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Short Report

L-Arginine Supplementation Influenced Nitrite but Not Nitrate and Total Nitrite in Rabbit Model of Hypercholesterolemia

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ABSTRACT

Background: The assessment of altered nitric oxide (NO) availability is of potentially important diagnostic and prognostic significance. The present study is aimed to investigate the effect of L-arginine (as a natural NO donor) supplementation on NO metabolite in a rabbit model of hypercholesterolemia to find a reliable marker for endothelial NO production. **Methods:** White male rabbits (n = 30) randomly assigned to 2 groups. Rabbits were fed 1% high-cholesterol diet (HC group, n = 15), or HC diet with oral L-arginine (3% in drinking water) (HC + L-arginine group, n = 15) for 4 weeks. The serum levels of lipids, L-arginine, total NO metabolites (NOx), nitrite and nitrate were measured before and after the study. **Results:** In this study, L-arginine supplementation led to a significant increased plasma level of L-arginine. The serum level of nitrite was significantly higher in L-arginine treated group while serum level of nitrate and NOx was significantly lower than HC group. **Conclusion:** As the result of our study showed, nitrite is a useful marker of endogenous endothelial NO production and although frequently used, neither nitrate nor NOx are reliable markers of acute changes in endothelial NO synthase activity. *Iran. Biomed. J. 12 (3): 179-184, 2008*

Keywords: Nitric oxide (NO), L-arginine, Nitrite, Nitrate, Total nitrite

INTRODUCTION

Nitric oxide (NO) is a widespread signaling molecule involved in numerous biological functions, such as regulation of vascular tone, control of thrombosis, the interaction of platelets and leukocytes with the vessel wall, and growth [1, 2]. NO is synthesized from the amino acid L-arginine by the constitutive calcium- and calmodulin-dependent endothelial isoform of endothelial NO synthase (eNOS) in vascular system. Disturbance of eNOS is suggested to play a key role in endothelial dysfunction and the development of atherosclerosis [3, 4].

Because endothelial dysfunction has a crucial role in atherosclerosis and is at least in part reversible [5], the assessment of an altered NO availability is of potentially important diagnostic and prognostic significance. Identification of such alterations may help targeting asymptomatic individuals who are at risk of cardiovascular diseases and would likely benefit from preventive measures. Therefore, establishing a biomarker to assess NO bioavailability in the form of a blood test is highly desirable and important.

NO is unstable and rapidly oxidized to nitrite and nitrate *in vivo* and *in vitro*, so, the measurement of plasma nitrite and nitrate or the sum of them called NOx, has been noticed as index of endogenous NO production [6, 7]. However, it has been shown that nitrite is a good marker for endothelial NO production while plasma nitrate levels are influenced by a variety of NOS-independent factors [7-10].

Traditionally, the endothelial L-arginine-NO pathway has been indirectly assessed via the flow response to endothelium-dependent vasodilators locally administered into the coronary, pulmonary or forearm circulation [11, 12]. However, the plasma level of NOx and urinary nitrate have also been used to assess NO bioavailability *in vivo* [11, 13, 14].

Many studies which have used L-arginine as a natural NO donor to regain endothelial function have

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not measured NO metabolites [15-20]. Several other studies that measured NOx interestingly reported that despite of beneficial effect of L-arginine on some aspect of endothelial function, L-arginine supplementation did not result in significant increased level of NOx [21-23]. Despite of much effort which has been made to validate markers of NO production or bioavailability, there is no convinced marker to monitor endothelial NO production in conditions such as L-arginine supplementation [7].

The present study is aimed to investigate the effect of L-arginine as a NO donor supplementation on nitrite, nitrate, NOx in a rabbit model of hypercholesterolemia to shed some light on the direct effect of L-arginine on NO metabolites.

MATERIALS AND METHODS

Animals and experimental design. This study was reviewed and approved by the Ethics Committee of Isfahan University of Medical Sciences (Isfahan, Iran). White male rabbits (n = 30) weighing 1.8 ± 0.3 kg were obtained from the Razi Vaccine and Serum Research Institute of Iran (Karaj). After a 1-week acclimation period and an overnight fasting, blood samples were taken as pre-experiment sampling. Collected blood samples were centrifuged (10,000 ×g), and the resulting serum was stored at -70°C until measurements. The animals were then randomly assigned to 2 groups. The rabbits were fed rabbit chow supplemented with 1% cholesterol. (Hypercholesterolemic diet) (high-cholesterol [HC] group, n = 15) or HC diet with oral L-arginine (3% in drinking water) (HC+L-arg group, n = 15) for 4 weeks. By the end of 4 weeks, the blood samples were taken and the animals were euthanized by an overdose of sodium pentobarbital and exsanguinated. The serum was stored again for further measurement.

Lipid and lipoproteins measurements. Total cholesterol levels were measured by standard enzymatic kit according to manufacturer's instruction (Pars Azmoon Co., Iran).

Serum nitrite measurement. The serum level of nitrite (stable NO metabolite) was measured using a colorimetric assay kit (R&D Systems, Minneapolis, USA) that involves the Griess reaction. Briefly, serums were added into wells (a 96-well enzymatic assay plate). A sulphanilamide solution was added to all experimental samples, and after incubation, *N*-1-naphtylethylenediamine dihydrochloride solution was added. Then, absorbance was measured by a microreader at 540 nm wavelength. The sample of nitrite concentration was determined by comparison to nitrite standard reference curve. The detection limit was 0.25 µM nitrite.

Serum NOx and nitrate measurement. For total nitrite (NOx) measurement in serum samples, total NO/nitrite/nitrate Assay kit (R and D Systems, Minneapolis, USA) was used that is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. Briefly, serums were added into wells (a 96-well enzymatic assay plate). Then, equal volume of nicotinamide adenine dinucleotide-hydrogen and diluted nitrate reductase were added to all wells. The samples were incubated at 37°C for 30 minutes. By the end of this step, all the nitrate in samples was reduced to nitrite. Then, the concentration of nitrite in samples was measured as mentioned above. For determining the nitrate concentration in the sample, the endogenous nitrite concentration was subtracted from the NOx concentration.

Serum L-arginine measurement. For measurement of L-arginine, the plasma was deproteinized with sulfosalicylic acid (30%) containing 1 mmol/L ß-(2-thienyl) (±) alanine as an internal standard. The samples were stored at 4°C for 30 minutes and centrifuged at 12,000×g for 5 minutes, and the supernatant was analyzed for L-arginine by HPLC. After dilution of plasma extract in membrane-filtered water (Waters Millipore, USA) and 2-minute derivatizations with *o*-phthaldialdehyde, a 10-µL sample was injected into the column by an autosampler (Spark Triathlon, Switzerland). Separation of derivitized amino acids was obtained by HPLC (Beckman, Australia) equipped with a 3-mm particle size and 125 × 4.6-mm octyl dodecyl sulphate Hypersil column (Bischoff, Germany), using two-buffer-system gradient elution. L-arginine measurement was done in Masoud Medical Laboratory (Tehran, Iran).

Statistical analysis. The data are reported as the mean ± SEM. A statistical software package, SPSS (version 13), was used to perform statistical analysis. The data were tested for normality and homogeneity of variance. Otherwise, paired student's *t*-test was

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Table1. The serum levels of total cholesterol, nitrite, nitrate, and NOx in two groups of the study at baseline and after 4 weeks.

	Before experiment	**after 4 weeks**	***P* (before and after)**
Total cholesterol (mg/dl)			
HC	109.4 ± 12.04	2082.7 ± 182.0	<0.05
HC + L arg	127.8 ± 12.30	2195.6 ± 153.3	<0.05
P (between groups)	NS	NS	
Nitrite (µ mol/l)			
HC	10.52 ± 0.75	12.21 ± 0.78	NS
HC + L arg	11.38 ± 0.55	14.70 ± 0.50	<0.05
P (between groups)	NS	<0.05	
Nitrite (µ mol/l)			
HC	46.65 ± 5.6	118.04 ± 7.6	<0.05
HC + L arg	48.54 ± 12.3	90.15 ± 8.2	<0.05
P (between groups)	NS	<0.05	
NOx (µ mol/l)			
HC	58.91 ± 5.6	131.00 ± 12.4	<0.05
HC + L arg	58.37 ± 5.1	104.16 ± 6.7	<0.05
P (between groups)	NS	<0.05	

Data are expressed as mean ± S.E. HC, hypercholesterolemic animals; HC + L-arg, animals who received hypercholesterolemic diet with oral L-arginine; NOx, *total nitrite;* NS, no significant difference.

used to assess the significance of any change within groups, while an unpaired student's *t*-test (equal or unequal variance assumed accordingly) was used to assess the significance of any change among groups. Statistical significance was accepted at $P<0.05$.

RESULTS

Serum cholesterol. The cholesterol-rich diet induced a significant increase of total cholesterol, in both HC and HC+L-arginine groups ($P<0.05$) (Table 1). After 4 weeks of cholesterol-rich diet, there was no significant difference in serum levels of total cholesterol between the animals of the HC and the HC+L-arginine groups ($p>0.05$) (Table 1).

The serum level of L-arginine. L-arginine supplementation (3%) for 4 weeks resulted in significant increased serum level of L-arginine in HC + L- arginine group from 55.62 ± 12.1 µg/dl to 112.37 ± 14.8 µg/dl ($P<0.05$).

The serum level of nitrite, nitrate and NOx. The mean serum levels of nitrite, nitrate and NOx in animals of HC and HC+L-arginine groups are summarized in Table 1. As shown in the Table, L-arginine supplementation led to significant increased level of nitrite ($P<0.05$), while the serum level of nitrite remained unchanged in HC group ($P>0.05$). The serum level of nitrate and NOx increased significantly in both groups through the study ($P<0.05$). By the end of the study, the serum level of nitrite was significantly higher in HC+L-arginine group than the HC group ($P<0.05$); while despite of 4 weeks L-arginine supplementation both the serum level of NOx and nitrate were significantly higher in HC group ($P<0.05$) (Table1).

DISCUSSION

Researches in the field of L-arginine supplementation as a natural NO donor have been largely developed in recent years [11-23]. The main assumption of L-arginine efficacy has been based on increased NO bioavailability [11]. Despite of this fact, direct assessment of NO metabolites has been overlooked in many studies [15-20] and it is not explicit that which one of NO metabolites is suitable for monitoring the NO bioavailability [7, 24].

In our study, L-arginine supplementation increased significantly the plasma level of L-arginine showing that the intervention has been successful. Among the NO metabolites, the serum level of nitrate and NOx was significantly lower in L-arginine treated group. In agreement with our results, Theilmeier *et al.* [21] did not found increased NOx levels in hypercholesterolemic humans who received dietary L-arginine. Another study in hypercholesterolemic rabbits showed that treatment with L-arginine partly restored urinary nitrate excretion [22]. In Evans *et*

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al. [23] study, increasing dietary arginine intake did not result in increased level of NOx in healthy human subjects. Furthermore, Plasma nitrate and total NOx levels did not change during pharmacological modulation of L-arginine-NO pathway in healthy subjects [10].

It has been shown that endogenous sources of NOx in mammals are derived from: (1) oxidation of endogenous NO, (2) enzymatic reduction of salivary nitrate by bacteria in the mouth and gastrointestinal tract, (3) nutritional sources such as meat, vegetables, and drinking water [25]. In our study, all animals received same diet (cholesterol enriched rabbit chow), so the dietary and bacterial sources of NOx were not influential. Therefore, we focused on endogenous NO production by NOS isoforms: constitutively active forms eNOS, neuronal NO synthase (nNOS), and the inducible isoforms (iNOS). The iNOS isoforms are induced mainly by cytokines in inflammatory states and produce large amounts of NO for an extended period of time [2]. Because neuronal NOS is predominantly expressed in neurons and skeletal muscle, this NOS isoform is not expected to release relevant amounts of NO into the vascular lumen [13]. eNOS is the major NOS isoform expressed in the cardiovascular system and produces a small amount of NO in a pulse tile manner, while iNOS is barely detectable in the normal vasculature, but it is abundantly expressed by smooth muscle cells and macrophages in atherosclerosis lesions [26].

The increased serum level of nitrate and NOx may be because of increased iNOS activity in atherosclerotic lesions in hypercholesterolemic group. Significantly, lower nitrate and NOx concentration in L-arginine treated group may suggest that L-arginine supplementation as a natural NO substrate enhanced eNOS activity, thus restrained the inflammatory state and inhibited iNOS activity.

Our study shows the serum level of nitrite was significantly higher in L-arginine treated group. Consistent with our finding, it has been shown that dietary L-arginine restored the levels of plasma nitrite in hypercholesterolemic monkeys [27]. Metzger *et al.* [7] have reported that nitrite or cGMP concentrations in plasma may be useful as clinical markers of NO formation in healthy subjects. Furthermore, it has been shown that activation or inhibition of NO synthase activity is associated with parallel increases or decreases in circulating nitrite concentrations [8, 9].

Among the NO metabolites, nitrite is major oxidative metabolites which was implicated to be an indicator of NOS activity. It has been shown that up to 70-90% of plasma nitrite is derived from eNOS activity in fasted humans and other [25]. Other studies demonstrated that plasma nitrite levels progressively decreases with increasing cardiovascular risk load [9]. Furthermore, NOS-inhibition in humans, pigs, dogs, and mice significant decrease the plasma nitrite concentration [28]. As it was mentioned, the assumption of L-arginine efficacy theoretically has been based on eNOS activation and nitrite production enhancement [11]. Our results were in the line of this assumption, L-arginine supplementation led to significantly higher plasma nitrite concentration.

In conclusion, determination of NO radical itself is difficult because of its radical nature and very short half-life; so NO metabolites i.e. plasma nitrite, nitrate and NOx determinations are increasingly being mentioned as markers for determining NO bioavailability. In this study, if we measured nitrate or NOx, it may result in a big misunderstanding that L-arginine supplementation had no augmenting effect on NO bioavailability. Our study and others [7-10] showed that nitrite is a useful marker for endogenous endothelial NO production and although frequently used, neither nitrate nor NOx are reliable markers of acute changes in eNOS activity. Further studies are needed to validate nitrite as a biochemical marker for NO production under other pathophysiological conditions in experimental and clinical studies.

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