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Full Length Research Paper

Plasma malondialdehyde, bilirubin, homocysteine and total antioxidant capacity in patients with angiographically defined coronary artery disease

Ali Movahed[1], Dariush Iranpour[2*], Iraj Nabipour[3], Mojtaba Jafari[3], Samad Akbarzadeh[1], Majid Assadi[4], Kamran Mirzaei[3] and Najmeh Hagian[1]

[1]Department of Clinical Biochemistry, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran.
[2]Department of Cardiology, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran.
[3]Department of Healthy Heart, The Persian Gulf Tropical Medicine Research Center, Bushehr University of Medical Sciences, Bushehr, Iran.
[4]The Persian Gulf Nuclear Medicine Research Center, Bushehr University of Medical Sciences, Bushehr, Iran.

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Oxidative stress has been implicated in coronary artery disease (CAD). Malondialdehyde (MDA) is lipid peroxidation end product. Bilirubin may act as an antioxidant that suppresses lipid oxidation. The role of MDA and antioxidant capacity and their inter-relationship in patients with and without CAD was investigated. Thirty-eight consecutive patients with angiographically diagnosed CAD were compared with 60 age, and sex-matched controls. The controls had completely normal coronary arteries in angiograms. Plasma MDA, serum bilirubin, total homocysteine and total antioxidant capacity (TAC) levels were measured. Risk factors of CAD were determined for all subjects using National Cholesterol Education Program (NCEP)-Adult Treatment Panel (ATP)-III criteria. Serum MDA and total homocysteine concentration were significantly higher, but TAC, total bilirubin and direct bilirubin levels were lower in CAD patients when compared to the controls. Age, and sex-adjusted plasma MDA levels had negative correlations with TAC (r = -0.30, p = 0.001) and total bilirubin (r = -0.30, p = 0.002) concentrations. In multivariate analysis by the multiple logistic regression method, serum MDA was significantly associated with CAD (OR = 1.15, 95% CI, 1.25 to 1.82; p < 0.0001)) after adjustment for lipid status parameters and traditional risk factors in this study population. Increased serum MDA concentration, as a biomarker of lipid peroxidation, low serum bilirubin and antioxidant capacity were observed in patients with angiographically defined CAD. The significant inverse correlation of the serum bilirubin and MDA levels demands further in-depth investigations to clarify the association between them in the development of CAD.

Key words: Oxidative stress, bilirubin, malondialdehyde, coronary artery disease, antioxidant capacity, homocysteine.

INTRODUCTION

It has been suggested that oxidative stress, especially oxidative modification of low-density lipoproteins (LDL), may play a causative role in the pathogenesis of a variety of vascular diseases including atherosclerosis, hypertension and coronary artery disease (Chisolm and Steinberg, 2000; Kaur, 2008). Through the oxidative modification hypo-thesis, it is well predicted that oxidized LDL contributes to atherosclerosis (Stocker and Keaney, 2004). Clinical research has used different markers to investigate the consequences and complications of lipid peroxidation process. Thiobarbituric acid reactive

*Corresponding author. E-mail: Iranpour.dr45@yahoo.com, Mobile no:+98-917-371-2273. Fax: 98-771-2520657

substances (TBARS) mainly MDA are the end products of peroxidation of poly unsaturated fatty acids in the cell membranes which accumulate when lipid peroxidation increases (Ceconi et al., 1992). These substances have the ability to interact with lipoproteins, which are then taken up by macrophages, and transformed into foam cells that contribute to atherosclerotic plaque development and progression of atherogenesis (Cavalca et al., 2001). Lopresti et al. (2008) has also used TBARS mainly MDA to evaluate the role of oxidative stress in patients with acute myocardial infarction. So one of the most frequently used biomarker providing an indication of the overall lipid peroxidation level is the plasma concentration of MDA (Nielsen et al., 1997). Hence, a great deal of interest has focused on investigating the role of different anti oxidants including internal and external to prevent the oxidation of lipids in the cell membrane especially the oxidation of LDL.

Bilirubin is now recognized as being a potent antioxidant manufactured by the body. Normal human serum bilirubin concentrations are high enough to provide a substantial portion of the total known antioxidant capacity of serum (Morse and Choi, 2005). The biological actions of bilirubin may be especially relevant to the prevention of oxidant-mediated cell damage (Kushida et al., 2002). It has also been proved that bilirubin at low concentration scavenges reactive oxygen species *in vitro,* thereby reducing oxidant-mediated cellular damage and attenuate oxidative stress *in vivo* (Stocker et al., 1987). Neuzil and Stocker, (1994) has reported that bilirubin may inhibit oxidation of LDL lipids initiated within the lipoprotein core. The roles of bilirubin in counteracting oxidative stress have been reviewed previously (Abraham et al., 2008).

Schwertner et al. (1994) first reported a significant inverse correlation between bilirubin concentration and the prevalence of coronary artery disease (CAD). There are evidences showing that serum bilirubin concentrations at the upper level of the reference values (0.4 to 1.2 mg/dl) may provide protection against CAD, whereas the lower level indicates increased atherogenesis (Mayer et al., 2000). Serum bilirubin level was also suggested as a predictive biomarker for long-term outcomes in patients with cardiac syndrome X (Huang et al., 2010). Also reduced serum levels of bilirubin were associated with a higher prevalence of coronary artery disease (Ghem et al., 2010) and coronary artery calcification (Muhei et al., 2009). It has been reported that high plasma concentration of total homocysteine is associated with increased *in vivo* lipid peroxidation in men (Sari et al., 1999). Also significant increased plasma concentration of MDA and total homocysteine have been observed in the patients with CAD (Cavalca et al., 2001). More over the increase in the level of plasma MDA has been associated with inflammatory markers in patients with coronary artery calcification which is a marker of CAD severity Jung et al., (2004).

The aim of our study was to further understand the role of plasma MDA and the antioxidant system and their inter-relationship in patients with CAD compared with the controls. To our knowledge, this is the first study to investigate possible correlation of serum bilirubin and plasma MDA in patients with angiographically defined CAD.

MATERIALS AND METHODS

Subjects and definitions

Thirty-eight consecutive patients (22 men and 16 women, mean age, 52.47 ± 1.14 years) with CAD were compared with 60 age and sex-matched controls (26 men and 34 women, mean age, 50.58 ± 1.07 years). All these subjects underwent coronary angiography in the department of cardiology in Bushehr University of Medical Sciences, Iran. The total duration for recruitment of participants of the study was three months. In this study, selection of both patient and control groups was based on the results of angiography. The controls had a history suggestive of angina pectoris but completely normal coronary arteries (arteries with no wall irregularity in angiograms, Friesinger index = 0). Two well experienced cardiologists, unaware of the patients' clinical history and biochemical results, visually assessed all angiograms to observe the extent of the CAD. Patients with myocardial infarction within the previous 3 months, renal or hepatic diseases, malignancy, thyroid disease, connective tissue diseases were excluded. Fasting serum glucose of ≥ 126 mg/dl was defined as diabetes. Hypertension was defined as having systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg. We also measured liver enzymes in our subjects to separate apparent liver diseases.

The cut-off points for serum total cholesterol, HDL-cholesterol and LDL-cholesterol and serum triglycerides distributions used to assign subjects at different levels of risk were those derived from the National Cholesterol Education Program guidelines in the United States Bethesda, Maryland National Institute of Health (http://www.nhlbi.nih.gov/guidlines/cholesterol/,accessed) 10 December 2007.

The subjects did not receive antioxidant therapy such as ACE inhibiters or vitamin supplements at the time of investigation. All the patients and controls signed consent forms, and the procedures followed were approved by our institution's responsible committee.

Samples and analyses

Following a 12 h fasting period, blood samples were collected by venipuncture, and EDTA-plasma and sera were obtained by centrifugation and stored at -80°C until they were analyzed. Serum bilirubin was measured by a diazo method (Rand and diPasqua, 1962). Glucose was assayed by the enzymatic method of Pars Azmun - co, Iran (intra and interassay coefficients of variation (CVs) were 3.2 and 2.95%, respectively). Serum total cholesterol and HDL-cholesterol were measured using enzymatic method of cholesterol oxidase, pars Azemoon-CO- Iran, on an autoanalyzer (Vital scientific Selectra 2 Spankeren, Netherlands). Intra and interassay CVs for TC and HDL-C were 2.1, 2.4% and 1.5, 1.9%, respectively. Serum low density lipoprotein (LDL)-cholesterol was calculated using the Friedewald formula. An enzyme immunoassay for the determination of total homocysteine in blood was used; the quantification limit of the DRG Homocysteine EIA (DRG International, Inc. USA) was 1.0 µmol/L with a CV <20%.

Malondialdehyde was measured by using an improved Thiobarbituric Acid reactive substaces (TBARs) based method (Manceit and Copeland, 1992). The determination of total antioxidative capacity was performed using a commercial Kit from

Table 1. General characteristics and some basic biochemical parameters of patients and the controls.

Characteristics and biochemical parameters	Patients (n = 38)	Control (n = 60)	P values
Age (years)	52.47 ± 1.14	50.58 ±1.07	0.247
Hypertensive, n (%)	22 (57.9)	25 (41.7)	0.078
Hyperlipidemia, n (%)	29 (76.3)	24 (40.0)	<0.001
Diabetes, n (%)	12 (31.6)	6 (10.0)	0.008
Family history of CAD, n (%)	8 (21.0)	8 (13.3)	0.302
Fasting blood sugar (mg/dl)	157.65 ± 63.52	116.52 ± 4.88	<0.0001
Total cholesterol (mg/dl)	216.50 ± 13.02	217.01 ± 8.90	0.973
HDL-Cholesterol (mg/dl)	35.44 ± 1.47	43.24 ± 1.41	<0.0001
LDL-Cholesterol (mg/dl)	125.47 ± 10.13	191.63 ± 41.79	0.217
Triglyceride (mg/dl)	249.68 ± 37.78	206.62 ± 8.67	0.261
AST (IU/L)	15.11 ± 1.93	22.53 ± 3.93	0.153
ALT (IU/L)	9.72 ± 1.34	16.55 ± 3.54	0.137

Data is presented as mean ± SD, AST: aspartate aminotransferase ,ALT: Alanine aminotransferase HDL:High density lipoprotein, LDL: Low density lipoprotein, CAD: Coronary artery diseas n(%):numbers(percent of subgroup). and P Value is considered as <0. 05.

BioVision-Co, USA which uses Trolox equivalents to standardize the antioxidants.

Statistical analyses

The significance of the difference in the results of any two groups was determined by Chi-square analysis using 2 × 2 contingency tables. A two-tailed t-test was used to compare the mean values across groups. Absolute values were presented as mean ± standard deviation.

The correlations between markers of oxidative stress and biochemical measurements were evaluated using Pearson's partial correlation while controlling for the effect of age and sex. Multiple logistic regression analysis was used to ascertain the associations between MDA and CAD. Sex, age, lipid status parameters and traditional risk factors were considered as covariates, and CAD as the dependent variable. P<0.05 was considered statistically significant. Statistical analysis was performed with an IBM computer using the SPSS 9.05 statistical software package (SPSS Inc., Chicago, IL).

RESULTS

Demographic features of the subjects and comparison of the patients with CAD and the controls regarding cardiovascular risk factors are shown in Table 1. There were no significant differences between the two groups regarding age, sex, family history of CAD and hypertension. However, the patients with CAD had significantly higher history of hyperlipidemia and type 2 diabetes mellitus when compared to the controls.

The lipid profiles of the two groups are presented in Table1. Serum HDL-C levels were significantly lower in CAD group when compared to the controls. There was no significant difference between the two groups regarding serum concentration of LDL-C, total cholesterol, triglyceride and liver enzymes (Table 1). Serum homocysteine and fasting blood glucose concentrations were significantly higher in CAD patients than in the controls ($p < 0.0001$).

The parameters that show oxidative stress and antioxidant status are presented in Table 2. Serum MDA and total homocysteine levels were significantly higher, but TAC, total bilirubin and direct bilirubin values were lower in CAD patients when compared to the controls. Age, and sex-adjusted serum MDA levels had negative correlations with TAC ($r = -0.34$, $p = 0.001$), total bilirubin ($r = -0.30$, $p = 0.002$) and HDL-C ($r = -0.25$, $p = 0.01$) concentrations. Age, and sex-adjusted serum total bilirubin had positive correlations with TAC ($r = 0.25$, $p = 0.01$) and HDL-C ($r = 0.22$, $p = 0.03$). There were no correlation between MDA and LDL-C, total cholesterol and triglyceride levels. But serum total bilirubin had a significant negative correlation with homocysteine ($r = -0.25$, $p = 0.01$).

In multivariate analysis by multiple logistic regression method, serum MDA was significantly associated with CAD (OR = 1.15, 95% CI, 1.25 to 1.82; $p < 0.0001$) after adjustment for lipid status parameters and traditional risk factors in the study population. Likewise, serum total bilirubin was negatively associated with CAD (OR = 0.01, 95% CI, 0.01 to 0.24; $p = 0.004$).

DISCUSSION

The results of this study confirmed the role of oxidative stress in patients with angiographically defined CAD and discovered an expected inverse correlation of plasma MDA and antioxidant system.

The lipid peroxidation end product, MDA is one of the most reliable and widely used indexes of oxidative stress (Kaur et al., 2008). The involvement of lipid peroxidation in CAD patients was confirmed by the significant increase in the blood concentration of MDA in the case group

Table 2. Plasma malondialdehyde, total bilirubin, direct bilirubin, antioxidant status and homocysteine levels of the study groups.

Blood parameter	Patients (n=38)	Control (n=60)	P value
Total antioxidant capacity (µmol/L)	131.82 ± 5.27	170.05 ± 4.80	<0.0001
Malondialdehyde (nmol/mL)	12.19 ± 0.68	5.68 ± 0.45	<0.0001
Total Bilirubin (mg/dl)	0.48 ± 0.02	0.76 ± 0.03	<0.0001
Direct Bilirubin (mg/dl)	0.11 ± 0.01	0.17 ± 0.01	0.02
Homocysteine (µmol/L)	18.10 ± 1.64	10.63 ± 0.91	<0.0001

The values are shown as mean ± SD and P value is considered as < 0. 05.

when compared with the controls. Thus, our findings are in line with the results of other published studies, (Mendis, 1995; Kotur- Stevuljevic, 2006).

One of the main roles in the prevention of oxidative stress has been attributed to endogenous molecules such as bilirubin (Neuzil and stocker, 1994). Many studies have reported that low serum bilirubin concentrations are associated with an increased risk of atherosclerosis (Lin et al., 2006). The strength of the association is similar to that of smoking, elevated systolic blood pressure and low levels of high-density lipoprotein (HDL) cholesterol (Rantner, 2008).

In our study, we also demonstrated an inverse relationship between the presence of CAD and circulatory total bilirubin. This might be explained by *in vitro* findings that have shown bilirubin to have antioxidative and cyto-protective properties (Stocker, 1987; Neuzil and Stocker, 1994). The mechanism of action of these properties of bilirubin could be explained by the role of the enzyme heme oxygenase-1 (HO-1). This enzyme catalyzes the conversion of heme to biliverdin, carbon monoxide and iron. Subsequent to the reaction, biliverdin is converted to bilirubin by biliverdin reductase. Biliverdin and bilirubin have been shown to act as scavengers of reactive oxygen species and so they have anti inflammatory effects (Ryer and Choi, 2006). Oxidized LDL is known to act as a potent inducer of heme oxygenase-1 (HO-1) in vascular cells. In vascular endothelial cells, vascular smooth muscle cells, and macrophages, HO-1 is markedly up-regulated by oxidized LDL (Abraham and Kappas, 2008). The cytoprotective activity of this enzyme against oxidative injury and cellular stresses was attributable to bilirubin (Morse and Choi, 2005; Idriss, 2008). Accordingly, low bilirubin concentrations may be associated with increase in oxidized lipids and lipoproteins which consequently leads to atherogenic plaque formation (Mayer, 2000).

Given the antioxidant capacity of bilirubin, it is plausible that we found a strong inverse relationship between serum bilirubin concentrations and MDA, which is a highly reactive metabolite of free radical induced lipid peroxidation.

There are very limited studies about correlation of bilirubin and MDA in ischemic heart disease (Masini, et al., 2003; Abraham and Kappas, 2008). Serdar et al. (2006) demonstrated that an inverse correlation exists between plasma MDA, erythrocyte MDA and delta-MDA levels, and most of the antioxidant enzymes and vitamins in patients with CAD. This is in accordance with our findings that a significant inverse correlation exists between serum MDA and total antioxidative capacity.

Interestingly, we found a strong inverse correlation between MDA and HDL-C, and also a significant positive correlation between serum total bilirubin and HDL-C in this studied population. So our observations confirmed the data from previous studies (Kotur- Stevulijevic, 2007; Mahparta, 1998). This is to mention that even though in our study, there were correlations between serum MDA and the parameters including total bilirubin, TAC and HDL-C, but the r values were very small. These observations need a mechanistic explanation concerning the relationship between oxidative stress and lipoprotein particles.

Our study showed a significant increase in serum concentration of homocysteine in the patients with CAD than the controls. However, no correlation was found between homocysteine and MDA concentration. These results agree with those of Cavalca et al. (2001). As a limitation, due to the small number of patients, we were not able to evaluate the effect of MDA on the severity of coronary lesions and stratification of the study groups according to the serum bilirubin level could not be done.

In conclusion, this current study indicates that oxidative stress and low antioxidant capacity may be associated with the pathogenesis of CAD. The observed strong inverse correlation of the serum bilirubin level and MDA demands further in-depth investigations to clarify the connections of HO-1/bilirubin and reactive metabolites of free radical induced lipid peroxidation in the development of CAD. It is probable that we will see, in the not too distant future, human trials involving the heme oxygenase system to combat lipid peroxidation for the management of CAD.

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