LATE TREATMENT WITH L-ARGININE INCREASES 8-ISOPROSTAGLANDIN F2AAND OXIDIZED LDL IN HYPERCHOLESTEROLEMIC RABBITS

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Abstract

BACKGROUND: The role of L-arginine, the precursor of nitric oxide, on the oxidative stress and atherosclerosis has been previously studied; it has had inconsistent beneficial effects. The aim of this study was to investigate whether administration of L-arginine reduces oxidative stress and the progression of atherosclerosis in cholesterol fed rabbits.

METHODS: Eighteen white male rabbits were randomized into three groups. All of them received 1% high cholesterol diet for the first four weeks and normal diet for the second four weeks of the experiment. The early treatment (ET) group received L-arginine (3% in drinking water) in the first four weeks while the late treatment (LT) group received L-arginine for the second four weeks of the experiment. Control (C) group received no L-arginine. The plasma levels of lipids, 8-isoprostaglandin F2α, CRP and oxLDL were measured before, and at 4th and 8th weeks of the experiment. Aorta fatty streak formation was measured at the end of the experiment.

RESULTS: The plasma levels of lipids were increased significantly during the first 4 weeks and decreased significantly during the second 4 weeks with no significant differences between the groups. The plasma concentration of 8-isoprostaglandin F2α was significantly decreased in the ET group compared with the C group at the end of the experiment. The fatty streak formation in the ET group was significantly lower than that in the C group at the end of the experiment. The plasma concentration of CRP significantly increased after 4 weeks administration of hypercholesterolemic diet in all groups. Also, its amount was significantly smaller in ET group in comparison with other groups. The plasma concentration of oxLDL decreased significantly in the ET group compared with LT group at the end of the experiment. However, the plasma concentration of oxLDL increased in the C group and in the LT group at the end of the experiment.

CONCLUSION: L-arginine therapy from the very beginning of hypercholesterolemia reduced oxidative stress and the consequential irreversible vascular damage, and may be useful for primary prevention.

Keywords: Atherosclerosis, Oxidized Low Density Lipoproteins, Oxidative Stress, 8-Isoprostaglandin F2α, L-Arginine, Nitric Oxide.

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Introduction

Increased oxidative stress and associated oxidative damage are mediators of vascular injury in cardiovascular pathologies including atherosclerosis.[1,2] Oxidative stress can initiate inflammatory response in the vessel walls leading to endothelial dysfunction, main causal event in vascular damage.[3,4] Oxidative stress also leads to formation and accumulation of oxidized low density lipoprotein (oxLDL) in the vessel walls. OxLDL has many potential proatherogenic activities [5], and the cellular accumulation of oxidized LDL is considered a hallmark of atherosclerosis.[6]. Besides, it is a major factor contributing to decreased biological activity of nitric oxide (NO) associated with development of atherosclerosis.[7,8]

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NO is a vasodilator and antiatherogenic molecule, which if produced adequately can work against oxidative stress and LDL oxidation in the promotion of endothelial dysfunction and atherogenesis.[8] However, negative effects of NO, harmful as well as protective functions, have been observed too.[9] It can react with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) and acts to modify proteins and lipids and reduces antioxidant defenses.[10]

The oxidation of L-arginine, a semi-essential amino acid, by nitric oxide synthase (NOS) leads to production of L-Citrulline and NO.[11] L-arginine with no serious adverse effects can play an important role in improving endothelial dysfunction, the suppression of inflammatory processes, the enhancement of the endothelial NOS (eNOS) or inducible NOS (iNOS) expressions.[12] L-arginine has been shown to restore the biological activity of vascular NO and to reduce the progression of atheromatous lesions in cholesterol-fed rabbit aorta.[12,13]

There are some clinical studies that have shown oral administration of L-arginine improve endothelial dilatation in hypercholesterolemic patients.[14-16] However, some other studies have shown that oral supplementation of L-arginine was not associated with improvement of endothelium-dependent vasodilatation in patients with atherosclerosis.[17,18]

The fact is that not every study has shown the same beneficial effects of supplementary L-arginine. Why some studies showed benefits with L-arginine administration and others did not, is not quite well understood. These controversial findings suggest that under specific conditions, L-arginine could even be dangerous to vascular health.[17,18] It is still unclear whether administration of L-arginine could recover endothelial function in atherosclerotic arterial wall.

Oral supplementation of L-arginine has been used mostly in the advanced stages of atherosclerosis for treatment purposes in both human and animal models [14-18]. In fact there are few evidences that confirm the beneficial effects of oral L-arginine in the early treatment of atherosclerosis.[12,13] Therefore, this question that whether L-arginine would be more effective at earlier stages of disease development or as a treatment for risk factors remains to be answered.

As Lipid peroxidation is the initial event in the process of atherogenesis, measuring the concentration of lipid peroxidation byproducts would give a reliable estimate of the severity of the damage.[19]

8-isoprostaglandin F2α (8-iso-PGF2α) is a prostaglandin like compound and it is one of the stable end products of lipid peroxidation that has been proposed to be the most accurate indicator of oxidative stress.[20,21]

Previous studies demonstrated that 8-iso-PGF2α levels are elevated under conditions of increased oxidative stress both in animals and humans.[22,23]

The present work was undertaken to assess the effects of administration of L-arginine on vascular oxidative stress in cholesterol-fed rabbits, as assessed by measuring plasma 8-iso-PGF2α and ox-LDL. Also the effects of early treatment (ET) rather than late treatment (LT) with L-arginine on CRP level as a known inflammatory biomarker and fatty streak formation in hypercholesterolemic rabbits were studied too. ET with L-arginine was initiated at the same time as hypercholesterolemic diet while treatment was initiated 4 weeks after the induction of hypercholesterolemia, thereby mimicking a more closely potential clinical use of these compounds in humans.

Materials and Methods

Animals and study design

Eighteen male white rabbits weighing 1.95 ± 0.1 kg were obtained from the Pasteur Institute of Iran. Prior to the experiment, all animals were kept three per cage in the housing facilities of Isfahan School of Medicine, Applied Physiology Research Center (APRC) for one week with free access to food and water. This study was reviewed and approved by the ethics committee of Isfahan University of Medical Sciences.

The rabbits were grouped randomly into 3 groups, six rabbits each. Groups were named control group (C), early treatment group (ET), and late treatment group (LT). All groups were ex-

posed to a high cholesterol diet, rabbit chow supplemented with 1% cholesterol (Sigma, USA), for first four weeks of the experiment. The ET group received L-arginine (Ajinamoto, Japan) 3% in drinking water in the first four weeks of the experiment and normal diet for second four weeks (a total study duration of 8 weeks). The LT group received L-arginine 3% in drinking water during the second four weeks. The C group received no L-arginine during the experiment.

The blood was taken from the animals at the beginning of the study as a baseline, at the end of the 4th week, and at the end of the 8th week, by the time study was finished.

At the end of the study the rabbits were anesthetized with sodium pentobarbital (50 mg/kg) and sacrificed and the aorta artery was excised for pathological investigation to determine fatty streak lesion formation.

Lipid parameters

After an overnight fasting, blood samples were taken at the beginning of the study, and after completion of 4 weeks and 8 weeks. Collected blood samples were centrifuged (10,000 _ g), and the resulting serum was stored at -70°C until the time of the assay. Plasma total, LDL, and HDL cholesterol levels of all subjects were assessed by standard enzymatic kit (Pars Azmoon Co., Tehran, Iran).

Plasma 8-iso-PGF2α

Plasma 8-iso prostaglandin F2α levels were measured with an enzyme-linked immuno-sorbent assay kit (Cayman, USA) according to the manufacturer's instruction.

CRP

CRP was measured using enzyme-linked immuno-sorbent assay kits (IBL, Co., Germany) according to manufacturer's instruction.

OxLDL

OxLDL was measured using enzyme-linked immuno-sorbent assay kits (Promokine, Co., Germany) according to manufacturer's instructions.

Fatty streak measurement

Fatty streak measurement was done as previously described.[24] Briefly, the entire aorta from the aortic arch to external iliac arteries was dissected out and cleaned of excess adhering fat and connective tissue. The tissue were fixed in buffered 10% formalin for 24 hours, and then embedded in paraffin. The paraffin-embedded specimens were sectioned at 5 μm (20 sections in succession) and stained with haematoxylin and eosin, and examined by light microscopy to measure fatty streaks by two pathologists in a double blinded manner. Fatty streak formation was determined by intima thickness and media thickness measurement in all sections. The data were averaged and were used to obtaine the Intima thickness/Media thickness (IMT) ratio.

Data analysis

Repeated measures analysis of variance was performed on the measured parameters in order to detect their changes over time. $P < 0.05$ is considered as significant throughout the study. Least significant difference was used as multiple comparison criterion (post hoc) to determine the significance of pair wise differences.

Results

Lipid parameters

There were no significant differences between the groups in plasma total, LDL, and HDL cholesterol concentrations at baseline as shown in table 1 ($P > 0.05$). Plasma total, LDL, and HDL cholesterol concentrations significantly increased after 4 weeks of administration of hypercholesterolemic diet with no significant difference between the groups. They were decreased after 4 weeks of applying normal diet but never reached the baseline. Supplementation of L-arginine had no significant effect on plasma total, LDL, and HDL cholesterol levels in both ET and LT groups compared with C group (Table 1).

Plasma 8-iso-PGF2α

There were no significant differences between the groups in plasma 8-iso-PGF2α concentrations at baseline (Table 1) ($P > 0.05$). After 4 weeks of administration of cholesterol-enriched diet,

plasma concentration of 8-iso-PGF2α significantly increased from the baseline with no significant differences between the groups (Table 1) ($P > 0.05$). The plasma 8-iso-PGF2α concentration was significantly decreased and almost reached the baseline in C group by the end of the experiment. In fact there was no significant difference between its concentration at the baseline and at the end of the study in C group (Table 1) ($P > 0.05$). The plasma 8-iso-PGF2α concentration was significantly decreased at the end of the experiment in ET group rather than C group ($P < 0.05$). Besides, the plasma 8-iso-PGF2α concentration was significantly lower in ET group compared to LT group at the end of the study (Table 1) ($P < 0.05$).

The plasma 8-iso-PGF2α concentration remained elevated in LT group during the following second four weeks. Also the plasma 8-iso-PGF2α concentration was significantly higher in the LT group in comparison with its amounts in C group ($P < 0.05$) and ET group ($P < 0.05$) at the end of the experiment (Table 1).

CRP

There were no significant differences between the groups in plasma CRP concentration at baseline. The plasma concentration of CRP significantly increased after 4 weeks of administration of hypercholesterolemic diet in all groups. Also its amount was significantly smaller in ET group in comparison with other groups (Table 1) ($P < 0.05$). The plasma concentration of CRP decreased significantly while the normal diet were being applied but there were no significant differences between groups at the end of the experiment (Table 1) ($P > 0.05$).

OxLDL

The plasma concentration of oxLDL decreased significantly in the ET group rather than LT group at the end of the experiment ($P < 0.05$) (Figure 1). However the plasma concentration of oxLDL increased in the C group and in the LT group at the end of the experiment.

Fatty streak formation

IMT ratio in ET group was significantly less than its amount in C group by the end of the experiment (Figure 2) ($P < 0.05$). There were no significant differences in IMT ratio between the control group and the LT group and also between the ET group and LT group at the end of the study.

Table 1. Plasma total, LDL, and HDL cholesterol, 8-iso-PGF2α, CRP concentrations

Parameters	Groups	0th week	4th week	8th week
Total cholesterol (mg/dl)	C	122.09 ± 23	2181.30 ± 295†	1148.27 ± 226‡
	ET	139.73 ± 25	2063.18 ±361†	1275.00 ± 169‡
	LT	98.52 ± 25	1839.09 ± 0†	1336.82 ± 143‡
HDL (mg/dl)	C	20.25 ± 3.47	86.40 ± 14.65†	79.33 ± 2.40
	ET	14.83 ± 1.30	89.33 ± 19.33†	71.50 ± 13.50
	LT	11.00 ± 0.58	104.00 ± 16.23†	76.00 ± 16.00‡
LDL (mg/dl)	C	19.28 ± 12.47	1569.03 ± 324.76†	559.49 ± 251.24‡
	ET	24.95 ± 1.30	936.77 ± 137.91†	623.19 ± 126.56‡
	LT	55.00 ± 11.24	1132 ± 72.58†	683 ± 145.53‡
8-iso prostaglandin F2α (pg/ml)	C	509.95 ± 241.43	3452.92 ± 1410.90†	517.70 ± 116.8‡
	ET	318.10 ± 75.86	2846.58 ± 855.25†	327.37 ± 54.35*‡
	LT	567.97 ± 31.81	2368.94 ± 554.60†	1440.67 ± 485.7
CRP (µg/ml)	C	2.9 ± 0.08	3.52 ± 0.08†	2.7 ± 0.12‡
	ET	2.9 ± 0.1	3.17 ± 0.19†	2.94 ± 0.09*‡
	LT	3.0 ± 0.1	3.58 ± 0.04†	3.09 ± 0.04‡

***** Indicates significant difference with control group
† Significant difference with baseline
‡ Significant difference with 4th week

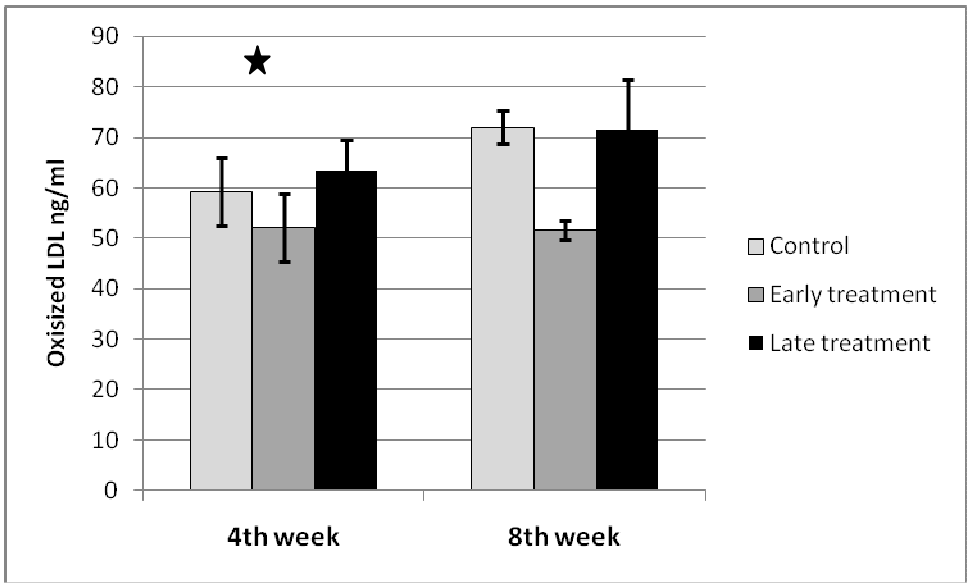

Figure 1. Plasma concentrations of oxLDL (ng/ml) after 4 weeks and at the end of the study in all groups.
Rabbits were fed 1% cholesterol and 3% L-arginine in drinking water for four weeks followed by normal rabbit chow for another four weeks (ET), or cholesterol in first four weeks and L-arginine in the second four weeks (LT), or just cholesterol in the first four weeks following by normal rabbit chow (C).
***** Indicates significant difference with control group ($P < 0.05$).
OxLDL: oxidized low density lipoprotein, ET: early treatment group, LT: late treatment group, C: control group.

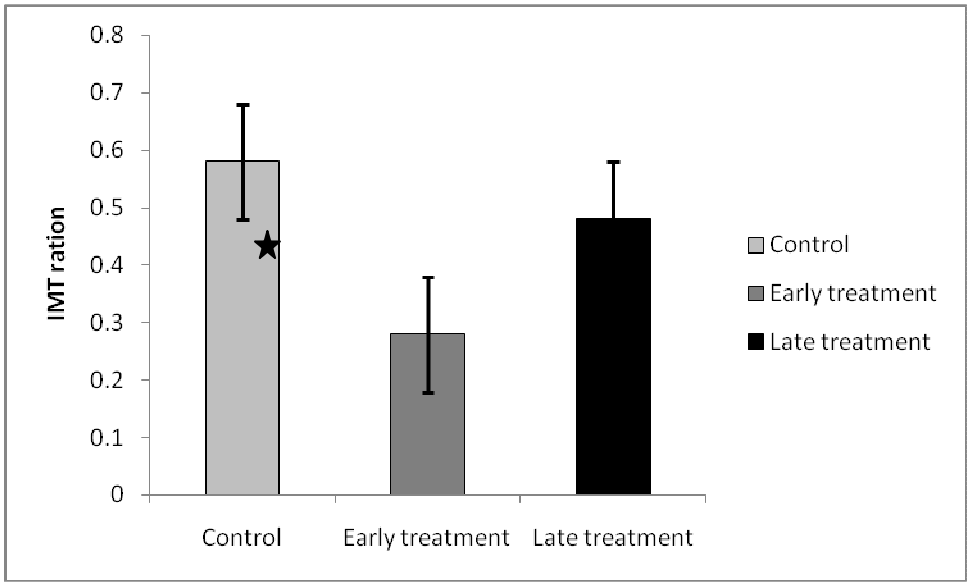

Figure 2. Illustrated the IMT ratio in C group, ET group and LT group at the end of the study
***** Indicates significant difference with control group ($P < 0.05$).
IMT ratio: Intima/media thickness, ET: early treatment group, LT: late treatment group, C: control group.

Discussion

Hypercholesterolemic rabbits were provided with oral supplementation of L-arginine either at the same time they were receiving the hypercholesterolemic diet (early treatment) or after the establishment of hypercholesterolemia (late treatment). It was found that in the ET group L-arginine supplementation could lead to decreased 8-iso-PGF2α concentration, decreased oxLDL, decreased CRP and decrease in the size of fatty streaks.

Interestingly, it was found that in the LT group not only the L-arginine supplementation had not benefited the hypercholesterolemic rabbits but also it caused increase in all the oxidative stress markers that was measured (8-iso-PGF2α and oxLDL), CRP and fatty streak size.

8-iso-PGF2α is a marker for oxidative stress in vivo and either its plasma levels or urinary excretion are increased in hypercholesterolemic humans as well as animal models of hypercholesterolemia.[21,22,23] The present study confirmed those findings by showing increased 8-iso-PGF2α plasma levels in cholesterol fed rabbits. Early studies have shown e that long-term supplementation of L-arginine hypercholesterolemic rabbits from the very beginning of cholesterol-feeding could bring the endothelial function back and reduce progression of intimal lesions formation as well.[12,13] The present data along with these findings represented an effective decrease in the oxidative stress markers as well as a significant reduction in fatty streak size when L-arginine is applied early from the beginning in the ET group. However, evidences resulted from several animal and human studies representative of late treatment with L-arginine have shown some controversial findings. In some experiments, subjects have benefited from late treatment with L-arginine in either animal model of hypercholesterolemia or human clinical trials.[11,24] But interestingly there are some other studies in which late treatment with L-arginine have shown no safe end results in reducing oxidative stress damage in vivo.[17,18,25]

It has been shown that late treatment with L-arginine starting 4 weeks after the induction of cholesterol-enriched diet resulted in an effective reduction in the urinary 8-iso-PGF2α excretion along with an elevation in the urinary nitrate excretion in rabbits.[26] Late treatment with L-arginine also reduced the progression of intimal thickening in the proximal and distal aorta.[27] Clarkson et al has shown that oral L-arginine administration improved endothelium-dependent dilatation in hypercholesterolemic individuals.[28] However Blum et al has demonstrated that oral supplementation of L-arginine did not improve endothelium-dependent dilation or the inflammatory process in patients with CAD.[17] It has been shown that 2 weeks of oral L-arginine supplementation was not associated with improvement of endothelium-dependent vasodilatation, oxidative stress or exercise performance in patients with stable angina.[18] The role of L-arginine administration in patients with CAD, as a treatment during the post-myocardial-infarction period, has recently been examined. This study found that L-arginine was associated with higher post infarction mortality than placebo.[29]

The results of the present study showed that late treatment with L-arginine not only represent no beneficial effects in the LT group but also lead to significant increase in oxidative markers and CRP as well as fatty streak lesion formation. In fact administration of L-arginine in the LT group was started after induction of hypercholesterolemia and the resulting inflammatory state of it. It has been confirmed that pro inflammatory molecules can induce the expression of iNOS. The large amounts of NO produced by iNOS react with superoxide radicals, leading to the synthesis of the peroxynitrite radical, which promotes atherogenesis.[30,31] Besides, it has been shown that in the presence of atherosclerosis, the reduced amount of L-arginine inhibits iNOS expression which leads to a decrease in the production of peroxynitrite and other reactive oxygen species. Supplementation of L-arginine while oxidative stress is present causes an increase in iNOS expression and enzyme activity which can explain the negative effect of L-arginine after establishment of the atherosclerosis in the present study.[32] Conversely, the eNOS-derived NO represents vasodilatory and antithrombotic properties.[7] This can explain why early treatment with L-arginine from the very beginning of the experiment improves reduction of oxidative markers and regression of the fatty streak lesion.

In addition, deficiency of tetrahydrobiopterin (BH4), an essential cofactor for eNOS function, which is commonly induced by reactive oxygen species leads to the uncoupling of the enzyme turning this enzyme to a source of superoxide anions instead of NO.[33,34] On the other hand, administration of high cholesterol diet for 4 weeks

with no L-arginine supplementation to oppose the initiation of the oxidative stress can lead to reduction of BH4 and uncoupling of the enzyme, a possible explanation for why late treatment with L-arginine didn't reduce the oxidative stress in the LT group.

This study has several limitations. First, the rabbit model in this study is a rapidly progressive model and short-time outcome in animal model is not completely the same as human disease. A second potential limitation of the present study is the relatively small sample size. A third potential limitation of the study is using only one dose of oral L arginine. Furthermore, as the present study was designed to assess the effects of L-arginine for 4 weeks, the long-term effects of L-arginine need to be studied.

Conclusion

These findings suggest that L-arginine is effective before exhaustive endothelial dysfunction and damage. It seems that L-arginine supplementation is useful in vascular bed with healthy and functional eNOS. It might even be harmful if supplemented in situations with eNOS uncoupling or iNOS over expression.[35]

It seems that the available evidence do not support safe conclusion about the effect of oral L-arginine supplementation for the treatment of coronary atherosclerosis.

Additional studies are necessary to describe the exact temporal relations of the inflammatory process and the preventive effects of L-arginine supplementation.

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Conflict of Interests

Authors have no conflict of interests.

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