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Effects of prolonged oral supplementation with L-arginine on blood pressure and nitric oxide synthesis in preeclampsia

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Abstract

Background Several lines of evidence point to the dysfunction of the endothelial L-arginine–NO system in preeclampsia. We investigated the influence of dietary supplementation with L-arginine on blood pressure and biochemical measures of NO production in women with preeclampsia in prospective, randomized, placebo-controlled study.

Design The 61 preeclamptic women on a standardized low nitrate diet received orally 3 g of L-arginine ($n = 30$) or placebo ($n = 31$) daily for 3 weeks as a supplement to standard therapy. The differences between the two groups in systolic (SBP), diastolic (DBP) and mean arterial blood pressures (MAP) as well as in plasma levels of selected aminoacids, plasma concentrations of nitrates/nitrites (NOx) and in 24-h urine NOx excretion were determined.

Results After 3 weeks of treatment, values of SBP, DPB and MAP were significantly lower in the group taking L-arginine as compared with the placebo group (SBP: 134·2 ± 2·9 vs. 143·1 ± 2·8; DBP: 81·6 ± 1·7 vs. 86·5 ± 0·9; MAP: 101·8 ± 1·5 vs. 108·0 ± 1·2 mmHg, $P < 0·01$). Importantly, treatment with exogenous L-arginine significantly elevated 24-h urinary excretion of NOx and mean plasma levels of L-citrulline. Exogenous L-arginine did not influence plasma concentrations of L-arginine, L-ornithine and methylated arginines (ADMA, SDMA, L-NMMA).

Conclusions We conclude that in women with preeclampsia, prolonged dietary supplementation with L-arginine significantly decreased blood pressure through increased endothelial synthesis and/or bioavailability of NO. It is tempting to speculate that the supplementary treatment with L-arginine may represent a new, safe and efficient strategy to improve the function of the endothelium in preeclampsia.

Keywords Endothelial dysfunction, L-arginine, nitric oxide, preeclampsia.
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Introduction

Preeclampsia, described clinically as the occurrence of hypertension and proteinuria after 20 weeks' gestation in previously normotensive women, complicates approximately 6–8% of all gestations and is the leading cause of foetal growth restriction, infant mortality, premature birth and maternal complications [1]. Although the aetiology and pathogenesis of the disease are still disputed, it becomes clear that a compromised function of maternal endothelium contributes at least to haemodynamic features of preeclampsia, i.e. vasoconstriction with increased peripheral resistance and hypertension [2]. Isolated small arteries from women with preeclampsia demonstrate impaired endothelium-dependent vasodilatation [3,4]. Moreover, recent *in vivo* studies have reported that preeclamptic patients have impaired flow-mediated vasodilatation as well as a decreased response of vessels to acetylcholine [5–7]. Nitric oxide (NO), a potent endothelial-derived vasodilator synthesized by constitutive nitric oxide synthase (NOS-3) from L-arginine, was shown to modulate peripheral vascular tone [8]. Increased production of NO has been claimed to be responsible for physiological vascular adaptation to gestational stimulation of renin-aldosterone axis and an increase of blood volume [9]. On the other hand, decreased synthesis and/or bioavailability of NO has been suggested to play a role in preeclampsia

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[10]. Pharmacological inhibition of NOS in pregnant rats produces preeclampsia-like symptoms, which can be reversed by an infusion of L-arginine [11,12]. In patients with preeclampsia, plasma levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthesis, were found to be elevated [13,14]. The recent understanding of the role of NO in preeclampsia raises new possibilities of treatment of this dangerous disorder. Although, exogenous NO, i.e. application of NO-donors, was shown to improve fetoplacental circulation in preeclamptic women, recent reports demonstrated impaired endothelial function after prolonged use of these drugs [15,16]. In this regard, stimulation of synthesis of endogenous NO, by use of a substrate for NOS, L-arginine seems safer. Intriguingly, whether exogenous L-arginine promotes endothelial synthesis of NO, reverses endothelial dysfunction and therefore improve clinical status of patients is still uncertain [17]. Several authors have demonstrated such a possibility in patients suffering from atherosclerosis, hypertension or diabetes; however, studies carried out on preeclamptic women have been conflicting [18,19]. Infusion of 30 g of L-arginine was reported to reduce blood pressure and increase plasma levels of L-citruline and nitrite; noteworthy, these effects were significantly greater in uncomplicated than preeclamptic pregnancies [20]. On the other hand, a recent study on preeclamptic patients denied a hypotensive effect of L-arginine given in a daily oral dose of 12 g, up to 5 days [21]. Importantly, in both studies L-arginine was given in high amounts over short periods of time. However, taking into consideration the complex nature of endothelial dysfunction one should expect a significantly longer time for its reversal [22].

The aim of present placebo-controlled study was to determine whether L-arginine, given orally in a relatively low daily dose (3 g) over long period of time (3 weeks), might affect blood pressure in patients with preeclampsia. To study the biochemical effects of exogenous L-arginine, plasma levels of selected aminoacids as well as plasma levels and 24-h excretion of nitrite/nitrate (NOx) in urine were assessed.

Methods

Subjects

The investigation was carried out in the Department of Gynecology and Obstetrics of Jagiellonian University Medical College in Krakow, Poland, over a period of 4 years (2000–03). Initially, 83 women with preeclampsia were included in the open, prospective, placebo-controlled trial (61 patients completed the study). The diagnosis of preeclampsia was made by strict ACOG criteria: onset of hypertension during late gestation with systolic and diastolic pressure greater than 140–90 mmHg on at least two occasions and urinary protein excretion greater than 300 mg L^{-1} after the 20th week of pregnancy; subjects were normotensive during the first trimester and had no history of chronic hypertension, and pregnancies were singleton. Women who smoked were excluded from the study, as were women with any underlying chronic illnesses such as hypertension, coronary heart disease, renal disease or diabetes mellitus. All patients received hypotensive treatment daily containing dihydralazine (4 × 25 mg) and methyldopa (250 mg), as well as an intravenous infusion of magnesium sulphate (6 g of $MgSO_4 \times 7H_2O$ in 500 mL sterile saline). During the course of the study the doses of hypotensive drugs were decreased or increased whenever blood pressure was lower than 130–90 mmHg (50% of patients from the L-arginine group vs. 12·9% of patients from the placebo group) or was higher than 150–100 mmHg (16·7% of patients from the L-arginine group vs. 48·4% of patients from the placebo group), respectively. Steroid treatment to accelerate lung maturation was used in all patients between the 26th and 34th weeks of pregnancy.

Study protocol

The protocol for the use of L-arginine in preeclampsia was approved by the Ethics Committee of Jagiellonian University and all patients gave written informed consent. The study was carried out in the Department of Gynaecology and Obstetrics. The treatment with L-arginine or placebo was initiated 1–4 weeks after diagnosis of preeclampsia and the start of hypotensive treatment and was continued until the 36th week of pregnancy.

Reduced nitrate diet was enforced for 1 week before, as well as for 1 week after 3 weeks of treatment with L-arginine (Curtis Healthcare, Poznan, Poland, 3 g per day, $n = 42$) or placebo ($n = 41$). Blood and urine samples were harvested on the 3rd day of the diet period. Systolic and diastolic blood pressure values (SBP, DBP) were measured according to WHO-ISH guidelines [23], four times daily (8·00; 12·00; 18·00; 22·00) for 4 days starting from the 2nd day of the reduced nitrate diet period, on the right hand with a standard sphygmomanometer. Mean arterial blood pressure (MAP) was calculated according to formula:

$$MAP = [\text{systolic pressure} + (2 \times \text{diastolic pressure})]/3$$

and expressed as an average value from all time-points collected during the 4-day observation. Additionally, as a part of everyday care, blood pressure was measured regularly throughout the study period. Other clinical data collected on every patient included the following: age, body weight, obstetric history, proteinuria, creatinine clearance, liver function tests and coagulation profile.

Reduced nitrate diet

The subjects were requested to forgo foodstuffs rich in nitrate (cured meats, fish, cheese, vegetables and malted beverages) and all medications, which may interfere with the measurement of nitrate/nitrite (NOx) (esp. Vitamin C). Food and beverages, the same for all patients, were prepared by the kitchen of the Department of Gynaecology and Obstetrics in consultation with a registered Dietician.

Sample collection

Peripheral venous blood was drawn into 5-mL tubes containing heparine/EDTA. The plasma was separated from the blood by centrifugation 8000 g for 5 min at room temperature and kept frozen (–80 °C) until assayed.

Each subject was instructed on the proper collection of 24-h urine. Urine containers were kept in the refrigerator. When the 24-h urine collection was finished, urine samples were collected, immediately frozen and kept at –80 °C until assayed.

Measurements of NOx

The total amount of nitrite and nitrate (NOx) in blood was quantified colourimetrically by a modified Griess method [24]. Micromolar concentrations of NOx were calculated from the standard curve of sodium nitrate. Urinary excretion of NOx was expressed as the amount of NOx per amount of excreted creatinine (μmol μmol^{-1}).

Measurements of plasma amino acid levels

Plasma samples were diluted with saline solution (1 : 1 v/v) and deproteinized by ultrafiltration through a low-binding cellulose membrane with nominal weight cut-off 10 kDa (Millipore, Vienna, Austria). Separation of aminoacids were determined by a reverse-phase HPLC TSP system coupled with a mass spectrometer LCQ (Finnigan, San Jose, CA) using a Purospher RP-C18e column with a Lichrosphere B C-18 precolumn (Merck, Darmstadt, Germany) with a mobile phase consisting of 0·1% formic acid in water-acetonitrile (95 : 5 v/v). Detection of examined compounds was performed using a mass spectrometer with ion-trap, equipped with an electrospray ionization (ESI)-positive ions mode. As in this method, peaks of ADMA and SDMA cannot be separately calculated, and the total concentration of methylated arginines (ADMA + SDMA) was used in the analysis.

Statistics

Data are presented as means ± standard deviation (SD) and were compared using a Student's *t*-test, as well as one-way ANOVA with Tukey's *post hoc* test whenever appropriate. Additionally, a paired *t*-test as well as ANOVA for repeated measurements were performed. All statistical analyses were performed with STATISTICA vs. 5·0 (StatSoft, Krakow, Poland). $P < 0·05$ was considered statistically significant.

Results

Although initially 83 subjects met the criteria for enrolment, 61 women completed the study. Owing to instability of maternal and/or foetal conditions and the necessity of a termination of pregnancy, 12 and 10 patients dropped out during the study from the L-arginine and placebo groups, respectively. There were no observed or reported adverse effects attributable to the use of L-arginine.

There were no initial differences between the groups with regard to mean age (29·3 ± 6·7 vs. 29·2 ± 5·9 years), mean length of gestation on admission (29·3 ± 3·4 vs. 29·1 ± 3·4 weeks), creatinine clearance (136 ± 46 vs. 142 ± 61 mL min^{-1}), body weight (74·9 ± 12 vs. 73·2 ± 11·9 kg) and previous history of pregnancies and labours. Table 1 summarizes selected data of patients at the beginning of the study.

There were no significant differences between groups in SBP, DBP as well as in mean arterial blood pressure (MAP) at the beginning of the study (SBP: 143·9 ± 5·7 vs. 145·5 ± 4·8; DBP: 87·8 ± 1·7 vs. 87·7 ± 0·9; MAP: 107·0 ± 1·0 vs. 106·0 ± 1·3 mmHg, NS).

However, after 3 weeks of treatment, values of SBP, DPB and MAP were significantly lower in the group taking L-arginine than in the placebo group (SBP: 134·2 ± 2·9 vs. 143·1 ± 2·8; DBP: 81·6 ± 1·7 vs. 86·5 ± 0·9; MAP: 101·8 ± 1·5 vs. 108·0 ± 1·2 mmHg, $P < 0·01$).

There were no significant differences between the groups in the amounts of NOx calculated per amount of excreted creatinine in 24-h urine at the beginning of the study (0·063 ± 0·01 vs. 0·077 ± 0·014 μmol μmol^{-1}, NS, Fig. 1A). Importantly, after 3 weeks of treatment, 24-h urinary excretion of NOx was significantly elevated in the group of patients taking L-arginine (0·162 ± 0·045 vs. 0·07 ± 0·0098 μmol μmol^{-1}, $P < 0·05$, Fig. 1A). Interestingly, although plasma NOx concentrations tended to be higher in the L-arginine group, the difference did not reach statistical significance (Fig. 1B).

Initially, there were no significant differences between the groups in plasma levels of L-citrulline, L-ornithine and L-arginine, as well as its methylated derivatives (ADMA + SDMA, L-NMMA) (Table 2). Treatment with exogenous

Table 1 Comparison of selected demographic data of patients enrolled in the analysis. All data were collected at the beginning of the study, after randomization, just before L-arginine or placebo administration

Variables	L-Arginine ($n = 30$)	Placebo ($n = 31$)	*t*-test
Maternal age (years)	29·3 ± 6·7	29·2 ± 5·9	NS
Maternal weight (kg)	74·9 ± 12·0	73·2 ± 11·9	NS
Gestational age (weeks)	29·3 ± 3·4	29·3 ± 6·7	NS
Creatinine clearance (mL min^{-1})	136 ± 46	142 ± 61	NS
Urine Protein excretion (g 24 h^{-1})	2·56 ± 2·5	2·89 ± 3·0	NS

Table 2 Plasma aminoacid levels (μM) in patients with preeclampsia before and at the end of 3 weeks' treatment with placebo or L-arginine (3×1 g day^{-1}, P.O.)

	Before treatment		3 weeks' treatment	
	Placebo ($n = 31$)	L-Arginine ($n = 30$)	Placebo ($n = 31$)	L-Arginine ($n = 30$)
L-ARG	63·2 ± 23	61·9 ± 22	54·5 ± 20	63·9 ± 27
L-CIT	23·0 ± 16	20·3 ± 12	18·9 ± 11	30·0 ± 13*
L-ORN	47·4 ± 16	46·3 ± 14	44·0 ± 16	50·0 ± 18
ADMA + SDMA	0·65 ± 0·5	0·68 ± 0·6	0·51 ± 0·3	0·82 ± 0·5
L-NMMA	0·24 ± 0·1	0·21 ± 0·1	0·243 ± 0·2	0·298 ± 0·15

L-ARG, L-arginine; L-CIT, L-citrulline; L-ORN, L-ornithine; ADMA, asymmetric N^G, N^G-dimethylarginine; SDMA, symmetric N^G, N^G-dimethylarginine; L-NMMA, L-N^G-monomethylarginine.
*$P < 0·05$ as compared with the placebo group.

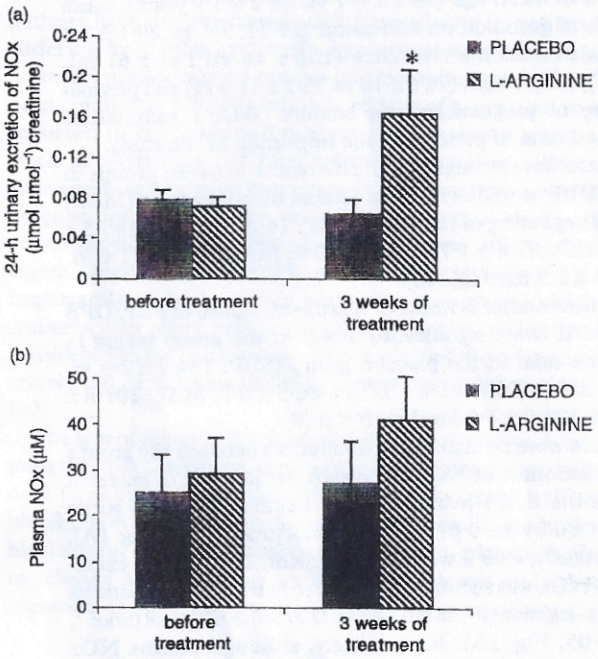

Figure 1 Influence of 3 weeks' treatment with L-arginine (3×1 g, $n = 30$) vs. placebo ($n = 31$) on 24-h urine nitrate/nitrite (NOx) excretion (a) as well as on plasma levels of NOx (b) in patients with preeclampsia. Urinary excretion of NOx was expressed as the amount of NOx per amount of excreted creatinine (μmol μmol^{-1}). *$P < 0·01$ vs. placebo group.

L-arginine did not significantly influence plasma levels of L-arginine, L-ornithine, ADMA + SDMA and L-NMMA; however, after 3 weeks of treatment mean plasma levels of L-citrulline were higher in patients receiving L-arginine (30·0 ± 13 vs. 18·9 ± 11 μmol L^{-1}, $P < 0·05$; Table 2). Interestingly, treatment with L-arginine slightly increased plasma levels of all methylated arginine derivatives (Table 2).

There were no influences of L-arginine treatment on urine protein excretion, as compared with the placebo group (3·32 ± 2·8 vs. 3·18 ± 3·74 g 24 h^{-1}, NS).

Discussion

Hypertensive disorders are essentially related to endothelial damage [25]. Importantly, antihypertensive drugs, which may reverse the dysfunction of the endothelium cannot be used in pregnancy either because they are teratogenic, like ACE inhibitors, or because their adverse effects are augmented when administered in combination with magnesium sulphate, such as calcium-channel blockers [26]. In this work we demonstrated that in women with preeclampsia the addition of oral L-arginine but not placebo to standard hypotensive treatment caused a significant decrease of blood pressure without any adverse reactions. Moreover, prolonged administration of L-arginine significantly improved foetal conditions and infant outcome (Rytlewski *et al.*, unpublished data).

By far, none of the studies about L-arginine administration in hypertension or in preeclampsia, including our preliminary report, describes any adverse reactions attributable to the use of L-arginine; however, its influence on blood pressure is more controversial [27]. Short-time infusions of large amounts of L-arginine were shown to transiently decrease blood pressure in patients with hypertension and preeclampsia through nitric oxide release, as evidenced by elevations of serum L-citrulline and nitrite levels [20]. On the other hand, in a recent study, dietary supplementation with 12 g of L-arginine up to 5 days did not reduce diastolic or mean blood pressure in women with preeclampsia [21]. Surprisingly, only a few studies addressed the question of the effects of L-arginine administered over a prolonged period of time. A single study demonstrated slight hypotensive effects of oral L-arginine given to hypertensive patients over 4 weeks [18]. To our knowledge, the present study is the first in which a relatively low dose of L-arginine was administered for longer than 1 week in a placebo-controlled manner to a relatively numerous group of women with preeclampsia.

Controversies about mechanisms of antihypertensive action of exogenous L-arginine remain. In our hands, after 3 weeks of treatment with L-arginine but not with placebo, there was an increased 24-h urine nitrite/nitrate (NOx) excretion, which was accompanied by elevated levels of

plasma L-citrulline. Apparently, exogenous L-arginine increased the formation of NO. Caveats and pitfalls of use of NOx assays as a measure of systemic NO synthesis in clinical studies were thoroughly described [28]. In order to minimize the influence of a dietary intake of nitrate, we measured urine and plasma NOx in patients on the standardized diet containing a reduced amount of NOx [29]. In this setting, L-arginine treatment tended to elevate plasma NOx levels; however, the difference did not reach statistical significance. This is in keeping with previous reports that 24-h urine NOx excretion is a more sensitive and reliable method for estimation of steady-state NO production than NOx plasma levels, influenced also by NOx clearance [30]. Despite the lack of differences between the placebo and L-arginine groups in the initial and final values of creatinine clearance, we normalized 24-h excretion of NOx in urine to excretion of creatinine, which eliminated 'kidney function' bias, and made our results more reliable.

It should be noted that urine or plasma NOx as well as plasma aminoacid measurements reflect global NO production in the human body, and cannot discriminate between particular tissues as sources of NO. Importantly, many studies have pointed to endothelial NOS-3 as the main source of basal levels of NOx in plasma as well as basal 24-h excretion of NOx in urine in patients on a low nitrate diet [31]. Thus, we hypothesize that in our patients a decrease of blood pressure depended on the increased production of NO by endothelial NOS-3.

There are several possible ways by which exogenous L-arginine may increase production of NO by NOS-3.

Some authors have demonstrated lower plasma levels of L-arginine in preeclamptic patients [32]. Theoretically, exogenous L-arginine could compensate for a shortage of endogenous substrate. However, in our study, plasma levels of L-arginine remained stable irrespective of treatment with exogenous aminoacid, which makes this line of explanation unlikely.

Another possible way of action of exogenous L-arginine could depend on the attenuation of the inhibitory influence of methylated arginines on NOS-3 activity. It was demonstrated that in preeclampsia, plasma levels of endogenous inhibitors of NOS, assymetric (ADMA) and symmetric dimethylarginines (SDMA) are elevated [14,33]. One could argue that an excess of L-arginine might restore NOS-3 activity by competitively displacing endogenous inhibitors from the active site of the enzyme [34]. However, neither plasma concentrations of L-arginine nor levels of methylated arginines (ADMA + SDMA, L-NMMA) changed significantly in the patients treated with L-arginine.

Although speculative, another line of explanation of observed phenomena may depend on the ability of L-arginine to inhibit the formation and/or action of reactive oxygen species (ROS) within the vascular wall. The role of increased oxidative and nitrosative stress in preeclampsia was well-documented [35–37]. L-arginine, as a substrate for NOS-3, might not only increase NO synthesis but also prevent NOS-3-dependent formation of ROS [38] and improve NO bioavailability; interestingly, L-arginine also could be a scavenger of ROS [39]. Whether the antihypertensive action of L-arginine depends on such mechanisms remains an attractive hypothesis to be tested.

In summary, we have demonstrated that in women with preeclampsia prolonged dietary supplementation with L-arginine significantly decreased blood pressure through increased synthesis and/or bioavailability of NO. It is tempting to speculate that the supplementary treatment with L-arginine may represent a new, safe and efficient strategy to improve the function of the endothelium in preeclampsia.

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