Platelets Oxidative Stress in Indian Patients with Ischemic Heart Disease

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Background: Oxidative stress has been implicated in the development of atherosclerosis and vascular tissue injury. Both platelet activation and lipid peroxidation are known to play major role in ischemic heart disease. The purpose of this study was to investigate the status of platelets oxidative stress in Indian patients with ischemic heart disease. **Methods:** We measured platelets aggregation, malonyldialdehyde (MDA), plasma-ionized Ca^{2+} , and antioxidant enzymes, i.e., glutathione peroxidase and superoxide dismutase in healthy volunteers and patients with myocardial infarction, unstable and stable angina 40 subjects each. **Results:** Platelets aggregation, MDA,

and plasma-ionized Ca^{2+} have increased significantly across the patients groups compared with controls, this increase was accompanied by an overall decrease in the antioxidant enzymes activity; except for the slight increases in the glutathione peroxidase levels among the myocardial infarction patients. **Conclusions:** The current results suggest that platelet lipid peroxidation as marked by increased MDA level is augmented in ischemic heart diseases. The increased oxidative stress seen in these patients was accompanied by platelet activation and impaired antioxidant enzymes activity. J. Clin. Lab. Anal. 24:49–54, 2010. © 2010 Wiley-Liss, Inc.

Key words: cardiovascular disease; atherosclerosis; oxidative stress; heart disease; platelets

INTRODUCTION

Cardiovascular diseases (CVD) remain the major cause of morbidity and mortality in developed countries, and have joined the infection diseases in the developing countries as one of the leading causes of death in these countries (1). There is a growing body of evidence which indicate that oxidative stress plays an important role in the initiation and progression of atherosclerosis and cardiovascular diseases (2–7). Studies on human and animal models have documented that excessive free radicals production can result in decreased cell survival, altered membrane phospholipid asymmetry, hypercoagulability, and increased adhesion to endothelial cells (6,8–10). Platelet involvement in the pathogenesis of thrombosis-related complications is well documented. Increased lipid peroxidation in platelets has been closely reviewed with the metabolism of arachidonic acid and thromboxane A_2 (TXA_2) (11), where glutathione peroxidase (GPx) is understood to have an inhibitory effect on cyclooxygenase by eliminating hydroperoxides

depletion. The enzyme belongs to a family of selenocysteine-containing proteins, four of which have peroxidase activity. Each of these peroxidases reduces hydrogen peroxide and lipid peroxides to its corresponding alcohol (12,13). The importance of GPx in the regulation of platelet-dependent thrombus formation rests on the fact that activated platelets are rich sources of reactive oxygen species such as superoxide and variety of biologically active eicosanoids associated with platelets oxidative stress such as 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12(S)-HpETE) (14,15). Superoxide dismutase (SOD) on the other hand plays

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a major role for defenses against superoxide anions (16). GPx and SOD appear to have decreased activity in coronary artery diseases (17–22). This study was designed to investigate the status of platelets oxidative stress in patients with ischemic heart diseases (IHD).

MATERIALS AND METHODS

All the chemicals and reagents were of analytical grade. *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, albumin, and ADP were purchased from Sigma Chemical Co. (St. Louis, MO), ionized calcium reagents were purchased from Radiometer (Bronshøj, Denmark), SOD and GPx kits were obtained from Randox Laboratories Ltd., (Antrim, UK), remainder chemicals obtained from Merck (Darmstadt, Germany).

Study Design

Recruitment and analysis were completed at G. B Pant Hospital and Mulana Azad Medical College, New Delhi, India.

Study population

All subjects completed and signed consent form explaining the voluntary nature of the study. The patients' population for this study consisted of 120 patients diagnosed with IHD during their visit to the New Delhi city metropolitan Emergency Unit of LNJP Hospital and the Department of Cardiology at G.B. Pant Hospital. The patients initially complained of chest pain. Patients were later diagnosed with IHD cases according to the criteria of the New York Heart Association. All cases 40 from each group of MI, stable (SA) or unstable angina (UA) were newly diagnosed cases; however, some of the patients with SA or UA visit regularly the Department of Cardiology, G.B. Pant Hospital for followup. Care was observed during the selection and enrollment. Patients with primary coagulopathy, bleeding diathesis, diabetes, or any known disease that alter platelet activity were excluded from the study. Also those who underwent angiographic studies and their findings failed to match with the criteria of the disease or on drugs that may affect platelet function were dropped from the study. A group of 40 apparently healthy individuals, belonging to similar socio-economic status of the patients, and without any history suggestive of coronary artery disease or its risk factors, were selected for the study from volunteers visiting the Out Patients Department of G.B. Pant Hospital.

Blood sample

One time blood from fasting subjects was collected from a forearm vein of patients and controls without stressing using a 20-gauge needle, or larger. The first

2 ml of blood was collected in plain vials and then blood collection continued with a fresh draw which was later transferred to vials containing 3.8% trisodium citrate anticoagulant and mixed by gentle inversion of the polyethylene container avoiding any frothing which may activate the platelets. This method for blood collection was followed to eliminate platelets activation due to stress and injury. Platelet count was performed immediately as previously described (23).

Blood samples in plain vials were immediately centrifuged at 2500 rpm for 10 min and serum aliquots were stored in polyethylene tubes at -70°C until assayed. Platelet rich plasma (PRP) was prepared by centrifugation of the citrated blood at 1000 rpm for 15 min at 37°C using polyethylene test tubes. The process of PRP preparation was completed within the first hour of the blood collection; aliquots of the PRP were immediately used for the platelet aggregation studies. The remainder PRP was further centrifuged for the preparation of the washed platelets suspensions according to the method described earlier (24) Washed platelet suspensions were transferred to fresh polyethylene tubes, and platelet count was performed in platelet suspensions and later the count was adjusted to $150\text{--}400 \times 10^9/\text{l}$ using Tyrode's buffer. Containers were then stored at -70°C until assayed.

Biochemical assays

Using enzymatic methods serum total cholesterol (TC), triglycerides (TG), and high-density lipoproteins (HDL) were determined by Olympus AU400 chemistry analyzer.

Determination of platelets aggregation

Platelet aggregation was determined in the PRP by a previously described method (23). Briefly, the absorbance of platelet-rich plasma falls as platelets aggregate; the amount and rate of the fall depends on platelet reactivity to a known concentration of platelet agonist; ADP solution ($100\text{ }\mu\text{M}/\text{l}$) was used and other variables, such as temperature, platelet count, and mixing speed, were controlled. Optical aggregation was performed using Chrono Log aggregometer model 560-CA (Chrono Log Corporation, Havertown, PA). Stirring speed was set at 1000 rpm. Five hundred microliter of PRP and PPP were pipetted in two clean and dry polyethylene cuvettes and a stir bar was inserted in the PRP containing cuvette. Aggregation was recorded on a chart and calculated accordingly.

Measurement of ionized calcium (Ca^{2+})

Ionized calcium Ca^{2+} in plasma was measured using ICA2 ionized calcium analyzer (Radiometer, Copenhagen, Denmark).

Oxidative stress markers measurements

Measurement of malonyldialdehyde (MDA)

Plasma and platelets MDA levels were measured by a modified method (25) for the quantification of thiobarbituric acid reactive substances (TBARS) in biological samples briefly; the samples containing MDA and standards were first reacted with TBA at 95°C. After a brief incubation, the samples and standards were read on spectrophotometer at 532 nm and unknown samples concentrations were determined from a predetermined MDA standard curve.

Determination of GPx and SOD

The activity of GPx and SOD in washed platelets suspensions ($U/10^9$) platelets was determined by commercial kits.

Statistical Analysis

Data were analyzed with a computer software (S-plus 6 for Windows and SAS Software Release 8.2 packages). The results were expressed as mean $\pm$ SD, and the significance of the difference between the mean values of both sexes was determined by the Student's *t* test employing analysis of variance program. Spearman's rank correlation was calculated to assess the association between the markers of oxidative stress and lipids or platelets function. Linear regression models were run to examine the relationship between oxidative stress and platelets, after adjustment for other factors.

RESULTS

A total of 120 patients and 40 healthy controls (HC) enrolled in the study. Patients were divided into three groups with 40 patients each; the IHD groups consisted of patients with myocardial infarction, UA, and SA. The basic characteristics of the subjects are given in Table 1. The subjects' age mean range from 40 to

51 years. The study population has small number of females' enrollment, across all the groups.

There is a significant increase in serum TC and TG ($P < 0.001$) among the IHD and a significant decrease in HDL ($P < 0.01$) compared with HC; the calculated LDL was significantly ($P < 0.001$) higher among the IHD compared with the controls.

Platelets counts notably higher in IHD patients with UA or SA than that of patients with MI or the HC, the differences between the means of platelets counts ($\times 10^9/l$ platelets) in HC vs. UA or SA are significant ($P < 0.01$), the same is seen between the means of MI vs. UA or SA ($P < 0.01$). However, the difference between the means of HC and MI is not significant. Platelets aggregation in IHD patients groups and HC increased immensely compared with the platelets aggregation in HC. The differences between the means of platelets aggregation (%) in MI, UA, or SA patients and the HC are highly significant ($P < 0.001$). The respective means and ranges for the platelets aggregation (%) in HC, MI, UA, and SA are 50.85 ± 10.76 , 70.95 ± 15.85 , 65.87 ± 20.16 , and 73.85 ± 16.67 (Table 2). In order to assess the effect of external ionized calcium on platelet activation in IHD we measured plasma-ionized calcium in all subjects. Abreast with the platelet aggregation, the plasma-ionized Ca^{2+} levels in IHD patients and HC demonstrated a similar trend. Platelets Ca^{2+} in IHD groups significantly increased ($P < 0.001$) compared with HC. The means $\pm$ SD of Ca^{2+} (mmol/l) in the study population were 1.25 ± 0.19 , 1.41 ± 0.18 , 1.40 ± 0.14 , and 1.34 ± 0.15 for HC, MI, UA, and SA, respectively. The ionized Ca^{2+} levels in SA patients were slightly low compared with the remaining IHD groups. However, the differences between their means were not significant. Ca^{2+} levels demonstrated a notable correlation with platelet counts ($r = 0.2527$, $P < 0.05$) among the combined IHD patients population. Similar correlation was seen between aggregation and TC ($r = 0.2801$, $P < 0.05$).

Results of the various oxidative stress markers measured in plasma and platelets are summarized in

TABLE 1. Subjects Basic Characteristics

Subjects/variables	Age (years)	Sex	T Chol Mean $\pm$ SD (mg/dl)	TG Mean $\pm$ SD (mg/dl)	LDL Mean $\pm$ SD (mg/dl)	HDL Mean $\pm$ SD (mg/dl)
Healthy controls	40	M (35) F (5)	153.50 ± 39.09	137.45 ± 46.98	83.17 ± 35.08	43.7 ± 6.85
Myocardial infarction	51	M (36) F (4)	240.87 ± 51.98	177.45 ± 50.04	169.05 ± 44.43	38.75 ± 8.48
Unstable angina	50	M (35) F (5)	213.05 ± 61.13	164.82 ± 45.51	140.85 ± 56.92	38.85 ± 7.63
Stable angina	49	M (36) F (4)	214.42 ± 56.64	193.22 ± 42.34	132.35 ± 37.24	38.27 ± 8.42
P (HC vs. MI, UA, and SA) ^a N = 40			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

^aHC, Healthy controls; MI, Myocardial infarction; UA, Unstable angina, SA, Stable Angina; T Chol, Total cholesterol.

TABLE 2. Platelet Aggregation and Plasma-Ionized Calcium Levels

Subjects/variables	Platelet counts (10^9 /l platelet)	Platelet aggregation (%)	Plasma-ionized Ca^{2+} (mmol/l)
Healthy controls	262.12 ± 65.87	50.85 ± 10.76	1.25 ± 0.19
Myocardial infarction	265.02 ± 63.85	70.95 ± 15.85	1.41 ± 0.18
Unstable angina	344.52 ± 100.86	65.87 ± 20.16	1.40 ± 0.14
Stable angina	323.85 ± 69.68	73.85 ± 16.67	1.34 ± 0.15
<i>P</i>	$P < 0.001$	$P < 0.001$	$P < 0.001$

TABLE 3. MDA and Antioxidants Enzymes

Groups/variables (<i>N</i> = 40)	Plasma MDA Mean $\pm$ SD ($\mu\text{mol/l}$)	Platelets MDA Mean $\pm$ SD ($\mu\text{mol/l}$)	Platelets GPx Mean $\pm$ SD (U/ 10^9 platelets)	Platelets SOD Mean $\pm$ SD (U/ 10^9 platelets)
Healthy controls	1.77 ± 0.48	0.45 ± 0.22	170.87 ± 79.99	20.15 ± 10.28
MI	3.32 ± 0.98	0.87 ± 0.48	182.57 ± 77.63	5.37 ± 2.38
Unstable angina	3.42 ± 0.91	0.80 ± 0.30	132.52 ± 63.04	5.50 ± 2.43
Stable angina	3.00 ± 0.73	0.68 ± 0.37	150.67 ± 91.37	6.92 ± 3.45
<i>P</i>	$P < 0.001$, HC vs. MI, UA, or SA	$P < 0.001$, HC vs. MI, UA, or SA, $P < 0.05$, MI vs. SA	$P < 0.01$ MI vs. UA $P < 0.02$ HC vs. UA	$P < 0.001$ HC vs. MI, UA, or SA $P < 0.05$, SA vs. MI or UA

HC, Healthy controls; MI, Myocardial infarction; UA, Unstable angina, SA, Stable Angina; GPx, Glutathione peroxidase; SOD, Superoxide dismutase; MDA, Malonyldialdehyde.

Fig. 1. Platelet aggregation and SOD in MI. Increased platelet aggregation reversely associated with SOD activity in MI patients $r = -0.38$, $P < 0.01$.

Table 3. As it can be seen from the table, plasma MDA levels increased significantly ($P < 0.001$) in IHD groups compared with HC. Among IHD groups, UA patients have greater plasma MDA levels; in addition plasma MDA seems to correlate with cholesterol ($r = 0.2749$, $P < 0.05$), TG ($r = 0.2544$, $P < 0.05$), platelets aggregation ($r = 0.3250$, $P < 0.05$), plasma-ionized Ca^{2+} ($r = 0.3082$, $P < 0.05$) and inversely with SOD ($r = -0.4909$, $P < 0.01$); however, MDA did not show any correlation with GPx. The platelets MDA increased significantly ($P < 0.001$) in patients compared with HC. It is also observed that SA patients showed lower platelets MDA levels compared with the remainder IHD group; however, only the differences between the means of SA platelets MDA and that of MI is significant ($P < 0.05$). Activity of platelets SOD immensely decreased ($P < 0.001$) among IHD patients compared with HC, and inversely correlated with platelet aggregation among MI patient (Fig. 1).

DISCUSSION

It has been explicitly established that free radicals can cause metabolic disturbances and cell injury in a variety of ways, including lipid peroxidation, hydroxyl radical-induced modification of proteins and nucleic acids, changes in enzyme activity, and carbohydrate damage. Oxidative modification of lipids can be induced *in vitro* by a wide array of pro-oxidant agents and occurs *in vivo* during atherosclerosis and several other disease conditions (2). Alterations in the superoxide and glutathione oxidation–reduction system may lead to depleted antioxidant capacity and may result in oxidative stress. Previous studies have suggested that platelets and the vascular endothelial cells could be the central target as well as the origin of oxygen-free radicals or its metabolites (26). Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. These aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress.

The major strength of this study is the use of highly standardized procedures for the measurement of oxidative stress biomarkers. In addition, we were able to control for a number of factors that influence platelets function, including antioxidants vitamin and other related drugs.

Although the specificity of TBARS toward compounds other than MDA has been controversial, the assay continues to be the most widely employed format for monitoring lipid peroxidation. Lipids with higher

degrees of unsaturated bonds produce higher TBARS values, and also biological samples may contain a mixture of TBARS, such as hydroperoxides and aldehydes, which increases in response to oxidative stress. The plasma and platelets MDA content, in this study, demonstrated a clear elevation in its content among IHD patients, compared with controls. The increase in plasma MDA levels observed in this study correlated significantly with cholesterol, TG, and platelets aggregation. Both antioxidant enzymes determined in platelets have demonstrated a decreased activity among the IHD groups compared with HC, except for the GPx activity in MI patients, which exhibited a mild elevation, when compared with HC. GPx activity demonstrated tangible decreases among UA, and SA patients compared with MI patients, and also increased platelet aggregation reversely associated with SOD in MI. Increased plasma and platelets MDA levels, and decreased platelets GPx and SOD activity observed in this study among IHD groups are in concordance with the reported studies of Dousset et al. (26) who reported increases in plasma MDA levels in MI; similarly, Nanda et al. have shown increased plasma MDA levels among IHD patients (27). Platelets MDA levels reported to increase during aggregation (28,29). SOD was studied either in red blood cells or in serum; however, only few reports are available on SOD activity in platelets on some types of IHD patients. Interestingly, plasma-ionized Ca^{2+} estimated in this study enormously elevated among IHD groups compared with HC ($P < 0.001$). However, patients with MI, and UA, have shown similarity in their plasma-ionized Ca^{2+} increases. Elevation in plasma-ionized Ca^{2+} may have contributed to the increased platelets aggregation as suggested earlier by Joseph et al. (30) that the free calcium concentration in the extracellular fluid, mediate and propagate the intracellular Ca^{2+} influx, which may lead to platelet activation.

Increased plasma and platelets MDA levels in IHD patients can be attributed to the oxidative status of platelets (26). Increased MDA levels also ascribed to hyperaggregability of platelets induced by TXA_2 synthesis, which is supported by the significant correlation between MDA levels and aggregation, and enhanced platelets aggregation among IHD groups (31,32). Increased free radicals damage, which is linked to the lipid peroxidation, may have overwhelmed the platelets GPx and SOD and resulted in decreased activity in majority of IHD groups. In conclusion, the current results suggest that platelet lipid peroxidation as marked by increased MDA level is augmented in IHD. The increased oxidative stress seen in these patients was accompanied by platelet activation and impaired antioxidant enzymes activity.

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