

Smoking is associated with reduced serum paraoxonase, antioxidants and increased oxidative stress in normolipidaemic acute myocardial infarct patients

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ABSTRACT

Background Paraoxonase is a high-density lipoprotein (HDL)-associated enzyme that protects lipoproteins from oxidative modifications and from becoming atherogenic in nature. Smoking is a well-known major cardiovascular risk factor that promotes lipid peroxidation (LP). The present study examined the hypothesis that smoking modulates the activity of paraoxonase and depletes antioxidants.

Aim The present study evaluated paraoxonase activity, antioxidant status and LP in smoking and non-smoking normolipidaemic acute myocardial infarct (AMI) patients, and results were compared with controls.

Settings and design The serum paraoxonase activities, antioxidants and LP were determined in 86 normolipidaemic patients diagnosed of AMI, and 86 age—sex-matched healthy volunteers served as control.

Material and methods Serum paraoxonase activities were measured by enzymatic kit. The glutathione peroxidase, superoxide dismutase and catalase activity was determined by standard methods. Malondialdehyde was measured by the thiobarbituric acid reaction, and conjugated diene levels by the Recknagel and Glende method. Serum uric acid, total bilirubin, serum albumin and lipid profiles were analysed by standard methods.

Statistics The values were expressed as mean \pm SD, and data from the patients and control were compared using the Student t test.

Results and conclusion The total cholesterol/HDL cholesterol ratio, triglycerides, low-density lipoprotein cholesterol, low-density lipoprotein/HDL cholesterol ratio and triglyceride/HDL cholesterol ratio were significantly higher, and HDL cholesterol significantly lower in smokers compared with non-smoking AMI patients. Superoxide dismutase, glutathione peroxidase and catalase were significantly higher in non-smokers compared with smokers. Serum albumin, uric acid and bilirubin were higher in the control compared with smoking AMI patients. The malondialdehyde and conjugated dienes were significantly higher, and paraoxonase activities were significantly lower in smokers compared with non-smokers.

INTRODUCTION

Paraoxonase (PON1) is a serum enzyme complexed to high-density lipoprotein cholesterol (HDL-C).¹ High density lipoprotein (HDL) associated paraoxonase (PON1) enzyme is known to have protective effects on lipid peroxidation (LP) and oxidative modifications further delaying the

atherogenesis.² The verdict is justified by developing a PON knockout mouse model that demonstrated a greater susceptibility of lipoproteins to oxidation.³ In humans, PON is an independent, genetic risk factor for coronary artery disease (CAD).^{4 5} This observation has been confirmed in independent studies^{6–10} although not uniformly.^{11 12} Numerous cohort studies and clinical trials have confirmed the association between a low HDL cholesterol concentration and increased risk of CHD. HDL is one of the most important independent protective factors for the arteriosclerosis which underlies coronary heart disease. Though many factors may play a role in its pathogenesis, low PON1 activity could be an independent risk factor.¹³ PON1 activity is inversely related to the risk of developing an atherosclerotic lesion, which contains cholesterol-loaded macrophage foam cells. Although experimental studies have demonstrated the reduction in PON1 activity due to oxygen free radicals in ischaemia and reperfusion,¹⁴ there are controversial data on the correlation between PON1 HDL-C and the ischaemia process. Under oxidative stress, not only low-density lipoprotein (LDL) but also other serum lipids are exposed to oxidation. As it is well established that dyslipidaemia is an important contributory factor for acute myocardial infarct (AMI), and PON1 activity is decreased in dyslipidaemia, the present study was undertaken with the objective of studying the PON1 activity in normolipidaemic AMI patients and also to observe whether PON1 activity could be an independent risk factor in this group of patients. Also, the study aimed to observe the relationship between smoking, PON1, antioxidants and free radicals in normolipidaemic AMI patients.

Smoking is a widely known and firmly established major cardiovascular risk factor. Oxidative stress is considered to be the major, pathological mechanism associated with smoking, leading notably to LP.^{15–17} Several studies have demonstrated increased susceptibility of LDL to oxidation and higher levels of oxidised LDL in smokers.^{18–20} This would provide an important causal mechanism that links smoking with vascular disease given the numerous pathological effects of oxidised LDL.^{2 21}

Smoking may enhance oxidative stress not only through the production of reactive oxygen radicals in smoke but also through a weakening of the antioxidant defence mechanisms. In this context, a recent in vitro study showed that extracts of

cigarette smoke inhibited the enzymatic activity of PON.²² Given its hypothesised, antioxidant role, this could also contribute to the increased oxidation of LDL in smokers. The present study examined the hypothesis that smoking is associated with lower serum PON activity and antioxidant status in normolipidaemic AMI patients causing the severity of vascular disease.

MATERIALS AND METHODS

Eighty-six patients with AMI and 86 age–sex-matched healthy volunteers were taken for this study. The study was conducted for a period of 3 years from September 2005 to August 2008. The design of this study was preapproved by the institutional ethical committee board of the institution, and informed consent was obtained from the patients and controls.

Biochemical parameters and other parameters such as smoking habits, systolic and diastolic blood pressure, and family history were recorded after clinical confirmation of AMI.

Diagnostic criteria of patients

All the patients had their first episode of MI with diagnostic criteria: typical chest pain, specific abnormalities for MI on electrocardiogram and elevated serum creatine phosphokinase (CPK-MB) levels.

Exclusion criteria

Patients with diabetes mellitus, renal insufficiency, hypertension or hepatic disease, or those taking lipid-lowering drugs or antioxidant vitamin supplements were excluded.

Criteria for normolipidaemics

A normal lipid profile was defined thus if the LDL was <130 mg/dl, HDL \geq 35 mg/dl, total cholesterol (TC) <200 mg/dl and triglycerides (TG) <150 mg/dl.²³

Venous blood was collected after an overnight fast of 12 h, EDTA was added, and samples were processed for lipid profiles.

Ten millilitres of blood was collected after overnight fasting in different containers.

Preparation of erythrocytes for antioxidant studies

Five millilitres of blood was taken. Red cells were washed three or four times with ice-cold normal saline and used for estimation of glutathione peroxidase (GPx), superoxide dismutase and catalase.

Serum for lipid profile, lipid peroxides, endogenous antioxidants and conjugated dienes measurement

The remaining blood was taken, and the serum was separated. Serum was used to determine the lipid profile, albumin, uric acid, bilirubin, malondialdehyde and conjugated dienes (CD).

For PON1 activity studies, blood samples were collected in patients who were admitted to the intensive care unit 4–6 h after AMI. Only normolipidaemic AMI patients were included in the study. The lipid profile (total cholesterol, triglycerides and HDL cholesterol) was analysed enzymatically using a kit obtained from (Randox Laboratories, Crumlin, UK). Plasma LDL cholesterol was determined from the values of total cholesterol and HDL cholesterol using the following formulae:

$$\text{LDL-c} = \text{TC} - \text{TG}/5 - \text{HDL-c} \text{ (mg/dl)}$$

For PON1 studies, an assay kit (Zeptomatrix Corporation, Buffalo, New York; ZMC catalogue 0801199) was used.^{24 25} The assay is based on the principle that PON1 catalyses the cleavage of phenyl acetate, yielding the phenol. The rate of formation of

phenol is measured by monitoring the increase in absorbance at 270 nm at 25°C. One unit of arylesterase activity is equal to 1 μ M of phenol formed per minute. The activity is expressed in kU/l, based on the extinction coefficient of phenol of 1310/M/cm at 270 nm, pH 8.0, and 25°C. Blank samples containing water are used to correct for non-enzymatic hydrolysis.

Glutathione peroxidase

The glutathione peroxidase (GPx) activity was determined by the procedure of Paglia and Valentine.²⁶ Briefly, the oxidised glutathione produced during the GPx enzyme reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was monitored as a measure of formation of oxidised glutathione. Results were expressed as units of GPx per gram of haemoglobin.

Superoxide dismutase (SOD)

Superoxide dismutase enzyme activity was measured using the SOD assay kit using the rate of inhibition of 2-(4-indophenyl)-(4-nitrophenol)-5-phenyltetrazolium chloride reduction method (modified method of Sun *et al.*²⁷) using the assay Ransod kit SD 125 (Randox Laboratories). One unit of SOD activity was defined as the amount of protein that inhibits the rate of 2-(4-indophenyl)-(4-nitrophenol)-5-phenyltetrazolium chloride reduction by 50%. Enzyme activity was expressed as units per gram of haemoglobin. Haemoglobin was measured by Drabkin's method.

Catalase

Catalase activity was measured spectrophotometrically as described by Beutler *et al.*^{28 29} One unit of enzyme activity was expressed as (μ mol) hydrogen peroxide decomposed per minute per gram of haemoglobin.

Thiobarbituric acid-reactive substances

Malondialdehyde (MDA) levels were estimated by thiobarbituric acid reaction.³⁰ Using 40% trichloroacetic acid, proteins were precipitated from 0.5 ml of serum, and precipitated proteins were incubated with thiobarbituric acid reagent in a boiling water bath for 1 h. After bringing the mixture down to room temperature, the coloured complex formed was measured using a spectrophotometer at 532 nm. 1,1,2,3-Tetraethoxypropane (1 nmol/l) was used as a standard for MDA estimation. Concentrations were expressed in nmol/l.

Conjugated dienes (CD)

CD levels were measured by Recknagel and Glende's method³¹ with little modification. Briefly, the principle of the assay is based on the rearrangement of double bonds in polyunsaturated fatty acids leading to the formation of CD, which absorb light at 233 nm. The oxidation index of the lipid sample at 233 nm and 215 nm is computed, which reflects the diene content and the extent of peroxidation. The LP products measured in serum were treated with antioxidant butylated hydroxy toluene twice, immediately after obtaining and before adding the test reagents to suppress artefactual changes during handling and assay procedures. The first stage of LP consists of the molecular rearrangement of the double bonds in polyunsaturated fatty acid residues of lipids, which leads to CD formation and conversion of CD into hydroperoxide (LOOH). Serum was chosen to avoid possible influences of substances required for plasma preparation. Serum sample (150 μ l) and (150 μ l) of 0.9% saline (reagent blank contains only isotonic saline) were incubated at 37°C for 25 min. Butylated hydroxyl toluene (0.25%, 150 μ l) was added, and the lipids were extracted by heptane/isopropanol (1:1).

Table 1 Lipid profiles of smoking and non-smoking acute myocardial infarct patients (mean±SD)

Variables	Acute myocardial infarct patients (n=86)		p Value (95% CI)
	Smokers (n=43)	Non-smokers (n=43)	
Age (years)	62.32±3.35	60.25±2.76	0.0037 (61.61 to 63.02)
TC (mg %)	173.36±11.84	157.67±13.56	<0.001 (170.85 to 175.86)
HDL cholesterol (mg %)	37.87±5.73	53.83±7.89	<0.001 (36.65 to 39.08)
TC:HDL cholesterol*	4.55±0.67	2.86±0.47	<0.001 (4.40 to 4.69)
TG (mg %)	135.47±15.34	117.36±13.53	<0.001 (132.22 to 138.71)
LDL cholesterol (mg %)	121.46±13.62	81.73±12.61	<0.001 (118.58 to 124.33)
LDL:HDL cholesterol*	3.13±0.72	1.47±0.36	<0.001 (2.97 to 3.28)
TG:HDL cholesterol*	3.53±0.37	2.13±0.53	<0.01 (3.45 to 3.60)

*Ratio.

All values are mean±SD. Values in parentheses indicate the number of subjects.

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglycerides.

Then, samples were acidified by 5 mol/l HCl and extracted by cold heptane (1600 µl). After centrifugation for 5 min at 3000 rpm, the absorbance of the heptane fraction were measured spectrophotometrically at an absorbance maximum between 220 nm and 250 nm. The amount of hydroperoxides produced was calculated using a molar coefficient of $2.52 \times 10^4 / \text{m}^{-1}$.

Other assays

For estimation of other biochemical parameters, standardised reagents and chemicals of analytical grade were obtained from Sigma-Aldrich (New Delhi). Serum uric acid was estimated by the method of Brown, based on the development of a blue colour due to tungsten blue as phosphotungstic acid is reduced by uric acid in alkaline medium.³² Serum total bilirubin was estimated by the method of Jendrassik and Grof,³³ and serum albumin by the bromocresol green dye binding method.³⁴

Statistical analysis

Data on lipid profile and PON1 activity were entered into Microsoft Excel for Windows 2003. The mean±SD was obtained, also using Excel software. The two-sample-t test value between the patients and the control was obtained. A p value of <0.001 was considered as highly significant.

Table 2 Lipid profiles of smoker and non-smoker acute myocardial infarct patients and healthy controls (mean±SD)

Variables	Control (n=86)	Acute myocardial infarct patients (n=86)		p Value†
		S vs NS	Mean±SD	
TC (mg %)	155.58±12.16	S (43)	173.36±11.84	0.001
		NS (43)	157.67±13.56	0.067
HDL cholesterol (mg %)	55.51±6.78	S (43)	37.87±5.73	0.001
		NS (43)	53.83±7.89	0.079
TC:HDL-C*	2.78±0.36	S (43)	4.55±0.67	0.001
		NS (43)	2.86±0.47	0.069
TG (mg %)	114.84±11.51	S (43)	135.47±15.34	0.01
		NS (43)	117.36±13.53	0.037
LDL cholesterol (mg %)	80.59±11.95	S (43)	121.46±13.62	0.001
		NS (43)	81.73±12.61	0.021
LDL:HDL-C*	1.42±0.31	S (43)	3.13±0.72	0.001
		NS (43)	1.47±0.36	0.137
TG:HDL-C*	2.11±0.35	S (43)	3.53±0.37	0.01
		NS (43)	2.13±0.53	0.073

*Ratio.

†Significance between control and smoker (S) and non-smoker (NS) groups separately. All values are mean±SD. Controls are healthy subjects. Values in parentheses indicate the number of subjects.

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglycerides.

RESULT

Total cholesterol, TC/HDL-C ratio, triglycerides, LDL cholesterol, LDL/HDL-C ratio and TG/HDL-C ratio were higher in smoking AMI patients when compared with non-smoking AMI patients (table 1) ($p < 0.001$). The HDL-C levels were significantly decreased in smokers when compared with non-smoking AMI patients. Also, significant differences were observed in total cholesterol, TC/HDL-C ratio, triglycerides, LDL cholesterol, LDL/HDL-C ratio and TG/HDL-C ratio in smoking AMI patients when compared with healthy controls, but the differences were not significant when comparing non-smokers with healthy controls (table 2) ($p < 0.001$).

The superoxide dismutase, glutathione peroxidase and catalase were significantly higher (table 3) ($p < 0.001$) in non-smokers when compared with the observations among smokers AMI patients. Similar findings were observed in the levels of albumin, uric acid and bilirubin (table 3) ($p < 0.001$) when compared with smokers and non-smoking AMI patients. The oxidative stress indicators, namely MDA and CD, were significantly higher, and paraoxonase activity was significantly lower in smokers when compared with non-smokers, indicating the extent of free radicals generated in smokers (table 3, figure 1) ($p < 0.001$).

Table 3 Antioxidant and paraoxonase activities in smoker and non-smoker acute myocardial infarct patients (mean±SD)

Variables	Acute myocardial infarct patients (n=86)		p Value (95% CI)
	Smoker (n=43)	Non-smoker (n=43)	
Superoxide dismutase (U/g haemoglobin)	793.59±78.53	1826.47±31.86	<0.001 (776.98 to 810.19)
Glutathione peroxidase (U/g haemoglobin)	35.28±7.92	61.29±3.94	<0.001 (33.60 to 36.95)
Catalase (k/g haemoglobin)	174.28±28.73	256.15±26.65	<0.001 (168.20 to 180.35)
Albumin (mg %)	3.98±0.47	4.43±0.31	<0.01 (3.88 to 4.07)
Uric acid (mg %)	4.21±0.83	5.82±1.26	<0.001 (4.03 to 4.38)
Bilirubin (mg %)	0.58±0.27	0.77±0.16	<0.01 (0.52 to 0.63)
Malondialdehyde (nmol/l)	16.98±2.87	5.71±0.97	<0.001 (16.37 to 17.58)
Conjugated diene (µmol/l)	56.93±6.78	31.04±2.68	<0.001 (55.49 to 58.36)
Paraoxonase (kU/l)	58.89±10.37	98.42±6.15	<0.001 (56.69 to 61.08)

All values are mean±SD. Values in parentheses indicate the number of subjects.

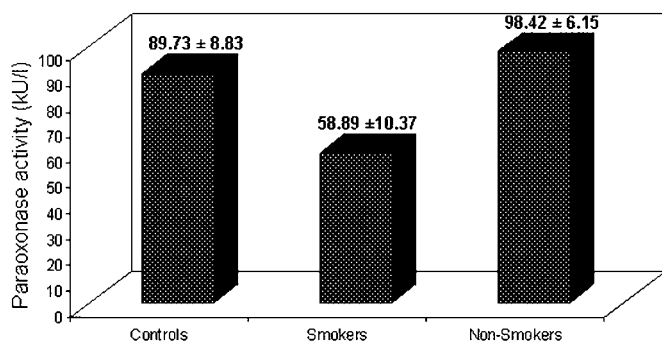


Figure 1 Paraoxonase activities in controls and smoking and non-smoking acute myocardial infarct (AMI) patients. p Value significant (<0.001) between control and smoker. p Value significant (<0.001) between smoker and non-smoker. p Value not significant (0.087) between control and non-smoker.

Although there was a statistically significant difference in observations among smokers and non-smokers in the levels of antioxidants and LP along with paraoxonase activity, even though the difference between healthy controls and smokers were highly significant, the difference among healthy controls and non-smokers AMI were insignificant.

DISCUSSION

Cigarette smoking is widely established as a primary risk factor for atherosclerosis and cardiovascular disease. Increased oxidative stress is one of the principal mechanisms by which it may exert its pathological influence.¹⁶ Earlier studies conducted provide supportive arguments for PON as an antioxidant function,^{4 35 36} which has antiatherogenic potential. In this context, the results of the current study are agreeable with the hypothesis of oxidative modifications in PON due to smoking thus causing changes in its activity further accelerating the atherogenic process. Dyslipidaemia and myocardial infarction may also be associated with a lower activity of serum PON,³⁷ which could be linked to HDL and shows significant correlations with apoA-I

concentrations.^{38 39} Myocardial infarction is also associated with lower serum levels of HDL concentrations, which could therefore explain the alterations in PON activities. Antioxidants and free radicals could conceivably protect PON through augmentation of the overall antioxidant capacity, and so the results from the current study show significant differences among smokers when compared with non-smokers. However, the results were not significantly different among non-smokers to those of healthy controls as PON's antioxidant capacity is not altered in non-smokers thus remarkable difference is not observed and hence the results.

The question is whether the observations are of relevance to the occurrence of cardiovascular disease. Smoking has such a powerful impact on the risk of disease as it per se immensely decrease the PON activity so much that PON's activity could not be differentiated when smoking is associated as a risk of disease. The rationale was that if serum PON were of relevance, lower levels could be observed in more severe cases of disease. The analysis demonstrated that enzyme activities and concentrations were significantly lower in patients with three-vessel disease.

More importantly, perhaps, we demonstrate that differences in PON concentrations, of the order of those observed in the present study, can influence the ability of HDL to protect LDL from oxidation. Thus, incremental increases in HDL PON are associated with incremental decreases in the level of LDL hydroperoxides generated under oxidation conditions.

In vitro studies are providing a wealth of data on the functions of PON, but observations that concern the clinical consequences of modifications to serum PON are less abundant. A limited number of studies have reported lower PON activities in pathologies associated with a higher risk of vascular disease. PON has also been identified as a genetic risk factor for vascular disease. The current study observed the association between smoking, a pro-oxidant phenomenon with a demonstrated inhibitory effect on PON, and serum PON activities and its concentrations remain unaltered in non-smokers. These data also indicate that lower serum PON levels are associated with

Table 4 Antioxidant and paraoxonase activities in smoker (S) and non-smoker (NS) acute myocardial infarct patients and healthy controls (mean±SD)

Variables	Control (n=86)	Acute myocardial infarct patients (n=86)		p Value*
		S vs NS	Mean±SD	
Superoxide dismutase (U/g haemoglobin)	1867.43±97.19	S (43)	793.59±78.53	0.001
		NS (43)	1826.47±31.86	0.089
Glutathione peroxidase (U/g haemoglobin)	66.13±6.36	S (43)	35.28±7.92	0.001
		NS (43)	61.29±3.94	0.124
Catalase (k/g haemoglobin)	293.06±35.87	S (43)	174.28±28.73	0.001
		NS (43)	256.15±26.65	0.722
Albumin (mg %)	4.61±0.33	S (43)	3.98±0.47	0.01
		NS (43)	4.43±0.31	0.234
Uric acid (mg %)	5.76±0.38	S (43)	4.21±0.83	0.001
		NS (43)	5.82±1.26	0.141
Bilirubin (mg %)	0.76±0.23	S (43)	0.58±0.27	0.01
		NS (43)	0.77±0.16	0.031
Malondialdehyde (nmol/l)	5.67±1.66	S (43)	16.98±2.87	0.001
		NS (43)	5.71±0.97	0.065
Conjugated diene (µmol/l)	28.31±3.43	S (43)	56.93±6.78	0.001
		NS (43)	31.04±2.68	0.253
Paraoxonase (kU/l)	89.73±8.83	S (43)	58.89±10.37	0.001
		NS (43)	98.42±6.15	0.087

*Significance between control and S and NS groups separately. All values are mean±SD. Controls are healthy subjects. Values in parentheses indicate the number of subjects.

increased severity of CAD and reduced capacity to protect LDL from oxidation. They are consistent with the hypothesis that smoking modifies serum PON such that there is an increased risk of CAD, which may be due to a diminished capacity to protect lipoproteins from oxidative stress.

Research into paraoxonases has flourished in the last decade. It seems evident now that PON1 has the ability to degrade lipid peroxides in lipoproteins and in cells, and this plays a protective role against oxidative stress and inflammation, which are key processes involved in the pathophysiology of atherosclerosis, leading to myocardial infarction. In future, PON1 measurement can be included in the battery of routine analyses in clinical biochemistry laboratories.

Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by the Ethical Review Board of Jawaharlal Nehru Memorial Hospital.

Contributors AK designed the study and wrote the manuscript.

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