

## THE EFFECTS OF DIETARY NITRATE, pH AND TEMPERATURE ON NITRATE REDUCTION IN THE HUMAN ORAL CAVITY

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**Abstract.** *Dietary nitrate is metabolized to nitrite by bacterial flora on the posterior surface of the tongue leading to increased salivary nitrite concentrations. In the acidic environment in the stomach, nitrite forms nitrous acid, a potent nitrosating agent. The aim of this study was to examine the effects of dietary nitrate, pH and temperature on nitrate reduction in the human oral cavity. Nitrate reduction was monitored by nitrate reduction assay, based on incubation of nitrate test solutions in the mouth. Results show that all investigated factors significantly influence the reduction of nitrate in the oral cavity. With increasing levels of nitrate the amounts of nitrite in reduction assay increased, but reduction ratio ( $\text{NO}_2^-/\text{NO}_3^-$ ) decreased in this conditions. Influence of pH on nitrate reduction is specific and in line with nature of nitrite-producing bacteria. The greatest reduction activity of bacteria is at pH 8. Variations of temperature of the oral cavity due to outdoor conditions also influence its reduction activity, which is twice lower in January than in July.*

**Key words:** *nitrate, nitrite, oral cavity, reduction, pH, temperature*

### INTRODUCTION

Human nitrate and nitrite intake and metabolism have received considerable interest because nitrite formation in saliva is suspected to be involved in infantile methaemoglobinaemia and carcinogenesis [1,2,3]. Dietary nitrate mainly originates from vegetables (the main source of nitrate in food) [4] and nitrate polluted drinking water. Nitrate in drinking water should be more hazardous than nitrate in vegetables because the latter is counterbalanced by vitamin C and polyphenols that inhibit nitrosation [5]. Ingested nitrate is adsorbed from the stomach or intestines and about 25% is secreted in the saliva by anion transport system [4,5]. As a result, salivary nitrate concentrations are approximately 10 to 20 times those found in plasma [7,10]. Saliva collected directly from salivary ducts contains nitrate but no nitrite. However, saliva collected at other locations in the oral cavity also contains nitrite [10]. Oral bacterial flora, mainly from the posterior surface of the tongue, reduce about 20% of salivary nitrate (5% of ingested nitrate) to nitrite, which is mostly swallowed in the saliva and is normally the main

source of gastric nitrite [5,6]. Nitrite reacts with amines and amides in the stomach to form *N*-nitrosamines and *N*-nitrosamides. This reaction can occur by acid catalysis involving conversion of nitrite anion to nitrous acid ( $\text{HNO}_2$ ) and its further reaction to form nitrous anhydride ( $\text{N}_2\text{O}_3$ ) [7], or by bacterial catalysis at neutral pH, for example in the achlorhydric stomach [8,9]. Hence, saliva nitrite is an important factor determining the extent of gastric nitrosation, and may contribute to the aetiology of cancer of the lung, stomach, esophagus, nasal cavity, urinary bladder and oral cavity [11], of leukemia [12] and non-Hodgkin's lymphoma [13].

Therefore, it is important to characterise the microbial nitrate reductase activity as quantitative measurement of the ability of nitrate to nitrite reduction in the oral cavity. The level of salivary nitrite is dependent on dietary nitrate, the enterosalivary nitrate circulation including salivary flow rate, redox potential in the mouth and the stomach, pH value of the oral cavity and the stomach, absorption of nitrate in the small intestine, etc. In general, *in vitro* and *in vivo* studies have been used to measure nitrate reduction in the oral cavity. In *in vitro* methods, nitrite formation is measured in original or diluted saliva incubated with nitrate addition [14,15]. These methods are easily performed, but saliva represents only a part of bacterial flora present in the oral cavity. *In vivo* studies are usually based on nitrite formation in saliva at different times after low or high nitrate intake [4,7], and their main characteristic is the large spread of the results, both between individuals and between sampling date and time.

In this study, the effects of dietary nitrate, pH and temperature on reduction of nitrate to nitrite in the human oral cavity were investigated *in vivo*, by method based on incubation of nitrate solutions directly in the mouth for a specific period of time.

## EXPERIMENTAL

### Chemicals

The chemicals used were of *p.a.* grade.  $\text{KH}_2\text{PO}_4$ ,  $\text{NaNO}_2$ ,  $\text{KNO}_3$  and sulfanilic acid were obtained from Merck (Darmstadt, Germany),  $\alpha$ -naphthylamine and glycine from Sigma (Deisenhofen, Germany), and  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{NaOH}$  and Cd granules from Zorka (Šabac, Serbia). Deionized distilled water was used to prepare all solutions.

### Subjects

This study was performed in 6 subjects, 3 males and 3 females. They were healthy persons, aged from 30–40 years old. Most of them were nonsmokers, or persons who avoided smoking for few days before experiments. All the volunteers had medical examinations and all undertook dietary questionnaires for determination of their suitability for the study. Exclusion criteria were: high alcohol intake, consumption of food/vitamin supplements, pregnancy, oral contraceptives, individuals who require antibiotic or anti-inflammatory treatment and those with chronic diseases or conditions affecting the stomach.

### Nitrate reduction assay

Nitrate reduction assay was performed by incubation of nitrate solutions in the oral cavity [16]. The oral cavity was initially rinsed with deionized distilled water. Immediately after that,  $15.0 \text{ cm}^3$  of a specific test solution was incubated in the oral cavity. Solution was gently mixed at the beginning of incubation and at every 15–20 seconds. After 2 min, the total mouth content was expectorated into sterile plastic tube containing  $0.60 \text{ cm}^3$   $1.0 \text{ mol dm}^{-3}$   $\text{NaOH}$

solution. NaOH was added to the samples in order to stop further reduction of nitrate and to destroy vitamin C in the incubates [17]. The samples were kept at  $-20^{\circ}\text{C}$  for a maximum one month until analysis.

### General procedures

During and for one day before every test, the subjects avoided foods rich in nitrate and nitrite [5,19]: green leafy vegetables (lettuce, broccoli, spinach, celery), red meat and processed meats (bacon, sausages, salami, and other cured meats), foods rich in vitamin C, vitamin-fortified foods and vitamin supplements. They did not chew gum or hard candy, or take aspirin. Also, the subjects did not use mouthwashes during and for 3 days before the test [14]. The tests were performed in the morning before subjects brushed their teeth.

At first, the control test was performed with all subjects, to determine mean concentrations of nitrate and nitrite in the oral cavity. Each subject was rinsed the mouth and incubated a  $15.0\text{ cm}^3$  of deionized distilled water in period of 2 min, in accordance with nitrate reduction assay. The test was repeated on three different days.

In order to investigate the effects of dietary nitrate on nitrate reduction in the oral cavity, series of nitrate solutions were prepared by diluting sodium-nitrate stock solution, concentration  $1000\text{ mg}$  of nitrate nitrogen per  $\text{dm}^3$  ( $\text{mg-N dm}^{-3}$ ). Concentrations of test solutions were:  $1.0$ ,  $10.0$ , and  $100.0\text{ mg-N dm}^{-3}$ . The test with series of test solutions, consecutively in rising order of nitrate concentrations, was performed once a day according to the nitrate reduction assay. Before incubation of every test solution, subjects were repeatedly rinsed the mouth with deionized distilled water. The time lapse between assays was minimum 5 min, owing to stabilize conditions in the oral cavity. The test was examined in three consecutive days.

The investigation of pH effects on nitrate reduction in the oral cavity was examined with series of the test solutions at pH 5.5, 6.0, 7.0, 8.0 and 8.5, buffered with  $0.1\text{ mol dm}^{-3}$  phosphate buffers. Every test solution containing  $10.0\text{ mg-N dm}^{-3}$  nitrate. Subjects performed the test with all test solutions once a day, with minimum interval between assays 10 min. After every assay, subjects were closely rinsed the mouth and before incubating of next solution they were gargle solution of identical pH without nitrate, to stabilize pH of the oral cavity. Test was repeated during three consecutive days.

To study influence of temperature on nitrate reduction in the oral cavity two subjects, one male and one female performed the tests in January and July. Each subject performed the 2-minute reduction assay with nitrate solution containing  $10.0\text{ mg-N dm}^{-3}$  and repeated it consecutively three times a day, in outdoor conditions. Subjects repeated both tests on three different days with similar temperatures characteristic for specific period of year:  $5.6 \pm 0.3^{\circ}\text{C}$  in January and  $35.1 \pm 0.7^{\circ}\text{C}$  in July.

### Determination of nitrate and nitrite

The mouth samples were thawed and well mixed.  $5.0\text{ cm}^3$  of sample, which already contained NaOH, was deproteinized by adding  $0.06\text{ cm}^3$  of  $1.5\text{ mol dm}^{-3}$   $\text{ZnSO}_4$  solution, stirring, and after 30 min centrifuging at 5000 rpm for at least 10 min at room temperature. The purpose of proteins removal was reduction of turbidity, which may interfere with spectrophotometrical measuring [17]. Nitrite was analysed directly with the Griess reagent [20,21]. Reagent A was prepared by dissolving of  $0.60\text{ g}$  of sulfanilic acid in  $20\text{ cm}^3$  of concentrated hydrochloric acid, and diluting to  $100\text{ cm}^3$  with the deionized distilled water [21]. Reagent B was prepared by dissolving of  $0.060\text{ g}$  of  $\alpha$ -naphthylamine in deionized distilled water con-

taining 1 cm<sup>3</sup> hydrochloric acid and dilute to 100 cm<sup>3</sup> [21]. In 1.0 cm<sup>3</sup> of the mouth sample was added 0.5 cm<sup>3</sup> of reagent A and the absorbance at 543 nm was measured using a Varian Cary 50 UV/VIS spectrophotometer (Mulgrave, Victoria, Australia). The data were referred to as A1. After that, the same volume of reagent B was added to the sample. After 15 min the absorbance (A2) was measured. The final absorbance was calculated as A2–A1 for each sample. Nitrite concentrations were calculated by comparison with a standard curve prepared with sodium-nitrite calibrators.

Nitrate was determined after reduction to nitrite by cadmium [18,20]. Cadmium granules (2–2.5 g) were activated by rinsing three times with deionized distilled water and swirling in a 5·10<sup>-3</sup> mol dm<sup>-3</sup> CuSO<sub>4</sub> solution in glycine-NaOH buffer (pH 9.7) for 5 min. The copper-coated granules (2-3) were transferred to 1.0 cm<sup>3</sup> of deproteinized mouth sample buffered with 0.5 cm<sup>3</sup> glycine-NaOH buffer [20]. After 15 min granules were removed and nitrite was determined as above. After use, the granules are rinsed and stored in 0.1 mol dm<sup>-3</sup> H<sub>2</sub>SO<sub>4</sub> solution; they can be regenerated by repeating mentioned steps. The calibrators were made by diluting of nitrate stock solution with glycine buffer and reducing nitrate by cadmium just as the mouth samples were.

#### RESULTS AND DISCUSSION

Nitrate reduction assay, applied in this work, is very simple and effective *in vivo* method for investigation of nitrate reduction in the human oral cavity. In this assay, reduction process is investigated in test solutions of nitrate exposed to whole surface of the oral cavity, i.e. all presented nitrite-producing bacteria. This is great advantage of nitrate reduction assay according to *in vitro* methods, in which reduction process is investigated in isolated saliva [15] which contain about 10<sup>8</sup> viable bacteria/cm<sup>3</sup>, against about 10<sup>11</sup>/cm<sup>3</sup> on the surface of the mouth [19,22]. Also, many anaerobic nitrite-producing bacteria from posterior part of tongue can not survive in *in vitro* conditions. In contrast with other *in vivo* methods, this assay enable very precise controlling of start conditions (nitrate concentration, pH, etc.), independently of differences between subjects. Many studies show that composition of saliva is very different between subjects, whereby concentrations of nitrate and nitrite fluctuate from 5 to 20 mg-N dm<sup>-3</sup>, and from 1 to 4 mg-N dm<sup>-3</sup>, respectively [6,15,17,18]. Control test in this paper shown that after mouth rinsing and removing of residual nitrate and nitrite, in test solution based on deionised distilled water mean concentration of nitrate for all subjects was 0.17 ± 0.06 mg-N dm<sup>-3</sup> and for nitrite 0.05 ± 0.02 mg-N dm<sup>-3</sup>, measured at three different days. These results are very significant for application of nitrate reduction assay, especially in the meaning of reproducibility of measurements. Also, the fact that subjects avoided food reach in nitrate and nitrite before tests lowered possibility of test solutions contamination by these ions. Moreover, nitrate reduction assay is safe for the subjects according to other *in vivo* methods [4,7], because they not need to ingest test solutions, but only to incubate it in the mouth for a few minutes.

Influence of dietary nitrate on reduction of nitrate in the human oral cavity was investigated on test solutions with nitrate concentration 1.0, 10.0 and 100.0 mg-N dm<sup>-3</sup>, incubated 2 min in the oral cavity. Applied concentration range simulated increase of nitrate concentration in the oral cavity with increase of nitrate amount by oral ingestion [4,7,24]. Results are shown in Fig. 1, as mean of triplicate measuring ± SD.

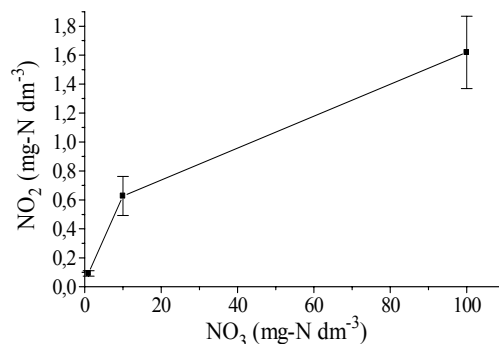


Fig. 1. The influence of the dietary nitrate on reduction of nitrate in the human oral cavity. Test solutions with nitrate concentrations 1.0, 10.0 and 100.0 mg-N dm<sup>-3</sup> were incubated in the mouth for a 2 min. Error bars represent the standard deviation of means values of triplicate sample

Curve shows that nitrite formation significantly increased with increasing levels of nitrate in the test solutions. However, it can be seen that increase of nitrite in the mouth samples, is not linear function of nitrate concentration, i.e. reduction efficacy decline with nitrate levels increasing. Dependence of reduction efficacy, expressed as  $[\text{NO}_2^-]/[\text{NO}_3^-]$  ratio in the mouth samples after 2 min assay, of nitrate concentration is shown in Fig. 2.

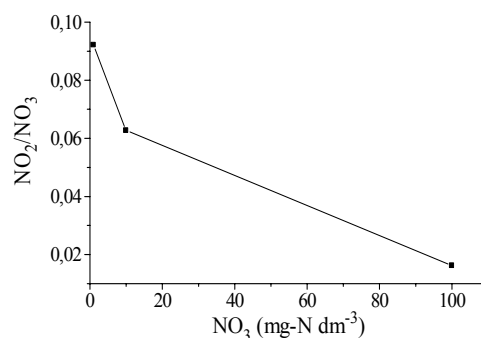


Fig. 2. Dependence of reduction efficacy,  $[\text{NO}_2^-]/[\text{NO}_3^-]$ , of nitrate concentration

Reduction efficacy was the greatest for NO<sub>3</sub><sup>-</sup> concentration of range 1 mg-N dm<sup>-3</sup>, and decreased with increase of concentration to 100 mg-N dm<sup>-3</sup>. In the investigated NO<sub>3</sub><sup>-</sup> concentration range, the overall decrease of reduction was over six times. It is assumed that the cause of this event is the enzymatic nature of nitrate reduction process in the oral cavity, so its kinetic depends on concentration of substrate – NO<sub>3</sub><sup>-</sup> ion. It is obvious that the rate of process decreased due to saturation of nitrate reductase enzyme by substrate, bearing in mind that the bacteria amount in the oral cavity was constant. The obtained result is in agreement with the results of Li and coworkers [22], which deal with investigation of reduction activity of bacteria from the rats tongue surface. This paper shows that the rate of NO<sub>3</sub><sup>-</sup> reduction, in *in vitro* conditions, decreases with increase of NO<sub>3</sub><sup>-</sup> concentration, and average value of Michaelis constant,  $K_m$ , is around 10<sup>-3</sup> mol dm<sup>-3</sup>. Additionally, reduction of nitrate in the human oral

cavity happen in heterogeneous system: aqueous nitrate solution – surface of the oral cavity colonized by bacteria, which can be approximated as a solid reductant with developed specific surface due to pores and papilla on mucosa [24]. In order to be reduced,  $\text{NO}_3^-$  ions have to come to the surface of solid reductant by diffusion, which can be also limiting factor of process efficacy. Over all, supposition is that the process is under mixed, diffusion-kinetic control, although the kinetic control predominates.

Obtained results are of great importance about nitrate toxicity, because its concentration in saliva linearly increases with increase of dietary  $\text{NO}_3^-$  amount [4,7]. Although concentration of nitrite in the oral cavity do not increase linearly with nitrate, danger from dietary nitrate is still great, due to the fact that the significant part of non reduced  $\text{NO}_3^-$  is resorbed in stomach and intestine and returned to the oral cavity [1,4].

Investigation of pH influence on nitrate reduction in the oral cavity was performed at pH 5.5, 6.0, 7.0, 8.0 and 8.5, and  $\text{NO}_3^-$  concentration  $10 \text{ mg-N dm}^{-3}$ . The obtained results are shown in Fig. 3.

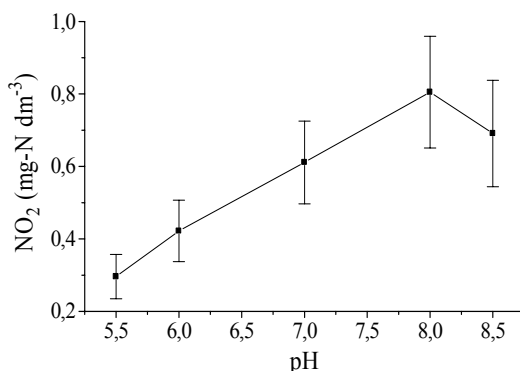


Fig. 3. The influence of pH on nitrate reduction in the human oral cavity. Investigation was performed by incubation of test solutions with pH 5.5, 6.0, 7.0, 8.0 and 8.5, and  $\text{NO}_3^-$  concentration  $10 \text{ mg-N dm}^{-3}$  over a period of 2 min

It could be seen that after 2 min nitrate reduction assay  $\text{NO}_2^-$  concentrations in the mouth samples are very differ in great deal according to particular pH values. Reduction activity of the oral cavity intensively increased with increase of pH from 5.5 to 8.0, and increasing trend is almost linear. In this pH range, nitrite concentration increases even for 3 times. However, with further increase of pH to 8.5, there was decrease of reduction activity. It is obvious that the greatest physiological activity of nitrite-producing bacteria of the oral cavity is at pH 8.0, indicating that they belong to neutrophiles [23]. Shown results are in agreement with many papers [10,17,19], which prove that the pH value of the greatest reduction activity of the oral cavity is between 7 and 8. It is assumed that the difference in results of cited papers is due to variation of saliva pH of different subjects. Nitrate reduction assay enables good control of experimental conditions, giving this study high reproducibility of measurements.

Considering that the greatest activity of nitrite-producing bacteria is at pH 8.0 and that it decreases with pH change, using of mouthwash solutions or other reagents which can lower pH of the oral cavity, is approved. Also, it is important to emphasize that pH value should not be lower than 5.5 to 6.0, because there is a risk of tooth enamel erosion [24].

In Fig. 4, there are results of investigation of temperature influence on nitrate reduction in the oral cavity.

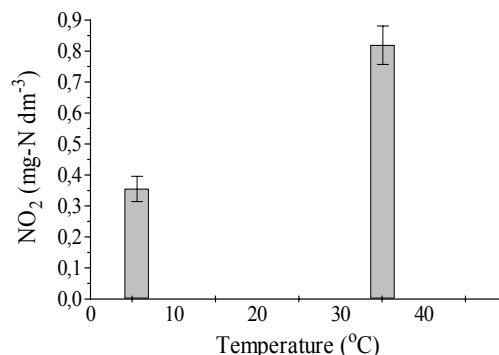


Fig. 4. The influence of temperature on nitrate reduction in the human oral cavity. The tests were performed in January at average temperature  $5.6 \pm 0.3^{\circ}\text{C}$  and in July at  $35.1 \pm 0.7^{\circ}\text{C}$ , by incubation  $10.0 \text{ mg-N dm}^{-3}$  nitrate solution in the mouth of subjects exposed to outdoor conditions

Chart shows that nitrite concentrations in the mouth samples incubated in winter period, on average temperature of about  $5^{\circ}\text{C}$ , was more than twice lower than in the mouth samples incubated in summer period, at about  $35^{\circ}\text{C}$ . Those results are in agreement with investigation of Eisenbrandt *et al.* [6], which also show the same reduction activity dependence of temperature. Considering that the oral cavity is open system, its temperature depends of outdoor conditions, as well as physiological activity of mouth bacteria. The average temperature in summer period in which this investigation was performed is  $35^{\circ}\text{C}$ , which is close to normal body temperature,  $37^{\circ}\text{C}$ , where mesophile bacteria show the greatest activity. With decreasing of temperature, the amount of microorganisms is not changed, although rate of all physiological processes of bacteria decrease, affecting their reduction activity.

#### CONCLUSION

- Nitrate reduction assay is very simple and effective method for investigation of  $\text{NO}_3^-$  reduction in the human oral cavity. Comparing to other *in vivo* methods it enables very precise controlling of start conditions and high reproducibility of measurements. In regard to *in vitro* methods this assay is performed in natural conditions – the oral cavity. Also, method is fully safe for the subjects.
- With increasing levels of nitrate the amounts of nitrite in reduction assay increased. However, reduction ratio,  $\text{NO}_2^-/\text{NO}_3^-$ , significantly decrease with increasing of nitrate concentration, because of saturation of nitrate reductase by substrate.
- pH has specific influence on nitrate reduction in the human oral cavity. The greatest physiological activity of nitrate-reducing bacteria is at pH 8.0 and decreased with increase or decrease of pH.
- Seasonal variations of temperature significantly influence reduction activity of the oral cavity, because it is open system exposed to outdoor conditions.

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## UTICAJ EGZOGENOG NITRATA, pH I TEMPERATURE NA REDUKCIJU NITRATA U USNOJ DUPLJI ČOVEKA

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*Egzogeni nitrati se prevode u nitrite delovanjem bakterija zadnjeg dela površine jezika, što dovodi do povećanja koncentracije nitrita u ustima. U kiseloj sredini želuca nitriti formiraju nitritnu kiselinu, potencijalni nitrozujući agens. Cilj ovog rada je ispitivanje uticaja egzogenog nitrata, pH i temperature na redukciju nitrata u usnoj duplji čoveka. Redukcija nitrata je merena redukcionim esejem, koji se bazira na inkubaciji rastvora nitrata u ustima. Rezultati pokazuju da svi ispitivani faktori imaju značajan uticaj na redukciju nitrata u usnoj duplji. Sa povećanjem koncentracije nitrata količina nitrita u redukcionom esejem raste, međutim, stepen redukcije ( $\text{NO}_2^-/\text{NO}_3^-$ ) opada u ovim uslovima. Uticaj pH na redukciju nitrata je specifičan i u skladu je sa prirodom nitrato-redukujućih bakterija. Najveća redukciona aktivnost bakterija je na pH 8. Oscilacije temperature usne duplje, izazvane spoljašnjim uticajem, takođe utiču na njenu redukcionu aktivnost, koja je dvostruko manja u januaru u odnosu na izmerenu vrednost u julu.*