Oral L-arginine does not improve endothelial dysfunction in children with chronic renal failure

KATY J. BENNETT-RICHARDS, MIA KATTENHORN, ANN E. DONALD, GILLIAN R. OAKLEY, ZAC VARGHESE, K. RICHARD BRUCKDORFER, JOHN E. DEANFIELD, and LESLEY REES

Departments of Nephrourology and Vascular Physiology, Great Ormond Street Hospital for Children NHS Trust, and The Institute of Child Health; Royal Free Hospital Renal Research Unit, Royal Free and University College Medical School; and Department of Biochemistry Molecular Biology, University College, Royal Free Campus, London, England, United Kingdom

Oral L-arginine does not improve endothelial dysfunction in children with chronic renal failure.

Background. Cardiovascular disease is a major cause of mortality amongst patients with chronic renal failure (CRF). L-arginine has been used to improve endothelial function by increasing nitric oxide (NO) bioavailability and in animal models this in turn has attenuated the progression of atherosclerosis. We examined whether dietary L-arginine supplementation improved endothelial function in children with CRF.

Methods. A randomized, double-blind, placebo-controlled, crossover trial of L-arginine was conducted in 21 normotensive children aged 11.5 ± 3 (7 to 17) years with CRF (GFR 27.4 ± 13.2 mL/min/1.73 m²) in whom endothelial dysfunction had previously been demonstrated. We examined the effect of L-arginine on the endothelial response to shear stress (NO-dependent) using a non-invasive technique of high-resolution ultrasound. Each subject was studied before and after 4 weeks of L-arginine (2.5 g/m² or 5 g/m² × 3/day) or placebo, separated by a rest period of 4 weeks. Brachial artery diameter was measured at rest, during increased flow (endothelial-dependent dilation) and after 25 μg of glyceryl trinitrate (endothelial-independent dilation) at each visit.

Results. After oral L-arginine, plasma L-arginine levels rose from 82 ± 20 to 179 ± 110 μmol/L (P < 0.001). No significant change in endothelial-dependent dilation during L-arginine (7.96 ± 2.35 to 7.71 ± 3.22%; P > 0.05) or placebo (8.2 ± 2.89 to 8.3 ± 3.14%; P > 0.05) was noted. There was no change in endothelial-independent dilation.

Conclusion. Endothelial function was not improved with L-arginine, suggesting that dietary supplementation is not a useful clinical approach in children with CRF.

Key words: atherosclerosis, renal failure, childhood ESRD, dietary supplements, uremic toxicity, nitric oxide synthesis, cardiovascular disease.

Premature atherosclerosis is the most important cause of death in adults with chronic renal failure (CRF), and is believed to be due to a high incidence of the classical risk factors such as hypertension, hypercholesterolemia, hypertriglyceridemia and diabetes in this population [1]. However, we have previously demonstrated that conduit artery endothelial function is already abnormal in children with CRF even in the absence of these risk factors, suggesting a toxic effect of uremia itself [2]. Endothelial dysfunction is a key early event that precedes the formation of atherosclerotic plaques, and involves reduced bioavailability of nitric oxide (NO), an important anti-atherogenic agent [3].

The semi-essential amino acid L-arginine is the only substrate for NO synthesis. Levels of L-arginine have been reported as being low in CRF but this is not a universal finding [4].

In CRF, however, decreased clearance results in high levels of circulating analogs of L-arginine, asymmetrical dimethylarginine (ADMA) and its stereoisomer, symmetrical dimethylarginine (SDMA) [5]. ADMA has been characterized as an endogenous inhibitor of NO synthase (eNOS or NOS III), the enzyme that catalyses the production of NO in vascular endothelial cells from L-arginine [5]. Also, NO may be inactivated by increased oxidative stress and free radical production in CRF [7]. Therefore, there are several ways in which supplementation with L-arginine might improve the bioavailability of NO in CRF: it may correct substrate deficiency, overcome competitive antagonism, and increase production to overcome rapid removal. Dietary L-arginine supplementation has already been shown to improve endothelial function in the brachial artery of young people with hypercholesterolemia [8], and experimentally to inhibit atherogenesis in animal models [9]. We hypothesized that arginine supplementation may improve NO bioavailability and endothelial function, and therefore lead to a simple preventative treatment strategy to decrease the risk of premature vascular disease. Our technique of high-resolution ultrasound was used to measure endothelial function non-invasively by
assessing the change in brachial artery diameter in response to endothelium dependent and independent stimuli, in a randomized, double blind, placebo-controlled cross-over trial of oral l-arginine supplementation in children with CRF.

METHODS
Subjects
Twenty-five children (11 girls and 14 boys, mean age 12 ± 3 years; range 7 to 17) with CRF [glomerular filtration rate (GFR) <50 mL/min/1.73 m²] were studied. Twenty-four had congenital structural and one acquired (neonatal cortical necrosis) causes of CRF. Exclusion criteria were children who were smokers, hypertensive, diabetic or nephrotic or on vasoactive medication. We did not preselect children on the basis of endothelial function in order that the study group should be representative of all children with moderate to severe CRF.

The local research ethics committee approved the study and informed consent was obtained from the parents or guardian or from the patient in those >16 years.

Study design
The trial was randomized, placebo controlled, double blinded and cross over in design with two four-week treatment periods separated by a four-week washout period. Blood samples were taken following a six hour fast. l-arginine was given at 5 g/m² surface area to a maximum of 7 grams three times daily. This dose was selected to provide a twofold increase in plasma l-arginine levels and was based on previous studies performed by our group [8]. After three children with low GFR complained of nausea and were found to have an elevated urea on completion of the first treatment phase, the dose of the treatment syrup was subsequently adjusted in those with a GFR <35 mL/min/1.73 m² to 2.5 g/m² three times daily (without unblinding the study). The active drug preparation and the placebo were prepared as syrup by South Devon Healthcare (Paignton, Devon TQ4 7TW, UK). Dietary intake of nitrates and nitrites were not restricted as it was thought not to be practical in this age group and we anticipated poor compliance. As each child acted as his or her own control, it was not believed to be a confounding variable. However, this places limitations on the interpretation of the nitrate/nitrite data.

Assessment of vascular function
Endothelial function was determined by recording the dilator response of the brachial artery to increase blood flow generated during reactive hyperemia of the downstream forearm, flow mediated dilation (FMD). Subjects lay supine in a temperature-controlled laboratory (22 to 25°C). The brachial artery was scanned in longitudinal section using a 7 MHz linear array transducer and an XP 128/10 (Acuson), magnified using a resolution box function and gated with the R wave of the ECG. End-diastolic images of the vessels were acquired every 3 seconds using data-acquisition software (Information Integrity, Boston, MA, USA) and stored off-line for later analysis. Arterial diameter over a 1 to 2 cm segment was determined for each image using automatic edge detection software (Information Integrity). Analyses were performed by an experienced vascular technician who was blinded to the phase of the study. Using pulsed wave Doppler, blood flow was recorded continuously throughout the study and was expressed as the velocity time integral (VTI; area under the blood velocity/time curve for a complete cardiac cycle). Baseline recordings of arterial diameter were made for one minute before inflation of a blood pressure cuff placed distal to the site of arterial imaging. Recording continued for 5 minutes during cuff inflation to 300 mm Hg and for 5 minutes after deflation. Endothelium-independent dilation of the brachial artery was assessed by measuring the dilator response to a 25 µg dose of the NO donor, glycercyl trinitrate given sublingually. This elicited vascular dilation of the same order of magnitude as that of the endothelium-dependent flow stimulus. Results are expressed as percentage maximum change in vessel diameter from baseline.

Laboratory assays
Urea, creatinine, bicarbonate and electrolytes were measured (Vitros 750; Ortho-Clinical Diagnostics, Rochester, NY, USA). Fasting lipid analyses were performed for total cholesterol (TC), high-density lipoprotein (HDL) and triglycerides (TGs) using colorimetric assays (Vitros 750, Ortho-Clinical). Both very low-density lipoprotein (VLDL) cholesterol and LDL were calculated. LDL subfractions were measured using high-resolution polyacrylamide gel electrophoresis (PAGE; Quantimetrix, Redondo Beach, CA, USA) and reported as the ratio of less dense to more dense (LDL1 +2:LDL3 +4+5). LDL lag times were measured by isolating LDL using density gradient ultracentrifugation and desalted by gel filtration. Oxidation was promoted using copper; conjugated diene production was monitored; and lag times generated [10]. Antioxidant activity was measured using a chemiluminescent assay [11]; antibodies to oxidized LDL by enzyme linked immunosorbent assay (ELISA; Olab, Biomedica Gruppe, Germany); and total plasma lipid peroxides (reaction products of oxidative damage) by a lipid hydroperoxide assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Free l-arginine levels were measured by an automated amino analyzer (Pharmacia, Milton Keynes, UK); plasma nitrite and nitrate (NO oxidation products) by chemiluminescence; nitrated proteins (nitrotyrosines: NO and superoxide result in peroxynitrite, which combines with tyrosine residues and is present in atherosclerotic plaques)
RESULTS

The primary study endpoint was the change in FMD of low density to high density LDL or lipid peroxides. Each subject served as his or her own control. There was no effect of L-arginine used produced a significant rise in nitrate but not significantly. There was no significant change with treatment. There was no effect of L-arginine on the ratio of low density to high density LDL or lipid peroxides.

Lipid analysis

Baseline total cholesterol was normal but triglycerides were elevated (Table 3). There was a significant fall in TGs on treatment versus placebo (−0.06 ± 0.54 vs. 0.19 ± 0.57 mmol/L, \( P = 0.005 \)) otherwise lipid profiles remained unchanged.

Oxidative stress

A highly significant fall in total antioxidant activity of the serum was noted during the treatment period versus the placebo phase (−41 ± 30 vs. 16 ± 54 μmol/L trolox Eq; \( P < 0.001 \); Table 3). LDL lag times (a measure of the susceptibility of LDL to oxidation) and antibodies to oxidized LDL were above the normal range for adults at baseline and there was no significant change with treatment. There was no effect of L-arginine on the ratio of low density to high density LDL or lipid peroxides.

Nitrate chemistry

Plasma nitrate was elevated but plasma nitrite was normal at baseline (Table 4). Plasma nitrated protein (nitrotyrosine) levels were increased significantly at entry to the study when compared with data available on normal children (0.126 ± 0.07 vs. 0.02 ± 0.007 nitroso-bovine serum albumin (BSA) equivalents μg/mL/mg protein, \( P < 0.001 \)). No significant changes in plasma nitrate or nitrite were observed with L-arginine treatment. Protein nitrotyrosine concentrations increased with L-arginine treatment but not significantly. There was no relationship between nitrotyrosine concentrations and those of nitrate and urea. Both L-arginine analogs ADMA and SDMA were elevated at baseline, but there was no significant change on treatment or placebo.

A negative correlation between SDMA and FMD was the only relationship noted with \( r^2 = 0.6 \) and \( P < 0.05 \). Otherwise no significant relationships between NO metabolites and GFR were seen.

Effect of oral L-arginine on vasomotor function

There was no relationship between baseline FMD and any clinical and biochemical parameters measured (Table 5). There was no effect of L-arginine on baseline arterial diameters, baseline blood flow or reactive hyperemia (\( P = \) NS) on within subject analysis (hence no
Table 2. Biochemical effect of L-arginine supplementation

<table>
<thead>
<tr>
<th>Urea nr 2.2–5.7 mmol/L</th>
<th>Baseline</th>
<th>Post-L-arginine</th>
<th>Baseline</th>
<th>Post-placebo</th>
<th>Change on L-arginine</th>
<th>Change on placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.2 ± 7.8</td>
<td>16.1 ± 11.1</td>
<td>11.6 ± 6.0</td>
<td>12.5 ± 7.4</td>
<td>−3.9 ± 4.9</td>
<td>0.9 ± 2.7</td>
<td>0.015</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Biochemical effect of L-arginine and placebo

<table>
<thead>
<tr>
<th>Antibodies to oxidized LDL (&lt;250 mU/mL) LDL lag times (&gt;60 min) Lipid peroxides (μmol/L) Antioxidant activity (&gt;440 μmol/L trolox Eq) Lipoprotein ratios TC (3.1–5.4 mmol/L) TG (0.4–1.4 mmol/L) HDL (&gt;0.91 mmol/L) LDL (&gt;3.3 mmol/L)</th>
<th>Baseline</th>
<th>Post-L-arginine</th>
<th>Baseline</th>
<th>Post-placebo</th>
<th>Change on L-arginine</th>
<th>Change on placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>631 ± 501</td>
<td>579 ± 468</td>
<td>609 ± 406</td>
<td>562 ± 478</td>
<td>−23 ± 72</td>
<td>−47 ± 91</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Effect of L-arginine on NO chemistry

<table>
<thead>
<tr>
<th>Nitrates (32.1 ± 4.3 μmol) Nitrates (1.4 ± 0.2 μmol) L-arginine (n80–120 μmol/L) ADMA (0.7 ± 0.1 μmol/L) SDMA (0.3 ± 0.1 μmol/L) Nitrotyrosines (0.02 ± 0.007 NT-BSA equivs, μg/mL/g)</th>
<th>Baseline</th>
<th>Post-L-arginine</th>
<th>Baseline</th>
<th>Post-placebo</th>
<th>Change on L-arginine</th>
<th>Change on placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>68.6 ± 63.1</td>
<td>67.2 ± 37.4</td>
<td>70.0 ± 55.6</td>
<td>62.9 ± 39.3</td>
<td>−1.13 ± 59.4</td>
<td>−7.12 ± 67.1</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Replication error), using the linear model repeated measures analysis.

Flow mediated dilation:
Endothelial dependent dilation

No significant difference in the change in flow mediated dilatation (endothelial dependent dilatation) during the treatment phase versus the placebo phase was noted (P = 0.84; Fig. 1).

Response to GTN: Endothelial independent dilatation

No significant difference in the change in response to GTN during the treatment phase versus the placebo phase was noted (P = 0.2; Fig. 1).
There was no significant change in resting heart rate, supine blood pressure during treatment or placebo phase, suggesting that there was no hemodynamic effect of the l-arginine.

DISCUSSION

Our results show that oral supplementation with L-arginine did not improve brachial artery FMD, a measure of endothelial derived NO bioavailability, in children with CRF, despite achieving plasma levels of twice normal. Indeed, it would not have been possible to increase the dose any further as the children developed a significant increase in plasma urea, a fall in venous bicarbonate and a fall in total antioxidant activity.

Increased cardiovascular mortality and morbidity is well recognized in adults with CRF [1]. The adverse impact of CRF on cardiovascular mortality and morbidity in the young is however even greater with a 500 times higher rate of cardiovascular deaths than a control population [12]. The initiation of vascular damage begins very early during the course of CRF, and involves the vascular endothelium. We have previously shown impaired brachial artery FMD in children with CRF from as young as 7 years of age. Use of this non-invasive ultrasound technique, which is NO dependent [13, 14], enables the study of the early atherogenic process, and permits serial assessment of therapeutic interventions at a stage when maximum benefit might be expected. The biological activity of NO is impaired in CRF [15], which leads to reduced vascular relaxation, increased platelet aggregation, increased leukocyte adhesion and smooth muscle proliferation, all processes which precede the formation of atherosclerotic plaques [3]. L-Arginine is the substrate for NO synthase and has been shown to increase endothelial function in animal models and in clinical studies of subjects with hypercholesterolemia and coronary artery disease [8, 16–18]. We postulated that L-arginine supplementation in children with CRF might increase NO bioavailability by three main mechanisms: (1) by correcting a possible substrate deficiency; (2) by reducing the effect of endogenous inhibitors such as ADMA, known to be elevated in CRF; and (3) by overcoming increased oxidative inactivation. We did not demonstrate substrate deficiency, as baseline serum L-arginine levels were normal. However, intracellular levels may not correlate with serum levels in CRF, as elevated levels of oxidized LDL (which are present in CRF) are associated with defective cellular transport for L-arginine [19]. L-Arginine supplementation might overcome the effect of elevated methylated analogs, ADMA and SDMA in CRF. ADMA is an inhibitor of eNOS [5] and although SDMA is not a direct inhibitor, it competes with the cationic amino acid transporter in the endothelial cell membrane [20] and therefore could heighten any intracellular arginine deficiency. In our children, both SDMA and ADMA were elevated at baseline when compared with previously published data in children [2]. Levels were, however, not as high as those previously reported in adults with CRF [4, 5]. The modest elevation of ADMA in our study group may have contributed to the lack of vascular benefit seen with L-arginine supplementation.

There was, however, no correlation between the response of FMD and levels of ADMA or those of SDMA. Markers of oxidative stress (antibodies to oxidized LDL, antioxidant activity) were elevated in our study. Increased oxidative stress might be responsible for reducing NO bioavailability by conversion of NO to nitrate, nitrate, and peroxynitrite (a potent cytotoxic agent) by superoxide. L-arginine supplementation could overcome this as in vitro studies have shown that L-arginine supplementations prevents preferential superoxide production in endothelial cells exposed to near physiological levels of oxidized-LDL [21]. In our CRF children, there was no biochemical evidence of increased NO production (nitrate and nitrite levels remained unchanged) despite a doubling of the L-arginine levels. In view of the unrestricted diet this observation should be interpreted with caution. De Nicola et al achieved similar L-arginine levels in adult CRF patients with oral supplementation and also were unable to demonstrate enhanced NO production by measuring urinary cyclic guanosine mono-

<table>
<thead>
<tr>
<th>Table 5. Vascular responses to L-arginine and placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Brachial artery diameter mm</td>
</tr>
<tr>
<td>Baseline flow VTI, m</td>
</tr>
<tr>
<td>Maximum reactive hyperemia %</td>
</tr>
<tr>
<td>FMD %</td>
</tr>
<tr>
<td>Systolic blood pressure mm Hg</td>
</tr>
<tr>
<td>Diastolic blood pressure mm Hg</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.
phosphate (cGMP, an intracellular mediator of NO) [22]. It is possible that important co-factors for eNOS such as tetrahydrobiopterin may have been deficient in our patients [16, 23]. Under these conditions, NOS preferentially reduces molecular oxygen to superoxide even in the presence of adequate substrate concentrations. L-Arginine at the dose used had adverse metabolic effects including elevated urea and extracellular acidosis. These could have affected pH dependent signaling pathways and, therefore, eNOS activity. There was no correlation between these fully reversible effects and endothelial function, however. The reduction in total antioxidant activity of the serum suggests that increased oxidant stress was generated during the treatment phase.

Fig. 1. Flow-mediated dilation (FMD) pre-(left) and post- (right) treatment with L-arginine (A) and placebo (B). P = NS for group means.
We chose to evaluate children because this allowed us to study the process of atherosclerosis early in its natural history when it is potentially more responsive to intervention. In addition, the young population provided an opportunity to minimize the unquantifiable impact of life-long confounding risk factors on endothelial function. Children with CRF secondary to inflammatory diseases, diabetes and hypertension were excluded, as these factors are known to have a major impact on vascular function, even in the absence of renal impairment. Our study population was not preselected on the basis of FMD or clinical severity of disease so that they would be representative of the effect of CRF in young subjects.

The technique of FMD developed by our group is ideally suited to this study. It is noninvasive, reproducible and well validated as a measure of NO-dependent vaso-dilation and hence endothelial function in conduit arteries [14, 24]. There is good correlation between the brachial artery and the coronary and carotid circulations [25, 26]. The impact of a range of interventions on FMD is well reported both by our group and others in both children and adults with cardiovascular risk factors.

L-Arginine supplementation in this group of children with CRF did not improve endothelial function. Oral supplementation at a dose producing a twofold rise in serum levels resulted in adverse metabolic sequelae in several children, and may have contributed to the negative outcome. Investigation into other mechanisms of impaired vascular biology in CRF is required.

ACKNOWLEDGMENTS

Funding for this study was from The British Heart Foundation. Research at the Institute of Child Health and Great Ormond Street Hospital for Children benefits from R&D funding received from the NHS executive. Dr. Bennett-Richards was supported by a grant from the British Heart Foundation. Mrs. Kattenhorn was supported by the British Heart Foundation, and Mrs. Donald was funded by CORDA (Coronary Artery Disease Research Association) through a legacy from the late Marian Silcock. A portion of these data was presented at the Renal Association UK (April 2000) and Royal College of Paediatries and Child Health UK (April 2000). We thank Mr. Michael Beau-champ (laboratory technician, Great Ormond Street Hospital) for his assistance in preparation of the many samples for biochemical analysis.

Reprint requests to Dr. Katy Bennett-Richards, Vascular Physiology Unit, 34 Great Ormond Street, London WC1 3JH, England, United Kingdom
E-mail: KatyIBR@aol.com

REFERENCES