J.Agric.&Env.Sci.Dam.Univ.,Egypt Vol.15 (1) 2016

ACETYLCHOLINESTERASE: A UNIVERSAL TOXICITY BIOMARKER

ATEF MOHAMED KHEDR NASSAR
Plant Protection Department, Faculty of Agriculture, Damanhour University, Damanhour, Albeheira, 22516, Egypt
E-Mail; atef.nassar@dmu.edu.eg, **Tel.**; +201021833040

ABSTRACT

Abamectin, indoxacarb, and sumithion are widely applied against large number of insect and mite pests worldwide. More often, these pesticides are involved in integrated pest management, yet their side effects to human are not fully studied. Current study aimed to study their effects on acetylcholine esterase (AChE) as a quick toxicity indicator. Four groups of rats were orally-injected with 1/20 LD_{50} doses of abamectin (ABM) and indoxacarb (IND), and 1/40LD_{50} dose of sumithion (SUM) for 30 days. Results showed that SUM had pronounced reduction of AChE specific activity compared to ABM and IND. Both ABM and IND showed some similar suppression of AChE activity. The organophosphate insecticide (SUM) irreversibly inhibited the AChE (63%) while the effects of ABM (19%) and IND (28%) were reversible. Yet, AChE would be a good bio-indicator for environmental pollution of pesticides.

Keyword: Neurotransmitters; pesticides; White Albino Rats

INTRODUCTION

Acetylcholinesterase (AChE: EC 3.1.1.7) is a crucial enzyme in the nervous system of vertebrates and invertebrates. It degrades the neurotransmitter acetylcholine (ACh) into the choline and acetic acid. Mainly, AChE is concentrated at the neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity is to stop the synaptic communication. It is well documented that the organophosphorus (OP) and carbamate (CR) pesticides' mode of action is the inhibition of the enzymatic activity of AChE in the

central and peripheral nervous system of pests. Binding between OP and AChE is a quasi-irreversible, which leads to complete inactivation of the enzyme. Then, the function of nervous system is distorted and results in the death of pests and mammals (O'Brien, 1976; Fournier and Mutero, 1994; Pohanka, 2014).

Also, AChE enzyme has been reported to be involved in the development of resistance in pests to OP and CR pesticides, where mutations changed the sensitivity of AChE (Bourne *et al.,* 2003). Also, conformational molecular structures of AChE from resistance *Torpedo californica, Drosophila melanogaster,* and other organisms to OP revealed the change of key amino acid residues located at the allosteric effects on AChE (Villatte *et al.,* 2000). Acute and chronic mammalian toxicities and the development of resistance are major disadvantages of the use of OP and CR chemicals in pest control (Tong *et al.,* 2013). Accordingly, there is a need to discover and develop alternatives to conventional AChE inhibitors.

Alternatives of OP and carbamate insecticides within the integrated pest management is the avermectins and oxadiazine. Abamectin and indoxacarb are the lead members of the avermectins and oxadiazine pesticide groups, respectively. They have been used as insecticides, acaricides, and/or nematicides (Tomlin, 2004; Kolar *et al.*, 2008). Avermectins mode of action is interference of the function of γ-aminobutyric acid (GABA) receptor in the peripheral nervous system and blocking the electrical activity in nerves and muscles (Clark *et al.,* 1995; McCavera *et al.,* 2007; Abd-Elhady and Abou-Elghar, 2013). On the other hand, the indoxacarb exerts its mode of action by through blocking of the voltage-dependent sodium channel (IRAC, 2013).

Although these two pesticides are being extensively used in pest control, few studies have been conducted to assess their effects on mammalian AChE. Thus, current investigation was conducted to evaluate the effects ABM and IND on AChE activity compared to the organophosphate insecticide, SUM. Also, the study tried to examine if AChE would be used as a universal bio-indicator of pesticides poisoning in general.

MATERIALS AND METHODS

Chemicals and Pesticides

Acetylthiocholine iodide (ATChI; Sigma order # A5751-250MG), 5,5-dithiobis-2-nitrobenzoic acid (DTNB-Ellman's reagent: Sigma order # D218200), brilliant (Coomassie) blue G-250 (Sigma order #: B0770-5G), and bovine serum albumin (BSA: Sigma order # A2153-10G) were provided from Sigma-Aldrich, USA. The commercial formulation of abamectin (Vertemic®, 1.8% EC), indoxacarb (Avaunt®, 15% SC) were supplied by Syngenta Agro Services AG, Egypt. Active ingredient (95%) of sumithion was provided by Dr. Ismail El-Gendy (Plant Protection Research Institute, Agriculture Research Center, Giza, Egypt).

Animals and Treatments

Male albino rats (*Rattus norvegicus*) (115±10 g) were obtained from the Research Institute of Ophthalmology, Giza, Egypt. Animals were housed in small groups (5 each) inside propylene cages (25 X 50 cm) at 25±2 °C, 12 h dark/light photoperiod, and 70±10 % RH. Rats were fed with commercial pelleted rodent feed and drank water *ad libitum*. The animals were acclimatized to laboratory conditions for two weeks, and then divided into four groups of five adult males each. Rats in groups 1 and 2 were orally given 1/20 LD_{50} of abamectin and indoxacarb, respectively. Group 3 rats were administered daily doses of 1/40LD_{50} of sumithion and group 4 was the control and given equal volume of distilled water. Rats were given daily oral doses for 30 days. Handling of the experimental animals was consistent with the international principles on the care and use of experimental animals (National Research Council, 2011).

Blood Collection

The animals from the four groups were sacrificed 24 hours after the last treatment. Rats were anesthetized using diethyl ether for 10 s before the neck vessels were aseptically severed. Blood was collected in 15 mL screw cap test tubes. Serum was separated through centrifugation of blood samples at 4,000 rpm for 10 min (Universal 32R, Hettich Zentrifugen model D-78532, Germany). Serum was divided into 200 µL portions in Eppendorf tubes and stored at -20ºC until analysis.

Biochemical Estimations

Acetylcholine Esterase (AChE) Enzyme Activity

The assay of AChE activity was quantified following a modified method of Ellman *et al.* (1961). Enzymatic reactions were performed at 25 °C in phosphate buffer (100 mM sodium phosphate, pH 7.5) containing 0.5 mM acetylthiocholine iodide and 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in a total volume of 200 µL using 96-well VICAM microplate reader with DigiRead Software at 405 nm. The specific activity of AChE was expressed as µ moles of ATChI that hydrolyzed mg protein. The AChE activity was calculated as µmol ATChI/ mg BSA min^{-1}. The samples were measured in triplicates.

Protein Determination

Protein concentration of the plasma samples was determined following the method of Bradford (1976) using Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) as a standard at 595 nm. Protein concentrations of the BSA were adjusted to provide 0, 0.2, 0.4, 0.8, 1.2 and 1.6 mg mL^{-1}.

Method Validation

Accuracy and precision were evaluated using five concentrations (75, 150, 300, 600, and 1200 ng mL^{-1} of BSA within the same day to obtain repeatability (intra-assay precision) and over 5 consecutive days to obtain intermediate precision (inter-assay precision). The accuracy and precision were calculated and expressed in terms of percent recovery and coefficient of variation (CV %), respectively (Ermer, 2005).

Statistical Analysis

The present study was designed as an oral sub-chronic toxicity study. Results were analyzed using the general linear model (GLM) procedure of statistical analysis system (SAS) as completely randomized design. Means were compared using Tukey-Kramer *post-hoc* multiple comparison test ($P<0.05$) (SAS, 2009).

RESULTS AND DISCUSSIONS

Method Reliability

The reproducibility of the analysis methods of AChE was presented in Table 1. The inter-assay (n = 25) value was 6.58% and intra-assay (n = 5) value was 7.04. These results were within the

acceptable range and confirm that the analytical techniques were reliable.

Table 1 Summary of statistical analysis and inter- and intra-assay percentages of acetylcholine esterase

Statistical Parameter	Value
Mean	31.21
R^2	97.23
CV	5.90
F value	117.21
Pr>F	<.0001
Inter-Assay	6.58
Intra-Assay	7.04

Toxicity Symptoms and Mortality

Toxicity symptoms of poisoning with ABM, IND, and SUM were recoded every week for 4 weeks and presented in Table 2. There were no observed effects on rats in control group. For rats in other groups, toxicity symptoms appeared starting from the first week of administration. Toxicity symptoms were loss of weight, excessive sweating and salivation, pupillary dilation, heavy breathing, tremors, ataxia, recumbence, blindness, and eventually death. Sumithion and indoxacarb were more toxic compared to abamectin, where about 40% of treated rats died after the 3rd and 4th week.

Acetylcholine Esterase (AChE) Enzyme Activity

Acetylcholinesterase is a biomarker in the toxicological examinations of exposure to cholinesterase-inhibiting pesticides (organophosphates and carbamates) **(**Hill and Fleming, 1982; Lima *et al.*, 2013). Results of acetylcholinesterase specific activity in µM ATChI mg^{-1} protein were presented in Table 3 and Figure 2. ABM, IND, and SUM insecticides significantly reduced the activity of AChE with 33.4, 29.6, 15.3 µM ATChI mg^{-1} protein compared to 41.3 for the control. Abamectin and indoxacarb showed similar trend of the reduction of AChE activity, where they were not significantly different (Figure 1). Sumithion was the most potent inhibitor of AChE with 62% compared to ABM and IND with 19 and 28% reduction in AChE activity of control, respectively. Current results were in

agreement with Mansour *et al.* (2008) who reported that abamectin decreased the AChE activity.

A plethora of compounds inhibit the AChE activity (Schwarz *et al.*, 1995). AChE is a member of the serine hydrolases family (Ollis *et al.*, 1992). Moreover, serum AChE was reported to be suitable to study the AChE suppression by anti-AChE insecticides and as a measure of brain AChE inhibition by such compounds (Lotti, 1995; Chen *et al.*, 1999). Mode of binding of the inhibitors with different structural motives that can bind to the esteratic part of the active site by esterification of serine hydroxyl, or interact with the alpha anionic part of the active site, the aromatic gorge and the peripheral anionic site (Weiner *et al.*, 2009).

Table 2. Toxicity symptoms and mortality in male rats after repeated oral administration of abamectin, indoxacarb, and sumithion for 30 days

Dose (mg kg^{-1} bw)	Toxicity Symptoms (week)				Mortality (%)
	1	2	3	4	
Control	NOE*	NOE	NOE	NOE	0
Abamectin (15)	NOE,	WL, A	WL, A, T	WL, PD, T, A, HB, ES, S, R	0
Indoxacarb (86.6)	WL	WL, A, S, T	WL, A, HB, ES, S, T, PD	WL, PD, B, R, ES, HB, S, T, D	40
Sumithion (42.5)	WL	WL, A, HB, S, T	WL, A, WL, HB, ES, S, T, PD	WL, A, PD, B, R, ES, S, HB, T, D	40

*NOE: no observed effects, WL: weight loss, ES: excessive sweating, S: salivation, PD: pupillary dilation, HB: heavy breathing, T: tremors, A: ataxia, R: recumbency, B: blindness, D: death.

Table 3. LS Mean values of acetylcholinesterase (AChE) specific activity in µM acetylthiocholine iodide (ATChI) min-1 mg-1 protein (BSA)

Dose (mg kg^{-1} bw)	AChE	±SE	Pr > |t|	% of Change*
Control	41.25^{a}	0.921	<.0001	
Abamectin (15)	33.40^{b}	0.824	<.0001	-19.03
Indoxacarb (86.6)	29.55^{b}	1.303	<.0001	-28.36
Sumithion (42.5)	15.28^{c}	1.064	<.0001	-62.96

* % of change: percent of increase or decrease compared to control value = (Control – Treatment)/Control * 100

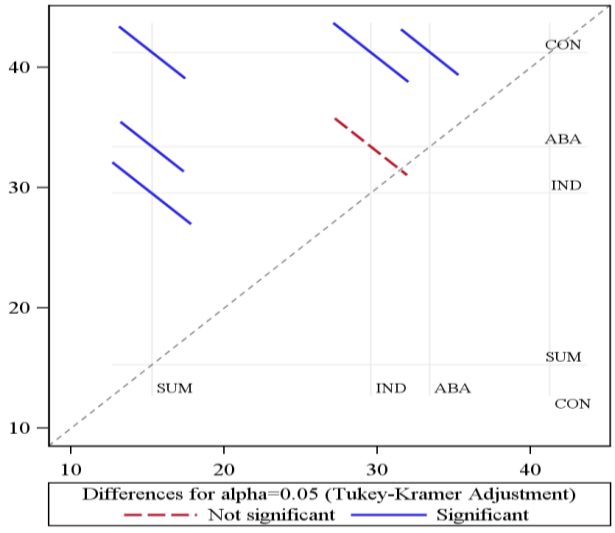

Figure 1. Adjustment for multiple comparisons of acetylcholine esterase specific activity (AChE; µM mg^{-1} protein min^{-1}) based on Tukey-Kramer Significant Difference *post-hoc* multiple comparison test (*P*<0.05).

OP pesticides class bind covalently to the AChE active site with extreme sensitivity to active site geometry and block the next reactions. OPs inactivate AChE by covalent attachment at the active site serine. This binding could be reversible according to Ludke *et al.* (1975), they reported that the level of the risk depends on the inhibition percentages, where percentages of 0 to 20% show normal variation, 20 to 50% refer to reversible effects, and 50 to 100% show

life-threatening or irreversible effects (Hill and Fleming 1982; Trudeau and Sans Cartier 2000). So, results reported herein about the adverse effects of abamectin and indoxacarb were not life threaten but sumithion is.

Results reported herein, that sumithion is life threaten while abamectin and indoxacarb were not, could be interpreted by looking into the mechanism of binding between organophosphate and carbamate insecticides and the AChE protein. Sumithion is not a strong inhibitor of AChE and it has to be converted from P═S to P═O in the animal body to be an active esterase inhibitor. So, oxidation of fenitrothion to fenitrooxon [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphate] by the microsomal mixed function monooxygenase in liver and other tissues convertes it to be more toxic to mammals (Miyamoto *et al.,* 1963; Miyamoto, 1964). Moreover, it was reported that plasma ChE was susceptible to acute and short-term administration of fenitrothion in rats, guinea-pigs, dogs, rabbits, and humans (Miyamoto *et al.,* 1963; Gallo and Lawryk, 1991; Thompson *et al.,* 1992; FAO/WHO, 2001).

In comparison, abamectin is a member of the group avermectins, which is derived from *Streptomyces avermitilis* and comprises at least 80% of avermectin B1a and 20% of avermectin B1b with a large molecular structure. Similarly, indoxacarb structure formula is different from that of OPs but the presence of the oxadiazin group might have some advantages while binding to AChE compared with abamectin. However, its effects on AChE was reversible and not life threaten. So, binding of AChE protein with ABM and IND might occur or it might affect another biochemical process that affect AChE activity, which might explain why reversible effect on AChE was reported in current study.

CONCLUSIONS

Abamectin, indoxacarb, and sumithion pesticides inhibited the activity of AChE with different potencies. Sumithion was a specific suppressor with irreversible action of AChE, while ABM and IND were not. Besides, current investigation concluded that toxicity of new pesticides (ABM and IND) could be monitored quickly using AChE

enzyme. However, the inhibition of the AChE enzyme depends on several factors including chemical groups available for binding, isomerization of chemicals to bind to available amino acid residues, or some other factors that could be studied.

ACKNOWLEDGEMENTS

The author would like to thank Dr. Yehia M. Salim (Assistant Lecturer at Pesticide Chemistry and Toxicology, Plant Protection Department, Faculty of Agriculture, Damanhour University) for his help with the laboratory assays.

REFERENCES

Abd-Elhady, H.K. and G.E. Abou-Elghar (2013). Abamectin induced biochemical and histopathological changes in the albino rat, *Rattus norvegicus.* Journal of Plant Protection Research 53: 263-270.

Bourne, Y., P. Taylor, Z. Radic, and P. Marchot (2003). Structural insights into ligand interactions at the acetylcholinesterase peripheral anionic site. EMBO J. 22: 1–12.

Bradford, M.M.A. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Chen, W.L., J.J. Sheets, R.J. Nolan, and J.L. Mattsson (1999). Human red blood cell acetylcholinesterase inhibition as the appropriate and conservative surrogate endpoint for establishing chlorpyrifos reference dose. Regul. Toxicol. Pharmacol., 29, 15–22.

Clark, J.M., J.G. Scott, F. Campos, and J.R. Bloomquist (1995). Resistance to avermectins: extent, mechanisms, and management implications. Annu Rev Entomol 40: 1–30.

Ellman, G.L., K.D. Courtney, V.Jr. Andres, and R.M. Featherstone (1961). A new and rapid colorimetric determination

of acetylcholinesterase activity. Biochem Pharmac 7: 88-95.

Ermer, J. 2005. Performance parameters, calculations and tests. In: Method Validation in Pharmaceutical Analysis. A Guide to Best Practice, ed. J. Ermer and J.H. McB Miller. Weinheim: WilleyVCH Verlag GmbH & Co. KGaA. Ch 2.

FAO/WHO (2001) Fenitrothion. In: Evaluations of some pesticide residues in food: The monographs, Geneva, World Health Organization, pp. 335-381 (WHO Pesticide Residues Series).

Fournier, D. and A. Mutero (1994). Modification of acetylcholinesterase as a mechanism of resistance to insecticides, Comp Biochem Physiol. 108C: 19–31.

Gallo, M.A. and N.J. Lawryk (1991). Organic Phosphorus Pesticides. Academic Press, San Diego

Hill, E.F. and W.J. Fleming (1982). Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. Environmental Toxicology 1: 27-38.

IRAC (Insecticide Resistance Action Committee) (2013). IRAC Mode of Action Classification Scheme. Version 8.1. www.irac-online.org (April 2013).

Kolar, L., N.K. Erzen, L. Hogerwerf, and C.A.M. Van Gestel (2008). Toxicity of abamectin and doramectin to soil invertebrates. Environ Pollut 151: 182–189.

Lima, S.C., A.C. Dutra-Tavares, F. Nunes, A.L. Nunes-Freitas, A. Ribeiro-Carvalho, C.C. Filgueiras, A.C. Manhães, A. Meyer, and Y. Abreu-Villaça (2013). Methamidophos exposure during the early postnatal period of mice: immediate and late-emergent effects on the cholinergic and serotonergic systems and behavior. Toxicological Sciences 134(1): 125–139.

Lotti, M. (1995). Cholinesterase inhibition: complexities in interpretation. Clin. Chem., 41, 1814–1818.

Ludke, J.L., E.W. Hill, and M.P. Dieter (1975). Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. Arch Environ Contam Toxicol 3: 1-21.

Mansour, S.A., T.M. Heikal, A.H. Mossa, and A.A. Refaie (2008). Toxic effects of five insecticides and their mixture on male albino rats. J Egypt Soc Toxicol 39: 85-94.

McCavera, S., T.K. Walsh, and A.J. Wolstenholme (2007). Nematode ligand-gated chloride channels: an appraisal of their involvement in macrocyclic lactone resistance and prospects for developing molecular markers. Parasitology 134: 1111–1121.

Miyamoto, J. (1964). Studies on the mode of action of organophosphorus compounds. Part III. Activation and degradation of Sumithion and methylparathion *in vivo*. Agric. biol. Chem., 28: 411-421.

Miyamoto, J., Y. Sato, T. Kadota, and A. Fujinami (1963). Studies on the mode of action of organophosphorus compounds. Part II. Inhibition of mammalian cholinesterase *in vivo* following administration of Sumithion and methylparathion. Agric. biol. Chem., 27: 669-676.

National Research Council (2011). Guide for the Care and Use of Laboratory Animals, 8th ed.; the National Academies Press: Washington, DC, USA.

O'Brien, R. (1976). Insecticides Action and Metabolism, Academic Press, New York.

Ollis, D.L., E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Vershueren and A. Goldman (1992). The α/β hydrolase fold. Protein Engin., 5, 197–211.

Pohanka, M. (2014). Inhibitors of acetylcholinesterase and butyrylcholinesterase meet immunity. Int. J. Mol. Sci. 15: 9809–9825.

SAS (2009). (Statistical Analysis System, Version 9.2): SAS Institute Inc., Cary, NC 27513-2414 USA.

Schwarz, M., D. Glick, Y. Loewenstein, and H. Soreq (1995). Engineering of human cholinesterases explains and predicts diverse consequences of administration of various drugs and

poisons. Pharmacol. Ther. 1995, 67, 283–322.

Thompson, C.M., S.M. Ryu, and C.E. Berkman (1992). Consequence of phosphorus stereochemistry upon the postinhibitory reaction-kinetics of acetylcholinesterase poisoned by phosphorothiolates. J. Am. Chem. Soc., 114, 10710–10715.

Tomlin, C.D.S. (Ed.) (2004). A world compendium. The e-Pesticide Manual. Thirteen's Edition. Version 3.0 2003-04. British Crop Protection Council (BCPC).

Tong, F., R.M. Islam, P.R. Carlier, M. Mab, F. Ekström, and J.R. Bloomquist (2013). Effects of anticholinesterases on catalysis and induced conformational change of the peripheral anionic site of murine acetylcholinesterase. Pesticide Biochemistry and Physiology 106: 79–84.

Trudeau, S. and G.S. Cartier (2000). Biochemical methods to determine cholinesterase activity in wildlife exposed to pesticides. Technical Report Series No. 338. Canadian Wildlife Service, Headquarters, Hull, Québec, Canada.

Villatte, F., P. Ziliani, V. Marcel, P. Menozzi, and D. Fournier (2000). A high number of mutations in insect acetylcholinesterase may provide insecticide resistance. Pest Biochem Physiol 67: 95–102.

Weiner, L., V.L. Shnyrov, L. Konstantinovskji, E. Roth, Y. Ashani, and I. Silman (2009). Stabilization of *Torpedo californica* acetylcholinesterase by reversible inhibitors. Biochemistry 2009, 48, 563–574.

J.Agric.&Env.Sci.Dam.Univ.,Egypt Vol.15 (1) 2016

الملخص العربى

إنزيم الاسيتيل كولين إستيريز (AChE) كدليل حيوي لرصد التسمم بالمبيدات

عاطف محمد خضر نصار
قسم وقاية النبات، كلية الزراعة، جامعة دمنهور، دمنهور، البحيرة، ص ب 22516 مصر
E-Mail; atef.nassar@dmu.edu.eg, Tel.; +201021833040

تم إجراء الدراسة علي مبيدات الاباميكتين والاندوكسيكارب والثوميثيون التي تستخدم بكثرة في برامج مكافحة الآفات المختلفة. تلك المبيدات يتم استخدامها بكثرة في برامج المكافحة الا أن تأثيراتها الضارة للإنسان لم يتم الكشف عنها كليا. تهدف الدراسة الحالية الي دراسة استخدام انزيم الـ AChE كدليل حيوي للكشف عن التلوث بالمبيدات. تم استخدام أربعة مجموعات من الفئران. تم إعطاء المجموعات الاولي والثانية جرعات تساوي 20/1 من الجرعة النصف قاتلة من مبيدي الاباميكتين والاندوكسيكارب في حين المجموعة الثالثة تم إعطاءها جرعات متكررة تساوي 40/1 من الجرعة القاتلة. المجموعة الرابعة أعطيت ماء واستخدمت ككنترول. تم إعطاء الجرعات لمدة 30 يوم. أوضحت النتائج أن مبيد الثوميثيون مثبط قوي لانزيم AChE بالمقارنة بالمبيدين الاخريين. كان تثبيط مبيد الثوميثيون للانزيم غير عكسي بنسبة 63% من الكنترول بالمقارنة مع 19 و28% لمبيدي الاباميكتين والاندوكسيكارب. أوضحت نسب الخفض للمبيدين الاخريين أن تأثيرهما عكسي أي أن تأثيرهما يزول بعد فترة من الزمن. وبالرغم من ذلك فنجد أن إنزيم الـ AChE فعال كمقياس حيوي لرصد التلوث بالمبيدات.