Assessment of DNA Damage in Leukocytes of Patients With Coronary Artery Disease by Comet Assay

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SUMMARY

DNA damage in the peripheral blood leukocytes (PBL) of patients with coronary artery disease (CAD) was investigated using the sensitive alkaline single cell gel electrophoresis (SCGE)/comet assay.

This case-control study consisted of CAD patients (n = 200; mean age, 59.04 ± 0.75 years) undergoing treatment at local hospitals and age-, sex-, and ethnicity-matched healthy controls (n = 200; mean age, 57.88 ± 0.96 years) from the general population.

CAD patients had significantly (P < 0.001) increased DNA damage (tail DNA percent (T-DNA %) 22.45 ± 0.50 versus 5.81 ± 0.28; tail moment (TM) 89.35 ± 3.16 versus 9.98 ± 0.69; Olive tail moment (OTM) 60.50 ± 1.79 versus 10.94 ± 0.63; damage frequency (DF) 91.12 ± 0.93 versus 41.78 ± 2.04, damage index (DI) 173.68 ± 3.36 versus 48.53 ± 2.59) compared to controls. Patients with acute myocardial infarction (AMI) showed significantly higher DNA damage than patients with unstable angina (UA) (T-DNA % 24.05 ± 0.87 versus 21.06 ± 0.90; TM 100.02 ± 6.19 versus 81.61 ± 5.84; OTM 66.19 ± 3.20 versus 56.47 ± 3.33; DF 94.02 ± 0.84 versus 91.10 ± 1.16, DI 184.13 ± 5.33 versus 166.42 ± 5.89). Moreover, DNA damage was found to be significantly (P < 0.05) elevated in patients receiving ecosprin, ramipril, and metoprolol therapy compared to aspirin and nitrocontin.

The increased DNA damage in CAD patients may be the consequence of disease and/or drug therapy. These observations are of concern because unrepaired DNA can lead to malignancy, and the likelihood of increasing mortality and morbidity rates in CAD patients. (Int Heart J 2017; 58: 271-274)

Key words: Genetic damage, SCGE assay, CAD subtypes, Peripheral blood leukocytes, Drug therapy

Coronary artery disease (CAD) has emerged as one of the major causes of morbidity and mortality worldwide. Pathophysiological mechanisms of CAD include oxidation of lipids, plaque rupture, and thrombus formation.1-2 It has also become clear from previous studies that DNA damage may initiate a number of chronic degenerative diseases, including CAD.3-5 There is increasing evidence of the presence of DNA damage in patients with CAD.6-10 DNA damage in human atherosclerotic plaques ranges from ‘macro’ damage (deletions or additions of whole or parts of chromosomes) to ‘micro’ damage (DNA strand breaks, mutations of single bases, base modifications, or DNA adducts).11-16 Factors that may contribute to DNA damage in CAD include age,17 diabetes mellitus,18 dyslipidemia,19 mechanical reperfusion therapy,20 and drug therapies.21 However, there is only one study on CAD sub-types.22 Therefore, the present study aimed to evaluate DNA damage in peripheral blood leukocytes (PBL) of CAD patients stratified by sub-types using a single cell gel electrophoresis (SCGE) assay which is a sensitive technique for assessment of DNA damage.23

METHODS

Participants: The study subjects were 200 patients manifested with CAD, as documented by electrocardiographic (ECG) changes, echocardiographic evidence of myocardial infarction, positive treadmill test, and elevated serum creatine phosphokinase (CPK-MB) levels. Patients with renal, liver, thyroid, or lung disorders, or malignancy were excluded from the study. The control group consisted of 200 age-, sex- and ethnicity-matched healthy adults from the same area with no present or past family history of CAD or any other disease. The study was approved by the Institutional Ethics Committee of Guru Nanak Dev University, Amritsar and was carried out in accordance with the Declaration of Helsinki.

Data and blood sample collection: The demographic and disease-related information of each participant was documented on a pre-designed questionnaire. Anthropometric measurements (height, weight) were taken following standard methodology.24 Body mass index (BMI) was calculated using the criteria of WHO.25 Physiometric measurements were taken after the subject had rested for 10 minutes as per the recommendations of the American Heart Association.26 The average of 3
measurements was taken as the blood pressure values and the guidelines of the Joint National Committee (JNC VII) were used to categorize participants as normotensive or hypertensive. Blood samples (~0.5 mL/participant) were taken in pre-labelled microcentrifuge tubes containing heparin sodium (3U, Biological E Limited, India) and processed within 3-4 hours for DNA damage assessment.

**SCGE/Comet assay:** Prior to the SCGE assay, the blood samples were assessed for cell viability by the trypan blue dye exclusion method and since cell viability was 92-100%, the alkaline version of SCGE assay was performed on PBL according to the method of Singh, et al with slight modifications which included use of local chemicals, agarose-precoated slides in lieu of frosted slides, and silver-staining of comets instead of using ethidium bromide.

In brief, 110 μL of molten low melting point agarose (LMPA; 0.5%, SRL, India) mixed with 20 μL of whole blood was poured over slides pre-coated with 1% normal melting point agarose (NMPA, SRL, India) and immediately covered with cover slips while avoiding air bubbles and kept at 4°C for 5 minutes to allow the gels to solidify. After removing the cover slips, a third layer (100 μL of 0.5% LMPA) was poured over the blood-agarose layer and again covered with fresh cover slips and kept at 4°C for 5 minutes allowing the gels to solidify. All further steps were carried out under subdued light to prevent any additional DNA damage. After removing the cover slips, the slides were treated with freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO; pH = 10) for at least one hour at 4°C to facilitate the lysis of membranes, cytoplasm, and nuclear proteins, leaving the DNA as nucleoids. Slide preparations were placed for 30 minutes in an electrophoretic chamber filled with freshly-prepared electrophoretic buffer (0.3 M NaOH, 1 mM EDTA, pH ≥ 13) for unwinding of DNA, expression of single-strand DNA breaks, and alkali-labile sites. Electrophoresis was carried out using the same electrophoretic buffer (25V 300 mA, 0.8V/cm, 25 minutes). After electrophoresis, the slides were washed 3 times with neutralization buffer (0.4 M Tris; pH7.5) for 5 minutes each to remove the alkali and detergents, and were finally stained with silver nitrate (0.2% AgNO3, SRL, India).

Images of 100 random nucleoids (50 from each of the two replicate slides) per individual were captured with a Jenoptik camera (Progress Capture Pro, version 7.8; Germany) fitted onto an Olympus microscope (BX43Model, Tokyo) and analyzed blindly using the freely-available Comet Assay Image Analysis Software Programme (CASP, http://www.casplab.com). The various endpoints scored for DNA damage assessment included tail DNA percent (T-DNA %; the amount of DNA in comet tail); tail moment (TM, the product of per cent tail DNA and tail length in arbitrary units), and Olive tail moment (OTM; the difference between tail mean and head mean multiplied by per cent DNA in tail in arbitrary units). The damage frequency (DF; arbitrary units) was calculated on the basis of number of nucleoids with tails. The damage index (DI, arbitrary units) is the sum of classes of 100 cells analyzed per individual ranging from nucleoids with no tails (class 0), little damage (class 1), medium damage (class 2), more damage (class 3), high damage (class 4) types, so the total comet score can range between 0 and 400 arbitrary units (Supplemental Figure).

**Statistical analysis:** All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 16.0 for Windows 7, Chicago, IL, USA) and data are expressed as the mean ± SEM. Differences between the continuous variables were evaluated using Students' t-test, whereas categorical variables were compared by the chi-square test or Fisher’s exact test as appropriate. Comparisons of means among 3 groups of CAD sub-types were performed by using one-way analysis of variance (ANOVA). Associations of DNA damage with other variables were obtained by Pearson’s correlation analysis. A multivariate linear regression analysis was performed to identify independent predictors of DNA damage. A two tailed P-value < 0.05 was considered as statistically significant.

**RESULTS**

The demographic and clinical characteristics of the patients (n = 200; mean age, 59.04 ± 0.75 years) and controls (n = 200; mean age, 57.88 ± 0.96 years) have been previously described in detail. No case or control subjects smoked while alcohol consumption was more prevalent among controls. General obesity was ~81% among cases and central obesity was ~100%. Patients and controls were matched for age, gender, waist-hip-ratio, and socioeconomic status. Dyslipidemia was present in ~74% of cases and ~22% of cases were hypertensive (Supplemental Table I). The patients were comprised of 36.50% males and 63.50% females diagnosed with disease between 31-88 years (males, 57.08 ± 1.27 years; females, 53.75 ± 0.92 years). The disease spectrum included stable angina (SA) in 43.50% of the patients, unstable angina (UA) in 26%, and acute myocardial infarction (AMI) in 30.50%. The onset-of-disease was at 54.97 ± 0.75 years and the patients had been on prescribed medications for 4.04 ± 0.33 years (range, 0-20 years) with males (patients for 3.18 ± 0.53 years and females for 4.53 ± 0.41 years). The treatment time was the same as duration of disease since the patients were on prescribed medicines from the time of diagnosis. Drug therapy included the categories of beta-blockers (metoprolol), antiplatelet therapy (aspirin, ecosprin), ACE-inhibitors (ramipril), and nitrates (nitrocontin) in various combinations.

Leukocyte DNA damage (Supplemental Table II) was significantly (P < 0.001) higher in the patients than in healthy controls (T-DNA % 22.45 ± 0.50 versus 5.81 ± 0.28; TM 89.35 ± 3.16 versus 9.98 ± 0.69; OTM 60.50 ± 1.79 versus 10.94 ± 0.63; DF 91.12 ± 0.93 versus 41.78 ± 2.04 and DI 173.68 ± 3.36 versus 48.53 ± 2.59). However, on stratification by gender, no significant differences were observed for these damage parameters while male and female patients separately also had significantly (P < 0.001) elevated all DNA damage indices than their parallel control counterparts. AMI compared to CAD patients had significantly (P < 0.05) increased genetic damage (T-DNA % 24.05 ± 0.87 versus 21.06 ± 0.90; TM 100.02 ± 6.19 versus 81.61 ± 5.84; OTM 66.19 ± 3.20 versus 56.47 ± 3.33; DF 94.02 ± 0.84 versus 91.10 ± 1.16 and DI 184.13 ± 5.33 versus 166.42 ± 5.89). However, DNA damage did not differ between the ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction patients (NSTEMI) or between the patients with stable or unstable angina (Supplemental Table III).
On stratification by drug-therapy for the 11 drug-combinations (Supplemental Table IV), DNA damage was significantly ($P < 0.05$) higher (1.3x T-DNA%, 1.5x each TM and OTM and 1.5x DI) in group 4 (ramipril and metoprolol combination) than in group 3 (aspirin, metoprolol). The comparison of group 10 (ecosprin, ramipril, metoprolol) with group 11 (ecosprin, metoprolol, nitrocontin) revealed that DF was significantly ($P < 0.05$) higher in group 10. No statistically significant differences were observed in other pair-wise comparisons.

Correlation and regression analyses were performed with DNA damage indices as dependent variable and age, obesity, hypertension, dyslipidemia, alcohol, family history, and medications as independent variables (Supplemental Table V). No significant correlations were observed between DNA damage indices and these variables. On multivariate regression analysis, none of the variables emerged as independent predictors of DNA damage.

**DISCUSSION**

Patients in the AMI sub-group had significantly ($P < 0.001$) increased leukocytic DNA damage with elevated DNA damage compared to that in the UA group. Indices of DNA damage scored in the present study included T-DNA%, TM, OTM, DF, and DI. In the literature, these parameters have also been studied; tail DNA per cent is the relative amount of DNA in tail. Tail moment is the product of tail length and percentage of DNA in tail. Olive tail moment is the product of tail length and the fraction of total DNA in tail. Damage frequency is the frequency distribution of the number of nuclei with tails. Damage index, in which each cell was assigned to one of 4 classes (from no damage = 0 to maximum damage = 4) according to tail size and shape. In the present study, these damage indices were significantly higher in patients implying significantly increased DNA damage in patients. Reports in the literature have also documented such observations. Botto, et al reported increased levels of DNA damage levels in patients with CAD, as have Demirbag, et al and Rajesh, et al. Bhat, et al in their previous studies also observed significantly increased levels of T-DNA %, TM, and OTM as well as binucleated cells with micronuclei and micronuclei in patients with CAD. The other observation was the significantly increased DNA damage in patients with AMI compared to patients with UA, implying that the levels of DNA damage increase with the severity of CAD. In the literature, earlier studies have also supported the relation between DNA damage and severity of CAD.

Prescribed medications, despite having antioxidant properties, also have been reported to have genotoxic potential, implying that treatment with these medications can induce genetic damage. The medications prescribed for the management of CAD in the present study included metoprolol (beta-blocker), aspirin, ecosprin (antiplatelet therapy), ramipril (ACE-inhibitor), and nitrocontin (nitrates). Patients on the ecosprin-ramipril-metoprolol combination had significant ($P < 0.05$) increases across all scored indices, implying that the observed DNA damage in CAD patients may also be from these drugs. In the literature, the genotoxicity of these drugs has also been studied in bacterial cells as well as in mice bone marrow cells and human peripheral blood lymphocytes. Metoprolol was positive for genetic damage in algae *Pseudokircheriella subcapitula* and in the bacteria *Vibrio fischeri*. Genetic damage was also observed in Chinese hamster lung cells treated with aspirin. Ramipril induced agranulocytosis in chronic renal failure patients, confirmed by a lymphocyte cytotoxicity test. Andreassi, et al have documented significantly higher micronucleus frequency in the lymphocytes of ischemic and CAD patients on treatment with nitrates. On the basis of the genotoxic nature of aspirin, ramipril, metoprolol and nitrocontin, the patients taking these drugs could also be susceptible to DNA damage from these drugs. Therefore, the genotoxic nature of these drugs cannot be ignored, like the carcinogenicity of cardiovascular drugs, including diuretics, beta blockers, calcium antagonists, and ACE-inhibitors has also been debated.

**Conclusion:** Patients with CAD showed significantly increased levels of DNA damage in their peripheral blood leukocytes, which might be due to the pathological consequences of the disease and/or the prescribed medications. These findings highlight the need for appropriate management and preventive strategies to circumvent any dire outcomes.

**ACKNOWLEDGMENTS**

The help of Dr. Ajinder Pal Singh (A. P. Heart-Care Hospital) and Dr. Sudhir Abrol (Mata Kaulan Ji Bandi Chod Charitable Hospital) Amritsar, Punjab for the diagnosis of patients is gratefully acknowledged. MAB is grateful for the UPE fellowship from GNDU for his PhD programme.

**DISCLOSURE**

**Conflict of interest:** None declared.

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