Oxidative and nitrosative stress in association with DNA damage in coronary heart disease

Rajesh K G, Surekha R H, Mrudula S K, Prasad Y, Sanjib K S, Prathiba N

ABSTRACT

Introduction: Oxidative and nitrosative stress caused by a disturbance in the homeostasis of pro-oxidants and antioxidants play a vital role in the pathogenesis of coronary heart disease (CHD). Enhanced formation of reactive oxygen species/reactive nitrogen species may also affect the oxidation/nitration of biomolecules such as lipids, proteins and DNA. The present study was undertaken to estimate oxidative and nitrosative stress, and to evaluate oxidative DNA damage.

Methods: The study population consisted of 120 patients with angiographically documented CHD and an equal number of age- and gender-matched healthy controls. Lipid profiles were estimated using Glaxo kits. Estimation of plasma malondialdehyde (MDA), nitrite/nitrate and comet assay were carried out using previously published methods.

Results: Lipid profiles were significantly different in patients with coronary artery disease compared to the controls (p-value less than 0.01). The levels of MDA, nitrite/nitrate and DNA damage in the patients were significantly higher compared to the controls, and a strong correlation was found between the comet tail length and the MDA and nitrite/nitrate levels. Further analysis revealed that the influence of nitrite/nitrate was greater than that of MDA.

Conclusions: Our results indicate that abnormal levels of lipid profiles, along with increased oxidative and nitrosative stress as well as somatic DNA damage, could be important pathogenic factors that act as additional prognostic predictors. They may also serve as potential targets for therapeutic strategies in CHD for early management and prevention of the disease.

Keywords: coronary heart disease, DNA damage, nitrosative stress, oxidative stress

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INTRODUCTION

Coronary artery disease (CAD) is one of the most frequent causes of death and disabling symptoms worldwide. Epidemiological studies have indicated the rising prevalence of atherosclerosis globally.[1] Formation of atheromatous plaques in the arteries obstructs the supply of oxygen and nutrients to the myocardium, resulting in coronary heart disease (CHD).[2] Studies have shown that the imbalance between the production of reactive oxygen species (ROS) and antioxidant defense results in oxidative stress.[3] Elevated ROS ensures cell proliferation, hypertrophy, growth arrest, apoptosis and oxidation of lipids, proteins and DNA.[4] There is increasing evidence to suggest that DNA damage to cells within the atheromas plays an important role in both atherogenesis and the behaviour of established lesions. DNA damage ranges from 'macro' damages (including microdeletions, insertions in chromosomes) to 'micro' damages (including DNA strand breaks, point mutations, modified bases [includes oxidation] or DNA adducts).[5]

The present study was undertaken to assess oxidative and nitrosative stress by estimating the levels of malondialdehyde (MDA) and nitrite/nitrate, as well as to evaluate the oxidative DNA damage in the peripheral lymphocytes of CHD patients by comet assay (single-cell gel electrophoresis), a sensitive method to analyse the extent of DNA strand breaks.

METHODS

A total of 120 patients with clinical evidence of CAD, as defined by clinical presentation (myocardial infarction) and angiographic documentation of CAD, were recruited for the study. Patients presenting with systemic inflammatory disease, renal and liver disease, cardiomyopathy, malignancy and AIDS were excluded from the study.[6,7] The controls were healthy individuals matched for age, gender and socioeconomic status, and were representative of the normal population with no

Department of Genetics, Osmania University, Hyderabad 500007, Andhra Pradesh, India

Rajesh KG, MSc, PhD
Junior Research Fellow

Surekha RH, MSc, PhD
Assistant Professor

Mrudula SK, MSc, PhD
Junior Research Fellow

Prasad Y, MSc
Biostatician

Prathiba N, MSc, PhD
Professor

Durga Bai Deshmukh Hospital and Research Centre, Vidyanagar, Hyderabad 500007, Andhra Pradesh, India

Sanjib KS, MBBS, DNB, MD
Consultant Cardiologist

Correspondence to:
Dr Surekha Rani H
Tel: (91) 986 662 0067
Fax: (91) 402 709 5178
Email: surekharanih@gmail.com

Table I. Demographic and clinical data in the control and patient groups.

Variable	Controls (n = 120)	CAD (n = 120)
Female/male	55/65	31/89
Mean age ± SD (yrs)	54.55 ± 14.39	56.731 ± 12.28
Mean SBP ± SD (mmHg)	124 ± 4.92	136.10 ± 25.87
Mean DBP ± SD (mmHg)	81.83 ± 3.89	85.87 ± 15.02
Mean BMI ± SD (kg/m^2)	23.8 ± 3.27	24.96 ± 3.05
Diabetes mellitus*	-	54 (45)
Smoking*	-	57 (47.5)
Non-vegetarian*	60 (50)	90 (75)

* Data indicates no. of patients (%).
SD: standard deviation; CAD: coronary artery disease; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index

Table II. Lipid profiles in the control and patient groups.

Variable	Mean ± SD	
	Controls (n = 120)	CAD (n = 120)
CHO (mg/dL)	160.49 ± 4.44	244.32 ± 6.08*
TG (mg/dL)	146.73 ± 4.45	180.53 ± 6.87*
HDL (mg/dL)	45.7 ± 1.6	28.09 ± 1.52*
LDL (mg/dL)	85.55 ± 4.34	179.22 ± 6.12*
VLDL (mg/dL)	29.30 ± 0.9	36.38 ± 1.56*

*All the variables are significant at $p < 0.01$.
SD: standard deviation; CAD: coronary artery disease; CHO: cholesterol; TG: triglycerides; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein

Table III. Oxidative/nitrosative stress and DNA damage markers in the control and patient groups.

Variable	Mean ± SD	
	Controls (n = 120)	CAD (n = 120)
MDA (n moles/ml)	1.78 ± 0.091	7.23 ± 0.33*
Nitrite/nitrate (µ moles/ml)	1.74 ± 0.0935	4.44 ± 0.2142*
Comet (µm)	10.29 ± 0.277	21.149 ± 0.852

*All the variables are significant at $p < 0.01$.
SD: standard deviation; CAD: coronary artery disease; MDA: malondialdehyde

risk factors for CHD or clinical symptoms of any other systemic disease. The study protocol was approved by the Ethical Committee of the department. As per the selection criteria in each group, informed consent of the patients was obtained. Detailed information regarding their demographic status, clinical history, family history and medication was noted.

All reagents used were of analytical reagent grade and were obtained from Merck (Mumbai, India) and Qualigens (Mumbai, India). The kits were obtained from Glaxo (Mumbai, India). Body mass index was calculated by dividing weight (kg) by the square of the height (m^2), and blood pressure (BP) was measured ≥ 30 minutes after the last caffeine intake or cigarette smoked. Three measurements were taken at five minutes of the initial rest and subsequently at two-minute intervals, and any increase in diastolic BP (DBP) or systolic BP (SBP) was recorded (mean DBP ≥ 90 mmHg and/or mean SBP ≥ 140 mmHg).

Tests for total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were conducted using commercially available kits and measured with the Shimadzu UV-240 spectrophotometer (Shimadza Corporation, Kyoto, Japan). Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald's equation: LDL-C = total cholesterol (TC) – HDL – TG/5. Very low-density lipoprotein cholesterol (VLDL) was calculated using the formula LDL-C = TG/5.

Estimation of plasma MDA was carried out according to the method of Gavino et al.[8] An equal amount of 0.9% saline and trichloroacetic acid were added to 0.5 ml of freshly obtained plasma. It was then incubated at 37°C for 20 minutes and centrifuged for ten minutes at 3,000 rpm. 0.25 ml of thiobarbituric acid was added to 1 ml of protein-free supernatant and incubated for 60 minutes at 95°C, and then measured using the spectrophotometer.

The nitrite/nitrate concentrations present in the reaction mixture were determined using a Griess reagent (a 1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% N-[1-napthyl] ethylene diamine) by the method of Lepoivre et al.[9] 0.5 ml of serum was precipitated with 50 µl of 70% sulfosalicylicacid, mixed well for five minutes, vortexed and then centrifuged at 3,000 rpm for 20 minutes. 200 µl of supernatant was taken, and 30 µl of 10% NaOH, 300 µl of 50 mM tris buffer and 530 µl of Greiss reagent were added and incubated for 10 minutes in the dark. The absorbance was read against blank (double distilled H_2O) at 540 nm using the spectrophotometer. The concentration of nitrite/nitrate in serum was determined based on the standard curve generated.

The single-cell gel electrophoresis technique proposed by Singh et al,[10] with alterations suggested by Ahuja and Saran,[11] was followed in the present study, with the exception of silver staining of comets, as described by Gandhi,[12] which was carried out instead of ethidium bromide. Approximately 2 ml of venous blood was taken from each individual in a sterilised eppendorff tube containing EDTA, and processed for assay within 2–4 hours. 30 µl of fresh blood (around 60,000 cells) mixed with 130 µl of 0.7% low-melting point agarose

Table IV. Multiple regression analysis of MDA and nitrite/nitrate on comet assay in the control and patient groups

Variable	Controls: R-square 0.0136		Patients: R-square 0.8183	
	Parameter	Standardised	Parameter	Standardised
Intercept	11015889	0	3.82632*	0
MDA	−0.26682	−0.8725	0.61088*	0.23531
Nitrite/nitrate	−0.22488	−0.07564	2.90466*	0.73034

* $p < 0.01$
MDA: malondialdehyde

(LMPA) in phosphate buffered saline at 37°C was layered onto a slide that was pre-coated with thin layers of 1% normal melting point agarose. The slides were left for five minutes at 4°C in order to allow the agarose to solidify, and this was followed by another layer of LMPA. The slides were then subjected to the lytic process in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris-HCl, pH 10–10.5, 1% Triton X-100 and 10% dimethyl sulfoxide added just before use) for at least 8–12 hours[11] and then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/l NaOH, and 1 mmol/l Na_2EDTA, pH > 13) at 4°C for unwinding (40 minutes), followed by electrophoresis (25 V/300 mA, 25 minutes). All the steps were carried out under minimal illumination and stained with silver nitrate ($AgNO_3$) solution. For each sample, the slides were prepared in duplicate, and a total of 100 cells were scored under binocular microscope at 40 times magnification using an oculometer. DNA damage was calculated as the difference between the length of the comet and the diameter of the comet head.

Statistical analysis was performed using SAS version 9, (SAS Institute Inc, Cary, NC, USA) and the data was expressed as mean ± standard deviation. The Student's *t*-test was performed for lipid profiles, lipid peroxidation (MDA), nitrite/nitrate and comet in order to determine the difference between the controls and CAD patients. Multiple regression analysis and Pearson's correlation test were performed to assess the relationship between MDA and nitrite/nitrate and DNA damage.

RESULTS

The demographics and clinical data of the patients and controls are presented in Table I. There were more male than female subjects in the patient population. The risk factors for coronary atherosclerosis, including BP, obesity, diabetes mellitus, smoking and being non-vegetarians, were higher among the patients compared to the controls.

Total cholesterol (160.49 ± 4.44 vs. 244.32 ± 6.08), TG (146.73 ± 4.45 vs. 180.53 ± 6.87), LDL-C (85.55 ± 4.34 vs. 179.22 ± 6.12) and VLDL (29.30 ± 0.9 vs. 36.38 ± 1.56) values were significantly higher ($p < 0.01$) in CAD patients compared to the control group, while HDL-C (45.7 ± 1.6 vs. 28.09 ± 1.52) levels were lower in CAD patients than the controls ($p < 0.01$), as shown in Table II. Plasma MDA levels (1.78 ± 0.091 vs. 7.23 ± 0.33) were found to be significantly higher in CAD patients compared to the controls ($p < 0.01$) (Table III). Serum nitrite/nitrate tended to be higher in CAD patients (4.44 ± 0.2142) than the controls (1.74 ± 0.0935), with the difference being statistically significant at $p < 0.01$ (Table III). Comet tail length (oxidative DNA damage) of CAD patients (21.149 ± 0.852 vs. 10.29 ± 0.277) was found to be significantly higher ($p < 0.01$) than that of the controls (Table III).

Table IV shows the stepwise multiple regression analysis carried out for all variables, which showed that

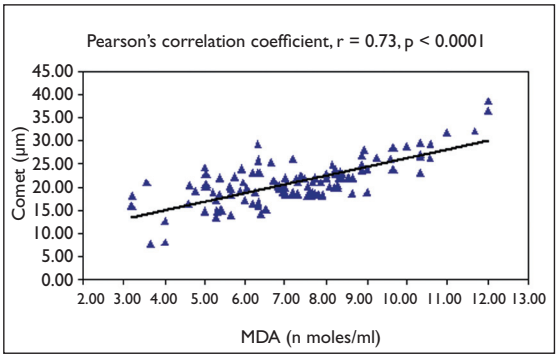

Fig. 1 Graph shows correlation between malondialdehyde and comet in the patient group.

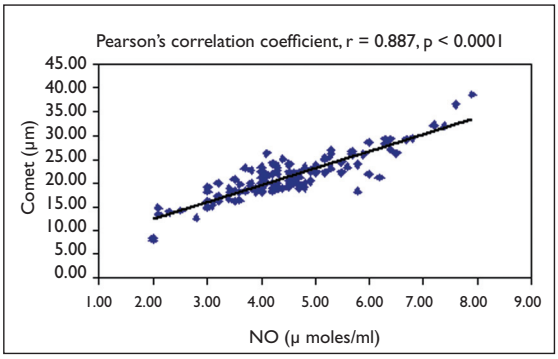

Fig. 2 Graph shows correlation between nitric oxide and comet in the patient group.

MDA and nitrite/nitrate significantly influence the length of comet in CAD patients. This was seen in the variation of patient comet values by the factor at 81.83% compared to the controls at 1.36% (i.e. R-square value), which was found to be significant at $p < 0.01$. MDA was found to be associated with oxidative DNA damage (comet) in CAD patients when compared to controls ($p < 0.01$), and the increase in oxidative DNA damage (comet) was parallel to the increase in MDA, which was statistically significant ($p < 0.01$, $r = 0.723$) (Fig. 1). Stepwise multiple regression analysis also showed an association between nitrite/nitrate and DNA damage (comet) ($p < 0.01$, $r = 0.887$) (Fig. 2). The influence of nitrite/nitrate was much greater than that of MDA in the patient group ($r = 0.887$ for nitrite/nitrate vs. $r = 0.723$ for MDA).

DISCUSSION

CAD is one of the common multifactorial disorders involving both environmental and genetic risk factors.[13] The present study focused mainly on the consquences of oxidative and nitrosative stress on DNA damage and lipid profiles. A number of studies have shown that TG serves as an excellent biomarker for CHD, in part because of its association with atherogenic cholesterol-enriched remnant particles,[14] which may be directly incorporated by macrophages[15] and may participate in pro-atherothrombotic inflammatory signaling pathways.[16]

Elevated levels of VLDL correlate with accelerated rates of atherosclerosis. It is well known that a high level of HDL-C seems to be able to protect against cardiovascular diseases, and an inverse relationship between plasma levels of HDL-C and the risk for CAD has also been well established.[17] In the present study, similar observations were made; higher levels of TG, VLDL and low levels of HDL-C were found in our CHD patients compared to the controls (Table II).

Evidence has shown that excess deposition of cholesterol in coronary arteries contributes to narrowing and blockages that cause CAD, and this has been considered to be atherogenic cholesterol that exists mainly in the form of LDL. Recent studies suggest that it is not the LDL itself but a modified LDL that is the problem, and this supports the hypothesis that oxidised lipids (including ox-LDL) contribute to CAD and that the process is enhanced by ROS.[18,19]

Oxidative degradation of lipids is known as lipid peroxidation, and one of the most abundant carbonyl products of lipid peroxidation is MDA, which is an index of oxidative damage.[20] Increased lipid peroxidation may occur as a result of increased free radical generation and suppressed scavenging mechanism.[21] In our study, the mean values of MDA in CAD patients were significantly higher than that of the controls (Table III). Similarly, higher levels of MDA have been observed in atherosclerotic patients compared to control patients.[20,22-24] Oxidative modification of LDL by free radicals results in negative effects on vascular function, such as endothelial apoptosis, increase in smooth muscle cell proliferation and synthesis of proinflammatory molecules.[25]

There is growing evidence that demonstrates the importance of nitric oxide (NO) in the pathogenesis of CAD,[26] including blood pressure regulation, inhibition of thrombocyte aggregation, leucocyte adhesion, smooth muscle cell proliferation and LDL oxidation.[27] Under pathophysiological conditions, ROS and NO react to generate toxic reactive nitrogen species, particularly dinitrogen trioxide and peroxynitrite (ONOO–), and cause significant damage to cellular components (proteins, membranes, nucleic acid), leading to chromosomal alterations, protein nitration, lipid peroxidation, subsequent cellular dysfunction and cellular death.[28-30]

Soydinc et al observed that CAD patients have higher levels of NO compared to healthy controls.[27] Similarly, we observed higher levels of nitrite/nitrate in CAD patients compared to healthy controls (Table III) in the present study, which is in accordance with an earlier report.[31] ROS and their by-products are capable of causing oxidative damage that may be cytotoxic, and it has also been speculated that ROS may cause extensive DNA damage and DNA strand breaks.[32] Oxidative DNA base modification can also reflect exposures to both endogenous and exogenous oxidants such as oestradiol and lipid peroxidation products.[33-36] The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination and deamination.[37,38]

Recent evidence has indicated that oxidative DNA damage is present in all cells within the atherosclerotic plaque and in the circulation of patients.[5,6,39] Botto et al reported the presence of chromosomal damage in the peripheral blood lymphocytes of 53 patients with coronary ischaemia heart disease by using a micronucleus test.[6] Demirbag et al, who documented DNA damage in 53 CAD patients using comet assay, also observed negative correlation with total antioxidant capacity.[3] An earlier study on 150 CAD patients also reported significantly higher levels of MDA and nitrite/nitrate (markers of oxidative stress) but lower levels of TAC in CAD patients compared to healthy controls.[31]

In the present study, which is the first of its kind, we assessed DNA damage in the peripheral lymphocytes of

CHD patients using comet assay as well as the markers of oxidative stress (MDA and nitrite/nitrate) and lipid profiles on a large sample, and confirmed the observations of earlier reports. Multiple regression analysis performed on all variables showed that MDA and NO significantly influenced the length of comet in patients (as evident by the R-square value [81.83% in patients vs. 1.36% in controls, Table IV]) and that the interactions of MDA and NO with ROS may lead to DNA damage. The study also analysed the association of comet length with MDA and NO, and found that the influence of NO is greater than that of MDA ($r = 0.887$ and $r = 0.723$ for NO and MDA, respectively). NO, or more likely, reactive products derived from it (e.g. NO_2, ONOO–, N_2O_2 and HNO_2), with the potential to produce nitration, nitrosation and deamination reactions on DNA bases, may be one of the causes of DNA damage, as observed in the present study (Figs. 1 & 2).

We suggest that the extent of damage caused by free radicals may be reduced through lifestyle modifications and dietary intervention, such as increasing the intake of vegetables, fruits and antioxidant supplements (e.g. beta-carotene, vitamins C and E).[40] We also advocate that further research in this area be given high priority. This is because increased oxidative and nitrosative stress as well as somatic DNA damage could be important pathogenic factors that act as additional prognostic predictors and potential targets for therapeutic strategies in CAD for early management and prevention of the disease.

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