

The effect of oral L-arginine supplementation on fasting glucose, HbA1c, nitric oxide and total antioxidant status in diabetic patients with atherosclerotic peripheral arterial disease of lower extremities

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Abstract. – Introduction: Numerous studies indicate hyperglycemia and oxidative stress as factors responsible for endothelium dysfunction and the following development of angiopathy. Increased production of free radicals by vascular endothelium causes disturbance in production and/or decreases bioaccessibility of nitric oxide (NO). It has been suggested that L-arginine supplementation is a reasonable method to increase endothelium NO production and lower free radicals formation. There is a growing number of evidence showing that dietary supplementation of arginine reverses endothelial dysfunction associated with major cardiovascular risk factors and ameliorates many common cardiovascular disorders.

Objective: The aim of the study was to evaluate the potential influence of two-months oral L-arginine supplementation on fasting glucose, HbA1c, nitric oxide and total antioxidant status (TAS).

Materials and Methods: 38 patients with atherosclerotic peripheral arterial disease of lower extremities at Fontaine's stage II and coexisting type 2 diabetes and 12 healthy volunteers as control group were studied. All patients were treated with oral L-arginine (3 x 2 g/day) for two months. Fasting glucose, HbA1c, nitric oxide and total antioxidant status (TAS) were measured before and after the study.

Results: Fasting glucose and HbA1c did not change significantly after L-arginine treatment. Statistically significant increase in NO concentration and TAS level was found.

Conclusions: Oral two-month supplementation with L-arginine (3 x 2 g/day) had no effect on fasting glucose and HbA1c level in diabetic patients with atherosclerotic peripheral arterial dis-

ease of lower extremities at Fontaine's stage II. The supplementation of L-arginine led to substantial increase in NO concentration and TAS level in these patients, suggesting its indirect antioxidative effect.

Key Words:

L-arginine, Diabetes mellitus, Atherosclerosis, Nitric oxide, Oxidative stress.

Introduction

The endothelium is more and more frequently perceived as the largest organ of human organism. Endothelial cells not only act as a barrier between blood and vascular membrane but also are an important endo- and paracrine organ^{1,2}.

The key function of endothelium is to sustain correct vascular tone by production and secretion of vasodilating and vasospastic factors. In physiological conditions endothelium ensures maintaining balance between factors regulating vascular tone and plays basic role in regulation of hemostasis. Proper function of endothelium guarantees free blood flow, secretion of NO and proper thickness of vascular wall. However, hyperglycemia, lipid disorders, oxidative stress and/or arterial hypertension change the endothelium functioning, which leads to vasospastic, proliferating, prothrombic and inflammatory activity^{3,4}.

Therefore, the endothelium is an essential mediator of the pathogenic effects of these conditions on the blood vessels^{5,6}.

Dysfunction of proper endothelium activity and structure results in early development of atherosclerosis and is the cause of major health problems, especially in patients with incorrect glucose tolerance and type 2 diabetes⁷⁻⁹. Numerous studies indicate hyperglycemia and oxidative stress as factors responsible for endothelium dysfunction and development of angiopathy (including atherosclerosis)^{5,10-13}. The relationship between hyperglycemia and oxidative stress was confirmed in a study performed by Giardino et al¹⁴ who observed substantial increase of free radicals synthesis and increased concentration of lipid peroxidation products in the culture of endothelial cells bred in environment containing 30 mM of glucose.

The atherosclerosis of lower limbs is one of the symptoms of systemic atherosclerosis. It is a chronic disease that results from narrowing or occlusion of arteries which supply lower limbs' tissues with blood^{15,16}. Significant role in development of this condition is assigned to endothelium dysfunction associated with nitric oxide deficiency¹⁷.

Endothelial L-arginine/NO pathway is a physiological vasodilating mechanism which influences peripheral vascular resistance thus influencing the blood pressure. Moreover NO effectively inhibits the adhesion of thrombocytes, neutrophil leucocytes and monocytes to vascular wall^{13,17}. Such activity may protect from atherosclerosis and thrombosis.

Learning the arginine – NO pathway revealed another therapeutic use of L-arginine, as it is the only endogenous source of nitric oxide in the organism. Numerous studies delivered more and more information concerning effective use of L-arginine in treatment and prevention of many diseases associated with endothelium dysfunction, such as hypercholesterolemia, atherosclerosis^{18,19}, pulmonary hypertension²⁰, diabetes^{19,21-23} and its chronic complications (including atherosclerosis of lower limbs).

It is worth noticing that only few studies assessed the effect of oral L-arginine supplementation on carbohydrate balance in patients with atherosclerotic peripheral arterial disease of lower extremities and coexisting type 2 diabetes and the results were inconclusive.

Therefore, the aim of our study was to estimate the effect of two-months oral supplementa-

tion with L-arginine (6 g/24 hours) on fasting glucose concentration, HbA1c level, total antioxidant status (TAS) and concentration of nitric oxide in patients with atherosclerosis of lower limbs and coexisting type 2 diabetes.

Patients and Methods

The Patients

38 hospitalized patients (18 women and 20 men) with diagnosed atherosclerotic peripheral arterial disease of lower extremities at Fontaine's stage II and newly diagnosed type 2 diabetes were studied. To estimate the Fontaine's stage of peripheral arterial disease, the pain-free walking distance was measured on the horizontal Cambridge 9800 walking treadmill at 3.5 km/h. Oral glucose tolerance test was carried out to diagnose carbohydrate abnormalities.

All patients were treated with standard stable pharmacotherapy (according to European Society of Cardiology 2008 guidelines and Polish Diabetologists Association guidelines) during and after hospitalization. All patients were treated with:

- Comparable diet and exercise
- ASA-acidum acetylsalicylicum dosed 75 mg/24 hours or ticlopidine dosed 2 × 250 mg (in case of contraindications)
- Statins (simvastatin dosed 20 mg/24 hours, atorvastatin dosed 40 mg/24 hours); all patients were successfully treated with stable dose of statin.

Exclusion criteria were: congestive heart failure, hypertension, thyroid disease, alcohol abuse, cigarette smoking, serious liver failure (aminotransferases two times the normal value), serious kidney failure (serum creatinine 1.5 times the normal value), acute or chronic inflammation, electrolyte imbalance.

The control group consisted of 12 healthy volunteers (6 men and 6 women), who showed no signs of organ pathology, especially associated with cardiovascular system, liver, kidneys and inflammation.

All patients and healthy volunteers underwent medical interview, physical examination, biochemical examination (blood morphology, erythrocyte sedimentation ratio (ESR), lipid profile, glucose concentration, general analysis of urine)

and additional examinations (blood pressure measurement, treadmill test, ankle-brachial index) on the beginning, after one month and at the end of the study.

Informed consent was obtained from all subjects and the study was approved by the Research Ethics Committee of Poznan University of Medical Sciences and registered as nr 290/06. It conformed to all ethical issues included in the Helsinki Declaration.

Study Design

The study had prospective character. Duration of the study was two months, during which patients were orally supplemented with L-arginine (manufactured by Curtis Healthcare of Poland) 3×2 g/day. Blood tests were carried out before L-arginine supplementation and after two months of treatment.

Biochemical Measurements

The examinations were carried out in standard hospital conditions. Blood samples at baseline were obtained between 8:00 and 10:00 after overnight fasting. 10 ml of blood was taken for analyses from the elbow vein. The blood was then distributed to tubes containing K₂EDTA or heparin. Samples for the analysis of NO and TAS were immediately centrifuged (10 minutes with RCF = $1000 \times g$) in order to minimize the loss of labile NO. Samples of plasma were stored in -70°C until the analysis.

Nitric oxide was analysed with Oxis NO-kits (OXIS International, Inc., Cutter Circle, Portland, OR, USA) in Hyperion MicroReader (International Immuno-Diagnostics, Foster City, CA, USA). TAS was assessed with Randox NX2332 test (Randox Laboratories, Crumlin, United Kingdom). The glucose level was measured with Dade Behring Inc. Dimension[®] GLU test (Dade

Behring; Milton Keynes, U.K.). In vitro quantitative analysis of the percentage of HbA1c was carried out with HA1c test in Dade Behring Inc. Dimension[®] system (Dade Behring; Milton Keynes, U.K.).

Statistical Analysis

Statistical analysis was carried out using Statistica 6.0 software (StatSoft). The normality of distribution was estimated with W. Shapiro-Wilk test. The significance of differences in mean values was estimated with *t*-test for independent samples with separate evaluation of the variance.

Results

50 subjects completed the study – 38 patients and 12 healthy volunteers. The characteristic of studied groups is presented in Table I.

There were no significant difference between patients and control group in age, gender, height, weight, BMI systolic and diastolic blood pressure and lipid profile.

The patients' HbA1c level and fasting glucose concentration significantly exceeded these observed in the control group (Table II, Figures 1 and 2).

There was no statistically significant decrease of the fasting glucose concentration in patients supplemented with L-arginine (8.63 ± 1.50 nmol/l at the beginning vs. 8.48 ± 1.44 nmol/l after two months of L-arginine supplementation).

Similar results (no statistically significant changes) were obtained in the analyses of HbA1c level ($7.28 \pm 0.78\%$ at the beginning and 7.23 ± 0.75 after two months of L-arginine supplementation).

Table I. Characteristic of studied groups.

Parameter	Patients N = 38	Control N = 12	Significance
Gender (male/female)	20/18	6/6	ns
Age (years)	56.5 ± 7.6	54.0 ± 6.9	ns
Height (cm)	172.6 ± 5.9	172.8 ± 5.2	ns
Weight (kg)	74.4 ± 8.1	71.2 ± 6.1	ns
BMI (kg/m ²)	24.9 ± 2.0	23.8 ± 1.2	ns
SBP (mmhg)	138.2 ± 11.3	135.3 ± 9.4	ns
DBP (mmhg)	85.1 ± 6.1	83.2 ± 5.7	ns

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; *p*: statistical probability; ns: not significant.

Table II. Fasting glucose concentration, HbA1c level and lipids profile in the control and patients group before L-arginine supplementation.

	Patients N = 38	Control N = 20	Significance
Fasting glucose (mmol/L)	5.37 ± 0.70	8.63 ± 1.50	<i>p</i> < 0.05
HbA1c (%)	5.43 ± 0.44	7.28 ± 0.78	<i>p</i> < 0.05
Total cholesterol (mmol/L)	4.96 ± 0.92	4.96 ± 0.92	ns
LDL-cholesterol (mmol/L)	4.96 ± 0.92	5.02 ± 0.67	ns
HDL-cholesterol (mmol/L)	1.63 ± 0.35	1.56 ± 0.29	ns
Triglycerides (mmol/L)	1.70 ± 0.48	1.69 ± 0.47	ns

HbA1c: hemoglobin A1c; *p*: statistical probability; ns: not significant.

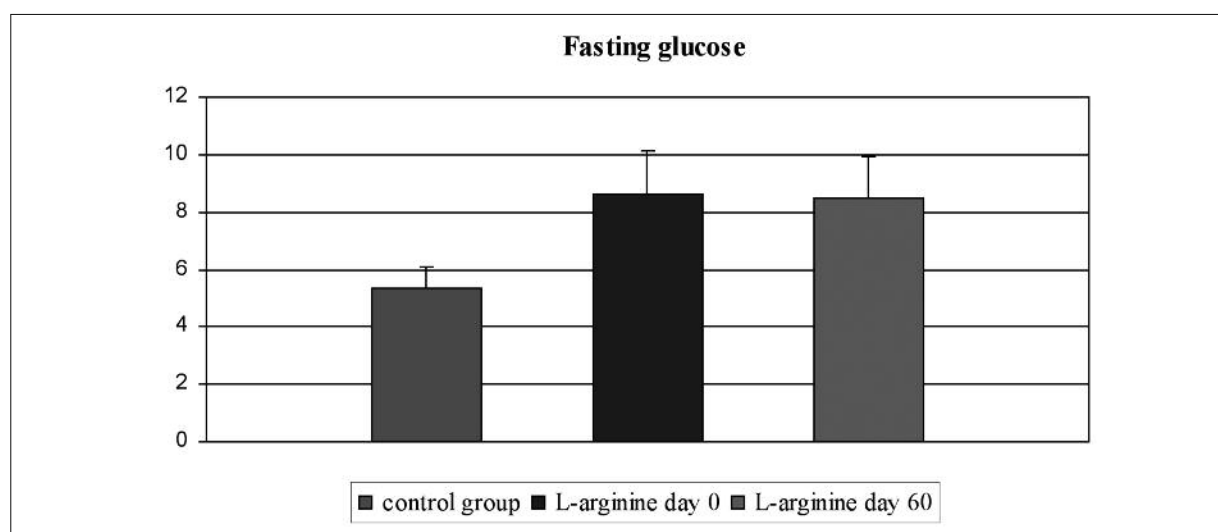


Figure 1. Concentration of fasting glucose in examined groups.

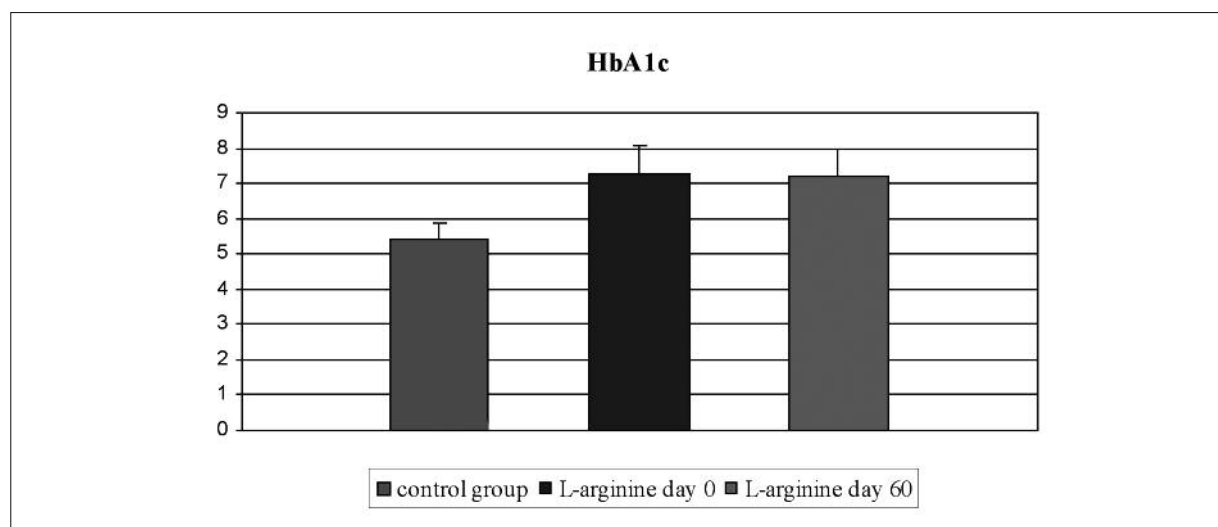


Figure 2. Level of HbA1c in examined groups.

The changes in level of TAS and NO are presented in Table III and Figures 3 and 4.

The patients showed substantially lower total antioxidant status at the beginning of the study, compared to the control group (1.19 ± 0.38 mmol/l vs 1.79 ± 0.21 mmol/l; $p < 0.05$). Statistically significant increase of TAS level was observed in patients after two months of L-arginine supplementation (Table III).

Statistically significant higher nitric oxide concentration was noticed in the control group (2.07 ± 0.70 μ mol/l) compared to the patient group (1.28 ± 0.64 μ mol/l) at the beginning of the study. Statistically significant increase of NO level was observed in patients supplemented with L-arginine (Table III).

Discussion

The prevalence of diabetes is increasing dramatically. Chronic hyperglycemia results in micro- and macrovascular complications, which reduce life expectancy up to 8 years. Diabetes is among the most important risk factors for premature atherosclerosis²⁴⁻²⁶. Peripheral vascular disease concerns 17% diabetics²⁷ and causes ulceration of lower limbs, gangrene and amputation. The DCCT data reports that the risk of amputation of lower limbs because of atherosclerosis is 7-fold greater in type 2 diabetic patients²⁸. Diabetics with peripheral vascular diseases have greater risk of death (70-80%) than diabetics without this disease^{29,30}.

Numerous researches^{29,31,32} indicate hyperglycemia as the key mediator of disadvantageous influence of diabetes on origin and progression of arterial's pathology. Hanefeld et al³³ were the first to reveal particular role of postprandial hyperglycemia in development of sclerotic changes. This observation was then confirmed in DECODE study³⁴. It was proven that fasting glucose concentration greater than 6.1 mmol/l increases

by 38% the risk of morbidity and mortality caused by cardiovascular diseases while postprandial glycemia (glucose concentration > 7.8 mmol/l) increases the risk by 58%. EPIC-Norfolk results³⁵ showed that 1% increase of HbA_{1c} increases by 30% the risk of death caused by ischaemic heart disease.

Because vascular disease in diabetic patients is the main cause of morbidity and mortality, there is an ongoing search for new effective therapeutic strategies to limit vascular complications caused by diabetes. The discovery of L-arginine – NO metabolic trait and antioxidative properties of this amino acid proved to be very significant in that matter^{36,37}.

The level of total antioxidant status is a specific determinant of activity of all antioxidative systems of the organism. The TAS level is influenced by non-enzymatic low-molecular antioxidants³⁸. The range of norm of TAS values is 1.30-1.80 mmol/l according to Randox data. The TAS values obtained in this study were normal for control group (1.79 ± 0.21 mmol/l) while being initially decreased in the patients (1.19 ± 0.38 mmol/l) and reaching 1.60 ± 0.51 mmol/l after 2-month supplementation with L-arginine. Observed increase of TAS in the patients supplemented with L-arginine was statistically significant ($p < 0.05$). The patients' TAS level after 2-month L-arginine supplementation was similar to values observed in the control group which indicates an indirect antioxidative role of L-arginine. Counteraction of oxidative stress by L-arginine in patients with atherosclerosis of lower limbs and type 2 diabetes may be explained by inhibition of aldose reductase, which activity is a significant source of reactive oxygen species (ROS) in hyperglycemia³⁹.

Böger et al⁴⁰ were one of the first noticing antioxidative role of L-arginine, showing the inhibitive effect of L-arginine on superoxide radicals in *in vivo* material. Moreover, L-arginine increased the amount of available antioxidants, which were protecting native forms of LDL from oxidation, by decreasing production of O₂⁻.

Table III. Statistically significant increase in TAS and NO in patients with peripheral artery disease and diabetes type 2 supplemented with L-arginine (from beginning of the study – patients day 0, to the end of the study – patients day 60).

	Patients day 0 N = 38	Patients day 60 N = 38	Significance level
TAS (mmol/l)	1.19 ± 0.38	1.60 ± 0.51	$p < 0.05$
NO (μ mol/l)	1.28 ± 0.64	2.93 ± 0.42	$p < 0.05$

TAS: total antioxidant status; NO: nitric oxide; p : statistical probability.

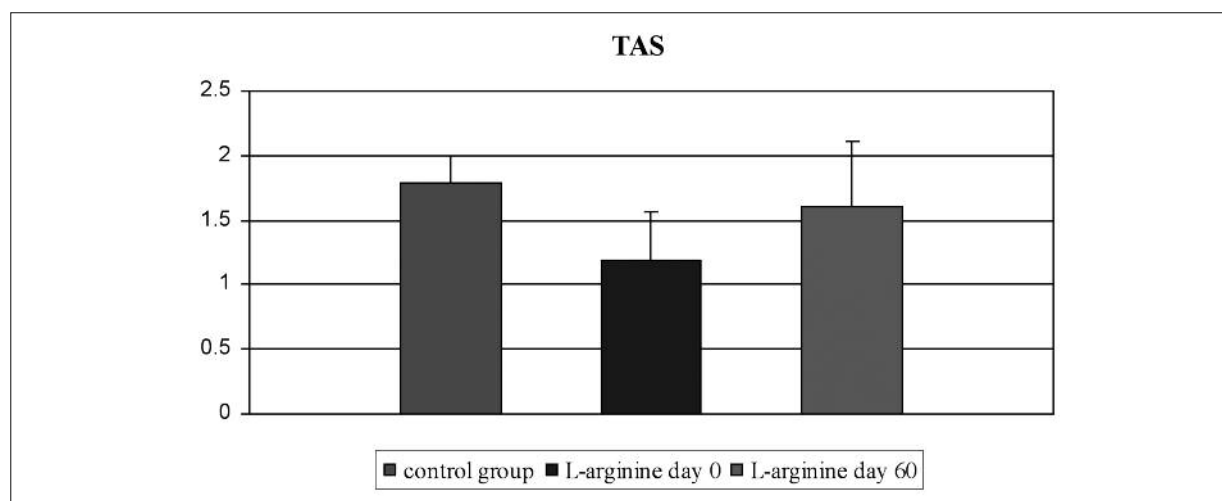


Figure 3. Level of TAS in examined groups.

Chęciński et al⁴¹ also noticed significant increase of total antioxidant status after 7, 14 and 28 days L-arginine supplementation. These results agree with those of our study.

Cassone-Faldetta et al⁴² proved that even short-lived intravenous application of arginine decreased the intensity of oxidative stress in diabetic patients and had favorable effect on blood pressure.

Nitric oxide plays a fundamental role in the mechanism of L-arginine antioxidative activity. Reduced synthesis and increased oxidative inactivation of NO by free radicals are the main ways leading to the loss of NO biological activity^{43,44}. Free radicals are produced in great amounts in such cases as cardiovascular diseases (often followed by endothelium dysfunction), hyper-

glycemia and hypercholesterolemia. It is assumed that reduction of free radicals synthesis may reduce oxidative inactivation of NO.

Intensification of NO synthesis is an alternative way of increasing the amount of biologically active NO and improving the activity of endothelial cells. This can be achieved by the increase of availability of agonists stimulating releasing of NO or by the delivery of additional amounts of eNOS substrate which is L-arginine^{19,43-45}.

Potential vasodilating, antiaggregating and antiproliferating capabilities of NO enabled the use of nitric oxide in treatment of lower limbs ischaemia. Sławiński et al⁴⁶ observed the clinical improvement after 7 days of intravenous application of L-arginine in patients with atherosclerosis

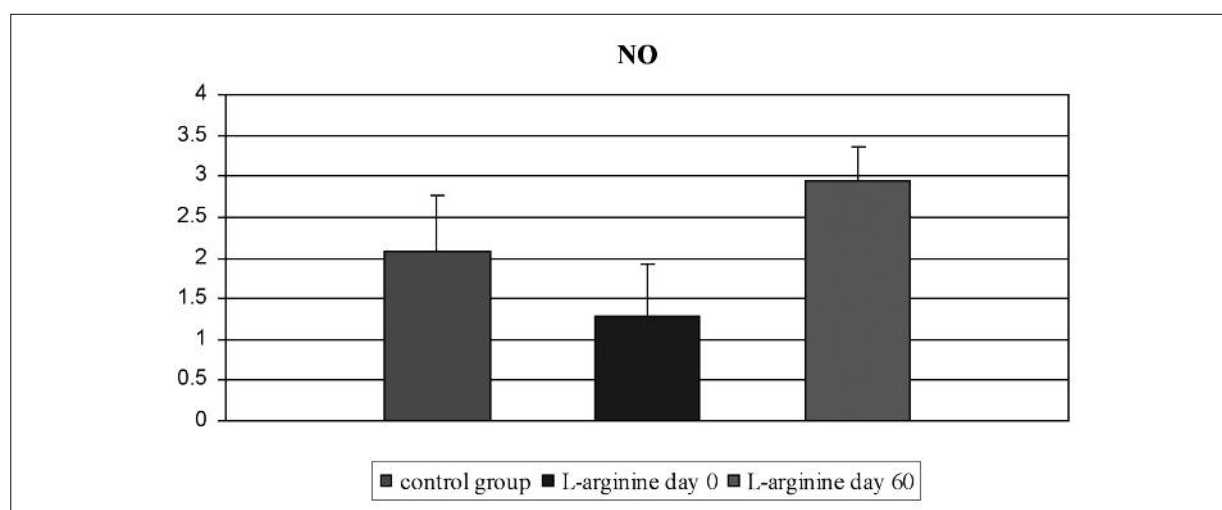


Figure 4. Concentration of NO in examined groups.

of lower limbs at the Fontaine's 2a and 2b stage. The improvement manifested in extended walking distance in intermittent claudication, shortened duration of pain after walking the maximum distance, increased shank blood flow and increased partial pressure of oxygen in ischaemic limb. These observations were confirmed by other studies⁴¹.

In our investigation, significantly lower NO concentration was observed in patients with atherosclerotic peripheral arterial disease of lower extremities at Fontaine's stage II and newly diagnosed type 2 diabetes compared to the healthy control. After 2-month supplementation with L-arginine the patients' NO level significantly increased.

Despite the lack of measurement of L-arginine serum concentration, significant increase of plasma NO concentration was observed, which can indicate a positive correlation between increased availability of exogenic L-arginine and increased concentration of NO.

It should be noticed that there are assumptions available undermining above statement. The study performed by Wierusz-Wysocka et al⁴⁷ showed significantly higher concentration of NO in patients with type 1 diabetes compared to healthy population. Such results can be explained by activation of protein kinase, activated by even short-lived increase of glucose concentration, which is followed by the increase of intracellular calcium level leading to activation of endothelial nitric oxide synthase⁴⁸. Similar mechanism takes place in labile type 2 diabetes, where increased glucose level is a common phenomenon. There are also hypotheses that assume increased NO synthesis as a mechanism protecting from its degradation by free radicals⁴³.

The interpretation of obtained results (especially concerning the NO level) is difficult due to some discrepancies in the literature. However, recent studies delivered many prerequisites indicating the positive role of L-arginine in pathology of persistent diabetic complications. Decreased arginine level, endothelial tetrahydrobiopterin (BH₄) level and decreased synthesis of NO were proven in streptozotocin-induced diabetic rats. It was also found that supplementation with L-arginine normalized these disorders and led to improvement of endothelium function⁴⁹.

Piatti et al³⁷ found out that a monthly supplementation with L-arginine could cause improvement of peripheral and hepatic sensitivity to insulin in patients with well-controlled type 2 dia-

betes due to the normalization of the NO/cGMP pathway. Other studies have also confirmed the examples of decreased vasodilating response in patients with insulin resistance as well as the relation between insulin resistance and endothelial response to inhibition of NO synthesis⁵⁰.

There are some prerequisites supporting the thesis that increased NO availability can also improve glucose metabolism, independently from its vasodilating properties. Reverse relation between concentration of cGMP and basic endogenous synthesis of glucose was observed⁵⁰. Above hypothesis is supported with studies carried out on animals and *in vitro*, which showed the presence of NO synthase in muscles and direct influence of NO on muscular glucose metabolism⁵¹.

These previous reports encouraged us to form a hypothesis about potential hypoglycemic activity of L-arginine. Assuming that supplementation with L-arginine improves peripheral and hepatic sensitivity to insulin in patients with type 2 diabetes, the parameters of carbohydrate balance can be improved by supplementation with this amino acid. The decrease of glucose blood level could also reduce disadvantageous effects of oxidative stress, improving secretory activity of pancreas β -cells. However, our findings did not confirm this hypothesis.

The patients' glucose concentration after 2-months supplementation with L-arginine did not change significantly nor did the level of glycated hemoglobin. High values of carbohydrate balance parameters prove bad metabolic control of diabetes or new diagnosis. However, these factors do not seem to distort the final result of the study. Also, Piatti et al³⁷ did not observe a statistically significant lowering of glucose and glycated haemoglobin level in their study concerning the influence of L-arginine on insulin sensitivity of 12 patients with a good metabolic control of type 2 diabetes.

Conclusions

We conclude that 60-days supplementation with 3 × 2 g of L-arginine does not influence basic parameters of carbohydrate balance in patients with diagnosed atherosclerotic peripheral arterial disease of lower extremities at Fontaine's stage II and coexisting type 2 diabetes, although it protects the endothelium indirectly by increase

of TAS and NO level. Application of L-arginine in treatment of varying forms of atherosclerosis, coexisting with disorders of carbohydrate balance, need further clinical studies carried out on large population.

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