original article

Assessment of the role of paraoxonase gene polymorphism (Q192R) and paraoxonase activity in the susceptibility to atherosclerosis among lead-exposed workers

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BACKGROUND AND OBJECTIVE: Lead exposure is a well known cause of cardiovascular damage, including atherosclerosis. Paraoxonase 1 (PON1), a high-density lipoprotein-associated antioxidant enzyme, is capable of hydrolyzing oxidized lipids and thus it protects against atherosclerosis. The mechanism by which heavy metals inhibit serum PON1 activity is still not clear. Our aim was to detect the association between lead exposure and serum PON1 activity and lipid profile and also to study the polymorphism of the PON1 gene.

DESIGN AND SETTING: A case-control, cross-sectional study conducted from June 2008 until May 2009.

SUBJECTS AND METHODS: Male workers (n=100) in a lead battery manufactory were recruited for this study. They were compared with 100 male age-matched workers not exposed to lead (control group). Serum lipid profile, paraoxonase activity and lead were measured in blood samples. The DNA was extracted for detecting the Q192R polymorphism of the PON1 gene by polymerase chain reaction followed by restriction fragment length polymorphism.

RESULTS: There was significant difference in triglycerides, total cholesterol and high-density lipoprotein cholesterol (HDL-C) (P=.01,.05 and.04, respectively) between cases and controls. Multiple linear regression analysis showed that blood lead levels were significantly associated with decreased serum paraoxonase activity (P=.03) in lead workers. The paraoxonase genotype QR was the most prevalent in 34/53 subjects (64%) among the lead-exposed groups, while the genotype QQ was more prevalent in the control group, in 15/25 subjects (60%), with a significant difference between the control and other groups (P<.05).

CONCLUSION: Lead exposure is associated with increased triglycerides, total cholesterol and low-density lipoprotein cholesterol and decreased HDL-C. Because of the protective role of PON1 in the development of atherosclerosis, a decrease in serum PON1 activity due to lead exposure may render individuals more susceptible to atherosclerosis.

Lead exposure is the oldest known occupational health hazard. Continuous lead overexposure and lead poisoning remain a serious problem in Egypt.[1] Multiple morphological and biochemical changes in the cardiovascular system, various organs and blood are documented with chronic lead exposure.[2] Also, it is associated with altered lipid metabolism, especially serum cholesterol and lipoprotein levels in both humans and animals.[3-9] An inverse correlation between the serum concentration of high-density lipoprotein cholesterol (HDL-C) and the development of atherosclerosis has long been known.[10] Several laboratories have reported that HDL-C protects against low-density lipoprotein cholesterol (LDL-C) oxidation,[11,12] which is the main step in initiation and progression of atherosclerosis.[13] The anti-atherogenic properties of HDL-C are partially due to the activity of HDL-associated enzymes, which prevent and/or reverse LDL oxidation.[14] One of those enzymes is the calcium-dependent ester hydrolase paraoxonase 1 (PON1), which

is found tightly associated with the HDL particle.[15]

Paraoxonase-1 (PON1) is a protein of 354 amino acids with a molecular mass of 43 kD. The PON1 gene is located on the long arm of chromosome 7 between q21 and q22 with other members of its supergene family. PON1 has two amino acid polymorphisms, one at position 55 (methionine/leucine, M/L) and the other at position 192 (arginine/glutamine, R/Q).[16,17] Multiple factors can affect PON levels and thus interfere with its protective function.[18] Previous studies have shown that various metals, including lead at concentrations <1 μg/dL cause significant inhibition of PON1 activity in vitro[19,20] and in vivo.[21]

The aims of the present study were to investigate the relationship between chronic occupational lead exposure and the occurrence of atherosclerosis through altered lipid profile, detect the association between lead exposure and serum PON1 activity as one of the mechanisms of atherosclerosis, and to study the polymorphism of the PON1 gene that encodes for this enzyme.

SUBJECTS AND METHODS

This current case-control cross-sectional study was conducted from June 2008 until May 2009. The study participants included 100 male workers from a lead-acid battery manufactory in Cairo, Egypt (cases). Subjects with diabetes mellitus, thyrotoxicosis, renal disease, as well as smokers, were excluded from the study. Controls were healthy 100 male workers who were not exposed to lead in the workplace and were matched with cases with regard to age and social class. Approval by all workers through verbal consent was obtained before the commencement of study. Both groups were subjected to a full history with special emphasis on age, duration of their occupation, and an examination, specifically blood pressure measurements (hypertension being considered as systolic blood pressure [SBP] ≥135 mm Hg and diastolic blood pressure [DBP] ≥85 mm Hg, according to the guidelines of the American Heart Association.)[22]

The exposed and control groups underwent tests for blood lipid and blood lead. A subsample was drawn from all the workers for the genetic study (53 cases and 25 controls). Ten milliliters of fresh blood was withdrawn as a sample and collected on-site during the health examination. Each sample was divided into 3 tubes: one in an EDTA (lead free) tube for measurement of blood lead. The second aliquot was obtained in a sterile EDTA tube. The buffy coat was isolated for genomic DNA preparation. All samples were stored at -20°C until measurement. Plain tubes were used for the third aliquot. The sera were separated for analysis of lipid profile and PON1 activity immediately on the same day. All lab tests were done in the Chemical Pathology Department, Faculty of Medicine, Cairo University. Total cholesterol <200 mg/dL; triglycerides <150 mg/dL; HDL >40 mg/dL; and LDL <100 mg/dL were considered a favorable profile according to the guidelines for risk factors for cardiovascular disease given by the American Heart Association.[22] Tests for total cholesterol, triglycerides and HDL-C were done for all workers by an automated enzymatic assay on Hitachi 917[23] using a kit purchased from Roche (Roche Diagnostics GmbH, D-68298 Mannheim.). LDL-C was calculated using Friedwald's formula.[24] Blood lead was analyzed by a Zeeman-Effect graphite furnace atomic absorption spectroscopy; PerkinElmer AS 800 auto-sampler (PerkinElmer, Wellesley, MA, USA).[25] PON1 paraoxonase activity was measured spectrophotometrically[26] using 1.2-mM paraoxon (O, O-diethyl p-nitrophenyl phosphate) in 50-mM Tris/HCl buffer (pH, 6.8) containing 1.0-mM CaCl2. These chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rate of generation of p-nitrophenol was monitored at 25°C with a continuously recording spectrophotometer at 405 nm (RA 50, Chemistry Analyzer, Bayer, Chemical Heritage Foundation, 315 Chestnut Street, Philadelphia, PA 19106). PON1 activity was expressed as micromoles of hydrolysis product formed per minute per liter.

The calculation of PON activity (nmol) = $[(\Delta Abs/min)\times(1/\varepsilon)(tv/sv)\times(1/d)]\times10^9$, where ΔAbs/min is blank-corrected change in absorbance/minute, ε is the molar absorbance coefficient (7480 liter· mol-1· cm-1), tv is the total reaction volume (0.3 mL), sv is the sample reaction volume (0.04 mL) and d is the light path (0.6 cm). The conversion of molar into nanomolar absorbance coefficient (in liter· mol-1· cm-1) was achieved by multiplying by 109. For PON1 genotyping, genomic DNA was extracted from buffy coat using salting-out technique[27] with reagents supplied by Fermentas International Inc. (830 Hurrington Court, Burlington Ontario). The 192 polymorphism of the PON1 genotype was determined by polymerase chain reaction amplification followed by polymorphism-specific restriction enzyme digestion and gel analysis. The Q192R polymorphisms, in 53 samples, were determined following a protocol developed by Humbert et al.[28] The sense primer 5'TATTGTTGCTGTGGGACCTGAG3' and antisense primer 5'CACGCTAAACCCAAATACATCTC3', which encompass the 192 polymorphic region of the human PON1 gene, were used. These primers were designed by Metabion (Metabion-International AG, Lena-Christ-Str.44, Martinsried, Germany). After denaturing the DNA for 5 minutes at 95°C, the reaction

mixture was subjected to 46 cycles of denaturing for 1 minute at 94°C, 30 seconds annealing at 61°C and 1 minute extension at 72°C using Hybaid thermal cycler from Promega Corporation (Promega Corporation, 2800 Woods Hollow Road, Madison, WI, USA). Detection of the band amplified in agarose gel 2% was done by performing E/P on the gel electrophoresis apparatus supplied by Pharmacia EPS 500/400 (Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, PO Box 1327, Piscataway, NJ, USA) and ultraviolet (UV) transillumination. The 99-bp PCR product was digested with 8U Alw1 restriction endonuclease overnight at 50°C. This enzyme was also supplied by Fermentas International Inc. The digested products were separated on 4% agarose gel with ethidium bromide staining and UV transillumination. The R-genotype (arginine) contains a unique Alw1 restriction site which results in 66- and 33-bp products. This site is not present in the Q-genotype (glutamine), thus allowing the PON 192 genotype to be determined (**Figure 1**). According to the U.S. Occupational Safety and Health Administration [OSHA], 29 workers were classified into three groups with blood levels <40 µg/dL, 40-59 µg/dL, and ≥60 µg/dL.

Data obtained from the study was coded and entered using the software SPSS (Statistical Package for Social Sciences), version 11.0. Quantitative parametric data was summarized using mean and standard deviation, while nonparametric data was summarized as median and percentiles. Frequency and percentages were used for qualitative variables. Comparison between groups was done using chi-square test and Fischer exact test for qualitative variables. For quantitative data, the t test and Mann-Whitney U test were used to compare two groups, while ANOVA and Kruskal-Wallis test were used to compare multiple groups. The correlation between blood lead level and PON1 activity, as well as blood lead level and serum lipids, was assessed by Pearson coefficient of correlation. Multiple linear regression analysis was used to test the association between PON1 activity on the one hand and blood lead level, age, exposure duration, total cholesterol, HDL-C and LDL-C, and triglycerides, respectively, on the other hand. A *P* value <.05 was considered significant.

RESULTS

No significant difference was detected between the 100 male occupationally lead-exposed workers with a mean and the 100 healthy workers not known to be exposed occupationally to lead in age and blood pressure values, but higher systolic and diastolic values were observed among cases. The cases had higher blood lipids than controls although both had normal values. There was a significant difference in levels of triglycerides, cholesterol and HDL-C (*P*=.01, .05 and .04, respectively), while LDL-C was not significantly different (*P*=.7) (**Table 1**). The mean blood lead levels of the lead-exposed group was significantly different from the controls. The median value of paraoxonase activity was also significantly higher among cases than among controls (987 vs. 367.2 µmol/min/L) respectively. The clinical and biochemical parameters of the workers by level of blood lead are presented in **Table 2**. The mean blood pressures of the cases in these three groups were in the normotensive range. Workers in group 3 had a borderline high systolic blood

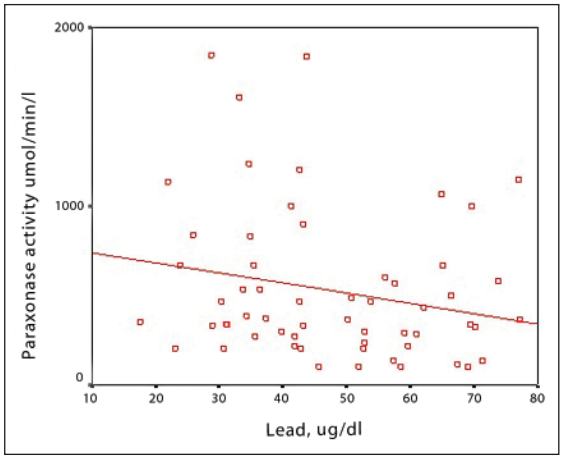

Figure 1. PON1 192 amplified fragment cleaved by Alw1. The 8 samples shown in the figure are QQ:1,7,8; QR:2,3,5,6; and RR:4.

Table 1. Basic characteristics of the case and control groups.

Variable	Control	Cases	*P* value
Number (%)	100 (100)	100 (100)	
Age (years)	32.5 (13.9)	34.6 (11.5)	.3
SBP (mm Hg)	130.9 (13.9)	132.2 (12.9)	.9
DBP (mm Hg)	78.8 (9.5)	80.4 (10.1)	.5
Triglycerides[a] (mg/dL)	75 (64-160)	101 (32-325)	.01
Total cholesterol (mg/dL)	115.4 (21.3)	167.1 (35.7)	.05
HDL-C (mg/dL)	47.2 (6.1)	44.9 (6.9)	.04
LDL-C † (mg/dL)	78.3 (32.6)	99.9 (28.4)	.7
BPb (µg/dL)	12.5 (3.3)	45.7 (15.3)	.0001
Paraoxonase † (µmol/min/L)	987 (600-2576)	367.2 (200.3-1645.6)	.001

All data are presented as mean (SD) unless otherwise indicated.
[a]Median (25th percentile-75th percentile).
BPb: blood lead level, DBP: diastolic blood pressure, SBP: systolic blood pressure.

Table 2. Basic characteristics of the lead-exposed workers in relation to their blood lead level.

Variable	Group 1 BPb <40 µg/dL	Group 2 BPb 40-59 µg/dL	Group 3 BPb ≥60 µg/dL	*P* value
Number (%)	39 (39)	42 (42)	19 (19)	
Age (years)	33.3 (10.6)	34.7 (12)	37.1 (12.4)	.3
Duration of work (years)*	5 (2-24.2)	7 (5.6-34)	14 (7.5-40.3)	.4
SBP (mm Hg)	131.3 (12.9)	130.7 (16.5)	134.3 (24.4)	.9
DBP (mm Hg)	80 (7.2)	80 (11.8)	81.1 (9.6)	.5
Triglycerides* (mg/dL)	85 (32-190)	101 (65.4-248.9)	102.3 (54.7-352)	.5
Total cholesterol (mg/dL)	124.4 (22.3)[ab]	170.5 (26.1)[ac]	180.3 (53.2)[bc]	.01
HDL-C (mg/dL)	46.1 (7.7)	45.2 (5.3)[a]	41.7 (7.4)[a]	.02
LDL-C* (mg/dL)	97.4 (27.4)[a]	98 (32.9)	112 (43.1)[a]	.01
BPb (µg/dL)	30.6 (5.8)[ab]	49.8 (6.6)[ac]	68.9 (4.8)[bc]	.0001
Paraoxonase* (µmol/min/L)	634.6 (200.3-1654.6)[ab]	366 (170-1420.8)[ac]	300 (100.6-1087)[bc]	.001

All data are presented as mean (SD) unless otherwise indicated.
*Median (25th percentile-75th percentile).
BPb: blood lead level, DBP: diastolic blood pressure, SBP: systolic blood pressure.
[abc]Same symbol indicates significant differences between each pair of groups.

pressure (134.3 [24.4] mm Hg), but the differences were not statistically different.

The values of blood lipids in the case subjects were within the reference ranges prescribed by the American Heart Association.[22] Total cholesterol showed a gradual increase among groups, reaching its highest (high normal) level in group 3, with a significant difference between group 1 and the other two groups, as well as between groups 2 and 3. HDL-C showed gradual decrease as the blood lead level increased, with a significant difference between groups 2 and 3. LDL-C was above the reference range in group 3; meanwhile, a significant difference was detected between groups 1 and 3. On the other hand, triglyceride concentrations were not significantly different between the three groups (P>.05). A positive significant correlation was observed between blood lead and total cholesterol (r= 0.3; P=.05) and between blood lead and LDL-C (r= 0.3; P=.04), while a nonsignificant negative correlation was found between blood lead and HDL-C (r= –0.2; P=.1).

Paraoxonase activity and genotypes

A significant difference was observed between the three groups with regard to the paraoxonase activity. A gradual decrease in enzyme activity was obvious as the lead level increased, reaching its minimum value [300 (100-1087) µmol/min/L] when lead level was ≥60 µg/dL (**Table 2**). A significant negative correlation was detected between blood lead level and paraoxonase activity, as shown in **Figure 2** (r=–0.2; P=.03). Lead level was the only significant predictor of paraoxonase activity in a multiple linear regression (P=.03) (**Table 3**). An increase in the blood lead of 1 µg/dL was associated with 5.7 µmol/min /L decrease in the paraoxonase activity.

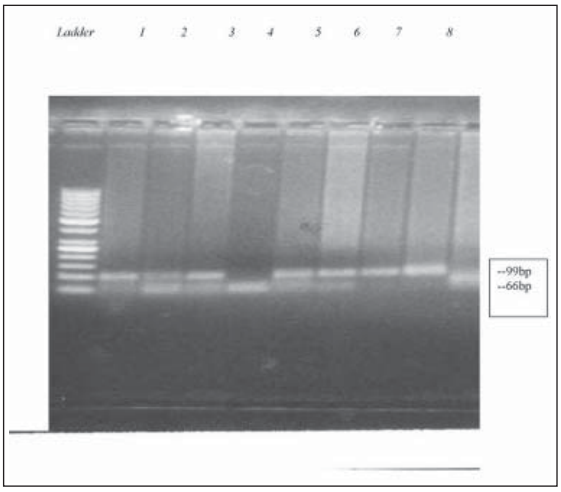

Figure 2. Correlation between paraoxonase activity and blood lead level.

The paraoxonase genotype QR was the most prevalent among the lead-exposed groups, while the genotype QQ was more prevalent in the control group, with a significant difference existing between the control and other groups (**Table 4**), while no significant difference was found between the lead-exposed groups. The Q allele was significantly more prevalent among the control group and group 1. In the lead-exposed groups, the paraoxonase activity within the different genotypes was recorded to be the lowest in RR genotype; and median (25th-75th percentile) [484 (183-10002) vs. 450 (318-668) vs. 334 (254-894)] in genotypes QQ vs. QR vs. RR, respectively. However, no significant difference in the paraoxonase level was found among the three genotypes (P=.237).

DISCUSSION

The present study aimed to elucidate the effect of lead exposure on the lipoprotein pattern of exposed workers and to explain one of the mechanisms by which lead can affect the lipid profile. The study recruited 100 workers from a lead-acid battery manufactory and 100 control subjects with no known occupational exposure to lead. OSHA rules include the following guidelines: "If a worker's blood lead level is found to be greater than 40 µg/dL, the worker must be notified and should be

provided with a medical examination. If a worker's one-time blood lead level reaches 60 µg/dL (or averages 50 µg/dL or more on three or more tests), the employer is obligated to remove the employee from excessive exposure, with maintenance of seniority and pay, until the employee's lead level falls below 40 µg/dL."[29] The most striking observation in this study was that the workers exhibiting blood lead levels greater than 60 µg/dL were still working in their position.

During the past decade, a vast amount of evidence has confirmed that lipid and lipoprotein abnormalities play a major role in the initiation and progression of atherosclerosis and, consequently, cardiovascular diseases. These chronic degenerative disorders have become a growing health problem worldwide.[30-32] Studies in both humans and animals indicate that chronic lead exposure is associated with altered lipid metabolism.[3-7] The findings of the present study support the findings of previous ones and indicate that exposure to lead alters significantly the lipid levels, as demonstrated by comparing cases and controls. Also, this study exhibited the cumulative effect of the blood lead level. An increase in the duration of lead exposure and consequent gradual increase in the blood lead level was associated significantly with an increase in cholesterol, LDL-C and a decrease in HDL-C. Although correlations do not imply causality, the observation of a significant positive relationship between lead and total cholesterol on one hand and lead and LDL-C on the other hand, seems to support these findings. The result is in concordance with the results of previous studies.[1,8,9,21,33]

The paraoxonase gene family contains at least three members, including PON1, PON2 and PON3, which are located on chromosome 7q21.3-22.1. The PON1 gene product is serum paraoxonase, which is expressed mainly in the liver and which hydrolyzes organophosphates.[34] PON1 resides on high-density lipoprotein (HDL,'good cholesterol') and is involved in the prevention of atherosclerosis by protecting against LDL-C oxidation.[15] A significant gradual decrease in PON1 activity as the blood lead level increases and also a significant negative correlation between the enzyme activity and the blood lead level were the main findings in this present study. This represents reduced protection against LDL oxidation, thereby increasing the accumulation of lipid peroxides and eventually promoting atherosclerosis. This was consistent with the study previously performed by Debord et al,[20] Ito et al[6] and Li et al,[21] respectively. The mechanism by which heavy metals, including lead, inhibit serum PON1 activity was discussed by Gonzalvo et al.[35] They suggested that these metal ions, such as those of lead, copper and mercury, bind to the free sulfhydryl group of the enzyme, and this will reduce not only the hydrolytic activity of PON1 but also its antioxidant function.

The activity of the PON1 enzyme is a more important factor in atherosclerosis and coronary heart disease than that of the PON1 genotype.[12] Our study supports this finding, as no significant difference was observed with regard to the activity of the enzyme in relation to its genotypes.

Previous studies reported that the inhibitory effect of lead on PON1 activity is influenced by Q192R polymorphism, as they found that subjects who are homozygous for the R allele are more susceptible to lead tox-

Table 3. Prediction of paraoxonase activity using multiple linear regression.

	Paraoxonase activity				
	B	SE	95% confidence interval for the coefficient		*P* value
			Lower	Upper	
Lead	–5.7	2.7	-44.5	-1.04	.03
Exposure duration	–10.6	11.9	-17	29	.4
Age	–19.6	12.1	-40.8	3.4	.1
Cholesterol	4.4	2.7	-3.5	10.4	.1
HDL-C	6.3	7.9	-6.1	25.9	.4
LDL-C	–5.3	3.1	-10.1	4.7	.8
Triglycerides	–1.1	.8	-2.1	3.0	.2
Intercept	1552.2	514.2	242.9	2292.5	.03
R2			**0.38**		

Table 4. PON1 genotype distribution among the population.

Variable	Control group	Group 1 BPb <40 µg/dL	Group 2 BPb 40-59 µg/dL	Group 3 BPb ≥60 µg/dL	*P* value
Genotype					
QQ	15 (60)	4 (22.2)	4 (16)	1 (10)	
QR	7 (28)	12 (66.7)	17 (68)	5 (50)	.004
RR	3 (12)	2 (11.1)	4 (16)	4 (40)	
Total (%)	**25 (100)**	**18 (100)**	**25 (100)**	**10 (100)**	
Allele					
Q	37 (74)	20 (55.5)	25 (50)	7 (35)	.012
R	13 (26)	16 (44.5)	25 (50)	13 (65)	
Total (%)	**50 (100)**	**36 (100)**	**50 (100)**	**20 (100)**	

icity than are subjects of other genotypes.[17,21] This study showed that QR polymorphism was more prominent among the lead-exposed groups. In contrast, Bauters et al[36] suggested that the Q allele, rather than the R allele, is more important. This study demonstrated that the Q allele was significantly more prevalent among the control group and group 1. The reason for these conflicting results may be the wide inter-ethnic variability in PON1 polymorphism observed in those studies.[37] An important study conducted before is that of Jarvik et al,[38] who found that intake of vitamins C and E is associated with increased paraoxonase activity. Hence, the workers may be advised to take these antioxidant vitamins to protect them from lead toxicity.

In conclusion, lead exposure is associated with increased levels of triglycerides, total cholesterol and LDL-C and decreased levels of HDL-C. However, the association of these findings with profound cardiovascular damage in lead workers has yet to be explored. Also, lead exposure is associated with decreased serum PON1 activity, regardless of the PON1 genotype, which is more profound with increased duration of exposure to lead. Because of the protective role of PON1 in the development of atherosclerosis, a decrease in serum PON1 activity due to lead exposure may render individuals more susceptible to atherosclerosis.

We recommend that all industrial plants with lead exposure implement the OSHA rules. Additional information is required, particularly about nutritional and pharmacological effects of serum PON1 activity, which might lead to intervention trials to enhance this activity. The role of supplementation with vitamins C and E should be considered. More research should be conducted to demonstrate the clinical association between lead exposure and cardiovascular diseases. Finally, genotype screening of suspected lead-exposed workers before their employment should be taken into consideration.

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