RESEARCH ARTICLE

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Organophosphate Pesticide Exposure Reduced Serum Paraoxonase1 (PON1) Activity Which Correlated with Oxidative Stress in Pesticide Factory Workers

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Background & Aims of the Study: Serum paraoxonase (PON1) is a potent antioxidant that is associated with the pathogenesis of several diseases. Also, it is reported that environmental factors can modulate the PON1 activity. In this study, the association between the Organophosphates (OP) exposure and plasma Paraoxonase/Arylesterase (PON1) activity and also OP-induced oxidative stress was investigated among pesticide manufacturing factory workers.

Materials and Methods: In this cross-sectional study, Plasma cholinesterase (PChE) and RBC Acetylcholinesterase activity (AChE) were measured as a biomarker of OP exposure. Also, the amount of malondialdehyde (MDA) was evaluated as a biomarker for lipid peroxidation and oxidative stress. The PON1activity was measured by two distinct substrates, Paraoxson and Phenylacetate, to measure paraoxonase (PONase) and arylesterase (AREase) activities of PON1 enzyme respectively.

Results: A significant decrease was observed inPChE and AChE activities in the blood sample of factory workers which confirmed OP exposure ($p<0.001$). Furthermore, the level of MDA increased that shown lipid peroxidation induced by OP ($p<0.05$). PONase and AREase activities decreased significantly in factory workers ($p<0.05$, $p<0.001$_). The reduction in AREase activity shown a correlation with the decrease in PChE and AChE activities ($p<0.01$), also a negative correlation was observed with AREase activity and lipid peroxidation ($p<0.01$). The Ponase activity only negatively correlated with lipid peroxidation ($p<0.01$).

Conclusion: Our study indicates that occupational OP pesticide exposure in factory workers causes a decrease in serum PON1 activity which is associated with oxidative stress. Decreased PON1 activity is involved in the pathogenesis of several diseases such as cardiovascular disease then it is recommended that PON1 activity of OP-pesticide workers has been periodically monitored.

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Background

Pesticides are synthetic chemicals that use worldwide for controlling the agricultural and domestic pest. The active component of pesticides is broad and includes Organochlorine, Organophosphate, Carbamate, Pyrethroid and Neonicotinoid compounds. Of all these compounds, Organophosphates are the most widely used insecticides today. They are

used in agriculture, the home, gardens, and veterinary practice. More than 100 different OP compounds are formulated as a pesticide which mainly used as insecticide and herbicide (1) Chronic exposure to pesticides especially organophosphates cause many severe problems for human health (2). Studies indicated that the prevalence of diseases such as diabetes, cardiovascular diseases, congenital disabilities, and reproductive disorders had been related to long term exposure to pesticides. Also, chronic neurologic effects such as neurodegenerative disorders include Parkinson, Alzheimer, and Amyotrophic lateral sclerosis (ALS) are suspected of being linked to chronic OP exposure, creating a real concern in the health community (3-6) Occupational exposure to the pesticide is a major concern. Agricultural and pesticide manufacturing factory workers are at risk of exposure to high level of pesticides and its consequence health effect. (7-9).

Paraoxonase-1 (PON1) is a serum esterase which is synthesized by the liver. It is a high-density lipoprotein-associated enzyme that hydrolyzes oxidized phospholipids and capable of detoxifying OP compounds in human serum (10,11). Also, PON1 has a protective effect against free radicals that is considered as a powerful antioxidant (12). Human serum PON1 enzyme has two distinct enzyme activities: arylesterase (AREase) and paraoxonase (PONase) activities toward specific substrates phenylacetate and paraoxone respectively. There is a genetic polymorphism in PON1 gene which severely affects paraoxonase activity of the enzyme, and wide individual variation is observed on paraoxonase activity, Humans can be categorized in three different serum PON1 phenotypes by using enzymatic analysis include AA: low activity, AB: moderate activity, BB: high intensity. The AREase activity has not been affected by genetic polymorphism and usually shows the content of the enzyme in the serum. (13).

In addition to genetic factors that affect PON1 activity, other factors such as disease conditions and environmental pollutants affect PON1 activity. Inorganic arsenic reduced serum PONase and AREase activity in rats after chronic low dose exposure (14). Studies have been indicated that in several chronic diseases such as diabetes, cancer, and Alzheimer disease, associated with increased systemic oxidative stress, reduced serum PON1 activity has been observed(15,16). Then it is postulated that oxidative stress is associated with reduced PON1 activity (17-19).

One of the proposed mechanisms of OP toxicity is oxidative stress. It is reported that the OP compounds interact with cellular antioxidant defense and also produce oxygen free radicals which lead to oxidative tissue damage. The oxidative stress markers have been found in the serum of OP pesticide manufacturing factory workers (20,21).

The metabolism of organophosphates may be affected by the genetic polymorphic variation (Polymorphism) in serum paraoxonase activity in individuals at risk of OP exposure. Then several types of research have been studied the relation between the PON1 polymorphism and the risk of OP poisoning (19,22,23) but little is known about the effect of OP occupational exposure on the plasma PON1 activity and also the relation between OP-induced oxidative stress and PON1 activity.

Aims of the study:

In the present study PChE activity and RBC AChE activity as a biochemical marker of exposure, lipid peroxidation as a biomarker of oxidative stress and serum paraoxonase /arylesterase activity were measured in OP pesticide manufacturing factory workers and also a correlation between these biomarkers were investigated.

Materials & Methods

Chemicals

Acetylthiocholine iodide, 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), Malondialdehyde bis dimethyl acetal, phenylacetate, 2-thiobarbituric acid (TBA), paraoxon were purchased from Sigma-Aldrich (USA)

Human subjects

In this cross-sectional study, 21 male workers of a pesticide manufacturing factory at the age range of 23-55 years, with a minimum work history of 1 year and a maximum of 5 years were included. Age- and sex-matched control subjects (n=40) were volunteers from Pathology Laboratory clients that they visited for a routine checkup who were not directly exposed to the pesticides. Every subject was asked to fill out a questionnaire about his health status, the use of medications, occupational history, exposure to pesticides, and so forth, and signed a consent form to participate in this study. Based on the questionnaire, subjects with a history of chronic diseases such as diabetes mellitus and hypertension, cigarette smoking and use of drugs were excluded from the study. A blood sample was obtained from the brachial vein of each subject by an expert person. The project was approved by the Ethics Committee of Tarbiat Modares University (Ethical code: IR.TMU.REC.1394.14), and informed consent was received from all workers and healthy subjects.

Sample preparation

Venous blood was collected into dried heparinized tubes. After blood centrifugation, plasma was separated from the erythrocytes and stored in -70 ℃ until assay. The erythrocytes were washed with normal saline then were suspended in de-ionized water in a volume corresponding to the initial volume of the whole blood and then were frozen for hemolysis.

PChE and RBC AChE activity assay

The activities of AChE and PChE were measured by the Ellman spectrophotometric method (24,25). In this method AChE hydrolyzed acetylthiocholine to produce thiocholine and acetate. The thiocholine reduces the Dithiobis-Nitrobenzoic Acid (DTNB) to nitrobenzoate (TNB) anions, a yellow color compound, which has an absorbance at 405 nm. The increase in absorbance at 405 is proportional to the enzyme activity. The RBC lysate was diluted 60-fold with a buffer (0.1 M phosphate buffer, pH=7.4). The activity of the enzyme was calculated by the use of the extinction coefficient of TNB anions. The PChE activity in plasma was also measured in the same way except butyrylthiocholine was used as a substrate.

Lipid peroxidation assay

Plasma Lipid peroxidation was estimated by the measurement of Thiobarbituric acid reactive substances (TBARS) concentration which was performed by thiobarbituric acid reaction (TBAR) colorimetric assay. One volume of plasma was mixed with two volumes of TBA reagent (containing 3.75% TCA and 0.0925% TBA) and the mixture incubated at 90°C for 60 min. After rapidly cooling on ice, centrifuged at 1000 g for 10 min and the optical density of supernatant was measured in 540 nm in a plate reader. Because the principal member of TBARS is malondialdehyde (MDA); MDA standard curve was established with using the stable MDA precursor, (Malondialdehyde bis dimethyl acetal) and then the concentration of TBARS is expressed in nmol MDA/mL in plasma (26)

Determination of PON1 enzymatic activities

Paraoxonase activity assay

Ponase activity was measured with paraoxon as a substrate as previously described (27). Briefly, 40 µl paraoxon (6mM) was added to 150 µl assay buffer (containing 0.132 M Tris-HCl pH 8.5 and 1.32 mM $CaCl_2$) then immediately after addition of 10µl sample, absorbance was monitored for 5minute at 405

nM by a plate reader. In this assay paraoxonase hydrolysis, the paraoxone to produce p-nitrophenol and the paraoxonase activity was calculated by using the extinction coefficient of p-nitrophenol (18050M^{-1} cm^{-1}) and units were expressed as micromoles of paraoxon hydrolyzed per minute. The salt stimulated activity was measured by the addition of 1M NaCl in the assay buffer. The percent stimulation of enzyme activity was calculated as follows:

$$\frac{\text{Paraoxonase activity with 1M NaCl – Basal paraoxonase activity}}{\text{Basal paraoxonase activity}} \times 100\%$$

The percent stimulation is a qualitative property of paraoxonase that separates the AA phenotype from the combined AB and BB phenotypes. The percent stimulation above 60% refers to AB and BB phenotypes, and less than 60% relates to AA phenotype (28). Individuals with AA phenotypes which had low enzyme activity were excluded from the study to reduce the within-group variation and partly to reduce the effect of polymorphism; then mean paraoxonase activity was compared between groups.

Arylesterase activity assay

Arylesterase activity was measured with the phenyl-acetate substrate as described earlier (29). Briefly,5µl of the sample was added to a 195µl substrate solution containing 3.26 mM phenyl-acetate, 100mM Tris–HCl pH=8 and 2 mM $CaCl_2$ and then the rate of generation of phenol was determined at 270 nm at 37°C, using a continuously recording spectrophotometer. The molar extinction coefficient of 1310 M^{-1} cm^{-1} was used for calculations of enzyme activity and units were expressed as micromoles of phenyl-acetate hydrolyzed per minute.

Statistical analysis

Student's t-test was used to determine the significance of differences observed between groups. F-test was done to determine the normal distribution of variances between groups. The correlation between groups was measured by the Pearson test which measures the statistical relationship, or association, between two continuous variables. p-values of 0.05 or less were considered as significant differences.

Results

Paraoxonase Phenotype in the Studied individuals

According to the percent stimulation of paraoxonase activity by salt, the studied individuals were categorized in AA phenotype or combined AB and BB phenotypes. Table 1 shows the phenotype distribution of studied individuals. Only 11 persons from a total 61 persons have AA phenotype with low paraoxonase activity. The data of persons with AA phenotype were excluded from the study to reduce the within-group variation and eliminate the effects of polymorphisms.

Table 1) The phenotype distribution of studied individuals

	Phenotype	
	AA	**AB and BB**
Control	6	34
Workers	5	16
Total	11	50

AA: low activity, BB: high activity, AB: Moderate activity

PChE and RBC AChE activity

PChE activity and RBC AChE activity were measured by the Ellman method and results indicated in Figure 1. The significant reduction in both enzyme activity was observed in factory workers ($p<0.001$). The PChE activity inhibition was about 31.7±5.9%while RBC AChE activity inhibition was 14.7±2.2% (Table 2).

Table 2) Comparison of biochemical parameters between organophosphate pesticide manufacturing factory workers and control unexposed groups

Biochemical parameters	Control(Mean±SD)	Workers(Mean±SD)
PChE activity(U/L)	7482±1466	5105±1599***
AChE activity(U/grHb)	128.5±15.31	109.6±12.22***
Ponase activity(U/L)	86.38±46.28	53.72±37.36*
AREase activity(U/L)	7490±1545	4592±1337***
MDA (nM/ml)	10.42±2.04	16.05±2.5*

PChE(Plasma cholinesterase), AChE(RBC Acetylcholinesterase), Ponase(Paraoxonase), AREase(Arylesterase),MDA(malondealdehyde).

(*) represents significant difference versus control. (t-test,*p<0. 05, ***p<0.001)

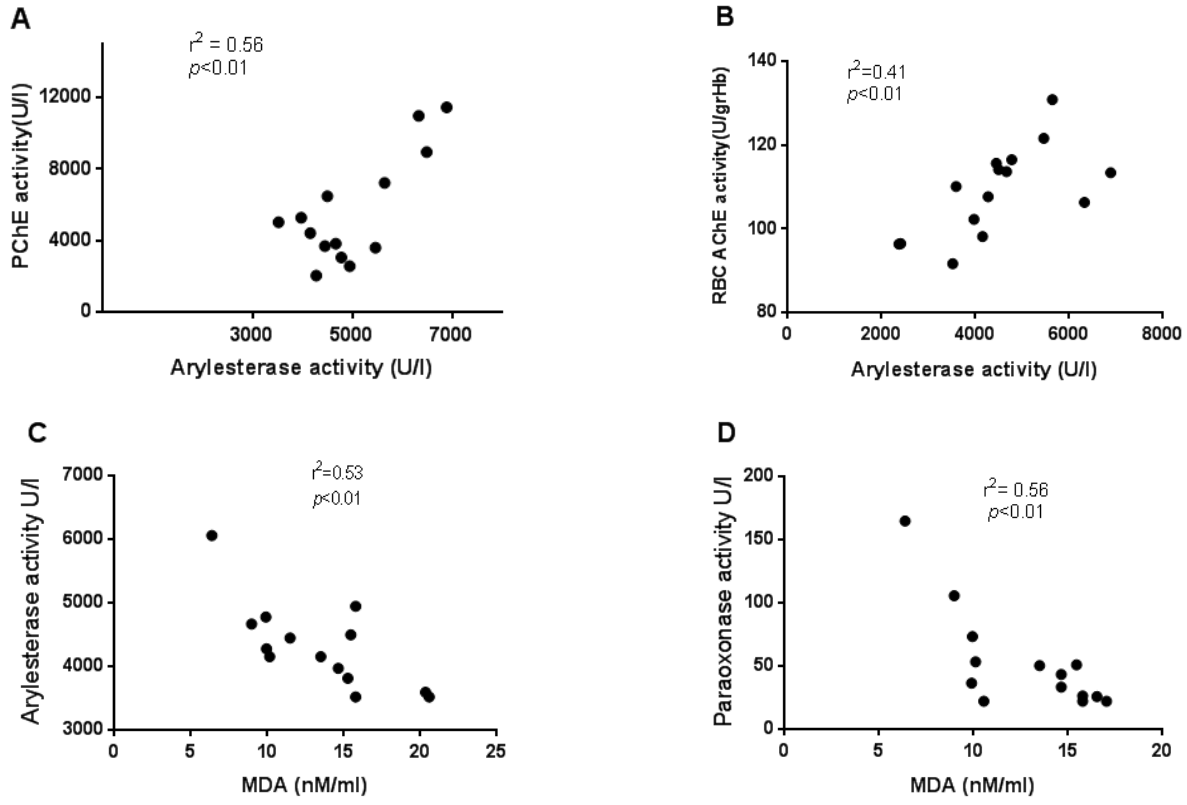

Figure1) correlation between plasma cholinesterase (PChE) activity and plasma Arylesterase activity(A), RBC acetylcholinesterase (AChE) activity and plasma Arylesterase activity(B), plasma lipid peroxidation and plasma Arylesterase activity(C) and plasma lipid peroxidation and plasma Paraoxonase activity(D) (Pearson test, p<0.01)

Lipid peroxidation

The amount of TBARS production was measured as an index of lipid peroxidation. Results indicated that the TBARS concentration was increased in factory workers' blood samples (Table 2) and statistical analysis showed significant differences between workers and control groups ($p<0.05$).

Paraoxonase and Arylesterase activity

Ponase activity was measured with Paraoxone as a substrate. The basal Ponase activity ranged between 43.33-233.4 U/l in the control group and 22.16-115.23 U/l in factory workers and a high variation between individuals was observed. Statistical analysis indicated a significant reduction in Ponase activity in factory workers in comparison to the control group ($p<0.05$). AREase activity of the enzyme was measured with phenylacetate as a substrate. A significant reduction in plasma AREase activity was also observed in factory workers ($p<0.001$) (Table 2).

Assessment of correlation between measured parameters

The correlations between plasma Ponase and AREase activity with plasma lipid peroxidation, PChE activity and RBC AChE activity in factory workers were evaluated. Results indicated that plasma AREase activity positively correlated with PChE ($r^2=0.56$, $p<0.01$) and RBC AChE activities ($r^2=0.41$, $p<0.01$). Low AREase activity associated with low PChE and low AChE activities (Figure 1A and 1B respectively). Also, plasma AREase activity negatively correlated with plasma lipid peroxidation ($r^2=0.53$, $p<0.01$) then low plasma AREase activity was associated with high plasma lipid peroxidation (Figure 1C). Moreover, plasma Ponase activity negatively correlated with plasma lipid peroxidation ($r^2= 0.56$, $p<0.01$) (Figure 1D) but significant correlations with Ponase and PChE activity and also Ponase and RBC AChE activity were not observed.

Discussion

In this study, we compared the paraoxonase and arylesterase activity and lipid peroxidation as a biomarker of oxidative stress in the plasma sample of pesticide manufacturing factory workers with control (unexposed person) group. The PChE activity and RBC AChE activity were measured as a biomarker of OP pesticide exposure (30). The results indicated that both enzymes were inhibited in factory workers which confirmed the OP exposure. The PChE activity was more inhibited than RBC AChE activity. This finding is in accordance with previous studies reported that PChE activity is more susceptible to inhibition by OPs. Indeed, inhibition of this enzyme indicates short term exposure to OPs. The inhibition of AChE has a lower recovery rate than PChE inhibition and then reflects the chronic exposure to OP (31,32) All workers who included in this study were actively working in the organophosphate production line at the time of sampling. Thus, the inhibition of PChE in these workers is expected. Several studies indicated reduced activity of both enzymes in OP-exposed farm and manufacturing factory workers (20,33). Oxon metabolites of OPs inhibit these enzymes irreversibly that leads to the reduction in enzyme activity. Measuring enzyme activity in blood samples is a valid biomarker to assess exposure with OP pesticides, and also it can be used in monitoring occupational exposures (32).

The detrimental effects of OP compounds are not limited to the inhibition of cholinesterase enzyme, but non-cholinergic effects such as free radical production and impairment of the antioxidant system which resulted in oxidative stress can damage the tissues (34). The oxidative damages such as lipid peroxidation, protein oxidation and damage to DNA in consequence of OP exposure have been observed in individuals who occupationally exposed to OP pesticides (21,35).

In this study, an increased amount of plasma lipid peroxidation was observed in factory workers in comparison to the control group which implied to OP-induced oxidative stress. On the other hand, our study indicated that Ponase and AREase activity significantly reduced in factory workers and also there was a negative correlation between the activity of these enzymes and amount of plasma lipid peroxidation which indicated that reduced activity of PON1 enzyme was associated with high amount of lipid peroxidation and oxidative stress. PON1is an essential extracellular enzyme that plays a role in antioxidant processes. In vitro studies have shown that PON1 has a peroxidase-like activity that can hydrolysis H_2O_2 and also prevents and reduce the production of hydroperoxide in the plasma. Reduced PON1 activity is associated with oxidative stress (18). PON1 protein has free thiol groups which are necessary for the antioxidant activity of the enzyme. It is postulated that in oxidative stress conditions, these free thiols may be oxidized that resulted in decreased enzyme activity (36,37). Then it is proposed that OP-induced oxidative stress has decreased the PON1 activity in factory workers.

On the other hand, decreased PON1 activity reduces the antioxidant capacity of plasma and worsens the OP-induced oxidative stress. The Ponase activity of the PON1 enzyme is affected by genetic polymorphism, and there is a high variation in plasma Ponase activity between individuals. But AREase activity of PON1 enzyme is not influenced by genetic polymorphism and indicates of level of enzyme in the plasma (29) also it is reported that AREase activity of PON1 enzyme is a better indicator of antioxidant activity of enzyme (38) then it is deduced that the level of plasma AREase activity can estimate the OP-induced oxidative stress.

Besides the antioxidant activity of PON1, this enzyme involves hydrolyzing oxon metabolites of the OP which are a potent inhibitor of cholinesterase enzyme. Thus PON1 plays a vital role in detoxification of OP. For this reason, several studies have investigated the relation between PON1 status and susceptibility to OP intoxication (22), but are few studies evaluated the effects of OP exposure on plasma PON1 level. Hernandez et al. have been investigated the relation between OP-pesticide exposure and PON1activity in farm workers. Results of their study have shown that short term exposure to OP-pesticide was associated with decrease PON activity in workers (39). Mackness et al. investigated the PON1 activity and its level in Persian Gulf war veterans who have been exposed to OP nerve gases and found low PON1 activity and low PON1 concentration in the serum which was independent of the effect of PON1 genotype (40). Our findings were in accordance with the results of these studies and indicated that both Ponase and AREase activity of PON1were reduced by OP exposure. Also, the results of our research showed a positive correlation between AREase and PChE activity and also AREase and AChE activity that indicated reduced AREase activity was associated with OP exposure. As a result, it is postulated that Op-induced oxidative stress decreased the PON1 activity that resulted in less OP-metabolite detoxification and more cholinesterase inhibition. This vicious cycle worsens the OP-induced toxication, oxidative stress, and tissue damages. Our results did not show a correlation between the Ponase activity of PON1 enzyme and cholinesterase activity. This may be because of the high variation in Ponase activity between individuals, and our sample size was not enough to indicate a significant correlation. According to this finding, we proposed that AREase activity of the PON1 enzyme is a better indicator than Ponase activity for measurement of PON1 level after OP exposure and also is a better indicator for OP-induced oxidative stress and could be

used as a biomarker of OP exposure and OP-induced oxidative stress.

Reduced PON1 level contributes to chronic diseases such as cardiovascular disorders, cancer, diabetes, and neurodegenerative diseases. In consequence, reduced PON1 activity as a result of OP exposure may predispose the OP manufacturing workers to such clinical conditions. Then monitoring of plasma PON1 activity has been recommended in OP manufacturing workers. Also, some studies indicated that natural polyphenols such as quercetin have increased plasma PON1 activity (41). It is suggested that the supplementation of the worker's diet with this polyphenol may restore PON1 activity and prevent from OP-induced oxidative stress and related diseases. This hypothesis needs further investigations.

Conclusion

In this study, a reduction in both Ponase and AREase activity of PON1 enzyme and also an increment in the amount of lipid oxidation were observed following OP exposure in pesticide manufacturing factory workers. Also, this study showed that AREase activity of the PON1 enzyme was positively had the correlation with PChE and AChE activity and negatively with plasma lipid peroxidation then we conclude that OP exposure reduces serum PON1 enzyme level which is associated with oxidative stress. Because reduced PON1 activity was related to oxidative stress-induced diseases, monitoring of PON1 activity is recommended as a routine checkup for factory workers and for this end measuring of AREase activity could be a better indicator of PON1 level, because it is independent of genetic variation.

Footnotes

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Conflict of Interest:

The authors declared no conflict of interest.

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