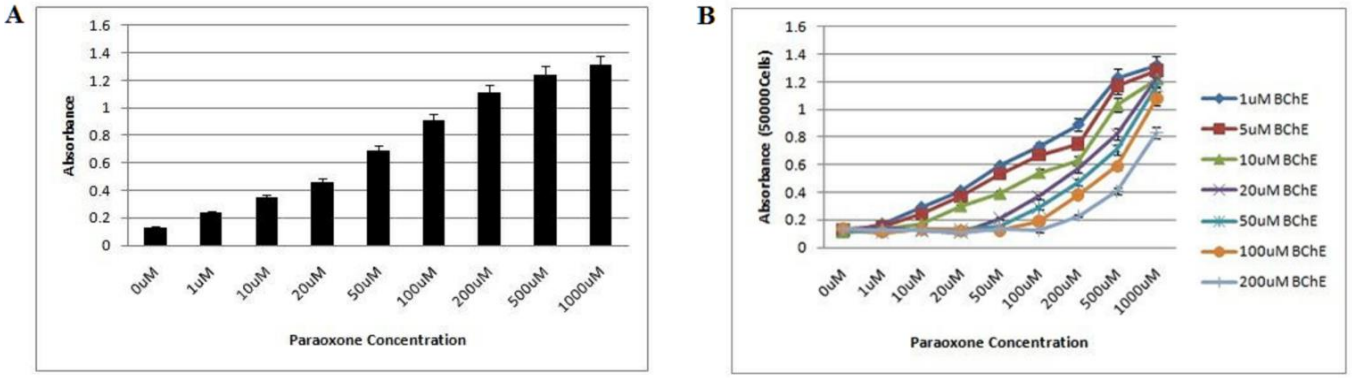

Figure 5: Caspase activity test. The caspase activity of PC12 cells in different treatments of paraxon (A). Caspase activity test. The caspase activity of the PC12 cells in the presence of different concentrations of paraxon and BCHE (B)

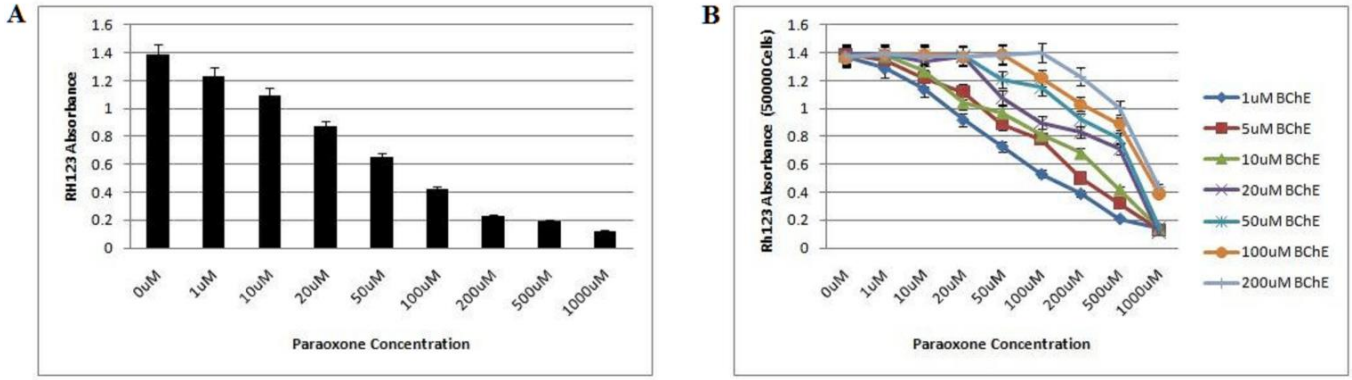

Figure 6: MMP. The MMP of PC12 cells in different treatments of paraxon (A). MMP. The MMP of the PC12 cells in the presence of different concentrations of paraxon and BCHE (B)

3.5 Caspase activity

Caspases are the most important proteins in the apoptosis cascade. Assessment of caspase activity shows us the apoptosis occurrence. Our results revealed that paraxon increases the caspase activity in PC12 cells. Caspase activity of paraxon-treated PC12 cells was significantly higher than the control group. As the concentration of paraxon increases activity of caspase increases ($Pv \leq 0.05$). Between the control group and paraxon-treated pc12 cells caspase activity, a significant difference was obvious. Also, a significant intragroup difference was observed in different treatments of paraxon (Figure 5A). BCHE enzyme significantly suppressed the activity of caspase in paraxon-treated PC12 cells. A high concentration of BCHE enzyme resulted in highly effective suppression of caspase activity which revealed that this enzyme decreases the occurrence of apoptosis in a dose-dependent manner ($Pv \leq 0.05$). A high concentration of paraxone causes the high activity of caspase and apoptosis occurrence and a high concentration of BCHE enzyme cannot overcome that (Figure 5B).

3.6 Mitochondrial membrane potential

The absorbance of rhodamine-123 decreases in paraxon-

treated PC12 cells in comparison with control cells. It means that the mitochondrial membrane potential (MMP) of paraxon-treated PC12 cells was lower than the control group significantly (Pv≤0.05). There is a significant difference between paraxon-treated PC12 cells and control groups (Figure 6A). BCHE enzyme improved MMP of paraxon-treated PC12 cells in a dose-dependent matter (Pv≤0.05). As the concentration of the enzyme increases, the MMP increases, and apoptosis decreases (Figure 6B).

4 Discussion

The removal of pests from agricultural and edible products is an important issue that has led to the development of chemical pesticides and mechanical methods in the last century. Although chemical pesticides can remove a wide range of pests, they can also have direct harmful effects on human and animal health (18-20). Today, the use of organophosphorus insecticides as the most important chemical pesticides has increased the volume of organophosphorus compounds in the world (21, 22). Therefore, it is necessary to investigate the effects of these compounds on human health and study their inhibitory factors. paraxon is the most important metabolite of a wide used organophosphorus insecticide, parathion (23, 24).

A. ray et al. studied the effects of paraxon on rat brain cells. They administrated the paraxon to the intracerebral part of the rat's brain and followed the function of cholinesterase. They revealed that paraxon inhibits the cholinesterase in a concentration-dependent manner and leads to the accumulation of acetylcholine in the intracerebral part of the rat's brain (25). Denis V. Abramochkin et al. used the microelectrode technique to investigate paraxon in the isolated atrial and ventricular myocardium preparations of cod (a fish), frog, and rat. Paraxon caused a significant reduction of action potential duration and made slow sinus rhythm. It is maybe due to the accumulation of acetylcholine through inhibition of acetylcholinesterase by paraxon (26).

Acetylcholinesterase inhibition indicates the presence of organophosphorus insecticides and exhibits toxicity induced by acetylcholine accumulation. Often, acetylcholinesterase family enzymes inhibit in a non-disruptive and competitive manner (27). As noted in previous studies, paraoxon competitively inhibits acetylcholinesterase (28). Jing Liu et al. studied on Anticholinesterases power of Organophosphorus compounds like paraxon. They revealed that paraxon inhibits the Acetylcholine Release in Rat Striatum which is mediated through Muscarinic Receptors (29).

In this study, we aimed to examine the Interaction of acetylcholinesterase and paraoxon in neuron-like cells, PC12. Our results revealed that paraxon inhibits the acetylcholinesterase competitively and doesn't disrupt the acetylcholinesterase structure. Therefore, it seems that if the concentration of acetylcholinesterase increases the harmful side effects of paraxon may disappear. We studied the effects of different concentrations of butyrylcholinesterase (BCHE) on the viability, cytotoxicity, cell death index, caspase activity, and mitochondrial membrane potential (MMP) of PC12 cells in the presence of lethal concentrations of paraxon. PC12 cells origin is adrenal medulla of rats. Pheochromocytoma, as the source of PC12 cells, is a mix of neuroblastic and eosinophilic cells and originated from the neuronal crest. It is a neuron-like cell line which is suitable for the study of neuron (30, 31). BCHE is a non-specific pseudocholinesterase or plasma cholinesterase which codes by the BCHE gene which is similar to the neuronal acetylcholinesterase and hydrolyses some choline esters in human (32). Gabriel Amitai AB et al. have examined the kinetics of paraxon inhibitory function on AChE and BChE (33). In this study, we examined the inhibitory potential of BCHE on paraxon lethal side effects because of the competitive relationship between paraxon and BCHE.

4 Conclusion

This study revealed that BCHE can inhibit paraxon-induced cell cytotoxicity and apoptosis in a dose-dependent manner. There was a competitive relationship between paraxon and BCHE, then we concluded that BCHE can also inhibit the paraxon function and suppress the paraxon-induced cell death. It can reduce the lethal effects of paraxon on human health and we introduce it as an appropriate alternative in the treatment of poisonings caused by these organophosphorus insecticides.

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Competing interests

The authors declare that there is no conflict of interest.

Ethical issue

Authors are aware of and comply with, best practices in publication ethics specifically about authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests, and compliance with policies on research ethics.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses, and manuscript writing.

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