Original Article

Short-Term Impact of Two Kinds of Vegetables to Exogenous Total Nitrate and Nitrite Intake: Is Antibacterial Mouthwash Influential?

Malihe Moazeni[1,2], Sahar Gholipour[1,3], Behzad Mahaki[4], Afshin Ebrahimi[2,5]

[1]Student Research Committee, School of Health, Isfahan University of Medical Sciences, Isfahan, Iran, [2]Department of Environmental Health Engineering, School of Health, Isfahan University of Medical Sciences, Isfahan, Iran, [3]Department of Environmental Health Engineering, School of Health, Kashan University of Medical Sciences, Kashan, Iran, [4]Department of Biostatistics, School of Health, Kermanshah University of Medical Sciences, Kermanshah, Iran, [5]Environment Research Center, Research Institute for Primordial Prevention of Non-communicable Disease, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Nitrate is a compound with adverse effects on human health that can exist also in vegetables. This study aimed to determine the intake of total nitrate/nitrite from lettuce and carrot as high and low nitrate content, respectively. Moreover, the effect of chlorhexidine mouthwash on the elimination of the nitrite level in saliva and urine was surveyed. **Methods:** This study was designed as a crossover based on the randomized selection method. Thirty-nine participants were divided into two groups; the lettuce and carrot juice consumers (control group). The case group was consumed these two vegetables, while they used antibacterial mouthwash. The background of nitrate/nitrite of the participants was determined before exposure. The intake of total nitrate and nitrite via lettuce and carrot juice consumption was investigated. The Griess colorimetric reaction was used for nitrate and nitrite determination in samples. **Results:** Total nitrate concentrations in case and control groups were detected 0.79 and 0.78 mM in saliva and 1.78 and 1.38 mM in urine after lettuce consumption, respectively. However, it was determined 1.55 and 2.43 mM in saliva and 2.92 and 3.04 mM in urine after carrot ingestion. Salivary nitrite concentration 0.53 mM was decreased to 0.45 mM after antibacterial mouthwash application (P-value <0.05). **Conclusions:** This study indicated that the intake of total nitrate/nitrite via leafy vegetables was higher than rooty ones. The chlorhexidine mouthwash is an appropriate recommendation to reduce the nitrite concentration in the human body for preventing the probable side effects of nitrate metabolites such as N-nitrosamines forming.

Keywords: *Antibacterial agents, mouthwashes, nitrates, nitrites, vegetables*

Introduction

Nitrate is a common substance in the environment as a part of the nitrogen cycle. Using fertilizers containing nitrogen and wastewater effluent through agricultural and urban activities has altered this cycle.[1] It can be found in soil, surface, and underground waters, as well biomass, and is considered one of the most common pollutants in food products.[2] Nitrate can accumulate in vegetables and fruits, so herbal foods are the main sources of nitrate (80–95%).[3] The main part of the daily intake of foods is related to vegetables consumption. Among them, leafy vegetables such as lettuce and spinach have the highest nitrate concentration, whereas carrot and potato have lower levels.[4,5] It is believed that the maximum nitrate level of lettuce, especially *Lactuca sativa*, which is one of the most consumed vegetables in the world, is about 2500–3500 mg kg^{-1} for summer harvest.[3,6]

It is observed that nitrate is concentrated and secreted into the oral cavity via salivary glands and converted to nitrite (NO_2^-) by oral bacteria after nitrate load diet consumption. Furthermore, it is reduced to nitric oxide (NO) in the acidic condition of the stomach and converted to nitrate and nitrite in the body. In addition, a nitrate diet can form N-nitrosamines in the stomach that can promote N-nitrosamines carcinogenic effects.[7] It is reported that nitrogenous compounds as a form of NO are beneficial for human health. So, they can prevent cardiovascular diseases such as hypertension and atherosclerosis, insulin resistance, and metabolic syndrome.[8,9] However, nitrate can be lead to methemoglobinemia in infants under 6 months,[10] and risk of type 2 diabetes in greater age groups.[11] Besides, there is a possible association between chronic exposure to nitrate, nitrite, and N-nitrosamines with some types of cancers.[12] Usually, the incidence of nitrate related diseases is high in low-income countries.[12] In 2010, the international

Address for correspondence:
Prof. Afshin Ebrahimi, Department of Environmental Health Engineering, Hezar Jerib Ave Isfahan University of Medical Sciences, School of Health, Isfahan, 81676 – 36954, Iran.
E-mail: a_ebrahimi@hlth.mui.ac.ir

Access this article online
Website: www.ijpvmjournal.net/www.ijpm.ir
DOI: 10.4103/ijpvm.IJPVM_192_20
Quick Response Code:

How to cite this article: Moazeni M, Gholipour S, Mahaki B, Ebrahimi A. Short-term impact of two kinds of vegetables to exogenous total nitrate and nitrite intake: Is antibacterial mouthwash influential?. Int J Prev Med 2021;12:168.

agency for research on cancer (IARC) had classified nitrate in group 2A, since nitrate and nitrite ingestion in foods has probably carcinogenic effects on humans.[13]

Mouthwashes such as chlorhexidine had been used for preventing the reduction of nitrate to nitrite by the oral bacteria in the exogenous nitrate-nitrite–NO pathway.[1,14] The oral bacteria are involved in the regulating of systemic nitrite levels can changes in human fluids nitrite when the oral microflora is disturbed by the use of some drugs such as antibiotics.[15,16] In addition, some studies have shown that chlorhexidine mouthwash prevents the conversion of nitrate to nitrite in saliva and plasma.[15,16]

Based on a study in Iran, lettuce and carrot are one of the high and low nitrate load vegetables, respectively.[11] In 2018, also a study showed that lettuce posed critical human health risks in Isfahan populations, Iran.[17] So, based on the risk/benefit of nitrate, the present study was designed to determine the intake of total nitrate and nitrite from lettuce and carrot as instances of leafy and rooty vegetables that consume by people as salad and juice, respectively, in a semi-arid region, Isfahan, the center of Iran for the first time. The effects of chlorhexidine mouthwash on the reduction of nitrite level in human saliva and urine after consumption of these vegetables to decrease the likely side effects of nitrate were also considered.

Methods

Study's participants

Thirty-nine healthy people participated in the study. All participants were screened for enrollment. They had not used any medicine and antibiotics, were non-smokers with no current use of mouthwash, and did not have a history of any illness such as diabetes, cancer, or cardiovascular disease.

Experimental protocol

This study had a crossover design based on a randomized selection. Based on some studies,[11,18] one day (24 hours) prior to each test, volunteers were asked to avoid high nitrate diets (including spinach, onion, meat, fast food, and so on), and also mouthwash. Participants were tested in the morning, and initially, their samples including 1 ml saliva and 3 ml urine were collected.[16] Following the baseline sample collection, 20 participants were asked to eat 300 grams of lettuce (*Lactuca sativa*), and 19 others drank 600 mL carrot juice. Eight volunteers after consumption of each lettuce or carrot juice were given the chlorhexidine mouthwash to gargle according to manufacturer's instructions (10 ml gargled twice for 1 min after eating). Then, 1 hour and 4 hours after intake, their saliva and urine samples were collected, respectively.[19] The samples were kept at -80°C until measurements (maximum 3 weeks).

The studied vegetables were provided from an agricultural field in Isfahan in autumn 2017. Recently, due to drought conditions, farmers have been interested in using local wastewater treatment plant effluents instead of water in agricultural fields for irrigation of food crops in Isfahan.[20] The cultivation of vegetables on soil contaminated with wastewater sludge may lead to the accumulation of nitrate in edible plant parts.[21]

Nitrate and nitrite analysis

Saliva and urine samples were diluted 1:1 and 1:10, respectively, and were centrifuged at 5000 rpm for 20 min within 60 s after sampling. Total nitrate and nitrite concentrations were measured in samples using the Griess colorimetric reaction.[22] In this method, saliva (40 μl), and urine (80 μl) samples were transferred to a 96-well ELISA plate after diluting (Cayman Chemical Co., Ann Arbor, MI, USA), and then determined.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL) software. The results of total nitrate and nitrite values are reported as means ± SEM. Multiple linear regression analysis was used to establish the relation of total nitrate and nitrite with characteristics of participants. Student's paired t-test was used to compare salivary and urinary nitrate and nitrite concentration between before and after exposure. The independent sample t-test was performed to evaluate the difference of total nitrate, and nitrite mean concentration in the case and control groups, and for lettuce and carrot consumptions. Difference between groups was considered significant at P value ≤0.05.

Results

The demographic characteristics of the study participants are shown in Table 1. Participants were age 20-45 years and BMI equal to 17.53-34.11 Kg/m^2. Multiple linear regression analysis exhibited that there was not a relevant relationship between the salivary and urinary concentrations of total nitrate and nitrite with of participants' characteristics (age and BMI) based on P value ≥0.05. Then, in this study, we did not consider the effect of these parameters.

Lettuce ingestion

The results of total nitrate and nitrite levels in saliva and urine samples before and after 60-240 min of lettuce and carrot consumption for case and control experiments (with and without antibacterial mouthwash) are presented

Table 1: Characteristics of study participants

Parameters	Male (16%)			Female (83%)		
	Min	Max	Mean±SEM	Min	Max	Mean±SEM
Tall (m)	1.74	1.82	1.78±0.02	1.54	1.73	1.62±0.01
Weight (Kg)	75.0	96.0	87.8±4.2	46.0	94.0	57.2±1.8
BMI (Kg/m^2)	24.8	31.4	27.7±1.1	17.5	34.1	21.7±0.6
Age (years)	41.0	45.0	43.0±0.9	20.0	40.0	26.3±0.8

in Figures 1 and 2. Based on Figure 1, the lettuce ingestion has increased total nitrate in samples and nitrite in saliva for the control group (*P*-value <0.05). Figures 1 and 2 show based on comparison of case and control groups, the antibacterial mouthwash had no effect on the reduction of total nitrate concentration (*P*-value >0.05). Moreover, nitrite concentration was nearly minimized immediately after antibacterial mouthwash; for example, in saliva from 0.53 ± 0.16 to 0.45 ± 0.1 mM in the lettuce consumers (*P*-value <0.05). Then, due to the use of antibacterial mouthwash, nitrate reduction to nitrite did not happen.

Carrot juice drinking

Figure 1 shows the carrot consumption increased total nitrate for both saliva and urine samples. Furthermore, there was no significant difference between total nitrate and nitrite concentration in both groups (*P*-value >0.05) [Figures 1 and 2]. As well, for carrot juice drinking, antibacterial mouthwash could not influence nitrite reduction.

Based on the results of this study, both the lettuce and carrot consumption increased total nitrate level. Though the lettuce ingestion could increase total nitrate and nitrite in saliva and urine compared with carrot ingestion (*P*-value <0.05).

Discussion

Effect of vegetables consumption

The beneficial health effects of nitrate exposure are mitigation of cardiovascular disease and hypotensive. Nitrate and nitrite exposure have been a concerning issue in terms of the incidence of some diseases. Furthermore, it probably leads to adverse effects on the nervous system, and carcinogenic effects in the stomach.[9] Several previous studies showed that the average daily nitrate and nitrite intake was 121 and 1.88 mg, respectively, for the human body. Moreover, 85% nitrate and 43% nitrite intake from the diet are due to vegetable consumption.[23] The nitrate content of vegetables depends on different factors, the main factors to increase nitrate amount of vegetables are the use of synthetic nitrogen fertilizers, livestock manure, biosolids in intensive and reuse of wastewater effluent in agricultural activities.[1,5,6,24] Ingestion of nitrate-loaded diet causes nitrate absorption and secretion in human body fluids such as saliva.[19] Besides, almost 75% of the total exposed nitrate was excreted via urine.[25] Then, in the present study, intake of total nitrate and nitrite via vegetables consumption in the saliva and urine of healthy subjects were surveyed. Haftbaradaran *et al*. reported the health risks of nitrate for the consumption of some vegetables in Isfahan, Iran. They indicated that the highest risk was predicted for lettuce consumption in the study area.[17] Thus, the lettuce and carrot were chosen as instances of leafy and root vegetables, respectively. The results of the present study [Figure 1] indicated that intake to nitrate from lettuce and carrot consumption leads to increased total nitrate and nitrite levels in both the saliva and urine samples, as well there were significant differences between the concentration of these compounds before and after vegetable consumption (*P*-value <0.05). Van Velzen *et al*. reported that the oral bioavailability of nitrate for lettuce was 114%.[24] Spiegelhalder *et al*. reported that the increased amount of nitrate and nitrite in saliva was related to the amount of ingested nitrate.[26] In addition, Ashworth *et al*. proved that the consumption of the high-nitrate load vegetables meaningfully increased plasma nitrate and nitrite concentrations.[27] The effect of dietary nitrate load on salivary, plasma, and urinary nitrate metabolism in human showed that high nitrate meals cause increased nitrate and nitrite levels in urine and saliva ranging from 53 mg to 223, and 241 to 243 mg for urine and from 204 to 302 μmol/l and 249 to 483 μmol/l for saliva after dietary intake.[25] Figure 1 shows the total nitrate and nitrate concentrations after lettuce ingestion are higher than after carrot juice drinking (*P*-value <0.05). In this regard, one study showed that the nitrate and nitrite amounts were high after a high load food of nitrate and nitrite; however, after 24 hours of dietary intake, these parameters reached the concentration close to the baseline limit.[19] Bahadoran *et al*. showed that nitrate and nitrite in leafy vegetables such as

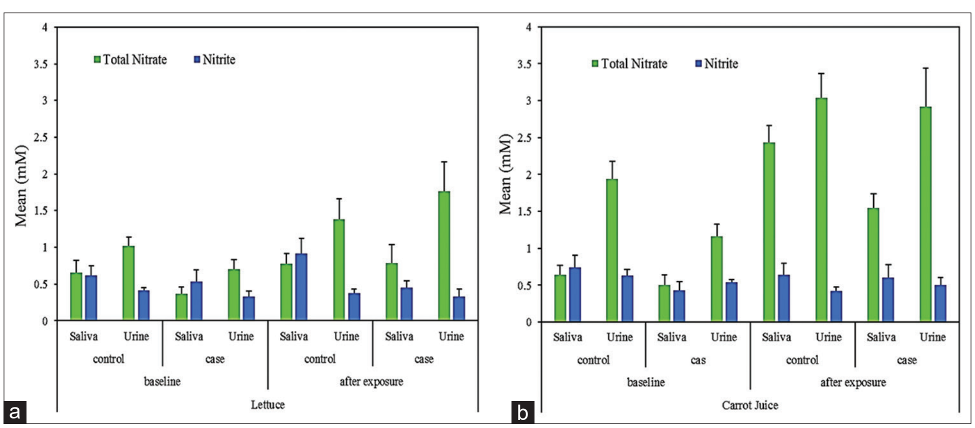

Figure 1: The mean of total nitrate and nitrite concentrations for; (a) lettuce and (b) carrot juice drinking in the biological fluid before and after intake in case and control groups

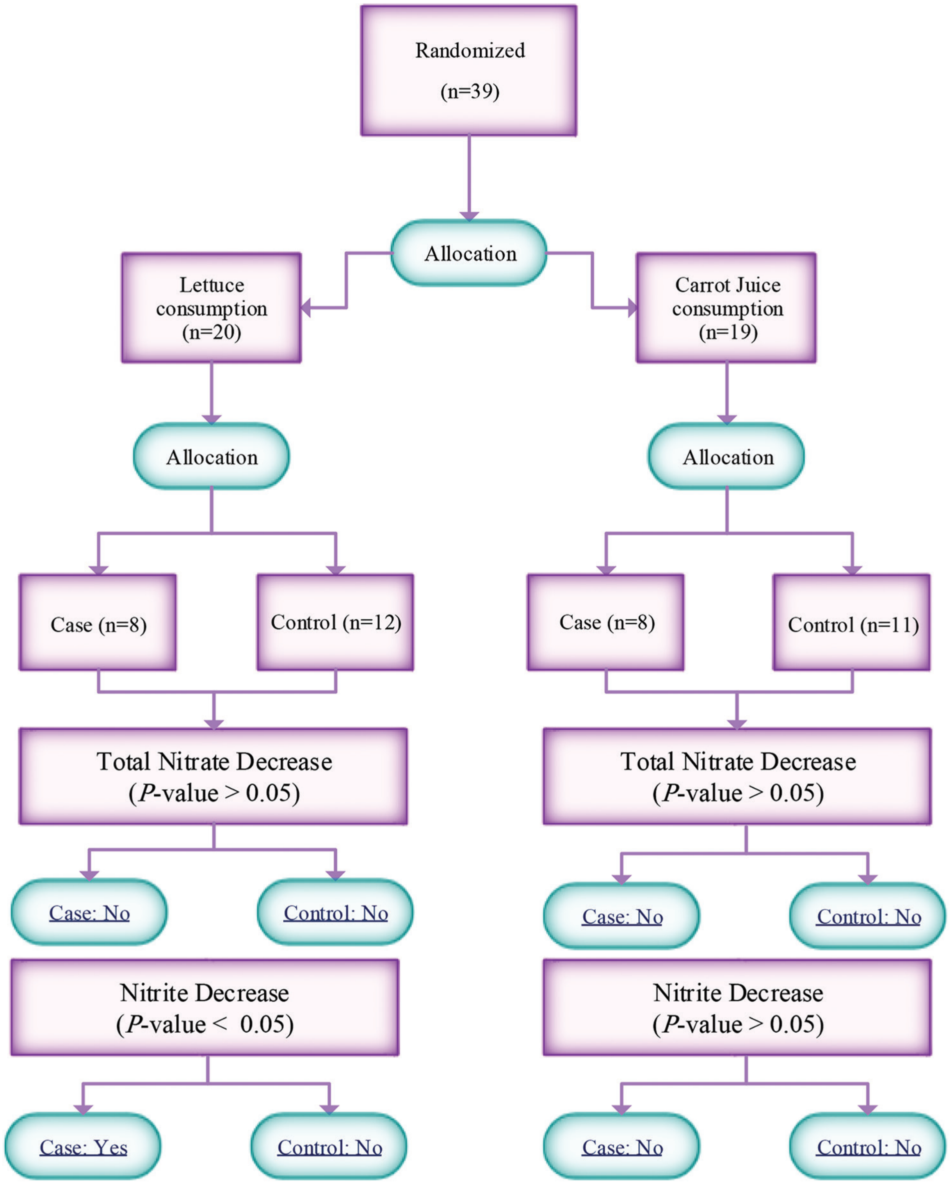

Figure 2: Study flow diagram and the comparison results of case and control groups

lettuce (365 mg 100 g^{-1}) are higher than rooty vegetables such as carrot (50.3 mg 100 g^{-1}).[11]

Effect of antibacterial mouthwash

The nitrate is reduced to its metabolites such as nitrite and then N-nitrosamines that can exert adverse effects on human health. In this study, we used an antibacterial mouthwash to reduce or prevent the activity of oral bacteria. Some previous studies have shown that this type of mouthwash can reduce the activity of nitrate-to-nitrite-converting oral bacteria by reducing nitrate accumulation in saliva.[16] Woessner *et al*. examined three types of mouthwashes: listerine antiseptic, cepacol antibacterial, and chlorhexidine to determine their impacts on salivary and plasma nitrate and nitrite concentrations. They reported that chlorhexidine mouthwash was a suitable option for the nitrate reduction in saliva.[15] Furthermore, Mitsui and Harasawa's study showed that the chlorhexidine mouthwash had a favorable effect on oral nitrate-reducing activity while essential oil and povidone-iodine mouthwash had an insignificant effect on it.[14] As well, the results of our study showed that chlorhexidine mouthwash reduced nitrite concentration generated after lettuce consumption, but it could not decrease nitrite concentration after drinking carrot juice [Figure 2]. Probably, it did not exert a perfect effect on nitrite after carrot juice drinking because it was in the solution phase with a rapid bioavailability for oral bacteria. These results presented a good idea to use an antibacterial mouthwash before food consumption that was also reported by Govoni *et al*.[16] The results of this and other studies indicate that the use of chlorhexidine mouthwash even at a low concentration, may affect oral bacteria and inhibit nitrate reduction to nitrite.[1,14,16]

Conclusion

In this study, total intake of nitrogenous compounds: total nitrate and nitrite, through lettuce and carrot consumption, were investigated via sampling of saliva and urine in subjects. The results showed that lettuce consumption caused a further increase in total nitrate and nitrite in saliva and urine samples compared to carrot juice drinking. Moreover, using chlorhexidine antibacterial mouthwash was investigated as a strategy to mitigate nitrite absorption. According to results of the comparison between the case and control groups the studied mouthwash could only reduce nitrite generation after consumption of lettuce; however, it did not influence total nitrate intake after lettuce and carrot juice consumption. Also, it did not affect the reduction of nitrite after carrot juice drinking. Thus, it is recommended to use antibacterial mouthwashes for the inhibition of nitrate to nitrite conversion in human fluids. Paying attention to appropriate vegetable cultivation methods and their storage also seems essential.

Acknowledgments

This study was approved by the Medical Ethics Committee of the Isfahan University of Medical Sciences (IR.MUI.REC.1395.2.210). This work was supported by the vice chancellery for Research and Technology at the Isfahan University of Medical Sciences (Grant No. 295210). The authors would like to thank the Environment Research Center, Research Institute for Primordial Prevention of Non-communicable Disease, and Department of Environmental Health Engineering, School of Health at Isfahan University of Medical Sciences.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Received: 16 Apr 20 **Accepted:** 21 Jan 21
Published: 14 Dec 21

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