



Effect of nitrate administration on plasma nitric oxide levels in patients with peripheral arterial disease at Dr. Sardjito General Hospital, Yogyakarta

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ABSTRACT

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Peripheral arterial disease (PAD) is a disease involving reduction of blood flow to the inferior extremities associated with atherosclerotic lesions. In 2010, more than 200 million people with PAD were reported globally, including 54.8 million in Southeast Asia. The high prevalence of PAD causes its management to become challenging for clinicians. Nitric oxide (NO) has a role in endothelial function that is associated with the appearance of symptoms in patients with PAD. Exogenous nitrate is usually used as a primary vasodilator in the treatment of angina pectoris and Prinzmetal angina. However, information concerning the role of nitrate administration to improve patients with symptomatic PAD is limited. This study aimed to evaluate the effectiveness of exogenous nitrates in increasing plasma NO levels and improving patients with PAD. A prospective pre-post clinical trial was conducted involving patients with PAD who were registered in the vascular registry at Dr. Sardjito General Hospital, Yogyakarta. Primary endpoint was the change in plasma NO levels after short term administration of nitroglycerin oral 2.5 mg once daily (4 h) and long term (7 d). Analysis of variance test with Bonferroni posttest was used for statistical hypothesis testing. Among 33 subjects who completed this study, no negative side effects, and only one hypotensive patient was reported at the first follow-up after 4 h of the nitroglycerin administration. Plasma NO levels increased in the post 4 h and in the post 7 d administration. However, no statistically significant difference was observed ($p > 0.05$). Administration of exogenous nitrates in patients with PAD increases the plasma NO levels, even though it is not statistically significant.

ABSTRAK

Penyakit arteri perifer atau *peripheral artery disease* (PAD) adalah penyakit yang ditandai dengan penurunan aliran darah ke anggota gerak bawah akibat lesi aterosklerosis. Pada tahun 2010, terdapat lebih dari 200 juta penderita PAD dilaporkan secara global dengan 54.8 juta di antaranya di Asia Tenggara. Prevalensi pasien PAD yang tinggi, menjadikan manajemen pasien ini sebagai tantangan bagi klinisi. Nitrit oksida (NO), berperan pada fungsi endotel dan dihubungkan dengan timbulnya gejala pada pasien PAD. Nitrat eksogen biasa digunakan sebagai vasodilator primer pada terapi angina pectoris dan angina Prinzmetal. Namun, peran pemberian nitrat dalam memperbaiki gejala pasien PAD terbatas. Penelitian ini bertujuan mengkaji efektivitas pemberian nitrat eksogen dalam meningkatkan kadar NO plasma dan dalam memperbaiki gejala klinis pasien. Uji klinik pre-post prospektif yang melibatkan pasien PAD yang terdaftar di pencatatan vaskular di RSUP Dr. Sardjito dilakukan. Luaran utama penelitian ini adalah perubahan kadar NO plasma setelah pemberian nitroglicerine oral 2,5 mg satu kali sehari yang dinilai dalam jangka pendek (setelah 4 jam pemberian) dan jangka panjang (setelah 7 hari pemberian). Analisis varian dilanjutkan Bonferroni *post test* digunakan untuk uji hipotesis. Di antara 33 pasien yang mengikuti penelitian hingga selesai, tidak ditemukan efek samping negatif, hanya satu pasien hipotensi pada kunjungan pertama setelah 4 jam pemberian nitroglicerine. Kadar plasma NO meningkat setelah pemberian nitroglicerine 4 jam dan 7 hari, tetapi tidak bermakna ($p > 0,05$). Dapat disimpulkan, pemberian nitrat eksogen pada pasien PAD meningkatkan kadar NO plasma, meskipun tidak bermakna secara statistik.

Keywords:
peripheral arterial disease
nitric oxide
acute limb ischemia
side effect
hypotension

INTRODUCTION

Peripheral arterial disease (PAD) is a disease with reduction of blood flow in the limbs associated with atherosclerotic lesions which cause stenosis or occlusion of the arteries.¹ The consequence of insufficient blood supply to the lower extremities is tissue ischemia leading to symptoms such as pain, pallor, pulseless, poikilothermia, paresthesia, paralysis and non-healing wounds or ulcers.^{2,3} In 2010, more than 200 million people with PAD were reported globally, including 54.8 million in Southeast Asia.⁴ Patients with PAD in Indonesia increased from 1.7% in 1985 to 5.8% in 2014.⁵ According to the American Diabetes Association (ADA) in 2004,⁸ PAD is a major risk factor for lower limb amputation, especially in patients with diabetes mellitus.

In endothelial dysfunction, the formation of the substance that functions as a vasodilator called nitric oxide (NO) is disturbed. NO is synthesized from L-arginine and is released from endothelial cells. It plays a role in the regulation of vascular tone and blood flow by relaxation of vascular smooth muscle cells by entering these cells and, activating guanylate cyclase that converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (GMP).⁹ NO also inhibits platelets aggregation, therefore the reduction of NO will facilitate the occurrence of stenosis, atherosclerosis, and thrombus.¹⁰ Exogenous nitrate will be converted into NO at or near the plasma membranes of vascular smooth muscle cells. It will then

give effect like physiological NO to repair endothelial damage, reduce arterial stiffness, and prevent thrombus while increasing the blood flow to hypoxic tissue.¹² Nitrate is used as a treatment of angina pectoris through its systemic venous dilatation mechanism to reduce preload and has been given to patients with coronary artery spasm (Prinzmetal angina) for dilating coronary arterioles.¹¹ Nitrate can also be given for patients with PAD to maintain normal blood NO levels and reduce claudication. However, it has not been routinely performed there are and not enough data to support its effectiveness.¹²

The high prevalence of PAD causes the management of this disease to become challenging for clinicians. Nitrate is a simple form of nitric acid esters and nitrites from polyalcohol which are excreted through the liver. Its action mechanism influences blood NO levels.¹³ Nitrate is easy to find, routinely used, and proven safe for patients with angina pectoris as one of the atherosclerosis manifestations. In addition, several studies reported administration of exogenous nitrates causes vasodilation of peripheral arteries and improves vascular function (Peak systolic velocity and 6 min walk test) in patients with PAD.¹⁴⁻¹⁶ This study aimed to evaluate the effectiveness of exogenous nitrates administration in increasing plasma NO levels and in improving vascular function in order to determine whether nitrate is a new effective treatment for patients with PAD.

MATERIALS AND METHODS

Study design

A prospective pre-post clinical trial was conducted involving patients with PAD who were registered in the vascular registry at Dr. Sardjito General Hospital, Yogyakarta, Indonesia. This clinical trial was approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta. Patients registration were conducted by a cardiologist and a researcher to record the data of patients with vascular disease from January 2016. The subjects were enrolled consecutively between March 2017 until November 2018.

The inclusion criteria were: 1) patients who have been diagnosed with peripheral arterial disease based on criteria from the ESC 2011 or AHA Guidelines 2016; 2) > 18 years old; and 3) agreed to participate in this research. The exclusion criteria were 1) patients with intolerance or contraindication to administration of nitrate; 2) patients with wounds in the lower extremities; 3) patients with average systolic blood pressure <100 mmHg; and 4) patients with a history of sildenafil administration within one week of study.

Subjects who met the inclusion and exclusion criteria were recorded in a case report form for demographic, clinical data, and Doppler ultrasound

examination results. Demographic data included age, gender and medicine being consumed by patients. Clinical data included risk factors for atherosclerosis (smoking, diabetes mellitus, family history, hypertension, and dyslipidemia). Other data information needed for research purposes was obtained from the medical records or from the history taking with patients. Sampling of data was conducted using non-probability sampling techniques.

Interventions and follow up

All patients under went the six-minute walk test in the rehabilitation room of Dr. Sardjito General Hospital to determine the pain-free walking distance. Doppler ultrasound test was conducted to measure peak systolic velocity of inferior extremity artery with standard protocol. Blood sample was then collected for measurement of plasma NO levels. The patient was given 2.5 mg oral nitroglycerin and the first follow-up was conducted after 4 h of administration. In the first follow up, the patients was evaluated for side effects of nitrate exposure and plasma NO levels. Patient was then given 2.5 mg oral nitroglycerin daily for 7 d. Second follow-up was performed in day 7 to evaluate the side effect of nitrate exposure, pain-free walking distance by the six-minute walk test, peak systolic velocity with Doppler ultrasound and plasma NO levels (FIGURE1).

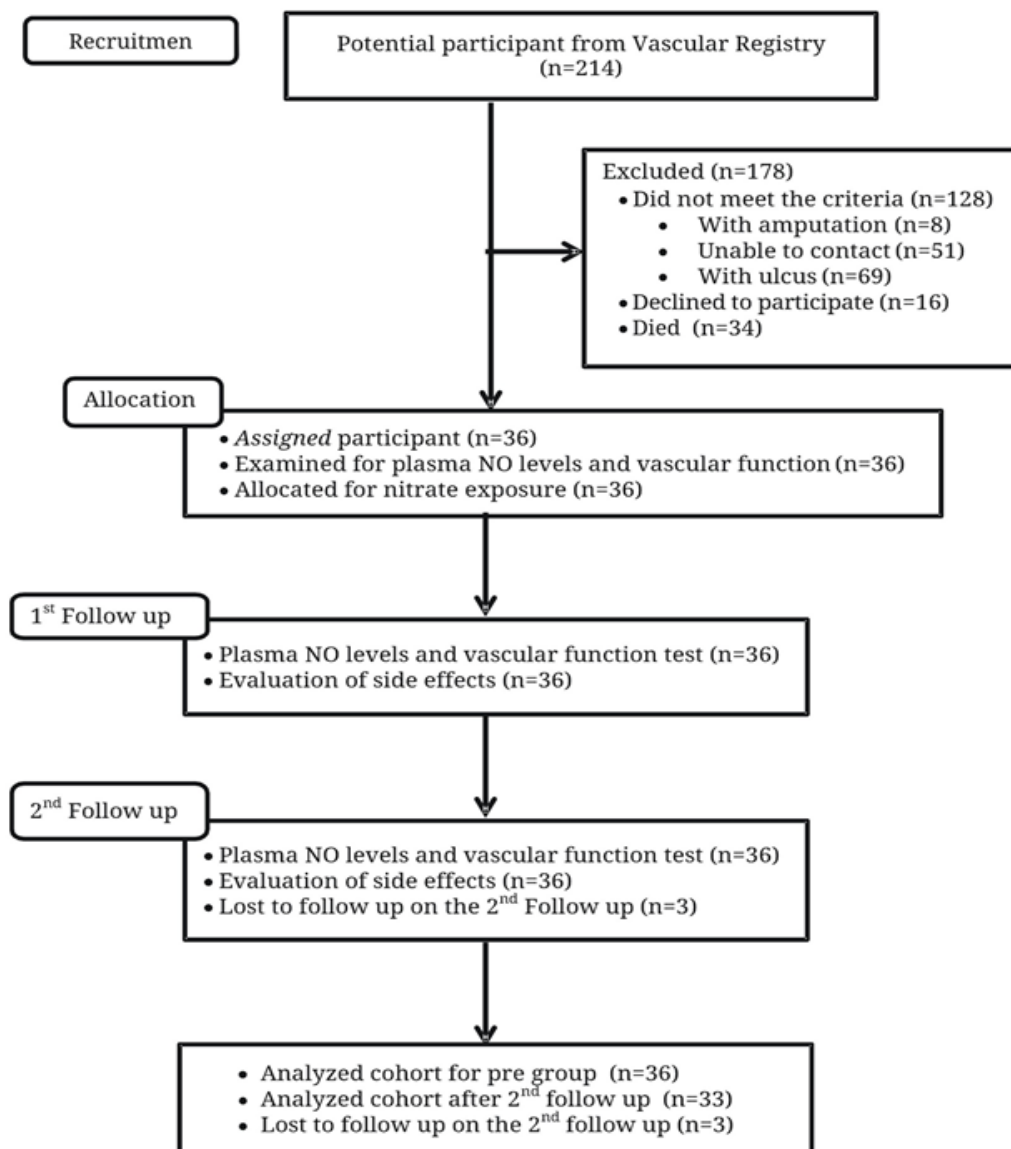


FIGURE 1. CONSORT diagram

In the six-minute walk test, the pain was measured using a quantitative Visual Analog Scale (VAS) which was categorized as pain relief when subjects gave score 0. Doppler ultrasound examination was conducted with high-frequency B-mode ultrasound (Philips 1, Vivid 7 and Vivid S6) with 7-12MHz linear transducers. Neither observer nor operator was blind to the patient group.

Plasma NO levels were measured using colorimetry method (StressMarq Biosciences). Blood samples were taken and put into EDTA tubes for plasma and centrifuged for 15 min at 3000 rpm. Plasma was stored in the freezer with a minimum temperature -70 °C and before

processing, the plasma was placed at room temperature for 30 min. Plasma sample was then filtered through 10,000 MWCO spin filters to remove the protein. After that, the sample and reagents were mixed and incubated. The optical density was then read at λ 540-570 nm to find the total NO for all samples.

Outcomes

The primary outcome of this clinical trial was plasma NO levels after 4 h and 7 d after exogenous nitrate administration. This study aimed to find if there is any significant increment of plasma NO levels after administration of exogenous

nitrate. The secondary outcome included the safety of nitrate treatment in patients with PAD, pain-free walking distance by six-minute walk test and peak systolic velocity.

Statistical analysis

The data were analyzed by SPSS version 20.0. (IBM Corp., Armonk, NY) Basic characteristics were described as means \pm standard deviation (SD) or total in number and percentage. Descriptive analysis of each variable was in numerical or interval for age, body mass index BMI, systolic blood pressure and in categorical or nominal for patient with hypertension, diabetes mellitus (DM), dyslipidemia, smoking, sex, and each interpretation of vascular function. For hypothesis testing, analysis of variance continued with Bonferroni post test was applied due to this study took repeated measurements from each subject. Analysis was conducted between two groups (pre and post 4 h; pre and post 7 d). For numerical variables, the

dependent t test was use for data with normal distribution or Mann-Whitney test was used if the data distribution was not normal. Number needed to treat and number needed to harm were analyzed to measure the effectivity and side effect.

RESULTS

Characteristics of patients

Thirty-six patients were enrolled in this study consisting of 26 (72.2%) male patients and 10 (27.8%) female patients. The average age of patients was 62.31 ± 9.2 y.o. with the youngest age of 31 y.o. and the oldest of 73 y.o. From the risk factors, 28 patients (77.8%) had hypertension, 16 (44.4%) had DM, 20 patients (55.6%) were active smoker, and 15 patients (41.7%) had dyslipidemia. The mean BMI was 24.51 ± 3.82 kg/m². Three patients dropped out due to not attending the second follow-up so that a total of 33 patients were involved in this study. No significant difference in the patients characteristics was observed (TABLE 1).

TABLE1. Basic characteristics of patients with PAD involving in this study

Variable	Pre (n=36)	Post ¹ (n=33)	p
Sex			
• Male [n (%)]	26 (72.2)	24 (72.7)	0.963
• Female [n (%)]	10 (27.8)	9 (27.3)	
Age (year) ²	62.31 \pm 9.19	62.63 \pm 9.46	0.968
Risk factor			
• Hypertension [n (%)]	28 (77.8)	26 (78.8)	0.919
• Diabetes mellitus [n (%)]	16 (44.4)	16 (48.5)	0.737
• Dyslipidemia [n (%)]	15 (41.7)	14 (42.4)	0.949
• Smoker [n (%)]	20 (55.6)	18 (54.5)	0.933
BMI (kg/m ²) ²	24.51 \pm 3.82	24.66 \pm 3.89	0.667
Systolic BP (mmHg) ²	140.4 \pm 17.50	141.51 \pm 17.42	0.979
Medication			
• Beta blocker [n (%)]	25 (26,3)	19 (57.6)	0.949
• ACEi/ARB [n (%)]	22 (62.8)	27 (81.8)	0.677
• Statin [n (%)]	36 (100)	33 (100)	-

BMI: body mass index; BP: blood pressure; ARB: angiotensin receptor blocker; ACEi: angiotensin converting enzyme inhibitor; ¹Post: from subjects who complete the second follow up; ²average \pm standar dviation (with the assumption that data within normal distribution)

Effect of nitrate administration on plasma NO levels

This study aimed to evaluate the effectiveness of the administration 2.5 mg oral nitroglycerin daily or for 7 d on plasma NO levels. Only one patient suffered from a hypotensive episode at the first follow-up or after 4 h administration. The mean plasma NO level before nitroglycerin administration (pre group) was $10.5 \pm 10.17 \mu\text{M}$, whereas after 4 h administration (post group), it increased to $21.99 \pm 32.38 \mu\text{M}$. However, it was not significantly different ($p > 0.05$). The mean plasma NO level post 7 d administration increased to $13.56 \pm 15.89 \mu\text{M}$. However, it was not significantly different ($p=0.865$). Furthermore, the mean plasma NO level post 7 d administration was lower compared to post 4 h administration, although it was also not significantly different.

The side effects or adverse effects were also evaluated using a 2 x 2 table analysis. One subject complained about having headaches at the first and second follow-up, but there were no other significant side effects in other subjects. It was decided that the post 7 d group was chosen as a controls assuming complaints appeared related to acute exposure.

Effect of nitrate administration on vascular function

The six-minute walk test showed a significant increase ($p=0.001$) of pain-free walking distance between the pre and post 7 d administration ($281.97 \pm 121.26 \text{ m}$ vs $262.56 \pm 123.34 \text{ m}$). In addition, the ratio of peak systolic velocity determined using Doppler ultrasound test significantly decreased after post 7 d administration compared to that in the pre-administration.

DISCUSSION

After 4h and 7d of nitrate administration, the plasma NO levels increased, even though it was not significantly different ($p > 0.05$). Whereas, the vascular function changed as demonstrated by the significantly increase in pain-free walking distance between the pre and post 7 d administration ($p=0.002$). Furthermore, the peak systolic velocity ratio was significantly decreased ($p=0.002$).

In this study, there were more male patients (72.2%) than female patients (27.7%). Previous study reported that gender is not associated with the incidence of PAD compared with other cardiovascular diseases.¹⁷ However, if claudication was used as a parameter as in Framingham, the incidence and prevalence of claudication are more common in men (7.1/1000) than in women (3.6/1000).¹⁸

In this study, average age of the patients was $62.31 \pm 9.19 \text{ y.o.}$. The youngest subject was 31 y.o., while PAD rarely occurs at the age $<40 \text{ y.o.}$ ¹⁷ The prevalence of PAD at the age $<50 \text{ y.o.}$ was under 5%, at the age of 65 y.o. it was around 10%, and at the age $>80 \text{ y.o.}$ $> 25\%$.^{2,19} In the Edinburgh Artery Study,⁴ the incidence of PAD was mostly found in the age range of 55-74 y.o.

The risk factors of PAD in this study were hypertension (77.8%), smoking (55.6%), DM (44.4%) and dyslipidemia (41.7%). Hypertension was reported to increase the odds ratio (OR) for the incidence of PAD by 1.32 and in other study, the OR was 1.5-2.2.^{17,20} The Framingham study reported that the risk of claudication in the population will increase by 30% with systolic blood pressure (BP) $>160 \text{ mmHg}$, and only systolic BP has a correlation with the incidence of PAD.²

Smoking also increased the OR incidence of PAD. In the Rotterdam study,²⁰ smoking increased the risk of PAD by 2.69 times compared with not smoking. Meanwhile, the Multi-Ethnic Study of Atherosclerosis (MESA) reported,²¹ that smoking increases the risk of PAD up to 3.42 times. Diabetes mellitus is strongly associated with the incidence of PAD with an OR varying from 1.89 to 4.05. In addition, patients with PAD and DM have an amputation risk of about 50% compared to the PAD population without DM. This study also demonstrated that the risk of death increased three fold higher. Arteries affected in the DM population are also more distal than the knee.¹⁷

Dyslipidemia was only related with PAD in a univariate analysis. However, in a multivariate analysis, lipid variables were not an independent risk factor. Therefore, the role of lipids as an independent risk factor for PAD is not yet clear.² High levels of low density lipoprotein-c reduce NO production in endothelial cells by regulation of caveolin proteins that inhibit interactions with eNOS.²²

In this study, 2.5 mg oral nitroglycerin administration as exogenous nitrate increased plasma NO levels by 11.42 μM after 4 h administration. Other studies reported that administration of organic nitrates such as beetroot juice increase plasma NO levels up to 2 μM . On the seventh day, plasma NO level increased 2.99 μM compared with before administration. This level was lower compared to after 4 h administration. Increased levels of NO in administration for less than 15 d were also found in transdermal nitrate studies up to 2.7 μM .²³ Acute increasing of nitrate maybe because its peak lasts between 2.5 to 4 h.¹³ From this study, we found that there was no significant statistical Difference in the evidence ($p = 0.865$ and $p = 0.643$).

Nitrite oxide plays a role in regulation of vascular tone and

blood flow by relaxation of vascular smooth muscle cells and also inhibits platelets aggregation. In this study, significant increase in pain-free walking distance between the pre and post 7 d administration was observed ($p=0.001$). Significant decrease in the peak systolic velocity ratio ($p=0.002$) between the pre and post 7 d administration was also observed ($p=0.002$). These results demonstrated that administration of exogenous nitrated affected vascular function significantly by improving the flow through stenosis arteries and reduced the pain.

Some limitations of the study were reported. First, there were small sample sizes, with no placebo control group and no blinding that could cause high bias in the observation result. Second, in this study the exogenous nitrate administration was only done once whereas in another study, it was administered for longer duration. Third, the clinical parameter for the quality of pain evaluation used a six-minute walk test which was subjective because the pain was only analyzed from history taking. Further studies are needed using larger samples, with controls blinding and longer duration of nitrate administration to avoid the potential for bias and confirm the findings.

CONCLUSIONS

In conclusion, administration of exogenous nitrates on patients with PAD for 7 days improves the vascular function as demonstrated by the significant increase of pain-free walking distance and the decrease of peak systolic velocity ratio. Moreover, the administration of exogenous nitrates also increases plasma NO level, although the result were not statistically significant.

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