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Salivarni dušikov oksid kao biomarker rizika za karijes kod djece

Salivary Nitric Oxide as Biomarker of Caries Risk in Children

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Sažetak
Svrha: Zubni je karijes infektivna bolest uzrokovana bakterijama, a ima veliku incidenciju u državama u razvoju. Važni čimbenici u njegovu nastanku mogli bi biti sastavni dijelovi salivarnog obrambenog sustava, kao na primjer organske i anorganske komponente u slini. Posljednjih godina sve je veće zanimanje za istraživanje utjecaja nitrata i nitrita u zaštiti od oralnih bolesti. Svrha ove studije bila je odrediti odnos između koncentracije NO-a u nestimuliranoj i stimuliranoj slini djece s niskim i visokim rizikom za karijes. **Ispitanici i postupci:** U sklopu studije ispitano je 123 djece s trajnom denticijom (srednja starost 13±0,3). Ovisno o indeksu DMFT-a sudionici su bili podijeljeni u skupinu niskoga karijesnog rizika (51 ispitanik) i visokoga karijesnog rizika (72 ispitanika). Od svakoga su bila uzeta dva uzorka – nestimulirana i stimulirana slina. Nakon toga se metodom Griessove reakcije mjerila koncentracija dušikova oksida te ukupni nitrati i nitriti. **Rezultati.** Rezultati studije pokazuju da je prisutnost dušikova oksida i njegovih metabolita u slini djece s prirodno zdravim zubima znatno viša u odnosu prema skupini visokog rizika, što ističe zaštitnu ulogu NO-a u odnosu prema karijesu. **Zaključak:** Veliko povećanje nitrata i nitrita u stimuliranoj slini kod skupine visokoga karijesnog rizika mogao bi biti odgovor organizma u sprječavanju bakterijskog rasta zbog indukcije sustava NO-a u žlijezdama slinovnicama. Dosadašnji rezultati podupiru tezu o dušikovu oksidu kao modulatoru bakterijske proliferacije i sugeriraju da povećani NO može kod djece pridonijeti nižoj incidenciji karijesa.

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Ključne riječi
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Uvod

Zubni karijes, kao najčešća bolest usne šupljine, infektivni je proces uzrokovan bakterijama i ima sve veću incidenciju u zemljama u razvoju. Istaknimo da se pacijenti u siromašnim državama često ne liječe, što završava gubitkom inficiranog zuba. Moramo reći da se povećava broj djece s visokim karijesnim rizikom, pa je to razlog zašto sve strategije, posebice one usmjerene na djecu, pokušavaju pronaći neki novi biobiljeg koji bi pomogao u preven-

Introduction

Dental caries, the most common disease in oral cavity, is an infectious process, caused by bacteria, with an increasing incidence in developing countries. Patients with dental caries often remain untreated in these countries in particular, which leads to the loss of the infected teeth. There is an increasing number of children with a high caries incidence. That's why all strategies aimed to prevent caries, especially in children, are directed to find the new biociji bolesti. Važni čimbenici u sprječavanju nastanka karijesa mogli bi biti sastavni dijelovi salivarnog obrambenog sustava, tj. organske i anorganske komponente u slini. Veće zanimanje za tu mogućnost potaknule su spoznaje o različitosti i međudjelovanju mnogih zaštitnih tvari u slini. Posljednjih godina raste zanimanje za ulogu nitrata i nitrita u zaštiti protiv oralnih bolesti (1,2).

Ljudska usna šupljina okolina je s neprekinutom opskrbom koncentriranih nitrata, a oni su metabolički produkt dušikova oksida (NO-a). U usnoj šupljini dušikov oksid i nitriti te stabilni metaboliti NO-a potječu ili iz fiziološke redukcije prehrambenih nitrata ili od reakcije L-arginina kataliziranih inducibilnom nitričnom oksidnom sintazom (iNOS-om) enzima izraženom u žlijezdama slinovnicama i epitelnim stanicama izvodnih kanala koji mogu biti stimulirani preko proupalnih podražaja (3-6).

Premda se već nekoliko godina dušikov oksid (NO) i njegovi metabolički produkti - nitrati i nitriti, povezuju sa štetnim utjecajem na ljude, nedavni dokazi upućuju na povoljne antimikrobne učinke NO-a u oralnoj šupljini (7).

Svrha ove studije bila je odrediti kod djece odnos ukupne koncentracije nitrata i nitrita u nestimuliranoj i stimuliranoj slini, u odnosu prema njihovu niskom i visokom karijesnom riziku.

Ispitanici i postupci

Ispitivana skupina sastojala se od 123 djece s trajnom denticijom (srednja dob 13,4±0,3). Svi sudionici bili su dobrog općeg zdravlja, bez drugih zubnih i oralnih bolesti te nisu uzimali nikakve lijekove koji bi mogli utjecati na sastav sline. Protokol studije odobrilo je lokalno Etičko povjerenstvo, a ispitanici i njihovi roditelji dali su suglasnost.

U skladu s kriterijima WHO-a, stomatološki status i razinu karijesa kod svih je ispitanika odredila ista osoba koristeći se indeksom DMFT-a. Ovisno o tome ispitanici su bili podijeljeni u dvije skupine - jednu s DMFT-om 0, to je bila skupina LCR (djeca s niskim karijesnim rizikom –51 sudionik) i druga s DMFT-om 7,7 (djeca s visokim karijesnim rizikom – 72 ispitanika) – to je bila skupina HCR. Slina se skupljala nakon doručka. Od svih se zahtijevalo da se najmanje tri sata prije nego što daju uzorak sline suzdrže od hrane i pića te da se ne koriste osvježivačem daha. U sterilnim epruvetama skupljeno je

markers which could help in prevention of this disease.

The significant factors in caries could be the elements of salivary defense system, i.e. organic and inorganic compounds present in saliva. Deep interest in these aspects grew out of the findings about the diversity and orchestration of the many protective agents in saliva. In the course of the last years there has been a growing interest in the role of nitrates and nitrites in protection against oral diseases (1,2).

Human oral cavity represents the environment with a constant supply of concentrated nitrates, the metabolic products of nitric oxide (NO). In oral cavity NO and nitrite, the stable NO metabolite, originate either from physiological reduction of dietary nitrates or from L-arginine undergoing the reaction catalyzed by inducible nitric oxide synthase (iNOS), the enzyme expressed in salivary glands and duct epithelial cells which may be induced by proinflammatory stimuli (3-6).

Although for many years nitric oxide and its metabolical products, nitrates and nitrites, have been associated with deleterious effects in humans, the recent evidence has suggested the beneficial antimicrobial role of NO in oral cavity (7).

The aim of the present study was to determine the relationship between total nitrate + nitrite concentration in unstimulated and stimulated saliva of children in correspondence to low and high caries risk.

Material and Methods

The study group consisted of 123 children with permanent dentition, with the mean age 13.4±0.3. All examined subjects were in good general health, without other dental and oral diseases and not taking the medications that might affect saliva composition. The study protocol was approved by the local ethical commitee and the subjects and their parents gave their informed consent.

Following the WHO criteria, the state of dentition and the level of caries in all individuals were determined by the same person, using DFMT index. According to DFMT index, the subjects were divided into two examined groups: one group with DFMT - 0 (children with low caries risk – 51 subjects) – LCR group, and, the other with mean DFMT – 7.7 (children with high caries risk – 72 subjects) – HCR group.

Saliva was collected after breakfast. The subjects were instructed to refrain from eating, drinkbilo 2,0 do 2,5 ml sline. Od svakog ispitanika uzeta su dva uzorka – nestimulirana i stimulirana slina. Uzorci nestimulirane sline bili su iz sline u usnoj šupljini, a uzorci stimulirane sline uzeti su nakon što je ispitanik pet minuta žvakao sterilni parafin, što je povećalo sekreciju.

Uzorci sline najprije su filtrirani kako bi se uklonile epitelne stanice i ostale čestice. Svaki filtrat bio je zamrznut jedan sat prije nastavka obrade. Koncentracija NO-a te nitrata i nitrita (NO_2+NO_3) bila je izmjerena metodom Griessove reakcije (8). Nakon deproteinizacije ocijenjeno je stvaranje dušikova oksida i to mjerenjem koncentracije nitrita. Premda se nitrati u kadmijskoj redukciji transformiraju u nitrite, oni su mjereni neposredno spektrofotometrijski na 543 nm koristeći se kolorimetrijskom metodom prema Griessu (Griess reagens: 1,5 % sulfanilamide u 1 M HCl uz 0,15 % N- (1-naphthyl)ethylendiamine dihydrokloride u destiliranoj vodi). Izračunati su srednja vrijednost i standardna devijacija te su podaci analizirani jednovarijantnom analizom varijance (SPSS verzija 10,0).

Rezultati

Slike 1. i 2. prikazuju koncentracije NO_2+NO_3 kod nestimulirane (UWS) i stimulirane (SWS) sline djece s niskim i visokim karijesnim rizikom te upućuju na veliku razliku između UWS-a i SWS-a.

Razine UWS-a u skupini LCR znatno su više (252,22 ± 47,9 μmol/L; p<0,001) u usporedbi s UWS-om u skupini HCR (116,45 ± 32,4). Stimulirana ukupna slina (SWS) pokazuje različiti raspored sa znatno višim vrijednostima u skupini HCR (211,76 ± 77,7; p<0,001) u usporedbi sa skupinom LCR (100,94 ± 28,6) (Slika 3.)

Rasprava

Fiziološka funkcija aktivne sekrecije salivarnih nitrata još nije potpuno razjašnjena. Nitrati nastaju oksidacijom dušikova oksida, jednim od najjačih antibakterijskih spojeva, te djeluju na inhibiciju bakterijskog rasta pojačanjem makrofazima inducirane citotoksičnosti. Salivarni nitrat reducira se u nitrite i dušikov oksid uz pomoć mikroorganizama u ljudima i životinjama (9-11). Kod kiseloga pH-a

ing and using breath fresheners for a minimum of 3 hours before saliva collection. Approximately 2.0-2.5 ml of saliva was collected by instructioning the subjects to spit into sterile tubes. Two saliva samples were taken from each subject – unstimulated and stimulated. Unstimulated whole saliva samples were taken by collection of saliva present in oral cavity and stimulated one after the subjects had chewed sterile paraffin for 5 minutes, which had led to abundant saliva secretion.

Saliva samples were first filtrated to remove epithelial cells and other particles. Each saliva filtrate was frozen within 1 hour of collection until further processing. Nitric oxide concentration was measured as total nitrates and nitrites (NO_2+NO_3) by the Griess reaction method (8). After deproteinization the production of NO was evaluated by measuring nitrite concentrations. However, nitrates were previously transformed into nitrites by cadmium reduction. Nitrites were assayed directly spectrophotometrically at 543 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl)ethylendiamine dihydrochloride in distilled water). Means and standard deviations were calculated and data were analyzed by univariate analysis of variance SPSS version 10.0.

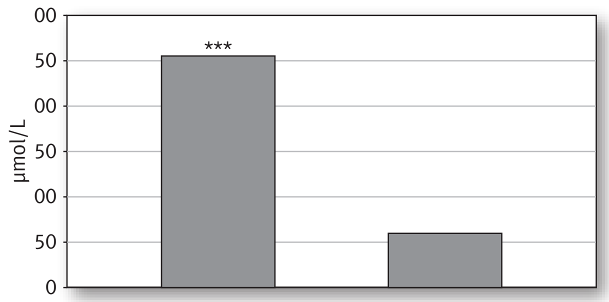
Results

Figures 1 and 2 show NO_2+NO_3 concentrations in unstimulated (UWS) and stimulated whole saliva (SWS) of the children with low and high caries risk, exerting the significant differences between UWS and SWS.

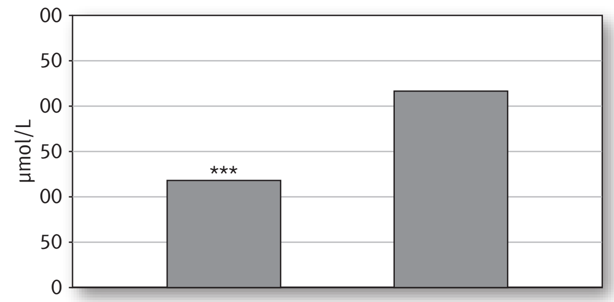
The levels in UWS of LCR group is significantly higher (252.22 ± 47.9 μmol/L; p<0.001) compared to UWS of HCR group (116.45 ± 32.4). Stimulated whole saliva (SWS) shows a different pattern with significantly higher values in HCR (211.76 ± 77.7; p<0.001) group compared to LCR one (100.94 ± 28.6) (Figure 3.)

Discussion

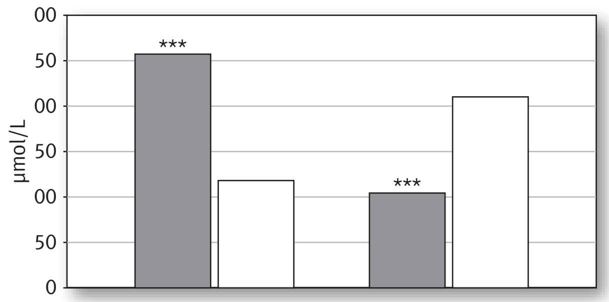
The physiological function of active salivary nitrate excretion is as yet unknown. Nitrate arises from oxidation of nitric oxide, one of the most powerful antibacterial compounds, acting through inhibition of bacterial growth or through enhancement of macrophage-induced cytotoxicity. Salivary nitrate is reduced to nitrite and nitrous oxide by oral microorganisms in humans and animals (9-11). At



Slika 1. NO_2+NO_3 koncentracije u slini kod skupine niskog karijesnog rizika (LCR-a); ***p<0,001 prema stimuliranoj slini
Figure 1 NO_2+NO_3 concentrations in saliva of low caries risk (LCR) group; ***p<0.001 vs stimulated saliva



Slika 2. NO_2+NO_3 koncentracije u slini kod skupine visokog karijesnog rizika (HCR-a); ***p<0,001 prema stimuliranoj slini
Figure 2 NO_2+NO_3 concentrations in saliva of high caries risk (HCR) group; ***p<0.001 vs stimulated saliva



Slika 3. NO_2+NO_3 koncentracije u slini testiranih ispitanika; ***p<0,001 prema većem riziku
Figure 3 NO_2+NO_3 concentrations in saliva of tested subjects; ***p<0.001 vs high risk

nitriti imaju antibakterijska svojstva, posebice kad je riječ o visoko karioznim sojevima bakterija (12). Dušikov oksid lako prolazi kroz staničnu membranu i uzrokuje oštećenja preko nekoliko mehanizama - kao inhibicija DNK sintetaze sa željeznim sadržajem, reakcija sa željezno-sumpornim centrom u mitohondrijskom respiratornom lancu enzima i kombinacija sa superoksidima u stvaranju visoko reaktivnog hidroksil-radikala (13-15). Zbog toga bi se moglo očekivati da će incidencija karijesa kod osoba s visokim koncentracijama NO-a biti niska.

Rezultati studije pokazuju da je količina NO-a i njegovih metabolita u stimuliranoj slini djece s prirodno zdravim zubima znatno viša u usporedbi sa skupinom visokoga karijesnog rizika, što pokazuje zaštitnu ulogu NO-a u odnosu prema karijesu.

Veliko povećanje nitrata i nitrita u stimuliranoj slini skupine visokoga karijesnog rizika mogao bi biti odgovor domaćina na bakterijski rast. Podaci iz literature za indukciju iNOS-a preko produkata mikroorganizama podupiru indukciju iNOS-a u tim uvjetima, što olakšava konverziju L-arginina u dušikov oksid. Jednom stvoren, NO se brzo pretvara u nitrate i nitrite (16).

Ova studija pokazuje da subjekti UWS-a s prirodno zdravim zubima imaju znatno više koncentracije dušikova oksida u usporedbi s ispitanicima iz

acid pH nitrite has been shown to be antibacterial, particularly against highly cariogenic species (12). NO can easily penetrate the cell membrane and induce microbial damage through several mechanisms, such as inhibition of iron-containing DNA synthases, reaction with iron-sulphur center of mitochondrial respiratory chain enzymes and combination with superoxide to highly reactive hydroxyl radical (13-15). Consequently, it could be considered that incidence might be low in subjects with high NO levels.

The results of this study show that the presence of NO and its metabolites in stimulated saliva of children with natural healthy teeth is significantly higher compared to high risk group, suggesting the protective role of NO in relation to caries.

The highly significant increase of nitrates and nitrites in stimulated saliva of high caries risk group could be the host defense response opposing bacterial growth. The literature data of iNOS induction by microorganism products support the induction of iNOS in these conditions, which promotes the conversion of L-arginine into NO. Once formed, NO is rapidly converted to nitrates and nitrites (16).

The present study shows that in UWS of subjects with naturally healthy teeth the levels of NO are significantly higher compared to those with HCR. High skupine HCR-a. Visoka incidencija karijesa povezana je s povećanjem NO-a kod skupine UWS-a. Obrnuti omjer između LCR i HCR koncentracije NO-a u stimuliranoj slini testiranih ispitanika zahtijeva indukciju salivarnog iNOS-a u tim uvjetima. Dobiveni rezultati podupiru ulogu NO-a kao modulatora bakterijske proliferacije i sugeriraju da povećanje dušikova oksida može pridonijeti manjoj incidenciji karijesa kod djece. To je u skladu s rezultatima Carossa i suradnika – oni su predložili dušikov oksid kao dio obrane u bakterijskoj proliferaciji u zubnom plaku (17).

Ako uzmemo u obzir rezultate Silva-Mendeza i njegovih kolega koji navode mogućnost da nitriti iz sline imaju snažan utjecaj na rast i preživljavanje kariogenih bakterija te dobro poznatu činjenicu da manjak sline pridonosi zubnom karijesu, smatramo da bi količina dušikova oksida u slini mogla biti važan čimbenik u obrani domaćina od mikroorganizama koji potiču tu bolest (18). Zubni karijes poseban je problem kod djece te bi se mogla postaviti hipoteza da je veći unos nitrata u toj skupini važan u suprimiranju bakterijskog rasta i zato štiti zube od karijesa. Ako se uzme u obzir nepravilna uporaba prehrambenih nitrata, potrebno je istaknuti kako je dokazano da se njihove koncentracije u plazmi ne mijenjaju prije nitratnog obroka i poslije njega (19). Na osnovi pretpostavke da nitrati u hrani loše djeluju na ljudsko zdravlje donedavno ih se smatralo vrlo štetnima, ali epidemiološke studije nisu uspjele to potvrditi. Dokazano je da se prehrambeni nitrati podvrgavaju enterosalivarnoj cirkulaciji - recirkuliraju se u krv, koncentriraju u žlijezdama slinovnicama, izlučuju sa slinom i reduciraju u nitrite uz pomoć fakultativnih gram-pozitivnih anaeroba (*Staphylococcus sciuri* i *S. intermedius*) na jeziku. Salivarni nitriti gutanjem se prenose u «kiseli» želudac gdje se reduciraju u velike količine dušikova oksida i ostalih oksida dušika te vjerojatno pridonose stvaranju sistemskih S-dušikovih soli i ulja. Premda su prehrambeni nitrati koji određuju produkciju skupine dušikovih oksida u želucu sve važniji u učinkovitoj obrani od gastrointerstinalnih patogena, oni su i modulator palete aktivnosti i možda čak promotori gasrtointerstinalne pokretljivosti i mikrocirkulacije (20).

Ova studija jedna je od rijetkih u kojoj je obrađen problem sline kod djece s trajnom denticijom te se došlo do podataka koji bi mogli znatno utjecati na spoznaje o važnosti prehrane u redukciji incidencije karijesa u djetinjstvu.

incidence of caries is associated with the increase of NO in UWS. The opposite ratio between LCR and HCR stimulated whole saliva NO of tested subject suggests the induction of salivary iNOS in these conditions. The obtained results support the role of NO as modulator of bacterial proliferation and suggest that increased NO production might contribute to lower caries incidence in children. This is in accordance with the results of Carossa et al. (17), who suggested the role of NO in the defense against bacterial proliferation in dental plaque.

Considering the results of Silva-Mendez et al. (18), which demonstrate the possibility that nitrite in saliva has a dramatic effect on cariogenic bacteria growth and survival, and the well known fact that the impairment of saliva excretion promotes dental caries, we believe that the amount of NO in saliva could be an important factor of host defense mechanisms against caries-producing microorganisms. Dental caries is a particular problem in children, so it could be hypothesized that increased nitrate intake in this group may be important in supressing the growth of bacteria and thereby protecting the teeth against caries. Taking into account the misuse of dietary nitrates, it is neccessary to mention that it has been proved that plasma nitrite concentrations pre and post nitrate meal were unmodified (19). Based on the premise that dietary nitrate is detrimental to human health, until recently nitrate has been perceived as a purely harmful dietary component, but epidemiological studies have failed to substantiate this. It has been shown that dietary nitrate undergoes enterosalivary circulation. It is recirculated in the blood, concentrated by the salivary glands, secreted in the saliva and reduced to nitrite by facultative Gram-positive anaerobes (*Staphylococcus sciuri* and *S. intermedius*) on the tongue. Salivary nitrite is swallowed into the acidic stomach where it is reduced to large quantities of NO and other oxides of N and, conceivably, also contributes to the formation of systemic S-nitrosothiols. Thus, nitrate in the diet, which determines reactive nitrogen oxide species production in the stomach, is emerging as an effective host defence against gastrointestinal pathogens, as a modulator of platelet activity and possibly even of gastrointestinal motility and microcirculation (20).

This study is one of few studies that investigate this problem in the saliva of children with permanent dentition, obtaining the results with the possible implications to dietary influence on caries incidence reduction in childhood.

Zahvala

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Abstract

Purpose: Dental caries is an infectious process, caused by bacteria, with an increasing incidence in developing countries. The significant factors in caries could be the elements of salivary defense system, i.e. organic and inorganic compounds present in saliva. The last years there has been a growing interest in the role of nitrates and nitrites in protection against oral diseases. The aim of the present study was to determine the relationship between NO concentration in unstimulated and stimulated saliva of children with low and high caries risk. **Material and Methods:** The study group consisted of 123 children with permanent dentition (the mean age 13.4±0.3). According to DFMT index, the subjects were divided into low (51 subjects) and high caries risk group (72 subjects). Two saliva samples were taken from each subject – unstimulated and stimulated one. Nitric oxide concentration was measured as total nitrates and nitrites by the Griess reaction method. **Results:** The results of this study show that the presence of NO and its metabolites in saliva of children with natural healthy teeth is significantly higher compared to high risk group, suggesting the protective role of NO in relation to caries. **Conclusions:** The highly significant increase of nitrates and nitrites in stimulated saliva of high caries risk group could be the host defense response opposing bacterial growth, due to induction of iNOS in salivary glands. The obtained results support the role of NO as modulator of bacterial proliferation and suggest that increased NO production might contribute to lower caries incidence in children.

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Key words
Dental Caries; Caries Detector; Biological Markers

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