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Review

Nitric oxide formation in the metabolism of nitrates in the oral cavity

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Nowadays, nitric oxide is recognized as a regulator of important vascular and metabolic functions. Nitric oxide is formed in the endothelium by converting essential amino acid L-arginine to L-citrulline with the participation of constitutional endothelial nitric oxide synthase. In addition to endogenous pathway of formation, dietary nitrate contributes to the nitric oxide generation through the successive stages (NO_3-NO_2-NO) mediated by salivary glands and bacteria of the oral cavity.

Purpose of the research – to demonstrate modern scientific data focused on a role of salivary glands and bacteria in nitrates metabolism and maintenance of nitric oxide homeostasis.

Results of the studies show that in the oral cavity, there are synanthropic facultative anaerobic bacteria which possess nitrate reductase enzymes and reduce nitrates to nitrites. In the acidic environment of the stomach, nitrites undergo non-enzymatic disproportionation, followed by the formation of nitric oxide and other nitrogen compounds which are involved in the regulation of important biological functions. Dietary nitrites and nitrates can be rapidly absorbed from the upper gastrointestinal tract into the systemic bloodstream and serve as effective donors of nitric oxide in a case of physiological hypoxia. This mechanism of nitric oxide formation is called "enterosalivary nitrate-nitrite-nitric oxide pathway". The review presents a cardioprotective effect of regular consumption of dietary nitrate-rich products. Diagnostic markers of nitric oxide metabolism in the oral fluid are shown.

Conclusions. Based on the scientific data, it was concluded that dietary nitrate and bacteria of the oral cavity play a significant role in the synthesis of NO by enzymatic conversion. Regular intake of dietary nitrate-rich products is able to provide a systemic and local vasodilating effect through enterosalivary pathway and conversion of nitrite to nitric oxide.

Key words: nitric oxide, oral cavity, oral bacteria, nitrate, nitrate reductase, nitrite, nitrite reductase, saliva, nutrition, systemic diseases.

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Утворення оксиду азоту при метаболізмі нітратів у порожнині рота

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Нині оксид азоту визнаний регулятором важливих судинних і метаболічних функцій. Оксид азоту утворюється в ендотелії шляхом перетворення незамінної амінокислоти L-аргініну в L-цитрулін за участю конституційного ферменту ендотеліальної синтази оксиду азоту. Крім ендогенного шляху утворення, нітрат в їжі сприяє генерації оксиду азоту через послідовні етапи (NO_3-NO_2-NO), опосередковані слинними залозами та бактеріями порожнини рота.

Мета роботи – подати сучасні наукові дані про роль слинних залоз і бактерій у метаболізмі нітратів, підтримці гомеостазу оксиду азоту.

Результати досліджень свідчать, що в ротовій порожнині є синантропні факультативні анаеробні бактерії, які відновлюють нітрати до нітритів за допомогою нітратредуцирувальних ферментів. У кислому середовищі шлунка нітрити піддаються мимовільному розкладанню з дальшим утворенням оксиду азоту та інших сполук азоту, які беруть участь у регуляції важливих біологічних функцій. Нітрити та нітрати, котрі не піддалися розкладанню, з кишечника всмоктуються в системний кровотік і в умовах фізіологічної гіпоксії можуть утворювати оксид азоту. Цей шлях утворення оксиду азоту називають «ентеросаліварною циркуляцією NO_3». В огляді показано кардіопротекторну дію регулярного вживання харчів із великою кількістю нітратів. Наведені діагностичні маркери метаболізму оксиду азоту в ротовій рідині.

Висновки. На підставі аналізу наукових даних зробили висновок: нітрати, що надходять з їжею, та бактерії порожнини рота відіграють значущу роль в утворенні оксиду азоту ферментативним шляхом. Регулярне споживання харчів із великою кількістю нітратів здатне виявляти системний і локальний вазодилятувальний ефект за допомогою ентеросаліварної циркуляції та перетворення нітритів в оксид азоту.

Ключові слова: оксид азоту, ротова порожнина, оральні бактерії, нітрати, нітратредуктаза, нітрити, нітритредуктаза, слина, харчування, системні хвороби.

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Образование оксида азота при метаболизме нитратов в полости рта

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В настоящее время оксид азота признан регулятором важных сосудистых и метаболических функций. Оксид азота образуется в эндотелии путем преобразования незаменимой аминокислоты L-аргинина в L-цитруллин при участии конституционального фермента эндотелиальной синтазы оксида азота. Помимо эндогенного пути образования, нитрат в пище способствует генерации оксида азота через последовательные этапы (NO_3-NO_2-NO), опосредованные слюнными железами, бактериями полости рта.

Ключевые слова: оксид азота, ротовая полость, оральные бактерии, нитраты, нитратредуктаза, нитриты, нитритредуктаза, слюна, питание, системные болезни.

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Цель работы – представить современные научные данные о роли слюнных желез и бактерий в метаболизме нитратов, поддержании гомеостаза оксида азота.

Результаты исследований свидетельствуют, что в ротовой полости присутствуют синантропные факультативные анаэробные бактерии, которые восстанавливают нитраты до нитритов посредством нитратредуцирующих ферментов. В кислой среде желудка нитриты подвергаются самопроизвольному разложению с последующим образованием оксида азота и других соединений азота, которые участвуют в регуляции важных биологических функций. Нитриты и нитраты, не подвергшиеся разложению, из кишечника всасываются в системный кровоток и в условиях физиологической гипоксии могут образовывать оксид азота. Данный путь образования оксида азота имеет название «энтеросаливарная циркуляция NO_3». В обзоре показано кардиопротекторное действие регулярного употребления продуктов питания с большим количеством нитратов. Приведены диагностические маркеры метаболизма оксида азота в ротовой жидкости.

Выводы. На основании анализа научных данных сделан вывод, что нитраты, поступающие с пищей, и бактерии полости рта играют значительную роль в образовании оксида азота ферментативным путем. Регулярное потребление продуктов питания с большим количеством нитратов способно оказывать системный и локальный вазодилятирующий эффект посредством энтеросаливарной циркуляции и преобразования нитритов в оксид азота.

Nitric oxide (NO) is a simple free-radical gas considered as a signaling molecule with numerous physiological functions in humans. In the human organism, NO production through the endogenous synthesis and exogenous nitrate conversion is occurred by the activation nitrate-nitrite-reducing complex. It had previously been thought that inorganic anions of nitrates (NO_3-) and nitrites (NO_2-) were non-active endproducts of endogenous NO metabolism, which was NO synthases-regulated and derived from classic L-arginine pathway. However, over the last decade, it has been established that NO_3-, NO_2-anions disproportionation *in vivo* is an aspect of additional NO formation as its source in hypoxic conditions.

Irrespective of an origin, circulating NO_3- for further NO generation is actively absorbed in the salivary glands. Then it is excreted into saliva, and oral commensal microorganisms gradually reduce it to NO_2-. This pathway has been regarded as a meaningful metabolic conversion in human organism. Enzymatic reduction of NO_3- to NO_2- by oral microbiota is a new dimension in healthcare and diseases development due to its essential role.

Sources of dietary nitrate intake:

1. Diet:

a) vegetable food products;

b) animal origin food.

2. Drinking water.

3. Medications.

Canned goods and fresh vegetables are the main sources of nitrates for humans (40–80 % of the daily nitrates intake). In bakery products and fruits, nitrates are found in much smaller amounts and dairy products contain approximately 1 % of nitrates (10–100 mg per liter) [1,2]. Thus, ingested in food and water nitrates are further converted to NO_2- in the saliva representing a significant source of NO_2- exposure by endogenous transformation of NO_3- to NO_2-.

Drinking water contains approximately 200 mg/l of nitrates from surface and ground water sources that is much more than in water from artesian wells. Nitrates contaminate groundwater with various chemical fertilizers (NO_3-, ammonium) from fields, with waste products from chemical companies producing these fertilizers. The highest level of nitrates is contained in groundwater that is in well water, containing about 20 mg/l of nitrate. People living in rural areas consume drinking water containing 20–80 mg/l of nitrates [3].

Moreover, nitrates can be found in animal origin food. Fish and meat products contain relatively small amounts of nitrate, 5–25 mg/kg and 2–15 mg/kg, respectively. Cured meats are other considerable sources of NO_2- which deserve the attention. NO_2 – salts are used as food additives, as a means of processed meats preservation for curing and to enhance color and flavor (especially in cured meats). NO_2- content in raw fermented and boiled sausages is about 150 mg/kg and 50–60 mg/kg, respectively [4]. Despite a long history of using, until recently, dietary inorganic nitrates were thought to be harmful for human health being precursors in the endogenous production of nitrosamines known for its highly carcinogenic effect [5–7]. Changes in the methods of meat processing lowered these possible risks and NO_2- remained in use for food technology. From that time, two opposite directions influence consumers' perceptions of dietary NO_3- and NO_2-.

Ideas of nitrates physiological role have evolved since an endogenous metabolic pathway, catalyzing the reduction of nitrate through NO_2- to NO had been discovered. A wide range of experimental and clinical studies has also demonstrated positive results of dietary nitrates intake, in particular with regard to cardiovascular and metabolic health. Nutritional inorganic NO_3- enters the enterosalivary circulation followed by rapid absorption in the upper gastrointestinal tract thereby increasing NO_3- plasma levels. This NO_3- is predominantly excreted into the urine; approximately 25 % is transported into the salivary glands raising salivary content (6–10 mg/l of NO_2- and 15–35 mg/l of NO_3-) [8–13].

Non-dietary sources of nitrates include tobacco products. Some tobacco crops were found to contain up to 500 mg of NO_3- per 100 g of dry matter [14].

Nitrate-reducing bacteria in the oral cavity. Enterosalivary circulation of dietary nitrates

For the global nitrogen cycle, oral bacteria metabolism is essential as they use various redox reactions to metabolize nitrogen for energy transduction, detoxification, or assimilation. The most oxidized state of nitrogen is NO_3- while the most reduced its state is ammonia (NH_3). NO_3- is a very stable molecule and, unlike microorganisms, mammalian cells cannot effectively metabolize this anion requiring organic nitrogen compounds as a source of nitrogen, amino acids and nucleic acids acquired from food for recycled synthesis of new nitrogen compounds [10].

There are synanthropic facultative anaerobic bacteria possessing nitrate reductase enzymes for NO_3- to NO_2- reduction in the oral cavity [15]. In the acidic gastric environment, NO_2- undergo non-enzymatic disproportionation followed by the formation of NO and other nitrogen com-

pounds influencing the vital biological functions regulation. From the upper gastrointestinal tract, dietary NO_2- and NO_3- are quickly absorbed into the systemic bloodstream and serve as powerful NO donors in different forms of hypoxia. This mechanism of NO formation is identified as "enterosalivary nitrate circulation" [16]. Based on the experimental studies, enterosalivary nitrate circulation is supported by the presence of symbiotic bacteria with abilities to reduce NO_3-, mainly *Veillonella* species, *Actinomyces*, *Rothia, Staphylococcus epidermidis* and *Propionibacterium.* According to the study results, the most prevalent among NO_3- reducers, located on the posterior third of the tongue in healthy people, are bacteria such as *Veillonella atypica* (34 %), *Veillonella dispar* (24 %), *Actinomyces odontolyticus* (21 %) which demonstrate the maximum activity under anaerobic conditions [17].

Later, scientists investigated several nitrate-reducing bacteria in the oral cavity using a full metagenomic analysis. They examined isolates of four species, studying that they have substantive nitrate- and nitrite-reduction properties:

1. *Actinomyces odontolyticus* – microorganisms with only nitrate-reductase encoding genes in genomes.

2. *Veillonella dispar* – microorganisms with both nitrate- and nitrite-reductase encoding genes.

3. *Fusobacterium nucleatum* – microorganisms with only genes encoding nitrite-reductase.

4. *Streptococcus mutans* – microorganisms with only nitrite-reductase encoding genes.

The researchers grew strains separately and then four strains in association. The study results showed that effective NO_3- reducers were both *A. odontolyticus* and *V. dispar*, as evidenced by 80 % reduction of medium NO_3-. *S. mutans* and *F. nucleatum,* in contrast, did not show the same nitrate-reducing properties, while hardly detectable NO_2- levels remained in the media. *V. dispar* reduced NO_2- not as actively as *S. mutans* or *F. nucleatu*. Four species in association demonstrated a high capability to reduce both NO_3- and NO_2-, as evidenced by low NO_3- and undetectable amount of NO_2- in the medium [18,19].

Earlier experimental studies reported about an antibactericidal effect of salivary NO_2- on oral pathogens including *Streptococcus mutans, Lactobacillus acidophilus, Porphyromonas gingivalis, Capnocytophaga gingivalis, Fusobacterium nucleatum, Candida albicans* at concentrations from 0.5 to 10 mmol/L and acidic pH. Herewith, the same concentrations of NO_3- at low values of pH did not exhibit an inhibitory effect on any these pathogens growth [20]. The results are at odds with each other, although confirm the presence of NO-dependent antibacterial mechanisms [21], because active NO generation is possible at acidic pH via reaction of chemical denitrification not involving bacteria. Dietary NO_3- and NO_2- in saliva, mostly from vegetables and fruits, might have antibacterial effects against the genus *Desulfovibrio*, which is regarded as an etiologic agent associated with chronic periodontitis [22].

Interesting experimental findings were obtained after anaerobic incubation of saliva containing a bacterial mixture with glucose and NO_3-/NO_2- presence. The values of pH at such conditions were higher suggesting that NO_2- and NO_3- enhancement contributed to acid scavenging, acid fermentation inhibition and promotion of alkali production resulting in lowering the oral fluid acidity [23].

Nitrate and nitrite metabolism in the oral cavity under the influence of bacterial enzymes and chemical reduction

Various bacteria encode specific enzymes, which catalyze transformations of each nitrogen compound. These microbe-induced transformations are vital important as they are crucial in nitrogen bioavailability maintaining. Microbial pathways start with NO_3- reduction, denitrification and reduction to NH_3 [24,25].

Some stages of this cycle are also typical for mammals, and oral cavity microorganisms have an equal importance. A peculiar range of bacterial nitrate reductase enzymes is included in the metabolic cascade "NO_3^-- NO_2^-- NH_3" when NO_2- serves as the only source of nitrogen [26]. Some oral bacteria contain urease yielding alkaline NH_3 products via urea hydrolysis. Hence, bacterial nitrate reductase is not active in this case. The process of NO_3- ammonification is known to be a strictly anaerobic. It occurs only in the depth of bacterial biofilm at a high viscosity of saliva and hyposalivation.

The oral fluid acid-base abnormalities are significant in the pathogenesis of periodontal diseases [27]. In most cases in gingivitis, the oral fluid has a weakly alkaline characteristic due to high content of urea and NH_3. It contributes to supragingival and subgingival calculus deposition. In the oral cavity, bacterial urease system is an alkali-generating mechanism that breaks down urea to NH_3 and carbon dioxide. This reaction partially neutralizes an acid-forming effect caused by glycolytic enzymes. NH_3 is assimilated into variety of nitrogenous compounds used by oral microorganisms in the process of vital activity. Microbial imbalance in the oral cavity pathology may occur with a predominance of urease-positive or glycolytic bacteria resulting in pH fluctuations in oral environment.

Most oral microorganisms are capable of complete NO_3- dissimilatory reduction to ammonium. This reaction is known to be completely anaerobic that is preferable to denitrification at anaerobic conditions. At peak ammonium concentrations in the oral fluid, heterotrophic denitrification by oral facultative anaerobic bacteria using NO_3- to respire organic matter is possible. Denitrification is the process of bacterial reductive respiration using NO_3- or NO_2- to NO under hypoxic conditions [28,29].

The first stage of denitrification involves enzyme nitrate reductase. Activity of nitrate reductase depends on the temperature and pH. A peak of nitrate reductase activity is registered at a temperature of 35–40 °C, further temperature increase causes inactivation of the enzyme. The enzyme is active in weakly acidic and neutral pH of the fasting oral fluid. Enzyme nitrite reductase completes the denitrification reaction promoting NO formation from oral fluid NO_2-. The enzyme is the most active at a temperature of 30–40 °C and weakly alkaline pH, its activity decreases at neutral or acidic pH.

Acidic conditions have been shown to favor non-enzymatic synthesis of NO by such a mechanism of chemical denitrification:

$2\ NO_2^- + 2\ H+ \leftrightarrow 2\ HNO_2$

$2\ HNO_2 \rightarrow N2O_3 + H_2O$

$N_2O_3 \rightarrow NO + NO_2$

The activity of NO synthesis depends on the oral cavity status, inflammatory processes presence, oral fluid acidity and concomitant acid-associated diseases of the upper

digestive tract including chronic gastritis, duodenitis, gastroesophageal reflux disease.

A high-nitrate diet effects

Regular consumption of nitrate-rich food could have cardioprotective effects via enterosalivar circulation and NO_2- conversion to NO. Intake of nitrate-rich vegetables and fruits reduces both blood pressure and risk of adverse cardiovascular events. Some vegetables contain considerable amounts of NO_3- serving as a source of vasoprotective NO [30]. Previously, it has been shown that NO bioavailability was quite important for regulation of ischemic-induced angiogenesis and, in ischemic tissues, inorganic NO_2- could also generate NO [31, 32]. In the course of a high nitrate diet, salivary and plasma NO_3- and NO_2- levels can be significantly increased. However, this effect was no lasting because elevated levels of NO_3- and NO_2- returned to baseline indicators in a week after cessation of high nitrate diet. There are data in the literature that newborn infants, in comparison to adult persons, ingest substantially lower amounts of NO_3- and NO_2- per kilogram of body weight accounting for approximately only 5 % and 0.6 % of the NO_3- and NO_2- intake in adults, irrespectively of whether they are natural breastfeeding, artificial or parenteral feeding [33]. Moreover, although *Veillonella* and *Actinomyces spp*. are present in the oral cavity of infants, their nitrate reductase activity is significantly lower [28]. Herewith, NO_3- and NO_2- are effective in supporting glycoproteins secretion and the gastrointestinal mucosal barrier integrity, suggesting that in the development of infant diseases, such as necrotizing enterocolitis and others, physiological decrease in concentration of these compounds might play an etiological role. Taking into account the importance of NO_3^-- NO_2^--NO axis in adults, infant vulnerability to a hypoxic stress and gastrointestinal tract pathology may also be associated with low activity of nitrate-reducing bacteria.

It has been established that oral bacteria favorably influence the cardiovascular system activity, modulating the level of blood pressure and supporting NO homeostasis through NO_3- reduction [34]. Firstly, it was shown that twice daily use of an oral antiseptic combined with a low-calorie diet for seven days in healthy volunteers may attenuate the dietary nitrates bioactivity as salivary and plasma NO_2- were reduced by ~25 % and ~90 %, respectively [35], but the levels of blood pressure and NO_3- were increased in comparison to baseline. It demonstrated a correlation between blood pressure level and nitrate-reducing bacteria effect. Secondly, excessive use of antiseptic mouthwashes exhibited effect to significantly reduce the circulating NO_3- level suppressing a gastroprotective effect of NO in the stomach. At the same time, salivary NO_2-, converted to NO in the acidic gastric lumen, increased mucosa thickness and improved the stomach blood supply [36].

The most toxic compound among reactive forms of nitrogen oxides is peroxynitrite ($ONOO^-$), which has a regulatory and cytoprotective effect in the physiological conditions. ONOO- is the product of the diffusion-controlled reaction of NO and superoxide radicals (O_2^-). $ONOO^-$ reacting with thiols at the physiological pH value in cells and tissues produces about 1–2 % of S-nitrosothiols (RSNO). There is local intracellular formation of RSNO which is strongly influenced by the bioavailability of thiols, in particular glutathione and cysteine, in acidic pH.

Saliva contains both substrates (NO_2- and thiol groups) needed for S-nitrosothiol formation in the acidic environment of stomach [37]. NO_2^--derived NO could partly be transported through the mucosa in the form of S-nitrosothiols which can function as stable NO carriers, thereby increasing its half-life and allowing for more lasting effects. As it can be seen from the experimental data, antihypertensive effects of oral NO_2- or NO_3- can be potentiated by gastric S-nitrosothiol formation. This fact has significant implications, especially for patients taking proton pump inhibitors as their administration attenuates the antihypertensive effects of these anions [38].

Level of nitrates and nitrites in biofluids as a biomarker of dental pathology

It has been proved that salivary levels of NO_2- and NO_3- were biomarkers for the early diagnosis of drug-induced gingival overgrowth, which is a side effect of various drugs including anticonvulsants, in particular phenytoin [39].

The comparative analysis of saliva and gingival crevicular fluid assessment in healthy subjects and periodontitis patients indicated that levels of NO_3-, NO_2- and NO were much lower in gingival crevicular fluid as compared to saliva. Also, the salivary NO increased in the order of healthy subjects, gingivitis, reaching the maximum in periodontitis patients, whereas healthy individuals presented the highest NO level in gingival crevicular fluid. In the authors' opinion, periodontal tissues destruction might be largely driven by NO, therefore, a greater diagnostic value of saliva compared to gingival crevicular fluid was emphasized [39]. However, gingival crevicular fluid seemed to be more diagnostically useful for gingivitis in comparison with saliva according to other study results, which also suggested that NO_2-, rather than NO_3-, concentration in gingival crevicular fluid could be used as an early marker for diagnosis of periodontal inflammation [40,41].

The analysis of salivary NO concentration and its metabolites in subjects with healthy teeth and in those with cariogenic status of the oral cavity showed that NO level was significantly higher in healthy subjects compared to that found in the group of patients with caries [42]. This situation suggests that increasing NO synthesis could contribute to the decreasing incidence of caries in the population. Conversely, some scientists observed that neither salivary NO concentration nor NO rate correlated with the dental state of a person [43].

Romanenko Ye. G. [44] has shown that children with gingival inflammation had increased salivary content of stable NO metabolites due to a low activity of NO_3^-- NO_2^--reductase complex. At the same time, in a group of children with gastroesophageal reflux, the content of stable NO metabolites was significantly higher than that in the group of healthy children, despite a high activity of both enzymatic and non-enzymatic NO_3- and NO_2- reduction.

An increased salivary level of NO metabolites not only reflects a presence of inflammatory reaction, but also plays a protective role for the mucous membrane against action of hydrogen ions through enhancement of NO-induced mucus generation, vasodilatation and blood supply [36].

Unlike saliva, dental plaque is considered to be a key factor of a cariogenic status. It was shown that microorganisms of dental plaque are essential element of NO_2^--dependent NO synthesis in the human body.

Studying the dental plaque *Streptococcus mutans*, NO levels and plaque pH in children of different ages, scientists found a statistically significant relationship between these indicators and dental caries degree in all age groups. A strong positive significant correlation between dental plaque NO levels and DMFT index, that describes the amount of dental caries in a person and is obtained by calculating the number of decayed (D), missing (M) and filled (F) teeth (T), were revealed. It allows using NO estimation as screening tool to predict the risk of dental caries [45].

A number of authors also studied NO as an inflammatory biomarker in chronic and aggressive periodontitis and revealed a direct positive correlation between the salivary and the serum NO levels [46]. An increase in salivary NO levels was observed in patients with chronic periodontitis. The highest NO levels were detected in smokers, showing an increased oxidative load on the periodontal tissue [20].

Conclusions

1. Thus, dietary nitrate and bacteria of the oral cavity play a significant role in the synthesis of NO by enzymatic conversion. Regular intake of dietary nitrate-rich products is able to provide a systemic and local vasodilating effect through enterosalivary pathway and conversion of NO_2- to NO.

2. Salivary levels of NO_3- and NO_2- could be biomarkers for the early diagnosis of upper gastrointestinal tract diseases.

Prospects for further research. Further studies should be focused on mechanisms of bacterial denitrification in the oral cavity, nitrates absorption and excretion in the salivary glands as well as the development of acidified inorganic nitrate-containing medications for mouth rinsing in oral cavity and upper gastrointestinal tract diseases.

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