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Original article

L-Arginine is effective in scavenging oxygen derived free radicals in the patients of ischemic heart diseases

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ABSTRACT

Ischemic heart is a leading cause of death all over the world. Therapeutic role of L-arginine in overcoming the oxidative stress during myocardial ischemia is still not clear. In the present work, effect of L- arginine supplementation to the patients of acute myocardial infarction has been studied. Subjects were randomly divided into four groups: control, control with L-arginine supplementation, patients with acute myocardial infarction (AMI) and patients with acute myocardial infarction with L-arginine supplementation. Subjects were given oral dose of L-Arg (3g/day) for fifteen days. Oxidative stress indices [Xanthine oxidase (XO) and malondialdehyde (MDA)] and antioxidant indices [Superoxide dismutase (SOD) and total thiols (T-SH)] in the erythrocytes and serum cardiac markers CPK-MB and Troponin-T were monitored before and at the end of L-arginine therapy. L-arginine supplementation increased T-SH content significantly in treated patients ($p<0.001$). XO activity, upon L-arginine supplementation, decreased by 31% ($p<0.005$) in the patients. Also, we noted significant reduction in the levels of serum cardiac markers; CPK-MB and Troponin T in the AMI patients receiving L-arginine therapy ($p<0.001$)

1. Introduction

Acute myocardial infarction (AMI) is one of the major causes of mortality and morbidity in the world [1]. The most common cause of AMI is atherosclerotic coronary artery disease (CAD) with erosion or rupture of a plaque causing transient, partial or complete arterial occlusion. Evidences suggest that reactive oxygen species (ROS) play important role in the pathogenesis of myocardial infarction [2]. It has been shown that L-arginine serves as a precursor to the synthesis of nitric oxide (NO), which has various physiological properties including vasodilatation, inhibition of platelet aggregation, neutrophil adhesion, scavenging superoxide ($O_2^{\cdot}$) radical and inhibition of xanthine oxidase (XO) activity [3]. L-arginine has been shown to have protective role against reactive oxygen species (ROS) attack as a result of its interaction with $O_2^{\cdot}$ [4, 5]. There are evidences that L-arginine is closely linked to protective properties against oxidative stress [6]. Oxygen derived free radicals, the major cause of oxidative stress, are capable of damaging the compounds of all biochemical classes, including nucleic acids, proteins, lipids, lipoproteins, carbohydrates and connective tissue macromolecules etc. [7]. Generation of reactive oxygen species and their neutralization by antioxidants are at equilibrium in healthy organisms and disturbance in this homeostasis causes numerous diseases including coronary vascular diseases. Reactive oxygen species can be produced extra-cellularly via the respiratory burst in activated neutrophils, leakage of electron transport chain and/or the enzymatic reactions such as that of xanthine oxidase [8]. In the whole blood, superoxide radicals $O_2^{\cdot}$ and H_2O_2 released into the plasma can easily diffuse into the erythrocytes. $O_2^{\cdot}$ can pass through the anion channel in the erythrocyte membrane [9] and H_2O_2 can cross the cell membrane readily by simple diffusion [10]. If erythrocytes really act as scavengers for ROS produced during myocardial infarction, antioxidant enzymes in erythrocytes are very important for preventing the ROS induced damage. The antioxidant activity in tissues is manifested by antioxidant scavenger system, which includes enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), non-enzymatic antioxidants like thiols, vitamins (C, A, E etc.) and carotenoids [11-13]. Increased oxidative stress and generation of oxygen derived free radicals results in modification of LDL that leads to atherosclerotic plaques [14]. Disruption of an atherosclerotic plaque in an epicardial coronary artery leads to a clotting cascade, which sometimes results in total occlusion of the artery. When a severe plaque rupture occurs in the coronary vasculature, it may lead to myocardial infarction [15]. One popular method for removal of the infarcts is reperfusion.

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Early reperfusion by thrombolytic drugs is now accepted as an effective treatment of acute myocardial infarction (AMI), both to restore coronary function and to limit myocardial damage [16]. However, reperfusion may result in transient or permanent myocardial injury (reperfusion injury) assumed to be free radical mediated [16-19].

Oxygen derived free radicals produce per-oxidation of membrane lipids and proteins with structural and functional changes. These mechanisms can explain some manifestations of the reperfusion injury such as myocardial stunning and reperfusion arrythmias [17-19]. In addition, accumulation of polymorphonuclear (PMN) cells may initiate the reocclusion of damaged vessels, explaining why there is only a partial success of revascularization procedures [18]. Enhanced lipid peroxidation products could measure increased free radical production. Some studies have reported that L-arginine supplementation could control enhanced lipid peroxidation [20] and attenuate xanthine oxidase activity, which is the major enzymatic source of oxygen, derived free radicals [8]. In the present study we have tried to find out whether L-arginine could really be beneficial to the patients of acute myocardial infarction.

2. Materials and Methods

Patients from Cardiology department, CSM Medical University and Balarampur hospital, Lucknow, U.P., India were the candidates enrolled in the study. The study was cleared by the departmental ethical committee. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 declaration of Helsinki. Patients hospitalized with confirmed diagnosis of myocardial infarction (ST segment elevation/ non ST segment elevation) before reperfusion therapy were included in the AMI group of the study (n=50). These patients had increased levels of CPK-MB and Troponin T. The criteria for exclusion included patients with previous cardiovascular or other organic diseases such as diabetes, chronic renal failure, left ventricular failure, chronic obstructive pulmonary diseases (COPD), previous history of surgery or trauma and those planned for coronary revascularization. After having obtained the consent from the patients, their baseline data were collected by staff nurses in standardized format (Tables 1 and 2).

Table 1. Personal profile of the cases included in the study.

Parameter	Control Subject	AMI Patients
N	65	65
Age (year)	58 ± 2.5	63 ± 3.9
Weight	60.4 ± 2.4	75.2 ± 5.9
Sex (Male/ Female)	43 / 12	34 / 16
Systolic blood pressure (mm of Hg)	117 ± 3	157 ± 4
Diastolic blood pressure (mm of Hg)	85 ± 2	98 ± 3

AMI =Acute myocardial infarction. Values are mean ± SE.

These patients were given oral dose of L-arginine (3g/day) for fifteen days along with regular medications which included β-blockers, statins, ACE inhibitors and aspirin. The patients receiving L-arginine therapy were included in the AMI+Arg group (n=40) of the study. The study also included 65 non- diabetic, non-smoking healthy subjects serving as control. The control subjects were also given L-arginine therapy (3g/day) and included in the C+Arg group (n=55) of the study. The dose selection for the study was as recommended by Fried et al [21] and Adams et al [22]. Higher doses lead to problems like dizziness, headache, nausea etc while low doses didn't provide significant results.

Table 2. Metabolic markers of the subjects under study.

Parameter	Control Subject	AMI Patients
Glucose (mg /dl)	87.29 ± 1.99	148.70 ± 6.15 [a]
Total cholesterol (mg/ dl)	170.40 ± 4.25	237 ± 5.15 [a]
Triglyceride (mg/dl)	79.81 ± 3.69	195.70 ± 5.58 [a]
HDL-cholesterol (mg /dl)	47.45 ± 3.12	31.73 ± 1.98 [a]
LDL-cholesterol (mg /dl)	108.30 ± 7.03	134.0 ± 2.58 [b]

AMI = Acute myocardial infarction.
Values are mean ± SE, n = number of cases.
a $p < 0.001$ vs Control, b $p < 0.01$ vs Control.

2.1 Chemicals

All the chemicals employed in the study were AnalaR grade of Qualigens and the biochemicals employed were procured from Sigma Chemical Co. USA.

2.2. Lysate preparation

4.0 ml venous blood was withdrawn and collected 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 2000xg for 20 min at 4^0C. The pellet containing RBCs was washed thrice with ice cold, 0.85% NaCl on a centrifuge at 2000xg for 10 min at 4^0C. The final pellet was taken up in 5.0 ml chilled water, left in cold for 1 hr for hemolysis and the suspension was then recentrifuged. The supernatant, thus obtained, was used for analysis after suitable dilution as and when needed. The following parameters were studied in the erythrocytes of the patients and the control subjects.

2.3. SOD activity

SOD (E.C.1.15.1.1) activity was determined by the method of Misra and Fridovich [23]. 3 ml of the reaction mixture consisted of 1.5 ml of 100 mM carbonate buffer, pH 10.3; 0.01ml of 30 mM EDTA (ethylene di amine tetra acetic acid), suitable aliquot of enzyme preparation and water to make up the volume to 2.94 ml. The reaction was started by addition of 0.06 ml of 15mM epinephrine. Change in absorbance was recorded at 480 nm for 1 min at 15 sec intervals. Control consisting of all the ingredients, except enzyme preparation, was run simultaneously. One unit of enzyme activity has been defined to cause 50% inhibition of auto-oxidation of epinephrine present in the assay system by 1 ml enzyme preparation.

2.4. XO activity:

XO (E.C.1.2.3.2) activity was determined by the method of Fried and Fried [24]. The reaction mixture consisted of 0.9 ml of 100 mM phosphate buffer, pH 7.8; 0.75 ml of 10mM EDTA; 0.15ml of 0.2mg/ml phenazine methosulphate (PMS); 0.45 ml of 4mg/ml solution of nitroblue tetrazolium (NBT); suitable aliquot of enzyme preparation in the linearity range and water to make up the volume to 3.0 ml. The reaction was started at 37^0C with the

addition of 0.5ml of 1mM Xanthine. Change in absorbance was recorded at 540 nm for 1 min at 15 sec intervals. Extinction coefficient of the reduced NBT at 540 nm is 7.2 μMcm^{-1}. One unit of enzyme activity has been defined as the amount of enzyme that converts 1µmole of xanthine to uric acid in one minute at specified conditions of assay.

2.5. LPO levels

Lipid peroxidation (LPO) was measured in the form of MDA by the method of Ohkawa, Ohishi and Yagi [25], using thiobarbituric acid (TBA) reagent. To 0.05 ml lysate, in a Folin tube, was added 0.2 ml, 8.1% (w/v) sodium dodecylsulphate (SDS); 1.5 ml, 20% (v/v) acetic acid; 1.1 ml, 0.8% (w/v) TBA and water to make up the volume to 3.0 ml. The tubes were heated in a water bath at 90^0C for 1 hr and cooled immediately under running tap water. To each tube, 1.0 ml chilled water and 5.0 ml butanol: pyridine mixture (15:1 v/v) was added, vortexed and centrifuged at 800xg for 20 min. The upper layer was aspirated out and color intensity measured at 532 nm. 1,1,3,3-tetra ethoxy propane (TEP) was used as reference.

2.6.T-SH content

Total thiols (T-SH) were estimated by the method of Hu [26] using Ellman's reagent. In final volume of 4.0 ml, was added 0.05 ml lysate and 0.6ml of 20mM tris-HCL buffer, pH 8.2, followed by addition of 0.04 ml, 10mM 5,5'dithiobis- (2-nitrobenzoic acid) (DTNB) in absolute methanol and 3.31 ml absolute methanol. The tubes were capped and left at room temp for 15 min to develop color. The tubes were then centrifuged at 3000xg for 20 min. Supernatant was aspirated and absorbance measured at 412 nm. Molar extinction coefficient of 13,600 M cm^{-1} was used to calculate the total thiol content.

2.7. CPK-MB and Troponin T

Assay of these markers of cardiac damage were carried out in an ISO- 9001:2000 certified lab.

2.8. Protein estimation

Protein was measured by the method of Lowry, Rosebrough, Farr and Randal [27] using Folin phenol reagent. Bovine serum albumin was used as standard. Specific activity of the enzymes has been defined as activity/mg protein

2.9. Statistical analysis

The results are expressed as mean ± SE. One way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison tests has been applied to test the significance of the data. A p value <0.05 was considered to be statistically significant.

3. Results:

SOD activities of AMI group was found to be significantly reduced (27%) as compared to that of the control (C) group (p<0.001). The AMI+Arg group had SOD activity greater by 11% but not significant as compared to those of AMI group (Table 3). The XO activity of the AMI group was found to be elevated by 143%, as compared to that of C group (p<0.001). The AMI+Arg group had significantly lower XO activity (31%) compared to those with AMI group (p<0.005). As compared to C group, the AMI+Arg group showed a significant decrease in its XO activity (p<0.001) (Table 3).

The MDA levels in erythrocytes of AMI group showed an increase of 92% when compared with the C group (p<0.001). Furthermore, the AMI+Arg group had lower MDA levels (8%) when compared to the AMI group but the change was statistically not significant (Table 4). The T-SH content in erythrocytes of AMI group was found to be decreased by 76% when compared to the C group (p<0.001). L-arginine supplementation to patient group resulted in significant increase in T-SH (36%) when compared to the AMI group (p<0.001). However, the AMI+Arg group had significantly higher T-SH concentration as compared to the C group (p<0.001) (Table 4).

Table 3. Effect of L-arginine supplementation on antioxidant enzyme superoxide dismutase (SOD) and pro-oxidant enzyme xanthine oxidase (XO) activities in erythrocytes of the subjects

Parameter	Control Subject		AMI Patients	
	C (n = 65)	C+Arg (n = 55)	AMI (n = 50)	AMI+Arg (n = 40)
SOD (U/mg protein)	8.46 ± 0.34[a]	9.46 ± 0.50	5.4 7 ± 0.22[b]	6.16 ± 0.24 c
XO (U/mg protein)	1.45 ± 0.10	1.05 ± 0.15	3.53 ± 0.19[b]	2.46 ± 0.19[c, d]

Values are mean ± SE; n = no. of cases, AMI = Acute myocardial infarction, Arg = L-arginine
[a] p<0.05 vs C + Arg, [b] p<0.001 vs [C], c p<0.001 vs [C], [d] p<0.005 vs AMI

Table 4. Effect of L-arginine on the levels of malondialdehyde (MDA) and total thiols (T-SH) in erythrocytes of the subjects.

Parameter	Control Subject		AMI Patients	
	C (n = 65)	C+Arg (n = 55)	AMI (n = 50)	AMI+Arg (n = 40)
MDA (n mole/ml)	48.01 ± 2.45[a]	36.11 ± 2.63	91.70 ± 3.53[b]	84.35 ± 3.24[c]
T-SH (n mole/ml)	4.90 ± 0.28	5.45 ± 0.49	1.21 ± 0.10 b	1.91 ± 0.13[c, d]

Values are mean ± SE; n = no. of cases, AMI = Acute myocardial infarction, Arg = L-arginine.
[a] p<0.01 vs C+Arg, [b] p<0.001 vs C, [c] p<0.001 vs C, [d] p<0.001 vs AMI

Table 5 shows the levels of cardiac markers. There is significant increase in the levels of these markers in AMI patients when compared to the control subjects (p<0.001). However, following L-arginine therapy, significant reduction was observed in the levels of these markers in the AMI+Arg group when compared to the AMI group and this reduction was 45% (p<0.001) in case of CPK-MB and 56% (p<0.001) in case of Troponin- T respectively.

Table 5. Effect of L-arginine therapy on the levels of serum cardiac markers.

Parameter	Control Subject		AMI Patients	
	C (n = 65)	C+Arg (n = 55)	AMI (n = 50)	AMI+Arg (n = 40)
CPK-MB (ng/ml)	0.58 ± 0.018	0.35 ± 0.021	5.36 ± 0.34 a	2.92 ± 0.27[b, c]
Trop- T(ng/ml)	0.09 ± 0.004	0.05 ± 0.008	0.93 ± 0.021[a]	0.41 ± 0.023[b, c]

Values are mean ± SE; n = no. of cases, AMI = Acute myocardial infarction, Arg = L-arginine.
[a] p <0.001 vs [C], [b] p<0.001vs AMI, [c] p<0.001vs C
Lab reference range for serum CPK-MB is 0.100-4.9 ng/ml.
Lab reference range for serum Troponin-T is <0.40 ng/ml.

4. Discussion

Myocardial ischemia results from inadequate oxygen supply to the myocardium. A number of studies have shown free radical mediated damage during ischemia-reperfusion induced oxidative stress [28]. The present study assessed the effect of oral L-arginine supplementation on myocardial ischemia (represented by acute myocardial infarction in the present study) and on modulations of cardiac oxidant-antioxidant homeostasis. Inadequate oxygen supply to the myocardium during ischemia leads to the conversion of xanthine dehydrogenase in to xanthine oxidase, which upon reperfusion catalyzes the conversion of hypoxanthine to xanthine, with the concomitant production of oxygen free radicals. The importance of this pathway is related primarily with the level of ATP in heart tissue. Under normal conditions, xanthine dehydrogenase (XOD) form of the enzyme uses NAD+ as the electron acceptor in the conversion of hypoxanthine to xanthine and uric acid. During ischemia, ATP is degraded to AMP, inosine and hypoxanthine successively and xanthine dehydrogenase undergoes selective proteolytic conversion to form xanthine oxidase leading to excessive generation of superoxide radicals [29-31]. In the present study activity of XO showed marked increase in the patient when compared with the healthy subjects. L-arginine is a physiological precursor of nitric oxide (NO). Various studies have shown that NO is a biologically important molecule and has many modulatory functions for cells and tissues. NO has been shown to be cardioprotective in ischemia and reperfusion [32, 33]. Furthermore, NO may exert direct effect on cardiac myocytes [34]. The inhibition of XO activity by NO may be mediated through direct binding of NO to the enzyme's Fe-S moiety [35]. Another study also supported the notion that NO may suppress XO activity [36]. Hence, the protective role of NO could be due to its property of scavenging free radicals and inhibiting XO, this is in accordance with the present study.

SOD, the free radical scavenging enzyme, provides the first line of cellular defense against oxidative injury, decomposing $O_2^{\cdot}$ before they interact to form more reactive hydroxyl radicals. The enzyme protects the red blood cells against free radical mediated lipid per-oxidation [37]. We observed decreased activity of SOD in the erythrocyte of AMI patients. This observation is again in accordance with some earlier findings [38, 39]. During ischemia, the lowered activity of SOD fails to cope with excess free radical production which in turn causes the degradation of heme rings of hemoglobin, releasing iron that is capable of stimulating $OH^{\cdot}$ production and lipid per-oxidation in erythrocytes [40].

Extent of lipid per-oxidation, measured in the form of MDA levels, was found to be increased in the erythrocytes of the patients when compared to the healthy subjects clearly indicating the free radical mediated damage in the patients. Decrease in the activity of SOD in the patient's erythrocytes may be due to the inactivation of the enzyme by cross linking or due to exhaustion of the enzyme by increased lipid per-oxidation [41]. L-arginine supplementation, however, increased the activity of SOD in the treated groups and hence the MDA level was found to be decreased upon L-arginine supplementation. This again shows that L-arginine protects the myocardial tissue from ROS-mediated damage. Similar findings have been reported earlier also [42]. Since SOD, CAT and GPx constitute a mutually supportive team of enzymes that provide defense against the intermediaries of ROS, hence increased SOD activity in arginine supplemented patients would be beneficial to overcome oxidative stress.

Thiols are major non-enzymatic antioxidant and have been reported to play an important role in protecting the myocardium from ischemia and reperfusion induced injury [42]. Major part of thiols in RBC's are derived from proteins. During ischemia the increased formation of free radicals causes excess protein oxidation and hence the total thiol content was found to decrease in the patients. L-arginine supplementation improved the levels of total thiols in the patients under consideration. This finding is again in accordance with our previous work [43, 44]. Creatine kinase (CK) and particularly its isoenzyme CPK-MB normally exist in cellular compartments and leaks out into the plasma during myocardial injury due to disintegration of contractile elements and sarcoplasmic reticulum [45, 46]. Troponin T and I are the proteins of troponin regulatory complex involved in cardiac contractility. Both CPK-MB and Troponins are highly sensitive and specific markers of myocardial damage and therefore are preferred for diagnosis of myocardial infarction [46, 47]. In the present study, we observed the increased levels of CPK-MB and Troponin T in the patients of AMI when compared to the healthy control. L-arginine supplementation resulted in a decrease in the levels of these markers. This indicates that L-arginine therapy tends to reduce the damage caused during myocardial infarction.

5. Conclusion

Present study shows a significant increase in pro-oxidant indices and cardiac markers in the patients with acute myocardial infarction. A significant decrease in antioxidant status has been observed in AMI patients, thus causing an imbalance between oxidant and antioxidant parameters during AMI. The present study also indicates that several oxidant and anti-oxidant parameters are positively improved upon administration of L-arginine to the patients of AMI. L-arginine administration, therefore, may be used as an adjuvant therapy. Moreover, the present study also shows that the parameters studied are favorably improved upon administration of L-arginine to the healthy persons therefore its administration, in low doses, can be used as a preventive measure in elderly persons against precipitation of ischemic myocardial syndromes without any untoward side effects as L-arginine is a naturally occurring conditionally essential amino acid to human beings.

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