ANTIOXIDANT STATUS IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

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ABSTRACT

Free radicals play an important role in the pathogenesis of tissue damage in many clinical disorders, including atherosclerosis. Antioxidants protect the body from damage caused by free radicals. In this study we investigated oxidative stress, antioxidants and inflammatory molecules in patients with acute myocardial infarction. This study has been carried out on 106 patients with acute myocardial infarction, (89 men and 17 females). The control group consisted of 50 healthy, age-matched subjects (40 men and 10 females). Levels of Glucose, lipid profile, glutathione reduced, glutathione peroxidase, Superoxide dismutase, Glycosylated hemoglobin, fibrinogen, vitamin C, vitamin E, malondialdehyde, ceruloplasmin, adenosine deaminase, lysozyme and sialic acid were measured. Malondialdehyde and ceruloplasmin levels were significantly high and antioxidants such as vitamin C, vitamin E, glutathione reduced, glutathione peroxidase and superoxide dismutase were significantly decreased in diabetic and non-diabetic AMI patients as compared with control ($p < 0.001$). Inflammatory markers showed significant rise in diabetic patients as compared with controls. Our results clearly show increased inflammation and oxidative stress in patients with acute myocardial infarction. Depression of antioxidant system in these patients confirms this conclusion.

KEY WORDS

Oxidative Stress, Antioxidant, Inflammation and AMI.

INTRODUCTION

Free radicals contain one or more of unpaired electrons. They play an important role in the pathogenesis of tissue damage in many clinical disorders. Oxygen free radicals are capable of damaging compounds of all biochemical classes; including nucleic acids, proteins, lipids, lipoproteins, carbohydrates and connective tissue macromolecules (1). Normally, there is a balance between tissue oxidant and antioxidant activity. The latter is achieved by the antioxidant scavenger system, which includes enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and antioxidant vitamins (C, A, E and other carotenoids) (2). Oxidative stress is a condition in which oxidant metabolites exert their toxic effect because of an increased production or an altered cellular mechanism

of protection (3). Increased oxidative stress and the generation of the free oxygen radicals can result in modification of LDL to oxidized LDL that could lead to atherosclerotic lesions. Also, inflammation occupies a very important central position in all phases of atherosclerosis, which is underlying cause of Acute Myocardial Infarction (4).

In our study, many areas are covered which may have important role in the pathophysiology of Acute Myocardial Infarction (AMI), such as oxidative stress molecules (MDA and ceruloplasmin), antioxidant molecules (reduced glutathione), antioxidant enzymes (glutathione peroxidase and superoxide dismutase), antioxidant vitamins (vitamin C and vitamin E), inflammation-related molecules (fibrinogen, sialic acid, lysozyme and adenosine deaminase), metabolic control (glucose, glycosylated hemoglobin (GHb) and lipid profile), anthropometric parameters (body mass index and waist to hip ratio). Thus, we are able to get a nearly complete biochemical view of AMI, which is rarely found in previous studies. Though AMI is extensively studied, role of some possible inflammatory molecules like ADA, lysozyme and ceruloplasmin are poorly explained with reference to AMI. Therefore, in our study, we investigated role of ADA, lysozyme

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and ceruloplasmin in AMI-related inflammation and oxidative stress.

MATERIALS AND METHODS

Study design and patients : The study consisted of 106 patients (89 men and 17 women) with a mean age of 43.5 ± 10.1 years and the diagnosis of AMI, admitted to the Intensive Cardiac Care Unit, L.T.M. Medical College and General Hospital, Sion, Mumbai. Patients were further sub grouped as diabetic (58 patients) and nondiabetic (48 patients) according to their history and glucose and GHb levels. The diagnosis of AMI was established according to clinical criteria: chest pain, which lasted for up to 3 hours, ECG changes (ST elevation of 2 mm or more in at least two leads) and CPK elevation. The control group consisted of 50 healthy, age-matched subjects, 40 men and 10 women, recruited from an annual check-up program. L.T.M. Medical College and General Hospital, ethics committee gave approval.

Inclusion criteria:- Patients below 50 years of age, with diagnosis of AMI and free from any kidney or liver diseases.

Exclusion criteria:- Patients above 50 years of age, with any kidney or liver diseases and pregnancy.

Blood collection and biochemical methods used:-

10 ml of venous blood was collected after overnight fasting in different containers-

- Sodium fluoride bulb: - 1.0 ml of blood for glucose estimation.
- EDTA bulb: - 5.0 ml of blood was added. 0.2 ml of blood was used for reduced glutathione estimation. Remaining blood was centrifuged. Separated plasma was used for the estimation of fibrinogen and vitamin C. Red blood cells were washed 3 times with ice-cold normal saline and used for the estimation of glutathione peroxidase, superoxide dismutase and glycosylated hemoglobin.
- Plain bulb:- Remaining blood was added and serum was separated. Serum was used for the estimation of lipid profile, malondialdehyde, vitamin E, ceruloplasmin, sialic acid, adenosine deaminase and lysozyme.

Glucose and plasma lipids (total cholesterol and HDL-cholesterol, triglycerides) were measured using commercial kits from Accurex, India, on automated analyzer. All chemicals of analytical grade were obtained from Sigma or Merck India.

Reduced Glutathione (GSH) : The level of reduced glutathione in erythrocytes was determined by a method of Beutler (5). This method is based on the development of a

relatively stable yellow color, when 5, 5'- dithio-bis-(2-nitrobenzoic acid) or DTNB, is added to sulfhydryl compounds. 1.0 ml of blood extract was mixed with 4.0 ml of 0.3 M Na_2HPO_4 and 1.0 ml of DTNB reagent [40 mg of 5, 5'-dithiobis (2-nitrobenzoic acid) in 100 ml of aqueous 1% tri-sodium citrate]. Absorbance was read at 412 nm immediately. Results were expressed as micromoles/liter.

Glutathione Peroxidase (GPx) : The glutathione peroxidase activity was determined by the procedure of Paglia et al (6). Briefly, the oxidized glutathione produced during GPx enzyme reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was monitored as a measure of formation of oxidized glutathione. Results were expressed as units of GPx per gram of hemoglobin.

Superoxide Dismutase (SOD) : Superoxide dismutase was determined by method of Winterbourn et al (7). This method is based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide which is generated by the action of photo reduced riboflavin and oxygen. The blue color formed was read at 560 nm. One unit of enzyme activity was measured as the amount of SOD, which causes half of the maximum inhibition of reduction of nitro-blue tetrazolium. Results were expressed as units of SOD per gram of hemoglobin. Hemoglobin was measured by Drabkin's method.

Glycosylated hemoglobin (GHb) : was determined by method of Fluckiger et al (8). The red blood cells were thoroughly washed with saline and then hydrolyzed with oxalic acid (i.e. hydrolysis of hexoses bound to hemoglobin) whereby 5-hydroxymethyl furfural is liberated. After precipitating proteins, the supernatant was allowed to react with 2 thiobarbituric acid (TBA) at 37°C for 40min. The yellow colored TBA – 5 – hydroxymethyl furfural adduct formed was measured at 443nm in a spectrophotometer. Results were expressed as percentage of GHb per gram of hemoglobin.

Fibrinogen : Fibrinogen was measured by tyrosine method, as given in Varley et al (9). The fibrin clot is formed in the diluted plasma by the addition of calcium ions. The clot is then separated from the solution and washed free of other proteins. The clot is dissolved in alkali and the protein is determined with the Folin-Ciocalteu reagent. Blue color developed was measured at 680 nm. Results were expressed as mg/dl of fibrinogen.

Vitamin C : Vitamin C was determined by titration method as given in Varley et al (10). This method employs the titration

with 2, 6-dichlorophenol indophenol in acid solution. On titration with an ascorbic acid solution, this compound is reduced to the colourless leucobase. The ascorbic acid is oxidized to dehydroascorbic acid. End-point was blue to red to colorless. Mg of vitamin C / 100 ml of plasma are 1.6/ml of titration.

Vitamin E : Vitamin E was determined by method of Baker and Frank as given in Varley et al (11). Serum vitamin-E reduce ferric to ferrous ions, which then form a red colored complex with α , α -dipyridyl. Tocopherols and carotenes are first extracted into Xylene and the absorbance is read at 460 nm to measure the carotenes. A correction for the carotenes is made after adding ferric chloride and reading at 520 nm. Results were expressed as mg/dl of vitamin E.

MDA Method : MDA levels were estimated by thiobarbituric acid (TBA) reaction. Using 40% trichloroacetic acid, proteins were precipitated from 0.5 ml serum, and precipitated proteins were incubated with TBA reagent in a boiling water bath for 60 minutes. The colored complex that occurred was refrigerated to room temperature and measured by using a spectrophotometer at 533 nm. 1,1,3,3-tetraethoxypropane (1 μ mol/L) was used as a standard for MDA estimation. Concentrations of MDA were expressed in μ M/l.

Ceruloplasmin : Ceruloplasmin was determined using its copper oxidase activity by method of Ravin (13). In this method, action of ceruloplasmin on p-phenylenediamine is used to measure the amount of ceruloplasmin present in the serum. Dark lavender color was read at 530 nm using control tube as blank. Concentration of ceruloplasmin in mg/dl is absorbance X 87.5.

Adenosine deaminase (ADA) : was determined by method of Martinek (14) based on Berthelot reaction. In an alkaline solution, and in the presence of hypochlorite, ammonia will condense with phenol, to give an intense blue colored indophenol. The reaction is usually supported by the presence of a catalyst like sodium nitroprusside. Color was read at 640 nm. 1 unit of adenosine deaminase activity is the amount that liberates 1 μ g of ammonia nitrogen per ml of serum per hour at 37°C.

Lysozyme : Lysozyme was determined by method of Harrison et al (15). The assay is based on the lysis of *Micrococcus luteus* cells by lysozyme. To 1.0 ml of substrate containing *Micrococcus luteus*, 0.1 ml of serum/standard was added and kept at 25°C. Readings were taken at 30 seconds and 10 minutes. Difference between two readings was calculated. Concentrations of lysozyme were expressed in μ g/ml.

Sialic acid : Sialic acid was determined by method of Cabezas et al (16). The principle employed involves precipitation of serum glycoproteins by 95% ethanol at 0°C: Hydrolysis and the reaction of N-acetylneuraminic acid (NANA) by a HCl-cupric-resorcinol reagent; extraction of the colored component by butyl acetate; and the measurement of the color intensity at 580nm. Concentrations of sialic acid were expressed in mg/dl.

Statistical analysis : The data from patients and controls were compared using Student's 't'-test. Values were expressed as mean $\pm$ standard deviation (SD). Sigma stat version 3.0 was used for statistical analysis. 'P' value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Demographic data of control and AMI group are shown in Table 1.

Age and Sex : Diabetic AMI patients had mean age 44.9 ± 10.3 years, with weight 65.2 ± 10.9 Kg. Non-diabetic AMI patients had mean age 42.1 ± 9.9 years, with weight 64.0 ± 12.6 Kg and Controls had mean age 45.6 ± 9.0 years with weight 56.4 ± 7.4 Kg.

Body mass index (BMI) : It was significantly high in diabetic group ($p < 0.05$) as compared with control. Compared with controls, there were no significant differences in non diabetic AMI.

Blood Pressure : Systolic blood pressure was significantly high ($p < 0.05$) in both the patients groups (DM & NDM) as compared with controls.

The metabolic characteristics of the two groups (AMI and Control) are illustrated in Table 2. Glucose and glycosylated hemoglobin levels were significantly high in diabetic AMI group

Table 1 : Demographic data in AMI and Control

Variable	Control n=50	AMI	
		DM (n=58)	NDM (n=48)
Age (Years)	45.6 ± 9.0	44.9 ± 10.3	42.1 ± 9.9
Sex (Male/Female)	40/10	45/13	43/5
Weight (Kg)	58.4 ± 7.4	65.2 ± 10.9	64.0 ± 12.6
Body mass index(Kg/m ²)	22.3 ± 1.9	25.5 ± 4.5	24.7 ± 5.1
Waist/Hip	0.82 ± 0.04	1.00 ± 0.09	0.99 ± 0.14
Systolic blood pressure (mm of Hg)	111 ± 9	137 ± 18	130 ± 22
Diastolic blood pressure (mm of Hg)	85 ± 4	95 ± 10	87.0 ± 13

Table 2 : Metabolic markers in AMI and Control

PARAMETER (Normal Range)	Control (n = 50)	AMI	
		DM(n = 58)	NDM(n = 48)
Glucose (mg/dl) (70-110 mg/dl)	90.1 ± 4.1	161.9 ± 44.1*	102.7 ± 17.1NS
GHb (of total Hb) (3-8% of total Hb)	6.91 ± 0.63	10.25 ± 1.39*	7.89 ± 0.88NS
Total Cholesterol (mg/dl) (150-250 mg/dl)	170.2 ± 10.2	220.7 ± 26.9*	195.8 ± 34.4NS
Triglycerides (mg/dl) (50-150 mg/dl)	91.8 ± 15.3	178.8 ± 39.3*	158.1 ± 26.4**
LDL-Cholesterol (mg/dl) (100-160 mg/dl)	105.4 ± 12.7	149.8 ± 15.3*	134.2 ± 21.0NS
HDL- Cholesterol (mg/dl) (40-60 mg/dl)	49.6 ± 6.1	37.1 ± 6.3NS	36.8 ± 9.4NS
VLDL-Cholesterol (mg/dl) (10-30 mg/dl)	23.5 ± 3.2	39.7 ± 6.7 *	37.4 ± 7.2 **

Values Expressed as Mean ± SD

*P < 0.001, ** P < 0.01, *** P < 0.05, NS- Not significant as Compared to Control

(p<0.001) as compared with control. Compared with controls, there were no significant differences in non diabetic AMI. Blood lipids showed a significantly higher concentration (p<0.001) of total cholesterol, triglyceride, low density lipoprotein and very low density lipoprotein in diabetic AMI group.

Malondialdehyde and ceruloplasmin levels in control and AMI group are shown in Table 3. MDA and CER levels were significantly high in diabetic and non diabetic AMI patients as compared with control (p<0.001). A negative correlation was observed between rise in MDA and fall in GSH levels in diabetic AMI (r=-0.62) and non diabetic AMI (r=-0.60).

Antioxidant status in control and AMI group is presented in

Table 3 : Malondialdehyde and ceruloplasmin levels in AMI and Control

Variable	CONTROL (n = 45)	AMI	
		DM(n = 58)	NDM(n = 48)
Malondialdehyde (µM/l)	2.35 ± 0.11	4.57 ± 0.88*	4.36 ± 0.85*
Ceruloplasmin (mg/dl)	19.1 ± 3.1	35.2 ± 3.4*	28.7 ± 4.9*

Values Expressed as Mean ± SD

Table 4. All antioxidants, superoxide dismutase, reduced glutathione, glutathione peroxidase, vitamin C, and vitamin E were significantly decreased in DM and NDM groups of AMI (p < 0.001) as compared with controls.

Table 4 : Antioxidant Status in AMI and Control

Variable	CONTROL (n = 45)	AMI	
		DM(n = 58)	NDM(n = 48)
Superoxide dismutase (U/gm Hb)	4461 ± 481	2300±276*	2626 ± 451*
Glutathione reduced (µM / l)	76.9 ± 4.7	48.2 ± 4.9*	53.5 ± 7.9*
Glutathione peroxidase (U/gm Hb)	1.51 ± 0.17	0.75 ± 0.13*	0.95 ± 0.11*
Vitamin-C mg/dl	1.28 ± 0.13	0.64 ± 0.10*	0.68 ± 0.13*
Vitamin -E mg/dl	1.39 ± 0.17	0.80 ± 0.09*	0.83 ± 0.14*

Values Expressed as Mean ± SD

*P < 0.001 as Compared to Control

Inflammatory parameters in AMI patients are shown Table 5. All inflammatory parameters were significantly raised in diabetic AMI patients as compared to controls (p < 0.001) In non-diabetic patients, fibrinogen and adenosine deaminase levels were significantly high as compared to controls (p< 0.001 and P<0.01).

Table 5 : Inflammatory parameters in AMI and Control

Variable	CONTROL (n = 45)	AMI	
		DM(n = 58)	NDM(n = 48)
Fibrinogen (mg/dl)	327.3 ± 32.0	590.8 ± 56.7*	514.3 ± 77.7*
Adenosine deaminase (U/L)	2.85 ± 0.33	4.79 ± 0.52*	3.98 ± 0.71*
Lysozyme (µg/ml)	12.3 ± 1.4	16.0 ± 1.4*	13.3 ± 2.0NS
Sialic Acid (mg/dl)	51.5 ± 3.8	62.2 ± 8.4*	54.5 ± 12.1 NS

Values Expressed as Mean ± SD

*P < 0.001, NS-Non-Significant as Compared to Control

DISCUSSION

The root cause of AMI is mainly atherosclerosis. Contrary to earlier belief, research in the last two decades has shown that atherosclerosis is neither a degenerative disease nor inevitable due to ageing. On the contrary, atherosclerosis seems to be a chronic inflammatory condition that is converted

to an acute clinical event by the induction of plaque rupture, which in turn leads to thrombosis. Hence Inflammation occupies a very important central position in all phases of atherosclerosis, although inflammation must smolder for decades before resulting in a clinical event, like AMI (4).

Significant rise in MDA levels ($p < 0.001$), a lipid peroxidation product, in our patients is indicative of elevated oxidative stress in AMI patients. This is similar to work of Dubois Rande et al (17) and Mc Murray (18) who showed a decrease in antioxidant enzyme activities and increase in lipid peroxidation products (MDA, TBARS) in patients with unstable angina and chronic heart failure.

An increased level of serum ceruloplasmin in AMI patients ($p < 0.001$) suggests that this molecule may act as an oxidative stress indicator, though mechanism remains unclear. It is an inflammation-sensitive protein (19) and an acute phase reactant. It was shown that ceruloplasmin exhibits pro-oxidant activity and causes oxidative modification of LDL. This indicates that ceruloplasmin is an independent risk factor for cardiovascular diseases (20). A positive correlation was observed between ceruloplasmin and sialic acid ($r = +0.64$) in diabetic AMI group. As sialic acid is a well known inflammatory marker, ceruloplasmin may have possible role in inflammation. In diabetic AMI group, we also found positive correlation between ceruloplasmin and fibrinogen ($r = +0.62$), total cholesterol ($r = +0.70$), triglycerides ($r = +0.62$). All these results indicate that ceruloplasmin may be considered as an inflammatory molecule.

In AMI patients, we found significantly lower levels of vitamins E and C, ($p < 0.001$) compared with controls. This is in accordance with studies of Singh et al (21), who demonstrated that there was a significant drop in vitamins C, E, A and β -carotene, whereas lipid peroxides were significantly higher in AMI patients, compared with controls. This indicates severe damage to antioxidant system, which is unable to combat oxidative stress and inflammation.

Our data showed that reduced glutathione levels and glutathione peroxidase activity are significantly lowered in AMI patients ($p < 0.001$), which is similar to Kharb (22). Negative correlation was observed between reduced glutathione and malondialdehyde levels in diabetic AMI groups ($r = -0.62$). This implies that glutathione system, which is the important protective system against oxidative damage, is severely impaired in AMI patients. Our findings indicate the existence of an abnormal balance between the oxidative and protective mechanisms in AMI patients, particularly in diabetic AMI

patients.

We also found that the activity of SOD was significantly lower in patients group ($p < 0.001$). Similar to our observation, Kumar and Das (23) have suggested that an increase in free radical generation and a simultaneous decrease in the production of NO and antioxidant enzyme such as superoxide dismutase and vitamin E occurs in essential hypertension which is a well known risk factor for AMI.

In AMI patients, we found significantly higher levels of fibrinogen ($p < 0.001$) in patients group. Fibrinogen is a circulating glycoprotein that acts at the final step in the coagulation response to vascular and tissue injury. Fibrinogen is an acute phase reactant that is increased in inflammatory states (24). Hepatic synthesis of fibrinogen can increase up to 4-fold in response to inflammatory or infectious triggers (25). Some earlier studies have identified fibrinogen as a major independent risk factor for cardiovascular diseases (26, 27). Fibrinogen is directly associated with AMI and is an independent short-term predictor of mortality (28). It also indicates role of fibrinogen as an acute phase reactant and its role in response to inflammation.

Our data indicates that ADA can be considered as an inflammatory molecule, as we found significantly increased ADA levels in patients ($p < 0.001$), as compared with controls. Similar observations have been noted by some workers in other conditions, such as rheumatoid arthritis and pleurisy (29, 30). ADA may play a role in insulin effect and glycemic control, as increased activity of ADA was found in type 2 diabetes patients (31).

Lysozyme activity was observed significantly increased ($p < 0.001$) in diabetic patients, but non-diabetic patients, did not show any significant changes. Two studies showed that lysozyme activity was increased in AMI patients (32, 33). Hickey et al (34) suggested that increased lysozyme activity may be considered as a part of systemic inflammatory response. Hence, increased lysozyme activity may reflect greater inflammation in type 2 diabetic patients.

Sialic acid showed significant increase ($p < 0.001$) in diabetic AMI patients, which is similar to a previous study, which showed that serum sialic acid levels are elevated in type 2 diabetic patients, suggesting important role of diabetes in the development of atherosclerosis and finally AMI (35).

We found greater increase in inflammatory markers in diabetic AMI patients. One link between inflammation and the incidence

of type 2 diabetes may be insulin resistance. Several mechanisms may explain the relation between insulin resistance and inflammatory factors, such as the hypersecretion of proinflammatory cytokines like IL-6 and TNF- α , from adipose tissue. These cytokines exert stimulatory effects on the synthesis of acute phase proteins. Also, due to decreased insulin sensitivity, expression of inflammatory proteins is enhanced through the counteraction of the physiological effect of insulin on hepatic acute phase proteins synthesis, which may also play a role in diabetic inflammation (36).

Thus, our study indicates an imbalance between oxidant and antioxidant molecules in AMI patients, and magnitude of imbalance is greater in diabetic AMI patients, possibly because of greater inflammation in diabetic patients. Also, it is suggested that adenosine deaminase, lysozyme and ceruloplasmin can serve as markers of inflammation, which are poorly studied with respect to AMI.

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