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Protective Effect of L-Arginine in Experimentally Induced Myocardial Ischemia: Comparison With Aspirin

Alaaeldin I. Saleh, MSc¹, Sahar M. Abdel Maksoud, PhD¹, Shohda A. El-Maraghy, PhD², and Mohamed Z. Gad, PhD¹

Abstract

Objective: Coronary artery diseases including myocardial ischemia (MI) remain one of the leading causes of death worldwide. This study was designed to compare the protective effect of L-arginine versus aspirin from the biochemical changes associated with MI injury.

Experimental design: Four groups of male New Zealand white rabbits were investigated. Normal group (n = 8) rabbits were fed standard chow pellets, untreated MI group (n = 16), where hypercholesterolemia was induced by feeding the animals with a diet containing 2% cholesterol for 28 days, L-arginine group (n = 12) rabbits were fed a 2% cholesterol-enriched diet in conjunction with L-arginine (2.25 g %) in drinking water for 28 days, and aspirin group (n = 12) rabbits were fed 2% cholesterol-enriched diet in conjunction with aspirin administered orally (0.7 mg/kg per d) for 28 days. After 28 days, MI was induced in all groups, except the normal group, by a single subcutaneous (sc) injection of isoproterenol hydrochloride (0.2 mg/kg body weight [bw]). Animals were sacrificed 6 hours later. Results: Our results showed that L-arginine was more effective than aspirin in reducing platelet aggregation, reducing low-density lipoprotein (LDL) oxidizability, preventing aortic intimal thickening, and maintaining histological architecture of the myocardium. Both drugs, however, had similar positive effects on plasma fibrinogen levels and on the prevention of myocardial release of cardiac troponin I and creatine kinase-MB. The effect on hypercholesterolemia was insignificant for both drugs. Aspirin was more effective than L-arginine in prolonging prothrombin time. Conclusion: L-arginine supplementation represents a potentially novel nutritional strategy for preventing and treating coronary artery diseases especially in cases of aspirin resistance and/or hypersensitivity.

Keywords
myocardial ischemia, hypercholesterolemia, isoproterenol, aspirin, L-arginine

Introduction

Myocardial ischemia (MI) is caused by an insufficient supply of oxygen-rich blood to the myocardium caused by increased myocardial substrate demand and/or the narrowing or closure of coronary arteries.¹ Lack of oxygen and metabolic substrates rapidly decreases the energy available to the cell and leads to cell injury that might be reversible or irreversible in nature. One of the most widely used experimental models to study MI and the therapeutic effects of drugs is isoproterenol-induced MI.² Isoproterenol (isoprenaline) hydrochloride is a synthetic sympathomimetic amine that is structurally similar to epinephrine but selective for β receptors, activating β₁ and β₂ receptors equally, and exerting positive inotropic and chronotropic effects on the heart.³ Indeed, pathophysiological changes following isoproterenol administration are comparable to those taking place in human MI/infarction.⁴

L-Arginine is a semi-essential amino acid found in most mammals. Under conditions of stress and injury, L-arginine is considered an essential amino acid. It is the precursor of endothelial nitric oxide (NO) that plays a critical role in cardiovascular protection⁵ and in maintaining an active vasodilator tone in healthy blood vessels.⁶ Several studies in humans and experimental animals indicated that oral L-arginine intake has multiple beneficial cardiovascular effects when taken in doses larger than normally present in diet (reviewed in ref 7). The positive role of L-arginine in cardiovascular health is due to both NO-dependent and NO-independent effects.⁸ It has been shown that both oral and parenteral administration of L-arginine can
increase NO synthesis in various tissues.\textsuperscript{9} In hypercholesterolemia, the synthesis and/or release of NO is severely impaired, leading to attenuated endothelium-dependent vasodilation, enhanced platelet aggregation, intimal thickening, and subsequent development of atherosclerosis.\textsuperscript{10} The impairment of endothelial NO liberation in hypercholesterolemia is related to low-density lipoprotein (LDL) cholesterol uptake into the endothelium,\textsuperscript{11} and both native and oxidized LDLs are reported to interfere with the biological activity of NO in vitro.\textsuperscript{12} Evidence suggests that L-arginine may play a therapeutic role in tissue preservation during reperfusion after a period of hypoxia, as animal studies showed protective effects of L-arginine administration against ischemia/reperfusion injuries in several tissues, including the myocardium.\textsuperscript{13}

Among the traditional medicines used for cardiovascular disease (CVD) is aspirin, the prototype of traditional nonsteroidal anti-inflammatory drugs (NSAIDs), which had been officially approved by the US Food and Drug Administration (FDA) in 1939. The history of aspirin use in protection from CVD started in the 1950s, after the appearance of evidence that aspirin prolongs prothrombin time (PT).\textsuperscript{14} During the 1960s, aspirin was found to inhibit thromboxane A\textsubscript{2} synthesis that enhances platelet aggregation.\textsuperscript{15} Today, use of low-dosage aspirin is a common practice in the prophylaxis and treatment of CVD.\textsuperscript{16} This study is among the few studies that address the potential role of L-arginine in the prevention of MI injury. The molecular mechanisms behind this prevention were investigated and compared to the widely used cardiovascular protective agent, aspirin.

Materials and Methods

Animals
A total of 48 male New Zealand white rabbits weighing 1.55 to 1.95 kg were used in the study. They were kept in standard rabbit cages in the animal house of the Faculty of Pharmacy, Cairo University. The animals were adapted for 10 days prior to experimental use. All rabbits were maintained at almost constant environmental conditions throughout the study with free access to food and water. The study protocol was approved by the local ethics committee.

Drugs and Chemicals
L-Arginine powder (NOW FOODS, Bloomingdale, Illinois), Cholesterol fine powder (Sigma-Aldrich, Germany), Isopropyl alcohol hydrochloride ampules (0.2 mg/mL, Monico SPA, Italy), Aspirin tablets (acetylsalicylic acid 75 mg, CID, Egypt), and Tween 80 (Advic, Egypt) were used from the indicated manufacturers. All other reagents and chemicals were of high analytical grade.

Experimental Protocol
After the initial adaptation, rabbits were divided into four groups: group I, normal group of 8 rabbits fed standard chow pellets; group II, myocardial ischemic (untreated MI) group of 16 rabbits fed 2\% cholesterol-enriched diet for 28 days\textsuperscript{17}; group III, L-arginine group of 12 rabbits fed 2\% cholesterol-enriched diet in conjunction with L-arginine in drinking water (2.25 \text{g} \% ) for 28 days\textsuperscript{18}; and group IV, aspirin group of 12 rabbits fed 2\% cholesterol-enriched diet in conjunction with orally administered aspirin (0.7 mg/kg per d) for 28 days.\textsuperscript{19} After completion of the experimental period, animals in the untreated MI, L-arginine, and aspirin groups were then injected with a single bolus subcutaneous (sc) injection of isoproterenol hydrochloride (0.2 mg/kg body weight [bw])\textsuperscript{20} and sacrificed 6 hours later. Control group rabbits were injected with vehicle and were sacrificed 6 hours later. All animals were subjected to a 12-hour fast, with free access to water and were anesthetized with intravenous injection of 20 mg/kg bw thiopental sodium\textsuperscript{21} before killing.

Sample Collection
Fasting blood samples from each rabbit ear vein were drawn before killing, for the preparation of serum or plasma. Later, hearts were removed and used for histopathological examination and determination of aorta intimal thickness.

Biochemical Analysis
Commercially available kits were used for the determination of serum total cholesterol ([TC]; Diamond Diagnosis Co, Germany),\textsuperscript{22} serum creatine kinase-MB activity ([CK-MB]; Greiner Diagnostics GmbH, Germany),\textsuperscript{23} cardiac Troponin I ([cTnI]; DRG International Inc, Mountainside, New Jersey),\textsuperscript{24} PT, and fibrinogen measurements (Diagnostica stago, France).\textsuperscript{25} Spectrophotometric measurements were performed using a Jasco UV/VIS spectrophotometer (Japan) and a microtiter well reader Perkin Elmer (Perkin Elmer, Waltham, Massachusetts). In the LDL oxidizability experiment, metal ion copper was used for measuring the susceptibility of LDL and very-low-density lipoprotein (VLDL) to become oxidized.\textsuperscript{26} Plasma was used for the determination of platelet aggregation using a dual channel platelet aggregometer (Chrono-log, Havertown, Pennsylvania).\textsuperscript{27}

Histological Assessment
Autopsy samples were taken from the heart’s myocardium, coronaries, and aorta of rabbits and fixed in 10\% formal saline. Samples were then subjected to histopathological examination and intimal thickness measurement.\textsuperscript{28} For each animal, 10 specimens from the aorta were subjected to measurement of intimal thickness (\textmu m) by a Leica image analyzer. The mean of the 10 sections represented the intimal thickness of the animal. The average intimal thickness for all animals of the group was then calculated to represent the mean of intimal thickness for each group. These measurements were done in the Histology department, Faculty of Medicine, Cairo University.
differences were observed between the results of aspirin and PT was significantly lowered by 51% of total cholesterol (mg/dL). Data are expressed as mean ± SEM. Number of animals were 8, 16, 12, and 12, respectively, for normal, untreated MI, aspirin, and L-arginine groups. a indicates significant difference from normal at P < .05; MI, myocardial ischemia; SEM, standard error of the mean.

Statistical Analysis
Results are expressed as mean ± standard error of the mean (SEM). For statistical analysis of the data, multiple comparisons were carried out using 1-way analysis of variance (ANOVA) followed by Tukey-Kramer test for post hoc analysis. Statistical significance was accepted for a level of P < .05.

Results
Biochemical Changes

Hypercholesterolemia. Administration of 2% cholesterol-enriched diet resulted in significant rise in serum levels of TC in untreated MI, aspirin, and L-arginine groups as compared to the normal group.

No significant difference was observed between the means of untreated MI, aspirin, and L-arginine groups (Figure 1).

Markers of MI. Cardiac markers CK-MB and cTnI in the untreated MI group were significantly increased over the normal group (Figure 2A, B). Administration of aspirin or L-arginine produced a significant decrease in cTnI level by 48% and 50%, respectively (P = .016 and P = .0153) and in CK-MB activity by 43% and 44%, respectively (P = .048 and P = .04), as compared with untreated MI group. No significant differences were observed between the results of aspirin and L-arginine; both drugs reduced CK-MB and cTnI to control group levels (Figure 2A, B).

Blood clotting parameters. Platelet aggregation and fibrinogen levels were significantly elevated by 57.4% and 52.5% in the untreated MI group as compared with the normal group (P = .006 and P = .00127, respectively; Figure 3A, B, C). However, PT was significantly lowered by 51% (P = 7 × 10⁻⁵).

Administration of aspirin produced a decrease in platelet aggregation by 49% (P = .0014), a decrease in fibrinogen by 30% (P = .002), and an increase in PT by 122% (P = 2 × 10⁶) as compared with untreated MI group. All changes were statistically significant (P < .05). L-arginine administration also resulted in significant changes of these parameters. Platelet aggregation decreased by 61% (P = 3 × 10⁻⁵), fibrinogen decreased by 24% (P = .0713), and PT increased by 59% (P = .00142). Comparing the results of aspirin with those of L-arginine revealed that L-arginine was more effective in lowering platelet aggregation (P = .0196), whereas aspirin in the dosage used was more powerful in extending PT (P = .0079). No significant differences were observed for the decrease in fibrinogen. Platelet aggregation in the L-arginine group was even below the normal group, despite cholesterol-rich diet (Figure 3A), whereas PT in the L-arginine group improved compared to the untreated MI group but remained below normal levels (Figure 3C). Differences from normal group was observed for L-arginine in platelet aggregation results (P = .0346) and in PT (P = .0254). Other results for aspirin and L-arginine in blood clotting parameters were insignificantly different from those of normal results.

Low-density lipoprotein oxidizability. Low-density lipoprotein oxidizability in the MI group showed an increase compared to the normal group, which was, however, not statistically significant (P = .45; Figure 4). Aspirin did not elicit significant changes in LDL oxidizability (P = .0633 and P = .201, respectively, as compared to normal and untreated MI groups). In contrast, administration of L-arginine produced a significant decrease in LDL oxidizability reaching 57% of normal (P = .017), 32% of untreated MI (P = .008), and 43% of aspirin (P = 6 × 10⁻⁶).

Histopathological Changes

Aortic intimal thickness. Aortic intimal thickness in the normal group was 5.96 ± 0.38 μm (Figure 5). A significant increase in the intimal thickness in the untreated MI group was observed with a mean of 71.59 ± 11.3 μm (P = 6.6 × 10⁻⁶). Administration of aspirin or L-arginine reduced aortic intimal thickness significantly to 52.88 ± 7.5 and 9.86 ± 1 μm as compared with untreated MI group (P = .0056 and P = 9.6 × 10⁻⁶, respectively). In the L-arginine group the decrease of the intimal thickness was more pronounced than in the aspirin group (P = 5.3 × 10⁻⁶). Indeed, aortic intimal thickness in the L-arginine group was close to normal levels.

Histopathological examination of heart, coronaries, and aorta. Examination of sections from the normal group revealed no histopathological findings in the heart, coronaries, and aorta (Figures 6-8).

In contrast, sections from the untreated MI group showed ischemia in myocardial muscle cells characterized by granular degeneration and hyalinization (d in Figure 6) as well as focal inflammatory cell infiltration and fatty changes (v in Figure 6) between the myocardial bundles. Examination of coronaries showed severe congestion (Figure 7) and swelling of the...
The current study aimed to investigate the protective effect of L-arginine in comparison to aspirin from the biochemical changes caused by hypercholesterolemia-associated MI. Several studies have shown that acute hypercholesterolemia increases the severity of MI. Experimental ischemia and hypercholesterolemia were induced in our study by sc injection of isoproterenol to rabbits that had been fed a 2% cholesterol-enriched diet for 28 days. Isoproterenol produces relative ischemia or hypoxia due to myocardial hyperactivity and coronary hypotension, associated with cytosolic Ca^{2+} overload. This method seems to have an advantage over the method using coronary ligation and reperfusion, as it is nonsurgical and results in very small morbidity or mortality. The cholesterol-rich diet caused a significant increase in serum TC in the untreated MI, aspirin, and L-arginine groups as compared to the normal group. Similar results have been reported previously by many other groups.

Isoproterenol-induced MI was verified by significant increases in the serum levels of the ischemic markers CK-MB and cTnI in the untreated MI group. Hypercholesterolemia and MI are associated with Ca^{2+} overload and generation of reactive oxygen species (ROS), which lead to cardiac muscle injury, thus raising the levels of cTnI in the blood. The observed increase in serum cTnI after isoproterenol injection in our study is in agreement with previous reports. In support of our cTnI results, serum CK-MB also significantly increased in the untreated MI group over the normal group. Like cTnI, CK-MB is a well-known marker for MI; it is even more specific to cardiac muscle than to skeletal muscle.

Low-density lipoprotein oxidizability was increased, but not to the level of statistical significance, after the induction of hypercholesterolemia and MI. Hypercholesterolemia is associated with increased production of ROS and increased oxidation of LDL cholesterol. Production of ROS decreases the bioavailability of NO, thus leading to inadequate endothelium-dependent responses. It is also known that isoproterenol itself generates free radicals and stimulates lipid peroxidation. Indeed, this may be a causative factor for the irreversible damage to the myocardial membrane. All the blood-clotting parameters (platelet aggregation, fibrinogen, and PT) exhibited significant changes in the untreated MI group as compared to the normal group. The increase in platelet aggregation might be induced by thromboxane A_2, which is synthesized by platelets and other cells. Hypercholesterolemia was found to enhance the sensitivity of platelets to agonists such as ADP, collagen, or epinephrine. The increase in plasma fibrinogen in the untreated MI group is in support of the reported role of fibrinogen as a major risk factor in cardiovascular disease.

Prothrombin time, however, was significantly reduced in the untreated MI group, as expected, because hypercholesterolemia is one of the factors known to decrease PT. Furthermore, it has been reported that PT was significantly decreased in MI. Histopathological examination of the hearts of untreated MI group animals revealed an abnormal histological structure of the myocardial bundle. The aorta intimal thickness of untreated MI group was dramatically increased as compared to the normal group. These results are in agreement with a study showing...
that patients with hyperlipidemia had significantly lower levels of endothelium-dependent vasodilation and higher intima media thickness of the common carotid artery when compared to controls.46

Protective Effect of Aspirin

It was evident in the current study that hypercholesterolemia itself was not corrected by oral administration of aspirin. This result is in accordance with that of Yi et al,47 which showed no effect of aspirin in decreasing the cholesterol level in atherosclerotic rabbits. They concluded from their study that the mechanism of atherosclerosis suppression by aspirin in cholesterol-fed rabbits is related to the inhibition of cyclooxygenase 2 (COX-2) expression together with the reduced inflammation followed by but not related to the hypolipidemic effects.

In our experiments, aspirin showed only an insignificant effect on LDL oxidizability. This was opposite to the findings by Yada et al,48 who reported that patients with acute myocardial infarction (AMI), taking aspirin for 4 weeks experienced significant decrease in oxidized LDL. Aspirin was found to decrease the progression of atherosclerosis by protecting LDL from oxidative modification49 and also improves endothelial dysfunction in atherosclerotic vessels.50

However, aspirin administration has demonstrated significant improvement for all blood-clotting parameters. It normalized platelet aggregation and PT and decreased plasma fibrinogen levels significantly. Aspirin action is supposed to involve acetylation of platelet COX and subsequent inhibition of the synthesis of prostaglandins and thromboxane A2.51 Aspirin promotes inhibition of platelet aggregation by neutrophils, an effect that appears to be mediated by a nitric oxide (NO/cyclic guanosine monophosphate [cGMP])-dependent process.52 It was also found that aspirin acetylates fibrinogen and enhances fibrinolysis.53 Aspirin affects clotting factors including thrombin, fibrinogen, factor XIII, and tissue plasminogen activator, thereby potentially having a direct effect on clot formation and lysis.54 Furthermore, it has been suggested that aspirin modifies thrombin generation by acetylating macromolecules of platelet membrane and/or prothrombin.55

In a clinical study by Jacek et al,56 75 mg daily aspirin had no effect on decreasing PT in patients with plasma cholesterol levels >5.2 mmol/L (200 mg/dL). This indicates that individuals with hypercholesterolemia might receive less benefit than others from preventive aspirin treatment.

Histopathological examination of the aspirin group showed ischemic myocardial bundles with the appearance of severe congestion in the blood vessels. However, a significant decrease in aorta intimal thickness was observed when compared with the untreated MI group. Guo et al57 found that aspirin treatment of cholesterol-fed rabbits resulted in a significant decrease in maximum plaque thickness, the degree of artery stenosis and the proportion of the intimal circumference occupied by artheroma. Furthermore, a study found a 20% reduction in ischemic heart disease events in men at increased risk after taking low-dose aspirin.58

Protective Effect of l-Arginine

l-Arginine currently receives increased attention in the therapeutic field. Numerous studies have shown a beneficial role of l-arginine administration when taken in amounts greater than those taken in diet. In the current study, administration of l-arginine resulted in insignificant changes in hypercholesterolemia, similar to aspirin. It was also reported that l-arginine
supplementation in a model of hypercholesterolemia had no effect on serum lipids and lipoproteins.59,60 Nevertheless, L-arginine administration was associated with a remarkable reduction in aortic intimal lesions. Similar results were previously demonstrated in another study from our laboratory.34

In this study, L-arginine showed remarkable positive results with regard to the prevention of MI. Both serum cTnI and CK-MB were significantly lower in the L-arginine group than in the untreated MI group, both returning to levels of the normal group. This is in agreement with a study by Carrier et al,61 where serum cardiac troponin T level was reduced in patients receiving L-arginine–enriched blood cardiology solution, suggesting an overall improvement in myocardial protection. Furthermore, Kumar et al62 also found a significant decrease in serum CK level in L-arginine–treated rabbits compared to hypercholesterolemic untreated animals, both subjected to experimental MI.

The antioxidant property of L-arginine is evident in this study from the remarkable reduction of LDL oxidizability as compared to all other groups in the study. This antioxidant property of L-arginine was previously described in a number of reports.7,63 In these studies, L-arginine was found to elicit anti-inflammatory effects by scavenging ROS and to prevent the oxidation of LDL cholesterol, thereby retarding the progression of atherosclerosis. Reduced superoxide production by endothelial cells has been shown to be specific for L-arginine but not for D-arginine,64 suggesting that it may be related to NO synthase activity.

One of the most prominent effects of L-arginine in this study was its effect on platelet aggregation. L-arginine reduced platelet aggregation dramatically to a value even below that of the normal group. In comparison to aspirin, the reducing effect of L-arginine was more pronounced, demonstrating a platelet aggregation of 64% of that observed for aspirin. This result is in accordance with many investigations, which show that chronic dietary supplementation with L-arginine decreased platelet aggregation in hypercholesterolemic rabbits,33,34 and in individuals with hypercholesterolemia.65 In addition, L-arginine was previously shown to inhibit platelet aggregation in vitro, indicating that its effects are exerted directly on luminal platelets and not on the platelets attached to the vascular endothelium.66 The antiplatelet activity of L-arginine may be secondary to the increase in NO production.

L-arginine also stimulates fibrinogenolysis by inhibiting the formation of thromboxane B2 and the platelet–fibrin complex while enhancing plasmin generation and fibrin degradation.67,68 Indeed, Cylwik et al68 showed that fibrinogen concentration decreased after both acute and long-term administration of L-arginine. Both results support our observations. L-arginine also produced a significant increase in PT as compared to untreated animals; however, aspirin was more effective in increasing PT than L-arginine.

Superior to aspirin treatment, histopathological examination of the L-arginine group showed no abnormal histopathological findings in the heart. Oral administration of L-arginine to animals69 and humans70 has been demonstrated to slow the progression of atherosclerosis and its component processes. The initial stages of atherosclerosis and plaque formation are both inhibited by treatment with L-arginine.71

In conclusion, the salient findings of this study can be summarized in the following points:

1. L-arginine exerts protective effects against myocardial ischemic injury through different mechanisms. Evidence includes reduction in cardiac, coronary, and aortic histopathological changes, prevention of LDL oxidation, reduction in myocardial injury, and improvement in blood-clotting parameters.
Figure 6. A photomicrograph of a section in heart of a rabbit in the normal, untreated MI, aspirin and L-arginine group. Hx and E, ×64. Hx and E indicates hematoxylin and eosin.

Figure 7. A photomicrograph of a section in coronary blood vessel of a rabbit in the normal, untreated MI, aspirin, and L-arginine group. Hx and E, ×40. Hx and E indicates hematoxylin and eosin.
(2) L-arginine and aspirin have no effect on blood cholesterol. L-arginine demonstrated better effect in the reduction of LDL oxidizability, platelet aggregation, and aortic intimal thickness. Prolongation of PT is the only parameter in this study where aspirin was found to be superior to L-arginine.

(3) Given that L-arginine is a nutritional supplement with a relatively high safety margin and no tolerance on long-term use, we recommend considering L-arginine supplementation as a potentially novel nutritional strategy for preventing and modulating CVD, especially in cases of aspirin resistance or hypersensitivity.

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