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Method

# Profound differences between humans and rodents in the ability to concentrate salivary nitrate: Implications for translational research

Marcelo F Montenegro[a], Michaela L Sundqvist[a,b], Carina Nihlén[a], Michael Hezel[a], Mattias Carlström[a], Eddie Weitzberg[a], Jon O Lundberg[a,*]

[a] Dept. of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden
[b] Function Area Clinical Nutrition, Karolinska University Hospital, Stockholm, Sweden

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ABSTRACT

In humans dietary circulating nitrate accumulates rapidly in saliva through active transport in the salivary glands. By this mechanism resulting salivary nitrate concentrations are 10–20 times higher than in plasma. In the oral cavity nitrate is reduced by commensal bacteria to nitrite, which is subsequently swallowed and further metabolized to nitric oxide (NO) and other bioactive nitrogen oxides in blood and tissues. This entero-salivary circulation of nitrate is central in the various NO-like effects observed after ingestion of inorganic nitrate. The very same system has also been the focus of toxicologists studying potential carcinogenic effects of nitrite-dependent nitrosamine formation. Whether active transport of nitrate and accumulation in saliva occurs also in rodents is not entirely clear. Here we measured salivary and plasma levels of nitrate and nitrite in humans, rats and mice after administration of a standardized dose of nitrate.

After oral (humans) or intraperitoneal (rodents) sodium nitrate administration (0.1 mmol/kg), plasma nitrate levels increased markedly reaching ~300 μM in all three species. In humans ingestion of nitrate was followed by a rapid increase in salivary nitrate to > 6000 μM, ie 20 times higher than those found in plasma. In contrast, in rats and mice salivary nitrate concentrations never exceeded the levels in plasma. Nitrite levels in saliva and plasma followed a similar pattern, ie marked increases in humans but modest elevations in rodents. In mice there was also no accumulation of nitrate in the salivary glands as measured directly in whole glands obtained after acute administration of nitrate.

This study suggests that in contrast to humans, rats and mice do not actively concentrate circulating nitrate in saliva. These apparent species differences should be taken into consideration when studying the nitrate-nitrite-nitric oxide pathway in rodents, when calculating doses, exploring physiological, therapeutic and toxicological effects and comparing with human data.

## 1. Introduction

The nitrate-nitrite-NO pathway has emerged as an important additional source of NO generation in mammals complementing the classical NO synthase (NOS)-dependent pathway [1,2]. Inorganic nitrate formed following endogenous oxidation of NOS-derived NO or from exogenous dietary sources can be serially reduced to form nitrite and subsequently NO and a variety of other potentially bioactive nitrogen oxides. Ingestion of inorganic nitrate results in measurable NO-like bioactivity in both humans and animals including reductions in blood pressure [3,4], inhibition of platelet aggregation [5] as well as improvement of metabolic functions [6,7]. The fact that nitrate and nitrite can be substrates for generation of NO has challenged the dogma that dietary nitrate has detrimental health effects. Prior to the discovery of the nitrate-nitrite-NO pathway, nitrate was mostly known for being a substrate for the generation of potentially carcinogenic nitrosamines [8]. Interestingly, the route to nitrosamine formation from nitrate is the same as that leading to NO since both require intermediate formation of the more reactive nitrite anion.

A central process in the bioactivation of nitrate in humans is its active uptake from blood by the salivary glands and excretion in saliva. In the oral cavity commensal bacteria reduce salivary nitrate to the more reactive nitrite anion [9]. Nitrite is then swallowed and can form a number of nitrogen oxides locally in the acidic gastric environment and systemically in blood and tissues after absorption [1]. In humans the degree of nitrate accumulation in saliva is remarkable and it is believed that 25% of all circulating nitrate is taken up by the salivary glands with resulting salivary levels that are up to 20 times higher than

* Corresponding author.
*E-mail address:* Jon.Lundberg@ki.se (J.O. Lundberg).

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in plasma [10–12]. The requirement of salivary accumulation of nitrate and reduction to nitrite for the nitrate-nitrite-NO pathway to operate is clearly illustrated in experiments where the test persons either avoids swallowing saliva during a period following nitrate ingestion [5,11] or uses an antibacterial mouthwash to prevent bacterial nitrate reduction [12]. In both cases the physiological effects of nitrate are abolished.

While most of the favorable effects of dietary nitrate observed in humans occur also in rodents, the required doses to observe similar effects in rodents are typically considerably higher [13]. There have been suggestions that rodents do not concentrate nitrate in saliva [14–16] but direct measurements and comparisons with humans are currently missing. In this study we set out to directly compare salivary nitrate concentration in humans, rats and mice by administering a standardized amount of sodium nitrate followed by repeated blood- and saliva samples over time.

## 2. Material and methods

The human part of the study was approved by the Karolinska Institute regional ethics committee in Stockholm, Sweden. The experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the Karolinska Institute in Stockholm, Sweden, and performed according to the National Institutes of Health guidelines for the conduct of experiments in animals.

### 2.1. Study participants and experimental animals

Five healthy volunteers aged 27–57 years (3 females) participated in the study. The subjects arrived in the lab in the morning after an overnight fast and after having been instructed to avoid nitrate-rich food the preceding day. A catheter was placed in the antecubital vein for sampling. After collection of basal blood and saliva samples, sodium nitrate (0.1 mmol/kg dissolved in 10 mL of drinking water) was ingested and blood and saliva were collected repeatedly during 60 min.

Rats (Sprague Dawley) weighting 280–500 g were anesthetized using Inactin (60 mg/kg) and ketamine (60 mg/kg) and tracheostomy was carefully performed to avoid any damage to the submandibular glands. A catheter (PE50) was inserted in the femoral vein for blood collection. After this, a low dose of pilocarpine (64 μg/kg) was intraperitoneally administrated to induce a low flow of saliva sufficient to allow for collection using a capillary tube. After baseline collection of saliva and blood, sodium nitrate was administrated intraperitoneally (0.1 mmol/kg) and saliva and blood were collected at 15, 30, 45 and, 60 min. In mouse experiments, animals (C57Bl/J6) were anesthetized using ketamine (100 mg/kg) and then pilocarpine (64 μg/kg) was administrated intraperitoneally. Saliva and blood were collected before (baseline) and 60 min after an intraperitoneal injection of sodium nitrate (0.1 mmol/kg). In another set of experiments, 6 mice received a single gastric gavage of sodium nitrate (0.1 mmol/kg). After 60 min the mice were anesthetized and blood was collected along with excision and rapid removal of the sublingual and parotid salivary glands. No pilocarpine was used in these experiments.

### 2.2. Sample preparation

Blood samples were immediately centrifuged at +4 °C (4700×*g*, 5 min) and plasma was stored at −80 °C until analyses. The plasma samples were extracted using HPLC grade methanol (CROMASOLV, Sigma-Aldrich) and the methanol was pre chilled in the freezer using a glass bottle to avoid nitrite/nitrate contamination from different plastic materials. In brief, 100 μL methanol was added to 100 μL plasma in 1.5 mL Eppendorf tubes tested for nitrite/nitrate contamination. The tubes were vortexed and then centrifuged for 10 min 4 °C 10,000*g*.

The saliva samples were centrifuged for 10 min at 4 °C and 10,000*g* before dilution with carrier solution containing 10% HPLC grade methanol.

The salivary glands removed from the animals were weighted, cut in smaller pieces and immediately placed in ice-cold methanol 1 mL/g tissue. The tissue was then homogenized using a bullet blender with Zirccox beads. After homogenization, the samples were centrifuged for 10 min at 4 °C 10,000*g*. The supernatant was then transferred to another tube and stored overnight at −20 °C.

### 2.3. Nitrate and nitrite assessment

A HPLC system dedicated to assessment of nitrite and nitrate (ENO-20; EiCom, Japan) and attached to an auto-sampler (840, EiCom, Kyoto, Japan) was used. The method is sensitive and specific to nitrite and nitrate, and is based on the separation of nitrate by reverse-phase/ion exchange chromatography, followed by inline reduction of nitrate to nitrite with cadmium and reduced copper. Derivatization of reduced nitrate was performed with Griess reagent, and the level of diazo compounds was measured at 540 nm. The reactor solution was freshly prepared before analysis. A standard curve was prepared from sodium nitrite and sodium nitrate, diluted with carrier solution in the range of 0.1–20 μM. Aliquots of standards were stored at −20 °C. The slope was examined and used to calculate the concentration in the samples.

Immediately before running the samples in the HPLC, 100 μL of the samples were transferred to a 96 well plate with conical wells (Costar, nitrate- and nitrite-free). To avoid evaporation and also contamination from the auto sampler that contains a lot of electrical components releasing NO to the surrounding air, we sealed the plate with a film that was sterile and easy for the auto sampler needle to penetrate. The samples were kept at 4 °C by a cooling device in the auto sampler. The data collected and analyzed using the PowerChrom software (V 2.7.9, eDAQ).

### 2.4. Drugs and solutions

All drugs and reagents used were purchased from Sigma Chemical Co. (St Louis, MO, USA). All drugs were dissolved in saline solution immediately before use.

### 2.5. Statistical analysis

The results are expressed as means ± S.E.M. The comparisons between groups were assessed by *t* tests, two-way analysis of variance using Bonferroni correction or one-way analysis of variance followed by Dunnett's multiple comparison tests. A p-value $< 0.05$ was considered significant.

## 3. Results

Plasma and salivary levels of nitrate after administration of nitrate are shown in Fig. 1. In humans, plasma and salivary nitrate increased as expected after ingestion of sodium nitrate. At 60 min, plasma nitrate was 299 ± 14 μM whereas the levels in saliva had reached 6022 ± 485 μM, giving a ratio saliva to plasma of around 20:1 (Fig. 1E). In rats and mice injected intraperitoneally with nitrate, plasma nitrate also greatly increased during the observation period. At 60 min, plasma nitrate levels had increased to 357 ± 72 μM in rats and 261 ± 54 μM in mice (Fig. 1C) and were similar to those found in saliva (343 ± 91 and 260 ± 29 for rats and mice, respectively, Fig. 1D). Thus, in rats and mice, the ratio saliva:plasma for nitrate was close to 1:1 (Fig. 1E). Representative HPLC tracings of nitrate levels in saliva samples from the three species studied are shown in Fig. 2.

The levels of nitrite in plasma and saliva increased markedly in humans after administration of nitrate (Table 1). This increase occurred also in rodents but to a much lesser extent.

In whole salivary glands obtained from anesthetized mice having received nitrate 60 min earlier, the levels of nitrate were no different

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