Chapter 2

Effect of Environmental Toxic Molecules on Paraoxonase1 Activity
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CHAPTER 2

EFFECT OF ENVIRONMENTAL TOXIC MOLECULES ON PARAOXONASE1 ACTIVITY

2.1 AGRICULTURAL PESTICIDES

2.1.1 Introduction

Pesticides are the most important xenobiotic pollutants because of their widespread use in agriculture and they become an undeniable part of human life. Consequent to their indiscriminate use, humans are subjected to chronic and sometimes to acute toxicity by these toxic chemicals. In many developing countries, unregulated use of pesticides contributes not only to environmental pollution but also to health problems. Earlier studies have reported that cancer, birth defects, fetal death and neuro-developmental disorder are the health problems caused by pesticide exposure (Jurewicz & Hanke, 2008; Sanborn et al., 2007).

Throughout the world, humans are exposed to highest levels of pesticide in a direct or indirect way. However, human body has evolved to withstand or detoxify low-level toxic insults with the aid of XMEs. In a manner similar to that of the majority of xenobiotics, OPs, after entering an organism, are metabolized and hydrolyzed by PON1 (Tina & Metka, 2011).

PON1 protection against pesticide poisoning was validated by many studies. Studies in transgenic mice clearly demonstrated that low plasma PON1 activity is associated with greater AChE inhibition after exposure to chlorpyrifos-oxon and diazoxon. They also observed that administration of PON1 was found to abolish cholinergic signs and significantly protected against AChE inhibition. However, the in vivo efficacy of PON1 to OP exposure depends on the catalytic efficiency of hydrolysis (Li et al., 2000).

Studies have reported the induction of PON1 gene expression by xenobiotics. PCBs, dioxins (TCDD) and PAHs such as benzo-α-pyrene and 3-methylcholanthrene are known to induce PON1 gene via aryl hydrocarbon receptor (AhR) dependent pathway. The xenobiotic responsive element like (XRE-like) sequence in promoter
regions of PON1 gene is responsible for the regulation of its transcription (Gouédard et al, 2004). Some organochlorine pesticides are known to stimulate lipid peroxidation in rats (Yamano & Morita, 1995). In addition, elevated level of lipid hydroperoxides found in chlorothalonil treated rat hepatocytes were about 23 times higher compared with control rats (Suzuki, 1997).

In contrast, modulation of PON1 activity was observed in subjects exposed to acute and chronic pesticide. Therefore, it is important to examine the effect of pesticides on PON1 activity to understand the underlying mechanisms and assessing the aggregate risk. The purpose of this study was to investigate the in vitro effects of commonly used pesticides on PON1. These pesticides are currently used in Mysore district, India.

In this study, in vitro effect of chlorpyrifos, chlorothalonil, cypermethrin, methomyl, pyrethrin and metribuzin towards PON1 was determined. Occurrence of pesticide residues in randomly chosen fruits and vegetables (grapes and cauliflower) were analyzed. Effect of chlorothalonil on rat liver ApoA1 expression was studied.

2.1.2 Materials and Methods

Paraoxon, chlorpyrifos, chlorothalonil, cypermethrin and pyrethrin were purchased from Sigma Aldrich Co., St. Louis, USA. Methomyl, metribuzin, were purchased from local commercial store, Mysore. All other reagents used were of analytical grade and solvents were distilled before use. Antibody to oxidized Apo A1 was given by Mrs. Mamatha AM. The antibodies cross react with HDL.

**Isolation of serum PON1 (HDL)**

**Collection of blood and preparation of serum**

Blood was collected from healthy volunteers (age group 20-30 years; either gender). The blood was allowed to clot and centrifuged at 5000rpm for 20min. The serum obtained was pooled and stored at -20 °C and it was used for subsequent experiments. All the experiments involving human subjects were carried in accordance with the protocol approved by Institutional Human Ethical Committee, University of Mysore, Mysore [Sanction order no. IHEC-UOM No. 38/PhD/2009-10].
**Isolation of HDL by density centrifugation**

HDL was isolated from serum by density gradient centrifugation according to the method of Redgrave et al (1975). Briefly, 6ml of serum was mixed with solid KBr (0.5g/ml). 18ml of saline was layered on top of the serum sample. The tubes were centrifuged using ultracentrifuge in a fixed angle rotor for 3h at 45000 rpm (2,00,000g) at 4 °C. After centrifugation, the tubes were placed in the vertical position. The fractions were aspirated from the top. HDL appears as an orange-layer at the bottom of the tube. HDL containing fractions were dialyzed in the dark for 6 to 8h against 100mM phosphate buffered saline. PON1 activity of HDL was determined spectrophotometrically by using paraoxon as substrate.

**PON1 Activity**

PON1 activity was measured (using paraoxon as substrate) according to method of Beltowski et al (2003) with some modifications. The activity was measured in Tris buffer (100mM, pH 8.0) containing 2mM CaCl₂ and 1mM paraoxon in 1ml total reaction volume. HDL fraction (100µl of 1:10 dilution) was added to initiate the reaction. The rate of generation of p-nitrophenol was determined at 412 nm at 25 °C, with the use of a continuously recording spectrophotometer (Shimadzu). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1µmol of substrate per minute.

**Pesticide residue analysis in grape and cauliflower**

In order to analyze pesticide residue in fruit and vegetables, we randomly chose grapes and cauliflower sample from the local markets of Mysore India. Extraction and analysis of pesticide residue in those samples was done according to the method of AOAC [Association of Official Agricultural Chemists] Gas chromatography/mass spectrometry (GC/MS) was used for quantitative studies (“Pesticide and Industrial Chemical Residues,” 2005).

**In vitro effect of pesticides on PON1**

**Preparation of pesticide solutions**

Standard pesticide stock solutions (100mM) of chlorpyrifos, cypermethrin and pyrethrum were prepared in methanol, whereas, chlorothalonil (25mM) was prepared in acetone. Three commercially available pesticide brands were purchased from
Effect of Environmental Toxic Molecules on PON1

Mysore, India. Based on the information on the product labels, had pesticides such as methomyl, metribuzin and thiomethoxon respectively. 1g of pesticide powder of each brand was weighed in separate vials. Pesticide residues were extracted with chloroform and solvent was removed. Extracts were re-dissolved in 1ml of methanol. These samples were used for various experiments.

**PON1 inhibition by pesticides**

For *in vitro* studies, different concentrations of each pesticide (chlorpyrifos, chlorothalonil, cypermethrin, methomyl, pyrethrum, metribuzin) were incubated with PON1 separately. Effect of each pesticide on PON1 activity was determined by following the hydrolysis of paraoxon. Percentage activity of PON1 was determined by linear regression analysis. PON1 activity without inhibitor was taken as 100 %. IC$_{50}$ value for each pesticide was determined from the graphs. IC$_{50}$ was defined as that concentration of pesticide causing 50% of inhibition of PON1 activity.

**Effect of chlorothalonil on rat liver PON1**

*Liver perfusion and expression of PON1 in liver slice*

The rat was anesthetized and the liver was perfused with saline until all the traces of blood were removed. Liver was dissected out and cut into slices. Liver slices (200mg) were taken in separate dishes containing 1ml RPMI-1640 medium. Chlorothalonil pesticide solution (2µl of 0.5g/10ml) in acetone was added to the medium. Dishes without pesticide were taken as control. PON1 activity was determined for every 1h for up to 5h using phenyl acetate as substrate. Similarly, formation of HDL was monitored by quantitating the ApoA1 by Sandwich Elisa.

**Quantification of chlorothalonil induced HDL (ApoA1) by ELISA**

Chlorothalonil induced HDL was measured by sandwich-type ELISA in a time dependant manner. Ox ApoA1 antibodies of each assay (approximately 5µg/ml in carbonate/bicarbonate buffer, pH 9.6) were coated overnight at 4 ºC onto 96-well plates (Maxisorp, Nunc, Denmark), and free coating sites were blocked with phosphate-buffered saline (PBS) containing 1% (w/v) casein, followed by washing with PBST (10mM PBS containing 100mM NaCl, 2mM KCl and 0.05% Tween 20), 100µl of antigen in PBST were then incubated. Antigen binding was detected by with HRP conjugated antibody (1:5000 dilution). About 100µl per well of substrate ABTS
[2, 2_-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 1mg/ml; in 0.1M citrate phosphate buffer pH 5.5] with 3% hydrogen peroxide was used. The reaction was terminated with 0.2M citrate buffer and absorbance was read at 450nm using Elisa instrument TICON megallon F50 Sweden (Kohno et al., 1989).

2.1.3 Results

In this study, the in vitro effects of commonly used pesticides, namely chlorpyrifos, chlorothalonil, cypermethrin, methomyl, pyrethrum, metribuzin on PON1 was investigated. In order to do this study human serum PON1 was isolated by density gradient ultracentrifugation. A summary of the yield and fold purity is mentioned in Table – 2.1.

| Table – 2.1 : HDL associated PON1 isolation from serum by ultracentrifugation |
|------------------|-----------------|-----------------|-----------------|----------------|
|                  | Volume (ml)     | Total protein(mg) | PON1activity (U/ml) | Total activity (µmol/min)/mg) |
| Serum            | 25              | 1800             | 203              | 5075               |
| HDL fraction     | 8               | 64               | 85.96            | 687.68             |

Multi pesticide residues analysis was done on randomly selected grape and cauliflower samples according to the method of AOAC. The GC-MS analysis of standard and sample is shown in the Figure 2.1a and 2.2a. Figure 2.1b shown the GC-MS of grape sample, whereas Figure 2.2b shown GC-MS of cauliflower sample. Grape sample showed presence of chlorpyrifos residues with a concentration of 19ppm. Cauliflower sample showed presence of cypermethrin residue with a concentration of 0.2ppm. The pesticide concentration in the grape sample and cauliflower sample is shown in Table – 2.2.

<table>
<thead>
<tr>
<th>Table – 2.2 : Contamination results of pesticide residues in analyzed grape and cauliflower sample.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
</tr>
<tr>
<td>Grapes</td>
</tr>
<tr>
<td>Cauliflower</td>
</tr>
</tbody>
</table>
Figure – 2.1a : GC-MS spectra of pesticide standards for grapes sample (from left: chlorpyrifos, endosulfan I, endosulfan II, endosulfan sulfate and cypermethrin)

Figure - 2.1b : GC-MS spectra of grape sample; identified pesticide chlorpyrifos.
Figure – 2.2a : GC-MS spectra of pesticide standards for cauliflower sample (from left: Diclorvos, Methomyl, Cymoxanil, Chlorpyrifos, Chlorothalonil, Cypermethrin)

Figure – 2.2b : GC-MS spectra of cauliflower sample; identified pesticide cypermethrin

Inhibition of PON1 by pesticides is shown in Figure 2.3. Cypermethrin, metribuzin and pyrethrin did not inhibit PON1 in a dose dependant manner, whereas, other pesticides inhibited PON1 in a dose dependant manner. Chlorothalonil and chlorpyrifos showed the maximum inhibition. The IC50 values of inhibitors are shown in Table – 2.3.
Figure – 2.3 : Effect of pesticides on PON1 activity
Table – 2.3 : IC₅₀ values of pesticides with their groups on inhibition of PON1

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Group</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methomyl</td>
<td>Carbamates</td>
<td>316.7mM</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>Organophosphate</td>
<td>0.242mM</td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>Organochlorine</td>
<td>0.187mM</td>
</tr>
</tbody>
</table>

Rat liver slices were treated with chlorothalonil and PON1 activity was measured. Quantification of HDL was done by sandwich ELISA. Figure 2.4a indicates that chlorothalonil inhibited the PON1 activity in the liver slice. However HDL synthesis stimulated by chlorothalonil increased with time (Figure – 2.4b).

![Figure 2.4a](image1.png)

**Figure 2.4a Effect of chlorothalonil on liver PON1 activity.** Kinetic analysis of PON1 activity of the chlorothalonil incubated liver slice. Activity was determined using phenyl acetate as substrate.

![Figure 2.4b](image2.png)

**Figure 2.4b Effect of chlorothalonil on HDL expression.** Kinetic analysis of HDL synthesis in the chlorothalonil treated liver slice was measured by Sandwich Elisa method using APO A1 antibody.
2.1.4 Discussion

Many research works were validates the role of PON1 states in minimizing susceptibility towards acute pesticide poisoning. However, some studies reported declining PON1 activity in acute OP exposure and recovery of PON1 found after six months of the exposure.

Despite reports on the lower PON1 activity in serum of persons or animal model with the acute pesticide poisoning, there has been limited knowledge concerning cause for lower PON activity (Berkowitz et al., 2004; Hofmann et al., 2009; Łukaszewicz-Hussain, 2012; E. Y. Sözm en et al., 2002). Even polymorphism and substrate-dependent experiments could not account for the low serum PON activity in pesticide exposed individuals (Mackness et al, 2000). Our findings may provide one of possible explanations for a lower PON1 activity in acute poisoning, which has been observed in the assay condition routinely used.

In this study, effect of different classes of pesticide on PON1 activity was investigated. In order to do this, we selected chlorpyrifos, chlorothalonil, cypermethrin, methomyl, pyrethrum, metribuzin pesticides. Among them, chlorpyrifos, chlorothalonil and methomyl were found to inhibit PON1 activity.

Our results have a number of similarities with previous findings. Lukaszewicz-Hussain(2012) found significant decrease in PON activity in serum of chlorpyrifos intoxicated rats in vivo. However, a very few in vitro studies reported inhibitory effect of pesticides on PON1 activity. Nahit & Oktay(2011), showed the inhibition of PON1 isoenzymes using some pesticides available in Turkey (Nahit & Oktay, 2011).

In this study, we have shown the inhibitory effect of methomyl, chlorpyrifos and chlorothalonil on PON1 activity. Our present results may provide possible explanations for the decrease of PON1 activity, which has been observed in the assay condition routinely used. Chlorpyrifos and chlorpyrifos oxon are promiscuous substrates of PON1. Li et al 2000 reported that catalytic efficiency of PON1 towards hydrolysis of chlorpyrifos-oxon is much greater than paraoxon. In other words, PON1 have higher affinity towards chlorpyrifos than paraoxon. Higher affinity towards chlorpyrifos made PON1 less available for paraoxon hydrolysis and this might be the reason for decreased PON activity in our assay system.
Chlorothalonil is an organochlorine pesticide. Studies have been reported on chlorothalonil induced lipid peroxidation. Moreover, organochlorine residues have more affinity for lipoprotein in the blood than other carriers, during transportation (Gómez-Catalán et al, 1991). It may possible that chlorothalonil may affect the protein conformation or binding to HDL altering the activity of PON1. However in the present study found, chlorothalonil stimulated HDL synthesis in a time dependent manner.

Pyrethrin decreased the PON1 activity and cypermethrin has no effect. In contrast metribuzin showed the activation of PON1 enzyme. The mechanism behind this activation is unknown. However, even small structural differences can affect the paraoxon-hydrolyzing activity. There is an evidence to support this hypothesis, i.e higher sensitivity to the NaCl-induced stimulation of R PON1\[191\]Q isoenzyme differing in only one residue (Eckerson et al., 1983a).

Pesticide residue analysis on randomly chosen grapes and cauliflower showed the presence of pesticide residues, indicating the indiscriminate use of pesticide in our routine life. However, PON1 protection against dietary pesticide exposure needs to be studied.
2.2 DOMESTIC PESTICIDES

2.2.1 Introduction

*Mosquito coil repellent and PON1*

Burning mosquito coils indoor produce smoke that can prevent mosquitoes effectively. This practice is currently used in Asia, Africa and South America. Although the smoke can efficiently prevent mosquito bites, the smoke may contain pollutants that can affect human health (Sharma, 2001). Allethrin and prallethrin are the chief constituents of various mosquito repellent- insecticides in India. Other common ingredients are wood flour, coconut shell powder and starch/binders. When a mosquito coil is burnt, the insecticides evaporate with the smoke. Sub micrometer particles, carbonyl compounds (acetaldehyde, formaldehyde) and polycyclic aromatic hydrocarbons (PAHs) are also released during combustion (Liu et al., 2003). Accumulating evidence suggests that exposure to pollutants and ultrafine particles present in ambient air could elevate the risk of cardiovascular disease and even cardiovascular mortality (Brook et al., 2004; Dockery, 2001).

Plasma profile of chronic exposure to mosquito repellent showed increase in concentration of VLDL cholesterol, triglyceride and also elevated lipid peroxides. Thus it can be indicative to coronary and cardiovascular risk (Narendra et al, 2008). Several studies have shown a strong inverse relationship between the serum concentration of HDL and incidence of coronary heart diseases (Toth, 2004). HDL particle have anti-atherothrombotic action including anti-inflammatory, antioxidant, and profibrinolytic activities (Assmann & Gotto, 2004; Litvinov et al, 2012). This protective ability of HDL comes from the enzyme PON1 associated with it (Watson et al., 1995). Therefore, in this study we investigated the protective role of PON1 against modification of HDL caused by mosquito coil smoke.

2.2.2 Materials and Methods

*Materials*

Paraoxon (O.O-diethyl-O-p-nitrophenylphosphate), allethrin, were from Sigma Chemical Co, St. Louis, USA. All other chemicals and solvents used were of analytical grade. Tested mosquito coil brand purchased from Mysore India.
Collection of Mosquito coil repellent smoke extract (MSE)

Experimental apparatus

The experimental apparatus is shown in Figure – 2.5. The smoke was collected in 20ml of Tris buffer (100mM, pH 8) from the outlet tube of the apparatus setup shown below. Tested mosquito coil brand was purchased from Mysore, India. Based on the information on the product had active ingredients such as D-trans-allethrin, wood flour, coconut shell powder and starch/binders.

![Experimental apparatus for the collection of Mosquito smoke exhaust.](image)

Preparation of MSE

Smoke from a mosquito coil was passed through 20ml of 0.1M Tris buffer pH 8; contain 2mM CaCl₂ by aspiration for about 2h.

Preparation of HDL

HDL was isolated from serum by density gradient centrifugation according to the method of Redgrave et al (Redgrave et al., 1975) described in the section 2.1.2.

Treatment of HDL with MSE

HDL (200μl) was added to 2ml Tris buffer (pH8, 0.1M) containing CaCl₂ (2mM) with or without MSE. The reaction mixture with or without MSE was incubated at 37 °C for different time intervals.

PON activity

PON1 activity was measured according to the method of Beltowsk et al, (2003) described in the section 2.1.2.
Lipid peroxidation assay

The extent of lipid peroxidation was measured by the formation of malondialdehyde (MDA) by the method of Buege & Aust, (1978). One milliliter of MSE treated HDL (1:6 v/v) was taken in a test tube to which 1.5ml of 20% acetic acid (pH adjusted to 3.5 with NaOH), 1.5ml of 0.8% TBA and 0.05ml of 20% TCA are added, kept in boiling water bath for 15min and the contents were allowed to cool and then centrifuged at 1000g for 10min. The supernatant was transferred into a separate test tube and the absorbance of the sample was read at 535nm by a UV/Visible spectrophotometer against the reagent blank assuming the molar extinction coefficient to be 1.56 X 10^5.

Conjugated Dienes measurement

HDL oxidation kinetics was monitored by following the conjugated dienes (CD) formation. Briefly, 1ml of HDL was incubated with 5ml of MSE for 5h. Recording of conjugated dienes formation at 234nm was started immediately after the addition of MSE with interval of 1h up to 5h (Abuja & Albertini, 2001).

2.2.3 Results
MSE treated HDL associated PON1 activity

Incubation of HDL with MSE reveals decreasing PON1 activity at initial hours (1-2h) and activity was found to be recovered later on (3-5h). As shown in the Figure – 2.6, MSE slightly inhibited the PON1 activity in the 1h. On prolonged incubation the PON1 activity regained probably by overcoming the inhibitory effect of MSE.

Figure – 2.6 : HDL associated PON1 activity in the presence or absence of MSE at various time periods. 200μl of HDL is incubated with 1.8ml MSE for 5h.
The effect of allethrin on PON1 activity was investigated. Allethrin is an active component of mosquito coil repellent. The HDL was incubated with different concentration of allethrin in dose dependent manner for 1h. It was found that allethrin inhibited PON1 significantly (p<0.05) but the quantum of inhibition was only 5% (Figure 2.7).

![Figure 2.7: Effect of allethrin on PON1 activity of HDL.](image)

HDL associated PON1 activity at various concentrations of allethrin after 1h incubation at 37 °C.

**Effect of MSE on HDL oxidation**

The extent of HDL oxidation was analyzed by determining the amount of TBARS formed (measured as MDA equivalents), and conjugated dienes formation were by observing OD at 234nm (Figure 2.8 and Figure 2.9). MSE increased the oxidation of HDL within 1h. Subsequently the amount of MDA formed progressively decreased. The decrease in the MDA production was due to formation of stable protein oxidation products. The conjugated dienes formation paralleled the MDA formation.

![Figure 2.8: Effect of MSE on HDL oxidation.](image)

Kinetic analysis of MDA in the MSE incubated HDL. The extent of HDL oxidation was analyzed by TBARS assay by measured as MDA equivalents. 1ml of HDL was incubated with 5ml of MSE for 5h, prior to assay.
2.2.4 Discussion

Pesticides are an undeniable part of modern life, used to protect get protection from insects and pest, extensively used in houses as well as in agricultural fields. In houses, mosquito coil is the common source pesticide which is used in most parts of the world without considering its ill effects on health. It has been reported that, burning one mosquito coil released the same amount of particulate matter as burning 75 to 135 cigarettes. The amount of formaldehyde emitted from burning one mosquito coil is as high as that emitted from burning 51 cigarettes (Weili Liu et al., 2003).

Narendra et al.,(2008) studied the biochemical profile of those who were chronically exposed to mosquito repellent. They found elevated levels of lipid peroxides, VLDL, triglyceride, SGOT and SGPT in their profile. Elevated lipid peroxidation is also observed in rats exposed to allethrin-based mosquito coil smoke for 15–180 days (Madhubabu & Yenugu, 2012). Kale et al, (1999) undertook study to determine pyrethroid-induced lipid peroxidation and found the increase of antioxidant enzyme such as catalase (CAT) and superoxide dismutase (SOD) in response to the increased oxidative stress (Kale et al, 1999). The relation between increased lipid peroxidation and insecticides is not clear.
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Oxidation of HDL and LDL is considered as risk factor for cardiovascular diseases. Although HDL has anti-inflammatory, antioxidant and pro-fibrinolytic activities, it can undergo oxidative modification thereby losing its protective role (Panzenboeck et al., 1997). But, there are enzymes associated with HDL which can revert back the changes and help in gaining back HDL to its normal function (Navab et al., 2001). In this regard, contribution of PON1 is imperative compared with other HDL associated enzymes. PON1 may cleave oxidized lipids and thereby inhibit HDL from the effect of oxidation (Aviram et al., 1998).

In the present study, we found elevated lipid peroxidation and decreased PON1 activity at initial hours (1-2h) of MSE incubation. However, PON1 activity was recovered at later hours (3-5h) along with the declining MDA level and lipid peroxidation. These observations point out the protective role of PON1 against HDL oxidation induced by mosquito coil repellent.
2.3 PESTICIDE POISONING AND PON1

2.3.1. Introduction

Pesticides and insecticides are synthetic chemicals used worldwide in controlling agricultural as well as domestic pests. Consequent to their indiscriminate use, humans are exposed to chronic and sometimes to acute exposures to these toxic chemicals. The chronic exposure is mainly through pesticide residues in fruits and vegetables (Department of Agriculture and Cooperation & Ministry of Agriculture, 2012). Hence, not only the pesticide manufacturers and agricultural workers are exposed to pesticides, but even the general public is at a risk of chronic exposure to pesticides.

Acute exposure to pesticide occurs frequently through accidental exposure in few cases, but in most cases, it occurs due to consumption of pesticides during suicide attempts (Figure 2.10). According to World Health Organization, about 17,000 deaths in India occur by suicide every year. About 50% of suicidal deaths are due to consumption of pesticides (Patel et al., 2012).

![Graph showing the percentage of poisoning agents in Mysore, India](source)

**Figure – 2.10 : Agents commonly used for poisoning in Mysore, India**
(Source: Jesslin, Adepu, & Churi, 2010)

In India, mainly four types of pesticides are used, namely: organophosphates (OPs), carbamates, organochlorine and pyrethroid pesticides. The mode of action of OP and carbamate pesticides is through inhibition of AChE activity. In fact, measurement of cholinesterase activity is used as a diagnostic and prognostic tool in pesticide poisoning (Thiermann et al., 2007).
PON1 activity of an individual can vary to a large extent. It is reported that PON1 protein levels can vary by at least 13-fold and the activity up to 40-fold (Costa, Vitalone, Cole, & Furlong, 2005). This implies that the ability of individuals to detoxify OP would depend on their PON1 concentration and activity. In this study, we have compared the PON1 activity and cholinesterase activity of subjects who attempted to commit suicide by consuming pesticides.

2.3.2 Materials and methods

Materials

Phenylacetate, CaCl$_2$, and Tris were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Liqui Cholinesterase kit (catalog no. 2105) was from Futura Systems (Rome, Italy). All other reagents used were of analytical grade and solvents were distilled before use.

Methods

Subjects

Study group consisted of 58 pesticide poisoned cases who were admitted to KR hospital Mysore, India, from August 2009 to January 2010. They were all the cases admitted for acute poisoning with pesticides, and formed the ‘convenience sample’. The period of the study was arbitrarily chosen to represent half a year. Among them, 43 subjects were males and 15 subjects were females (age group from 14 to 70 years old; mean 30.26 ± 12.36). They were diagnosed as OP poisoning by an emergency room physician using clinical signs and symptoms. Blood of these subjects were drawn immediately after admitting, processed to obtain serum, which was used for enzyme assays or kept frozen at -20 °C for future use. All the experiments involving human subjects were carried out in accordance with the protocol approved by Institutional Human Ethical Committee, University of Mysore, Mysore [Sanction order no. IHEC- UOM No. 38/PhD/2009-10], India.

PON1 status

The level of PON1 activity was determined by measuring arylesterase (AREase) activity in serum using phenyl acetate as a substrate. A 10μl of diluted serum (1:10 v/v) was added to 10mM Tris-HCl buffer, pH 8.0 containing 2mM CaCl$_2$ and 2mM phenyl acetate. The rate of generation of phenol was determined at 270nm
at 25 °C, using a continuously recording spectrophotometer (La Du & Eckerson, 1984). One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1μmol of substrate per minute.

**Cholinesterase measurement**

Cholinesterase activity of serum was determined using Futura Systems Liqui Cholinesterase kit (catalog no. 2105), which is based on a colorimetric assay technique. Thiocholine, released from butyrylcholine, reacts with Hexacyanoferrate III (yellow) to form Hexacyanoferrate II (clear). The decrease in absorbance was determined photometrically at 405nm, and is directly proportional to cholinesterase activity in the serum. One unit of enzyme catalyzes the production of 1μm of thiocholine per minute under the assay conditions (pH 7.7 and 37 °C) (normal values 4200-11200 U/L).

**Statistical analysis**

Correlation between PON1 and cholinesterase was made using Pearson product moment correlation coefficient. Difference between groups was tested using student’s t test at $p \leq 0.05$.

**2.3.3 Results**

PON1 activity and cholinesterase activity were determined in serum of the subjects who had consumed pesticides in an attempt to commit suicide. The findings of enzyme levels are summarized in Table 2.4. There was a significant difference in cholinesterase activity between the groups with low PON1 activity and high PON1 activity. Serum PON1 activity positively correlated with cholinesterase activity ($r = 0.2843$; critical value for 56 df = 0.258) in suicidal subjects (Figure 2.11). Figure 2.12 shows the cholinesterase level among low PON1 activity group and high PON1 activity group. Relative to the high serum PON1 group (group 2), low serum PON1 group (group 1) had higher inhibition of cholinesterase, and the results were significant ($p \leq 0.05$).
Table – 2.4 : Mean activities of serum PON 1 and cholinesterase in experimental groups

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Activity</th>
<th>PON1[AREase activity (U/l)]</th>
<th>Cholinesterase activity (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total subjects</td>
<td>PON1 activity of all the subjects</td>
<td>77759 ± 3482</td>
<td>3474.1 ± 330.48</td>
</tr>
<tr>
<td></td>
<td>Median=79389</td>
<td>Median=2961</td>
<td></td>
</tr>
<tr>
<td>Group1</td>
<td>Subjects with low PON1 activity</td>
<td>43893 ± 2922</td>
<td>2528 ± 433.82</td>
</tr>
<tr>
<td></td>
<td>(Lowest 1/3rd)</td>
<td>Median= 44275</td>
<td>Median = 2102</td>
</tr>
<tr>
<td>Group2</td>
<td>Subjects with high PON1 activity</td>
<td>108283 ± 3340</td>
<td>4576 ± 745.53</td>
</tr>
<tr>
<td></td>
<td>(Highest 1/3rd)</td>
<td>Median=104326</td>
<td>Median=3927.5</td>
</tr>
</tbody>
</table>

Figure 2.11 Correlation between PON1 and cholinesterase activities determined using Pearson product moment correlation coefficient (r = 0.2843, Critical value of 56 df = 0.258).
Figure – 2.12: Cholinesterase level among PON1 activity groups [Group1: low PON1 activity; Group2: high PON1 activity]. Results are significant ($p \leq 0.05$) when compared with corresponding values of group 1.

2.3.4 Discussion

Cholinesterase, also known as plasma cholinesterase or pseudocholinesterase or BuChE, belongs to the same structural class of proteins as AChE. AChE is a serine protease that hydrolyzes neurotransmitter acetylcholine at the neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission. AChE, primarily found in the neural synapses and also on the surface of erythrocytes, maintains the integrity of erythrocytes (Aloni & Livne, 1974).

Cholinesterase is synthesized in liver and secreted into plasma. It preferentially acts on butyrylcholine and hydrolyzes acetylcholine (Chatonnet & Lockridge, 1989). Cholinesterase activity can be measured in serum as surrogates for neuronal AChE activity. Cholinesterase measurement is used as diagnostic tool for pesticide poisoning because of advantages such as simple detection procedure, stable nature, easy-to-sample and reproducibility (Xu et al, 2010). Each person has a certain normal basal level of activity for the proper functioning of the nervous system. Variations in cholinesterase activity in blood are observed in various clinical conditions including the entry of natural (snake venom) or synthetic toxins (pesticides) into the human serum (Pohanka, 2012; Rodriguez et al, 1983).
Effect of Environmental Toxic Molecules on PON1

Inhibition of AChE activity in the central and peripheral nervous systems is considered to be an important mechanism of OP toxicity. AChE inhibition prevents the breakdown of acetylcholine, resulting in excessively increased cholinergic activity at the nerve synaptic gaps (Pope et al., 2005). Excess accumulation of acetylcholine at muscarinic receptors causes clinical complications such as visual disturbance, tightness in chest, wheezing due to broncho-constriction, increased bronchial secretion, increased salivation, lacrimation, sweating, peristalsis and urination (Eskenazi et al., 1999; Leibson & Lifshitz, 2008).

OPs are bio-activated in vivo via oxidative desulphuration and dealkylation to form its oxygen analogues (Oxon’s) and active metabolites (Tina & Metka, 2011). These OP-oxygen analogues are potent inhibitors of the enzyme AChE. Serum PON1 can hydrolyze the oxygen analogs of OP and it is very important in OP detoxification process (Costa et al., 2003). The substrate specificity of PON1 is unusually broad and not fully understood (Draganov et al., 2005). AREase activity is considered to be a good surrogate for PON1 concentration in plasma/serum (Furlong et al., 1993 and 2006). In our study, PON1 activity was significantly correlated with levels of cholinesterase in subjects who consumed pesticides. The individuals with lower PON1 activity (group1) also had lower cholinesterase activity, suggesting the inhibition of cholinesterase by pesticides. In contrast, individuals with higher PON1 activity (group2) had higher cholinesterase activity, indicating the involvement of PON1 in detoxification of pesticide poisoning (Figure – 2.12)

The findings of our study indicated the association between low plasma PON1 activity and cholinesterase inhibition and are consistent with the results of Sözmen et al., (2002) and Hofmann et al.,(2009). They found that PON1 activity was lower among subjects with low cholinesterase activity upon hospital admission relative to subjects with higher cholinesterase activity. Studies in transgenic mice clearly demonstrated that low plasma PON1 activity was associated with greater brain AChE inhibition after exposure to chlorpyrifos-oxon and diazoxon (Li et al., 2000). They also found that administration of PON1 abolished cholinergic signs and significantly protected it against AChE inhibition (Li et al, 1995). Supporting our data, Akgur et al (2000) found positive correlation between AChE and PON1 activities in a study with 18 agricultural male workers who were exposed to OP poisoning in Turkey (Akgür et al., 1999).
Limitations

Although the study has achieved its aims, there was a limitation. It was not possible to follow up the outcome of the treatment of suicide subjects since some of them were shifted to private clinics. Hence, the survival or otherwise of the subjects was not known.

Conclusion

PON1 and cholinesterase in the serum of subjects with pesticide poisoning were measured and found to correlate positively. Our study suggests that patients with higher PON1 activity may have a better chance of detoxifying the poisoning effects of pesticides and may have a positive effect on survival even though this could not be verified by this study.