Investigating the Cardio-Protective Abilities of Supplemental \( \text{l-arginine} \) on Parameters of Endothelial Function in a Hypercholesterolemic Animal Model

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Summary  Endothelial dysfunction is now widely recognized as an early marker of cardiovascular disease, making its treatment, or complete avoidance, an emerging, interesting therapeutic target. This study investigated the ability of the highly intriguing amino acid \( \text{l-arginine} \) to influence endothelial function. Its therapeutic potential is also compared to that of known cardiovascular medications, namely nitroglycerin [a nitric oxide (NO) donor] and enalapril [an angiotensin-converting enzyme (ACE) inhibitor]. Fifty male New Zealand rabbits were included in the study, divided into 5 equal groups: control, hypercholesterolemia (untreated), hypercholesterolemia (+l-arginine), hypercholesterolemia (+enalapril), and hypercholesterolemia (+nitroglycerin). Biochemical investigations included measurement of circulating NOx, malondialdehyde (MDA), and lipid profile markers, as well as dimethylarginine dimethylaminohydrolase (DDAH) and ACE activities. Furthermore, aortic ACE activity and blood platelet aggregation were estimated. A histopathological examination and intimal thickness measurement were also conducted. Compared to the untreated hypercholesterolemic group, all agents were capable of positively influencing MDA levels, platelet aggregation and intimal thickness; however, only the l-arginine group was capable of beneficially and significantly altering both NOx levels and serum and aortic ACE activities. No agents were capable of modulating serum DDAH activity inhibited by hypercholesterolemia. Based on the results of this study, l-arginine appears to be a novel cardio-protective agent, illustrated by its ability to ameliorate the deleterious effects of hypercholesterolemia on endothelial function, in a manner comparable to, and sometimes more potent than, commonly used cardiovascular medications.

Key Words  \text{\textit{l-arginine, cardio-protection, endothelial function}}

Endothelial dysfunction is a hallmark of the ageing process that eventually leads to cardiovascular disease, the leading cause of death in many countries (1). It is characterized by an impaired ability of endothelial cells to mediate dilatation either due to a decrease in endothelial nitric oxide (NO) synthase (eNOS) expression, caused by ageing, or due to oxidative stress (1). Nonetheless, according to countless reports, NO is a key player implicated in the development of cardiac pathologies (2).

l-Arginine, a semi-essential amino acid now known to influence several systems of the human body, has a pivotal role in regulating NO homeostasis (3). l-Arginine acts as the substrate for the eNOS enzyme, which is responsible for producing NO for maintenance of cardiovascular health (3). Clinical data on the efficacy and relevance of exogenous l-arginine supplementation have been disparate. Numerous studies highlighting its ability to restore endothelial function in patients with cardiovascular diseases as well as in patients with cardiovascular risk factors are now available (3). Simultaneously, the questioning of the need for exogenous l-arginine is raised by different notions such as the increase in the expression of arginase, the enzyme responsible for l-arginine degradation, in response to exogenous supplementation of the amino acid, thus rendering the initial supplementation useless (4).

In a clinical setting, l-arginine deficiency is rarely witnessed in patients and thus, concluding that its lack leads to vascular disorders is imprecise (5). However, studies have shown that eNOS activity is not merely subject to l-arginine levels, but is also influenced by circulating concentrations of asymmetric dimethylarginine (ADMA), a methylated derivative of l-arginine and a competitive inhibitor of eNOS (5). ADMA levels have been shown to be increased in a number of cardiac-related disorders (6, 7) and hence, to act as a novel marker for cardiovascular disease (5). A decrease in the l-arginine/ADMA ratio renders a decrease in NO pro-
duction, prompting the use of supplemental L-arginine to positively influence this ratio in favor of NO synthesis (5).

The aforementioned premise serves as an explanation for the “L-arginine paradox,” which raises the fact that circulating levels of L-arginine in both healthy subjects and patients with vascular diseases are several folds higher than the Michaelis-Menden constant for eNOS (5). In other words, the enzyme is constantly saturated with its substrate. These findings drastically conflicted with experiments concluding that exogenous L-arginine increases endothelial NO production, which raised the possibility of the presence of an endogenous inhibitor of eNOS that is displaced upon L-arginine supplementation, hence ADMA (5). Inactivation of the seemingly detrimental molecule ADMA is catalyzed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (8), which produces L-citrulline, making its production a marker for DDAH activity.

A magnification of the complexity of NO signaling may involve mentioning the regulatory role it exerts on angiotensin II signaling (9), as illustrated by previous studies which showed that NO donors, namely nitroprusside, and intravenous infusions of L-arginine, are capable of inhibiting ACE activity (10, 11). Additionally, chronic supplementation with nitro-L-arginine-methyl ester (L-NAME), a NOS inhibitor, was shown to increase both cardiac and aortic ACE activities (12).

This study aimed to examine the possibly protective effect of supplemental L-arginine on endothelial function by investigating its modulation of the activities of DDAH and angiotensin-converting enzyme (ACE), as well as levels of the oxidative stress marker malondialdehyde (MDA), along with circulating NO, determined total nitrate/nitrite (NOx) and blood platelet aggregation. Another major aim was to compare the L-arginine’s ability to influence the mentioned parameters to known cardiovascular agents such as the ACE inhibitor enalapril and the NO donor nitroglycerin.

**MATERIALS AND METHODS**

**Animals.** Fifty male New Zealand rabbits, weighing 1.25–1.5 kg, were used in this study. The animals were maintained under constant environmental conditions with unlimited access to water and food. The study protocol was approved by the local ethical committee responsible for overseeing such studies.

**Experimental protocol.** The animals were initially fed standard chow pellets for 10 d for adaptation purposes and then randomly divided into 5 equal groups. The first group was designated the control group, where animals continued feeding on standard chow pellets for 10 d for adaptation purposes and then randomly divided into 5 equal groups. The first hypercholesterolemic group was left untreated, whereas the other groups were treated by 2.25 g% L-arginine in drinking water (14). 3 mg enalapril maleate/kg body weight orally for the whole 28 d (15), or 175 μg nitroglycerin per day orally from day 15 to 27, on every other day to minimize the effects of nitrate tolerance. After 28 d, the animals were sacrificed and dissected, and blood was drawn for further investigations.

**Biochemical analyses and histopathological examinations.** Biochemical investigations conducted were NO, as total NOx (NO2−/NO3−), using the Griess reaction described elsewhere (16), as well as activities of ACE and DDAH using well-established techniques described sufficiently in the literature (17, 18). MDA levels were determined using a thiobarbituric acid test (19) whereas serum total cholesterol, LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and triacylglycerols (TG) were determined using commercially available kits (Bicon, Germany).

Spectrophotometric measurements were performed using a Jasco UV/VIS spectrophotometer (Tokyo, Japan) and a microtitler well reader Perkin Elmer (Perkin Elmer, Waltham, MA) whereas platelet aggregation determination was conducted using a dual channel platelet aggregometer (Chrono-log, Havertown, PA) (20). Briefly, plasma samples were incubated at 37°C in the aggregometer for 1 min before adding the agonist (adenosine diphosphate–2 μg/mL). The levels of the aggregation response were expressed as the percentage in change of light transmission 6 min after the addition of the agonist.

The aortas of all animals included in the study were isolated, washed with ice-cold isotonic saline, freed of fat and then divided into two portions, the first of which was homogenized in ice cold phosphate buffer pH 7.4 using a glass-glass homogenizer to produce a 10% homogenate for aortic ACE activity determination whereas the second was kept in formal saline for the histopathological examination, using hematoxylin and eosin stains, and intimal thickness measurement. With the purpose of avoiding the possibility of an error in intimal thickness measurement due to pathological heterogeneity, the image analyzer measured the thickness (in μm) of an aortic sample from 10 different sections, thus giving the final result of intimal thickness as a mean of the 10 measurements.

**Statistical analysis.** Analyses were performed using GraphPad Prism statistics software (GraphPad Software, Inc.). Comparisons between two measurements of an investigated parameter were done using one-way analysis of variance (ANOVA) where a p-value of less than or equal to 0.05 was defined as statically significant. All results, unless stated otherwise, are presented as mean±standard error of the mean (SE). Post-hoc analyses were performed using Tukey’s test. Values with different superscript letters show significant difference at p<0.05.

**RESULTS**

The 2% cholesterol enriched diet significantly influenced the animals’ lipid profiles in a detrimental fashion where total cholesterol, HDL-C and LDL-C were shown to be increased in the hypercholesterolemic rabbits group compared to the control group (Table 1).

Furthermore, the hypercholesterolemic diet also resulted in significant increases in platelet aggregation,
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Table 1. Complete lipid profile of all investigated animal groups.

<table>
<thead>
<tr>
<th></th>
<th>Normal rabbits</th>
<th>Hypercholesterolemic rabbits</th>
<th>( l )-Arginine-treated rabbits</th>
<th>Nitroglycerin-treated rabbits</th>
<th>Enalapril-treated rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>88.1 ± 4.7(^a)</td>
<td>1,375.9 ± 172.4(^{b*})</td>
<td>1,233.5 ± 112.2(^{b*})</td>
<td>1,045.9 ± 80.1(^{c*})</td>
<td>1,031.8 ± 74.9(^{c*})</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>27.1 ± 3.0(^b)</td>
<td>70.4 ± 15.3(^{b*})</td>
<td>52.1 ± 7.1(^{b*})</td>
<td>29.1 ± 2.9(^{a*})</td>
<td>37.1 ± 3.3(^{b*})</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>34.5 ± 6.1(^b)</td>
<td>1,280.0 ± 166.9(^{b*})</td>
<td>1,158.6 ± 107.9(^{b*})</td>
<td>990.4 ± 81.2(^{c*})</td>
<td>974.9 ± 76.1(^{c*})</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>132.9 ± 8.0(^b)</td>
<td>127.6 ± 15.5(^{b*})</td>
<td>133.9 ± 10.5(^{b*})</td>
<td>132.3 ± 7.2(^{a*})</td>
<td>99.0 ± 9.5(^{b*})</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of the mean; \( n = 10 \).

Values with different superscript letters show significant difference at \( p < 0.05 \).

Table 2. Serum and aortic ACE activities in all investigated animal groups.

<table>
<thead>
<tr>
<th></th>
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<th>Enalapril-treated rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ACE (unit/mg)</td>
<td>75.9 ± 0.9(^a)</td>
<td>169.2 ± 3.8(^{b*})</td>
<td>58.4 ± 4.1(^{c*})</td>
<td>129.8 ± 4.1(^{d*})</td>
<td>14.4 ± 1.3(^{c*})</td>
</tr>
<tr>
<td>Aortic ACE (unit/mg)</td>
<td>16.4 ± 1.0(^b)</td>
<td>40.6 ± 1.8(^{b*})</td>
<td>23.6 ± 2.0(^{e*})</td>
<td>36.7 ± 2.0(^{d*})</td>
<td>10.0 ± 0.8(^{e*})</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of the mean; \( n = 10 \).

Values with different superscript letters show significant difference at \( p < 0.05 \).

Table 3. Values of MDA and NOx levels, as well as DDAH activity and platelet aggregation in all investigated animal groups.

<table>
<thead>
<tr>
<th></th>
<th>Normal rabbits</th>
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</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mL)</td>
<td>2.1 ± 0.1(^a)</td>
<td>5.6 ± 0.2(^{b*})</td>
<td>2.5 ± 0.3(^{a*})</td>
<td>2.4 ± 0.2(^{a*})</td>
<td>2.3 ± 0.2(^{a*})</td>
</tr>
<tr>
<td>DDAH (unit/mL)</td>
<td>6.4 ± 1.2(^a)</td>
<td>0.5 ± 0.1(^{b*})</td>
<td>0.6 ± 0.1(^{b*})</td>
<td>0.9 ± 0.2(^{b*})</td>
<td>0.4 ± 0.1(^{b*})</td>
</tr>
<tr>
<td>Serum NOx (nmol/mL)</td>
<td>115.6 ± 6.2(^b)</td>
<td>38.5 ± 3.1(^{b*})</td>
<td>73.7 ± 3.6(^{e*})</td>
<td>53.4 ± 3.6(^{e*})</td>
<td>34.2 ± 2.2(^{b*})</td>
</tr>
<tr>
<td>Platelet aggregation (%)</td>
<td>26.8 ± 3.1(^b)</td>
<td>55.2 ± 3.0(^{b*})</td>
<td>38.1 ± 2.4(^{b*})</td>
<td>29.8 ± 2.5(^{a*})</td>
<td>40.5 ± 2.2(^{a*})</td>
</tr>
</tbody>
</table>

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Values with different superscript letters show significant difference at \( p < 0.05 \).

MDA levels and serum and aortic ACE activities (Tables 2 and 3). With regards to the histopathological examination (Fig. 1), the deleterious effects of hypercholesterolemia were apparent to such a degree that substantial alterations in the aortic walls were observed with the formation of multiple, large atheromatous plaques as well as intimal thickness. Contrary to this description are the observations witnessed in the normal group, where the aortic wall presented a uniform thickness with the absence of bulging in the lumen and the endothelium lining remained intact with no interruptions.

With reference to the animals’ lipid profile, \( l \)-arginine treatment was not able to significantly modify any of the investigated parameters. On the other hand, nitroglycerin was able to lower significantly total cholesterol, LDL-C, as well as HDL-C, whereas enalapril lowered total cholesterol and LDL-C only. None of the treatments significantly influenced TG (Table 1).

Results of histopathological investigations revealed the abilities of all agents to reduce intimal thickness under hypercholesterolemia, reaching the measurements of the normal group in the cases of \( l \)-arginine and enalapril (Fig. 2).

\( l \)-Arginine and enalapril managed to maintain the activity of the ACE enzyme at levels significantly lower than that observed in the untreated hypercholesterolemic group and comparable to that of the control group (Table 2). Noteworthy is that despite the demonstrated ability of nitroglycerin to maintain the activity of serum ACE at levels lower than that of the untreated, hypercholesterolemic group, it failed to do so for the aortic ACE. Additionally, aortic and serum ACE activities were significantly higher in the nitroglycerin group compared to the control group.

Despite the ability of \( l \)-arginine and nitroglycerin to significantly increase NOx levels in the corresponding groups compared to the untreated group, they were unable to influence DDAH activity. On the other hand, enalapril was unable to modulate any change in either NOx levels or DDAH activity. All agents were successful in decreasing MDA levels in spite of a hypercholesterolic environment (Table 3).

The drastic increase in platelet aggregation in the untreated hypercholesterolemia group compared to the
controls was significantly blunted by all agents; however, only nitroglycerin managed to maintain platelet aggregation at levels comparable to that of the control group.

**DISCUSSION**

Results of this study indicate that hypercholesterolemia largely influences several parameters regulating cardiovascular health via the ability of a cholesterol enriched diet to unfavorably modulate the animals’ lipid profiles, platelet aggregation, intimal thickness, NOx and MDA levels, DDAH activity, and finally, serum and aortic ACE activity. Our results are largely supported in this regard by several other studies that provided similar observations (21–27).

The nutritional value of L-arginine is a widely debated topic among the medical community. While several clinical studies have demonstrated the protective ability of this controversial amino acid in different cardiovascular scenarios (3), other investigators have questioned the

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Fig. 1. Photomicrographs of sections in ascending aortas of normal (A), untreated hypercholesterolemic (B), L-arginine-treated (C), enalapril-treated (D) and nitroglycerin-treated (E) rabbits. Hx and E 100×.
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relevance of its exogenous supplementation, with reference to the arginase paradox, which indicates that the more one is supplied with l-arginine, the more it is broken down by arginase (4).

While l-arginine was unable to influence any of the tested lipid profiles, it was successful in countering the adverse, atherosclerotic, endothelium-destructive effects of a hypercholesterolemic diet. We here demonstrate a significant increase in NOx levels in the l-arginine treated group compared to the untreated hypercholesterolemic group. The NO signaling pathway is a highly versatile, majorly complex system, influencing and regulating various mechanisms. Besides being a potent vasodilator, its most recognized effect, NO has also been implicated in ACE activity and angiotensin II signaling (9). Reporting of such effects dates back to 1995, when Higashi et al. (11) described the subsequent decrease in NOx levels in the l-arginine treated group compared to the untreated hypercholesterolemic group. The NO signaling pathway is in harmony with the report of Moritoki et al. (31), who showed that l-arginine treatment causes relaxation of rat aortic rings.

Remarkably, results of this study revealed the ability of l-arginine to elevate NO to levels greater than that achieved in the two groups receiving known and widely used cardiovascular drugs, nitroglycerin and enalapril, which tend to exert their beneficial effects by increasing NOx production. NO is known to down-regulate the expression of angiotensin II type 1 receptor mRNA (28), the means by which angiotensin II exerts both its physiological and pathological cardiac actions, such as the mediation of vasoconstriction, proliferation and inflammation of vascular smooth muscle cells (9).

Fig. 2. Intimal thickness of all investigated animal groups. Values are mean±standard error of the mean; n=10. Values with different superscript letters show significant difference at p<0.05.

cantly maintained the parameters at levels significantly lower than those observed in both the untreated hypercholesterolemic group and the control group, complementing the results of Higashi et al. (11). While the exact mechanisms by which this phenomenon occurs are unknown, a highly conceivable explanation would involve referencing the documented beneficial ability of NO on ACE activity and angiotensin II signaling, mentioned earlier. Naturally, the l-arginine-treated group had aortic and serum ACE activity means significantly greater than those observed in the group treated with the known ACE inhibitor, enalapril, but lower than those of the nitroglycerin treated group.

With the now recognized role of oxidative stress in endothelial dysfunction (2), the rise in MDA levels in response to the hypercholesterolemic diet was significantly ameliorated by all agents, which maintained levels of MDA similar to those found in the control group.

The intimal thickness observed in the untreated hypercholesterolemic group could be explained by the activation of ACE in hypercholesterolemia, which would increase the synthesis of angiotensin II, which in turn directly induces vascular smooth muscle proliferation (9). On the other hand, decreased NO formation is a possible mechanism for increased myointimal cell proliferation (30). While all agents maintained intimal thickness at levels significantly lower than that of the untreated group, the nitroglycerin group had an intimal thickness significantly higher than that of the normal group, despite the positive effect on total cholesterol. However, the low level of HDL-C (increased risk of atherosclerosis) was noticeable with nitroglycerin treatment. This raises the prospects of the presence of more than one regulating mechanism. The significant reduction of intimal thickness and improvement of endothelial function observed with l-arginine treatment is in harmony with the report of Moritoki et al. (31), who showed that l-arginine treatment causes relaxation of rat aortic rings.

Previous studies have highlighted the inability of l-arginine to influence ADMA levels, in other words, to modulate DDAH activity. Our observation complements such work and adds the inability of the other investigated agents to affect the DDAH-ADMA axis.

Platelet aggregation is one of the main contributors to atherosclerosis (2). It is well known that dietary fats alter platelet function, and that there is a relationship between aggregation and platelet cholesterol content (32). Decreased serum NO may be another possible explanation for the increased platelet aggregation (2). Reduced NO could promote platelet attachment and release of growth factors in the vessel wall (33). The observed anti-platelet effect of l-arginine, which happens to be in accordance with previous studies (29), may be secondary to increased NOx production. Similar to l-arginine, enalapril significantly reduced platelet aggregation compared to the untreated group; however, levels were still greater than those of the control group. The nitroglycerin-treated group managed to maintain platelet aggregation at levels comparable to that of the control group.
CONCLUSION

We provided in this study supporting evidence of the link of hypercholesterolemia to the development of atherosclerosis that could be reduced to a great extent by l-arginine supplementation, despite its inability to modify the lipid pattern and the ongoing debate about its clinical utility. Several indicators were demonstrated, including the reduction of aortic histopathological changes and intimal thickness, in a way comparable to the cardiovascular drugs NTG and enalapril. This study also shed more light on the biochemical mechanisms exerted by l-arginine in preventing the development of atherosclerosis. Mechanisms involved may include competitive displacement of ADMA, an endogenous NOS inhibitor, resulting in increased NOx formation, as well as decreased oxidative stress (as assessed by serum MDA level), inhibition of platelet aggregation and inhibition of serum and tissue ACE activities. Given that l-arginine is a nutritional supplement with a relatively high safety margin even in doses much higher than regular drugs, in addition to the absence of tolerance upon long-term exposure, in contrast to nitro-compounds, we recommend establishing a dietary arginine supplementation as a potentially novel nutritional strategy for preventing and/or modulating cardiovascular disease.

Large-scale, randomized-controlled trials are in order to provide conclusive evidence for the need of exogenously-supplemented l-arginine in different cardiovascular disease settings.

REFERENCES


