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Oxidative stress parameters in erythrocytes of post-reperfused patients with myocardial infarction

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Abstract
The effect of reperfusion of patients with myocardial infarction on the levels of some anti-oxidant enzymes, total thiols, malondialdehyde formation in erythrocytes and plasma ascorbate levels have been investigated. Significantly decreased activities of catalase and superoxide dismutase and decreased levels of total thiols in RBC's and ascorbic acid in plasma suggest that reperfusion of the infarcted myocardium leads to oxidative stress conditions wherein anti-oxidant mechanisms become less effective in coping with the oxidative insult. This view is further supported by the observation that in the post reperfused patients there is a highly significant enhancement in the levels of malondialdehyde.

Keywords: *Myocardial infarction, oxidative stress, ascorbic acid, antioxidants, lipid peroxidation, RBC's*

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Introduction

Myocardial ischaemia-reperfusion injury may occur following blood restoration after a critical period of coronary occlusion [1]. It is a clinical problem associated with procedures such as thrombolysis, angioplasty and coronary bypass surgery used to minimize the damage (infarct area) of the heart due to severe myocardial ischaemia.

During the past two decades, major improvements have been made in the management of the patients with acute myocardial infarction. Despite these developments, myocardial infarction (MI) remains the major issue from the clinical, psychological and social point of view.

Reperfusion of a ischaemic myocardium leads to tissue injury that includes a series of events viz. reperfusion arrhythmia, microvascular damage, myocardial stunning and cell death. Causative factors for all these appear to be due to a burst of oxygen consumption during reperfusion leading to oxidative stress. Oxidative stress is a condition in which oxygen metabolites exert toxic effects because of the increased production of oxygen derived free radicals or altered cellular defence mechanisms to neutralize these radicals. Two main hypotheses, namely oxidative stress and calcium overload, have been proposed to explain the pathogenesis of ischaemia-reperfusion injury [2,3]. Both mechanisms are most likely related to each other, but it is not known whether they operate simultaneously or one precedes the other. In man, there is evidence of oxidative stress during reperfusion of the heart or after thrombolysis [4]

It has been proposed that free radicals are involved in the initiation and progression of various cardiovascular diseases including atherosclerosis. Various mechanisms contribute to the generation of free radicals. The potential source of free radicals during reperfusion of ischaemic tissues, among others, is xanthine dehydrogenase/oxidase system. Under normal conditions, about 90% of this enzyme exists as dehydrogenase [5] which oxidizes hypoxanthine and xanthine to uric acid using NAD^+ as electron acceptor. Under ischaemic conditions, dehydrogenase is converted to the oxidase form [6]. This may be

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caused by depletion of ATP during ischaemia and subsequent loss of membrane Ca^{+2}-gradient. Increased cytosolic Ca^{+2} activates Ca^{+2} dependent proteases which cause selective proteolysis of the dehydrogenase to convert it into xanthine oxidase [7]. Xanthine oxidase oxidizes its substrates at the expense of molecular oxygen, with the resultant production of superoxide anions. Depletion of ATP, under these conditions, results in an increase in the levels of its metabolites, namely hypoxanthine and xanthine which are the substrates of xanthine oxidase. Upon reperfusion, with ample oxygen available, xanthine oxidase exhibits optimum activity with copious production of O_2^-.

The superoxide radical, though less toxic by itself, triggers the formation of other reactive oxygen species (ROS). These include OH^o, H_2O_2 and HOCl. The hydroxyl radicals, in particular, interact with lipids, proteins and nucleic acids resulting in the loss of membrane integrity, structural and functional changes in enzymes and proteins and genetic mutations respectively [8].

To counter the damaging effects of ROS, the body has evolved certain mechanisms to deactivate them before they can cause damage. The oxidative damage will occur only in situations when the defence mechanisms are deficient, made less active or production of ROS exceeds the capabilities of defence mechanisms or a combination of all these. The first line of defence is enzymatic free radical scavengers such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR). All these enzymes convert ROS to less toxic or non-toxic products [8] The second line of defence to ROS is provided by anti-oxidant compounds present in the body (such as thiols) or taken from external sources (such as α-tocopherol, β-carotenoids, flavanoids and ascorbic acid).

Non-enzymatic anti-oxidants prevent ROS-mediated tissue damage by a variety of mechanisms. For example, oxidized low density lipoporoteins are involved in the progression of an atherosclerotic lesion [9] which can be prevented by ascorbic acid. Moreover, there is evidence that ascorbic acid and vitamin E preserve arterial vasodilation even in the presence of oxidative stress [10,11]. The findings of Frei [12] also suggest that ascorbic acid is the only physiological anti-oxidant that can completely protect lipids and low density lipoproteins (LDL) against per-oxidative damage induced by oxidants. In most human epidemiological studies, an inverse relationship between ascorbic acid intake and occurrence of atherosclerosis has been reported [13].

Partial or complete restoration of blood flow during reperfusion leads to a sudden massive increase in oxygen concentration which results in an imbalance of oxidative/anti-oxidative processes with excessive production of ROS causing extensive damage that may result even in loss of contractile function of the heart and severe myocardial cell damage [14], myocardial haemorrhage and cardiac rupture [15].

A fine balance between ROS and various anti-oxidant mechanisms is crucial for avoiding myocardial injury. A study of various anti-oxidant parameters under pathophysiological conditions may provide a better understanding of the role of individual anti-oxidant systems in myocardial protection against oxidative insult.

Materials and methods

A total of thirty post- reperfused patients from between the age group of 35-70 years of both the sexes were included in the study. Blood samples from forty age and sex matched healthy persons served as a control. Informed consent was taken from each donor. The study was cleared by the departmental ethical committee. Patients were thrombolyzed with streptokinase as per the set protocol of the department of medicine, K.G's Medical University, Lucknow. All the chemicals employed were of AnalaR grade and biochemicals employed were obtained from Ms. Sigma Chemical Co., USA.

Venous blood (3.5 ml) was withdrawn within 6 h. after reperfusion from the patients and transferred into polypropylene tubes containing 0.5 ml. 3.8% sodium citrate, pH 7.2. The tubes were gently rotated to mix the contents and centrifuged at 2000 × g for 20 min at 4°C. The supernatant plasma was saved. The pellet containing RBC's was washed thrice with ice cold 0.85% NaCl using a centrifuge at 800 × g for 10 min at 4°C. The final pellet was taken up in 5 ml. chilled water and left in the cold for 30 min. The suspension was then centrifuged at 2000 × g for 20 min. The supernatant, thus obtained, was used for analysis after suitable dilution wherever needed.

SOD was assayed in the RBC's by the method of Misra and Fridovich [16]. The method uses the inhibition of auto-oxidation of epinephrine by SOD in the sample. Catalase was assayed following the method of Luck [17]. Ascorbic acid was estimated in the plasma by the method of Stanley et al. [18]. In this method ascorbic acid is oxidized by Cu (II) to form dehydroascorbic acid, which reacts with acidic 2, 4-dinitrophenylhydrazine (DNP) to form the red bis-hydrazone. The color developed was measured at 520 nm. Total thiols in RBC's were measured by the method of Hu [19] using Ellman's reagent. Malondialdehyde (MDA), a marker of lipid per-oxidation, was estimated following the method of Ohkawa et al. [20] The method is based on the coupling of the MDA formed, with thiobarbituric acid, to form a coloured complex which was measured at 532 nm. The reference used was tetraethoxypropane. Protein estimation was carried out by the method described by Lowry et al. using Folin phenol reagent [21]. Bovine

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serum albumin was used as the standard. Specific activity of enzymes has been defined as the unit of activity/mg. protein.

Statistical analysis was carried out using Student's t test.

Results

The results obtained are reported in Table I. Compared to healthy persons, the activities of SOD and catalase in RBC's of the patients showed a significant decrease (52% and 54% respectively; p values in both the cases <0.0005) along with significant elevation in MDA levels (93%; $p < 0.01$). Plasma ascorbate levels also exhibited a significant fall (63%; $p < 0.0005$) in the patient group. Total thiol levels in RBC's did not show any overall statistically significant change but was found to be lowered by 33% in the patients.

Discussion

While oxygen free radical (OFR) generation as well as lipid peroxidation remains at a low level during ischaemia [22], coronary recanalisation, i.e a sudden massive supply of oxygen to previously hypoxic tissue, leads to an uncontrolled burst of oxygen free radical production [4,23,24]. Subsequently, reperfusion injury, manifested by reperfusion arrhythmia, myocardial stunning or an increase in infarct size, may occur [25,26].

As the half life of oxygen free radicals is very short, the extent of OFR generation is usually measured indirectly by the level of stable by product of lipid-peroxidation i.e. MDA. MDA has become the most widely used marker of free radical activity [22]. Our results are in accordance with those of Horwitz et al. [27] showing an increase in the concentration of MDA after reperfusion which is due to burst of oxygen consumption and generation of reactive oxygen species in the first six hours of reperfusion of an ischaemic myocardium.

To evaluate the ability of the organism to counter balance the oxidative stress caused by a sudden excess of OFR's after reperfusion, the activities of the enzymatic defence system, SOD and catalase, was followed. Simultaneously, the levels of a non-enzymatic anti-oxidant defense system, ascorbic acid and total thiols, was also measured.

SOD is involved in the removal of superoxide anion. It catalyzes the dismutation of superoxide anion to H_2O_2 and molecular oxygen. Catalase, together with GPx, eliminates H_2O_2 as well as toxic hydroperoxides of unsaturated fatty acids formed [28] We have found low activities of anti-oxidant enzymes in erythrocytes of the patients. It is possible that these enzymes have been removed from the erythrocytes as a result of their lysis caused by reperfusion injury. It has been shown that during ischaemia-reperfusion injury, endogenous SOD, GPx and catalase activities seem unable to cope with excessive production of superoxide radical and H_2O_2 [29]. Excess H_2O_2 can cause degradation of the haem rings of haemoglobin, releasing iron which is capable of stimulating OH production and lipid peroxidation in erythrocytes [30]. There is a possibility that decrease in the activity of SOD is due to the inhibition of the enzyme by excess H_2O_2 formed [31] or by decreased production of SOD or damage of the enzyme, thereby rendering an individual more susceptible to oxidative damage due to non clearance of free radicals and their further propagation through chain reactions. The lowered activities of both free radical scavenging enzymes might be the consequence of the damage of these enzymes during ischaemia/reperfusion conditions and thus contribute to the decrease in the anti-oxidant defence system. This could lower the scavenging of OFR's, which might result in enhanced atherosclerosis. OFR's may render haemoglobin unstable since oxidative conditions tend to produce haemichromes which release their haem to the erythrocyte membrane with consequent lipid peroxidation and cell lysis [30].

Decrease in the capability of the anti-oxidant defence system is further suggested by the significantly lowered level of non-enzymatic anti-oxidants i.e. ascorbic acid and total thiols in the patients. Ascorbic acid is the only most effective anti-oxidant in human RBC's capable of completely inhibiting lipids against detectable peroxidative damage induced by aqueous peroxyl radicals. It has chain breaking properties, reacts directly with superoxide ions, hydroxyl radicals and singlet oxygen and also acts to regenerate tocopherol [32]. Ascorbic acid exerts a protective effect against the per-oxidative damage of lipids in hypercholesterolemic rats [33].

Table I. Levels of anti-oxidants and malondialdehyde (MDA) in the RBC's and ascorbic acid in plasma in healthy persons and patients.

Cases	**SOD** (unit/mg protein)	**Catalase** (unit/mg protein)	**MDA** (nMole/ml RBC lysate)	**Total thiol** (μM RBC lysate)	**Ascorbic acid** (mg/dl plasma)
Healthy (n = 40)	2.90 ± 0.68	3.66 ± 0.93	0.69 ± 0.30	560.79 ± 115.05	0.40 ± 0.26
Patients (n = 30)	1.37 ± 0.50	1.66 ± 0.58	1.33 ± 0.33	375.85 ± 149.19	0.15 ± 0.07
p value	<0.0005	<0.0005	<0.01	>0.01	<0.0005

The values reported are mean ± SD; n = number of cases studied; p = data between healthy individuals and patients; $p > 0.01$ not significant, $p < 0.01$ significant, $p < 0.05$ highly significant.

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It also increases the number of LDL receptors on arterial smooth cells, thereby facilitating lipoprotein cholesterol clearance [34] and thus decreasing atherosclerosis. A low level of ascorbic acid in patients reperfused after myocardial infarction was observed, and this may be linked to increased consumption of ascorbic acid due to increased oxidant stress, as evident from higher MDA level, or it may be oxidized into dehydroascorbate under reperfusion conditions. In such patients, ascorbic acid supplementation might help in providing the necessary protection needed for balancing the deleterious effects of free radicals.

Decreased levels of total thiols (though statistically not significant) in RBC's also indicate the effect of oxidative stress on protein molecules during reperfusion. Like plasma, a major part of the thiols in RBC's are derived from proteins and a smaller part from free thiols. Oxidative stress may modify phospholipids and proteins leading to lipid-peroxidation and oxidation of thiol groups [35]. These changes are considered to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins during reperfusion. Since it is well known that GSH can either chemically [36] or enzymatically [37] reduce dehydroascorbic acid (DHA) to ascorbate and this reaction has been shown to occur in erythrocytic lysate [38], the lower level of total thiols would be responsible for reduced conversion of DHA to ascorbate so that the observed level decreases after reperfusion conditions.

This study brings into focus the significance of ROS homeostasis in reperfusion injury. The biochemical evidence strongly suggests that the components of reperfusion injury, identified in the laboratory, are of practical importance. The exact mechanism of reperfusion injury is uncertain, but probably includes cellular overload of calcium, osmotic cell swelling and myocyte or microvascular damage from cytotoxic free radicals derived from oxygen. The evidence for this last mechanism comes from interventional studies designed to show whether agents targeting the formation of free radicals might protect against reperfusion injury [39].

There is a distinct trend which suggest that higher oxidant stress and diminished anti-oxidant status along with higher MDA levels constitute the key factors in the progression of reperfusion injury. Hence, a management strategy aiming at simultaneous maintenance of oxidant /anti-oxidant homeostasis and control of lipid per-oxidation in patients reperfused after myocardial infarction is envisaged which could be facilitated through supplementation with ascorbic acid.

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