A STUDY ON PHENOTYPIC POLYMORPHISM OF SERUM PARAOXONASE1 AND ACTIVITY OF SERUM CHOLINESTERASE IN ACUTE ORGANOPHOSPHOROUS COMPOUND POISONING

A Dissertation submitted to

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CHENNAI – 600 001

APRIL – 2015

CERTIFICATE BY THE GUIDE

This is to certify that the dissertation entitled, **"A STUDY ON PHENOTYPIC POLYMORPHISM OF SERUM PARAOXONASE1 AND ACTIVITY OF SERUM CHOLINESTERASE IN ACUTE ORGANOPHOSPHOROUS COMPOUND POISONING"** submitted by **Dr. ANANTHI. P** in partial fulfillment of the requirements for the award of Doctor of Medicine in Biochemistry by The Tamilnadu Dr. M.G.R. Medical University, Chennai is a bonafide record of the work carried out by her at the Department of Biochemistry, Govt. Stanley Medical College, Chennai-600 001, under my guidance and supervision, during the academic year 2012-2015.

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DECLARATION

I, **Dr. ANANTHI. P**, solemnly declare that this dissertation entitled, **"A STUDY ON PHENOTYPIC POLYMORPHISM OF SERUM PARAOXONASE1 AND ACTIVITY OF SERUM CHOLINESTERASE IN ACUTE ORGANOPHOSPHOROUS COMPOUND POISONING"** is a bonafide work done by me at the Department of Biochemistry, Govt. Stanley Medical College, under the guidance and supervision of **Prof. Dr. R. Mahalakshmi, M.D., DCH.,** Professor & HOD, Department of Biochemistry, Govt. Stanley Medical College, Chennai - 600 001. This dissertation is submitted to The Tamil Nadu Dr. M.G.R Medical University, in partial fulfilment of the requirements for the award of Doctor of Medicine in Biochemistry (Branch XIII).

Place: Chennai **Dr. ANANTHI. P**

Date:

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CONTENTS

ABBREVATIONS

4-AAP	-	4-aminoantipyrine
AChE	-	Acetyl Cholinesterase
ACS	-	Acute Cholinergic Syndrome
ADA	-	American Diabetic Association
AHL	-	Acyl Homoserine lactones
ANOVA	-	Analysis of Variance
AR	-	Analytical Reagent
BSA	-	Bovine Serum Albumin
BuChE	-	Butyryl Cholinesterase
CHD	-	Coronary Heart Disease
CHE	-	Cholinesterase
CV	-	Coefficient of Variation
CYP450	-	CytochromeP450
DFP	-	Diisopropyl fluoro phophonate
DGKC	-	Deutsche Gesellschaft fuer Klinische Chemie (German Society for Clinical Chemistry)
HDL	-	High Density Lipoprotein
IMS	-	Intermediate Syndrome
LDL	-	Low Density Lipoprotein
MM-LDL	-	Minimally Modified Low Density Lipoprotein
M_r	-	Relative Molar mass
NTE	-	Neuropathy Target Esterase
OPs	-	Organophosphates
OPC	-	Organophosphorous compounds
OPIDN	-	Organophosphorous Induced Delayed Neuropathy
OPIDP	-	Organophosphorous Induced Delayed Polyneuropathy

PONs	-	Paraoxonases
QC	-	Quality Control
RPG	-	Random Plasma Glucose
SD	-	Standard Deviation
SPSS	-	Statistical Package for the Social Sciences
TP	-	Total Protein

A STUDY ON PHENOTYPIC POLYMORPHISM OF SERUM PARAOXONASE1 AND ACTIVITY OF SERUM CHOLINESTERASE IN ACUTE ORGANOPHOSPHOROUS COMPOUND POISONING

Ananthi P, Uma Maheswari V, Mahalakshmi R.

Objective: Serum Paraoxonase1 (PON1) is involved in the metabolism of Organophosphorous (OP) compounds. PON1 is polymorphic and at least three phenotypes have been described so far. The phenotypic polymorphism of PON1 may affect the susceptibility of patients to OPC toxicity. This study is focused on the evaluation of phenotypic enzyme activities of serum Paraoxonase1 (PON1) and its relationship with serum Cholinesterase activity in 54 patients with Acute Organophosphorus compound (OPC) poisoning.

Materials and Methods: Serum paraoxonase and arylesterase activities of PON1 were estimated in dual beam spectrophotometer. Serum Cholinesterase activity was measured in Semiautoanalyser. The results were analyzed using SPSS version 16, where 'one way ANOVA' was used to compare mean values of variables among PON1 phenotypes and correlation analysis was done with 'Pearson coefficient of correlation'.

Results: Mean values of activity of Cholinesterase, Paraoxonase, Arylesterase were 2325 (SD ± 2027.19) U/L; 218.39 (SD ± 124.30) U/L; and 85.13 (SD ± 30.19) kU/L respectively. Activity of all three enzymes viz., Cholinesterase, Paraoxonase, Arylesterase varied with respect to PON1 phenotypes. PON1 BB phenotype has high Paraoxonase and Cholinesterase activity whereas AA phenotype has low Paraoxonase and Cholinesterase activity and the AB phenotype was reported with in between activity. Serum Cholinesterase was correlated positively and significantly with serum Paraoxonase (Coefficient of Correlation, $r = +0.767$, with p value < 0.0001) as well as with serum Paraoxonase/Arylesterase activity (P/A) (Coefficient of Correlation, $r = +0.955$, with p value < 0.0001) which was used to determine PON1 phenotypes.

Conclusion: To conclude, in case of Acute OPC poisoning, identification of PON1 phenotype can be useful to identify the susceptibility of patients to OPC toxicity.

Keywords: Organophosphorus compounds, Cholinesterase, Paraoxonase1, PON1 Polymorphism.

INTRODUCTION

Poisoning is one of the prevalent causes of poor health and mortality worldwide. Organophosphorus (OP) compounds used in agriculture as pesticides are increasing in developing countries like India. Intoxication to OP compounds leads to a major health problem globally[1].

Organophosphorous compounds are esters of phosphoric or thiophosphoric acids. Acute exposure to these compounds results in different grades of neuromuscular toxicity[2,3] due to inhibition of Acetyl Cholinesterase (AChE) and Neuropathy Target Esterase (NTE)[4,5]. Organophosphorous compounds also increase the oxidative stress and damage the nervous and muscular tissues[3,6,7]. Serum Cholinesterase activity is a sensitive diagnostic alternative for neural Acetylcholinesterase activity. Serial estimation of serum Cholinesterase activity is useful in monitoring the disease progress and grading the severity of toxicity[8].

Paraoxonase 1(PON1) is an aryldialkylesterase synthesized in the liver and secreted into the blood in association with high density lipoproteins (HDL)[9]. It is involved in the detoxification of organophosphorous compounds and has a protective role against OPC toxicity[10,11,12]. It reduces

the oxidative stress by clearing the oxidized low density lipoproteins (LDL), lactones and aromatic carboxylic acid esters from blood[13].

Paraoxonase1 is a polymorphic enzyme that has three phenotypes namely AA, AB, BB which has low, moderate and high level of enzyme activity respectively. It is determined by the genotypic polymorphism of PON1 at the coding region Q192R[14] of the genome.

The degree and the duration of inhibition of cholinesterase activity depend on the level of active 'oxon' form of OPC in blood and tissues which in turn is directly related to the activity of Paraoxonase1 enzyme[15]. Since serum PON1 is a polymorphic enzyme, the susceptibility of patients to the toxicity of OPC varies with the level of serum Paraoxonase1 (PON1) activity[15,16] .which is specific for a particular phenotype thereby the severity of poisoning and the recovery of the patients are affected by serum Paraoxonase1 phenotypic polymorphism.

The present study was undertaken to identify the role of serum Paraoxonase1 (PON1) phenotype in patients with Acute Organophosphorous compound (OPC) poisoning.

POISONING

Poison is a substance that causes harmful effects when administered, either by intentional or accidental, to a living organism by altering their biological structure or functions by acting at the molecular and biochemical level[17].The rapid development in medical, industrial and agricultural fields introduced a new range of synthetic chemical substances to improve the productivity[17]. In addition with the naturally occurring poisons, the easy availability and accessibility of the synthetic compounds has now become a major risk for poisoning[18].

According to World Health Organization (WHO) report in 2002-2005, there were more than 3 million poisoning cases with 2, 20, 000 deaths occurred annually all around the world[18]. A study done by Unikrishnan *et al* in 2005 has shown that poisoning is the fourth most common cause of mortality rate in India. The pattern of poisoning in a particular region is influenced by many factors such as availability and accessibility to the poison, educational and socioeconomic status of the people[19]. Pesticidal poisoning and self poisoning to them are more common in developing countries like India.[20].

Poisoning can be either acute or chronic based on their duration of exposure. Acute poisoning is a medical emergency which has been reported that it constitutes 10% of admissions in medical emergency departments in India[21]. Organophosphorus compounds (OP) are the most common pesticides which are being used in agriculture since late 1930s. The morbidity and mortality results from acute and chronic exposures of humans to OP pesticides have also become a major public health concern[22].

ORGANOPHOSPHOROUS COMPOUNDS

'Organophosphates' (OP) is a generic term given for the organic derivatives of phosphorus. OP compounds are generally esters, amides or thiol derivatives of phosphoric acids with two organic group and additional side chain groups. They are used as insecticides, nematocides, fungicides or herbicides and plant growth regulators worldwide[23].

GENERAL STRUCTURE AND PHYSICAL PROPERTIES OPCs

There are over 100 different OPCs produced for agricultural purposes. They usually differ in their physical, chemical and biological properties. OPCs are esters or thiols derived from phosphoric acid. They are slightly soluble in water. They have a high oil-water coefficient and low vapour

pressure. OPCs are lipophilic compounds and are degraded by hydrolysis yielding water soluble products[24].

The general structure of OP compound is shown in the Figure.1

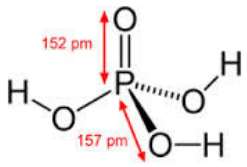


Fig.1: General Structure of Organophosphates[25].

The molecule of OP contains two lipophillic, aryl or alkyl groups (R1 and R2) which are bonded to a phosphorus atom either directly forming phosphinates, or through an oxygen or sulfur atom to form phosphorothioates. It contains highly electronegative –X group which can be a halide, aliphatic, heterocyclic or aromatic and it is known as 'leaving group' since it is the group which is released upon hydrolysis. The toxicity level depends on the –X group[25].

PHARMACOKINETICS OF OPCs

The pharmacokinetic activities of OPCs have been studied in animal models showed that the cholinergic toxic effects are similar to human symptoms which suggest that the AChE inhibition is an important determinant of the acute toxicity of OP intoxication[24,25].

1. ABSORPTION AND DISTRIBUTION OF OPCs

The OPCs are absorbed through skin, conjunctiva, oral mucosa, GIT, respiratory tract either by direct contact, ingestion, inhalation or injection[26]. Following absorption, the OPs are rapidly distributed in all tissues especially in liver, kidney, CNS and salivary glands and also in the fat stores. The lipophilic moiety of OP facilitates the absorption and redistribution. The elevated peak level is seen about 6 hours after absorption[24].

2. METABOLISM

The metabolism of OPCs occurs in the Liver by oxidation and hydrolysis which act on 'thioates' and 'oxons'. The oxidation products have different degree of toxicity[25]. The phosphorothioates (P=S) of OPCs are oxidized by desulphuration mediated by Cytochrome (CYP) 450 isoforms to their active oxon form which is the primary toxin. The active oxon forms which inhibit AChE are spontaneously hydrolyzed within 0.7 hours to 31 hours of exposure. The hydrolytic process is enhanced by hydrolases, such as the A-esterases, for example Paraoxonase1[16,26].

3. EXCRETION

The excretion mainly occurs via urine. A lesser quantity is eliminated through feces and exhaled air. The rate of excretion reaches its peak value within two days of intoxication and declines quickly[26,27].

MECHANISM OF TOXICITY

The basic mechanism of toxicity by OPCs is associated with the inhibition of Acetylcholinesterase (AChE) which occurs in a stepwise manner. The initial step is the nucleophilic attack of the serine hydroxyl moiety at the active site of cholinesterase on phosphorus group of OPCs. It results in the formation of reversible enzyme- inhibitor complex and then the cleavage of OPCs occurs. It results in the formation of a covalent P-O bond and the phosphorylated enzyme. This phosphorylated enzyme cannot hydrolyze acetylcholine[28,29]. The time interval between the exposure of OP intoxication and the formation of irreversible phosphorylated enzyme complex is referred to as 'aging' which differs with respect to the type of OPCs[29,30].

ACUTE POISONING

Clinical effects of acute OPC exposure are divided into three types depend on the onset and duration of clinical manifestations[22].

a. Initial acute cholinergic syndrome (ACS)

b. Intermediate syndrome (IMS)

c. Delayed polyneuropathy (OPIDN)

CLINICAL MANIFESTATIONS

The clinical manifestations of acute OPC poisoning and the onset of symptoms occur within 30 mins to 3 hrs. Depending upon the severity of exposure and the cholinesterase activity, the poisoning is classified as latent, mild, moderate and severe which is shown in Figure.2. The severity of poisoning is classified biochemically by using Namba., *et al* criteria for Serum Cholinesterase activity[8,24].

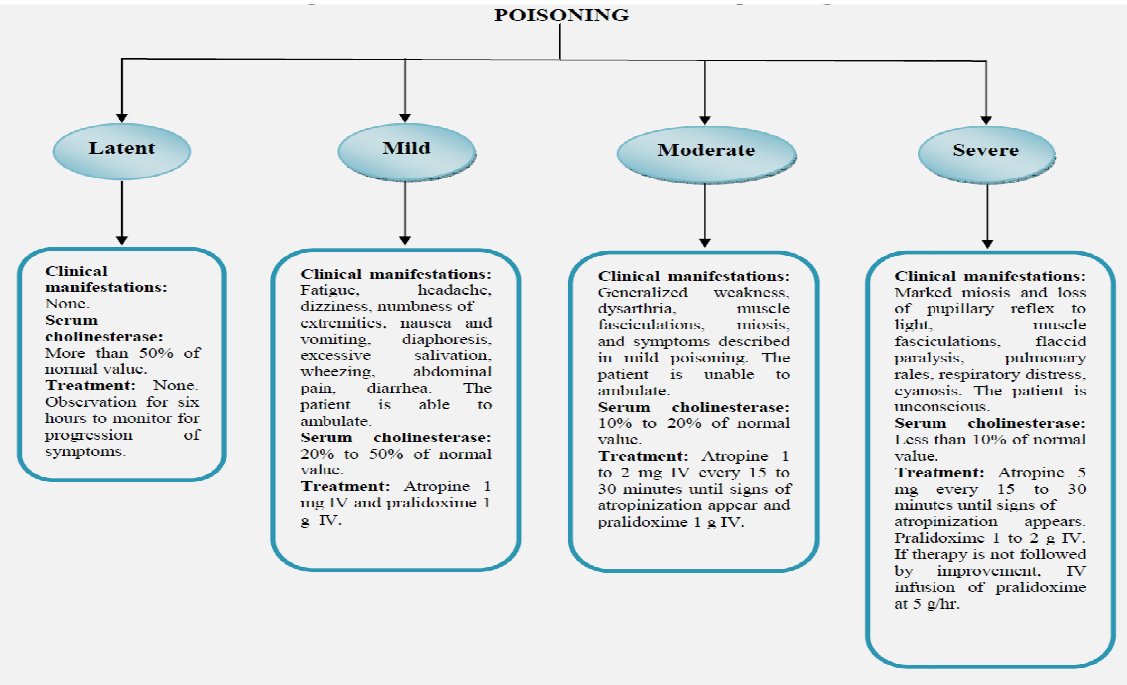


Fig.2: Classification of OPC poisoning based on the clinical manifestations on severity[24].

ACUTE CHOLINERGIC SYNDROME (ACS) (TYPE I PARALYSIS)

Acute Cholinergic crisis occurs within 48 hours of exposure[5].The accumulation of ACh due to the inhibition of AChE causes the initial stimulation of cholinergic synapses. On the basis of receptor stimulation, the clinical manifestations are classified into muscarinic, nicotinic, and central nervous system (CNS) effects[29].

Muscarinic	Nicotinic	Central
Miosis	Muscle Fasciculations	Unconsciousness
Blurred vision	Paralysis	Confusion
Nausea	Pallor	Toxic psychosis
Vomiting	Muscle weakness	Seizures
Diarrhoea	Hypertension	Fatigue
Salivation	Tachycardia	Respiratory Depression
Lacrimation	Mydriasis (rare)	Dysarthria
Bradycardia		Ataxia
Abdominal pain		Anxiety
Diaphoresis		
Wheezing		
Urinary Incontinence		
Fecal Incontinence		

Table.1: Clinical manifestations of OPC poisoning according to the receptor type[24].

INTERMEDIATE SYNDROME (TYPE II PARALYSIS)

The term intermediate syndrome (IMS) was first described by Senanayake and karallidde (1987) since it occurred in the interval between cholinergic crisis and the onset of organophosphorus induced delayed

polyneuropathy (OPIDP)[31]. It occurs 24 to 96 hours after the ingestion of an OP compound. This syndrome generally occurs among patients with severe and prolonged inhibition of AChE.

The clinical features are muscle weakness predominantly proximal muscles and neck flexors. Recovery from this type of paralysis takes place in 5 to 18 days unless it is complicated by infections and arrhythmias. The pathogenesis of intermediate syndrome is presumed to be due to dysfunction of neuromuscular junction and oxidative stress[24]. Although cholinergic crisis is the earliest manifestation, it is the intermediate syndrome which is responsible for the high mortality rate[31].

ORGANOPHOSPHATE INDUCED DELAYED NEUROPATHY (TYPE III PARALYSIS)

Organophosphate-induced delayed neuropathy (OPIDN) occurs usually by 7 to 21days after exposure due to the inhibition of neuropathy target esterase (NTE) enzyme in nervous tissues and spinal cord by certain OPCs. The neuropathy is found to be occurring due to phosphorylation and aging of enzyme NTE[6]. The clinical manifestations are associated with early symptoms, paraesthaesias and calf pain and symmetrical motor axonopathy with mild sensory loss[7].

DIAGNOSIS AND LABARATORY FINDINGS

Initial diagnosis is made from the history of exposure and detailed clinical examination. Miosis is a strong clinical indicator of OPC poisoning[29].

The diagnosis of exposure to OPC is biochemically confirmed by the estimation of Cholinesterase activity. Clinical manifestations appears when the AChE activity is <50% of normal and BuChE is <70% of normal. Since BuChE is relatively more sensitive to inhibition and rapid to recover, it can be used in monitoring the disease progression and response to therapy[8,24]. Metabolites of OPCs in urine can be tested to confirm the exposure and to estimate the level of absorption of OPC from stomach. Although the direct measurement of OPC in blood on admission gives the level of severity of poisoning is practically difficult for routine use[32].

MANAGEMENT

OPC poisoning is a medical emergency and the patient has to be hospitalized in the ICU. The initial management of OPC poisoning is to ensure a secured airway and the decontamination of skin and stomach. Further therapeutic management is based on the history of intoxication,

clinical assessment and level of serum CHE activity which is used for monitoring[32].

SPECIFIC ANTIDOTE THERAPY

The antidote therapy includes muscarinic antagonist usually atropine, and oximes. Atropine is an anticholinergic drug[33] whereas oximes are nucleophilic substances clinically used to reverse the activity of inhibited AChE before aging and are to be given as early as possible[34]. Despite the reactivation of AChE, the use of oximes has little effect on the outcome of OPC poisoning. However the animal studies have shown that oximes are useful in reactivation of AChE with low level of serum OPC[34].

XENOBIOTIC METABOLISM

The biotransformation of foreign substances (Xenobiotics) by enzymatic pathways which occurs primarily in the liver is known as xenobiotic metabolism[35]. There are two metabolic pathways: Phase I and Phase II metabolism. The phase I metabolism consists of oxidation and hydrolysis by cytochrome P-450-dependent mixed-function oxidase system and esterases[36].

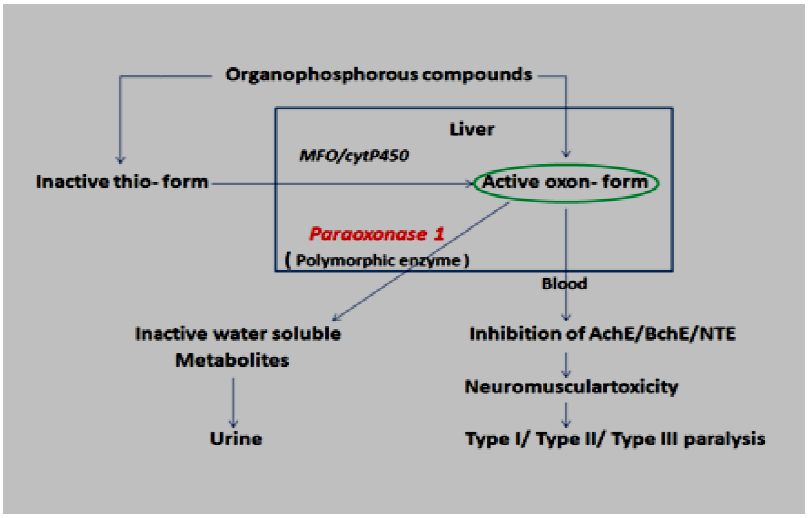


Fig.3: Xenobiotics of Organophosphorous Compounds

The inactive thio-organophosphates are activated by the action of phase I metabolism which consists of various Cytochrome P450s (CYPs), especially CYP2B6 and CYP2C19 are involved[37]. The active oxon forms of OPCs are then hydrolyzed by A-esterases especially the polymorphic Paraoxonase1 (PON1) enzyme.

SERUM ESTERASES

Esterases are classified into A-esterase and B-esterases depending upon the inhibition of the enzyme by Diethyl p-nitrophenyl phosphate (E600)[38,39]. A-esterases are not inhibited by E600 while B-esterases are sensitive to E600. Paraoxonases is a member of A-esterases. Cholinesterases belongs to the group of B-esterases[40,41].

CHOLINESTERASE

Cholinesterase (CHE) [EC 3.1.1.8], Acylcholine acylhydrolase contains a serine residue in the conserved region of –Gly-X-Ser-X-Gly motif at the active site of the enzyme[42]. It catalyzes the hydrolysis of ester of choline including acetylcholine (Ach). Cholinesterase is essential for the cholinergic neuron to return to its resting state after activation. It was first discovered and suggested by Dale in 1914 and was established by Loewi and Navratil in 1926[43].

CHE has been found in many tissues including central nervous system, peripheral nervous system, plasma and RBCs. CHE is classified into two types, Acetyl cholinesterase [EC 3.1.1.7] and Butyrylcholinesterase [EC 3.1.1.8] based on the ability of the enzyme to specifically hydrolyze the choline esters[42,43].

ACETYL CHOLINESTERASE [EC 3.1.1.7] AChE

Acetylcholinesterase is also known as true cholinesterase, specific cholinesterase or acetylcholine acetylhydrolase. It hydrolyzes the cationic neurotransmitter acetylcholine into inactive metabolites choline and acetic acid at the post-synaptic nerve ending and terminates the signal transmission[44]. It is synthesized by hematopoiesis and present in conducting

tissues such as neuromuscular junctions, central and peripheral nervous tissues, motor and sensory fibers, cholinergic and non- cholinergic fibers and also in RBC cell membranes[45]. AChE is a highly specific catalytic enzyme which diffuses 25000 molecules of Ach per second and responsible for the diffusion-controlled reaction[43].

STRUCTURE

The molecule of AChE is an ellipsoid with a homodimer containing 531 amino acids. Each subunit is of 69kDa. It contains two subsites namely 'anionic' and 'esteratic' corresponding to the catalytic and choline binding pocket respectively[46].

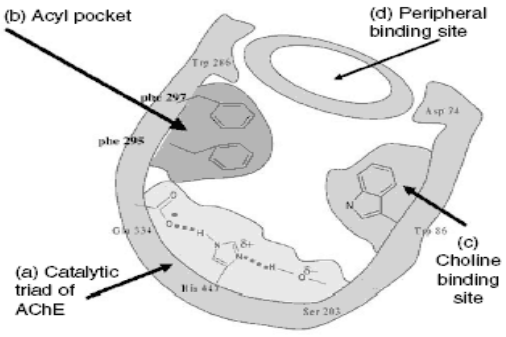


Fig.4: Active site of AChE

Figure.4 explains the active site of AChE containing the two binding subsites viz, 'esteratic' and 'anionic'sites. In addition to two subsites of active centre, AChE contains one or more peripheral anionic sites distinct from the choline-binding pocket of the active site. It acts as the binding site

for ACh and quaternary ligands[44]. The 'esteratic' site resembles the serine hydrolase and acts as a binding site for the choline molecule. The 'anionic' site interacts with the charged quaternary ammonium group of the choline residue[47].

MECHANISM OF ACTION of AChE

The anionic subsite of AChE is uncharged and lipophilic. The mechanism of enzyme involves the electrostatic attraction between positively charged quaternary amine of choline moiety of Ach[48].

The hydrolysis of acetylcholine is initiated by the acetylation of serine hydroxyl 200 in the esteratic subsite[46].

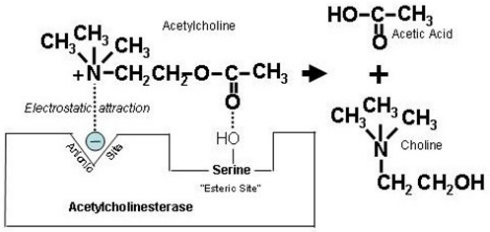


Fig.5: shows the hydrolytic degradation of Ach into Choline and acetic acid.

The enzyme binds to the acyl moiety and forms the acyl-enzyme and leaves the free choline. Then, the acyl-enzyme undergoes nucleophilic attack by a water molecule. It is assisted by histidine 440 and liberates acetic acid and finally the free enzyme is regenerated[44].

ISOFORMS OF AChE

AChE exists in various oligomeric forms which are identical in catalytic properties, but differ in their quaternary structure and physical properties. It is encoded by a single gene located in the long arm of chromosome 7 (7q22). The variation in isoforms results from alternative mRNA processing and post translational modifications of catalytic and structural subunits[49].

BUTYRYLCHOLINESTERASE [EC.3.1.1.8] BuChE

BuChE is also known as pseudo cholinesterase. It is an α-glycoprotein synthesized in the Liver and is found in blood plasma, smooth muscle, pancreas, adipocytes, skin, brain and heart. The normal value ranges from 5900-13,200 IU/L[50].

STRUCTURE

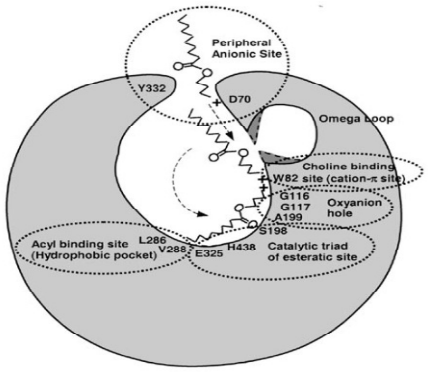


Fig.6: Structure of active site of BuChE

The gene for BuChE is lies on the long arm of chromosome 3(3q26)[51]. It occurs as a tetrameric form and contains 574 amino acids with Mr of 85kDa. The active site lies at serine 198 residue from the N- terminal[52].

MECHANISM OF ACTION

The mechanism of action of BuChE resembles that of AChE. BuChE has three subsites at the active centre of the enzyme viz, peripheral anionic site (PAS) at the entrance of enzyme surface, narrow aromatic gorge leading halfway inside the enzyme and active site found at the bottom of aromatic gorge[51]..

INHIBITORS OF AChE AND BuChE

The inhibitors of CHE are classified into two groups, Reversible and irreversible according to the mechanism of action. Carbomates are reversible whereas OP Compounds are Irreversible Inhibitors[28,54].

MECHANISM OF INHIBITION

The inhibition of AChE by OPCs is associated with the nucleophilic attack of serine residue to the alkyl phosphorus group of OPCs[29,54]. Organophosphates strongly phosphorylate the cholinesterases. Unlike the acetylated enzyme the phosphorylated form of enzyme is highly stable and is incapable of regenerating the enzyme activity. Initially the phosphorylation

process is reversible and further enzymatic reaction makes irreversible AChE[54].

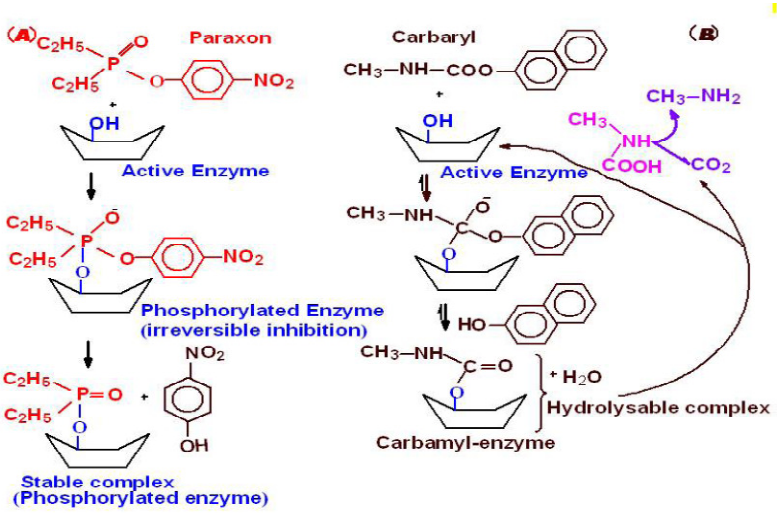


Fig.7: Mechanism of inhibition of OPCs.

Figure.7 explains the nucleophilic attack of serine residue at the active site of the enzyme to the phosphorus of OPCs[51].

AGING OF CHOLINESTERASE

The irreversible phosphorylation of enzyme forms a stable phosphorylated AChE. The time interval between the exposure of OPC intoxication and the formation of phosphorylated enzyme is known as 'aging'[34,55]. Once the aging process is achieved, the binding between OPC and serine moiety of active site is permanent. The aging process takes place at varied time intervals, depending upon the nature of the organophosphorus

compounds, for example dimethyl OP has half life of 3.7 hours after exposure while it is 33 hours in the case of diethyl OPCs[56].

COMPARISON OF AChE AND BuChE ACTIVITY

Characteristics	AChE	BuChE
Site	Nerve synapse, Neuromuscular Junction, RBC memebrane	Plasma, Liver, Kidney, Adipose tissue
Structure	Homo dimer	Tetramer
Active site	14 aromatic amino acids	8 aromatic amino acids
Substrates	Acetylcholine	Tributyrin, Tripropionin, Succinyl choline
Half life	120 days	7- 12 days
Clinical Features	Appear when >50% of enzyme is reduced	Appear when >70% of enzyme is reduced
Inhibition/Regeneration	Relatively slow but strong inhibition by inhibitors	Rapid inhibition and fast regeneration
Biochemical importance-Analysis	More Specific to confirm the exposure to inhibitors	More sensitive. Used in monitoring the response to therapy

Table.2: Comparison of AChE and BuChE[53]

REACTIVATION OF CHOLINESTERASE

The reactivation compounds of inhibited enzymes are cationic oximes formed from pyridine aldehydes which acts as a nucleophile. The mechanism of action of oximes involves the nucleophilic attack on phosphate group of OPC, thus forms a phosphorylated oxime and release the

enzyme activity before the process of aging[56]. Reactivation is effective when the oximes are given within one hour and the reactivation may vary with the type of OPCs used and the blood level of OPC[34].

PARAOXONASES

Paraoxonases are a group of enzymes belong to A-esterases. These enzymes act only catalytically and not stoichiometrically on their substrates where the active centre of the enzyme is not altered in their hydrolytic activity. The name "Paraoxonases" is derived from its commonly used in-vitro substrate Paraoxon (formerly as E600) which is used for the analysis of its enzyme activity[39,41].

ISOENZYMES OF PARAOXONASES

Human Paraoxonase has three isoenzymes namely PON1, PON2, PON3 and the genes are located on the long arm of Chromosome 7q21.3-22.1. It was found that PONs of mammalian species shared 60% and 70% of identity in their amino acid and nucleotide sequence respectively[12]. The isoenzymes differ in their tissue distribution, substrate specificity and degree of activity[57,58].

STRUCTURAL CHARACTERIZATION AND TISSUE DISTRIBUTION

PON1 is synthesized in the liver and secreted into the blood in association with High density lipoprotein (HDL) bound to Apo-A1. However HDL and ApoA1 concentrations are not related to the genotypes and activity of PON1[59]. Serum PON1is a heterodimer with 354 amino acid residues and the molar mass (Mr) of each subunits are 39kDa and 42kDa[60]. PON1 is dependent on Calcium ions for its maximum enzyme activity which can also be stimulated in-vitro by the addition of 1M sodium chloride[61,62].

PON2 is present in almost all tissues as a constitutive enzyme in humans with highest expression in liver, lung, heart and placenta. It is a homodimer with the Mr of 39kDa[12,60]. PON3 is synthesized primarily in the liver. It is also an HDL associated protein like PON1 with molecular mass of 40kDa.

ENZYMATIC ACTIVITIES OF PARAOXONASES

The isoenzymes of PONs are basically lactone hydrolases or lactonases [EC 3.1.8.1] with overlapping substrate specificities that hydrolyze lactones which are the oxidative products of arachidonic acid, docosahexaenoic acid, homoserine, homocyteine [12,59,60].

An N-acyl-L-homoserine lactone + H_2O →N-acyl-L-homoserine + lactone

They have two more allozymes activities such as arylesterase and paraoxonase that are governed and controlled by the presence of two alleles at same autosomal locus responsible for the polymorphism which affects the degree of enzyme activity[58,60].

Arylesterase [EC 3.1.1.2] activity is present in all PONs by which they metabolize the oxidative products of Low density Lipoprotein (LDL) and aromatic carboxylic acid esters with variable degree of activity. PON3 has limited arylesterase activity[60]. The reaction is written as

Aromatic carboxylic acid ester + H_2O →an aromatic alcohol +

carboxylic acid

PON1 is the only isoenzyme which has maximum paraoxonase [EC 3.1.1.81] activity that hydrolyzes a variety of OPCs[12,57,59,60]. PON3 has very low paraoxonase activity and PON2 does not have the following activity.

An aryl dialkyl phosphate +H_2O → an aryl alcohol + dialkyl phosphate

The bulky drugs like spironolactone and lipid lowering drug lovastatin are hydrolyzed only by PON3 by its lactonase activity[12,60].

PHYSIOLOGICAL ROLE OF PONs

Paraoxonase 1 and 2 mediate the enzymatic protection for all tissues (anti oxidant) mainly by preventing the endothelial damage to vascular

system caused by oxidative stress. They prevent Low density lipoprotein (LDL) lipid peroxidation and reverse the oxidation of minimally modified LDL (MM-LDL) and also inhibit the ability of oxidized LDL to induce monocyte chemotaxis and thereby the consequent series of events leading to atheroma formation[60].

PON2 has high lactonase activity towards Acyl homoserine lactones (AHL) which is produced by the quorum sensing bacterial species for the regulation and expression of their virulence genes. PON2 inactivates the AHL either by cleaving the ester bond or opening the lactone ring and disturbs the quorum sensing in pathogenic bacteria. Studies have shown that PON 2 expression was up regulated during infectious process and this aided the monocyte maturation to macrophages[59] that explained its role in innate immune response.

PON1 has an exclusive action in Xenobiotics for the detoxification of OPCs[15]. The clinical studies carried out so far on Paraoxonases revealed that any changes at the genetic level of PONs have effect on their enzymatic activity.

POLYMORPHISM OF PARAOXONASES

PON1 has more than 160 genetic polymorphisms, among which the two polymorphism in the coding region and five in the promoter region have

been studied extensively[10,12]. Promoter region polymorphism contributes to the variations in the expression and concentration of the enzyme[63]. The Leucine to Methionine polymorphism at coding region 55 (L55M) is associated with the variability in enzyme levels. The PON1 coding region Q192R (Glutamine/ Arginine) polymorphism has been widely investigated for its protective role in OPC toxicity and cardio vascular disease (CVD)[10,12,63].

PON2 has a clinically significant polymorphism at the codon 311 (Cysteine/Serine) which are associated with Coronary heart disease (CHD)[59,64]. Polymorphism of PON3 is the least studied compared to PON1 and PON2[64]. The frequency of all the polymorphic forms of PONs varies considerably in different races.

PARAOXONASE 1 (PON1)

Paraoxonase1, aryl dialkylphosphatase [EC 3.1.8.1] is a member of Paraoxonase hydrolase family which belongs to 'A-esterase' type of serum esterases[65]. Human PON1 is a glycosylated protein.

Hassett., *et al* (1991) explained that the hydrophobic leader sequence of the mature protein is retained from which only the starting amino acid methionine is cleaved. Sorenson., *et al* (1999) described that the retained N-

terminal signal peptide is the required structure for the association of PON1 with HDL. It was reported that PON1 has two Ca^{2+} binding sites needed for its stability and activity[59]. PON1 has histidine and tryptophan residues at its active site[66]. The 3D structure of PON1 enzyme has been described by Harel M., *et al* and it is a 6-bladed beta-propeller with a unique active-site lid which is also involved in binding to HDL[67].

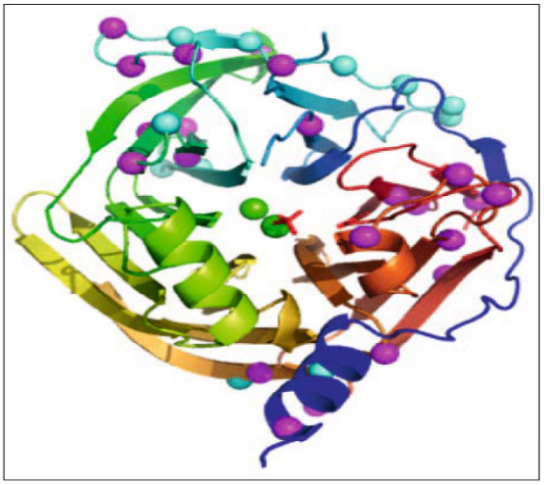


Fig.8: Cartoon image of the structure of rPON1 variant G2E6. The calcium ions are denoted as green balls at the center; phosphate ion as red stick model, reproduced from the work of Harel M., et al (2007).

Animal studies done by Lourdes Rodrigo., *et al* showed that Paraoxonase antigen is present in the endothelial cells of all tissues, in the centrilobular region of hepatocytes and within the endothelial cells of capillaries and arteriolar branches of hepatic artery. Paraoxonase1 is present

in the microsomes of a cell along with the line of CYP_{450} of xenobiotic system of the Liver[66].

Paraoxonase1 is basically a lactonase which also has two different allozyme activities namely paraoxonase and arylesterase due to the presence of two alleles at one autosomal locus which appears to be inherited as a simple dominant mendelian trait[61]. Paraoxonase activity is stimulated by high concentration of Sodium Chloride (1M). Both enzymes are dependent on calcium ions for their effective activity[62]. Serum PON1 activity is widely variable globally.

The catalytic mechanism of serum PON1 has been elucidated by Harel M., *et al* (2007) by determining its catalytic pH-rate profile. It has been shown that there is production of hydroxide ion by deprotonation of water molecule by the His-His dyad and calcium complex present at the active site of the enzyme without changing its structure stoichiometrically. This hydroxide anion attacks the carbonyl ester bond of the substrate to form an intermediate which is broken down into water soluble products and left the enzyme free[67].

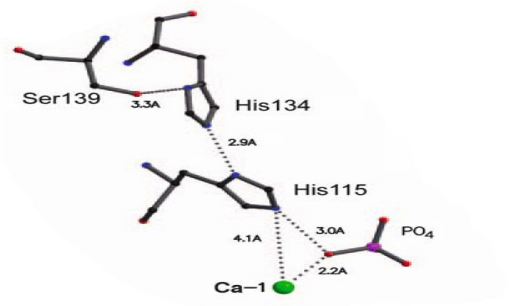


Fig.9: The postulated catalytic site of PON1

Figure.9 shows the postulated catalytic site of PON1 where the upper calcium atom (Ca-1) as green ball, the phosphate ion at the bottom of the active site, and the postulated His-His dyad, reproduced from the work of Harel M, et al (2007).

Catalysis occurs by the attack of ester bond by the hydroxide anion. This anion is formed by deprotonation of water molecule by His dyad in the presence of calcium and phosphate ions at the active centre.

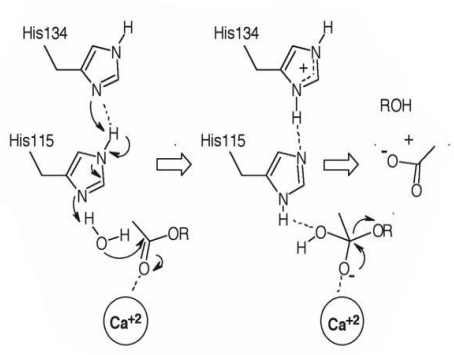


Fig.10: The postulated mechanism of action of Serum PON1, reproduced from the article of Harel M, et al (2007)

Serum PON1 degrades the toxic active forms of a variety of OPCs by its paraoxonase activity by which it plays a clinically significant role in OPC poisoning. It reduces the oxidative stress by reducing the peroxidation of membrane phospholipids caused by toxic substances. It was postulated that the protectivity and susceptibility to OPC toxicity is influenced by the level of activity[15]. The level of activity of the allozymes is affected mainly by the polymorphism of PON1 at the coding region192 where Glutamine is replaced by Arginine.

It was shown that paraoxonase activity is highly variable than arylesterase activity in Q192 R polymorphism and their ratio which clearly discriminates the enzyme activity into three types that exists as three phenotypes namely AA, AB, BB that has low, moderate and high level of activity respectively which affects the level of OPC in blood[61]. It was identified that these phenotypes are genetically determined as QQ, QR, RR of PON1 due to polymorphism at Q192R[61,68]. It was also found that though the frequency of phenotypes is widely variable worldwide, it is not significantly different in both gender and age groups.

Experimental studies on knockout mice of PON1 have shown that the susceptibility to OPC toxicity and atherosclerosis was increased in mice which was totally lacking of PON1[69]. It was shown in animal studies that

PON1 level was up regulated in adult mice after exposure to OPC[70] which has been evidenced by immunostaining by Harel M, *et al* (2007). Animal studies postulated that therapy with PON1 may prove beneficial in OPC poisoning by its metabolic capacity in lowering the concentration of OPC in blood and tissues[71].

RATIONALE FOR THE STUDY

Acute OPC poisoning is a medical emergency. It has been reported that approximately 10% of admissions in intensive medical care department in India accounts for acute OPC poisoning[21].

Early diagnosis and assessment of severity of poisoning is crucial in saving lives. Direct measurement of OPC in blood and its metabolites in urine is practically difficult as a routine procedure. Estimation of AChE is used to confirm the exposure of OPC. Although serum Cholinesterase activity has a significant role in monitoring the progression of disease and response to therapy, it could not predict the level of OPC in blood and extent of toxicity[34].

The reinhibition of CHE occurs with high level of OPC even with the high dose of oxime therapy. The reason for this failure of response to oxime therapy is the high concentration of OPC in the blood and tissues[71]. Paraoxonase1 (PON1) is a xenobiotic enzyme that degrades the active OPC and reduces the oxidative stress which is one of a cause for the intermediate syndrome thereby has a protective role in OPC poisoning[13,14]. Since Paraoxonase1 is a polymorphic enzyme, its phenotypes differ in their level of enzyme activity will affect the level of OPC in blood and tissues[12] and the severity of disease.

We therefore proposed to study and evaluate the relationship between the enzyme Cholinesterase that is inhibited by OPCs and the enzyme Paraoxonase1 that detoxifies the OPCs in ‘Acute’ OPC poisoning with the following objectives.

AIM

To identify the serum Paraoxonase1 (PON1) phenotype and its relationship with serum Cholinesterase activity in patients with acute Organophosphorous compound (OPC) poisoning.

OBJECTIVES

1. To determine the phenotype of serum Paraoxonase1 (PON1) by using the ratio of the allozymes Paraoxonase and Arylesterase activities of serum Paraoxonase1enzyme.
2. To estimate the serum Cholinesterase enzyme activity.
3. To assess the correlation between the phenotypic enzyme activity of serum Paraoxonase1 and serum Cholinesterase activity in acute OPC poisoning.
4. To establish the need for identification of serum Paraoxonase1 phenotype in patients with acute OPC poisoning for the early detection of their susceptibility to OPC toxicity.
5. To emphasize the significance of Serum PON1phenotyping for the extended medical management and follow up of those patients with susceptible phenotype so as to ensure a better prognosis.

MATERIALS AND METHODS

STUDY DESIGN

The study design is an experimental analytical observational correlation between two variables in acute OPC poisoning.

STUDY CENTRE

1. Department of Biochemistry, Govt. Stanley Medical College and Hospital, Chennai.
2. 24 hours Clinical Biochemistry - Central Laboratory, Govt. Stanley Hospital, Chennai.
3. Intensive Medical Care Unit (IMCU), Govt. Stanley Hospital, Chennai.

STUDY DURATION

Six months

STUDY POPULATION

Patients with acute OPC poisoning, admitted in Intensive Care Unit, Govt. Stanley Hospital, Chennai.

CRITERIA FOR SELECTION

Inclusion criteria

1. Patients who are exposed to Organophosphorous compound (OPC) and have been admitted within 24 hours of exposure.
2. Patients who are willing to participate in the study with informed consent.
3. Age: 18-65 years of age
4. Both genders

Exclusion criteria

1. Patients who have long standing co-morbid medical illnesses like chronic liver diseases, Diabetes mellitus.
2. Patients who are exposed to other poisons along with OPC.

SAMPLE SIZE

54 Patients with Acute OPC poisoning

STUDY PROCEDURE

The study was conducted at the Department of Biochemistry, Govt.Stanley Medical College, Chennai and in the 24 hours Clinical Biochemistry Laboratory, Govt. Stanley Hospital, Chennai. The sources of data were collected from the Intensive Medical Care Unit (IMCU),

Govt.Stanley Hospital, Chennai. The study was commenced with the approval obtained from the Institutional Ethics Committee, Govt. Stanley Medical College, Chennai.

ETHICAL CONSIDERATIONS AND RECRUITMENT OF STUDY POPULATION

All the Patients who were exposed to Organophosphorous compounds had been admitted within 24 hours of exposure were informed about the study purpose without interfering with their routine management. Only those patients who agreed to participate in the study were recruited. Informed consent was obtained from all the patients who participated in this study. All the procedures, potential risks and benefits related to the study were fully explained in their mother tongue. All their queries were answered to their satisfaction.

CLINICAL ASSESSMENT

A detailed history about OPC exposure and comorbid medical illness was taken and a complete clinical examination was done for the patients who participated in the study. The socio-demographic details along with their contact numbers were noted in the clinical assessment Proforma.

SAMPLE COLLECTION

5 ml of venous blood from Antecubital vein was collected under strict aseptic conditions from patients who fulfilled all the inclusion criteria. 2ml of blood was taken in Potassium oxalate, Sodium Fluoride vacutainer and the remaining 3ml in a plain vacutainer. Samples for measuring serial estimation of serum Cholinesterase activity were collected on Day 1, 3 and 5 of admission.

SAMPLE PROCESSING AND STORAGE

Samples for plasma were centrifuged within 20 minutes of collection at 2500 rpm for 10 minutes. Samples for serum were centrifuged after clot retraction at 2000 rpm for 15 minutes. Plasma and Serum were collected as two aliquots of 500µl. The samples were analyzed immediately for the following parameters.

ANALYTES

1. Serum Total Protein.
2. Random Plasma Glucose.

Samples containing values within the normal range were analyzed for the following enzyme activity.

ENZYME ACTIVITY

1. Serum Cholinesterase activity
2. Serum Paraoxonase activity of PON1
3. Serum Arylesterase activity of PON1

The serum CHE activity was measured in day 1, 3 and 5 samples and the average value was taken for statistical analysis. Remaining samples were stored at -20^{o}C to assess inter-assay variability.

COLLECTION AND PROCESSING OF QUALITY CONTROL SERA

200µl of serum was obtained from the blood collected from each healthy individual who attended the Master health check up at 24 hours Clinical Biochemistry Laboratory, Govt. Stanley hospital, Chennai and mixed well to obtain the 10ml of Quality Control (QC) Sera.

15% of Ethylene Glycol was added as a preservative to the Quality Control (QC) Sera and stored as 40 aliquots of 250 µl at -20^{o}C. All the three enzymatic activities mentioned above were analyzed before and after the addition of preservative to identify the effect of Ethylene glycol.

SAMPLE ANALYSIS

ESTIMATION OF SERUM TOTAL PROTEIN

Method

Biuret Method

Principle

The Cupric ions (Cu^{2+}) of Biuret reagent form purple coloured multivalent complexes quantitatively with peptide bonds of proteins under strong alkaline medium. The intensity of the purple colour is directly proportional to the concentration of serum total protein[72].

Reference range

Adults: 6.5 – 8.5 g/dL[72]

Criteria for inclusion in the study

Only patients who had total serum proteins above 6.5g/dL were included.

ESTIMATION OF RANDOM PLASMA GLUCOSE

Method

Trinder's method (Commercial Reagent Kit)

Principle

Glucose is oxidized to Gluconic acid and hydrogen peroxide by Glucose oxidase (GOD). The peroxidase (POD) enzyme catalyzes the oxidative coupling of 4-aminoantipyrine (4-AAP) with phenol to yield a coloured complex quinoneimine and its absorbance is directly proportional to the concentration of plasma glucose of the sample[72].

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{POD}]{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-AAP} \longrightarrow \text{Quinoneimine dye} + 2H_2O$$

Reference range

Adult (Fasting): 74 – 100 mg/dL[72]

Random Plasma Glucose: <200 (ADA Criteria)

Criteria to include in the study

Only patients with Random Plasma Glucose level below 200mg/dl were included in the study.

MEASUREMENT OF SERUM CHOLINESTERASE ACTIVITY

Method

Kinetic method as per the recommendation of DGKC (Commercial Reagent Kit)

Principle

Serum Cholinesterase (CHE) catalyzes the hydrolysis of butyrylthiocholine present in the reagent to form butyrate and thiocholine. Thiocholine reduces the yellow coloured hexacyanoferrate III to colourless hexacyanoferrate II. The change in absorbance is directly proportional to the activity of serum cholinesterase in the sample[72].

$$\text{Butyrylthiocholine} + H_2O \xrightarrow{\text{CHE}} \text{Thiocholine} + \text{Butyrate}$$

$$\text{Thiocholine} + \text{Hexacyanoferrate III} \longrightarrow \text{Hexacyanoferrate II}$$

Reagent Composition

1. Reagent 1

Active Ingredients	**Concentration**
Hexacyanoferrate III	2 mmol/L
Pyrophosphate Buffer (pH 7.6)	75 mmol/L

2. Reagent 2

Active Ingredients	**Concentration**
Butyrylthiocholine	15 mmol/L

Working Reagent preparation

4 parts of Reagent1 with 1 part of Reagent2 were mixed to prepare the working reagent which was stable for 3hours at 15 – 25^{o}C.

Procedure

Analysis was done in a semi-auto analyzer after Quality control analysis with the preserved QC sera. Linearity of the reaction was checked for 5minutes in kinetic mode. Levey Jenning's chart was plotted to monitor the performance of the test on a daily basis. To 1000µl of working reagent, 50µl of serum sample was added. Reagent and sample were mixed well and incubated for 30 seconds at 37^{o}C. The Change in absorbance per 30 seconds for the total measuring time of 90 seconds was measured at 405nm.

Assay Parameters

Mode of reaction - Kinetic mode

Slope of reaction - Decreasing

Wavelength - 405 nm

Temperature - 37^{o}C

Kinetic Factor	- 22653
Blank	- Distilled Water
Delay/ Lag time	- 30 seconds
No of readings	- 3
Interval	- 30 seconds
Reagent volume	- 1000 µl
Sample volume	- 50µl

Calculation

Serum Cholinesterase Activity, U/ L = Δ Abs/ minute **X** Factor

Where, Δ Abs/ minute - Change in Absorbance per minute

Factor = 22653

Reference range[72]

Adult Females	: 3930 – 10800 U/L
Adult Males	: 4620 – 11500 U/L

MEASUREMENT OF SERUM PARAOXONASE ACTIVITY OF PON1

Method

Eckerson., *et al* method[12,13].

Principle

Serum Paraoxonase1 hydrolyzes the pale yellow coloured substrate Paraoxon [*o*,*o* diethyl *o*- (4 –nitrophenyl phosphate)] into 4- nitrophenol and diethyl phosphate in the presence of a divalent cation ca^{2+} by its Paraoxonase activity. The increase in the intensity of yellow colour due to the formation of 4-nitrophenol is directly related to the enzyme activity which is stimulated by high concentration Sodium Chloride.

$$\text{Paraoxon} + H_2O \xrightarrow[Ca^{2+}/\ NaCl]{PON1} \text{4- nitrophenol} + \text{Diethylphosphate}$$

Chemicals

All the chemicals are reagent grade in purity.

1. Paraoxon
2. Tris (hydroxyl methyl) amino methane
3. Calcium chloride dihydrate

4. Sodium Chloride

5. Concentrated Hydrochloric acid

Reagent Composition

1. Reagent 1

Active Ingredients	**Concentration**
Tris- HCl Buffer (pH 8.0)	50 mmol/L
Calcium Chloride	0.9 mmol/L
Sodium Chloride	1 mol/L

2. Reagent 2

Active Ingredients	**Concentration**
Paraoxon- ethyl PESTANAL	2 mmol/L

Reagent preparation

1. Tris Base stock: 0.5 mol/L

6.057g of Tris (hydroxyl methyl) amino methane was dissolved in 80ml of distilled water and made up to 100ml which was stored in a polyethylene bottle at room temperature.

2. Preparation of 3N HCl: 3mol/L

25ml of Concentrated Hydrochloric acid (12N HCl) was dissolved with 60ml of distilled water and made into a final volume of 100ml with distilled water and stored at room temperature.

3. Tris – HCl Buffer: 50mmol/L, pH 8.0

10ml of Tris Base stock was dissolved in 60ml of distilled water. pH was adjusted to 8.0 with 3N HCl after calibration of pH meter with pH 7 standard solution . It was made into 100ml by adding distilled water with final pH adjustment of 8.0 ± 0.01. Solution was stored at 2-8^{o}C.

4. Preparation of Reagent 1: pH 8.0

13.23mg of Calcium chloride dehydrate was dissolved in 50ml of Tris-HCl buffer and mixed well until the solution become clear. 30 ml of the buffer was mixed. 5.844g of Sodium chloride was added and made into dissolve completely. pH was adjusted to 8.0 with 3N HCl. The solution was made into the final volume of 100ml with Tris-HCl buffer to obtain 0.9mmol/L of $CaCl_2$ and 1mol/L of NaCl. Stored at 2-8^{o}C in polyethylene bottle.

5. Preparation of Working Reagent (R1 + R2)

Working reagent was prepared freshly prior to analysis. The density of purchased Paraoxon –ethyl PESTANAL (Paraoxon) is 1.274g/ml (4.6mol/L) at 25^{o}C. 21.6 µl of Reagent 2 was dissolved which is equivalent to 27.52mg of substrate Paraoxon in 40ml of Reagent1 and mixed well. The solution was made into 50ml with Reagent1 to obtain 2 mmol/L of substrate concentration.

6. Sample preparation (1 in 4 dilutions)

50 µl of sample was diluted with 200 µl with Reagent1. Dilution factor was 4.

Standardization of the Procedure

Standardization of the procedure was done with the Quality Control sera in Dual beam Spectrophotometer.

1. Estimation of Non Enzymatic hydrolysis of the substrate

Non Enzymatic spontaneous hydrolysis of the substrate Paraoxon was measured by adding the substrate to Reagent1 in kinetic mode at 405 nm for 15 minutes. Reagent blank was done to tare the effect of spontaneous hydrolysis of Paraoxon during analysis.

2. Linearity of the reaction

To 2800 µl of working reagent, 80 µl of the diluted QC sera was added and mixed well for 30 seconds. The proportion of increase in absorbance was noted from 30 seconds to 15 minutes by analyzing the kinetic mode at 405 nm. Delay time and reading time were set according to the linearity of the reaction.

3. Accuracy and Precision of the procedure

The Accuracy and Precision of the procedure were analyzed by measuring the enzyme activities using QC sera with the standardized general assay parameters and plotting the values in the Quality control chart.

Assay Procedure

Analysis was started with blanking of the working reagent and monitoring the QC value. 80 µl of diluted serum sample was added and mixed well with 2800 µl of working reagent. Measurement was taken at 405 nm. The change in absorbance is directly proportional to the Salt stimulated enzyme activity.

Assay Parameters

Mode of reaction	- Kinetic
Slope of reaction	- Increasing
Wavelength	- 405 nm

Temperature	- 25^{o}C
Kinetic Factor	- 7873
Blank	- R1 + R2
Delay/ Lag time	- 60 seconds
No of readings	- 4
Interval	- 60 seconds
Read time	- 240 seconds
Reagent volume	- 2800 µl
Sample volume	- 80µl
Dilution factor	- 4
Unit	- U/L

Calculation

One unit of enzyme activity is equal to the formation of 1 nmol/min/ml of the product 4-nitrophenol at pH 8.0 which is equivalent to 1 µmol/min/L (U/L)[73].

Serum Paraoxonase Activity of PON1, U/ L = $\dfrac{\triangle \text{ Abs/min } \mathbf{X} \text{ TV } \mathbf{X} \text{ DF}}{\sum_{\mu mol} \mathbf{X} \text{ SV } \mathbf{X} \text{ Path length}}$

(Salt stimulated enzyme activity)

$= \triangle$ Abs/min x Kinetic Factor

Where

△ Abs/ minute	- Change in Absorbance per minute
TV (Total Reaction Volume)	- 2880 µl
SV (Sample volume)	- 80 µl
DF (Dilution Factor)	- 4
Path length	- 1 cm
$\sum_{\mu mol}$ (Micromolar absorptive of 4-nitrophenol at pH 8.0 at 405 nm)	- 18.29 x 10^{-3} L µmol^{-1} cm^{-1}
Derived Kinetic Factor	- 7873

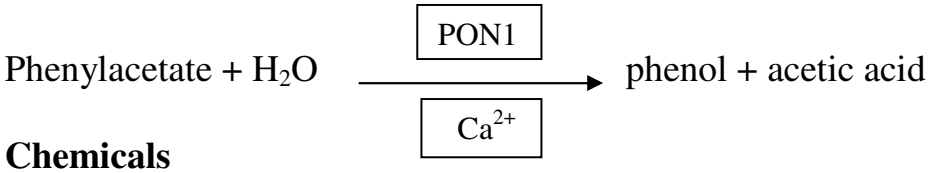
MEASUREMENT OF SERUM ARYLESTERASE ACTIVITY OF PON1

Method

Modified Zeller's method[12,13].

Principle

Serum Paraoxonase1 is a Calcium dependent enzyme that catalyzes the hydrolysis of the colourless substrate Phenylacetate to form phenol and acetic acid by its Arylesterase activity. The change in absorbance due to the formation of phenol is directly proportional to the enzyme activity.



Chemicals

All the chemicals are reagent grade in purity.

1. Phenylacetate
2. Tris (hydroxyl methyl) amino methane
3. Calcium chloride dihydrate
4. Concentrated Hydrochloric acid

Reagent Composition

1. Reagent 1

Active Ingredients	**Concentration**
Tris- HCl Buffer (pH 8.0)	20 mmol/L
Calcium Chloride	1 mmol/L

2. Reagent 2

Active Ingredients	**Concentration**
Phenylacetate	4 mmol/L

Reagent preparation

1. Tris Base stock: 0.2 mol/L

2.423g of Tris (hydroxyl methyl) amino methane was dissolved in 80ml of distilled water and made into a final volume of 100ml and stored in a polyethylene bottle at room temperature.

2. Preparation of 3N HCl: 3mol/L

25ml of Concentrated Hydrochloric acid (12N HCl) was dissolved with 60ml of distilled water and made it into a final volume of 100ml with distilled water and stored at room temperature.

3. Tris – HCl Buffer: 20mmol/L, pH 8.0

10ml of Tris Base stock was dissolved with 60ml of distilled water. pH was adjusted to 8.0 with 3N HCl. It was made into 100ml by adding distilled water with final pH adjustment of 8.0 ± 0.01 and stored at 2-8^{o}C.

4. Preparation of Reagent 1: pH 8.0

14.7 mg of Calcium chloride dihydrate was dissolved in 70ml of Tris-HCl buffer and mixed well until the solution become clear. pH was adjusted to 8.0 with 3N HCl. The solution was made to the final volume of 100ml

with Tris-HCl buffer to obtain 1mmol/L of $CaCl_2$ and stored at 2-8^{o}C in polyethylene bottle.

5. Preparation of Working Reagent (R1 + R2)

Working reagent was freshly prepared prior to analysis. The density of purchased Phenylacetate is 1.073 g/ml (0.79mol/L) at 25^{o}C. 25.4 µl of Reagent 2 which is equivalent to 27.23mg of substrate Phenylacetate was dissolved in 40ml of Reagent1and mixed well. The solution was made into 50ml with Reagent1 to obtain 4mmol/L of substrate concentration.

6. Sample preparation (1 in 4 dilution)

50 µl of Sample was diluted to 200 µl with Reagent1. Dilution factor was 4.

Standardization of the Procedure

Standardization of the procedure was done with the Quality Control sera in Dual beam Spectrophotometer.

1. Estimation of Non Enzymatic hydrolysis of the substrate

Non Enzymatic spontaneous hydrolysis of the substrate Phenylacetate was measured by adding the substrate to Reagent1 in kinetic mode at 270nm

for 10 minutes. Reagent blank was done to blank the effect of spontaneous hydrolysis of Phenylacetate during analysis.

2. Linearity of the reaction

To 2980 µl of working reagent, 20 µl of the diluted QC sera was added and mixed well for 30 seconds. The proportion of increasing in absorbance was noted from 30 seconds to 10 minutes by analyzing the reaction in kinetic mode at 270nm. Delay time and reading time were set according to the linearity of the reactions.

3. Accuracy and Precision of the procedure

The Accuracy and Precision of the procedure were analyzed by measuring the enzyme activities using QC sera with the standardized general assay parameters and plotting the values in the Quality control chart.

Procedure for analysis

Analysis was started with blanking of the working reagent and monitoring the QC value. 20 µl of diluted serum sample was added and mixed well with 2980 µl of working reagent. Measurement was taken at 270 nm. The change in absorbance is directly proportional to the enzyme activity.

Assay Parameters

Mode of reaction	- Kinetic
Slope of reaction	- Increasing
Wavelength	- 270 nm
Temperature	- 25^{o}C
Kinetic Factor	- 458
Blank	- R1 + R2
Delay/ Lag time	- 20 seconds
No of readings	- 2
Interval	- 60 seconds
Read time	- 120 seconds
Reagent volume	- 2980 µl
Sample volume	- 20µl
Dilution factor	- 4
Unit	- kU/L

Calculation

One unit of enzyme activity is equal to the hydrolysis of 1 µmol/min/ml of the substrate Phenylacetate at pH 8.0 which is equivalent to 1000 µmol/min/L of enzyme activity. The unit of serum Arylesterase activity is expressed in kU/L[73].

$$\text{Serum Arylesterase Activity of PON1, kU/ L} = \frac{\triangle \text{ Abs/min } \mathbf{X} \text{ TV } \mathbf{X} \text{ DF}}{1000 \mathbf{X} \sum_{\mu mol} \mathbf{X} \text{ SV } \mathbf{X} \text{ PL}}$$

$$= \triangle \text{ Abs/min } \mathbf{X} \text{ Kinetic Factor}$$

Where,

△Abs/ minute	- Change in Absorbance per minute
TV (Total Reaction Volume)	- 3000 µl
SV (Sample volume)	- 20 µl
DF (Dilution Factor)	- 4
PL (Path length)	- 1 cm

$\sum_{\mu mol}$ (Micromolar absorptive of phenol at pH 8.0 at 270 nm)

- 1310×10^{-6} L μmol^{-1} cm^{-1}

Calculated Kinetic Factor - 458

QUALITY CONTROL MANAGEMENT

The quality of the test procedures for analyzing the enzyme activities was monitored by doing Quality control sera analysis on daily basis.

The Mean and standard deviation (SD) values for plotting Levey Jenning's Chart (Quality Control Chart) were calculated by measuring the enzyme activities of the QC sera for 20 days with the standardized general assay parameters in Dual Beam Spectrophotometer. The reliability and repeatability of the test procedures were evaluated from the QC chart using Westgard rules for Quality Control Management. Analysis was preceded if the QC values are within ± 2 SD.

The intra assay variability of serum PON1allozyme activities was assessed by measuring them with same aliquots of QC sera and patients samples. The inter-assay variability of serum PON1allozyme activities was assessed by measuring the enzyme activity with parallel aliquots of same samples after one and two weeks from the first measurement.

DETERMINATION OF SERUM PARAOXONASE1 PHENOTYPE

The phenotypes of serum PON1 of OPC poisoning patients were determined from their allozyme activities such as Paraoxonase and Arylesterase activities. It was derived by calculating the ratio of serum Paraoxonase and serum Arylesterase activities[12].

$$\text{Phenotype of serum Paraoxonase1} = \frac{\text{Serum Paraoxonase activity}}{\text{Serum Arylesterase activity}}$$

REFERENCE RANGE FOR PHENOTYPIC CLASSIFICATION OF SERUM PON 1[68]

Phenotype of serum Paraoxonase1	**Ratio of PON 1 allozymes activity**	**Grades of Serum PON 1 Activity**
AA	< 3.0	Low
AB	3.0 – 6.9	Moderate
BB	>6.9	High

STATISTICAL ANALYSIS AND RESULTS

Socio demographic and biochemical data of 54 patients with acute OPC poising were set for statistical analysis. The Statistical analysis was performed using Statistical software SPSS (version 16) package.

The biochemical values obtained from standardization procedures were expressed as mean, standard deviation and coefficient of variation. The enzymatic activities of Quality Control (QC) sera were presented as Levey Jennings chart. Distribution of socio demographic data and analytical variables used to screen the co-morbid medical illnesses were calculated by frequency analysis. The level and degree of association between two dependent variables were assessed by Pearson Coefficient of correlation. The comparison of mean values of variables among more than two independent groups was analyzed by 'one way ANOVA' test.

The level and strength of correlation between serum paraoxonase1 phenotypes and serum Cholinesterase activity was calculated using 'Pearson coefficient of correlation'. A p value ≤ 0.05 was considered as statistically significant. The results of statistical analysis are presented as tables and charts. The socio demographic and biochemical data are attached in Annexure III.

Table.3: STANDARDISATION OF THE TEST PROCEDURES

Table.3.1: NONENZYMATIC HYDROLYSIS OF THE SUBSTRATE

Substrate	Mean	Standard Deviation
Paraoxon (U/L)	3.1	±0.65
Phenylacetate (kU/L)	0.26	±0.09

Table.3.1 shows the mean values of Non enzymatic hydrolysis of Paraoxon and Phenylacetate.

Table3.2: VARIABILITY IN ENZYME ACTIVITY

Table 3.2a: INTRA ASSAY VARIABILITY

Measures	Serum Paraoxonase activity (U/L)	Serum Arylesterase activity (kU/L)
Mean	294.34	72.14
Standard Deviation	±7.82	±1.52
CV (%)	2.66	2.11

Table.3.2a represents the intra assay variability in Serum Paraoxonase and Arylesterase activities of serum PON1. The Coefficient Variation (CV) for

serum Paraoxonase and Arylesterase activities are 2.66% and 2.11% respectively.

Table.3.2b: INTER ASSAY VARIABILITY

Measures	Serum Paraoxonase activity (U/L)	Serum Arylesterase activity (kU/L)
Mean	287.24	71.29
Standard Deviation	±9.36	±1.96
CV (%)	3.26	2.75

Table.3.2b represents the inter assay variability in Serum Paraoxonase and Arylesterase activities of serum PON1. The Coefficient Variation (CV) for serum Paraoxonase and Arylesterase activities are 3.26% and 2.75% respectively.

Table.4: ENZYME ACTIVITY VALUES OF QUALITY CONTROL SERA

Serum Enzyme activity	Mean	Standard Deviation
Cholinesterase (U/L)	7820	±33.13
Paraoxonase (U/L)	197.87	±4.68
Arylesterase (kU/L)	62.31	±2.32

Table.4 represents the mean and standard deviation values of Quality Control (QC) sera enzyme activities used to plot Levey Jennings chart for Quality Control management. The values were not influenced by the addition of 15% of ethylene glycol.

Figure.11: QUALITY CONTROL CHARTS FOR ENZYME ACTIVITIES

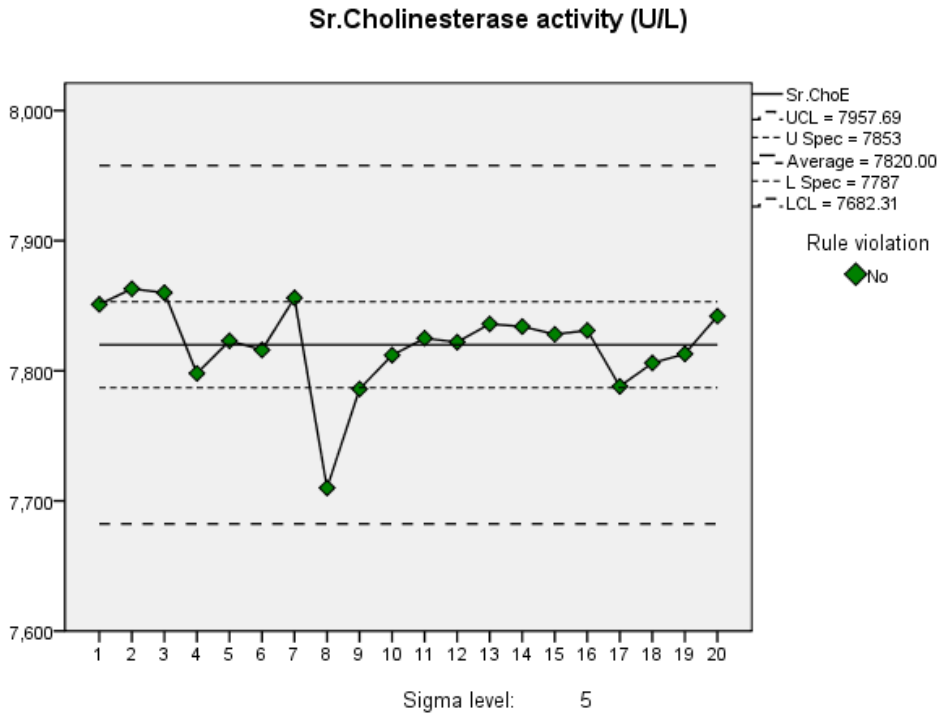


Figure.11.1: Levey Jennings Chart for serum Cholinesterase activity. x axis- Days; y axis- mean, ±1SD, ±2SD values of enzyme activity.

Figure.11.1 depicts the Levey Jennings Chart (LJ chart) for serum Cholinesterase activity of QC sera measured for 20 days which was used for Quality Control Management on daily basis.

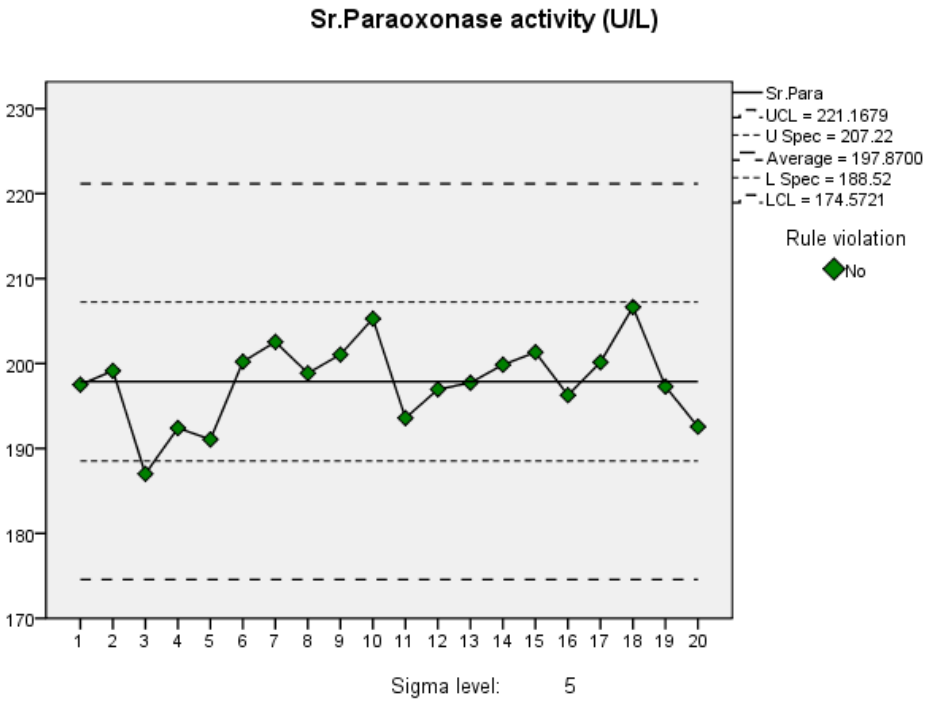


Figure.11.2: Levey Jennings Chart for serum Paraoxonase activity. x axis- Days; y axis- mean, ±1SD, ±2SD values of enzyme activity.

Figure.11.2 shows the Levey Jennings Chart (LJ chart) for serum Paraoxonase activity of QC sera measured for 20 days which was used for Quality Control Management on daily basis

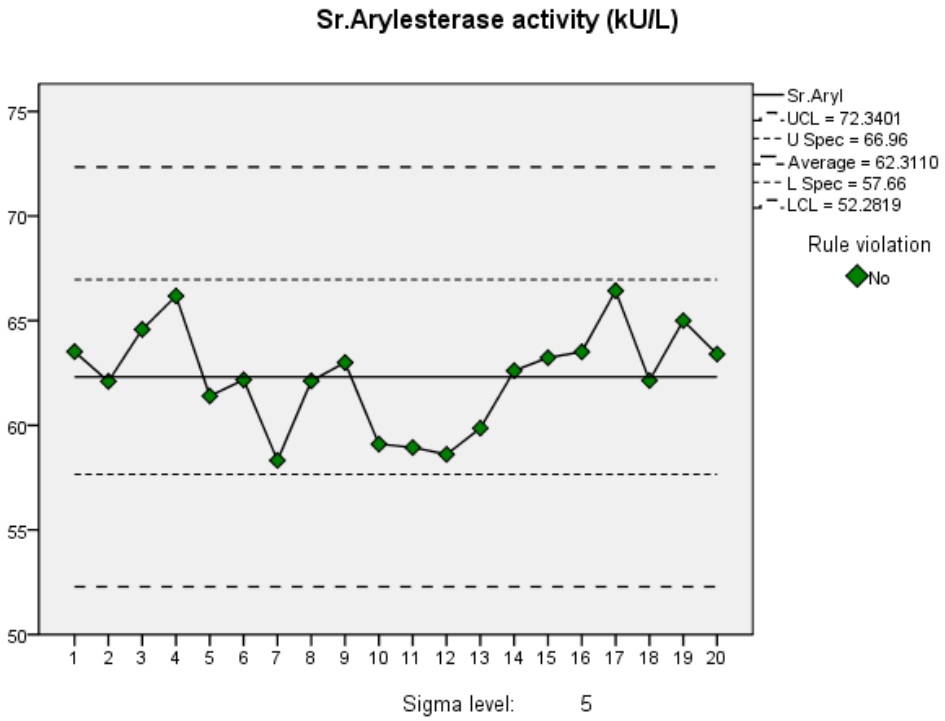


Figure.11.3: Levey Jennings Chart for serum Arylesterase activity. x axis- Days; y axis- mean, ±1SD, ±2SD values of enzyme activity.

Figure.11.3 shows the Levey Jennings Chart (LJ chart) for serum Paraoxonase activity of QC sera measured for 20 days which was used for Quality Control Management on daily basis.

Table.5: DISTRIBUTION OF VALUES OF ANALYTES FOR SCREENING AMONG STUDY POPULATION

Analytes	Minimum	Maximum	Mean	SD
Serum Total Protein (g/dL)	6.4	7.3	6.85	±0.16
Random Plasma Glucose (mg %)	74	126	89.63	±12.46

Table.5 shows the mean and standard deviation values of Serum Total Protein and Random Plasma Glucose of the study population. The minimum and maximum values are within the expected reference range.

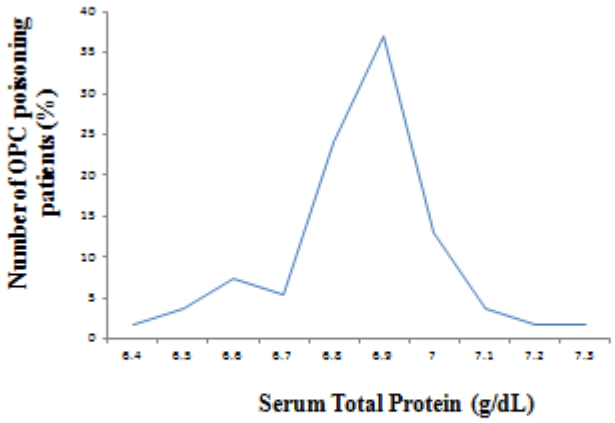


Fig.12: Distribution of Serum Total Protein amongst study population (OPC poisoning patients)

Figure.12 is the graphical representation of distribution range of serum total Protein values amongst the study population. It shows that 95% of the OPC poisoning patients have serum total protein values between 6.53 – 7.17 g/dL.

Table.6: AGE DISTRIBUTION AMONGST OPC POISONING PATIENTS

Age in years	No. of patients	Percent (%)
18-27	18	33.4
28-37	20	37
38-47	8	14.8
48-57	8	14.8
Total	54	100

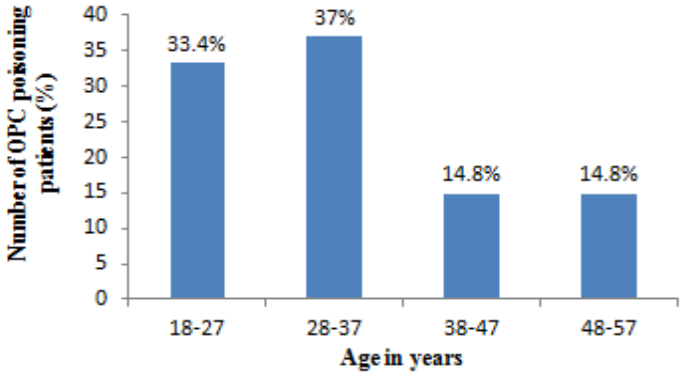


Fig.13: Age distribution of OPC poisoning patients

Table.6 and Figure.13 shows the Age distribution of study population which is expressed in percent. 70.4% of the study population belongs to the productive age group of 18 – 37 years .

Table.7: GENDER DISTRIBUTION AMONGST OPC POISONING PATIENTS

Gender	No.of patients	Percent (%)
Males	34	63
Females	20	37
Total	54	100

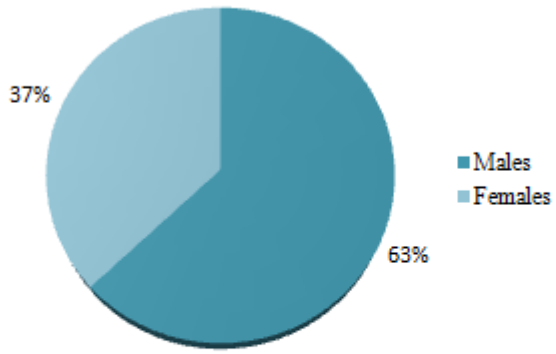


Fig.14: Gender distribution of OPC poisoning patients

Table.7 and Figure.14 shows the distribution of gender amongst the victims of acute OPC poisoning. 63% of acute OPC poisoning patients are males.

Table.8: EDUCATIONAL STATUS AMONGST VICTIMS OF OP POISONING

Educational status	No. of patients	Percent (%)
Illiterate	25	46.3
Primary	3	5.6
Secondary	26	48.1
Total	54	100

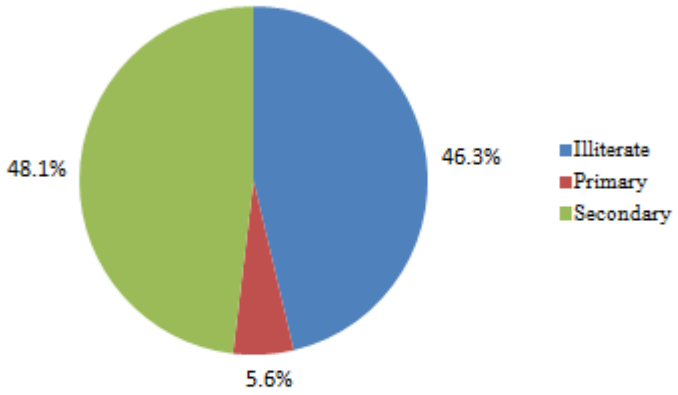


Fig.15: Educational status amongst the victims of OP poisoning

Table.8 and Figure.15 shows the distribution of Educational status amongst the study population. 53.7% of patients are literates.

Table.9: MARITAL STATUS AMONGT OPC POISONING PATIENTS

Marital status	No. of patients	Percent (%)
Unmarried	19	35.2
Married	35	64.8
Total	54	100

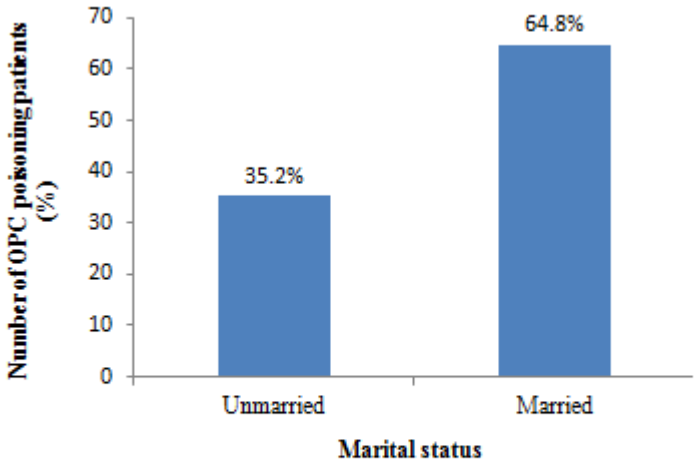


Fig.16: Marital status amongst OPC poisoning patients

Table.9 and Figure.16 represents the marital status of victims of OPC poisoning. 64.8% or more than two thirds of patients are married.

Table.10: OCCUPATIONAL STATUS OF VICTIMS OF OP POISONING

Occupational status	No. of patients	Percent (%)
Student	6	11.1
Own Business	9	16.7
Private job	14	25.9
Govt.Job	2	3.7
Farmer	9	16.7
House wife	9	16.7
Others	5	9.3
Total	54	100.0

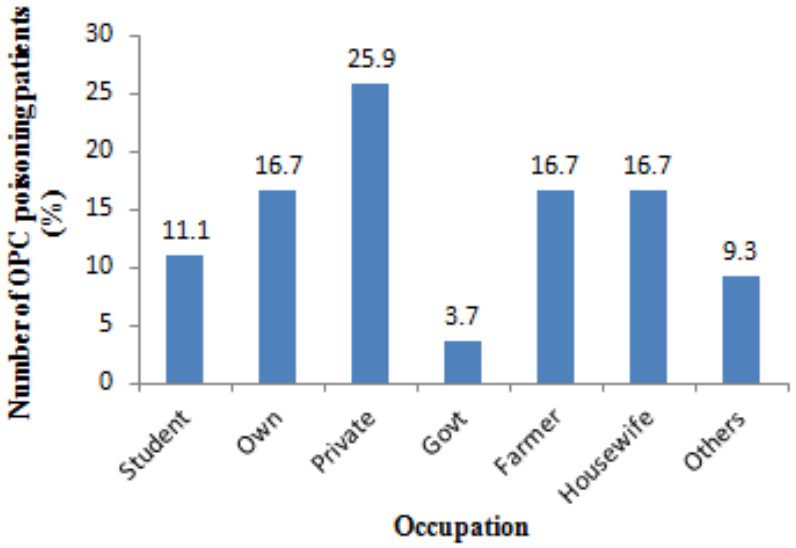


Fig.17: Occupational status in OPC poisoning patients

Table.10 and Figure.17 are the representation of Occupational status of acute OPC poisoning patients. 11.1% are students and 16.7% are farmers. 25.9% of patients are being in private job.

Table.11: SOCIOECONOMIC STATUS AMONGST OP POISONING PATIENTS

Socioeconomic status	No. of patients	Percent (%)
Lower	21	38.9
Middle	31	57.4
Upper	2	3.7
Total	54	100.0

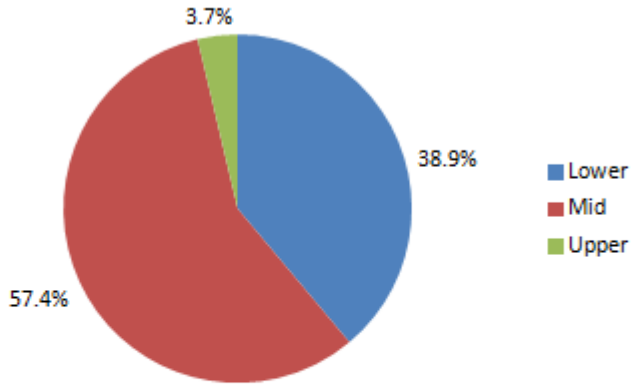


Fig.18: Socioeconomic status in OPC poisoning patients

Table.11 and Figure.18 shows Socioeconomic status amongst the study population.57.4% belongs to middle income group of socioeconomic status.

Table.12: DOMICILE DISTRIBUTION AMONGST OPC POISONING PATIENTS

Domicile	No. of patients	Percent (%)
Rural	12	22.2
Suburban	11	20.4
Urban	31	57.4
Total	54	100.0

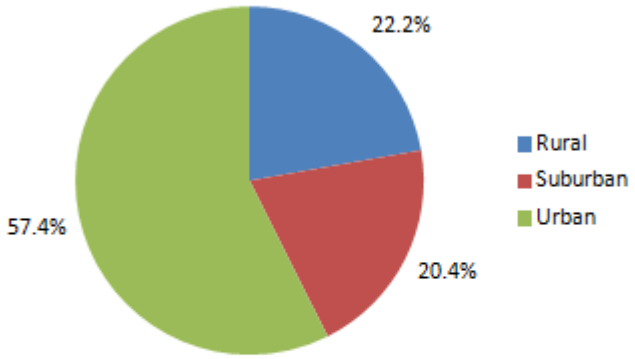


Fig.19: Domicile distribution of OPC poisoning patients

Table.12 and Figure.19 represents the domicile distribution amongst the study population.57.4% of patients are residing in urban areas.

Table.13: ASSOCIATION BETWEEN SERUM PARAOXONASE AND ARYLESTERASE ACTIVITIES OF SERUM PARAOXONSE1 ENZYME

Variables	Coefficient of Correlation (r)	Level of Significance (p)	Strength of Association	Interpretation
Sr.Paraoxonse vs Sr.Arylesterase	+0.301	< 0.05	Moderate association	Significant with positive correlation

Table.13 shows the level and strength of association of serum Paraoxonase activity with serum Arylesterase activity of serum PON1 enzyme. Both enzymes are positively correlated with weak strength of association. The coefficient of determination (r^2) is +0.09.

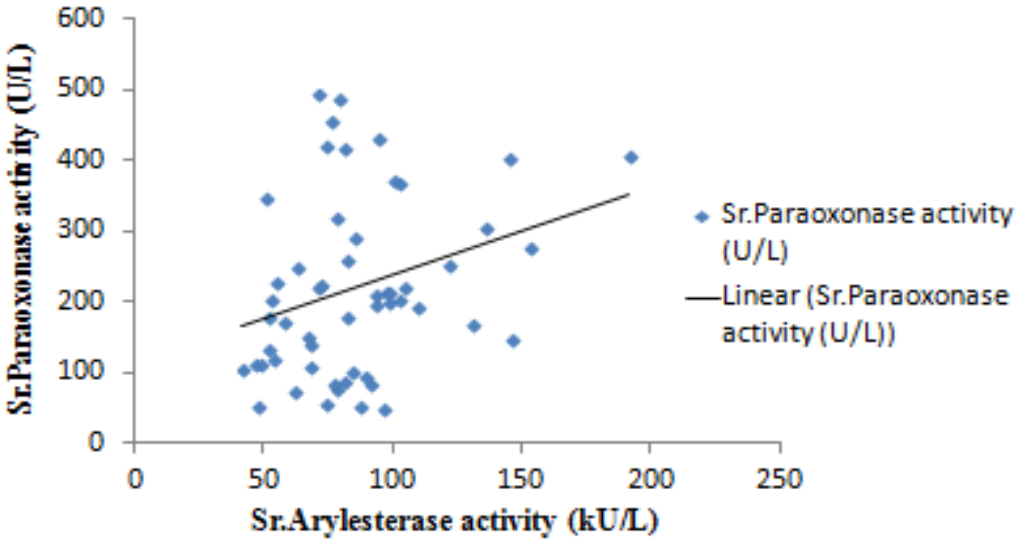


Fig.20: Serum Paraoxonase vs Arylesterase activity

Figure.20 is the graphical representation of association between Serum Paraoxonase and Arylesterase activities of Serum Paraoxonse1 enzyme.

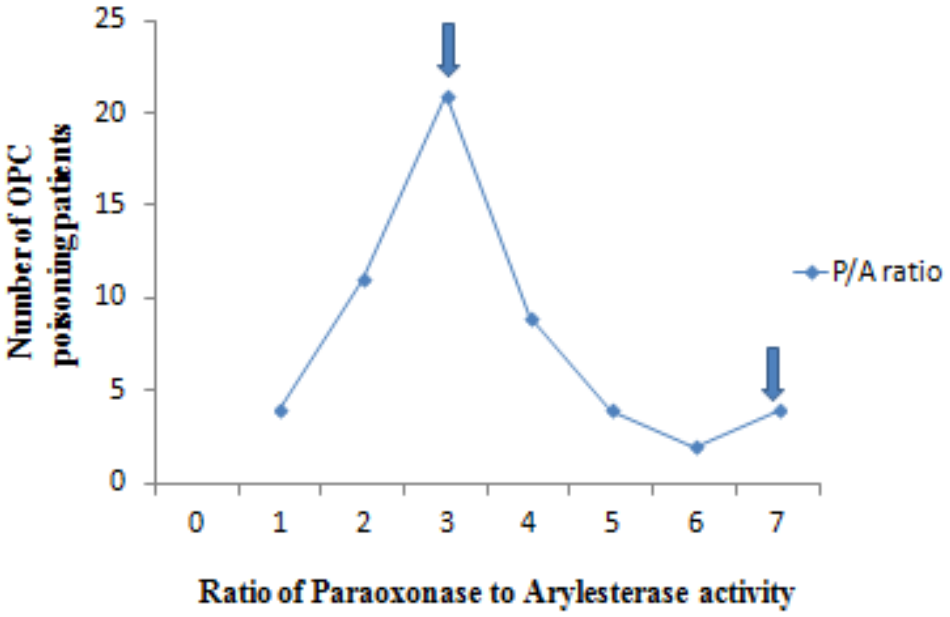


Fig.21: Population Distribution of Serum PON1 phenotypes amongst OPC poisoning patients

Figure.21 is the cumulative frequency graph which shows the trimodal distribution of study population by the ratio of serum Paraoxonase to Arylesterase activity. This trimodal distribution represents the phenotypes of Serum Paraoxonsae1 enzyme by the antimodes at 3.0 and 6.9 as AA, AB, BB respectively.

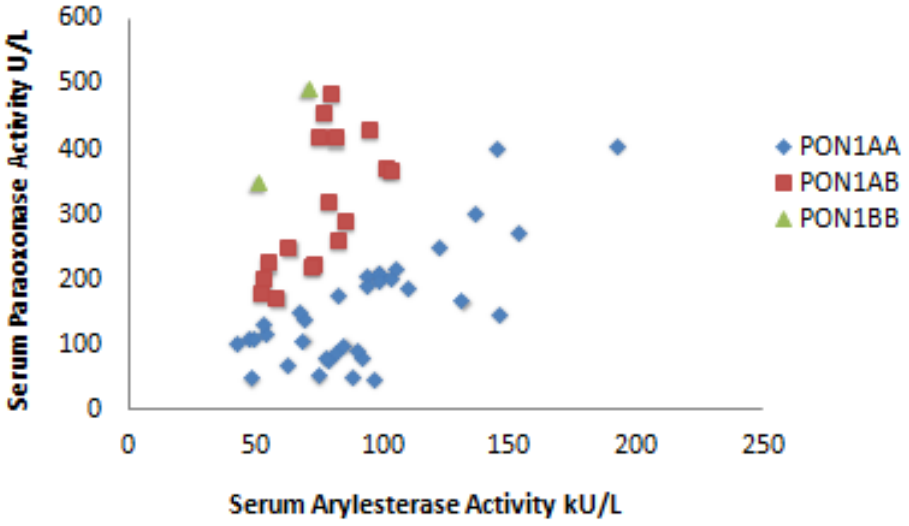


Fig.22: Serum Paraoxonase1 polymorphism

Figure.22 is the diagrammatic representation of the distribution of PON1 phenotypes among the study population due serum Paraoxonase1 polymorphism that affects the enzyme activity.

Table.14: DISTRIBUTION OF SERUM PON1 PHENOTYPES AMONGST OPC POISONING PATIENTS.

PON1 phenotype	No. of patients	Percent (%)
AA	35	64.8
AB	17	31.5
BB	2	3.7
Total	54	100.0

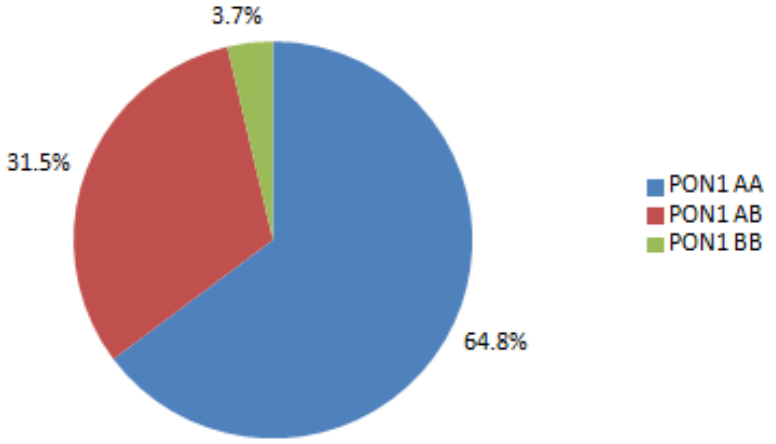


Fig.23: Distribution of Serum PON1 phenotypes amongst OPC poisoning patients

Table.14 and Figure 23 shows the distribution of serum PON1 phenotypes among the OPC poisoning patients. 64.8% of study population has shown to have AA phenotype of serum PON1 enzyme.

Table.15: DISTRIBUTION OF SERUM PON1 PHENOTYPES WITH RESPECT TO AGE GROUP OF THE STUDY POPULATION

Age in years	PON1 AA No. of patients	PON1 AB No. of patients	PON1 BB No. of patients	Total
18-27	12	5	1	18
28-37	14	6	0	20
38-47	4	4	0	8
48-57	5	2	1	8
Total	35	17	2	54

Table.15 shows the distribution of serum PON1 phenotypes with respect to age of the OPC poisoning patients. Among all age group, more than 60% of patients belong to PON1 AA phenotype.

Table.16: DISTRIBUTION OF SERUM PON1 PHENOTYPES WITH RESPECT TO GENDER OF THE STUDY POPULATION

Gender	PON1 AA No. of patients	PON1 AB No. of patients	PON1 BB No. of patients	Total
Males	21	12	1	34
Females	14	5	1	20
Total	35	17	2	54

Table.16 represents the distribution of serum PON1 phenotypes with respect to gender of the OPC poisoning patients. Serum PON1 phenotypes are equally distributed in both males and females.

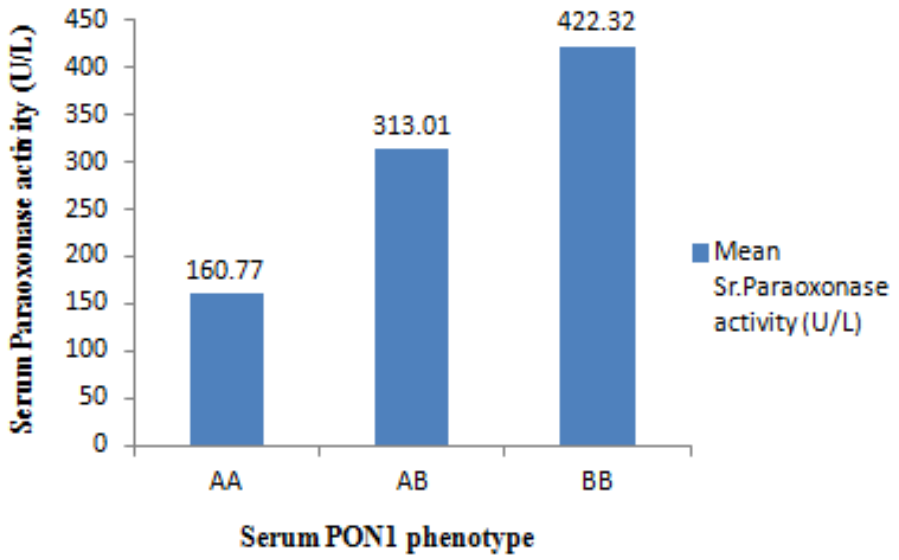


Fig.24: Serum Paraoxonase activity (U/L) amongst PON1 phenotypes

Figure.24 shows the serum Paraoxonase activity among the PON1 phenotypes. Phenotype BB has shown to have high paraoxonase activity.

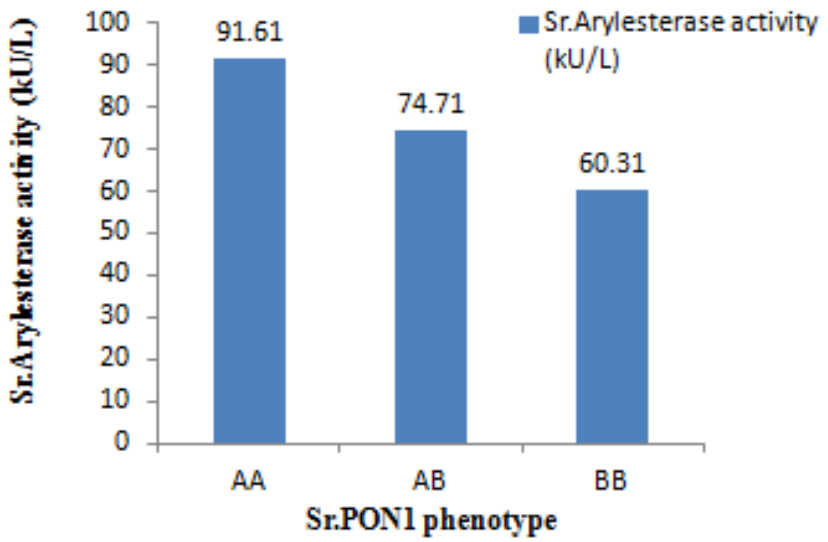


Fig.25: Serum Arylesterase activity amongst PON1 phenotypes

Figure.25 is the diagrammatic representation of serum Arylesterase activity among PON1 phenotypes. Phenotype BB has shown to have relatively lower Arylesterase activity.

Table.17: ASSOCIATION OF SERUM PARAOXONASE AND ARYLESTERASE ACTIVITIES WITH SERUM PARAOXONSE1 POLYMORPHISM

Variables	Coefficient of Correlation (r)	Level of Significance (p)	Strength of Association	Interpretation
Sr.Paraoxonse vs Sr.PON1polymorphism	+0.653	< 0.0001	Strong association	Significant with positive correlation
Sr.Arylesterase vs Sr.PON1polymorphism	-0.306	<0.05	Moderate association	Significant with negative correlation

Table.17 shows the level and degree of association of serum Paraoxonase and Arylesterase activities with serum PON1 phenotypic polymorphism.

Table.18: ENZYME ACTIVITIES OF STUDY POPULATION

Serum Enzyme activity	Minimum	Maximum	Mean	Standard Deviation
Cholinesterase (U/L)	342	9840	2325	±2027.19
Paraoxonase (U/L)	49.86	494.28	218.39	±124.30
Arylesterase (kU/L)	41.73	191.35	85.13	±30.19

Table.18 gives the values of serum Cholinesterase, Paraoxonase, Arylesterase enzyme activities of the study population.

Table.19: ASSOCIATION OF SERUM CHOLINESTERASE ACTIVITY WITH SERUM PARAOXONASE AND ARYLESTERASE ACTIVITIES

Variables	Coefficient of Correlation (r)	Level of Significance (p)	Strength of Association	Interpretation
Sr.Cholinesterase vs Sr.Paraoxonsae	+0.767	< 0.0001	Strong association	Significant with positive correlation
Sr.Cholinesterase vs Sr.Arylesterase	-0.239	> 0.05	Weak association	Not Significant with negative correlation

Table.19 shows the association of serum Cholinesterase activity with serum Paraoxonase and Arylesterase activities. It shows that serum CHE is strongly correlated with serum Paraoxonase allozyme activity of PON1 enzyme.

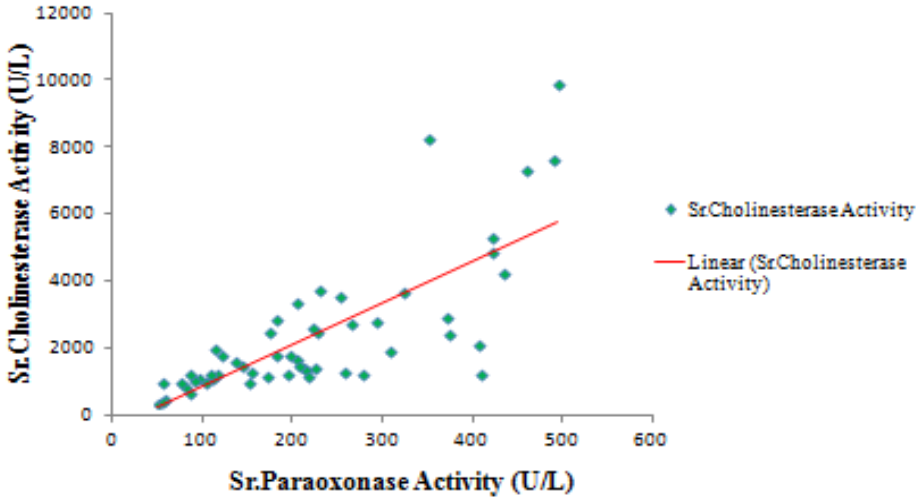


Fig.26: Serum Cholinesterase vs Paraoxonase Activity

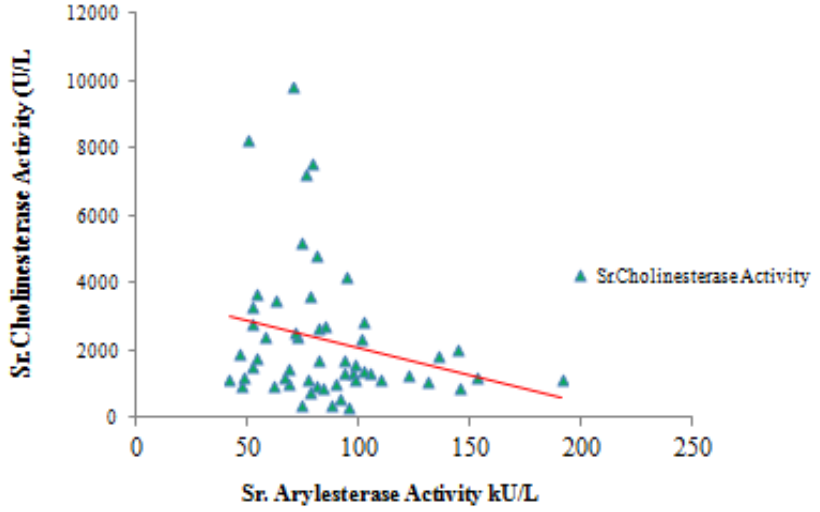


Fig.27: Serum Cholinesterase vs Arylesterase Activity

Figure.26 and 27 are the graphical representation of association of serum Cholinesterase activity with serum Paraoxonase and Arylesterase activities of PON1 enzyme.

Table.20: ASSOCIATION OF SERUM CHOLINESTERASE ACTIVITY WITH RATIO OF SERUM PARAOXONASE/ ARYLESTERASE ACTIVITY

Variables	Coefficient of Correlation (r)	Level of Significance (p)	Strength of Association	Interpretation
Sr.Cholinesterase vs Sr.P/A ratio	+0.955	< 0.0001	Strong association	Significant with positive correlation

Table.20 is the representation for the strong positive association of serum Cholinesterase activity with the ratio of serum Paraoxonase/ Arylesterase activity

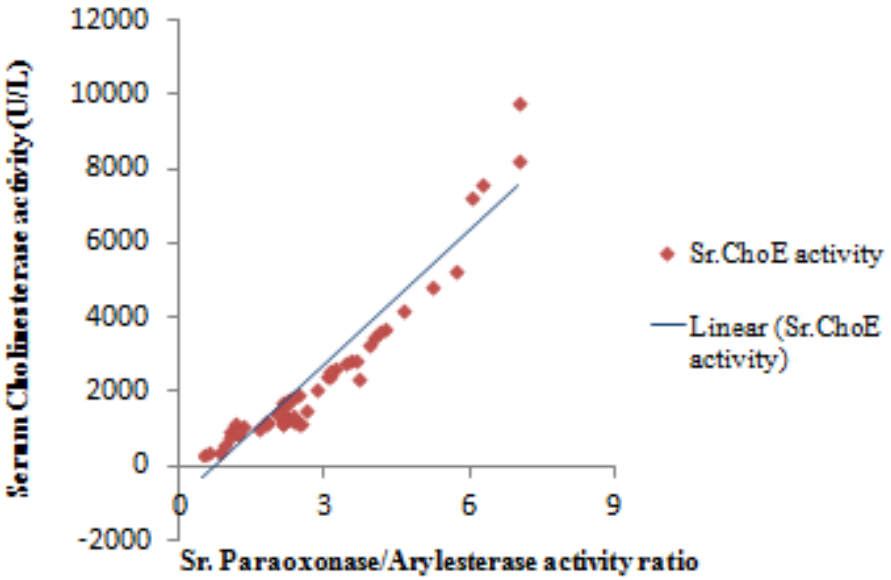


Fig.28: Serum Cholinesterase activity vs serum Paraoxonase to Arylesterase activity ratio

Figure.28 shows the strong linear positive relationship of serum CHE with the ratio of serum Paraoxonase / Arylesterase (P/A) activity.

Table.21: MEAN VALUES OF ENZYME ACTIVITIES AMONG SERUM PON1 PHENOTYPES OF THE STUDY POPULATION

Serum Enzyme activity	PON1AA	PON1AB	PON1BB
Cholinesterase (U/L)	1227.4	3794.94	9052.5
Paraoxonase (U/L)	160.77	313.01	422.32
Arylesterase (kU/L)	91.61	74.71	60.31

Table.21 shows the mean values of serum Cholinesterase, Paraoxonase, Arylesterase enzyme activities among serum PON1 phenotypes of acute OPC poisoning patients

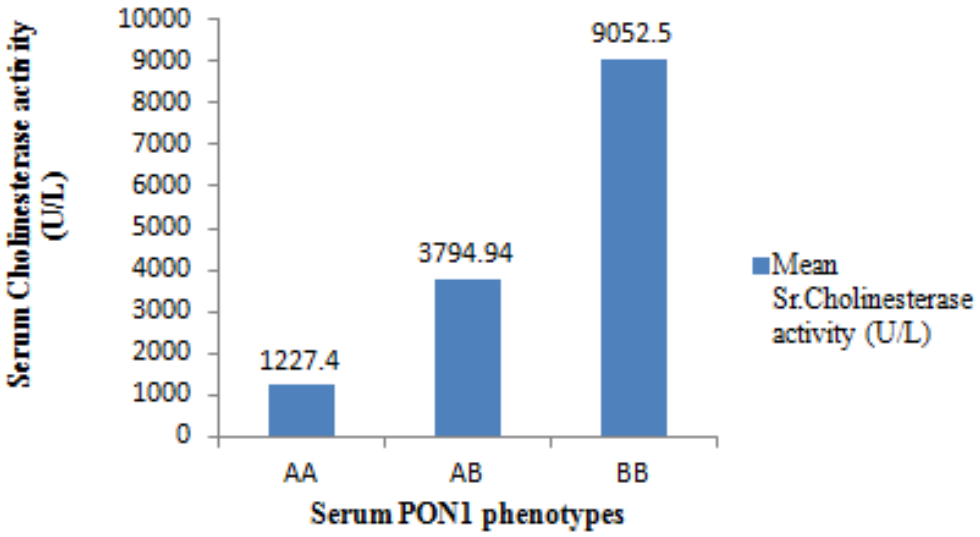


Fig.29: Serum Cholinesterase activity amongst PON1 phenotypes of OPC poisoning patients

Figure.29 represents the serum CHE activity among the PON1 phenotypes of acute OPC poisoning patients

Table.22: COMPARISON OF MEAN VALUES OF ENZYME ACTIVITIES AMONG SERUM PON1 PHENOTYPES

Variables (Activity)	F distribution	Level of Significance
Serum Cholinesterase	89.28	< 0.0001
Serum Paraoxonase	19.18	< 0.001
Serum Arylesterase	2.65	0.08
Serum P/A ratio	85.94	< 0.0001

Table.22 shows the 'one way ANOVA' test to compare the mean values of enzyme activities among serum PON1 phenotypes. Mean values of Serum Cholinesterase activity and ratio of serum Paraoxonase/Arylesterase activity are significant among PON1phenotypes at the < 0.0001 level.

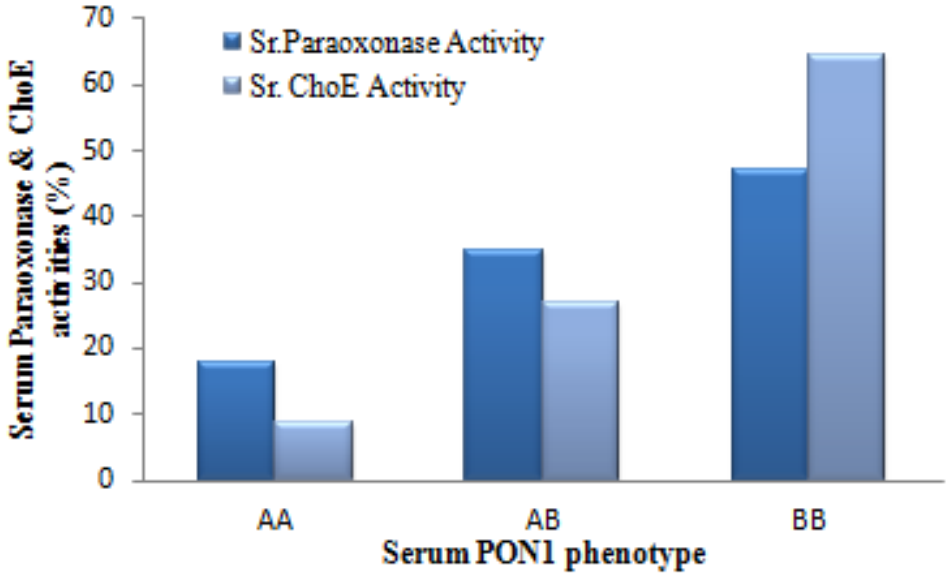


Fig.30: Relationship between serum Paraoxonase and Cholinesterase activities amongst PON1 phenotypes

Figure.30 emphasizes the positive linear relationship of Serum Cholinesterase activity along with serum Paraoxonase activity among the serum PON1 phenotypes of acute OPC poisoning patients.

Table.23: CORRELATION OF SERUM CHOLINESTERASE ACTIVITY AND SERUM PARAOXONASE1 PHENOTYPES

Variables	Coefficient of Correlation (r)	Level of Significance (p)	Strength of Association	Interpretation
Sr.Cholinesterase vs Sr.PON1 phenotypes	+0.857	< 0.0001	Strong association	Significant with positive correlation

Table.23 shows the correlation between serum Cholinesterase activity and serum Paraoxonase1 phenotypes.

Pearson correlation coefficient (r) value of +0.857 denotes the strong positive correlation between serum Cholinesterase activity and serum Paraoxonase1 phenotypes among the acute OPC poisoning patients. The relationship between the two variables is significant at the <0.0001 level.

DISCUSSION

Organophosphorous (OPC) poisoning whether intentional or accidental is one of the leading causes of morbidity and mortality worldwide especially in developing countries like India[17,19]. Acute OPC poisoning is a clinical emergency in which the early diagnosis and treatment are crucial[72].

The main mechanism of OPC toxicity involves the inhibition of Cholinesterase and Neuropathy Target Esterase activity (NTE) which leads to different grades of neuromuscular toxicity and various stages of paralysis which occur in different time interval.[4,5]. It also increases the oxidative stress that damages the nervous and muscular tissues[6,7].

The exposure to OPCs can be diagnosed early by the detailed history and clinical features of OPC poisoning and confirmed by biochemical analysis of Cholinesterase activity and urine metabolites of OPCs[8]. The toxicity level of OPC poisoning can be detected from the direct measurement of blood level of OPCs which is practically difficult to follow as a routine procedure.

Though RBC cholinesterase is specific to confirm the exposure to OPCs, Serum Cholinesterase activity is a sensitive diagnostic alternative for neuronal AChE activity[9] and has an advantage in monitoring the disease

progression during management by serial estimation of enzyme activity[8].

In spite of the advances in diagnosis and treatment of OPC poisoning, the outcome is still guarded because of the three different phases of the poisoning[72]. Although the acute cholinergic crisis (Type I paralysis) is the earliest manifestation of acute organophosphorous compound poisoning, it is the intermediate syndrome (Type II paralysis) which has high degree of mortality[5]. Some patients may develop OPC induced delayed neuropathy (OPIDN), the type III paralysis which usually occurs after 10 days of exposure[6,7].

It has been postulated that there is a phenomenon called "reinhibition of oxime reactivated acetyl cholinesterases" due to persistent high concentration of OPC which nullifies the effectiveness of oximes[34]. Hence the severity of OPC poisoning is decided by the degree and duration of inhibition of cholinesterase activity. It depends on the level of active oxon forms of OPC and is directly related to the activity of serum Paraoxonase1 enzyme that detoxifies the active OP compounds. These findings have been found to be reported in chronic OPC poisoning studies[13,15].

Sogorb MA., *et al* conducted studies in experimental animals and reported that the blood and tissue concentrations of organophosphates were

lowered by the action of mammalian Paraoxonase1 which has a beneficial role in OPC poisoning[71,75]. Since the serum PON1 is a polymorphic enzyme, the inherent detoxifying ability is highly variable among individuals and it affects the susceptibility of a patient to OPC toxicity[15,16].

Eckerson HW, *et al* reported that the trimodal distribution of serum PON1 phenotypes can be identified by the ratio of allozyme activities of PON1[12]. Though it was shown that the classification based on the ratio was reliable, recent studies found that it can also be differentiated by individual Paraoxonase and Arylesterase activities of PON1[15].

In the present study, we assessed the relationship of serum PON1 phenotypes with serum cholinesterase activity among 'Acute' OPC poisoned patients. The individual allozyme activities of PON1 and their association with each other were analyzed to identify the reliability of serum PON1phenotyping based on their ratio. We also evaluated the socio demographic profile to assess the pattern of OPC poisoning in the area where the study was conducted.

From the present study, it has been observed that most of the study population belongs to the productive age group of 18 – 37 years . Among the study population, 63% were males. Around 53.7 % of the population were

educated and majority of them were married. Most of the patients were from middle income group residing in urban area. The above socio demographic data reflect the pattern of OPC poisoning commonly seen in our hospital setup.

From the results obtained, it has been observed that serum PON1 has two different activities based on the substrate 'Paraoxon' and 'phenylacetate' used for in vitro analysis of enzyme activity. Paraoxonase activity was highly variable with wide variation in values than Arylesterase activity of PON1 in an individual. The association between paraoxonase and arylesterase activities of PON1 in the study population was moderately correlated with each other.

It has been found that only 9% of variability in paraoxonase activity can be explained by the variations in arylesterase activity. This shows that PON1 has bimodal and unimodal distribution of phenotypes according to its paraoxonase and arylesterase activities respectively as reported in earlier studies[12,13]. This indicates that the PON1 activity is contributed by 'two' alleles of the genome.

The association between paraoxonase and arylesterase activities also substantiates the statement of Abessolo FA., *et al* (2012) that the substrate

'Paraoxon' is the one which differentiates the activities of the two alleles of a single enzyme[58].

It has been observed that the ratio of paraoxonase and arylesterase activities revealed the trimodal distribution of serum PON1 as having three degrees of enzyme activities which proved the presence of phenotypic polymorphism contributed by two alleles which are inherited as a 'codominant' mendelian trait[12]. The existence of three phenotypes of serum PON1 such as AA, AB and BB was observed at the level of antimodes 3.0 and 6.9 which has been recently reported in India by Mahesh Harishchandra Hampe et al (2014)[68].

Eckerson., *et al* reported that PON1 AA, PON1 AB, PON1 BB phenotypes have low, intermediate and high paraoxonase/arylesterase activity ratio respectively[12,13] . It was explained that the variations in the ratio of the enzyme activities was mainly influenced by the increase in the levels of 'paraoxonase' activity. The similar results were observed in the present study.

The Arylesterase activity of PON1 was observed to be in decreasing order from PON1 AA to PON1 BB phenotypes. However the variations in arylesterase activity were relatively low among the phenotypes which were also shown by various authors on PON1 polymorphism[58]. It was explained

that the variation in activity of arylesterase was relatively less affected compared to Paraoxonase activity by the serum PON1 polymorphism[12,13,68].

The distribution of PON1 phenotype is highly variable worldwide[60]. In the present study, PON1 AA phenotype was found to be the predominant form and accounts for 64.8% of study population whereas the PON1 AB and BB phenotypes were found in 31.5% and 3.7% of patients respectively. It showed that the PON1 AA was the principal form of phenotype in our region which was equally distributed among all the age group and gender of our study population which showed the inheritance of PON1 is 'autosomal' dominant[12,13].

It has been observed that the Cholinesterase activity has positive correlation with the paraoxonase activity and the ratio of allozyme activities of PON1. From the 'one way ANOVA' test, it has been shown that cholinesterae activity is stronghly associated with the ratio of paraoxonase/arylesterase than paraoxonase activty. It has also been observed by the Pearson coefficient of correlation analysis where the 'r' value for CHE vs Paraoxonase activity was +0.767 and for CHE vs Paraoxonase/arylesterase activity ratio was +0.955. It showed the reliability

of the allozymic activity ratio for PON1 phenotyping and in the prediction of cholinesterase activity[12,68].

In the present study we observed that PON1 AA has low level of serum paraoxonase and cholinesterase activity. PON1 AB has intermediate activity while PON1 BB has the highest level of paraoxonase and cholinesterase activity by estimating the ratio of allozyme activities of PON1 to determine the PON1 phenotypes. Akgür SA.,*et al* reported the positive correlation of cholinesterase activity with PON1 phenotypes by estimation of paraoxonase activity of PON1 in chronic OPC poisoning[15].

From the present study it has been observed that the Pearson correlation coefficient of Serum cholinesterase vs serum PON1 phenotypes was +0.857 with p value of <0.0001. It indicates the strong positive linear relationship of serum cholinesterase with PON1 phenotypic polymorphism.

It was evident that serum PON1 phenotype AA has less ability to detoxify the OPCs due their low level of paraoxonase activity leads to incresed inhibition of CHE therby the individuals with PON1 AA were more susceptible to OPC toxicity[15].

From this study, it has been shown that serum PON1 phenotyping is useful in the identification of susceptible patients to OPC toxicity. Hence the

dosage and duration of oxime therapy and the time period for follow up management can be modified according to their susceptibility so as to ensure better prognosis.

CONCLUSION

From the present study, it can be concluded that Phenotypic polymorphism of Serum Paraoxonase1 has strong association with serum Cholinesterase activity thereby has an influence on the patient's susceptibility to OPC toxicity in Acute organophosphorous compound poisoning. Serum PON1 AA phenotype has low paraoxonase activity with low serum Cholinesterase activity. Serum PON1 BB phenotype has high paraoxonase and Cholinesterase activity.

This relationship supports the need for routine identification of serum Paraoxonase1 phenotype along with the measurement of serum Cholinesterase activity in patients with acute OPC poisoning. This will help the early detection of susceptibility to OPC toxicity. Hence the management can be planned according to their susceptibility and to ensure better prognosis.

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ANNEXURE I

CLINICAL ASSESSMENT PROFORMA

Name : Age/Sex : IP. No :

Admission date : Discharge date : Data No :

History of presenting illness :

Past History :

Personal/Socio demographic History :

General Examination :

Consciousness : Orientation : Pupils :

Vital Signs :

Temp : PR : RR : BP :

Systemic Examination :

Nervous system: Cardiovascular system:

Respiratory system: Abdomen:

Investigations:

1. Serum Total Protein:
2. Random Plasma Glucose:
3. Serum Cholinesterase activity:
4. Serum Paraoxonase (salt stimulated) activity of PON1:
5. Serum Arylesterase activity of PON1:

ANNEXURE II

தகவல் படிவம்

ஆய்வின் தலைப்பு :

"ஆர்கனோபாஸ்பரஸ் பூச்சிக்கொல்லி மருந்தினால் நஞ்சேற்றம் அடைந்த நோயாளிகளின் இரத்தத்தில் பாராக்சோனேஸ் - 1 (PON-1) நொதியின் செயல்நிலை வகையையும் கோலினெஸ்டரேஸ் நொதியின் செயல்நிலையையும் கண்டறியும் ஆய்வு"

ஆராய்ச்சி நிலையம் : அரசு ஸ்டான்லி மருத்துவமனை, சென்னை.

ஆய்வு மேற்கொள்பவரது பெயர் : மரு. ஆனந்தி . பெ

மேற்குறிப்பிட்டுள்ள ஆய்வு ஆர்கனோபாஸ்பரஸ் பூச்சிக்கொல்லி மருந்தால் நஞ்சேற்றம் அடைந்த நோயாளிகளின் நலனுக்காக ஸ்டான்லி மருத்துவமனையில் உயிர் வேதியியல் துறையைச் சார்ந்த பட்டமேற்படிப்பு மருத்துவரால் மேற்கொள்ளப்படுகிறது.

ஆர்கனோபாஸ்பரஸ் (OPC) பூச்சிக்கொல்லி மருந்து மனித இரத்தம் மற்றும் நரம்பு திசுக்களில் உள்ள கோலினெஸ்டரேஸ் (Cholinesterase) நொதியின் செயல்நிலையை தடை செய்வதன் மூலம் உடனடி மற்றும் நீண்ட கால பாதிப்புகளை உண்டாக்குகிறது. ஆர்கனோபாஸ்பரஸால் நஞ்சேற்றம் அடைந்திருப்பதை இரத்தத்தில் உள்ள கோலினெஸ்டரேஸ் நொதியின் செயல்நிலையை அளப்பதன் மூலம் உறுதி செய்யப்பட்டு மருத்துவம் அளிக்கப்படுகிறது.

நோயாளிக்கு ஏற்படும் பாதிப்புகள் இரத்தத்தில் உள்ள OPC-ன் அளவையும், நோயாளியின் பாதிப்புக்குள்ளாகும் தன்மையையும் பொருத்து அமையும். இவ்விரண்டு குணங்களும் PON-1 நொதியின் செயல்நிலையை சார்ந்துள்ளது. ஆகவே PON-1 மற்றும் Cholinesterase நொதிகளின் செயல்நிலை சம்மந்தத்தை கண்டறிய இந்த ஆய்வு மேற்கொள்ளப்படுகிறது. இதன் மூலம் நோயாளிகளின் பாதிப்புக்கு உள்ளாகும் தன்மையை அறிந்து அதற்கேற்ற மருத்துவ சிகிச்சையை ஆவண செய்ய உதவிகரமாக இருக்கும்.

இந்த ஆய்வில் சுய விருப்பத்துடன் பங்கேற்கும் நோயாளிகளுக்கு மட்டும் பரிசோதனை செய்யப்படும். ஆய்வில் பங்கேற்கும் நோயாளிகளின் வழக்கமான மருத்துவ சிகிச்சையை பாதிக்காமல் இந்த ஆய்வு மேற்கொள்ளப்படும்.

ஒப்புதல் படிவம்

ஆய்வின் தலைப்பு :

"ஆர்கனோபாஸ்பரஸ் பூச்சிக்கொல்லி மருந்தினால் நஞ்சேற்றம் அடைந்த நோயாளிகளின் இரத்தத்தில் பாராக்சோனேஸ் - 1 (PON-1) நொதியின் செயல்நிலை வகையையும் கோலினெஸ்டரேஸ் நொதியின் செயல்நிலையையும் கண்டறியும் ஆய்வு''

ஆராய்ச்சி நிலையம் : அரசு ஸ்டான்லி மருத்துவமனை, சென்னை.

மேலே குறிப்பிட்டுள்ள மருத்துவ ஆய்வின் விவரங்கள் எனக்கு விளக்கப்பட்டது. என்னுடைய சந்தேகங்களைக் கேட்கவும் அதற்கான தகுந்த விளக்கங்களைப் பெறவும் வாய்ப்பளிக்கப்பட்டது.

நான் இந்த ஆய்வில் தன்னிச்சையாகத் தான் பங்கேற்கிறேன். எந்த காரணத்திலும் எந்த கட்டத்திலும் எந்த சட்ட சிக்கலுக்கும் உட்படாமல் நான் இந்த ஆய்வில் இருந்து விலகிக் கொள்ளலாம் என்றும் அறிந்து கொண்டேன். இந்த ஆய்வு சம்மந்தமாகவும் இதை சார்ந்த மேலும் ஆய்வு மேற்கொள்ளும் போதும், இந்த ஆய்வில் பங்கு பெறும் மருத்துவ / சமூக பணியாளர் என்னுடைய மருத்துவ அறிக்கைகளைப் பார்ப்பதற்கு என் அனுமதி தேவையில்லை என அறிந்து கொள்கிறேன். நான் ஆய்வில் இருந்து விலகிக் கொண்டாலும் இது பொருந்தும் என அறிகிறேன்.

இந்த ஆய்வின் மூலம் கிடைக்கும் தகவல்களையும் பரிசோதனை முடிவுகளையும் மற்றும் சிகிச்சை தொடர்பான தகவல்களையும் மருத்துவ/சமூக பணியாளர் மேற்கொள்ளும்ஆய்வில் பயன்படுத்திக் கொள்ள என் முழுமனதுடன் சம்மதிக்கிறேன்.

பங்கேற்பவர்/உறவினர் கையொப்பம்

இடம் :

தேதி : கைவிரல் ரேகை

பங்கேற்பவரின் பெயர் மற்றும் விலாசம் :

ஆய்வாளரின் கையொப்பம் :

ஆய்வாளரின் பெயர் : **Dr. ஆனந்தி . பெ**

ANNEXURE III

MASTER CHART - I

SOCIO DEMOGRAIC DATA

S.No	Age (yrs)	Gender (M/F)	Education (IL/P/S)	Marital status	Occupation Status	Socio economic	Domicile
1	32	M	S	M	private	M	Urban
2	24	M	S	UM	own	U	Suburban
3	26	M	S	UM	student	M	Urban
4	38	M	IL	M	others	M	Urban
5	52	M	IL	M	farmer	L	Rural
6	32	M	IL	M	private	L	Rural
7	20	M	S	UM	student	M	Urban
8	19	F	S	UM	student	L	Urban
9	26	M	S	M	own	M	Urban
10	18	F	S	UM	student	M	Urban
11	25	F	IL	M	farmer	M	Rural
12	27	M	IL	M	farmer	M	Rural
13	34	M	IL	M	others	M	Suburban
14	35	M	S	M	private	M	Suburban
15	38	F	IL	M	house	L	Rural
16	26	M	S	UM	private	L	Suburban
17	22	M	S	UM	Govt	M	Urban
18	28	F	S	M	house	M	Urban
19	39	M	IL	M	farmer	M	Rural
20	27	M	IL	UM	others	M	Suburban
21	46	F	IL	M	private	L	Suburban
22	35	F	S	M	house	M	Rural
23	57	F	IL	M	house	M	Urban
24	30	M	IL	M	own	U	Urban
25	29	M	S	UM	private	L	Urban
26	28	M	IL	UM	others	M	Rural
27	31	F	S	M	house	M	Urban
28	54	M	P	M	own	M	Urban
29	40	F	IL	M	farmer	M	Rural

30	28	M	S	M	student	M	Urban
31	29	M	S	UM	private	L	Urban
32	33	F	S	M	Govt	L	Urban
33	35	F	IL	M	farmer	L	Rural
34	40	M	IL	M	private	M	Urban
35	22	F	S	M	house	L	Urban
36	30	F	IL	M	house	L	Urban
37	48	M	P	M	own	M	Urban
38	26	M	S	UM	private	M	Rural
39	21	M	S	UM	private	L	Urban
40	35	M	S	UM	own	M	Urban
41	25	F	S	UM	private	L	Urban
42	50	M	IL	M	house	L	Urban
43	29	M	S	UM	private	M	Suburban
44	47	M	IL	M	others	L	Suburban
45	36	M	IL	M	own	L	Urban
46	24	F	S	UM	student	L	Urban
47	46	F	IL	M	private	L	Urban
48	56	F	IL	M	house	L	Urban
49	52	M	P	M	private	M	Suburban
50	48	F	IL	M	farmer	M	Suburban
51	35	M	S	M	own	M	Urban
52	37	M	S	M	own	M	Urban
53	23	M	IL	UM	farmer	L	Suburban
54	28	M	IL	UM	farmer	M	Urban

Age in years
Gender: M- Male; F- Female;
Educational status: IL-Illiterate; P- Primary; S- Secondary
Marital status: UM- Unmarried; M- Married
Socioeconomic status: L- Lower; M- Middle; U- Upper

MASTER CHART - II

ANALYTICAL VALUES OF STUDY POPULATION

S.No	Age (yrs)	Gender (M/F)	Sr.TP (g%)	RPG (mg%)	Sr.CHE (U/L)	Sr.Para (P)(U/L)	Sr.Aryl (A)(kU/L)	P/A ratio	PON1 pheno
1	32	M	6.6	90	2856	180.91	51.38	3.5	AB
2	24	M	6.9	126	634	83.93	90.83	0.9	AA
3	26	M	7	119	342	49.86	95.52	0.5	AA
4	38	M	7.2	82	3512	250.23	62.26	4	AB
5	52	M	6.6	88	2698	262.56	81.84	3.2	AB
6	32	M	6.8	90	2462	173.39	57.49	3	AB
7	20	M	6.9	104	947	148.92	145.28	1	AA
8	19	F	6.8	92	1232	277.47	153.04	1.8	AA
9	26	M	6.9	112	1198	192.64	109.45	1.8	AA
10	18	F	6.9	90	2386	372.15	100.47	3.7	AB
11	25	F	6.6	86	9840	494.28	70.43	7	BB
12	27	M	6.7	90	1056	108.91	67.81	1.6	AA
13	34	M	6.9	92	2912	370.47	102.25	3.6	AB
14	35	M	6.9	80	1112	170.78	130.37	1.3	AA
15	38	F	6.4	84	1364	210.86	93.14	2.3	AA
16	26	M	6.8	80	1346	213.78	97.44	2.2	AA
17	22	M	7	115	7583	487.92	78.68	6.2	AB
18	28	F	6.9	98	4206	433.23	93.97	4.6	AB
19	39	M	6.8	88	2451	225.58	72.02	3.1	AB
20	27	M	7.1	85	1285	254.88	121.72	2.1	AA
21	46	F	6.9	76	816	79.91	78.11	1	AA
22	35	F	6.8	78	2069	405.99	144.38	2.8	AA
23	57	F	6.9	94	1082	94.56	89.04	1.1	AA
24	30	M	7	93	1934	112.78	46.33	2.4	AA
25	29	M	7	80	995	90.22	80.47	1.1	AA
26	28	M	6.8	90	1468	142.31	67.96	2	AA
27	31	F	6.7	118	1361	222.25	104.54	2.1	AA
28	54	M	6.9	83	8265	350.37	50.18	7	BB
29	40	F	6.5	82	1156	214.57	98.02	2.1	AA
30	28	M	6.8	81	7265	458.1	75.86	6	AB
31	29	M	6.9	83	973	52.47	47.35	1.1	AA

32	33	F	6.9	123	2560	220.8	71.04	3.1	AB
33	35	F	6.9	85	1215	113.87	48.17	2.4	AA
34	40	M	6.8	74	3320	203.37	52.13	3.9	AB
35	22	F	6.9	76	1632	201.99	97.63	2.1	AA
36	30	F	6.6	78	1791	119.19	53.33	2.2	AA
37	48	M	6.8	76	1567	135.67	51.82	2.6	AA
38	26	M	7	88	3713	227.6	54.15	4.2	AB
39	21	M	7	87	435	56.1	73.53	0.8	AA
40	35	M	7.3	83	3682	322.14	77.96	4.1	AB
41	25	F	6.9	86	2781	292.06	84.36	3.4	AB
42	50	M	6.8	80	1872	306.02	135.71	2.3	AA
43	29	M	6.7	107	927	101.96	83.92	1.2	AA
44	47	M	6.9	85	4872	419.64	80.5	5.2	AB
45	36	M	7	84	398	54.71	87.26	0.6	AA
46	24	F	7.1	85	1772	196.22	93.08	2.1	AA
47	46	F	6.8	88	1173	85.24	76.38	1.1	AA
48	56	F	6.5	90	5255	421.03	73.78	5.7	AB
49	52	M	6.9	86	1739	181.29	81.8	2.2	AA
50	48	F	6.9	88	1250	152.89	66.41	2.3	AA
51	35	M	6.8	98	981	73.8	61.4	1.2	AA
52	37	M	6.9	90	1178	408.91	191.35	2.1	AA
53	23	M	6.9	76	1209	106.34	41.73	2.5	AA
54	28	M	6.8	78	1450	205.68	102.02	2	AA

P/A ratio: SerumParaoxonase/Arylesterase activities Ratio

PON1 pheno: Serum PON1 phenotype

INSTITUTIONAL ETHICAL COMMITTEE,
STANLEY MEDICAL COLLEGE, CHENNAI-1

Title of the Work : A Study on Phenotypic Polymorphism of serum Paraoxonase 1 and activity of serum cholinesterase in acute Organophosphorous compound poisoning.

Principal Investigator : Dr. Ananthi.P

Designation : PG in MD (Bio Chemistry)

Department : Department of Bio Chemistry
Government Stanley Medical College,
Chennai-01

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 02.07.2014 at the Council Hall, Stanley Medical College, Chennai-1 at 2PM

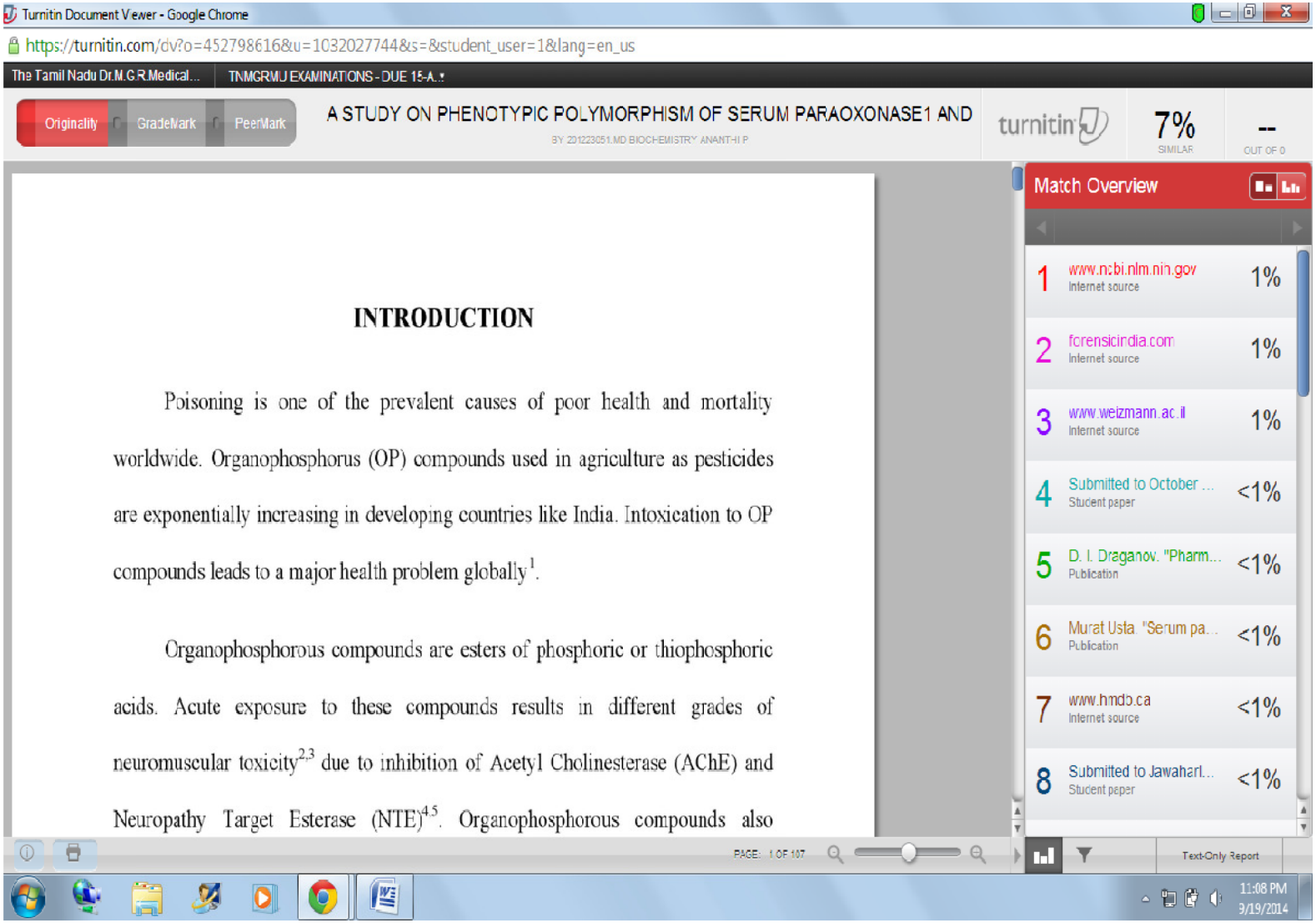
The members of the Committee, the secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The Principal investigator and their team are directed to adhere to the guidelines given below:

1. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
2. You should not deviate from the area of the work for which you applied for ethical clearance.
3. You should inform the IEC immediately, in case of any adverse events or serious adverse reaction.
4. You should abide to the rules and regulation of the institution(s).
5. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
6. You should submit the summary of the work to the ethical committee on completion of the work.

K Vasanthan

MEMBER SECRETARY,
IEC, SMC, CHENNAI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INTRODUCTION
Poisoning is one of the prevalent causes of poor health and mortality worldwide. Organophosphorus (OP) compounds used in agriculture as pesticides are exponentially increasing in developing countries like India. Intoxication to OP compounds leads to a major health problem globally[1].
Organophosphorous compounds are esters of phosphoric or thiophosphoric acids. Acute exposure to these compounds results in different grades of neuromuscular toxicity[2,3] due to inhibition of Acetyl Cholinesterase (AChE) and Neuropathy Target Esterase (NTE)[4,5]. Organophosphorous compounds also
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