ORIGINAL RESEARCH

NO Swish, No Caries - Nitric Oxide containing Salt for control of Cariogenic Microbes

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Abstract:

Introduction: Oral Nitric oxide (NO) is a colorless, water insoluble gas. At low concentrations it has important role in physiological functions. NO being a highly reactive radical, participates in the nonspecific natural defense mechanisms of the oral cavity to prevent bacteria from overgrowing. It also helps to improve vascular supply.

Aim: To study the effects of salt containing nitric oxide (Tri sodium mono nitrogen) against caries producing microorganisms.

Materials and Methods: Trisodium mono nitrogen salt was estimated for nitric oxide content. Common salt & Tri sodium mono nitrogen salt solution was tested for antibacterial efficacy by MIC and MBC & anti biofilm activity was determined by Time kill assay.

Results: The Nitrite Content in the sample is found to be 8.32 μM / 2mg and the NO_2 inhibition percentage of the sample is 44.4% (for 100μg/ml). Antibacterial effects of the test salt showed MIC of 50% against S. mutans and 60% against L. acidophillus. The time kill effect against biofilm organisms was 25 minutes.

Conclusion: The test salt (Tri sodium mono nitrogen) is able to release substantial quantities of nitric oxide, and has antibacterial efficacy against cariogenic pathogens, thus proving to be used as a potential mouth wash.

Keywords: Nitrous oxide, caries, anti cariogenic.

Introduction

Dental caries is defined as a "biofilm-mediated, sugar-driven, multifactorial, dynamic disease that results in the phasic demineralization and remineralization of dental hard tissues."[1] Mutans Streptococci (S. mutans) have been implicated in the pathogenesis of this biofilm based disease and they are considered to be the initial colonizers and were found in higher proportions and incidence in carious lesions than sound enamel. Another microorganism found in deep dentinal cavities is the Lactobacillus acidophilus (L. acidophilus); they are isolated from advanced lesions. According to a study conducted by Samaranayake, there are more than 1×10^6 mL^{-1} S. mutans and/or 1×10^5 mL^{-1} L. acidophilus in the saliva of people with high caries activity. On the other hand, less than 1×10^5 mL^{-1} S. mutans and/or 1×10^4 mL^{-1} L. acidophilus. were detected in the saliva of people with low caries activity.[2]

The functions of saliva are: Protection of the oral and perioral tissues, lubrication, dilution of sugars after food and drink intake, antimicrobial and cleansing activity, degrading some bacterial cell walls and inhibiting growth, buffering (neutralizing) acid production and controlling plaque pH with bicarbonate, remineralization of enamel with calcium and phosphates and tissue repair; apart from facilitating eating and speech, food preparation, enhancing chewing, the clearing of food residues and swallowing, digestion, food breakdown with enzymes, enhancing taste, enabling speech by lubricating the moving oral tissues.[3]

One of the salivary biomarkers is nitric oxide (NO) which is synthesized either chemically (by dietary nitrate metabolism) or enzymatically.[4] Nitric oxide (NO) is a free radical created from an extensively diverse group of cells and tissues in the human body. Remarkable differences have been detected in the levels and effects of NO in each oral cavity tissue.[5] NO at low concentrations plays role in important physiologic functions.[6] The nitrate and nitrites of saliva help in protecting against oral and gastrointestinal disease, which led us to consider the possible relevance of nitric oxide and its role in dental caries. Nitrate-rich fruits and vegetables, such as beetroot, spinach, lettuce, chervil, radish, and celery are natural and low-cost ways to contribute to health.[7]

Typically, saliva contains 1,500 micromoles nitrate (NO^3 -) and 100 micromoles nitrite (NO^2 -).[8] Consequently, salivary nitrate levels are 10 - 20 times higher than those levels found in plasma. In the human body, nitrate is a neutral substance and no enzyme exists to convert it. In the oral cavity, salivary nitrate is reduced to nitrite in contact with anaerobic bacteria present in the posterior regions of the tongue by the action of nitrate reductase enzyme during anaerobic respiration. Conventional alcohol based mouthwashes destroy favorable microbes along with pathogens depriving us of the natural NO production. The acidic secretion of dental plaque bacteria (eg. S. mutans, Lactobacilli, and Actinomyces) in a decaying environment leads to the acidification of nitrite and the formation of nitrous acid. Nitrous acid is an unstable acid that spontaneously decomposes and produces a combination of nitrogen oxides, especially nitric oxide (NO), which has bactericidal properties and inhibits and/or destroys a wide range of microorganisms.[9] The salivary glands and oral bacteria play an important role in maintaining NO homeostasis. In addition, it has been found that increasing NO concentration can play a defensive role against caries.[10]

Tri sodium mono nitrogen, a salt developed by Vacsons-BNT in association with Defence Research and Development organization (DRDO), claims to release NO and also have antimicrobial, anti-cancer properties. So, we wanted to test the claims of the salt for NO generation, explore its antimicrobial effects on the dual species biofilm with the organisms implicated in dental caries.

Aim:

Estimate the NO generation of the salt and study the effects of salt containing nitric oxide (Tri sodium mono nitrogen) against a dual species cariogenic biofilm containing S. mutans & L. acidophilus using the time kill assay, and compared it with sea salt solution as control.

Materials and Methods:

Nitrite Estimation

Reagents:

Sodium Nitrite Standard

Ammonium Chloride - 0.7M [pH 8.5]

Spongy Cadmium

Greiss Reagent [1% - Sulphanilamide, 0.1% - N-(1-napthyl) ethylene diamine hydrochloride in 10% orthophosphoric acid]

Standard Graph:

Sodium Nitrite is used as standard, 1mM of Stock

Solution is prepared by adding 690µg of Sodium Nitrite in 10ml of Distilled water. Then appropriate volume is transferred into working standard solutions.

Nitrite Estimation for Standard:

2ml of Working Standard Solution is taken in a test Tube and 0.1 ml of Greiss Reagent is added and incubated for 10 min in dim light at 20±5°C.

The absorbance is measured at 540nm.

Nitrite estimation for Sample (Tri sodium mono nitrogen):

25ml of known concentration of Sample is taken along with 5ml of Ammonium Chloride; 1g of Spongy Cadmium is added and incubated for 90 minutes in an orbital shaker. This reaction reduces all Nitrates into Nitrites while nitrite remains the same.

2ml of the reduced sample is taken in a test tube and 0.1 ml of Greiss Reagent is added and incubated for 10 min in dim light at 20±5°C.

The absorbance is measured at 540nm.

Confirmatory test for nitric oxide release in the test sample

Scavenging of Nitric Oxide Radicals

Principle:

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates Nitric oxide which interacts with oxygen to produce Nitrite ions, which can be measured at 550nm by spectrophotometer in the presence of Griess reagent (Kumar S et al., 2008).

Procedure:

Sodium Nitroprusside (5mM) in standard phosphate buffer saline (0.025M, pH 7.4) was incubated with 0.1 ml of sample; tubes were incubated at 29ºC for 3 hours. Control experiment without the test compounds but with equivalent amount of buffer was conducted in an identical manner. After 3 hours incubated samples were diluted with 1 ml of Griess reagent. The absorbance of the colour developed during diazotization of Nitrite with sulphanilamide and its subsequent coupling with Napthyl ethylene diamine hydrochloride was observed at 550nm on spectrophotometer. Same procedure was done with ascorbic acid which was standard in comparison to sample.

Calculations

$$\% \text{ inhibition} = \frac{\text{O.D. of control} - \text{O.D. of Test}}{\text{O.D. of control}} \times 100$$

Standard Bacterial Culture used in the Study:

Stock cultures of Streptococcus mutans (MTCC 890) and Lactobacillus acidophilus (MTCC 10307) was used for the study.

Revival of Bacterial Cultures:

The stock culture of S. mutans (MTCC 890) was revived on Mutans Sanguis agar (Hi Media laboratories Pvt Ltd, Mumbai, India). The plate was incubated overnight at 37°C in a candle jar, the growth obtained on the agar plate was checked for purity by Gram's staining- Gram Positive cocci in chains.

The stock culture of Lactobacillus acidophilus (MTCC 10307) was revived on Lactobacillus MRS agar (Hi Media laboratories Pvt Ltd, Mumbai, India). The plate was incubated overnight at 37°C in a candle jar, the growth obtained bacilli on the agar plates was checked for purity by Gram's staining- Gram Positive rods.

Preparation of Test Solutions:

Sodium Chloride:

Saturated solution of sodium chloride was prepared in sterile distilled water and the solution was allowed to stand undisturbed for 10 mins. The solution was filter sterilized using sterile disposable syringe filters (Membrane filters (0.45µm, Sartorius)

Tri Sodium Mono Nitrogen:

Saturated solution of tri sodium mono nitrogen was prepared in sterile distilled water and the solution was allowed to stand undisturbed for 10 mins. The solution was filter sterilized using sterile disposable syringe filters (Sartorius).

Inoculum Preparation:

L. acidophilus:

Isolated colonies (5-6) of L. acidophilus from Lactobacillus MRS (de Man, Rogosa & Sharpe) agar (Hi Media laboratories Pvt Ltd, Mumbai, India) plate was suspended into sterile Lactobacillus MRS Broth (Hi Media laboratories Pvt Ltd, Mumbai, India) and the turbidity was adjusted to match 0.5 McFarland standard (1.5×10^8 cfu/ml).

S. mutans:

Isolated colonies (5-6) of S. mutans from Mutans Sanguis agar (Hi Media laboratories Pvt Ltd, Mumbai, India) plate were suspended into sterile Brain heart infusion Broth (BHIB) (Hi Media laboratories Pvt Ltd, Mumbai, India) and the turbidity was adjusted to match 0.5 McFarland standard (1.5×10^8 cfu/ml).

Antibacterial Efficacy of saturated salt solutions on Planktonic Cells:

Broth microdilution assay were performed to assess the efficacy of Saturated sodium chloride solution and Saturated tri sodium mono nitrogen individually.

Minimal inhibitory concentration (MIC):

The lowest antimicrobial concentration that completely inhibits visible bacterial growth was recorded as the minimal inhibitory concentration (MIC). Doubling dilutions of the saturated salt solutions, Sat. Sodium Chloride & Sat. Tri sodium Mono nitrogen were done so as to obtain a concentration gradient. The assay was performed in duplicate for each test solution. BHI broth was dispensed in rows A, B, E and F. Lactobacillus MRS Broth was dispensed in rows C, D, G and H.

The test solutions were added to the respectively labelled wells - A1, B1, E1 & F1 (Sat. Sodium Chloride); C1, D1, G1 & H1 (Sat. Tri sodium Mono nitrogen). Doubling dilution was performed from well A1 through A11, well B1 through B11, well C1 through C11, well D1 through D11, well E1 through E11, well F1 through F11, well G1 through G11 and well H1 through H11. Wells A12, B12, C12, D12, E12, F12, G12, H12 served as culture controls (without the test solution). To all the wells in rows A (wells A1 to A12) & B (wells B1 to B12), E (wells E1 to E12) and F (wells F1 to F12), 10µl of S. mutans suspension was added, A & B (NaCl), C & D (Tri Sodium Mono nitrogen). Similarly, to all the wells in rows E & F (NaCl), G & H (Tri Sodium Mono nitrogen), 10µl of L. acidophilus suspension was added. The microtiter plate was incubated in a candle at 37°C for overnight. The MIC was determined by performing minimum bactericidal concentration (MBC).

Minimum Bactericidal Concentration:

Minimum Bactericidal Concentration was performed by inoculating 5µl of broth culture from all the wells onto respectively labelled plates - S. mutans on Mutans Sanguis agar, L. acidophilus on Lactobacillus MRS agar. The MBC of S. mutans and L. acidophilus was recorded.

In Vitro Biofilm Formation:

The sterile Lactobacillus MRS Broth was dispensed (100 µl/well) and all the wells were inoculated with 10µl of Lactobacillus acidophilus (MTCC 10307) broth culture and the microtiter plate was incubated at 37°C in a candle jar. To avoid nutrient depletion and accumulation of toxic end products sterile culture medium (Lactobacillus MRS Broth) was replaced every alternate day.

The sterile BHIB was dispensed (100 µl/ well) and all the wells were inoculated with 10µl of S. mutans MTCC 980 broth culture and the microtiter plate was incubated at 37°C in a candle jar. To avoid nutrient depletion and accumulation of toxic end products sterile culture medium (Lactobacillus MRS Broth) was replaced every alternate day. At the end of 1 week, culture purity was assessed by inoculating a loopful of the respective culture media (Lactobacillus acidophilus plated onto Lactobacillus MRS agar and S. mutans onto Mutans Sanguis agar) and by Gram staining.

Time-Kill Assay- Anti-biofilm activity:

The contents of the wells were decanted aseptically and the wells were washed thrice gently with sterile saline (200 µl/ well) in order to remove the planktonic cells. The in-vitro biofilm formed on the microtiter plate wells were exposed to the respective test. The viable count was assessed at regular time intervals (0, 5, 10, 15, 20 min) by spread plate method to estimate the viable bacterial count.

Briefly, after exposure to the test solutions, the biofilm from each well was mechanically disrupted and transferred to sterile Eppendorf tubes containing 1 ml of phosphate buffered saline (PBS) solution (Hi Media Laboratories Pvt Ltd, Mumbai, India). Spread plate

technique was adopted to enumerate the viable count by plating 10 µl of the solution from Eppendorf tube onto respective culture media (Lactobacillus acidophilus plated onto Lactobacillus MRS agar and S. mutans onto Mutans Sanguis agar). The plates were incubated in a candle jar at 37°C for 24 hours. The colony-forming units(cfu/mL) was counted using a digital colony counter.

Results:

The results of nitrite content of the standard and salt solutions are given in the Tables 1&2. The graphical representation of the nitrite content of the standard is represented as Figure 1.

The Nitrite Content in the sample is found to be 8.32 µM/ 2mg of sample. Thus the experimental salt has 4.16

Table 1: Nitrite content of the Standard

Sl.NO	Volume of Stock Solution (µl)	Concentration of Stock (µM)	Absorbance (O.D.)
1	40	20	0.771
2	80	40	1.393
3	120	60	1.855
4	160	80	2.282
5	200	100	2.443

Table 2: Nitrite content of the Sample

Sl.NO	Concentration of Sample(mg/ml)	Absorbance (O.D.)	Avg. O.D	Nitrite Content (µM)
1	1	0.407	0.407	8.32
2	1	0.405		
3	1	0.411		

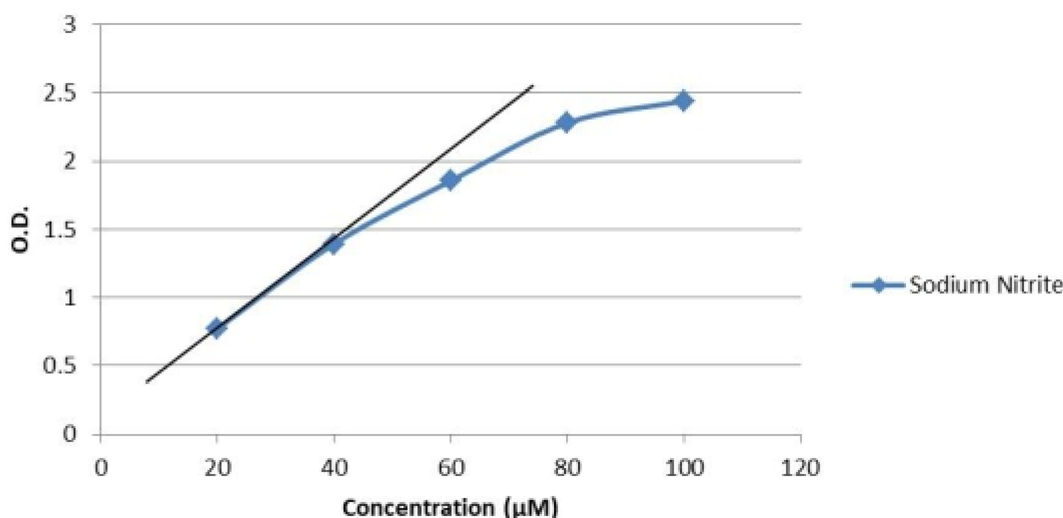

Fig 1 – Nitrite content of standard solution

µM/mg of NO. This is approximately 5 times less than that of the control (sodium nitrite).

The scavenging effect of nitric oxide radicals with control (Sodium nitroprusside) showed O.D of 0.171. For the test salt (Tri sodium mono nitrogen), the NO_2 inhibition percentage is 44.4% (for 100µg/ml) and given as a graphical representation in Figure 2.

MIC of the test salt was found to be 50mg/ml for S. mutans and 60mg/ml for L. acidophilus (Table 4)

Sl No.	Concentration (µg/ml)	O.D.	NO_2 Inhibiton %
1	20	0.155	9.356
2	40	0.143	16.37
3	60	0.13	23.97
4	80	0.121	29.23
5	100	0.095	44.44

Table 3: NO_2 inhibition % of the sample

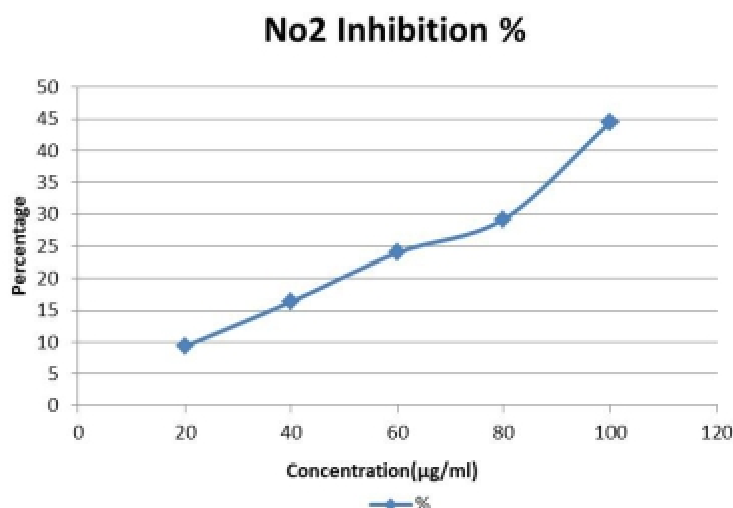

Fig 2 - NO2 inhibition % of the sample

Salt solution	Bacteria	MIC
NaCl	Set 1- *S. mutans*	50%
	Set 2- *S. mutans*	50%
Trisodium mononitrogen	Set 1- *S. mutans*	50%
	Set 2- *S. mutans*	50%
NaCl	Set 1- *L. acidophilus*	> 50%
	Set 2- *L. acidophilus*	> 50%
Trisodium mononitrogen	Set 1- *L. acidophilus*	> 50%
	Set 2- *L. acidophilus*	> 50%

Table 4:
MIC of Salt solutions against planktonic cells of S. mutans and L. acidophilus

Salt solution		**Cfu / mL**				
		0 min	5 min	10 min	15 min	20 min
NaCl	Set 1- *S. mutans*	268000	26600	2800	1600	0
	Set 2- *S. mutans*	196000	20400	2000	1000	0
Trisodium mononitrogen	Set 1- *S. mutans*	240000	86800	58800	33000	400
	Set 2- *S. mutans*	270000	115600	86200	53000	1200
NaCl	Set 1- *L. acidophilus*	122000	18800	2200	1400	600
	Set 2- *L. acidophilus*	111000	14000	2200	1000	200
Trisodium mononitrogen	Set 1- *L. acidophilus*	124000	36000	31600	16800	12600
	Set 2- *L. acidophilus*	130000	43200	36400	16600	10800

Table 5:
Time Kill assay against biofilm: Salt solution against S. mutans and L. acidophilus.

Time kill effect of the test salt Tri sodium mono nitrogen on biofilm organisms was found to be 25 minutes (Table 5)

Discussion:

The successful reduction of S. mutans count has been studied using various mouth rinses. Jothika et al, reported levels of S. mutans in the saliva of patients at moderate risk for developing dental caries significantly lower than those found prior to the use of a 0.2% chlorhexidine mouthwash and those found in the control group, 30 days after the end of the therapy.[11]

The use of a high-concentration chlorhexidine- based product as a mouth wash, exerts an immediate bacteriocidal effect followed by a prolonged bacteriostatic effect. It shows the ability to reduce the rate of formation of dental biofilm and its antibacterial action against different Streptococcus species, namely Streptococcus mutans. The bacteriocidal activity of chlorhexidine is particularly effective against gram-positive bacteria[12], but it has disadvantages such as staining, alteration in taste perception and tartar formation.

Salt water is a commonly used mouth rinse. It has been found efficacious against cariogenic microorganisms, Aravinth et al, in 2017 conducted randomized controlled trial to evaluate effect of salt water rinsing against oral microbes; they found MIC of salt water on S. mutans to be 0.7 M & L. acidophilus of 0.8 M. They also found that it was equivalent to CHX in reducing dental plaque. However CFU reduction of S. mutans & L. acidophilus was superior with CHX.[13]

Ballini et al, in 2021 compared the efficacy of sea salt mouth rinse containing Xylitol and lysozyme in improving oral health and reducing bacterial load. They found the mouthwash to reduce the levels of S. mutans significantly.[14]

In an experimental study by Lavaee et al, MIC and MBC for different concentrations of aqueous zinc sulfate and zinc acetate salt solutions for S. mutans in comparison with penicillin, chlorhexidine and diameters of zone of inhibition were detected. MIC and MBC of zinc sulfate solution were higher than penicillin and chlorhexidine.

In 25 and 50 μg/mL concentrations, the diameters of inhibition zone for zinc sulfate were more than zinc acetate. In the present study, we used the test salt and sea salt as control and conducted a time kill assay on a biofilm of S. mutans and L. acidophilus , resulting in total eradication of the viable organisms in 25min for both test salt and control salt.[15]

Miyasaki et al, examined MIC and MBC of hydrogen peroxide and sodium bicarbonate individually and in combination against Actinobacillus actinomycetemcomitans, Haemophilus aphrophilus, Eikenella corrodens, and Capnocytophaga gingivalis. These bacteria exhibited MBC (one hr) values ranging from 75 mumol/L to greater than 10 mmol/L and MIC from less than 5 to 500 mumol/L for H2O2. The tested bacteria exhibited MIC values for NaHCO3 of from 23 to 182 mmol/L, and the MBC (one hr) exceeded 728 mmol/L for most of the strains examined. In this present study, MIC of the test salt was found to be 50mg/ml for S. mutans and 60mg/ml for L. acidophilus[16].

Poluan et al in 2021 studied the effectiveness of 0.9m NaCl solution and 0.2% CHX gluconate on bacterial growth in oral cavity and concluded that both mouthwashes significantly reduced the number of bacterial colonies in the mouth. In our present study we conducted a time kill assay of the test salt and sea salt on a biofilm of S. mutans and L. acidophilus, resulting in total eradication of the viable organisms in 25min for both salts.[17]

The use of herbal alternatives such has triphala, Liquorice root & Tulasi have been tried and has shown good antibacterial properties. Tandon et al, in 2010 compared effect of Triphala and CHX mouthwash on prevention of caries, they found that there was no significant increase in DMFS score and incipient caries recorded at 3,6,9 months intervals in both the groups.[18] Rakshanaa et al, in 2017 studied the antibacterial effect of herbal mouthwash containing Liquorice root &Tulsi leaf against S. mutans, S. salivarius, S .sanguis & L. acidophilus. They found MIC of 50mg/ml showing good efficacy against S. mutans & 100mg/ml showing moderate efficacy against L. acidophilus.[19] These values are similar to the ones reported in the present study for the TSN salt used as a test agent.

Since CHX and other alcoholic mouthwashes produce various unwanted effects like dryness, staining, altered taste and eradication of commensals, there is an ongoing search for newer materials which have multiple benefits. In this context, we wanted to explore Tri sodium mono nitrogen salt used as a potential mouth rinse for preventing oral infectious diseases and cancer for its anti caries properties. The properties claimed by the manufacturer have been attributed to its ability to release Nitric Oxide. The investigations done in the present study confirmed the ability of the test salt to release nitric oxide. 4.16 μM/mg of NO was released from the salt. To the best of our knowledge there are no commercial or contemporary mouthwashes that release NO. Such mouthwashes act as NO boosters to the natural production by friendly microbes in tongue. Hence it may have beneficial effects on oral vascularity and disease control than only eradicating microbes.

Tri sodium mononitrogen salt was evaluated for its antibacterial efficacy against cariogenic bacteria. The results suggest that it is efficacious in controlling cariogenic organisms like S. mutans and L. acidophilus. The premise for testing this salt is based on its ability to release nitric oxide as a known antimicrobial agent. Though the salt was able to release a good quantity of nitric oxide compared to positive control sodium nitroprusside in the Griess analysis, the antimicrobial effect on cariogenic pathogens was not better than the control. This could probably be due to the labile nature of nitric oxide with a half-life of 2 minutes. However, further studies on other oral pathogens should be conducted to recommend its potential use as a mouth rinse.

Conclusion:

The test salt (Tri sodium mono nitrogen) is able to release substantial quantities of nitric oxide, and has antibacterial efficacy against cariogenic pathogens. Further studies on other oral pathogens are recommended to understand its usefulness as a potential mouth rinse.

Conflict of interest: None

Source of support: Nil

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