Oxidant and antioxidant status in coronary artery disease

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Abstract. Formation of atherosclerotic plaques is the major cause of coronary artery disease (CAD). Several lines of study have revealed the role of oxidative stress in CAD pathogenesis. In the present study the aim was to investigate the oxidative and antioxidative markers in CAD patients and a control population. The study sample comprised of acute coronary syndrome (ACS) patients, chronic CAD patients and healthy controls (n=30/group). Blood samples of patients and control subjects were collected to measure the concentrations of reduced glutathione (GSH), malondialdehyde (MDA) and the percentage of MDA release as well as the activity of erythrocyte glutathione peroxidase (GPx) and total antioxidant capacity (TAC) of plasma. All parameters were measured by spectrophotometric methods. Additionally, oxidant/antioxidant status was compared between CAD patients with single, double or triple-vessel stenosis and in comparison with controls. The results indicated a significant increase in MDA level and the percentage of MDA release ($P<0.05$), and a marked decrease in GSH concentration ($P<0.0001$), TAC ($P<0.0001$) and the activity of erythrocyte GPx ($P<0.0001$) in the patient groups compared controls. ACS patients exhibited a similar pattern of data when compared with the chronic CAD group. Similar results were also observed when chronic CAD patients with single, double or triple vessel stenosis and controls were compared. The present study indicates that the acute form of CAD is more susceptible to oxidative damage, suggesting that use of antioxidant therapy may be warranted to ameliorate oxidative stress in this condition.

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Introduction

Coronary artery disease (CAD) comprises the largest proportion of cardiovascular diseases (CVDs) and accounts for more than one third of all mortalities worldwide (1). Risk factors include hypertension, cigarette smoking, type 2 diabetes mellitus, increased cholesterol concentration and obesity (2). Atherosclerosis, the formation of plaque inside the arteries, is the main cause of CAD (3). Several pathological events contribute to atherosclerosis, including endothelial dysfunction, extensive lipid deposition in the tunica intima, exacerbated innate and adaptive immune responses, vascular smooth muscle cell proliferation and remodeling of the extracellular matrix (4).

Two major hypotheses have been proposed to describe the origin of atherosclerosis: i) the thrombogenic theory, which suggests that thickening of the intima layer of vessels is a result of the organization of fibrin by fibroblasts, associated with secondary lipid enrichment; and ii) the lipogenic theory, which suggests that the deposition of lipid inside the arterial walls is caused by an imbalance between the mechanisms responsible for lipid accumulation and removal (5-7). To date, several lines of study have indicated a role for oxidative stress in atherosclerosis and CVDs (8-11). Oxidative stress is the result of enhanced production of reactive oxygen species (ROS), which are the key molecules in the signaling pathways implicated in vascular inflammation in atherogenesis, starting from the initiation of fatty streak formation to lesion progression and plaque rupture (12). ROS are established to damage the fundamental biomolecules in cells including DNA, proteins and lipids (13). A previous report demonstrated that oxidative modification of low-density lipoprotein (LDL) is a key mechanism rendering lipoproteins atherogenic (14). Furthermore, it has been reported that lipid peroxidation produces unsaturated aldehydes including acrolein and malondialdehyde (MDA), which exert toxic effects due to their reactivity with nucleophile compounds and their ability to produce protein and DNA adducts without prior metabolic activation (15). These aldehydes are considered to function as mediators of inflammation and vascular dysfunction (15).

On the other hand, there are several antioxidant systems grouped as enzymatic and non-enzymatic antioxidant systems.

Antioxidant enzymes include catalase and glutathione peroxidase (GPx), superoxide dismutase and glutathione reductase (GR), while glutathione (GSH), vitamins A, E and C and uric acid are major non-enzymatic antioxidants (16).

The intent of the present study was to determine the oxidative and antioxidative markers in patients with CAD and to compare these parameters between patient and healthy volunteer groups. It also aimed to compare oxidant/antioxidant status in chronic CAD patients with single, double or triple vessel stenosis.

Materials and methods

Patients. The study sample consisted of 90 subjects who were divided into three equal groups: patients with acute coronary syndrome (ACS), patients with chronic CAD and healthy subjects as controls. Each group comprised of 30 subjects (20 male and 10 female aged 40-70 years). ACS subjects were selected from patients hospitalized at the Coronary Care Unit (CCU) of Modares Hospital in Tehran, Iran, due to angina pectoris or acute myocardial infarction. CAD patients were selected from patients referred to the Angiography Unit of Modares Hospital and healthy subjects from Modares Hospital were included in the study as controls. All patients were enrolled from June to November, 2012. All patients signed informed consent forms agreeing to their participation in the study. No subject had any other disease or was taking medications. The study was conducted following approval by the Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran; approval no. IR.SBMU.REC.1387.134). Clinical and laboratory data of the patients and controls are presented in Table I.

Table I. Biochemical parameters of patients and control subjects included in the study.

Parameter	Study group (n=30/group)		
	ACS patients	Chronic CAD patients	Healthy controls
Age	65	63	61
Cholesterol, mg/dl	246±21	194±22	178±16
Triglyceride, mg/dl	222±21	162±15	129±9
Creatine kinase, IU/l	295±20	118±15	94±11
Creatine kinase-MB, IU/l	63±10	345±7	20±8
High-density lipoprotein, mg/dl	43±7	48±5	52±5
Low-density lipoprotein, mg/dl	158±22	105±25	100±8
Diastolic blood pressure, mmHg	155±8	145±4	127±9
Systolic blood pressure, mmHg	111±7	96±8	85±7

ACS, acute coronary syndrome; CAD; coronary artery disease.

Sample collection. Blood samples (10 ml) were collected into EDTA sterile plastic tubes. All samples were centrifuged at 2,000 x g for 10 min at 4˚C, and were maintained at -70˚C until measurement of plasma total antioxidant capacity (TAC). For determination of GPx activity as well as GSH and MDA levels, the obtained packed red blood cells (pRBC) were washed with normal saline and phosphate-buffered saline (PBS), respectively, and then stored at -70˚C until further analysis.

Measurement of erythrocyte GSH concentration. GSH level was measured using 5, 5'-dithiobis-(2-nitrobenzoate) (DTNB; Merck KGaA, Darmstadt, Germany) according to the spectrophotometric method described by Beutler *et al* (16). Briefly, 0.2 ml of pRBCs was mixed with 8 ml PBS (0.2 M; pH 7.4) and centrifuged at 25,000 x g for 5 min at 4˚C. The sample was then mixed with 0.5 ml DTNB. For each GSH test, 0.1 ml pRBC suspension was mixed with 0.9 ml distilled water to provide hemolysate. Subsequently, the mixture was vortexed and absorbance was measured at 412 nm to detect the GSH content. The GSH levels were expressed per gram of hemoglobin (Hb; μmol/gHb). The quantity of GSH was determined by the known molar extinction coefficient of GSH ($1.36x10^4$ $mol^{-1}cm^{-1}$) (16).

Determining the susceptibility of RBC to oxidative stress. Plasma MDA is a naturally occurring product of lipid peroxidation usually measured based on levels of thiobarbituric acid (TBA) reactive substances or lipid peroxides (17). pRBCs were diluted (1:8 v/v) with 0.9% saline. According to a method reported by Stocks and Dormandy for induction of oxidative conditions, the RBCs were incubated with 4 mM H_2O_2 at 37˚C, in a shaking thermostatic bath for 120 min, either in the absence or presence of 2 mM sodium azide (as a potent inhibitor of RBC catalase; Merck KGaA). A 'zero time' sample was obtained by terminating the reaction [with arsenite-trichloroacetic acid (TCA) solution (Merck KGaA)] immediately following addition of 4 mM H_2O_2. This sample was also treated by H_2O_2 either in the absence or presence of 2 mM sodium azide. Healthy control samples were also incubated in the same conditions to assess the extent of spontaneous oxidation of RBC. Following addition of arsenite-TCA solution and centrifugation at 4,400 x g at 4˚C for 5 min, 2 ml supernatant was added to TBA-containing tubes. All tubes were then placed in a 100˚C bath for 15 min, and finally, the absorbance of the samples was measured spectrophotometrically at 535 nm. 1,1,3,3-tetraethoxy-propane was used as a standard. The results were reported as nmol/gHb. The concentration of blood Hb was measured by the cyanmethemoglobin method described by Amatuzio *et al* (18). The percentage of MDA release from erythrocyte membranes was calculated by the following formula: Concentration of MDA without sodium azide/concentration of MDA with sodium azide x100 (19).

Determination of erythrocyte GPx activity. The GPx activity of erythrocytes was measured by the modified method of Andersen *et al* (20), which is based on spectrophotometric monitoring of the decrease in absorbance of NADPH (Fluka Chemie GmbH; Sigma-Aldrich; Merck KGaA) at 340 nm in the presence of GR (Sigma-Aldrich; Merck KGaA). This method is based on GPx oxidizing GSH to oxidized GSH, which is then reduced by GR; finally, oxidation of NADPH

to $NADP^+$ leads to the steady decrease in absorbance of NADPH (21). GPx activities of erythrocytes were expressed in U/gHb in hemolysate.

TAC of plasma. TAC of plasma was measured by the method of Miller *et al* (22) using a Total Antioxidant Status kit (Randox Laboratories Ltd., Crumlin, UK). According to this method, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS; Merck KGaA) is incubated with metmyoglobin (Merck KGaA) and H_2O_2 to form the radical cation $ABTS^{+\bullet}$, the absorbance of which can be measured at 600 nm. In this method, the capacity of plasma antioxidants to inhibit this reaction is measured and compared with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Merck KGaA) as a standard. Briefly, 1 ml chromogen (601 μM metmyoglobin and 610 μM ABTS) was added to 50 μl plasma sample and mixed with 350 μl of 2,500 μM H_2O_2. The mixture was incubated at 37˚C for 3 min and its absorbance at 600 nm read. Values were expressed in mmol/l.

Statistical analysis. Data were expressed as the mean ± standard error of the mean. The data were analyzed using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). To compare the difference between the three groups, data were analyzed by one-way analysis of variance (ANOVA). Scheffe's test was used post hoc to assess the significance of differences among the groups. Additionally, to assess the association between the number of stenotic vessels and oxidative status in chronic CAD patients, the 30 patients with chronic CAD were divided into three equal groups (n=10/group) as follows: patients with one stenotic vessel, patients two stenotic vessels and patients with three vessel stenoses. The 30 healthy subjects were included in the analysis as the control group, and ANOVA and Scheffe's test were used as above. In all analyses P<0.05 was considered to indicate statistical significance.

Results

Comparison of erythrocyte GSH levels between the three subject groups. Fig. 1 depicts the level of GSH measured in RBCs from healthy controls and the groups of patients, where the capacity to overcome an oxidative stress has been evaluated. The concentrations of erythrocyte GSH in the three groups were as follows: ACS patients, 6.14±0.88 μmol/gHb; chronic CAD patients, 6.91±0.73 μmol/gHb and healthy controls, 10.63±0.48 μmol/gHb. As the results indicate, the levels of erythrocyte GSH in ACS and chronic CAD patients was significantly lower than in healthy controls (P<0.0001). In addition, the level in ACS patients was significantly lower compared with in chronic CAD patients (P<0.0001).

Comparison of RBC susceptibility to oxidative stress in the three study groups. Table II presents the levels of MDA measured in RBCs of the two patient groups and of healthy controls following incubation with H_2O_2 for 120 min and at zero time, either in the presence or absence of sodium azide. MDA levels in the presence of sodium azide at the incubation time of 120 min were as follows: in ACS patients, 988±52 nmol/gHb; in chronic CAD patients, 873±48 nmol/gHb; and in healthy controls, 409±24 nmol/gHb. As can be observed, these levels

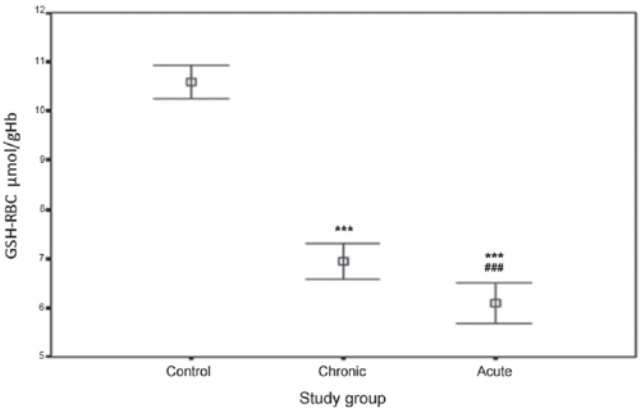

Figure 1. Concentration of erythrocyte GSH in patients with ACS and chronic coronary disease compared with healthy controls. Data are expressed as means ± standard error of the mean. ***P<0.0001 vs. control; ###P<0.0001 vs. chronic. GSH, reduced glutathione; RBC, red blood cell; gHb, gram of hemoglobin; ACS, acute coronary syndrome.

Table II. Levels of MDA in the presence or absence of sodium azide at zero time and 120 min after incubation with H_2O_2.

Group (n=30/group)	MDA-SA, nmol/gHb 120 min	MDA-SA, nmol/gHb Zero time	MDA, nmol/gHb 120 min	MDA, nmol/gHb Zero time
Control	409±24	246±20	173±19	108±9
Chronic CAD patients	873±48[b]	463±28	490±33	286±22
ACS patients	988±52[a]	512±37	633±39	312±26

All data are expressed as means ± standard error of the mean, n=30/group. [a]P<0.05 vs. chronic CAD and control groups; [b]P<0.05 vs. control group. MDA, malodialdehyde; MDA-SA, malodialdehyde with sodium azide; ACS, acute coronary syndrome; CAD; coronary artery disease.

of MDA in ACS and chronic CAD patients were markedly higher than in the control group (P<0.05). Furthermore, the MDA level in ACS patients was significantly higher than that in chronic CAD patients (P<0.05). The levels of MDA in the absence of sodium azide were as follows: in ACS patients, 633±39 nmol/gHb; in chronic CAD patients, 490±33 nmol/gHb and in healthy controls, 173±19 nmol/gHb, which revealed notable difference between the patient and control groups, and between the ACS and chronic CAD cases. A similar pattern of data were obtained with the zero time samples, revealing markedly high levels of MDA in ACS and chronic CAD patients compared with in the controls, and higher MDA levels in ACS patients in comparison with chronic CAD patients. Table III presents the percentage of MDA release from erythrocytes in patients and controls after the 120-min incubation and at zero time. The percentage of MDA release from erythrocytes, both at zero time and 120 min, was significantly increased in the ACS and chronic CAD patients compared with in the healthy controls (P<0.05). In addition, this percentage was consider-

Table III. Percentage of MDA release from erythrocytes at zero time and 120 min after incubation with 4 mM H_2O_2.

Group (n=30/group)	% MDA release (zero time)	% MDA release (120 min)
Control	35.0±5.00	42.0±4.80
Chronic CAD patients	42.0±4.70[a]	57.0±9.90
ACS patients	51.0±6.40[b]	64.0±9.30

All data are expressed as means ± standard error of the mean, n=30/group. [a]P<0.05 vs. chronic CAD and control groups; [b]P<0.05 vs. control group. MDA, malodialdehyde; ACS, acute coronary syndrome; CAD; coronary artery disease.

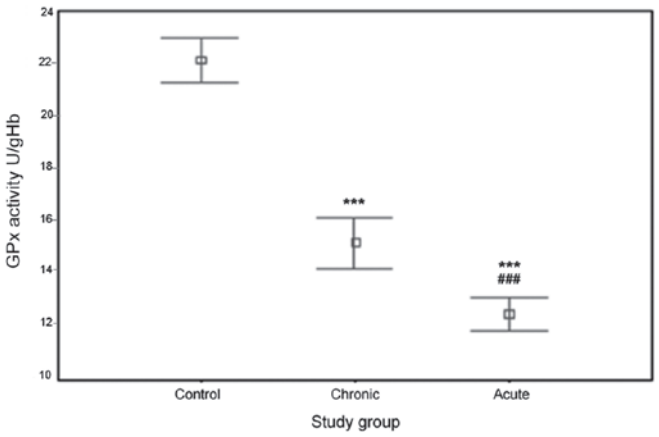

Figure 2. Evaluation of erythrocyte GPx activity in patients with ACS and chronic coronary disease compared with healthy controls. Data are expressed as means ± standard error of the mean. GPx, glutathione peroxidase; ACS, acute coronary syndrome. ***P<0.0001 vs. control; ###P<0.0001 vs. chronic.

ably higher in the ACS patients when compared with that in the chronic CAD patients (P<0.05).

Evaluation of erythrocyte GPx activity in patient groups and healthy controls. The results of erythrocyte GPx activity in the ACS/chronic CAD patients and controls are depicted in Fig. 2. GPx activity values in the three groups were 12.52±1.76 U/gHb for ACS patients, 15.14±2.52 U/gHb for chronic CAD patients and 22.12±2.12 U/gHb for controls. As illustrated in the Figure, the activity of erythrocyte GPx of ACS and chronic CAD patients was significantly lower than that in healthy controls (P<0.0001). Furthermore, the activity of this enzyme in ACS patients was markedly lower than that in chronic CAD patients (P<0.0001).

Comparison of plasma TAC in patient and control groups. Significant differences were observed between the TAC of plasma in the ACS, chronic CAD and healthy control groups. The values of plasma TAC were as follows: in ACS patients, 1.02±0.18 mmol/l; in chronic CAD patients, 1.24±0.16 mmol/l; and in controls, 1.52±0.23 mmol/l. Fig. 3 illustrates the significant reduction in the TAC of plasma in ACS and chronic CAD patients compared with that in

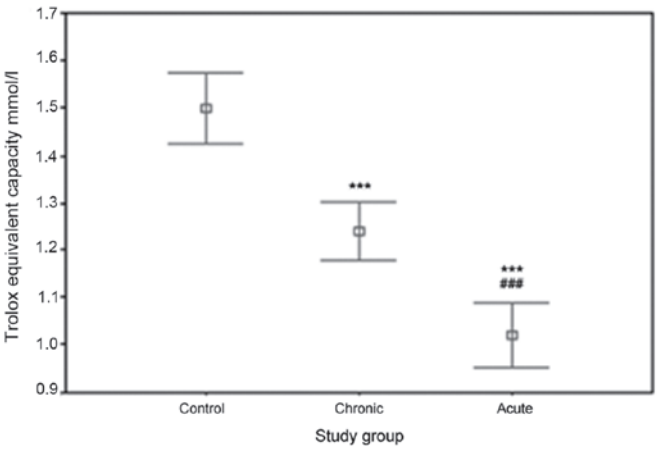

Figure 3. Representation of TAC of plasma in patients with ACS and chronic coronary disease compared with healthy controls. Data are expressed as means ± standard error of the mean. TAC, total antioxidant capacity; ACS, acute coronary syndrome. ***P<0.0001 vs. control; ###P<0.0001 vs. chronic.

Table IV. Association between the number of stenotic vessels and oxidative status in chronic coronary artery disease patients.

Group (n=30/group)	MDA nmol/gHb	Erythrocyte GPx, U/gHb	Erythrocyte GSH μmol/gHb	TAC mmol/l
Control	409±41	22.1±2.20	10.6±0.48	1.5±0.20
Patients with one-vessel disease[n]	660±28[a]	17.5±1.10	7.8±1.20	1.12±0.12
Patients with two-vessel disease[n]	900±35[b]	14.7±1.60	6.7±0.48	1.06±0.16
Patients with three-vessel disease[n]	991±43[c]	13.9±3.70	6.3±0.53	0.15±0.91

TAC, Total antioxidant capacity of plasma. All data are expressed as mean ± standard error of the mean; P-value <0.05. n, no. of subjects. [a]P<0.05, when compared with control group. [b]P<0.05, when compared with patients with one vessel disease and control group. [c]P<0.05, when compared with other three groups. MDA, malondialdehyde; GPx, glutathione peroxidase; GSH, reduced glutathione.

controls (P<0.0001). A significant reduction in the plasma TAC of ACS patients compared with that of chronic CAD patients was also revealed (P<0.0001).

Evaluation of association between the number of stenotic vessels and oxidative status in chronic CAD patients. The results of oxidative and antioxidative parameters measured in these three groups are presented in Table IV. From these data a significant increase in MDA (120 min) level (P<0.05) and notable decreases in GSH concentration, TAC and erythrocyte GPx activity were observed in patients with triple-vessel disease compared with the patients with double and single-vessel disease and healthy controls. Additionally, an augmented level of MDA (P<0.05) and reduced GSH and TAC levels as well as GPx activity were identified in patients with double stenotic vessels in comparison with the single

stenosis and control groups. Similar data were observed with single stenotic patients as compared with healthy subjects.

Discussion

The main purposes of the present investigation were to compare plasma oxidative status and RBC susceptibility to oxidative stress in patients with CAD and healthy controls. It was identified that the levels of erythrocyte GSH in ACS and chronic CAD patients was markedly lower than in healthy controls. Furthermore, the level was also lowered in ACS patients as compared with in chronic CAD patients. Similar results have been reported by other groups with regard to significant decreases in the level of GSH in CAD patients (23-25).

The present findings indicated that both the levels of MDA and the percentage of MDA release from erythrocytes in ACS and chronic CAD patients were significantly increased compared with in the healthy controls. In addition, these two parameters in ACS patients were significantly higher than in chronic CAD patients. These data suggest that erythrocyte membranes in ACS patients are more readily oxidized in comparison to those in chronic CAD patients and healthy subjects; and furthermore that the susceptibility of erythrocyte membranes to oxidation in chronic CAD patients is higher than that in healthy subjects. Generally, increases in MDA level and the percentage of MDA release from erythrocytes in patients appeared to be due to decreased erythrocyte GSH content relative to that in healthy subjects. Since a high concentration of polyunsaturated fatty acids in the phospholipid membrane of RBCs may lead to more extensive oxidation of the membrane lipids (26), it is necessary to consider the differences in erythrocytes membrane composition between patients groups and controls.

The present findings are also in accordance with a number of studies reporting an increased level of MDA in CAD and myocardial infarction patients compared with healthy control groups (25,27,28). Amaki *et al* (29) identified that the serum levels of circulating MDA-modified LDL in patients with CAD were significantly higher than in patients without CAD, indicating the use of this parameter as a diagnostic marker for advanced atherosclerosis.

The results of the present study revealed that patients with ACS and chronic CAD exhibited lower erythrocyte GPx and TAC activity compared with healthy controls. Decreased erythrocyte GPx and TAC activity in the patient groups appeared to be correlated with induced oxidative conditions, leading to extensive oxidative stress and increased susceptibility of the erythrocyte membrane to this oxidant status.

These present data are also concordant with the results of Serdar *et al* (30), who reported decreased activities of antioxidant enzymes including erythrocyte GPx and some other antioxidant enzymes as well as a decreased concentration of antioxidant factors in patients with CAD. In another study, Serdar *et al* (31) investigated the correlation between total sialic acid (TSA) concentration in serum and antioxidative and oxidative markers including plasma MDA, paraoxonase, GPx, vitamin C and vitamin E in CAD patients. They identified a positive correlation between TSA and these parameters. Furthermore, their study revealed a significant reduction of antioxidant parameters in the patients with CAD. Different groups have also reported an imbalance in the levels of peroxiredoxin-1 (an antioxidant enzyme) and GPx in the blood of patients with CAD, and existence of a direct correlation between low GPx activity and high levels of ROS in ACS patients (32,33). Collectively, these findings suggest a potential contribution of inefficient protection against oxidant-mediated damage to increased clinical risk of CAD. Further study has revealed marked increases in the levels of oxidized-LDL and GPx expression and activity in ACS patients in comparison with patients with stable CAD and healthy controls (34). According to this previous study, GPx level may be upregulated in response to an alteration in oxidative stress during ACS. According to the current data, patients with triple-vessel stenosis had significantly increased levels of MDA and notably decreased GSH, TAC and GPx activities in comparison with patients with double and single vessel disease and healthy controls. These parameters also exhibited the same patterns in double vessel disease patients when compared with the single vessel disease patients and controls. Such findings suggest that the number of narrowed vessels may have positive correlation with deteriorated oxidative condition in chronic CAD patients; the greater the number of stenotic vessels, the higher the oxidative stress induced in these patients.

It was apparent that ACS patients had deteriorated oxidant and antioxidant conditions compared with chronic CAD patients and healthy controls. The significant differences between ACS and chronic CAD patients suggested that the chronic form of disease had greater probability of improvement in antioxidative processes to ameliorate oxidative stress. Meanwhile, acute syndrome likely has insufficient time to enhance the protective processes and adaptive mechanisms.

This present study sheds light on the association between acute and chronic conditions in CAD and the oxidative status of affected patients. The results indicate that the chronic form of disease is more adapted to oxidative stress in comparison to the acute form, indicating the need to reduce oxidative status in ACS patients. However, in both ACS and chronic CAD patients, high erythrocyte membrane susceptibility, low antioxidant capacity and decreased function of antioxidative systems were detected compared with in the healthy controls. Therefore, the use of exogenous antioxidants may have potential therapeutic benefits in reducing oxidant status in these patients. However, there remains a need for detailed study of other key pathways involved in atherogenesis, including those associated with pro-inflammatory markers.

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Availability of data and materials

All datasets used or analyzed during this study are available from the corresponding authors on reasonable request.

Authors' contributions

AB designed the study and aided in writing the manuscript. SR assisted in drafting the manuscript and revising it critically for intellectual content. AD performed the experiments. HS was the co-supervisor together with AB and aided in collecting the samples and interpreting the results. FKB aided with manuscript writing and interpreting the data.

Ethics approval and consent to participate

Written informed consent was obtained and signed by all patients who agreed to participate in the study.

Patient consent for publication

The patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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