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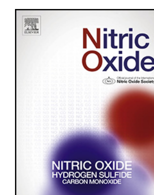

Dietary nitrate improves glucose tolerance and lipid profile in an animal model of hyperglycemia

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ABSTRACT

Reduction in nitric oxide (NO) production and bioavailability contribute to the pathogenesis of type 2 diabetes. Administration of nitrate has strong NO-like outcomes in both animals and humans. In this study, we examined the effects of dietary nitrate on glucose tolerance and lipid profile in type 2 diabetic rats. Type 2 diabetes was induced by injection of streptozotocin and nicotinamide. Thirty-two male Wistar rats were divided into 4 groups: controls (C), control+nitrate (CN), diabetes (D), and diabetes+nitrate (DN). For 8 weeks, the CN and DN groups consumed sodium nitrate (100 mg/L in drinking water) while the C and D groups consumed tap water. Serum nitrate+nitrite (NO_x), glucose, lipid profile, total antioxidant capacity (TAC), and catalase (CAT) activity were measured before and at the end of the study. Systolic blood pressure (SBP) was measured every 10 days. Intravenous glucose tolerance test (IVGTT) was performed at the end of the study. Serum NO_x decreased in diabetic rats and dietary nitrate restored it to normal values. Increases in serum glucose levels was significantly lower in the DN group compared to the D group (24.1% vs. 90.2%; $p < 0.05$). Nitrate therapy in diabetic rats significantly improved lipid profile, glucose tolerance (AUC: 20264 ± 659 vs. 17923 ± 523; $p < 0.05$ for D and DN groups respectively) and restored elevated SBP to normal values. Diabetic rats had lower TAC and CAT activity and dietary nitrate restored these to normal status. In conclusion, dietary nitrate prevented increase in SBP and serum glucose, improved glucose tolerance and restored dyslipidemia in an animal model of hyperglycemia.
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1. Introduction

Type 2 diabetes is characterized by hyperglycemia, insulin resistance, and dyslipidemia [1]. Almost 285 million individuals worldwide had diabetes in 2010, 90% of them having type 2 diabetes; it has been estimated that this figure will escalate to 439 million by 2030 [2]. Various treatments are available for type 2 diabetes, most of which, however, have insufficient efficacy, making further investigations imperative to provide more efficient treatments [3].

Nitric oxide (NO), a simple molecule with complex actions [4], has a specific role in regulating many biological functions in the body [5]. Reduced NO production and NO bioavailability has an essential role in the pathogenesis of many disorders [6] including type 2 diabetes [7]. NO is produced from L-arginine by enzymes known as nitric oxide synthase (NOS) [8], which have endothelial, neuronal, mitochondrial, and inducible isoforms [3]. Nitrate was previously considered a harmful ingredient in food and a useless product of the NO pathway [5]; however, in 1994, following the discovery of the non-enzymatic pathway of NO production from dietary nitrate highlighted the importance of nitrate/nitrite in many biological pathways [9,10]. During past decade, researches have focused on the beneficial effects of nitrate, reporting recently that administration of nitrate has strong NO-like outcomes in both animals and humans [8].

It has been shown that dietary nitrate may have protective effects against diabetes and cardiovascular disease (CVD) [5] and beneficial effects in disorders characterized by NO deficiency as well [6]. Useful effects of nitrate in protection against diabetes and CVD have been reported [5]. Nitrate rich diets, e.g. Mediterranean diets have cardioprotective effect via decreasing blood pressure and reducing the risk of stroke in humans [11]. Prevention of platelet aggregation and endothelial dysfunction are other beneficial effects of administration of dietary nitrate [12]; in addition, dietary nitrate could improve some features of metabolic syndrome including reducing serum triglycerides (TG) and improving intravenous glucose tolerance in eNOS-deficient mice [13]. To the best of our knowledge, there is no study addressing the

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effects of low dose long-term nitrate therapy in type 2 diabetes, which is why in this study, we aim to determine the effects of dietary nitrate on glucose tolerance, lipid profile, and systolic blood pressure (SBP) in rats with type 2 diabetes.

2. Materials and methods

2.1. Animals and experimental protocols

Male Wistar rats weighing 200–210 g were allowed to acclimatize at 21 ± 2 °C, relative humidity of 50 ± 6% with a 12 h light/dark cycle for 1 week and had free access to standard rat chow (Pars Co., Tehran, Iran) and water. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals provided by the ethics committee of the Research Institute for Endocrine Sciences, affiliated to the Shahid Beheshti University of Medical Sciences. Animals were housed in pairs, and body weight, food intake, and water consumption were determined every 3 days for 8 weeks. Preweighed food (using A&D Scale, EK-300i, Japan; sensitivity 0.01 g) and premeasured water were placed in the cages and measured on a per-cage basis. Food intake was determined as grams consumed/day and water consumption determined as milliliter consumed/day. Diabetes was induced in rats 10 days before administrating of sodium nitrate; day 10 of induction was designated as day 1 for sodium nitrate administration in diabetic rats. Animals were divided into 4 groups containing 8 rats each, as follows, control rats consuming tap water (C); control rats consuming sodium nitrate in drinking water (CN); diabetic rats consuming tap water (D); and diabetic rats consuming sodium nitrate in drinking water (DN). The CN and DN groups consumed 100 mg/L sodium nitrate in drinking water for 8 weeks while the C and D groups consumed tap water. Serum nitrate+nitrite (NO_x), glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), TG, total antioxidant capacity (TAC), and catalase (CAT) activity were measured before and at the end of the study. SBP was measured every 10 days. At the end of the study, intravenous glucose tolerance test (IVGTT) was performed in all groups.

2.2. Induction of type 2 diabetes

After overnight (12–14 h) fasting, diabetes was induced by intraperitoneal (IP) injection of 65 mg/kg streptozotocin (STZ) (Sigma Aldrich, Hamburg, Germany) 15 min following an IP injection of 95 mg/kg nicotinamide (NA) (Sigma Aldrich, Hamburg, Germany) [14,15]. STZ was dissolved in 0.1 mmol/L citrate buffer (pH 4.5) and NA was dissolved in normal saline [14]. Ten days after STZ-NA injection, rats with glucose levels ≥126 mg/dL were considered diabetic [16].

2.3. Intravenous glucose tolerance test

After overnight fasting, animals were anesthetized with an IP injection of 60 mg/kg pentobarbital sodium (Sigma Aldrich, Hamburg, Germany), following which, 20% glucose at a dose of 0.5 g/kg was administered via the femoral vein though a cannula filled with heparinized normal saline. Blood samples were collected from tip of the tail at 0 min and again 5, 10, 15, 20, 30, and 60 min after glucose administration. Equal volume of heparinized normal saline was administered for replacing blood volume [17,18].

2.4. Laboratory assessments

After 12–14 h fasting, blood samples were collected in anticoagulant-free tubes and then centrifuged at 3000 *g* for 15 min. Serum level of total NO_x was determined by the Griess method [19]. In brief, serum proteins were precipitated with zinc sulfate (15 mg/mL), centrifuged at 10,000 *g* for 10 min, and supernatants were removed for measurement of NO_x levels. Nitrate was reduced to nitrite by adding vanadium trichloride (8 mg/mL prepared in 1M HCl), after which 2% sulfanilamide, dissolved in 1M HCl, and 0.1% *N*-(1-naphthyl) ethylenediamine, in ddH_2O, was added to deproteinated serum samples. Samples were incubated for 30 min at 37 °C, and optical density was determined at 543 nm by a microplate reader (BioTek, MQX2000R2, USA). NO_x concentrations in the samples were determined using a standard calibration curve of 0–100 µmol/L of sodium nitrate. The intra-assay coefficient of variation (CV) was 4.4%.

Serum levels of glucose, TC, HDL-C, LDL-C, and TG were measured using a spectrophotometric assay with commercial kits (Pars Azmoon, Tehran, Iran) at 546 nm. Intra-assay CVs for glucose, TC, HDL-C, LDL-C and TG were 1.0%, 2.0%, 1.2%, 1.6%, and 1.4% respectively.

Serum levels of TAC and CAT were measured using a spectrophotometric assay with commercial kits (ZellBio, Germany, Veltlinerweg) at 520 nm and 405 nm respectively. Intra assay CVs for TAC and CAT were 3.3% and 4.1% respectively.

2.5. Measurement of systolic blood pressure

SBP was measured in conscious rats every 10 days using a non-invasive tail-cuff method (AD Instruments, MLT125R, Australia). The rats were individually controlled in a restrainer at an ambient temperature of 36–37 °C for 20 min. Three blood pressure values were obtained from each rat and the average of these was recorded as the animal's SBP.

2.6. Statistical analysis

Analyses were done using GraphPad Prism software (Version 5) and data are expressed as mean ± SEM. Paired samples t-test was used for comparing serum glucose, lipid profile, NO_x, TAC and CAT levels, before and after nitrate therapy. Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used for analyzing data of IVGTT, SBP, and body weight. One-way ANOVA was used for comparing the area under the curve (AUC) of IVGTT, SBP, and body weight. *P* values ≤0.05 were considered statistically significant.

3. Results

The diabetic group had significantly lower serum NO_x concentrations ($P < 0.05$) and dietary nitrate restored these decreased levels in the DN group ($p < 0.05$) (Table 1). Diabetic rats had impaired glucose tolerance, which significantly improved after nitrate administration ($p < 0.05$) (Fig. 1).

The effect of dietary nitrate on serum glucose, TC, HDL-C, LDL-C, TG, TAC, and CAT are shown in Table 1. As seen, serum glucose increased by 90.2% in the D group, compared with 24.1% in the DN group, a difference statistically significant ($p < 0.05$). Compared to baseline values, dietary nitrate significantly decreased serum TC by 23.6%, LDL-C by 28.8%, and serum TG by 24.2% in the CN groups and 22.9%, 54.2%, and 47.6% in the DN groups. Compared to baseline values, serum HDL-C significantly increased by 33.3% in CN and 42.4% in the DN group and by 15.7% in the D group. Nitrate administration significantly increased TAC and CAT by 47.1% and 39.8% in CN and significantly decreased in D group by 30.9% and 23.9%; dietary nitrate also restored decreased levels of TAC and CAT in the DN group ($p < 0.05$).

Rats in the D and DN groups, compared to the C and CN groups had significantly lower body weight throughout the study (Fig. 2A). In the D and DN groups, food intake and water consumption were significantly higher than in controls (Fig. 2B and C).

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