Relationship of oxidative stress with elevated level of DNA damage and homocysteine in cardiovascular disease patients

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Abstract: Amounts of DNA damage and homocysteine (Hcy) in heart patients blood may have strong function in the causation of cardiovascular disease (CVD). The main objective of this work was to know experimentally the role of total oxidants (produced by Reactive Oxygen species (ROS), clinical biochemical indices, their oxidized products and total antioxidant status (TAS) among such patients to find the association of homocysteine, total oxidation status (TOS) and oxidative DNA damage with other clinical parameters in sixty positive CVD patients compared with those of 60 normal subjects. As compared to healthy individuals, CVD patients had significantly higher concentrations of homocysteine ($p<0.0001$), total oxidants stress (TOS) ($p<0.0001$), serum total lipids ($p<0.04$), malondialdehyde (MDA) ($p<0.001$), high density lipoprotein-cholesterol (HDL-C) ($p<0.0001$), and low density lipoprotein cholesterol (LDL-C) ($p<0.01$), than those of healthy individuals. Plasma Hcy content, TOS and amount of DNA were positively and significantly associated with cholesterol, triglycerides, systolic blood pressure, urea, and albumin (p values<0.01). TOS, Hcy and oxidative DNA damage were negatively correlated with HDL-c, TAS and proteins. It is suggested that these parameters have pivotal role in diagnostic process of determining severity in CAD patients. Oxidized products of macromolecules in blood of CVD patients impart major functions in causing CVD disease.

Keywords: Antioxidants, heart disease, CVD, homocysteine, TOS, DNA damage, malondialdehyde, 8-Hydroxy-2'deoxyguanosine.

INTRODUCTION

Cardiovascular or heart disease is among the severe causative agents of death and disabling human systems globally. Epidemiological findings have shown the rising prevalence of CVD world-wide and are gaining a position of pestilence in all countries (Yusoff, 2002). Pakistan is a developing nation and is known to be a centre of CVD (Malik and Herbert, 2012). Built up of fibrin molecules in the heart muscles obstruct the flow of nutrients and oxygen to the muscular layer of the hearts' walls leading to cause CVD. It is generally caused by a combination of risk factors which include smoking, obesity, unhealthy poor diet, physical inactivity and others. New predictors are needed to detect the risk of CVD (Simon *et al*., 2013).

Hyperhomocysteinemia has become a leading cause of heart disease. Among dominant risk factors, Hcy can promote vascular disease by putting a direct cytotoxic effect on the endothelium and increases adhesiveness of the platelets and effects on clotting factors (Miguel *et al.,* 2001). Vitamin deficiencies and loss of methionine synthase activity may produce hyperhomocysteinemia which results in elevated oxidative stress and hence increases the risk for atherosclerotic diseases (Botto *et al*., 2002). Studies have shown the association of mild and severe hyperhomocysteinemia with the DNA damage at thymine and guanine residues respectively. Thus elevated Hcy content is linked with vascular disease (Botto *et al.,* 2002; Lentz, 2005).

Reactive nitrogen species (RNS) and ROS are free radicals and oxidants represent states which depict imbalance among rates of free radicals formation and degradation of such radicals by body's antioxidant system due to increased formation and accumulation of such radicals (Bukhari *et al*., 2010; Rajesh *et al*., 2011). Their formation takes place in mitochondria during consumption of oxygen (Khalili *et al*., 2016). This happens when electrons move away the main chain and reduce oxygen molecules to form superoxide anion (Bukhari *et al*., 2010). They are also synthesized in phagocytes' cells, vascular walls and other tissues by several enzymes, namely cyclo-oxigenases, lipo-oxygenases, myeloperoxidase, NAD (P) H oxidase and xanthine oxidase (Siow *et al*., 2006). At low concentrations, they are regulators of intracellular signaling cascades, apoptosis, modulation of immune system, and mutagenesis (Simon *et al*., 2013). If they are produced in phagocytes' bursting process, they impart

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defense against pathogens present in the environment (Shah and Channon, 2004). When ROS are produced in excess, they react with several molecules, namely, carbohydrates, lipids, DNA and proteins and alter not only their structures but also their functions (Malik and Herbert, 2012). These changes result in the damage to the cells and lead to formation of pathological processes including development of atherosclerosis in the cellular membranes (Rajesh *et al*., 2011). The deleterious effect of such damages is controlled by enzymatic and non-enzymatic anti-oxidants which can remove pro-oxidants and protect the effect of free radicals (Tomas *et al*, 2004). Alpha-tocopherol (vitamin E), ascorbic acid, glutathione, bioflavonoids, mixed carotenoids and thiols are non-enzymatic while catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase are enzymatic anti-oxidants and their combined impact is called anti-oxidant status (TAS) (Yusoff, 2002). Myocardial antioxidants have the ability to reduce the oxidative damage to carbohydrates, proteins, lipids, DNA and RNA. Anti-oxidants protect the heart from ROS-mediated damage (Demirbag *et al*., 2005). Their role in protecting the development of CVD is important since their presence may decrease the damage resulting from blood ROS during the process of reperfusion. DNA damage is emerging as a risk factor in CVD (Botto *et al*., 2002). If damages occur and are left un-attended, can cause mutations in DNA strands and can lead to diseased condition (Rajesh et al., 2011). To prevent such mutations and the production of altered DNA, lipids and proteins, cells may deploy their repair mechanisms to remove and replace defective nucleotides, rebuild broken strands, and stitch other kinds of damages (Tayal *et al*., 2012). Any change in DNA repair system may increase the risk for CAD. Cells strength to repair DNA damage is vital for maintenance of cellular genetic integrity (Andreassi *et al*., 2003).

Cellular DNA damage releases 8-Hydroxy-2'-deoxyguanosine (8-OHdG) as an abundant product of cellular aberrations (Botto *et al*., 2002). Several studies suggest the implication of TOS in the development of CVD and prove the role of DNA damage in CVD (Demirbag *et al*., 2012). Cells possess an efficient defense system which can scavenge the ROS (Malik & Herbret, 2012). A disturbed antioxidant/pro-oxidant profile is observed in CVD patients as discussed in previous studies (Simon et al., 2013). The present work was conducted to assess the extent of TOS, Hcy, TAS, and genetic material damage in Pakistani CVD patients compared with those of healthy individuals.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from Sigma Aldrich. Serum glucose kit (Fluitest Glu Biocon, Lot # H 265, Screen Master # 35510), Randox laboratory kit for measuring thiobarbituric acid reactive substances (TBARS), proteins, albumins and urea measuring kits (Randox, UK), Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measuring kits (Cat No. CZ 902 C; Cat No. CZ), EIA kit (589320) for measuring DNA damage and all other kits were supplied by Biorays Labs, Faisalabad-38800, Pakistan

Blood samples

We recruited 60 CAD cases (40 males and 20 females, age group: 45-76 years; Mean age =52.67±7.87) and 60 controls (35 males and 25 females, age group: 40-70 years; Mean age: 51.9±7.29) who visited Punjab Institute of Nuclear Medicine (PINUM), Faisalabad, Pakistan for examination and treatment. The diagnosis of CVD was performed using clinical history, chest X-ray, physical examination, echocardiography, electrocardiography and sintigraphy according to the current international guidelines available with PINUM. Diagnosis of CVD was confirmed by a senior cardiologist. Healthy blood donors with no clinical symptoms of coronary artery disease but belonging to patients' families were enrolled as controls. Data regarding smoking, alcohol use, diabetes, hyper-lipidemia and hypertension were recorded for all the subjects through questionnaires and medical forms. Hypertensive patients were those whose diastolic blood pressure was ≥90mm Hg and systolic blood pressure ≥140mm Hg. Hyper-lipidemic patients had total cholesterol level of >220mg/dL or triglycerides concentration >200mg/dL. Diabetes mellitus patients had a fasting plasma glucose concentration of ≥126mg/dL. Normal subjects were free of all above symptoms. Standard methods recommended for such studies were followed.

Collection of blood samples

Venous puncturing was applied for collecting 10mL blood from all patients in tubes containing heparin. Serum was obtained by centrifuging (5,000rpm for 2min) and kept at 4ºC. Ethical committee formulated by PINUM approved this study. Three supervisors looked after this work.

Measurement of risk variables and biochemical analyses

BP and body mass index (BMI) were recorded. Serum glucose was measured through enzymatic colorimetric test (GOD-PAP) using commercially available kit through spectrophotometer. Diabetic individuals were diagnosed according to the criteria of American Diabetes Association. Plasma thiobarbituric acid reactive substances (TBARS) were measured colorimetrically. Hitachi 704 analyzer using kit of Randox laboratory was used to determine the serum contents of cholesterol while triglycerides were determined enzymatically as described earlier (Bukhari *et al*., 2010). High-density lipoprotein (HDL)-cholesterol was measured using the enzymatic method after precipitation of the plasma with phosphotungestic acid in the presence of magnesium ions. Low-density lipoprotein (LDL)-cholesterol values were computed with the Friedewald formula.

Table 1: Physical, biochemical and health parameters of normal and cardiac patients (Mean ±SE)

Parameters	Normal subjects	Cardiac patients	p
Body mass index (BMI); (kg/m^2)	22.9±0.72	25.83±0.72	0.001
Systolic blood pressure (mm/Hg)	109.5±0.94	165.0±3.46	0.0001
Diastolic blood pressure (mm/Hg)	80.75±1.65	101.92±2.07	0.001
Glucose (mg/dL)	117.7±6.16	168.95±16.73	0.0036
Cholesterol (mg/dL)	112.35±4.5	144.5±4.44	0.0001
HDL-Cholesterol (mg/dL)	34.025±1.15	26.9±0.92	0.001
LDL-Cholesterol (mg/dL)	123.12±5.19	165.52±3.133	0.0375
Albumin (mg/dL)	3.68±0.26	5.75±0.36	0.001
Total Protein (gm/dL)	7.53±0.084	5.37±0.088	0.01
Urea (mg/L)	22.72±1.80	37.45±2.12	0.001
ALT (U/l)	24.92±1.67	67.17±3.46	0.0001
AST (U/l)	22.27±1.14	96.02±7.43	0.001
Homocysteine (Hcy; µmol/L)	8.97±1.46	35.60±1.55	0.0001
Total oxidant status (µmol H_2O_2 equiv./L)	9.85±0.33	31.7±0.37	0.0001
DNA damage (%)	7.42±0.072	34.22±0.11	0.001
MDA (µmol ./L)	0.56±0.04	1.10±0.05	0.0001
TAS (mmol/ Trolox Equiv./L	1.63±0.05	1.12±0.046	0.024

Table 2: Correlation of oxidative stress, DNA damage and homocysteine with other attributes

	Homocysteine (Hcy; µmol/L)	Total oxidant status (µmol H_2O_2 equiv./L)	DNA damage (%)
Homocysteine (Hcy; µmol/L)	1.000	0.994**	0.989**
		0.000	0.000
Total oxidant status(µmol H_2O_2 equiv./L)	0.994**	1.000	0.999**
	0.000		0.000
DNA damage (%)	0.989**	0.999**	1.000
	0.000	0.000	
Body mass index (BMI); (kg/m2)	0.901*	0.846*	0.827*
	0.014	0.034	0.043
Systolic blood pressure (mm/Hg)	0.997**	0.996**	0.993**
	0.000	0.000	0.000
Diastolic blood pressure (mm/Hg)	0.996**	0.980**	0.972**
	0.000	0.001	0.001
Glucose (mg/dL)	0.894*	0.844*	0.826*
	0.016	0.035	0.043
Cholesterol (mg/dL)	0.977**	0.946**	0.934**
	0.001	0.004	0.006
HDL-Cholesterol (mg/dL)	-0.853*	-0.906*	-0.921**
	0.031	0.013	0.009
LDL-Cholesterol (mg/dL)	0.991**	0.972**	0.964**
	0.000	0.001	0.002
Albumin (mg/dL)	0.969**	0.936**	0.923**
	0.001	0.006	0.009
Total Protein (gm/dL)	-0.964**	-0.988**	-0.993**
	0.002	0.000	0.000
Urea (mg/L)	0.979**	0.951**	0.939**
	0.001	0.004	0.005
ALT (U/l)	0.998**	0.991**	0.986**
	0.000	0.000	0.000
AST (U/l)	0.994**	0.986**	0.982**
	0.000	0.000	0.001
MDA (µmol ./L)	0.997**	0.982**	0.975**
	0.000	0.000	0.001
TAS (mmol/ Trolox Equiv./L	-0.914*	-0.954**	-0.964**
	0.011	0.003	0.002

Upper values indicate Pearson's correlation coefficient; Lower values indicatelevel of significance at 5% probability. * = Significant ($P<0.05$); ** = Highly significant ($P<0.01$)

Table 3: Correlation coefficients of physical and biochemical parameters with DNA damage of lymphocytes and serum Hcy of normal individuals

Parameters	DNA damage	Hcy
Body mass index (BMI; kg/m^2)	0.029 (0.857)	0.341 (0. 315)
Systolic blood pressure (mm/Hg)	0.217 (0.179)	0.099 (0.542)
Diastolic blood pressure (mm/Hg)	0.024 (0.885)	0.570 (0.0492)
Glucose (mmol/L)	0.076 (0.641)	0.087 (0.592)
Cholesterol (mmol/L)	0.198 (0.221)	0.596 (0. 226)
Triglyceride (mmol/L)	0.162 (0.317)	0.537 (0.0398)
HDL cholesterol (mg/dL)	0.243 (0.131)	0.177 (0.275)
LDL cholesterol (mg/dL)	0.136 (0.404)	0.582 (0.262)
Total proteins (gm/dL)	0.058 (0.721)	-0.549 (0.465)
Albumin (g/dL)	0.196 (0.225)	0.404 (0.210)
Urea (mg/dL)	0.169 (0.298)	0.630 (0.423)
Alanine aminotransferase (ALT; U/L)	0.041 (0.800)	0.046 (0.777)
Aspartate aminotransferase (AST; U/L)	0.268 (0.094)	0.041 (0.800)
TOS (µmol H_2O_2 equiv./L)	0.658 (0.226	0.768 (0.234)
TAS (mmol/ Trolox Equiv./L)	0.562 (0.334)	0.798 (0. 196)
MDA (µmol H_2O_2 equiv./L)	0.653 (0.446)	0.568 (0.334)

Values in parentheses show p value and open values show the correlation value

Serum proteins, albumins and urea were measured by using commercially available kits (Randox, UK). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also measured using commercially available kits. Plasma Hcy was measured by homocysteine micro-titer plate assay. DNA damage was measured using EIA kit (589320). The 8-OHdG content in unknown samples was determined by comparing with predetermined 8-OHdG standard curve. Plasma total oxidant (TOS) and antioxidant status (TAS) levels were determined using automated colorimetric measurement methods (Erel, 2004; Mahmood *et al*., 2009).

STATISTICAL ANALYSIS

Data were subjected to calculations of mean + SE (Steel *et al*., 1997). For this purpose two statistical tools, Graph PadInstat 3.0 and Minitab 15 softwares were used. Graph PadInstat 3.0 was used for analyzing table 1 while Minitab 15 was used for finding correlations among variables. Values having *P* less than 0.05 were considered statistically significant.

RESULTS

One hundred and twenty (120) participants formed the study sample. Physical, biochemical and other health parameters of all participants are presented in table 1. Patients had significantly higher BMI (p<0.001) as compared to that of control subjects. Similarly they (patients) had significantly higher systolic (p<0.0001) and diastolic ((p<.0.001) blood pressure Mean serum glucose concentration in CVD patients was significantly (p<0.0036) higher than that of control subjects. Similarly CAD patients had significantly higher serum cholesterol (p<0.001) and LDL-c (p<0.03751) as compared to that of control individuals. CVD patients also had significantly higher (p<0.0001 and <0.001 respectively) ALT and AST values respectively than those of the normal individuals. Total oxidant status (p<0.0001), MDA (p<.0001), Hcy (p<0.0001) and DNA damage (p<0.0001) were also significantly higher in CVD disease patients than those of healthy individuals while normal subject exhibited significantly higher HDL-c (p<0.001) and TAS (p<0.024).

Positive correlation existed between Hcy and other variables: cholesterol (*r*=0.977 and p<0.031), LDL-cholesterol (*r*=0.991, p<0.0001), urea (r=0.979, p<0.001), ALT (r=0.998 and p<.0001), AST (r=0.994 and p<.0001), MDA (r=0.997 and p<.0001) and TOS (r=0.994 and p<.0001) while inverse relation was observed between Hcy and some other variables: total protein (*r*=-0.964 p<0.002), HDL-c (r=-0.853, p<0.031) and TAS (r=-0.914 and p<0.011). Normal subjects did not show any correlation between Hcy and BMI, blood pressure, cholesterol, LDL-cholesterol, albumin, and TOS. DNA damage among coronary artery disease patients was strongly dependent on BMI (r=0.827, p<0.043), diastolic blood pressure (r=0.972, p<0.001), glucose (r=0.826, p<0.043), LDL-cholesterol (r=0.964, p<0.002), albumin (r=0.923, p<0.009), urea (r=0.939, p<0.005), ALT (r=0.968 and p<0.001), AST (r=0.982, p<0.001), and MDA (r=0.975, p<0.001 (table 2). Healthy subjects did not show significant correlation with DNA damage and other variables (table 3).

DISCUSSION

The mechanism involved in CVD development and progression is still not well documented but many studies show that hyperhomocysteinemia, high level of

thromboxane-mediated platelet aggregation, and increased LDL oxidation cause CVD (Erel, 2004). We tried to examine the relationship of BMI, lipid profile, lipid per oxidation product, and TAS with homocysteine level, TOS and DNA damage in both controls and diseased patients.

The hyperhomocystenemia is a result of inhibition of remethylation pathway or inhibition of trans-sulphuration pathway of homocysteine metabolism (Erel, 2004). Our results indicated significantly elevated levels of homocysteine in CAD patients. Our study showed a positive correlation of homocysteine with oxidative stress and DNA damage as reported earlier (Tomas *et al*., 2004). Possible mechanism behind homocysteine mediated oxidative stress might be due to NADPH oxidase-induced super oxide generation or due to auto-oxidation of homocysteine (Govindaiah *et al.,* 2009). Another probable mechanism is the increase in S-adenosyl homocysteine, which serves as DNA methyl transferases inhibitor (Siow *et al*., 2006).

DNA damage marker 8-OHdG/dG was significantly increased in CVD patients. We confirmed that 8-OHdG was a good biomarker for CVD risk assessment. Oxidative degradation of lipids is known as lipid per oxidation. MDA is one of the most abundant carbonyl products of lipid per oxidation which is an index of oxidative damage. The progression of CAD is positively correlated with TOS and can be represented by MDA measurements (Boto *et al*., 2002). In the present study, MDA levels were significantly increased in plasma of CVD patients as compared to those of controls. A possible reason for increased lipid per oxidation might be a poor antioxidant defense system possessed by cardiac patients. Among many components of antioxidant defense system, a deficiency in any of these may lead to reduce overall antioxidant status of an individual. This situation of antioxidant has been observed in cancer and heart diseases. TAS is an antioxidant marker which represents the total capacity of the antioxidant barrier of a cell, tissue or organ. It comprises of a wide range of low molecular weight antioxidants and antioxidant enzymes. It can be a helpful indicator of the pro-oxidative/antioxidative balance of organism resulting from a disease. Measurement of the TAS is a noninvasive method and measures the anti-oxidative defense system capacity. Quantitative measurements of the antioxidant capacity of each relevant compound might give the antioxidant capacity of an individual (Pezeshkian *et al*., 2001; Khalili *et al*., 2016). Oxidative stress and TAS are correlated with each other (Johnstone *et al*., 2006; Mahmood *et al.,* 2009) and disturbance in their proper ratio causes onset of not only heart disease but also other malignant diseases.

CONCLUSION

Cardiovascular patients showed positive link between homocysteine and cholesterol, LDL-cholesterol, cholesterol, and TOS respectively. They also had positive association between DNA damage and BMI, BP, cholesterol, triglycerides, LDL- cholesterol, and albumin, ALT, TOS and MDA respectively. There should be an increased TAS in Pakistani population so that negative effect of above variables may be minimized to eliminate DNA damage and lower Hcy in blood of individuals to have a healthy population.

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