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Genotyping Paraoxonase Polymorphisms in Iranian Farmers Exposed to Organophosphate Pesticides

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Abstract: Paraoxonase 1 is an esterase that associates with other enzymes to detoxify organophosphate pesticides. The expression and activity of paraoxonase is due to polymorphisms of the paraoxonase gene. Thus, the present study aimed to determine the activity of paraoxonase and paraoxonase genotypes along with their relationships, the risks and medical conditions in Iranian workers that were occupationally exposed to organophosphates. Workers (n = 80) exposed to organophosphates and controls (n = 160) that were not exposed to Organophosphates gave a blood sample in order to have two polymorphisms of paraoxonase, paraoxonase activity buthyrylcholinesterase activity and their interactions investigated. The results showed that the exposed group had significantly ($p<0.05$) less paraoxonase activity than the controls (90.04±4.632 and 149.8±5.236 nmol min^{-1} mL^{-1}, respectively). The Q/Q, Q/R and R/R genotypes (highest to lowest rates, respectively) significantly hydrolyzed paraoxon (i.e., paraoxonase activity) in both groups ($p<0.001$). Paraoxonase activity was higher in subjects with L/L genotypes and was the lowest in individuals with M/M genotypes in the exposed and control groups. The results indicate that individuals with paraoxonase R/R and M/M genotypes may be more susceptible toward organophosphate toxicity. Enzymatic activities were widely varied and it appears that the differences in genotypes can cause the changes in this activity. Thus, these different genotypes may be important biomarkers in protecting individuals and identifying individual risk factors in workers exposed to organophosphates.

Key words: Paraoxonase, polymorphism, organophosphate

INTRODUCTION

Organophosphates (OPs) are widely used in agriculture as insecticides, as therapeutic agents in the treatment of ophthalmic conditions, as nerve agents in chemical warfare and in industries such as solvents, plasticizers and flame retardants (Moshiri *et al*., 2012; Singh *et al*., 2011). OPs inhibit cholinesterase (ChE) which affects human health by causing neuropsychological syndromes, endocrine disorders, developmental anomalies, disruption of the immune system and hypersensitivity (Mansour, 2004). Specifically, exposure to OPs can cause lung and prostate cancers, non-Hodgkin's lymphoma and leukaemia (Bonner *et al*., 2010; Waddell *et al*., 2001). Human paraoxonase (PON1) is an esterase that is synthesized in the liver and it breaks down OPs before it binds to the ChEs which helps detoxify the OPs (Ferre *et al*., 2002). Further, PON1 associates with high-density lipoprotein (HDL) particles in the plasma (Gohari *et al*., 2012). Several polymorphisms have been identified in the PON1 gene. Substitutions of Glutamine (Q) for Arginine (R) at position 192 and Leucine (L) for Methionine (M) at position 55 are common polymorphisms in the coding sequence of PON1. Different isoforms of the Q/R alleles can vary the activity of PON1 and effect several biological processes

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(Hashemi *et al*., 2010). The L/M substitution affects the PON1 levels in the plasma (Hashemi *et al*., 2010). Previous studies have shown that several human diseases such as Alzheimer's and Parkinson's diseases (Erlich *et al*., 2006; Zintzaras and Hadjigeorgiou, 2004) as well as reproductive and cardiovascular disorders (Bhattacharyya *et al*., 2008; Chen *et al*., 2004; Perez-Herrera *et al*., 2008) occur with different PON1 genotypes. These same genotypes could be associated with various phenotypes since different expressions of the PON1 gene are expressed differently in individuals (Costa *et al*., 2005b).

Thus, the present study aimed to determine the activity of PON1 and PON1 genotypes along with their relationships, the risks and medical conditions in Iranian workers that were occupationally exposed to organophosphates.

MATERIALS AND METHODS

Subjects: Two hundred and forty subjects participated in this study. The control group (n = 160) consisted of individuals that went to Shafa hospital in Ahvaz, Iran to undergo genetic testing, prior to marriage. These individuals did not have a history of pesticide exposure. Every participant that worked in the fields signed n informed consent prior to giving a blood sample. These 80 workers were working in the fields for 18.89±1.47 years. During the study, the workers were spraying crops with pesticides, such as diazinon, parathion and 2,4 DEP. The test group (workers) and the control group had an average age of 40.70±1.37 and 38.85±1.01, respectively. This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran and all participants signed the informed consent prior to enrolment.

The questionnaires were completed by the test and control groups. Questions in the questionnaire included age, weight, height, presence or absence of disease, medication, smoking, alcohol, drug abuse, symptoms of illness (like vertigo, headache, nausea, etc.), years of farm work, use of protective equipment (such as mask, gloves, covering arms, etc.), type of pesticide used (diazinon, parathion, etc.) and the spraying method (use of tractors or hand spraying).

Blood sample: Ten milliliter of blood was collected from each subject 5 mL were transferred to a sterile tube that contained EDTA 2Na (Ethylenediaminetetraacetic acid, disodium salt) as anticoagulant for the genetic tests. The other 5 mL were transferred to a serum separator tube for enzyme analysis, total antioxidant capacity and stock. Blood samples were placed in a cooler and quickly transported to the Pharmacology and Toxicology Research Laboratory at the Jundishapur University of Medical Sciences Ahvaz, Iran.

BuChE activity: A ParsAzmoon kit that uses butyrylthiocholine as a substrate determined the serum BuChE activity by measuring the decrease in absorbance of Potassium hexacyanoferrate (II) at 405 nm. A yellow potassium hexacyanoferrate (II) was produced by combining thiocholine with colourless Potassium hexacyanoferrate (III). Absorbance was measured with a Beckman 640 spectrophotometer and the enzymatic activity was expressed in μmol min^{-1}.

Paraoxonase activity: Paraoxonase activity was measured by adding 20 μL of serum (diluted 1:10) to 180 μL of paraoxon (1.2 mM paraoxon in 1 M Tris-HCL and 1 M NaCl buffer containing 1 M $CaCl_2$, pH = 8.5) at 37°C, 405 nm (Richter *et al*., 2004). Paraoxonase activity was expressed as the number of nmol min^{-1} mL^{-1} of serum.

DNA extraction: A Roche kit was used to extract the genomic DNA from whole blood. The extractions were stored at -20°C.

PON1 genotyping: Genotyping was conducted with a Polymerase Chain Reaction (PCR) amplification using forward 5'-TAT TGT TGC TGT GGG ACC TGA G-3' and reverse 5'-CCT GAG AAT CTG AGT AAA TCC ACT-3' primers for the codon Q192R polymorphism and forward 5'-CCT GCA ATA ATA TGA AAC AAC CTG-3' and reverse 5'-TGA AAG ACT TAA ACT GCC AGT C-3' for codon L55M polymorphism. Each PCR reaction was performed using a final volume of 25 μL. The 25 μL consisted of distilled water (13.8 μL), 1000 mM PCR buffer (2.5 μL), 50 mM $MgCl_2$ (0.7 μL), 500 mM dNTP (0.5 μL), 5 U μL *Taq* polymerase (0.5 μL), 100 pmol μL^{-1} of each primer (1 μL) and genomic DNA (5 μL). Initial denaturation at 95°C for 4 min was followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 45 sec. The 35 cycles were followed by a final extension at 72°C for 4 min for the Q192R which was set up in a BIORAD icycler Thermal cycler (BIORAD CO, USA). The PON1 Q192R polymorphism was detected by AlwI digestion (Thermo Scientific CO, USA). Similarly, an initial denaturation at 95°C for 4 min was followed by 37 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 45 sec. The 37 cycles were followed by a final extension at 72°C for 4 min for the L55M which was set up in a BIORAD icycler Thermal

cycler (Applied BIORAD CO, USA). The PCR products were digested with NlaIII (Thermo Scientific CO, USA). Agarose gel (2.5%) electrophoresis was used to separate the digested products which were mixed with a DNA loading buffer (Thermo Scientific CO, USA) and placed in a UVitec gel documentation system (model 0519204 Uvitec-CO, UK).

Statistical analysis: A student t-test and a Mann-Whitney U test, Chi-square (χ^2) Kruskal-Wallis or a one-way analysis of variance (ANOVA) nonparametric test, multiple linear regression analysis, direct counting and χ^2 test and Pearson's correlation coefficient were performed with the SPSS 16.0 statistical package. Significance was considered as $p<0.05$.

RESULTS

The characterized information of the test and control groups is summarized in Table 1. The amount of years that the farmers worked the land ranged from 2-48 years with the average being 18.89±1.47 years. Protective measures such as gloves, face masks and covering the arms, were taken by 46.3% of the farmers. The 22.22% of farmers had symptoms like vertigo, headache, nausea, etc. 12.46% of farm workers used tractors for spraying. This study showed that the literacy rate of the farmers was significantly less than the control group ($p<0.05$).

Butyrylcholinesterase (BuChE) levels: The BuChE activity in the test and the control groups were 4558.81±277.223 and 6826.57±231.619 μmol min^{-1} (Mean±SEM), respectively. Figure 1a shows that this difference was significant ($p<0.0001$). The result showed that there were no significant correlations between BuChE activity and the Body Mass Index (BMI) and age in farmers as well as the controls. BuChE activity also had no correlation with working duration and smoking in exposure group.

PON1 activity: Figure 1b shows that the workers had significantly ($p<0.05$) less PON1 activity than the controls (90.04±4.632 and 149.8±5.236 nmol min^{-1} mL^{-1}, respectively). No significant differences were observed between the PON1 activity and BMI, or age in work farmer when compared with control group. There was no correlation between PON1 activity and working duration in farmer group.

Table 1: Characteristics of the exposed and control groups

Variables	Exposed group (n = 80)	Control group (n = 160)	p-value
Age (year)	40.70±1.37	38.85±1.01	0.4400
Sex (male) (%)	100	100	-
Alcohol drinking (%)	0	0	-
Smoking (%)	16.25*	0	<0.0001
Duration of farm working	18.89±1.47*	0	<0.0001
BMI (kg m^{-2})	25.66±0.55	25.36±1.80	0.6346
Symptom (%)	22.22*	0	<0.0001

*Significantly different from control group ($p<0.0001$)

Genotyping analysis: Table 2 shows the genotypes and allelic distributions for PON1 Q192R and L55M that were observed in the farmers and the controls. The data in Table 2 was the correlation of the allele frequencies (Table 3) with the PON1 activity and BuChE activity.

The different PON1 genotypes ($p<0.05$) did not significantly effect the Butyrylcholinesterase (BuChE) activity in the workers and controls. However, both the workers and the controls with PON1 192 Q/Q and PON1 55 L/L genotypes hydrolyzed paraoxon at higher rates (i.e., PON1 activity) than the PON1 192 R/R ($p<0.001$) and PON1 55 M/M genotypes. PON1 192 QR genotypes inversely influenced paraoxon (PONase activity) compared to PON1 55 LM genotypes ($p<0.05$). The polymorphisms of PON1 192 QR and PON1 55 LM effected PON1 activity in the following order: PON1192 Q/Q> Q/R> R/R and PON1 55 L/L> L/M> M/M. The effects of the PON1 genotypes on PON1 activity was also analysed. The PON1 QQ/LL genotype had the highest

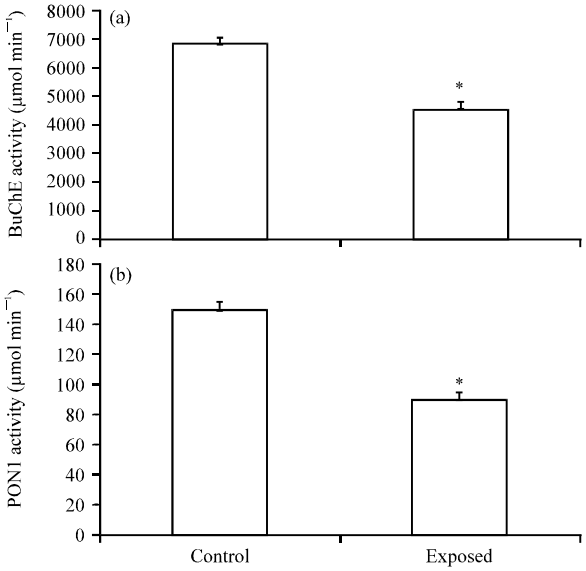

Fig. 1(a-b): Activities of (a) Butyrylcholinesterase and (b) Paraoxonase in exposed and control groups, *Significantly different from control group ($p<0.0001$)

Table 2: Effect of PON1 genotypes on biomarker values in exposed and control subjects

		Exposed group			Control group	
Genotypes	No.	BuChE (μmol min^{-1})	PON1 (nmol min^{-1} mL^{-1})	No.	BuChE (μmol min^{-1})	PON1 (nmol min^{-1} mL^{-1})
PON1 Q192R						
Q/Q	47	4508±393.3	97.32 (88.14,109.1)	86	5860±248.4	139.8 (145.9,206.9)
Q/R	27	4508±414.2	71.18 (61.28,99.69)	55	6477±288.9	121.5 (125.6,154.4)
R/R	7	5095±987.7	50.0 (30.92,107.7)	19	5886±452.8	80.10 (70.70,111.1)
p-value		0.8414	0.0103		0.2532	<0.0001
PON1 L55M						
L/L	27	4206±343.6	95.50 (80.77,114.5)	80	6104±255.5	152.6 (157.8,191.3)
L/M	36	4685±450.7	81.57 (73.58,105.8)	59	6212±300.8	121.2 (102.8,192.2)
M/M	18	4836±709.0	81.46 (68.05,90.56)	21	5580±359.2	87.70 (82.92,109.5)
p-value		0.6573	0.4107		0.5286	<0.0001
Random combination						
QQ/LL	11	3902±435.4	123.6 (105.5,135.9)	51	5893±330.4	157.3 (123.7,357.7)
QQ/LM	18	4551±708.8	105.3 (82.86,126.0)	28	5974±450	125.7 (113.1,146.7)
QQ/MM	18	4836±7.9	81.46 (68.05,90.56)	7	5571±516	91.4 (75.35,132.3)
p-value		<0.0001	0.0018		0.9183	<0.0001
QR/LL	11	3876±546.6	74.40 (50.73,115.7)	21	6409±480.4	153.4 (137.1,200.2)
QR/LM	16	4942±577.8	64.81 (51.86,105.3)	24	6568±505.2	121.3 (113.7,138.2)
QR/MM	n	-	-	8	6321±387.9	99. 4(82.69,138.2)
p-value		-	-		0.9521	0.0192
RR/LL	5	5598±928.6	64.37 (20.37,136.8)	6	7342±706.8	77.66 (53.62,118.0)
RR/LM	2	3836±3106	46.09 (-3.591,95.77)	6	5694±511.4	77.12 (40.71,180.1)
RR/MM	n	-	-	7	4803±789.1	80.10 (64.86,92.13)
p-value		-	-		0.0567	0.9570

Values are Mean±SEM or median (CI 95%); p-values were obtained by ANOVA nonparametric Kruskal-Wallis test compared within group, n: No genotype combination present; $p<0.05$ was considered significant

Table 3: Allele frequency of PON1

	Exposed group (%)		Control group (%)	
Allele	Heterozygote	Homozygote	Heterozygote	Homozygote
Q192R				
Q	34.2	58.2	36.3	51.3
R	34.2	7.6	36.3	12.4
L55M				
L	44.4	33.3	35.4	51.3
M	44.4	22.2	35.4	13.3

paraoxonase activity in both groups, whereas the lowest paraoxonase activity was in the PON1 RR/LM genotypes for the workers and control subjects, respectively.

DISCUSSION

Paraoxonases can decompose a number of OP compounds. Previous studies have shown that individuals with lower serum PON1 activity as well as individuals with higher serum PON1 activity cannot metabolize organophosphates (Mallinckrodt and Diepgen, 1988). Several studies have investigated the levels of paraoxonase and its genotype. The results of these studies indicate that there is a correlation between paraoxonase activity and healthy in people which suggests that low paraoxonase levels can cause diseases such as cancers and metabolic diseases (Marchesani *et al*., 2003; Nguyen and Sok, 2004). Previous studies have also demonstrated that OP exposure decreases BuChE activity (Sirivarasai *et al*., 2007). In this study, OP exposure significantly decreased BuChE activity and this decrease was not correlated to the duration of the exposure or to the distribution of PON1 genotypes and phenotypes.

Many studies have demonstrated that PON1 polymorphisms in the promoter and coding regions can cause changes in PON1 activity (Brophy *et al*., 2001; Davies *et al*., 1996; Suehiro *et al*., 2000). The PON1 Q192R polymorphism is responsible for the variations in PON1 activity. In this study, a wide variation in the PON1 activity between individuals in the exposed and control groups was observed. The Q/Q, Q/R and R/R genotypes increased PON1 activity from highest to lowest, respectively within the test and control groups. The R and M alleles are associated with reduced PON1 activity in Coronary artery disease (Mendonca *et al*., 2008).

In this study, the PON1 L55M substitution was not associated with PON1 activity in exposed group opposite control group. PON1 activity was more active in the L/L than in L/M and least active in M/M genotype. These results were consistent with earlier reports that indicated that the L allele has more PON1 activity than the M allele (Leviev and James, 2000; Sirivarasai *et al*., 2007). The PON1 polymorphisms effected paraoxonase activity as follows: PON1 192 Q/Q > Q/R > R/R and PON1 55 L/L > L/M>M/M. There were no variations in BuChE activity associated with PON1 Q192R and PON1 L55M polymorphisms which concurred with previous studies (Adkins *et al*., 1993; Lopez-Flores *et al*., 2009).

However, in this study, enzymatic activity did not vary with smoking. There was also no correlation between PON1 activity and BMI which was consistent with previous findings (Costa *et al*., 2005a). Further, there were no correlations between PON1 activity and the duration of OPs exposure. The correlation arises from the interaction of genotypes with environmental factors (i.e., exposure to OPs). In addition, different genotypes can change the activity of PON1 (Browne *et al*., 2006). Further, BuChE activity decreases in individuals that were exposed to pesticides. This decrease may increase the activity of PON1 which may compensate for the acute decline of the cholinesterase (Hodgson *et al*., 1991). So, genotypes, environmental factors and activity of BuChE can cause changes in paraoxonase activity. Thus, evaluation of individuals sensitivity to Ops implicate the determination of these 3 factors. But in our study both PONase and BuChE activity in farmer group were lower than control group. It is also clear that there are individuals that never have increased PON1 activity and therefore may be very sensitive to the OPs inactivation by PON1 (Costa *et al*., 1999).

An inefficient detoxification can cause the by-products of toxic agents to accumulate in the body which may produce tumors (Hodgson *et al*., 1991). A number of enzymatic isoforms contribute to the body's susceptibility to cancer (Bolognesi, 2003). Thus, the results of this study suggest that the paraoxonase genotype and its activity may separate individuals with higher and lower health-hazard risks due to OPs exposure.

CONCLUSION

Many studies showed that PON1 activity have a most suitable role in determination of the sensitivity to acute toxicity of OPs and pre-treatment with PON1 suggested protection against the toxicity of OP (dermally used, one of the major routes of occupational exposure) (Costa *et al*., 1999).

This study showed that PON1 activity in workers exposed to OPs was significantly lower than control group and the variation of PON1 activity was due to their polymorphisms. Further, individuals with PON1 R/R and M/M genotypes with lower activity of PON1 were more susceptible to the OP exposure.

Thus, exposed individuals such as farmers can be protected by providing baseline data of the PON1 status as a marker of susceptibility.

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