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Peer-reviewed author version

Cermak, Naomi; HANSEN, Dominique; Kouw, Imre; van Dijk, Jan-Willem; Blackwell, Jamie; Jones, Andrew; Gibala, Martin & van Loon, Luc (2015) A single dose of sodium nitrate does not improve oral glucose tolerance in patients with type 2 diabetes mellitus. In: NUTRITION RESEARCH, 35 (8), p. 674-680.

DOI: 10.1016/j.nutres.2015.05.017

Handle: <http://hdl.handle.net/1942/18920>

Accepted Manuscript

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PII: S0271-5317(15)00129-3
DOI: doi: [10.1016/j.nutres.2015.05.017](https://doi.org/10.1016/j.nutres.2015.05.017)
Reference: NTR 7489

To appear in: *Nutrition Research*

Received date: 27 February 2015
Revised date: 26 May 2015
Accepted date: 27 May 2015



Please cite this article as: Cermak Naomi M., Hansen Dominique, Kouw Imre W.K., van Dijk Jan-Willem, Blackwell Jamie R., Jones Andrew M., Gibala Martin J., van Loon Luc J.C., A single dose of sodium nitrate does not improve oral glucose tolerance in patients with type 2 diabetes mellitus, *Nutrition Research* (2015), doi: [10.1016/j.nutres.2015.05.017](https://doi.org/10.1016/j.nutres.2015.05.017)

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A single dose of sodium nitrate does not improve oral glucose tolerance in patients with type 2 diabetes mellitus

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ABBREVIATIONS

| | |
|----------------------------------|--|
| BMI; | body mass index |
| DPP-4; | dipeptidyl peptidase-4 |
| EDTA; | ethylene-diamineteraacetic acid |
| GLUT4; | glucose transporter 4 |
| HbA _{1c} ; | glycated hemoglobin |
| HOMA-IR; | homeostatic model of insulin resistance |
| iAUC; | incremental area under the curve |
| ISI-M; | Matsuda-DeFronzo insulin sensitivity index |
| NaNO ₃ ⁻ ; | sodium nitrate |
| NO; | nitric oxide |
| NO ₂ ⁻ ; | nitrite |
| NO ₃ ⁻ ; | nitrate |
| OGIS-index; | oral glucose insulin sensitivity index |
| OGTT; | oral glucose tolerance test |
| SUD; | sulfonylurea derivative |

ABSTRACT

Dietary nitrate supplementation has been proposed as an emerging treatment strategy for type 2 diabetes. We hypothesized that ingestion of a single bolus of dietary nitrate improves oral glucose tolerance in patients with type 2 diabetes. Seventeen men with type 2 diabetes (HbA_{1c}: 7.3±0.2%) participated in a randomized crossover experiment. Subjects ingested a glucose beverage 2.5 h after consuming either sodium nitrate (0.15 mmol NaNO₃⁻·kg⁻¹) or a placebo solution. Venous blood samples were collected before ingestion of the glucose beverage and every 30 min thereafter during a 2-h period to assess postprandial plasma glucose and insulin concentrations. The results show that plasma nitrate and nitrite levels were increased after sodium nitrate as opposed to placebo ingestion (treatment-effect: $P=0.001$). Despite the elevated plasma nitrate and nitrite levels, ingestion of sodium nitrate did not attenuate the postprandial rise in plasma glucose and insulin concentrations (time x treatment interaction: $P=0.41$ for glucose, $P=0.93$ for insulin). Despite the lack of effect on oral glucose tolerance, basal plasma glucose concentrations measured 2.5 h after sodium nitrate ingestion were lower when compared with the placebo treatment (7.5±0.4 vs 8.3±0.4 mmol/L, respectively; $P=0.04$). We conclude that ingestion of a single dose of dietary nitrate does not improve subsequent oral glucose tolerance in patients with type 2 diabetes.

Key words: cross-over studies; nitrites; nitrates; blood glucose; hyperglycemia; blood glucose; insulin

1. INTRODUCTION

Postprandial hyperglycemia has been associated with an increased risk for cardiovascular complications and mortality in patients with type 2 diabetes [1-4]. Therefore, proper management of postprandial blood glucose concentrations is an important goal in the treatment of type 2 diabetes [4]. Despite the application of oral blood-glucose-lowering medication and the consumption of a healthy diet, postprandial hyperglycemia remains a predominant feature in patients with type 2 diabetes [5, 6]. Therefore, additional treatment strategies are warranted to improve daily blood glucose homeostasis in patients with type 2 diabetes.

Epidemiological studies indicate that a diet rich in leafy green vegetables can reduce the risk of developing type 2 diabetes [7]. The health benefits from these particular vegetables may be, at least partly, attributed to their high nitrate content [8]. Nitrate and nitrite have historically been viewed as inactive end products of nitric oxide (NO) metabolism. However, recent work has revealed that a reverse pathway exists whereby nitrate and nitrite can be reduced back into NO which is a potent vasodilator and regulator of vascular tone and blood flow [8]. Nitrite has been shown to function as an endocrine reservoir of NO [9] and is generated from the conversion of dietary nitrate into nitrite by facultative anaerobic bacteria residing in the oral cavity [10]. This recent discovery complements the classical L-arginine-NO synthase pathway and is now identified as a phenomenon that may have major clinical relevance in both health and disease.

Several groups have investigated the potential physiological effects of dietary nitrate by supplementing subjects with sodium nitrate or foods high in nitrate such as beetroot juice. As such, there is now substantial evidence that dietary nitrate supplementation in humans improves postprandial endothelial function, microvascular perfusion of various tissues, and mitochondrial function [11-14]. These exciting findings may also be relevant for people suffering from type 2

diabetes who demonstrate impairments in NO-dependent endothelial function [14, 15]. In fact, endothelial dysfunction is particularly prominent in the postprandial state in people suffering from type 2 diabetes [16]. Improvements in postprandial endothelial function (e.g. vasodilatation and capillary recruitment) may enhance glucose and insulin delivery to skeletal muscle tissue, thereby facilitating improvements in insulin signaling and postprandial glucose uptake [17]. So far, it has not been established whether the physiological benefits induced by dietary nitrate ingestion translate to improvements in postprandial glucose metabolism. We hypothesized that ingestion of a single bolus of dietary nitrate prior to an oral glucose load attenuates the postprandial rise in plasma glucose and/or insulin concentrations in type 2 diabetes patients. For this purpose, we recruited 18 male patients with type 2 diabetes and evaluated the glycemic and insulinemic response to the ingestion of a glucose load following the administration of a single dose of sodium nitrate ($0.15 \text{ mmol NaNO}_3 \cdot \text{kg}^{-1}$ body weight) or a placebo.

2. METHODS and MATERIALS

2.1 Subjects

Eighteen male patients with type 2 diabetes using oral glucose lowering medication were included in this randomized crossover study (Figure 1). Exclusion criteria included self-reported renal failure or liver disease, morbid obesity ($\text{BMI} > 35 \text{ kg/m}^2$), insulin therapy, use of nitrate-containing medication, severe hypertension (systolic >160 or diastolic $>100 \text{ mm/Hg}$), and cardiovascular events within the last year. The use of blood glucose lowering medication was maintained as normal throughout the entire study. All subjects were informed about the nature and the risks of the experimental procedures before their written informed consent was obtained.

The experimental protocol and procedures were approved by the medical ethical committee of the Jessa hospital in Hasselt, Belgium.

2.2 Screening

After an overnight fast, subjects arrived at the laboratory at 08:00 h by car or public transportation. After 20 min of supine rest, a venous blood sample was collected by venepuncture from the antecubital vein for the assessment of HbA_{1c} content. Thereafter, body mass was measured to the nearest 0.1 kg using an analogue weight scale (Tanita model TBF-300, Tanita Corp., Tokyo, Japan) and height was measured to the nearest 0.1 cm. Body mass index (BMI) was calculated from the ratio of weight (kg) to height squared (m²). Next, blood pressure (Omron model HEM-907, Hoofddorp, The Netherlands) was assessed 5 times, and average blood pressure was calculated using the closest 3 values.

2.3 Study design

Subjects participated in a double blind cross-over study; consisting of two test days separated by a washout period of at least 14 d. Subjects were tested on the same day of the week for each of the test days. At the start of each test day, subjects ingested a single bolus of sodium nitrate (0.15 mmol NaNO₃⁻·kg⁻¹ body wt; ~12.75 mg NaNO₃⁻·kg⁻¹ body wt) or an equimolar amount of sodium chloride (placebo) dissolved in 250 mL water. The sodium nitrate and placebo beverages were provided in a randomized order according to a computer-generated randomization scheme. Exactly 2.5 h following sodium nitrate ingestion, subjects ingested a glucose beverage (75 g glucose dissolved in 250 mL tap water). The 2.5 h resting period was selected as plasma nitrite concentrations have been shown to peak 2-3 h following dietary nitrate ingestion [18]. Plasma

glucose and insulin concentrations were collected before and over the 2 h period following ingestion of the glucose load.

2.4 Study design and protocol

For the two main experimental test days, subjects reported to the laboratory at 08.00 h following an overnight fast for an oral glucose tolerance test (OGTT). Upon arrival at the laboratory, subjects were required to consume the experimental (sodium nitrate) or placebo (sodium chloride) solution ($0.15 \text{ mmol}\cdot\text{kg}^{-1}$ body wt) followed by catheter insertion into an antecubital vein for venous blood sampling. Subjects then rested comfortably for 2.5 h before the start of the OGTT. Following the 2.5 h of rest, a 75 g glucose bolus (dissolved in 250 mL water) was ingested ($t=0$ min). Before, and every 30 min after the ingestion of the glucose bolus, venous blood (8 mL) was sampled until $t=120$ min for the analysis of plasma nitrate, nitrite, glucose, and insulin concentrations.

2.5 Diet and physical activity

All subjects were required to refrain from exercise training or heavy labour during the 48 h prior to each experimental trial. In addition, subjects recorded their dietary intake for 48 h before the first experimental trial and replicated their diet during the 48 h period leading into the second trial. Furthermore, all subjects consumed the same standardized meal the evening prior to each experimental trial (621 kcal, with 19 energy % provided from protein, 48 energy % from carbohydrate, and 33 energy % from fat).

2.6 Blood sample analysis

During the OGTT, venous blood samples (10 mL) were collected in EDTA containing tubes and centrifuged at 1000 g at 4 °C for 10 min. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80 °C until analyses. Plasma nitrate (NO_3^-) and nitrite (NO_2^-) were analysed by gas phase chemiluminescence analysis. NO_2^- and NO_3^- are initially reduced to NO gas in a sealed purge vessel. For the reduction of NO_2^- , undiluted plasma was injected into a glass purge vessel containing 5 mL glacial acetic acid and 1 mL NaI solution. For NO_3^- reduction, plasma samples were deproteinised in an aqueous solution of zinc sulphate (10% w/v) and 1 M sodium hydroxide, prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). Quantification of NO was achieved by the detection of light emitted during the production of nitrogen dioxide formed upon the reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentration of NO_2^- was determined by plotting signal (mV) area against a calibration plot of 25 nM to 1 μM sodium nitrite. The concentration of NO_3^- was determined by plotting signal (mV) area against a calibration plot of 100 nM to 10 μM sodium nitrate. Plasma glucose concentration (Glucose HK CP, ABX Diagnostics, ref. A11A01667, Montpellier, France) was determined enzymatically with the COBAS FARA semi-automatic analyzer (Roche). Plasma insulin concentration was determined by radioimmunoassay (Millipore, ref. HI-14K, Billerica, USA). To determine HbA1c content, 3 mL blood samples were collected in EDTA containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany).

2.7 Oral glucose insulin sensitivity

Plasma glucose and insulin concentrations were used to determine the oral glucose insulin sensitivity (OGIS) index [19], the Matsuda-DeFronzo insulin sensitivity index (ISI-M) [20], and the homeostatic model of insulin resistance (HOMA-IR) [21], all of which have been validated against the glucose clamp in patients with type 2 diabetes.

2.8 Statistical analyses

Previous work has shown a robust increase in peripheral glucose uptake after administration of the NO donor sodium nitroprusside [22]. Based on this work and pilot work, we estimated moderately lower (20%) postprandial glucose responses (iAUC) in the sodium nitrate condition (600 mmol/L/120min) compared with the placebo condition (750 mmol/L/120min) with a group SD of 250 mmol/L/2h. Assuming a correlation of 0.7 between paired measurements, with a two-sided significance of 0.05 and a power of 0.8, 16 patients with type 2 diabetes were required (G*Power 3.1 software, Heinrich Heine Universität Düsseldorf, Germany). Taking a potential drop-out of 2 subjects into account, 18 subjects were recruited for this study.

Due to problems with the intravenous catheter, blood samples could not be taken properly in 1 of the subjects. Hence, this subject dropped out, and results are reported for the remaining 17 subjects (Figure 1). Plasma glucose and insulin responses are reported as incremental area under the curve (iAUC) above baseline values, as calculated by the trapezoidal rule. Repeated measures ANOVA with time x treatment interactions were conducted to assess whether changes in blood variables over time were different between conditions. The effect of dietary nitrate co-ingestion on non-time-dependent variables was assessed by paired student's *t*-tests. Differences were considered statistically significant when $P < 0.05$. All statistical calculations were performed using SPSS 20.0.0. Unless otherwise specified, results are reported as means \pm SEM.

3. RESULTS

3.1 Subjects

Subjects' characteristics are reported in **table 1**. Subjects were using the following glucose lowering medication: metformin only ($n=6$), metformin + sulfonylurea (SUD, $n=5$), metformin + DPP-4 inhibitor ($n=2$), metformin + meglitinide ($n=2$), metformin + DPP-4 inhibitor + SUD ($n=1$), and metformin + DPP-4 inhibitor + meglitinide ($n=1$).

3.2 Plasma nitrate and nitrite concentrations

Peak plasma nitrate values (480 ± 18 $\mu\text{mol/L}$) were reached 2.5 h after sodium nitrate ingestion, whereas peak plasma nitrite values (518 ± 66 nmol/L) were reached 3 h after sodium nitrate ingestion. Plasma nitrate and nitrite concentrations remained elevated throughout the entire 2-h postprandial period in the sodium nitrate condition compared with the placebo condition (treatment-effect $P<0.001$ for both variables: **Figure 2**).

3.3 Plasma glucose and insulin concentrations

Plasma glucose concentrations obtained during the 2-h postprandial period did not differ between the dietary nitrate and placebo treatments (time x treatment; $P=0.41$: **Figure 3A**). In accordance, no differences were observed between treatments in glucose responses expressed as incremental area under the curve (iAUC) ($P=0.17$: **Figure 3B**). Plasma insulin concentrations over the 2-h postprandial period also did not differ between treatments (time x treatment; $P=0.93$: **Figure 4A**). In agreement, no differences were observed between treatments in the insulin responses expressed as iAUC ($P=0.56$: **Figure 4B**). Despite the lack of effect on oral glucose tolerance, plasma glucose concentrations obtained prior to ingestion of the glucose beverage were

significantly lower in the dietary nitrate compared with the placebo treatment (7.4 ± 0.4 and 8.3 ± 0.4 $\text{mmol}\cdot\text{L}^{-1}$, respectively; $P=0.04$: **Figure 5**). The corresponding basal plasma insulin concentrations did not differ between the nitrate and placebo treatment (17.0 ± 1.8 and 17.2 ± 1.7 $\mu\text{U}\cdot\text{mL}^{-1}$; $P=0.79$).

3.4 Oral glucose insulin sensitivity

Oral glucose insulin sensitivity as determined by the OGIS-index was significantly higher in the nitrate compared with the placebo treatment (344 ± 17 vs 318 ± 15 $\text{mL min}^{-1} \text{m}^{-2}$, respectively; $p=0.02$). However, both other markers of insulin sensitivity did not differ between the dietary nitrate and placebo condition (ISI-M: 2.42 ± 0.33 vs 2.28 ± 0.26 , respectively; $P=0.44$, and HOMA-IR: 5.77 ± 0.89 vs 6.62 ± 0.92 , respectively; $P=0.41$).

4. DISCUSSION

In contrast to our hypothesis, acute dietary nitrate ingestion did not attenuate the postprandial rise in blood glucose or insulin concentrations following ingestion of an oral glucose load in patients with type 2 diabetes mellitus. Despite the lack of effect on oral glucose tolerance, we observed lower basal plasma glucose concentrations 2.5 h after ingestion of a single dose of sodium nitrate.

In the present study, ingestion of a single bolus of sodium nitrate (0.15 $\text{mmol NaNO}_3\cdot\text{kg}^{-1}$ body wt) effectively increased both plasma nitrate and nitrite concentrations in older patients with type 2 diabetes, with peak plasma nitrite values of 518 ± 66 nmol/L being reached 3 h after nitrate ingestion (OGTT time point 30 min). These findings are consistent with previous work in healthy subjects, investigating the impact of either sodium nitrate [10] or similar doses of nitrate provided through a vehicle such as beetroot juice [18, 23], with peak nitrite levels of ~ 350 - 600

nM being reached 2-3 h after nitrate ingestion. Although the consumption of a vegetable-based nitrate-rich drink (e.g. beetroot juice) would be more practical for subjects once in their own environment, in the present study we used sodium nitrate to prevent any confounding from the other components (including carbohydrates) present in beetroot juice on the glycemic and/or insulinemic response to the oral glucose load. Sodium nitrate was well tolerated and no discomfort was reported by the subjects during the hours following its administration.

Although plasma nitrate and nitrite levels remained elevated throughout the entire postprandial period in the dietary nitrate condition as opposed to the placebo condition, no differences in the postprandial rise in plasma glucose and insulin concentrations were observed between both conditions. Therefore, it can be concluded that a single dose of sodium nitrate administered before an oral glucose load does not improve oral glucose tolerance in patients with type 2 diabetes. Although we have no clear explanation for the null finding, a recent study by Larsen and colleagues also found no effect of dietary nitrate supplementation (3-day period) on glycemic and insulinemic responses to an oral glucose load in healthy volunteers [24]. Comparable findings were also reported by Joris and Mensink in overweight and obese individuals [11]. In that study, a single bolus of dietary nitrate did not improve the glycemic and insulinemic response to a mixed meal, despite an improvement in postprandial endothelial function [25]. Thus, it seems that improvements in postprandial endothelial function induced by dietary nitrate do not necessarily translate to improvements in postprandial glucose handling.

Despite the fact that a single dose of dietary nitrate did not improve oral glucose tolerance, we found that basal glucose concentrations measured before ingestion of the oral glucose load were lower in the dietary nitrate condition as opposed to the placebo condition (7.4 ± 0.4 vs 8.3 ± 0.4 mmol·L⁻¹, respectively; $P=0.04$). This unexpected finding is difficult to explain. It can be

speculated that NO acutely affects basal blood glucose homeostasis. Indeed, it has been shown that infusion of the NO donor sodium nitroprusside improves leg glucose uptake in resting conditions in individuals with and without type 2 diabetes [22, 26]. This finding has been attributed to NO-mediated increments in capillary recruitment and nutritive blood flow [22, 26]. These NO-mediated improvements in endothelial function may enhance the delivery of glucose and insulin to various tissues (e.g. skeletal muscle) thereby facilitating insulin signaling, GLUT 4 translocation and subsequent glucose uptake [17]. Alternatively, enhanced insulin delivery to the liver might inhibit hepatic glucose output, thereby lowering blood glucose concentrations. It is unclear, however, why these mechanisms would affect basal, but not postprandial blood glucose concentrations. Obviously, more research is needed to confirm the beneficial effect of dietary nitrate on basal glucose concentrations, along with the underlying mechanisms.

In the present study, we observed an improvement in the OGIS index during the dietary nitrate condition, which suggests an improvement in insulin sensitivity. However, this finding is attributed to the observed differences in basal blood glucose levels between treatments, rather than to true improvements in postprandial insulin release and/or glucose handling. In accordance, both other markers of insulin sensitivity (ISI-M and HOMA-IR), whose equations put less emphasis on basal blood glucose concentrations, did not show any improvements in whole-body insulin sensitivity. The apparent lack of improvement in whole-body insulin sensitivity after ingestion of a single bolus of sodium nitrate tends to be in line with two recent studies that assessed the impact of nitrate supplementation on insulin sensitivity by the application of a hyperinsulinemic euglycemic clamp [24, 27]. In those studies, respectively 3 days and 14 days of dietary nitrate supplementation appeared to be ineffective in improving insulin sensitivity in

healthy volunteers [24] and patients with type 2 diabetes [27]. Taken together, it can be concluded that supplementation with dietary nitrate does not improve insulin sensitivity.

Limitations of the current study include the relatively small sample size and the lack of a blood sample collection prior to sodium nitrate ingestion. Both factors would have increased the robustness of the present findings. Nevertheless, the study was well randomized and we optimized the test-retest reliability of the experiments by standardizing subjects' physical activity and food intake prior to testing.

In conclusion, a single dose of sodium nitrate does not improve subsequent oral glucose tolerance in patients with type 2 diabetes. The potential impact of sodium nitrate on basal blood glucose concentrations requires further investigation.

ACKNOWLEDGMENT

This study received no external funding. The authors have no conflicts of interest to disclose. The authors gratefully acknowledge the subjects for their participation in this study.

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TABLE 1

Subjects' characteristics

| | |
|---------------------------------|----------|
| <i>N</i> | 17 |
| Age, y | 66±2 |
| BMI, kg/m ² | 29.2±0.9 |
| HbA _{1c} , % | 7.3±0.2 |
| HbA _{1c} , mmol/mol | 56±2 |
| Diastolic blood pressure, mm/Hg | 71±1 |
| Systolic blood pressure, mm/Hg | 136±2 |

Data are reported as mean±SEM

FIGURE LEGENDS**Figure 1:** Study Flow Chart

Figure 2: Plasma nitrate (panel A) and nitrite (panel B) concentration over a 120-min period following ingestion of a glucose beverage. A single bolus of sodium nitrate or placebo was ingested 2.5 h prior to consumption of the glucose beverage. Data are expressed as means \pm SEM.

Figure 2A RM ANOVA: time-effect $P<0.001$; treatment-effect $P<0.001$; time x treatment $P<0.001$

Figure 2B RM ANOVA: time-effect $P=0.05$; treatment-effect $P<0.001$; time x treatment interaction $P=0.10$

Figure 3: Plasma glucose concentrations (panel A) and incremental area under the curve (panel B) over a 120-min period following ingestion of a glucose beverage. A single bolus of sodium nitrate or placebo was ingested 2.5 h prior to consumption of the glucose beverage. Data are expressed as means \pm SEM. No significant differences were observed between treatments.

Figure 3A: time-effect $P<0.001$; treatment-effect $P=0.69$; time x treatment $P=0.41$

Figure 3B: $P=0.17$

Figure 4: Plasma insulin concentration (panel A) and incremental area under the curve (panel B) over a 120-min period following ingestion of a glucose beverage. A single bolus of sodium nitrate or placebo was ingested 2.5 h prior to consumption of the glucose beverage. Data are expressed as means \pm SEM. No significant differences were observed between treatments.

Figure 4A: time-effect $P<0.001$; treatment-effect $P=0.62$; time x treatment $P=0.93$

Figure 4B: P=0.56

Figure 5: Basal plasma glucose concentration 2.5 h following the ingestion of a nitrate or placebo solution. Bars represent means \pm SEM, lines represent individual values. *Significant difference between treatments (P<0.05).

ACCEPTED MANUSCRIPT

Figure 1

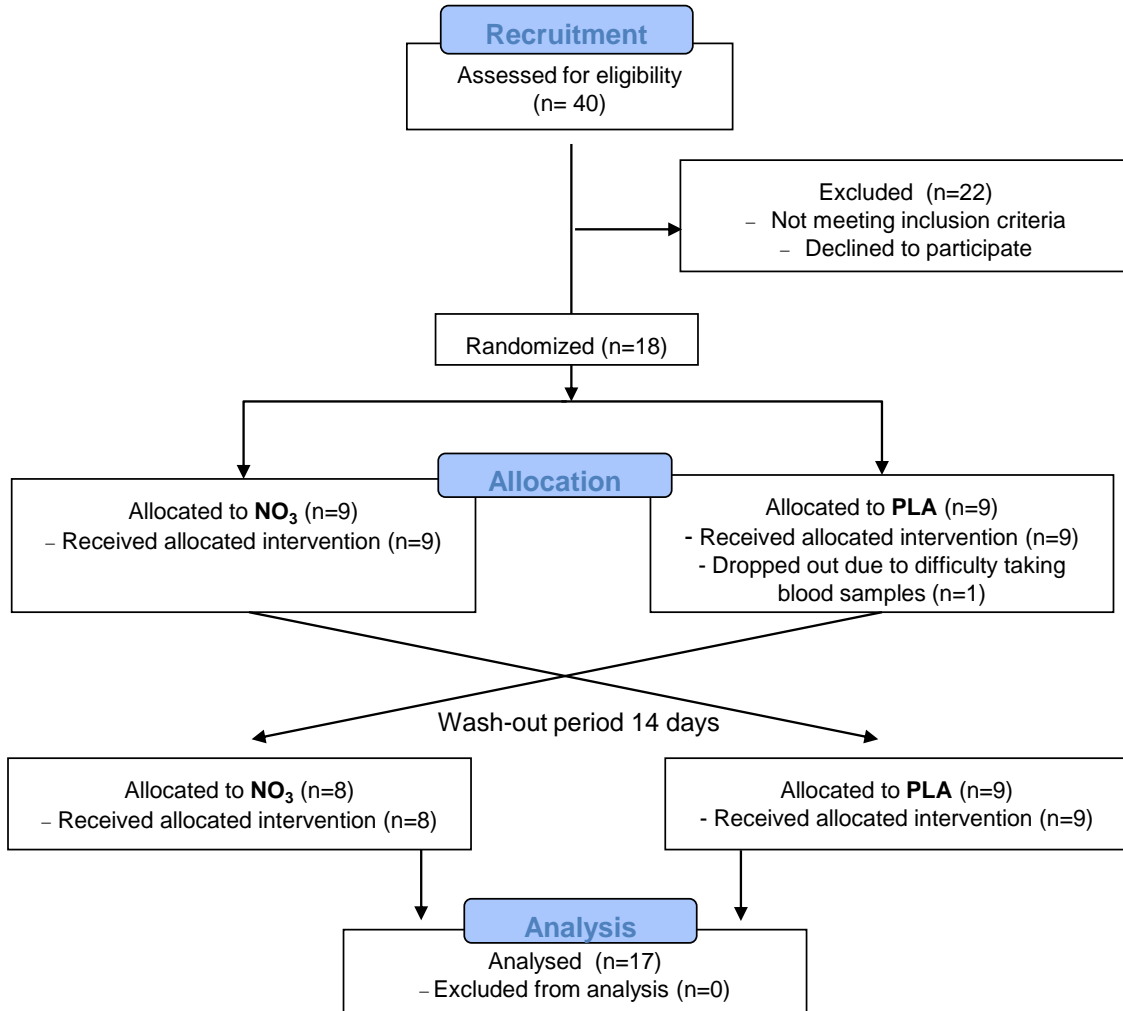


FIGURE 2

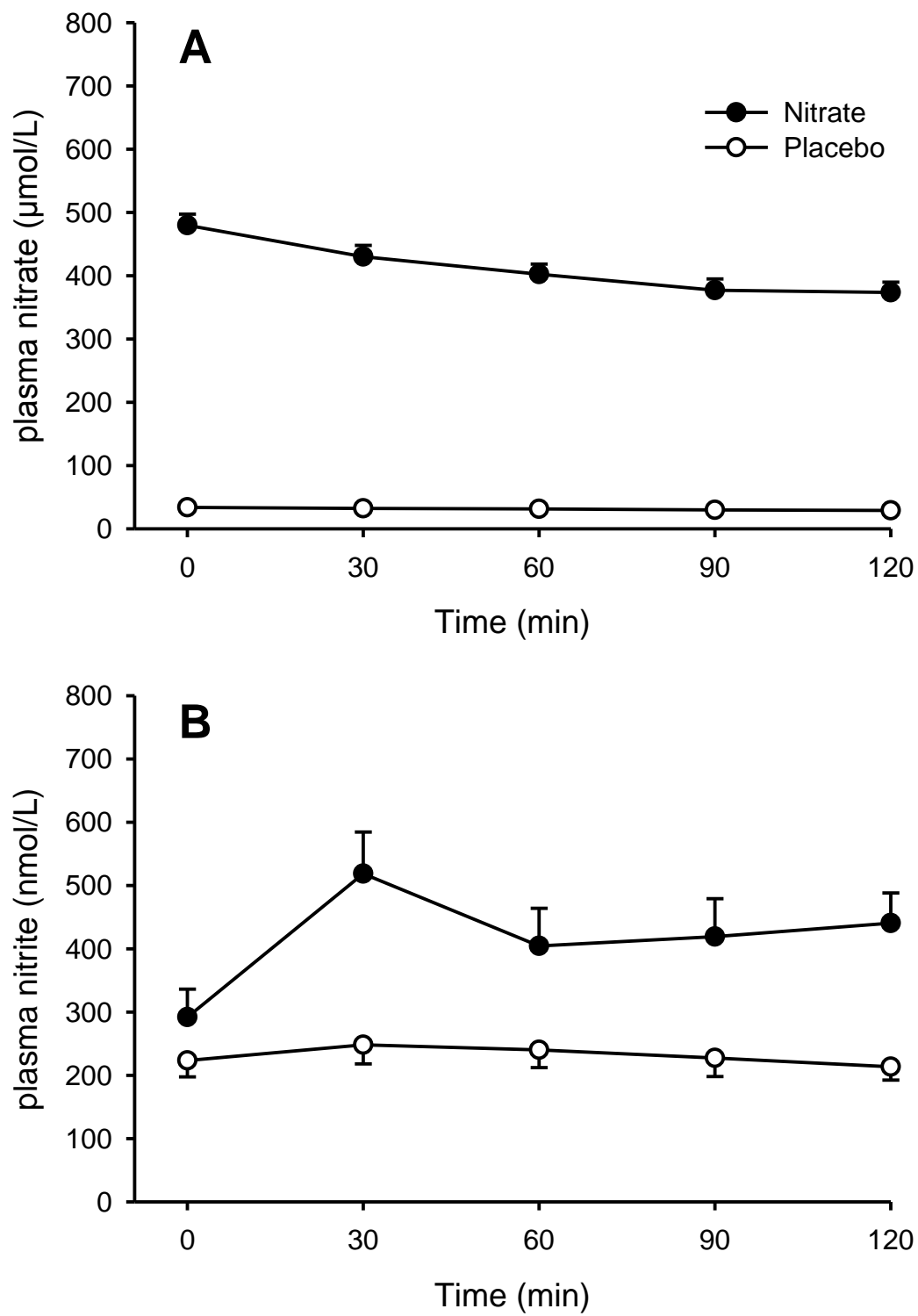


FIGURE 3

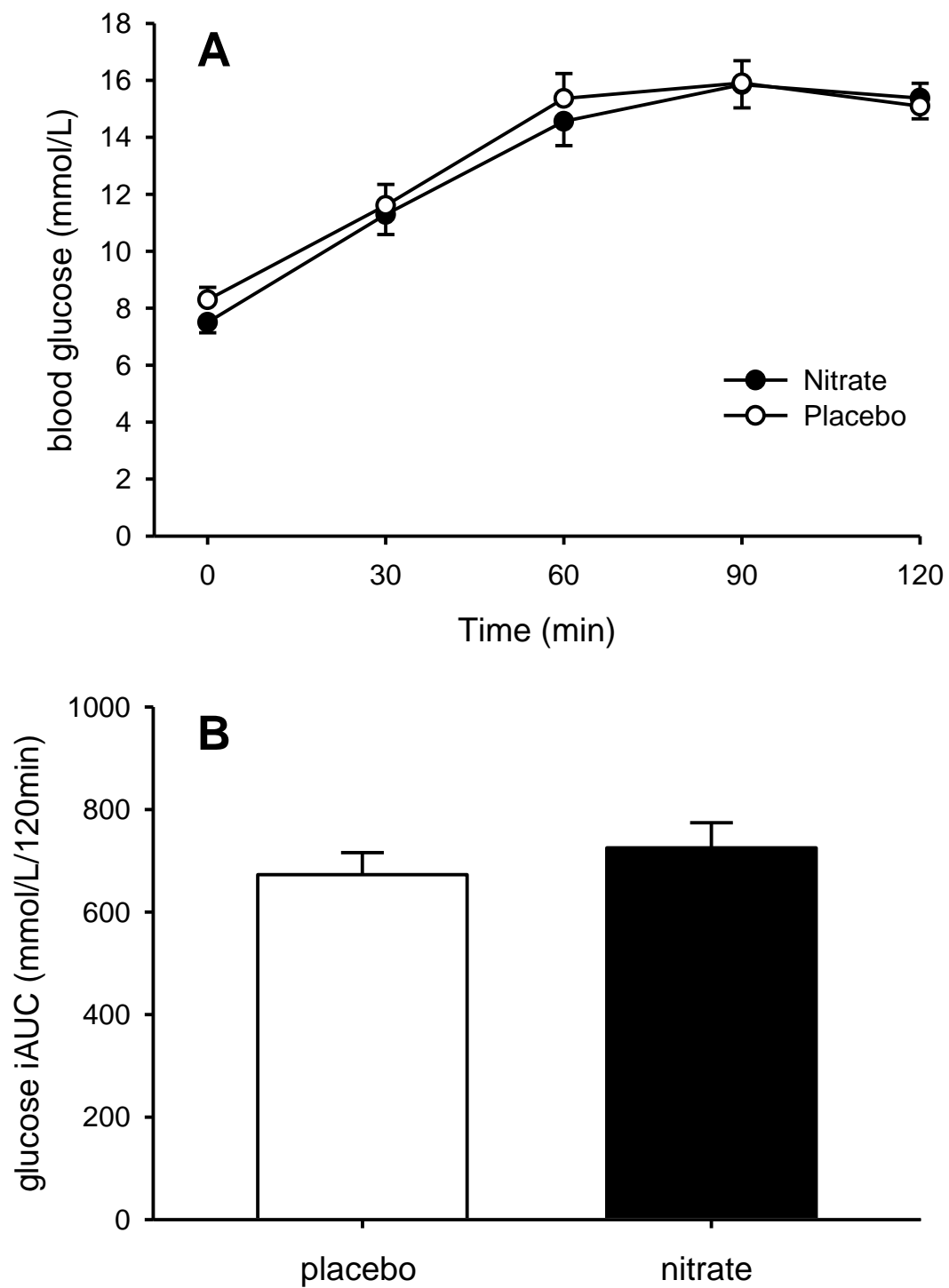


FIGURE 4

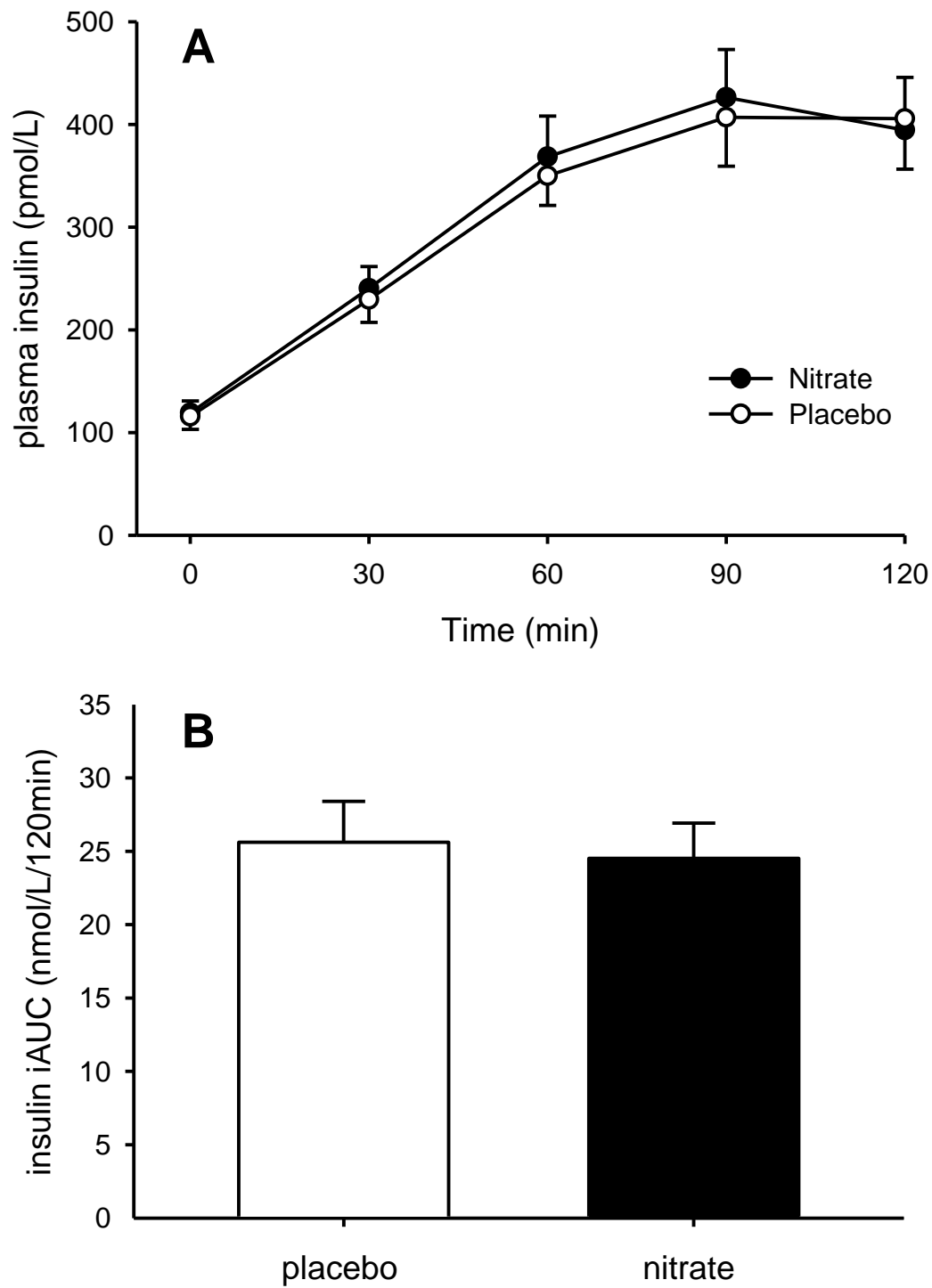
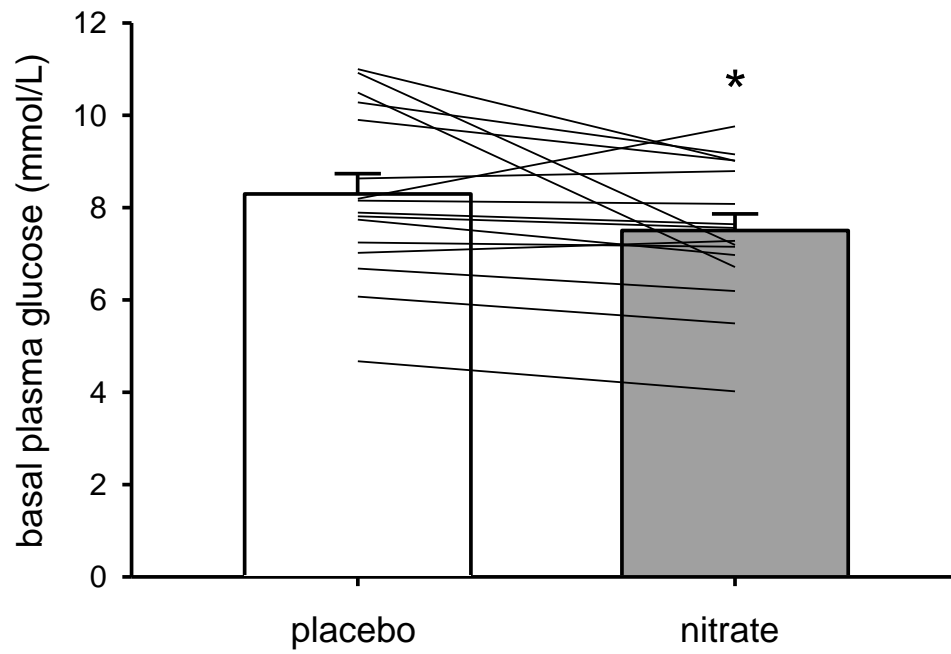


FIGURE 5



ACCEPTED