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Research Article

STUDY OF NITRITES IN THE SALIVA OF DIVERS LIKE A PREVENTIVE FACTOR OF DECOMPRESSION

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A B S T R A C T

Nitrates are a potent regulator of blood flow and vasodilatation via its metabolite nitric oxide. The aim of the study is to investigate nitrite levels in saliva of divers following intake of nitrate-poor food and their effect on the degree of gas bubbles in the bloodstream. Eighteen divers took part in the study, in which saliva was taken twice through 3 days to examine nitrite. In the period between the two studies, divers follow a strict non-nitrate diet. After each survey, the divers conduct a dive in hyperbaric chamber at 39 meters for 25 minutes, after which the Doppler monitoring is performed for evaluation the gas bubbles in the venous circulation. The results show that for divers with reduced levels of NO higher gas bubble levels are recorded, which supports our hypothesis that feeding with the presence of NO donors can have a positive effect by reducing decompression risk.

INTRODUCTION

Nitrate (NO_3^-) seems to be a potent regulator of blood flow and vasodilatation via its metabolite nitric oxide (NO) (also known as nitrogen monoxide), and has a greater relative affinity for areas of the body with poor oxygenation as the conversion of nitrite (NO_2^-, a metabolite of nitrate) to nitric oxide is undergone by deoxygenated blood. During the last 3-4 decades, many aspects of endogenous and exogenous nitric oxide synthesis have been revealed and the existence of the entero-salivary circulation has overturned the opinion of scientists about the role and importance of nitrate for the body.

International estimates of nitrate intakes from food are 31–185 mg/d in Europe and ≈ 40–100 mg/d in the United States [6, 10]. In people on a typical western diet, vegetables are the main dietary source of nitrate, account for 60–80% of the daily nitrate intake (35-44 mg/person per day for a 60-kg human) and 10-15% come through the water [7, 17]. Once ingested, nitrate is rapidly absorbed from the gastrointestinal tract and mixes with endogenously synthesized nitrate, which mainly comes from the L-arginine-NO pathway [8]. Up to 25% of plasma nitrate is actively taken up by the salivary glands and secreted with saliva [15]; the resulting salivary nitrate concentrations can be at least 10 times higher than the concentrations in plasma. Salivary nitrate is reduced to nitrite by commensal bacteria in the oral cavity. In the acidic environment of the stomach, salivary nitrite is reduced to NO and other reactive nitrogen intermediates (RNIs) [9].

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Nitric oxide is an important signal molecule that has a vasodilatory effect, affects endothelial and platelet function, vessel perfusion, promotes immune protection, is a transmitter in brain processes, he has a protective role over the mucous membrane of the gastrointestinal tract. For quantitative testing and assessment of nitric oxide production, the stable anions nitrate (NO_3^-) and nitrite (NO_2^-) obtained from the reaction of NO with superoxide are tested. Under conditions of reduced exogenous NO intake, normal function (no inflammatory reaction) and urinary excretion, they reflect the total production of NO from the body.

There are two ways to synthesize nitric oxide - endogenous and exogenous. In the endogenous pathway it is produced by L-arginine and molecular oxygen using three isoforms of NADPH-dependent enzyme - nitric oxide synthases (NOS) [5]. Two NOS isoforms are Ca^{2+} dependent enzymes constitutively expressed in neurons and endothelial cells (nNOS and eNOS) and produce NO in response to physiological stimulus. The third isoform of the NOS, called inducible (iNOS), is Ca^{2+} independent and requires cytokines or a microbial stimulus such as bacterial lipopolysaccharide to induce secretion. Excessive production of reactive nitrogen species is called nitrosative stress.

The second pathway is an exogenous and non-enzymatic process and depends on the intake of nitrates with food and their modification in nitrite in the saliva and stomach. Food-absorbed nitrates, after absorption, reach the salivary glands where they are secreted and partially reduced to nitrite from the oral microflora [4]. They themselves can be reduced to nitric oxide in the acid stomach contents, as well as in the

mouth at low pH. Some oral bacteria (Streptococcus salivarius, S. mitis and S. bovis) can contribute to the production of NO in human saliva as its formation occurs at neutral pH. This entero-salivary circulation has an important role in supplying the body with nitric oxide. The physiological values of nitrite are in the range of 20-200 µmol/l, which maintains the homeostasis balance in the oral cavity to suppress acid-producing and pathogenic microorganisms [12, 18]. Most of the nitrite in the saliva, however, does not convert to NO and is absorbed into the bloodstream. Plasma levels of nitrite are strongly dependent on the amount of salivary nitrate and its reduction. Besides the enzymatic and non-enzymatic pathways of nitric oxide synthesis, a number of molecules and enzymes such as hemoglobin, myoglobin, neuroglobin, xanthine oxido-reductase, carbonic anhydrase and enzymes in mitochondria play a role as nitrite reductases in the body.

On the other hand, SCUBA diving is characterized by several environmental stressors during dives with compressed air such as hyperoxia, formation of intravascular nitrogen bubbles, exercise, and psychological stress that may contribute to an oxidative stress situation (11). The volume of gas in bubbles is proportional to the risk of DCS (16), and the number of bubbles detected by ultrasound in the pulmonary artery is proportional to the number of vascular bubbles in the periphery (1). Prevention of bubble formation is a central goal in standard decompression procedures. Furthermore, nitric oxide may be involved in this protection; blocking the production of NO increases bubble formation [2].

Our hypothesis is that the lowering of nitrate levels, which leads to a decrease in nitric oxide production in tissues, could increase the decompression risk of divers.

Purpose

The aim of the study is to investigate the levels of nitrite in the saliva of divers following intake of nitrate-poor food and their effect on the degree of gas bubbles in the bloodstream.

MATERIAL AND METHODS

The study included 17 men and 1 woman, military divers, between 28 and 43 years of age, height 1.79 ± 0.11 meters, weight 84.2 ± 9.3 kg, and body mass index 24.9 ± 2.1 $kg.m^{-2}$. All participants have normal dental status, thus eliminating the presence of a local inflammatory process in the oral cavity.
They conduct two dives in the hyperbaric chamber, 3 days apart, to 39 meters for 25 minutes, with a total decompression time of 35 minutes. We have shown previously that this protocol produced a significant amount of venous bubbles. In the period between the two dives, divers follow a strict non-nitrate diet, the conduct of which is thoroughly instructed. Individuals have been instructed not to use toning drinks and cigarettes and have gone for 1 hour since the last tooth brushing.

Before each dive, saliva and blood are taken for examination. In serum, we investigate routine biochemical parameters, including CRP, to rule out systemic inflammation that can generate NO. To quantify the nitrite we use saliva collected on the principle of passive separation. The saliva was examined with a semi-quantitative test (ROSA test) and a Gray Colorimetric Test. This method is based on the conversion of sulphanilic acid from nitrite into acidic conditions into a diazonium salt that easily binds to N- (1-Naphthyl) ethylenediamine to form a strongly colored azo dye. Its absorption can be measured photometrically at 548 nm. The method was automated and adapted for the *Olympus AU 400* biochemical analyzer.

After each dive be performed Doppler monitoring by using a bi-directional Doppler (*BIDOP ES 100-V3 – HADECO,* Japan) with 2MHz transducer. Bubbles are detected in the precordial area with the usage of the Kisman-Masurel code. Monitoring was performed at 30, 60 and 90 min after surfacing and we observed for the maximum bubble grades - MBG (bubble peak). The bubble grade was subsequently used to calculate the Kisman integrated severity score (KISS) [3]. The KISS was assumed to be a meaningful linearised measure of post-decompression intravascular bubble activity status that may be treated statistically.

RESULTS

The measured levels of nitrite in the saliva and the values of the recorded gas bubbles of the divers are presented in Table 1. The values of nitrite and KISS obtained in the initial study are indicated as: saliva1 and KISS1, and those following the diet, respectively, with saliva2 and KISS2.

Table 1 Results for saliva and KISS

№	saliva1 µmol/l	saliva2 µmol/l	KISS 1	KISS 2
1	25	1.1	27.3	31.98
2	300	130	7.02	9.75
3	250	29	17.16	27.3
4	200	143	9.75	12.4
5	400	323	7.02	9.75
6	150	60	16.77	17.16
7	300	213	6.63	7.02
8	150	80	27.3	27.3
9	25	0	16.77	34.71
10	150	95	9.75	9.75
11	100	65	12.48	16.77
12	30	1.5	31.98	34.71
13	210	85	12.48	17.16
14	30	0.8	17.16	31.98
15	135	54	9.75	12.48
16	180	72	16.77	17.16
17	175	70	9.75	9.75
18	205	137	12.48	27.3

Our results showed a significant reduction in saliva nitrite values after observing a three-day non-nitrate diet and increase the values of KISS (Tables 2).

Table 2 Statistics for saliva and KISS

	Mean	Std. Deviation	Std. Error Mean
saliva1	169,7222	112,17728	26,44044
saliva2	87,3833	81,49832	19,20934
KISS1	14,9067	7,42093	1,74913
KISS2	19,6906	9,74868	2,29779

After the results were processed through the statistical program IBM SPSS Statistics - version 24.0, clear interrelations were established. There was a clear correlation between nitrite and KISS values before and after the diet (Tables 3).

Table 3 Correlations for saliva and KISS

Pairs	N	Correlation	Sig.
saliva1 & saliva2	18	,887	,000
KISS 1 & KISS 2	18	,813	,000

In the study of the differences in pairs, there was a significant positive difference between the nitrite values ($r = 6.366$ at $p < 0.001$). The differences in KISS values are negative and also statistically significant ($r = -3.563$ at $p \leq 0.002$).

DISCUSSION

In SCUBA diving, decreases in intravascular volume and cardiac preload have been reported commonly after diving. This is concomitant with a moderate increase in vascular resistance and may be the result of an inactivation of NO, probably through oxidative stress. The mechanisms responsible for the reduction in bubble formation by NO are not clear.

The present study demonstrates that in a state of reduced exogenous nitrite intake leads to an increase in the production of gas bubbles in the venous bloodstream. This effect is particularly pronounced in divers, where the endogenous synthesis of nitrates is less pronounced.

Salivary nitrate/nitrite concentration can be used as a biomarker of human exposure to nitrate. Nitrate, from green leafy vegetables, is involved in a plethora of physiological mechanisms. Although most of the biological effects of nitrate have been attributed to NO, it is clear that a complex network of chemical reactions culminates in the production of higher nitrogen oxides, some with the capability to modify both endogenous and exogenous macromolecules [13, 14] To maintain good oral homeostasis, nitrite levels in the saliva should be in the range of 130-220 μmol/l. In our study in four of the divers (numbers 1, 9, 12 and 14), comparatively lower baseline levels of nitrite in saliva were found, with their values after a three-day diet drastically reduced. It was with these divers that also the higher values of the bubbles were registered. In three divers (№ 8, 10 and 17), although there was a reduction in the nitrite level after the diet, in both studies, was measured the same degree of bubbles. The results showed a marked decrease in the level of nitrite in the saliva and, to a lesser extent, but significant increase in the amount of bubbles after a diet.

CONCLUSION

Our study showed that divers with reduced levels of NO have higher gas bubble levels, which supports our initial hypothesis that eating with the presence of NO donors can have a positive effect by reducing decompression risk and improving tolerance to aerobic and anaerobic loads.

The present results clearly demonstrated that limitations on exogenous nitrite may result in an increase in the amount of gas bubbles in the blood, which undoubtedly increases the decompression risk of divers. These results have considerable implications for the development of practical procedures for the reduction of bubble formation.

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