Nitrate in Type 2 Diabetes

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Beneficial metabolic effects of inorganic nitrate (NO3−) and nitrite (NO2−) in type 2 diabetes mellitus (T2DM) have been documented in animal experiments; however, this is not the case for humans. Although it has remained an open question, the redox environment affecting the conversion of NO3− to NO2− and then to NO is suggested as a potential reason for this lost-in-translation. Ascorbic acid (AA) has a critical role in the gastric conversion of NO2− to NO following ingestion of NO3−. In contrast to AA-synthesizing species like rats, the lack of ability to synthesize AA and a lower AA body pool and plasma concentrations may partly explain why humans with T2DM do not benefit from NO3−/NO2− supplementation. Rats also have higher AA concentrations in their stomach tissue and gastric juice that can significantly potentiate gastric NO2−-to-NO conversion.

Keywords: nitrate ; nitrite ; nitric oxide ; ascorbic acid ; type 2 diabetes

1. Introduction

Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) are considered storage pools for nitric oxide (NO)-like bioactivity that complement or alternate the NO synthase (NOS)-dependent pathway [1]. The biological importance of the NO_3^--NO_2^--NO pathway is more highlighted where the NOS system is compromised, e.g., in cardiometabolic diseases [2][3].

Type 2 diabetes mellitus (T2DM), a metabolic disorder complicated with disrupted NO metabolism [4][5], has recently been targeted for inorganic NO_3^--NO_2^- therapy. Supplementation of diets rich in inorganic NO_3^--NO_2^- has received increased attention as being effective in improving glucose and insulin homeostasis in animal models of T2DM [6][7][8][9][10]. Favorable effects of NO_3^- therapy on glucose and insulin homeostasis were surprisingly comparable to metformin therapy, a drug used as the first-line anti-diabetic agent [11].

In contrast to animal experiments, controversy surrounds the NO_3^--NO_2^- efficacy on metabolic parameters in humans with T2DM. These interventions have failed to show any beneficial effects on glucose and insulin parameters. Although some plausible explanations have been provided, the reason for this lost-in-translation remains an open question. Species-differences in NO_3^--NO_2^- metabolism, due to differences in the gut–oral microbiota, and the redox environment affecting the capacity of NO_3^- to NO_2^- to NO reduction (e.g., oral and stomach pH, reducing agents like ascorbic acid (AA), and NO_3^--NO_2^- reductase enzymes) may explain the failure of the data to translate from animals to humans. Furthermore, some confounding variables such as doses and forms of NO_3^- and NO_2^- supplementation, age of the experimental units [12], background dietary intake of NO_3^--NO_2^-, and use of anti-diabetic drugs in humans [11][13] can also influence the magnitude of the metabolic response to NO_3^--NO_2^- therapy in humans with T2DM.

2. A Brief Overview of NO_3^--NO_2^--NO Pathway

There are two major pathways for NO production in humans: (i) the classic l-arginine-NOS pathway, in which NO is produced from l-arginine by three isoforms of NOS, namely, endothelial (eNOS), neural (nNOS), and inducible (iNOS) NOSs, and (ii) NO_3^--NO_2^--NO pathway, in which NO_3^- is reduced to NO_2^- and then to NO [2]. The NO_3^--NO_2^--NO pathway has a compensatory role in maintaining basal levels of NO in the absolute absence of the NOS system (i.e., triple NOS-knockout model), thus keeping the animals alive [14]. There is negative cross-talk between the two pathways in maintaining NO homeostasis [1][15]. Chronic NO_3^- supplementation may reversibly and dose-dependently reduce eNOS activity; on the other hand, responses to exogenous NO_3^--NO_2^- depend upon the basal eNOS activity, and subjects with deficient eNOS activity and vascular NO deficiency may, therefore, have an augmented response to these anions [1][15]. Several dietary factors, including dietary antioxidants, polyphenols, and fatty acids, may affect the NO pathway in humans [16]. Furthermore, dietary antioxidant capacity and vitamin C intake may modify the potential effects of NO_3^--NO_2^- in cardiometabolic diseases [17][18].

Major sources of NO_3^- in humans are endogenously derived from NO oxidation and exogenously derived from the diet. About 50% of steady-state circulating NO metabolites are derived from dietary sources [19]; the acceptable daily intake (ADI) values are 3.7 and 0.06 mg/kg body weight for NO_3^- and NO_2^-, respectively [20]. Following ingestion, inorganic NO_3^- passes from the mouth into the stomach and is then absorbed into the blood from the proximal small intestine [21]. In humans, about 50–90% [22][23][24] (a mean of 75% [25]) of ingested NO_3^- is excreted in the urine, with negligible fecal excretion [26]. NO_3^- recovery from urine was reported to be about 35–65% of the oral doses in rats and rabbits [21][27]. About 25% of ingested NO_3^- is taken up from the plasma [28] by the salivary glands, probably via the sialin transporter [29], concentrated by 10–20 folds, and secreted in the saliva [29][30], a process that is called enterosalivary circulation of NO_3^- [28]. Unlike humans, the active secretion of NO_3^- into the saliva does not occur in rats and mice [31]; however, the entero-systemic cycling of NO_3^- may occur in these species by secreting from the circulation into the other parts of the gastrointestinal system, including the gastric and intestinal secretions via an active transport process [32].

Upon entering the mouth, oral NO_3^--reducing bacteria converts about 20% of the dietary NO_3^- to NO_2^- [28]. This pathway is the most important source of NO_2^- in the human body [33] and provides systemic delivery of substrate for NO generation. Oral NO_3^--reduction results in an average of 85.4 ± 15.9 nmol NO_2^- per min [34]. The oral NO_3^--reducing bacteria are mostly resident at the dorsal surface of the tongue both in humans and rats [34][35]. The critical role of NO_3^--reducing bacteria on the NO_3^--NO_2^--NO pathway and systemic NO availability is highlighted by the data showing that circulating NO_2^- is decreased and NO-mediated biological effects are partially or entirely prevented when the oral microbiome was abolished via antiseptic mouthwash [36][37][38]. Although the rat tongue microbiome is less diverse than the human, the physiological activity of the oral microbiome is comparable in both species [39].

Salivary NO_2^- reaching the stomach is rapidly converted to NO in the presence of acidic gastric juice and AA and diffuses into the circulation [40][41]. Inorganic NO_3^- can therefore act as a substrate for further systemic generation of bioactive NO [30]. The efficiency of sequential reduction of inorganic NO_3^- into NO_2^- and then into NO depends on the capacity of the salivary glands to concentrate NO_3^-, oral NO_3^--reducing bacteria, gastric AA concentration and the redox environment, O_2 pressure, pH in the peripheral circulation, and the efficiency of the enzymatic reductase activity (i.e., deoxyhemoglobin, aldehyde dehydrogenase, and xanthine oxidase) [1]; these factors may affect the metabolic response to oral dosing of inorganic NO_3^-.

3. Effects of Inorganic NO_3^- and NO_2^- in Type 2 Diabetes

Impaired NO metabolism, including decreased eNOS-derived NO bioavailability, over-production of iNOS-derived NO, and impaired NO_3^--NO_2^--NO pathway, are involved in T2DM development [42], hypertension [43], and cardiovascular diseases [44]. Increased NO bioavailability using NO precursors, including *L*-arginine [45][46], *L*-citrulline [47], or inorganic NO_3^- and NO_2^- has been suggested as complementary treatments in T2DM [48][49][50]. Due to lack of efficacy [51] and safety [52] of long-term *L*-arginine supplementation and undesirable side effects (i.e., induction of arginase activity [53][54], increased urea levels [55], suppression of eNOS expression and activity, and induction of cellar oxidative stress [56]), inorganic NO_3^- and NO_2^- have received much attention as NO-boosting supplements.

Inorganic NO_3^- and NO_2^- improve glucose and insulin homeostasis in animal models of T2DM [6][7][8][9][10]; supplementation with these anions decreases hyperglycemia and improves insulin sensitivity and glucose tolerance [9][10]. NO_3^- and NO_2^- increase insulin secretion by increasing pancreatic blood flow [57], increasing pancreatic islet insulin content [7], and increased gene expression of proteins involved in exocytosis of insulin in isolated pancreatic islets [58]. NO_3^- and NO_2^- increase insulin sensitivity by increasing GLUT4 expression and protein levels in epididymal adipose tissue [6], skeletal muscle [7], and its translocation into the cell membrane [9], increasing browning of white adipose tissue [59], decreasing adipocyte size [9], as well as improving inflammation, dyslipidemia, liver steatosis, and oxidative stress [3][7][60]. Table 1 summarizes the effects of NO_3^--NO_2^- therapy on glucose and insulin homeostasis and diabetes-induced cardiometabolic disorders in animal models of T2DM. More details about the favorable metabolic effects of NO_3^- and NO_2^- can be found in published reviews [2][3][61].

Table 1. The effects of NO_3^- and NO_2^- on glucose and insulin homeostasis, and cardiometabolic disorders in experimental models of type 2 diabetes mellitus and insulin resistance.

Author	Model	Treatment	Outcomes
Jeddi et al., 2021 [62]	High-fat diet + low-dose of STZ (30 mg/kg body weight), male rats	100 mg/L $NaNO_3$ in drinking water for 6 months	↓ Serum glucose by 13% ↓ Serum insulin by 23% ↑ cGMP level in epididymal adipose tissue by 85% ↑ Adipocyte density by 193% (epididymal adipose tissue) ↓ Adipocyte area by 53% (epididymal adipose tissue) ↑ Expression of browning genes in epididymal adipose tissue (↑ mRNA and protein levels of PPAR-γ, PGC1-α, and UCP-1 to their normal values)
Tian et al., 2020 [63]	High-fat diet + low dose of STZ (20 mg/kg body weight), male mice	255 mg/L $NaNO_3$ in drinking water for 8 weeks	↓ Fasting glucose Prevention of impaired glucose tolerance (measured by IP-GTT), Prevention of insulin resistance (measured by IP-ITT) ↓ Systolic blood pressure ↓Vascular oxidative stress (↓ROS formation) ↓ NADPH oxidase activity via induction of HO-1 and reduction in p47phox expression Improvement of endothelial function (ACh-mediated vascular relaxation) Improvement of inflammation and dyslipidemia ↓ Development of aortic atherosclerosis
Aggarwal et al., 2020 [64]	Insulin-resistant iNOS−/− male mice	50 mg/L $NaNO_2$ in drinking water for 5 weeks	Improved glucose tolerance (measured by IP-GTT) Improved insulin resistance (measured by IP-ITT) Partially reversed up-regulated gluconeogenesis (↓ expression of PEPCK, G6P, and PC) Restored total Akt (PKB) expression in the liver and adipose tissue Restored decreased Akt-1/2/3 phosphorylation (Ser473) in the liver Improved insulin signaling in the adipose tissue
Norouzirad et al., 2019 [65]	High-fat diet + low dose of STZ (30 mg/kg body weight), male rats	100 mg/L $NaNO_3$ in drinking water for 5 weeks	↓ Fasting glucose ↓ Gluconeogenesis (measured by IP-PTT) Improved glucose tolerance Restored CAT activity to near normal value Restored elevated TOS to near normal value Restored decreased TAC levels to near normal value ↑ Serum SOD, GSH, and GSH-to-GSSG ratio
Gheibi et al., 2018 [6]	High-fat diet + low dose of STZ (25 mg/g body weight), male rats	100 mg/L $NaNO_3$ in drinking water for 8 weeks	↓ Serum glucose and insulin, ↔ HbA1c ↑ Glucose tolerance (measured by IP-GTT) ↑ Insulin sensitivity (measured by QUICKI) ↓ Gluconeogenesis (measured by IP-PTT) ↑ GLUT4 mRNA expression and protein levels in the soleus muscle by 215% and 17% ↑ GLUT4 mRNA expression and protein levels in the epididymal adipose tissue by 344% and 22% ↔ GSIS, islet insulin content ↑ Serum CAT activity, ↓ Serum IL-1β ↔ Serum TBARS ↓ Elevated iNOS mRNA expression in the soleus muscle and epididymal adipose tissue
Gheibi et al., 2017 [7]	High-fat diet + low dose of STZ (30 mg/kg body weight), male rats	50 mg/L $NaNO_2$ in drinking water for 8 weeks	↑ GSIS (by 34%), ↔ BIS ↑ Protein levels of GLUT4 in the soleus muscle and epididymal adipose tissue by 22% and 26% Improved glucose tolerance (measured by IP-GTT) and insulin sensitivity (measured by IP-ITT and QUICKI) ↓ Insulin resistance (measured by HOMA-IR) ↓ Fasting serum glucose and insulin, ↔ HbA1c Restored pancreatic insulin content to 73% of controls (68.2 ± 6.4 vs. 117 ± 6.0 pmol/mg protein) Restored elevated serum levels of TC, TG, and LDL-C ↔ HDL-C
Ohtake et al., 2015 [9]	*KKAy* diabetic male mice	50 and 150 mg/L nitrite in drinking water for 10 weeks	↓ Fasting glucose ↓ Insulin resistance (measured by HOMA-IR) Improved glucose tolerance (measured by IP-GTT) ↑GLUT4 expression on the cell membrane of the skeletal muscle

Author	Model	Treatment	Outcomes
Khalifi et al., 2015 [8]	**STZ (65 mg/kg) + nicotinamide (95 mg/kg), male rats**	**100 mg/L $NaNO_3$ in drinking water for 8 weeks**	**Improved glucose tolerance (measured as IV-GTT)** **↓ Serum TC (23.6%), TG (24.2%), and LDL-C (28.8%)** **↑ Serum HDL-C (42.4%)** **Restored TAC and CAT levels to normal values**
Jiang et al., 2014 [66]	***db*/*db* diabetic male mice**	**50 mg/L $NaNO_2$ in drinking water for 4 weeks**	**↓ Fasting glucose (by 35%)** **↓ Plasma insulin**
Carlstrom et al., 2010 [10]	**eNOS-deficient female mice**	**85 mg/L $NaNO_3$ in drinking water for 8–10 weeks**	**↓ HbA1c, Fasting glucose** **↓ Pro-insulin to insulin ratio** **↑ Glucose tolerance (measured by IP-GTT)**

↔, no change; ↑, increase; ↓, decrease. ACh, acetylcholine; BIS, basal insulin secretion; CAT, catalase; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; G6P, glucose-6-phosphatase; GSH, reduced glutathione; GSIS, glucose-stimulated insulin secretion; GSSG, oxidized glutathione; HbA1C, glycated hemoglobin; HDL-C, high-density lipoprotein-cholesterol; HO-1; heme oxygenase-1; HOMA-IR, homeostasis model assessment of insulin resistance; IL-1β, interleukin -1β; iNOS, inducible NOS; IP-GTT, intraperitoneal glucose tolerance test; IP-ITT, intraperitoneal insulin tolerance test; IP-PTT, intraperitoneal pyruvate tolerance test; IV-GTT, intravenous glucose tolerance test; LDL-C, low-density lipoprotein-cholesterol; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1-α, PPAR-γ coactivator 1 alpha; PPAR-γ, peroxisome proliferator activated receptor gamma; phox, phagocyte oxidase; QUICKI, quantitative insulin-sensitivity check index; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TAC, total antioxidant capacity; TBARS, thiobarbituric reactive substances; TG, triglycerides; TOS, total oxidant status; TC, total cholesterol; UCP-1, uncoupling protein 1.

Despite being effective in animal models of T2DM, as it is summarized in Table 2, all acute [67], mid-term [68][69], and long-term [70][71][72] oral dosing of inorganic NO_3^- and NO_2^-, either as pharmacological forms (i.e., KNO_3, $NaNO_3$, and $NaNO_2$) or food-based supplementation (i.e., NO_3^--rich beetroot juice or powder) have failed to show beneficial effects on glucose and insulin parameters, including fasting and postprandial serum glucose and insulin concentrations, insulin resistance indices, and HbA1c levels in patients with T2DM. However, ergogenic [73][74] and beneficial cardiovascular effects of inorganic NO_3^- and NO_2^-, e.g., reducing peripheral and central systolic and diastolic blood pressures [75], have been highlighted in non-diabetic subjects by several clinical studies.

Table 2. Cardiometabolic effects of inorganic NO_3^--NO_2^- in patients with type 2 diabetes mellitus: findings of clinical trials.

Study	Intervention	Outcomes
Bahadoran et al., 2021 [76]	NO_3^--rich beetroot powder (250 mg/day NO_3^-), for 24 weeks	↔ Fasting glucose, HbA1c, insulin, C-peptide ↔ HOMA-IR, QUICKI ↔ Serum lipid parameters ↔ Serum ALT, AST, ALP, GGT ↔ Serum creatinine and uric acid ↔ Urinary creatinine and albumin
Faconti et al., 2019 [70] and Mills et al. [71]	NO_3^--containing beetroot juice (279 mg/day NO_3^-), for 24 weeks	↔ SBP, DBP ↔ Arterial stiffness ↔ Fasting glucose, HbA1c ↓ Left ventricular end-diastolic and end-systolic volume

Study	Intervention	Outcomes
Soin et al., 2018 [72]	40 and 80 mg/day sustained-release formulation $NaNO_2$, for 12 weeks	↔ HbA1c Improvement of neuropathic pain
Shepherd et al., 2015 [77]	70 mL/day NO_3^--containing beetroot juice (398 mg/day NO_3^-), for 4 days	↔ SBP, DBP ↔ Oxygen cost of exercise ↔ Walking performance (6-min walk test)
Cermak et al., 2015 [67]	An acute dose of $NaNO_3$ (12.75 mg/kg body weight)	↔ Postprandial glucose and insulin response to 75-g glucose ↑ OGIS index ↔ HOMA-IR
Mohler et al., 2014 [78]	40 and 80 mg/day $NaNO_2$, for 10 weeks	↑ FMD at a dose of 80 mg/day
Gilchrist et al., 2014 [68]	250 mL/day beetroot juice (465 mg/d NO_3^-), for 2 weeks	↔ Fasting glucose, HbA1c ↔ Cognitive function Improvement in simple reaction time
Gilchrist et al., 2013 [69]	250 mL/day beetroot juice (465 mg/d NO_3^-), for 2 weeks	↔ SBP, DBP ↔ Macro-(FMD) and micro-(ACh-induced vasodilation) vascular function ↔ Insulin sensitivity (hyperinsulinemic-euglycemic clamp technique)
Greenway et al., 2012 [79]	An acute dose of 80 mg of $NaNO_2$ (IR and EC formulation)	↓ SPB and DBP in IR ↔ SPB and DBP in EC

↔, no change; ↑, increase; ↓, decrease; ACh, acetylcholine; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C-peptide, connecting peptide; DBP, diastolic blood pressure; EC, enteric-coated formulation; FMD, flow-mediated dilation; GGT; γ-glutamyl transpeptidase; HbA1C, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; IR, immediate-release formulation; OGIS, oral glucose insulin sensitivity; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure.

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