Volume 1, No.1, September 2013, Pages 13-22

Environmental Studies of Persian Gulf

journal home page: espg.pgu.ac.ir

Cholinesterases Enzymes Activities as Biomarkers of Farm Workers Exposed to Organophosphates in Two Communities of Khuzestan, Iran

Gholamhossein Mohebbi [1,3,*], Heibatallah Kalantari [2], Mohammad Javad Khodayar [2], Alireza Jahangiri [4,5]

[1]*Persian Gulf Marine Biotechnology Research Center, Bushehr University of Medical Sciences, Bushehr, Iran.*
[2]*Department of Pharmacology and Toxicology, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[3]*Food and Drug Department, Bushehr University of Medical Sciences, Bushehr, Iran*
[4]*Department of Medicinal Chemistry, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[5]*Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*

ARTICLE INFO

Article History:
Received
May 18, 2013
Accepted
August 15, 2013

Keywords:
Cholinesterase
Biomarker
Organophosphates
Farm workers
Khuzestan

ABSTRACT

Organophosphate (OPs) compounds are widely used in intensive agriculture to improve production, protect crops and control diseases vectors. The main mechanism of toxicity of OPs is the inhibition of the Cholinesterase enzymes. In this study the acetylcholinestrase (AChE), butyrylcholinesterase (BChE) and specific acetylcholinestrase (SAChE) activities as potential biomarkers of exposure to OPs were evaluated in whole blood and plasma samples of exposed farm workers of Shushtar and Dasht-e Azadegan in Khuzestan, the south-west province of Iran based on the modified Ellman colorimetric method. Then, RBCs (Red Blood cell) and plasma cholinesterases, haemoglobin (Hb) and specific activity of acetylcholinestrase in Shushtar and Dasht-e Azadegan farm workers were compared with the control group. Results obtained in this study showed that the means of RBC AChE activities in Dasht-e Azadegan, the BChE activities in Shushtar as well as the SAChE activities in both groups were significantly ($p < 0.05$) lower than their means of activities in their control group. Also, means of haemoglobin concentration in samples obtained from Shushtar (p: 0.016) and Dasht-e Azadegan (p: 3×10^{-4}) were significantly ($p<0.05$) higher than the control group. Data obtained in this study indicated that cholinesterase enzymes inhibition provides a good biomarker of exposure to organophosphate pesticides in field studies of human population.

1 INTRODUCTION

Organophosphate (OP) compounds are diverse classes of pesticides (Nozha et al., 2006; Ruark et al., 2013) with the most well-known applications as insecticides and, to a lesser extent, as herbicides in agriculture; Moreover, several are registered for home, garden and veterinary practices (Science Group, 2004), and as chemical warfare agents

* *Corresponding author: Mohebbihsn@yahoo.com*

(Moore, 1998). Due to the fast rate of degradation of OPs in the environment, they have been a suitable replacement for organochlorines (Nozha et al., 2006; Bond et al., 2008; Peter et al., 2008; Kevin et al., 2013). In 2007, 15 million kg of OP pesticides, representing 36% of all insecticides were used in the United States (Lee, 2003; Grube et al., 2011). The toxic effects of OP compounds correlate well with their ability to Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). It is considered as the major mechanism of OP toxicity (Worek et al., 1999; Pesando et al., 2003; Worek et al., 2005; Mohammad Nejad et al, 2012; Alp et al, 2012; Ruark et al., 2013; Chaou et al., 2013) following cutaneous exposure, inhalation or ingestion (Lee, 2003). Anti cholinesterase activity effects induced by an OPs include two stages of the formation of enzyme inhibitor complex and the phosphorylation of the enzyme (Fukuto et al., 1990) which are provided in Fig. 1. Phosphorylated AChE is unable to perform its natural function of hydrolyzing Ach (Wadia et al., 1977; Yang et al., 2002; Kevin et al., 2013).

This results in an accumulation of acetylcholine in cholinergic synapses and excessive stimulation of acetylcholine receptors and can cause numerous malfunctions of the body (Worek et al., 1999; Reigart, and Roberts, 1999; Nigg, and Knaak, 2000; Nozha et al., 2006; Colovic et al., 2013; Ruark et al., 2013). The differences in the clinical courses depend on the properties of the different OP insecticide and their effect on the decrease of AChE activity (Wadia et al., 1977; Yang et al., 2002). OP poisoning is a major problem worldwide, especially in developing countries, with millions of cases and hundreds of thousands of deaths occurring each year (Nozha et al., 2006; Bond et al., 2008; Peter et al., 2008; Chaou et al., 2013).

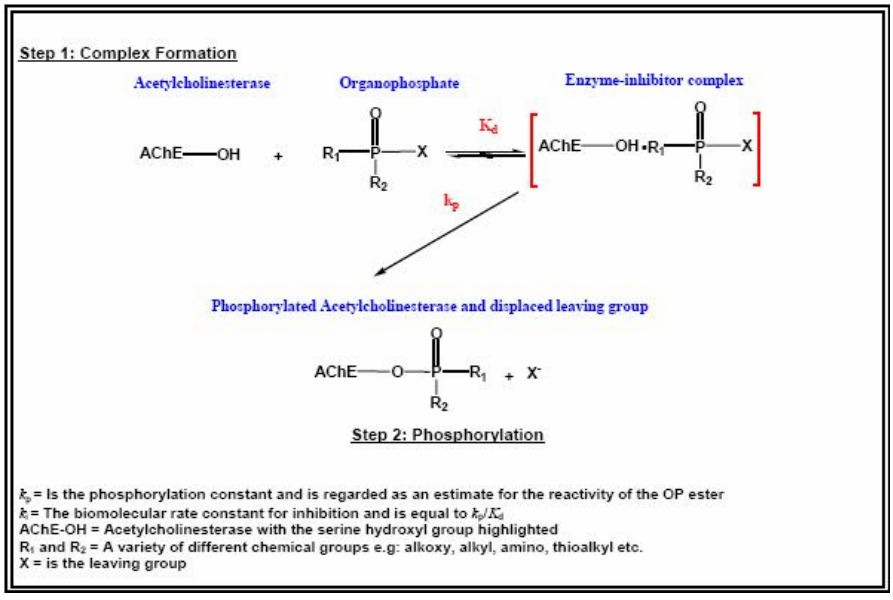

Fig.1. A Schematic equation for OP inhibition of AChE. Two steps of the formation of Enzyme- inhibitor complex and phosphorylation of the enzyme (Worek et al. 2005).

Overall, case fatality ranges from 10% to 20% (Eddleston et al., 2002; Gunnell and Eddleston, 2003), particularly in rural areas (Weerasinghe et al., 2008). In a study from China that examined a nationally representative sample of 518 suicides, 62% of deaths were due to pesticide ingestion and only 27% to physical methods (Phillips et al., 2002).

Agricultural workers are at greater risk of pesticide exposure than non-agricultural employees (Lee, 2003). Khuzestan province located in the southwest of Iran is surrounded by abundance of water resources such as Karun, Karkheh, and many other rivers and also water resources in forms of Creeks or" Khurs", lagoons and ponds. In addition, due to fertility of soil, this area is a rich and well-endowed land for the development of agriculture. OPs are widely used as pesticides by farmers at this province. According to the above-mentioned reasons and the probability of contamination, in this study the occupational exposure to OP pesticides and their effects on cholinesterase activities were investigated among farmers from two communities in Khuzestan, Iran.

2 MATERIALS AND METHODS

2.1. Chemicals and reagents

Acetylthiocholine iodide (ASCh), S-butyrylthiocholine iodide (BSCh), 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent), Triton X-100 were obtained from Sigma (Deisenhofen, Germany), Ethopropazine hydrochloride from Aldrich (Steinheim, Germany), $Na_2HPO_4.2H_2O$, KH_2PO_4, $NaHCO_3$, $K_3Fe[CN]_6$, KCN, HCl from Merck (Darmstadt, Germany). The reagents are prepared as follows: Phosphate buffer (PP, 0.1 mol/l, pH 7.4): Carefully-weighed 17.8 g of $Na_2HPO_4.2H_2O$ and 2.72 g of KH_2PO_4 were dissolved in 1000 and 200 ml of distilled water, respectively. The two solutions were mixed together until reaching the pH to 7.4, the PP was then filtered by HA filter, Millipore, and stored at 4°C. Color reagent (DTNB (Ellman's reagent, 10 mmol/l)) (Ellman et al., 1961): A 396.3 mg DTNB was dissolved in 100 ml of PP by magnetic stirring, and stored in 5 ml aliquots at −20°C. Substrate (ASCh, 28.3 mmol/l): An 82.24 mg acetylthiocholine was dissolved in 10 ml of distilled water and stored in 1 ml aliquots at −20°C. Substrate (BSCh, 63.2 mmol/l): A 200.47 mg butyrylthiocholine was dissolved in 10 ml of distilled water and stored in 1 ml aliquots at −20°C. Ethopropazine (6 mmol/l): A 20.94 mg ethopropazine was slowly dissolved in 10 ml of 12 mmol/l HCl, and stored in 500 µl aliquots at −20°C. Diluting reagent for whole blood samples: A 300 µl Triton X-100 was added to 1000 ml of PP and stored in an amber bottle at 4°C. Transformation solution: A 200 mg potassium ferricyanide, 50 mg potassium cyanide, and 1000 mg sodium bicarbonate were dissolved in 1000 ml of distilled water. After adding 500 µl Triton X-100, the solution was stored in an amber bottle.

2.2 Sample preparation

Because of temperate climate which makes agricultural products possible during the year, Shushtar and Dasht-e Azadegan, two agricultural areas located in south-west of Iran, were considered in this study. The whole blood samples were collected from the two-mentioned communities of farmers exposed to OP pesticide following the spraying. The number of samples in each group is mentioned in Table 2.

Table 1
Standard procedure for the determination of BChE and AChE activities

	AChE	BChE
Mix in polystyrol:		
Phosphate buffer (pH 7.4 , 0.1 mol)	2.000 ml	3.000 ml
DTNB (10 mmol/l)	0.100 ml	0.100 ml
Ethopropazine (6 mmol/l)	0.010ml	-
Hemolysate (whole blood 1:100)	1.000 ml	-
Plasma(undiluted)	-	0.010 m
Equilibrate at 37ºC for 10 min, then add:		
ASCh (28.4 mmol/ l)	0.050 ml	-
BSCh (63.2 mmol/ l)	-	0.050 ml

The color development was recorded for 3 min at 37°C and 436 nm (ε=10.6×10^{3}).

Demographical factors such as age, sex, medical history, type of OP and the other related factors were collected from the two groups and considered during this study.

All the individuals were males who were in the age range of 25-40 without any disease that might have affected enzyme changes. They had used diazinon, dichlorvos and chlorpyrifos more than the other OP pesticides, respectively. From the selected agricultural workers, 2-3 ml blood were sampled by venipunction in 2 tubes, one tube for determination of BChE activity and the other with EDTA for RBC AChE activity.

Samples collected in EDTA-tubes were diluted by adding 200 µl of blood to 20 ml ice-cold diluting reagent (v/v: 1/100). After careful mixing, the samples were immediately frozen in 1 ml aliquots at -20º C and kept until analysis. Plasma samples were obtained from the whole blood samples in EDTA after centrifugation with 500× g in 10 min, and stored as mentioned for the whole blood samples. The control group individuals were selected from the unexposed ones in the same communities.

2.3 Apparatus

The activities of cholinesterases and total haemoglobin were measured using a Shimadzu UV-265 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4 Methods

Prior to analysis, the whole blood dilutions were thawed by gentle shaking of the vials in cold water and kept on ice until analysis. Cholinesterases activities of whole blood, erythrocyte, and plasma were determined according to the Ellman kinetic method, modified by worek et al., (Worek et al., 1999), and are abbreviated in Table 1.

The RBC AChE activity was measured in diluted whole blood samples in the presence of the selective butyrylcholinesterase inhibitor ethopropazine (Worek et al., 1999). The assay measures the reduction of dithiobis-nitrobenzoic acid (DTNB) to thio nitro benzoate (TNB^{-}) by thiocholine, the product of acetylthiocholine hydrolysis (Ellman et al., 1961).The venous blood samples were diluted in 0.1 M phosphate buffer (pH: 7.4) and incubated with 10 mM of DTNB and 6 mM of ethopropazine for 20 minutes at 37 °C prior to addition of acetylthiocholine. The change in the absorbance of DTNB was measured at 436 nm. The AChE activity was calculated using an absorption coefficient of TNB^{-} at 436 nm (ε=10.6 mM^{-1} cm^{1}). The values were normalized to the haemoglobin (Hb) content

Table 2
Means of RBCs, plasma and specific cholinesterases activities and Hb concentration of farm workers exposed to organophosphates in two communities of Shushtar and Dasht-e Azadegan versus their control group.

Groups	Number	AchE activity (µmol/l/min)	Hb (µmol/ l)	Specific activity of AChE (µmol/l/min)	BChE activity (µmol/l/min)
Control					
Mean	34	4362.9267	59.0107	1188.6795	4847.6923
Std.Error of Mean		213.02043	3.27002	37.86374	411.64187
Range		2885.19	43.06	616.28	4907.00
Std.Deviation		928.53451	14.25368	165.04422	1484.19588
Shushtar					
Mean	35	4075.8652	72.4101	906.9015	3069.7222
Std. Error of Mean		193.84368	2.65197	46.78781	225.62806
Range		5549.96	67.87	1656.46	5405.00
Std. Deviation		1146.79467	15.68927	276.80042	1353.76836
Dasht- Azadegan					
Mean	31	3009.9855	55.5236	859.0726	3952.5455
Std.Error of Mean		223.14491	3.55983	45.48109	355.50409
Range		4327.45	90.83	1063.16	6160.00
Std. Deviation		1201.67211	19.17028	244.92314	1667.46196

(determined as cyanomethemoglobin) and expressed as mU/µM/Hb (Van Kampen et al., 1961). To determine total haemoglobin, 1.4 ml of the blood dilution was mixed with 1.4 ml of the transformation solution in polystyrol cuvets. After incubating at ambient temperature for 10 min, the absorbance was recorded at 546 nm against water blank.
All enzyme activities were expressed as a percentage of the baseline activity (100%).
The BChE, RBC AChE and Specific RBC AChE activities and the Hb concentration were done with the following equations (Worek et al., 1999).

1. Enz. Activity $_{BChE\ or\ Ache}$ (µmol/l.min) = ΔA /10.6

$\Delta A = (A_{sample} - A_{blank})$

2. Specif. Activity $_{AChE}$ (mU/µ mol Hb) = Enz. Activity $_{AChE}$(µmol/l.min)×1.58×1000/ [Hb] (µmol/l)

3. [Hb] (µmol/l) =Absorption (A) ×1000 /10.8

The detailed procedure is brought in Table 1.

2.5 Statistical analyses

The statistical analyses of all data were expressed as mean ± S.E.M by factorial ANOVA, followed by student test. The comparison of enzymatic activity was performed using Tukey HSD test. Differences between groups were considered significant when $p \leq 0.05$.

3 RESULTS

The activities of RBCs cholinesterase, plasma cholinesterase, haemoglobin and specific activity of acetylcholinestrase in Shushtar and Dasht-e Azadegan farm workers were measured and are shown in Table 2.

The means of AChE and BChE activities in samples from Shushtar, Dasht-e Azadegan and control groups were 4075.8652 and 3069.7222; 3009.9855 and 3952.5455; 4362.9267 and 4847.6923 (µmol/l.min), respectively. The means of Specific AChE activities in Dasht-e Azadegan and Shushtar were 859.0726 and 906.9015(mU/µ mol Hb), respectively. It was 1188.6795(mU/µ mol Hb) for control group. Moreover, means of haemoglobin concentrations in samples from Shushtar, Dasht-e Azadegan and control groups were 72.4101, 55.5236 and 59.0107(µmol/l), respectively.

The means of RBC AChE activities in Dasht-e Azadegan, the BChE activities in Shushtar as well as the SAChE activities in both of the groups were significantly ($p < 0.05$) lower than their means of activities in their control group as indicated in Table 2. However, the BChE activities in samples from Dasht-e Azadegan and the RBC AChE activities in Shushtar were lower than control group, but they were not significant. Also, means of haemoglobin concentration in samples from Shushtar (p: 0.016) and Dasht-e Azadegan (p: 3×10^{-4}) were significantly ($p < 0.05$) higher than the control group (Table 3).

4 DISCUSSION

OPs are widely used in intensive agriculture to improve production, protect stored crops and control disease vectors. Although application of OP pesticides offers benefits and advantages, health risks have been suggested in human that are occupationally and environmentally exposed to these compounds (OSHAS, 2003; Alp et al., 2012; Kevin et al., 2013). Exposure occurs during the preparation of the mixtures, loading and/or washing equipments and spraying on crops.

BChE activity is generally a more sensitive test of exposure because it is more rapidly inactivated by most of Ops. In addition, it regenerates more quickly than AChE. The full return to normal depends on its re-synthesis that is limited to about 0.8% per day (OSHAS, 2003). The comparison of RBCs cholinesterase, plasma cholinesterase, Hb and specific activities of acetylcholinestrases, in Shushtar & Dasht-e Azadegan farmers with control group were determined and the differences are shown in Table 2. The RBC AChE activities in Dasht-e Azadegan (p: 3×10^{-4}), the BChE activity in Shushtar (p: 1×10^{-3}) and the SAChE activities in Dasht-e Azadegan

Table 3
Comparison between the RBC, plasma and specific cholinesterases activities in addition to Hb concentration of Shushtar (2) & Dasht-e Azadegan (3) farmers groups with their control (1) group.

Activity	(I) control(1)	(J)Region Shutar(2)/ Dasht-azadegan(3)	Mean Difference (I-J)	Sig.
Plasma Activity (µmol/l/min)	1	2	1777.97009(*)	0.001
		3	895.14685	0.202
AChE activity (µmol/l/min)	1	2	287.06151	0.643
		3	1352.94115(*)	0.0003
SAChE activity (µmol/l/min)	1	2	281.778(*)	0.0003
		3	329.607(*)	0.00005
Hb conc. (µmol/ l)	1	2	-13.3994(*)	0.016
		3	-3.4871(*)	0.0003

(p: 5×10^{-5}) and Shushtar (p: 3×10^{-3}) were significantly ($p < 0.05$) lower than their means of activities in control group as indicated in Table 3. Also, means of haemoglobin concentration in samples from Shushtar (p: 0.016) and Dasht-e Azadegan (p: 3×10^{-4}) were significantly ($p< 0.05$) higher than control group. The Shushtar group had the highest mean of haemoglobin concentration. In this study, ChE activities in farmers exposed to OPs were lower than mean activity in control group. These results are consistent with those of other studies (WHO, 1993; Clarke et al., 1997).

In a similar study, Rendon et al. (Rendon et al., 2004) evaluated the AChE activities in four rural communities of farmers from Campeche, Mexico during insecticide use. Regarding the individuals from two communities, AChE activities were significantly lower than the mean activity of individuals in the control group.

In a study, Mohebbi and his colleagues (Mohebbi et al., 2011) measured the AChE activity in farmers from Ab-pakhsh, in Bushehr, Iran. Their findings showed that the mean of AChE activity in samples with amount of 4314.646 (μmol/l/min) was significantly lower ($p<0.05$) than mean activity in the control group (4055.111 μmol/l/min).

Regarding the method, the major disadvantage of the original Ellman procedure for the determination of erythrocyte AChE is the interference of massive haemoglobin absorbance at the absorption maximum of the colored indicator. High dilution of blood samples would reduce the haemoglobin absorption, but it would also decrease the sensitivity of the assay. Part of the problem could be resolved by using dual-wavelength photometers which, however, are likely to be unavailable in many laboratories. Alternatively, the problem could be circumvented by measuring at a wavelength distant from the sharp absorption band of oxyhaemoglobin. Some investigators changed the wavelength. We selected 436 nm, since this wavelength is also available in filter photometers with mercury lamps. At 436 nm, the haemoglobin absorption is reduced to one-fourth compared to 412 nm, while the indicator absorption is still 80% of its maximum at 37°C. The much lower haemoglobin absorption allows the use of low-tech photometers and of higher sample concentrations, which considerably improves the signal-to-noise ratio. The activities of human AChE and BChE are temperature-dependent. The erythrocyte AChE activity is firmly bound to the cell membrane and may be determined in blood samples after separation of red blood cells and plasma followed by repetitive washing of the erythrocytes with physiologic saline or buffer to remove residual plasma. This procedure is laborious and requires equipment which may not often be available.

In addition, this procedure may lead to erroneous results because part of the membrane-bound AChE may be detached without hemolysis of the erythrocytes. During centrifugation of the samples AChE may also be removed with the supernatant without loss of haemoglobin. By using specific substrates for AChE and BChE (e.g. acetylthiocholine and butyrylthiocholine) the enzymes can be determined separately in whole blood with little cross-reaction. Addition of Triton X-100 to the diluting reagent produces complete lysis of the erythrocytes, with reduced turbidity, allowing one to omit stirring during analysis. The inter-laboratory comparison of AChE activities is complicated by different methods of normalization. The enzyme activity is referred to hematocrit, haemoglobin, erythrocyte volume or whole blood volume.

Reference to hematocrit requires centrifugation of the sample, and normalization of AChE activity to blood or erythrocyte volume does not correct for possible dilution errors. Therefore, we refer the enzyme activity to the haemoglobin content of the sample, determined as cyanomethaemoglobin with a modified Zijlstra method at 546 nm, another mercury emission line. With this procedure, inaccuracies during sample dilution are corrected, giving excellent correlation between AChE activity and haemoglobin content. Direct recording of oxyhaemoglobin absorption, as preferred by other investigators, may lead to erroneous data if methaemoglobin is present in older or frozen samples (USEPA, 2006).

5 CONCLUSION

In conclusion, the present study was the first investigation to measure biochemical markers in farm workers engaged in pesticides use in Shushtar and Dasht-e Azadegan. The reluctance for giving blood samples among the farm workers reflects their unfamiliarity with such studies.

The exposure to OP compounds manifests a cholinergic crisis and the diagnosis is based on the clinical signs and symptoms. Measuring the inhibition of erythrocyte (RBC) and/or plasma cholinesterase (p-ChE) activities will help the diagnosis and the treatment of poisoned patients (USEPA; 2006; Alp et al., 2012; Kevin et al., 2013).

Environmental Protection Agency has provided Worker Protection Standard (WPS) for Agricultural Pesticides to reduce the risk of pesticide poisonings and injuries among agricultural workers and pesticide handlers (USEPA; 2006).Therefore, people involved in the production of agricultural plants and pesticide handlers or those who mix, load, or apply pesticides should be aware of the safe handling of pesticides, pesticide applications, use of personal protective equipment, restricted-entry intervals after pesticide application, decontamination supplies, and emergency medical assistance. In conclusion, our results suggest that small number of agricultural workers participating in the study may cause cumulative OP pesticides exposures to exceed a health protective reference value.

ACKNOWLEDGEMENT

The study was supported by the credit of approved research project No. 85 U 057. All of this research is reserved for The Deputy of Research of Ahvaz Jundishapur University of Medical Sciences at Ahvaz, Iran. Also this study was supported by grants from OTKA (k-72315), 78223, 83808, TAMOP 4.2.2-08/1-2008-0007,and TAMOP 4.2.1/B-09/KONV-2010-0007. The authors further would like to thank all the two mentioned communities of farmers and their families. Finally, the authors are particularly grateful to Mr. Saulat Raiessi.

REFERENCES

Alp, H., Aytekin, I., Hatipoglu, N. K., Alp, A. and Ogun, M. (2012). Effects of sulforophane and curcumin on oxidative stress created by acute malathion toxicity in rats. *Eur Rev Med Pharmacol Sci*. Vol. 16, pp. 144-148.

Bond, G. R., Pieche, S., Sonicki, Z. and et al. (2008). A clinical decision aid for triage of children younger than 5 years and with organophosphate or carbamate insecticide exposure in developing countries. *Ann Emerg Med.* Vol. 52, pp. 617–622.

Chaou, C. H., Lin, C. C., Chen, H.Y., Lee, C. H. and Chen, T. H. H. (2013). Chlorpyrifos is associated with slower serum cholinesterase recovery in acute

organophosphate-poisoned patients. *Clin, Toxicol*, Vol. 51, No. 5, pp. 402-408.

Clarke, E. K., Levy, I. and Spurgeon, A. (1997). The problems associated with pesticides use by irrigation workers in China. *Occu,* Vol. 47, pp. 301-308.

Colovic, M. B., Krstic, D. Z, Lazarevic-Pasti, T. D., Bondzic, A. M. and Vasic, V. M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Cur. Neuropharma,* Vol. 11, No. 3, pp. 315-335.

Eddleston, M., Szinicz, L. and Buckley, N. A. (2002). Oximes in acute organophosphorus pesticide poisoning: a systematic review of clinical trials. *Q. J. M*, Vol. 95. pp. 275-283.

Ellman, G. L., Courtney, K. D., Feather-Stone, R. M. and Andres, J. V. (1961). A new and rapid colorimetric determination of acetyl-cholinesterase activity. *Biochem. Pharmacol,* Vol. 7, pp. 88–95.

Fukuto, T. R. (1990). Mechanism of action of organophosphorus and carbamate insecticides. *Env. Health Perspect,* Vol. 87, pp. 245-254.

Grube, A., Donaldson, D., Kiely, T. and Wu, L. (2011). Pesticides Industry Sales and Usage: 2006 and 2007 Market Estimates. *Washington, DC: U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention.*

Gunnell, D. and Eddleston, M. (2003). Suicide by intentional ingestion of pesticides: a continuing tragedy in developing countries. *Int. J. Epidem*. Vol. 32, pp. 902-909.

Kevin, B. T., Alexander, P. T., Danett, K. B., Andrew, Y. L. and Adalberto, A. P. D. L. (2013). Acetylcholinesterases of blood-feeding flies and ticks. *Chem-Biol. Interac*, Vol. 203, pp. 319–322.

Lee, E. C. (2003). Clinical manifestations of sarin nerve gas exposure. *J.A.M.A.* Vol. 290, pp. 659-662.

Mohammad Nejad Shamoushaki, M., Soltani, M., Kamali, A., Imanpoor, M. R., Sharifpour, I. and Khara, H. (2012). Effects of organophosphate, diazinon on some haematological and biochemical changes in Rutilus frisii kutum (Kamensky, 1901) male brood stocks. *Iran. Jour. Of Fish. Sci*; Vol. 11 No. 1, 105-117.

Mohebbi, G. H., Jahangiri, A. and Hajeb, P. (2011). Inhibition of Acetyl Cholinesterase Activity Farmers Exposed To Organophosphate Pesticides in Bushehr Iran. *Americ. Eur. J. Agric. & Environ*; *Sci*, Vol. 3, No. 3, pp. 127-129.

Moore, A. and Waring, C. P. (1998). Mechanistic effects of a triazine pesticide on endocrine function of mature male Atlantic salmon (Salmo salar) parr. *Pest. Biochem. and Phys*, Vol. 62, pp. 41-50.

Nigg, H. N. and Knaak, J. B. (2000). Blood cholinesterases as human biomarkers of organophosphorus pesticide exposure. *Rev. Env. Contam. Toxicol*, Vol. 163, pp. 29-111.

Nozha, B., Amel, M., Nadia, K., Hayet, Gh., Youssef, B., Hafedh, T. H. and et al. (2006). Prognostic value of human erythrocyte acetyl cholinesterase in acute organophosphate poisoning. *Americ. Jour. of Emer. Med*, Vol. 24, pp. 822–827.

OSHAS. (2003). Occupational safety and health service. A Guideline to promote best practice with organophosphates, *Department of labor, wellington, New Zealand.*

Pesando, D., Huitorel, P., Dolcini, V., Angelini, C., Guidetti, P. and Falugi, C. (2003). Biological targets of neurotoxic pesticides analysed by alteration of developmental events in the Mediterranean Sea urchin. Paracentrotus lividus, *Marine Env. Res*, Vol. 55, pp. 39–57.

Peter, J.V., Moran, J. L., Pichamuthu, K., Chacko, B. (2008). Adjuncts and alternatives to oxime therapy in

organophosphate poisoning - is there evidence of benefit in human poisoning? A review. *Anaesth. Intens. Care*, Vol. 36, No. 3 pp. 339-350.

Phillips, M. R., Yang, G., Zhang, Y. and Wang, L. (2002). Risk factors for suicide in China: a national case-control psychological autopsy study. *Lancet*, Vol. 360, pp. 1728 – 1736.

Reigart, J. and Roberts, J. R. (1999). Recognition and Management of Pesticide Poisonings, Publication No. 735-R-98–003, *United States Environmental Protection Agency, Washington, D.C. USEPA.*

Rendon ven osten, J., Rolando, T. O. and Amaadeu, M. V. M. (2004). Effect of pesticide exposure on AChe activity in subsistence farmers from Campeche, Mexico. *Arch. Env. Health*, Vol. 59, No. 8, pp. 418-425.

Ruark, C. D., Hack, C. E., Robinson, P. J., Anderson, P. E. and Gearhart, J. M. (2013). Quantitative structure-activity relationships for organophosphates binding to acetylcholinesterase. *Arch. Toxicol*, Vol. 87, No. 2, pp. 281-289.

Science Group. (2004). A critique of the United States environmental protection agency's (US EPA) grouping of insecticidal organophosphates and N-methyl Carbamates into common mechanism groups.

USEPA. (2006). How to comply with the worker protection standard for agricultural pesticides, Manual Reprinted. *United State of America, Environmental protection Agency.*

Van Kampen, E. J. and Zijlstra, W. G. (1961). Standardization of haemoglobinometry. The haemoglobin cyanide method. *Clin. Chim. Acta*, Vol. 6, pp. 538-544.

Wadia, R. S., Bhirud, R. H. and Gulavanti, A.V. (1977). Neurological manifestations of three organophosphate poisons. *Ind. J. Med. Res*, Vol. 66, 460-468.

Weerasinghe, M., Pieris, R., Eddleston, M., Hoek, W., Dawson, A. and Konradsen, F. (2008). Safe storage of pesticides in Sri Lanka - identifying important design features influencing community acceptance and use of safe storage devices. *BMC. Pub. Health;* Vol. 8, pp. 276.

WHO. (1993). WORLD Health Chemical Safety 46th year Geneva Switzerland. WHO, 24-29

Worek, F., Koller, M., Thiermann, H. and Szinicz, L. (2005). Diagnostic aspects of organophosphate poisoning. *Toxicol*, Vol. 214, No. 3, pp. 182-189.

Worek, F., Mast, U., Kiderlen, D., Diepold, C. and Eyer, P. (1999). Improved determination of acetylcholinesterase activity in human whole blood. *Clin. Chem. Acta*, Vol. 288, No. 1-2, pp. 73-90.

Yang, D., Lu, X. and Zhang, W. (2002). Biochemical changes in primary culture of skeletal muscle cells following dimethoate exposure. *Toxicol*, Vol. 174, pp. 79 - 85.