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Biophysical and Spectrophotometric Evaluation of Nitrite in Human Saliva

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التقييم الفيزيائي الحيوي والقياس الطيفي للنيتريت في لعاب الإنسان

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Abstract

Salivary glands and oral bacteria play an essential role in converting dietary nitrate to nitrite and then to nitric oxide, which is a multifunctional signaling molecule involved in vascular and metabolic functions. In addition to the endogenous arginine pathway, dietary nitrate represents the main external source of nitric oxide through a sequential conversion from nitrate to nitrite to nitric oxide. Salivary nitrite normally remains below 7 milligrams per liter. Elevated levels may result from high intake of nitrate rich vegetables, poor oral health such as dental caries or periodontal disease, or systemic conditions including type 2 diabetes mellitus and kidney dysfunction. This cross sectional study aimed to quantitatively determine salivary nitrite concentrations in young adult students using a spectrophotometric method, compare the results with normal values, and discuss their physiological and pathophysiological significance. Fifteen saliva samples were collected from healthy volunteers aged 20–24 years after 8–10 hours of fasting; samples were stabilized with sodium hydroxide, deproteinized with zinc sulfate, centrifuged, and analyzed at 507 nm using a DR 900 spectrophotometer according to HACH method 8507. The results showed a mean nitrite concentration of 10.9 mg/L (range 6.7–24.3 mg/L). Only 26.7% of samples were within the normal limit (≤7 mg/L), while 73.3% exceeded it, with two samples showing markedly elevated levels above 20 mg/L. The most likely explanations for these elevations are recent consumption of green leafy vegetables (physiological) and undiagnosed oral health problems such as dental caries or gingivitis (pathophysiological), although early systemic conditions like diabetes or kidney disease cannot be completely excluded. The study concludes that salivary nitrite is a promising non invasive biomarker, but its interpretation requires careful control of dietary, oral hygiene, and circadian factors; larger controlled studies with standardized protocols are needed to establish population specific reference ranges and validate its clinical utility.

Keywords: Salivary nitrite, Nitrate, Nitric oxide, Spectrophotometer, Dental caries.

الملخص

تؤدي الغدد اللعابية وبكتيريا الفم دورًا أساسيًا في تحويل النترات الغذائية إلى نتريت، ثم إلى أكسيد النيتريك، وهو جزيء إشاري متعدد الوظائف يشارك في وظائف الأوعية الدموية والتمثيل الغذائي. بالإضافة إلى مسار الأرجينين الداخلي، تمثل النترات الغذائية المصدر الخارجي الرئيسي لأكسيد النيتريك من خلال تحويل متسلسل من النترات إلى النتريت ثم إلى أكسيد النيتريك. عادةً ما يبقى مستوى النتريت في اللعاب أقل من 7 ملليغرامات لكل لتر. قد تنتج المستويات المرتفعة عن الإفراط في تناول الخضراوات الغنية بالنترات، أو سوء صحة الفم مثل تسوس الأسنان أو أمراض اللثة، أو حالات مرضية جهازية بما في ذلك داء السكري من النوع الثاني واختلال وظائف الكلى. هدفت هذه الدراسة المقطعية إلى تحديد تركيزات النتريت في لعاب طلاب بالغين باستخدام طريقة قياس الطيف الضوئي، ومقارنة النتائج بالقيم الطبيعية، ومناقشة أهميتها الفيزيولوجية والمرضية. تم جمع خمس عشرة عينة من اللعاب من متطوعين أصحاء تتراوح أعمارهم بين 20 و 24 عامًا بعد صيام لمدة 8-10 ساعات؛ وتم تثبيت العينات باستخدام هيدروكسيد الصوديوم، وإزالة البروتين منها باستخدام كبريتات الزنك، ثم طردها مركزيًا، وتحليلها عند 507 نانومتر باستخدام مطياف DR 900 وفقًا لطريقة HACH 8507. أظهرت النتائج متوسط تركيز النتريت 10.9 ملغم/لتر (يتراوح بين 6.7 و24.3 ملغم/لتر). كانت 26.7% فقط من العينات ضمن المعدل الطبيعي (≤7 ملغم/لتر)، بينما تجاوزت 73.3% منها هذا المعدل، مع وجود عينتين أظهرتا مستويات مرتفعة بشكل ملحوظ تتجاوز 20 ملغم/لتر. التفسيرات الأكثر ترجيحًا لهذه الارتفاعات هي الاستهلاك الحديث للخضراوات الورقية الخضراء (فسيولوجي) ومشاكل صحة الفم غير المشخصة مثل تسوس الأسنان أو التهاب اللثة (مرضي)، على الرغم من أنه لا يمكن استبعاد الحالات المرضية الجهازية المبكرة مثل داء السكري أو أمراض الكلى بشكل كامل. خلصت الدراسة إلى أن النتريت اللعابي مؤشر حيوي غير جراحي واعد، لكن تفسيره يتطلب تحكمًا دقيقًا في العوامل الغذائية ونظافة الفم والإيقاع اليومي؛ هناك حاجة إلى دراسات مضبوطة أكبر ذات بروتوكولات موحدة لتحديد نطاقات مرجعية خاصة بكل فئة سكانية والتحقق من جدواه السريرية

الكلمات المفتاحية: نتريت اللعاب، نترات، أكسيد النيتريك، مطياف ضوئي، تسوس الأسنان.

Introduction:

Saliva is a complex biological fluid that not only moisturizes the oral cavity and aids digestion but has also emerged as a valuable non-invasive diagnostic tool for detecting various systemic and oral diseases. Among its important components are nitrate (NO_3^-) and nitrite (NO_2^-) ions, which are natural parts of the nitrogen cycle in the human body. These ions exist in water, soil, air, and plants, with food; especially green leafy vegetables, being the primary source of absorbed nitrate (Ma et al., 2018; Pawan et al., 2022). After ingestion, dietary nitrate is converted to nitrite by commensal bacteria in the oral cavity, and subsequently to nitric oxide (NO). NO is a multifunctional signaling molecule involved in biological processes such as regulation of mucosal blood flow, mucus generation, smooth muscle contraction, glucose homeostasis, and mitochondrial function (Lundberg et al., 2008). Recent evidence indicates that the nitrate-nitrite-NO pathway plays a protective role in the cardiovascular system and gastric mucosa, and helps prevent insulin resistance and the progression of type 2 diabetes mellitus (Bahadoran et al., 2015; Alkuraishy et al., 2017). Normal salivary nitrite concentrations in healthy individuals are generally below 7 mg/L. However, these values can be influenced by several factors including diet, oral hygiene, presence of dental caries, periodontal diseases, and even systemic conditions such as diabetes and kidney diseases (Ghasempour et al., 2014; Elyassi Gorji et al., 2020). Since approximately 25% of circulating nitrate is reabsorbed by the salivary glands and secreted into saliva at concentrations 10–20 times higher than in plasma (Lundberg, 2012), estimating salivary nitrite can indirectly reflect endogenous NO production and endothelial function.

Therefore, this study aimed to estimate salivary nitrite concentration in a sample of students from the College of Science, Sabratha University, compare the results with normal values, and investigate potential causes of elevated levels based on current scientific literature.

2. Materials and Methods

- **Study Design and Participants**

A cross-sectional study was conducted at the Department of Molecular Biology and Biochemistry, Faculty of Science, Sabratha University, during the year 2023. A total of 14 healthy volunteer students (age range: 20–24 years) participated after providing verbal informed consent. The study was approved by the departmental committee.

- **Sample Collection**

Participants were instructed to fast for 8–10 hours prior to collection (only water allowed). Whole unstimulated saliva samples (approximately 5 mL) were collected in glass tubes containing 0.5 mL of 1 N sodium hydroxide (NaOH). NaOH was added as a stabilizer because nitrite is unstable in acidic media; alkaline conditions preserve nitrite for at least 4 hours at room temperature (as per HACH method 8507).

- **Deproteinization and Preservation**

To prevent further reduction of nitrite after sampling, 0.2 mL of 0.5 M zinc sulfate ($ZnSO_4$) was added to a 3 mL saliva aliquot and mixed thoroughly. The mixture was then centrifuged at 3000 rpm for 10 minutes. $ZnSO_4$ treatment removes proteins and other interfering substances that could inhibit chromogen formation from nitrite. According to the method cited, omitting this step can underestimate nitrite concentration by up to 80%.

- **Spectrophotometric Nitrite Measurement**

The nitrite concentration was determined using a DR 900 spectrophotometer at a wavelength of 507 nm, following HACH method 8507 with NitriVer® 3 Nitrite Reagent Powder Pillows. The method is based on the diazotization reaction: nitrite reacts with sulfanilic acid to form a diazonium salt, which then couples with chromotropic acid (or similar compound) to produce a pink-colored complex. The intensity of the color is directly proportional to the nitrite concentration. Results were expressed as mg/L.

- **Circulation of nitrate in the body**

The recirculation of dietary nitrate occurs primarily within the salivary glands. In these glands, the transmembrane protein sialin facilitates the active transport and accumulation of nitrate. A portion of this nitrate is subsequently reduced to nitrite by commensal oral bacteria, and the resulting nitrite is then absorbed in the stomach and intestines, Figure 1. Approximately 25%

of the circulating nitrate is reabsorbed by the salivary glands, while the remainder is predominantly excreted by the kidneys. The physiological actions of nitrate are mediated through the exogenous pathway, which proceeds from nitrate to nitrite and finally to nitric oxide (NO). Abbreviations: NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate (Ma et al., 2018).

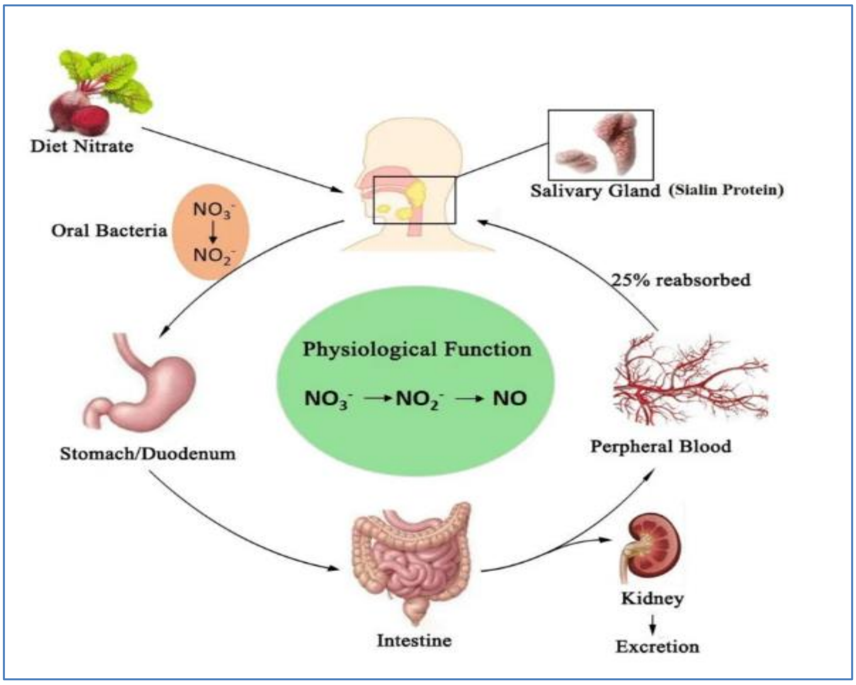

Figure 1: Circulation of nitrate in the body (Ma et al., 2018).

- **Data Analysis**

Descriptive statistics (mean, range, percentage) were calculated. The normal reference value was set at ≤ 7 mg/L based on Eisenbrand et al. (1980).

- **Collection and Preservation Procedures**
  - Approximately 5 mL of whole saliva was collected in glass tubes containing 0.5 mL of 1N sodium hydroxide (NaOH). NaOH stabilizes nitrite because nitrite is unstable in acidic media; alkaline conditions maintain stability for at least 4 hours at room temperature.
  - To prevent further reduction of nitrite after collection, 0.2 mL of 0.5M zinc sulfate ($ZnSO_4$) was added to a 3 mL saliva aliquot and mixed.
  - The mixture was centrifuged at 3000 rpm for 10 minutes. $ZnSO_4$ treatment removes proteins and other substances that could inhibit chromogen formation from nitrite. Omitting this step may underestimate nitrite concentration by up to 80%.
- **Nitrite Estimation**

Nitrite concentration was measured using a DR 900 spectrophotometer (Figure 2) at 507 nm according to HACH method 8507, using NitriVer® 3 Nitrite Reagent Powder Pillows.

Figure 2: DR 900 spectrophotometer.

3. Results Analysis

A total of 14 saliva samples were analyzed. The nitrite concentrations are presented in Table 1 and graphically in Figure 3.

Table 1: Salivary nitrite concentrations in study participants (N=15)

Sample ID	Percentage (%)	Nitrite Concentration (mg/L)
A	0.067	6.7
B	0.074	7.4
C	0.148	14.8
D	0.234	23.4
E	0.125	12.5
F	0.101	10.1
G	0.084	8.4
H	0.073	7.3
I	0.067	6.7
J	0.067	6.7
K	0.086	8.6
L	0.101	10.1
M	0.086	8.6
N	0.243	24.3
O	0.069	6.9

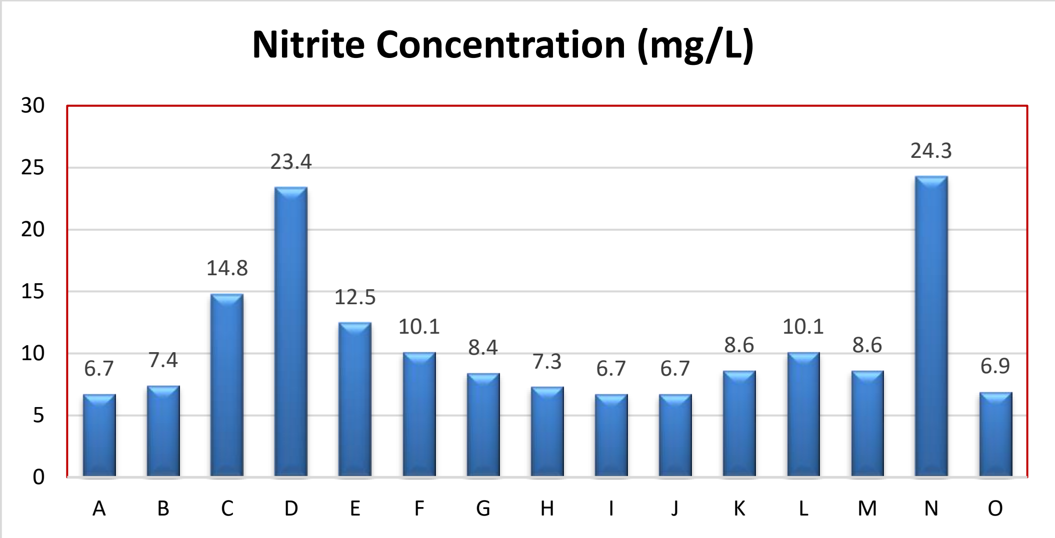

Figure 3: Distribution of salivary nitrite concentrations (mg/L).

The estimated nitrite concentrations in saliva samples (from Table 1) are as follows (converted to mg/L):

Summary statistics:

- Mean ± SD: 10.9 ± 5.7 mg/L
- Median: 8.5 mg/L
- Range: 6.7 – 24.3 mg/L

According to the references (e.g., Eisenbrand et al., 1980), the normal salivary nitrite concentration does not exceed 7 mg/L.

Categorization (normal ≤ 7 mg/L):

- Within normal limit: 4 samples (26.7%) – A (6.7), I (6.7), J (6.7), O (6.9)
- Above normal: 11 samples (73.3%)
- Markedly elevated (>20 mg/L): 2 samples (D=23.4, N=24.3) = 13.3% of total

4. Discussion

The present study quantitatively determined salivary nitrite concentrations in young adult students using a validated spectrophotometric method. The results showed that the majority (73.3%) of participants had levels above the normal limit of 7 mg/L, with a mean of 10.9 mg/L. Two participants had extremely high values (>20 mg/L), suggesting significant exposure or underlying pathology.

4.1 Physiological Significance

The most common cause of elevated salivary nitrite is recent consumption of nitrate-rich foods. Green leafy vegetables such as spinach, lettuce, arugula, and beetroot are major dietary sources (Ma et al., 2018). After ingestion, nitrate is rapidly absorbed, concentrated in saliva (10–20 times plasma levels), and reduced to nitrite by oral bacteria (Lundberg, 2012). Even after 8–10 hours of fasting, residual nitrite can remain elevated, especially if the previous meal contained high nitrate. The half-life of nitrate in plasma is 5–8 hours (Sobsey & Bartram, 2003), so a single high-nitrate meal can sustain elevated salivary nitrite for most of the next day.

From a physiological perspective, this is not necessarily harmful. The nitrate-nitrite-NO pathway is now recognized as an important alternative source of NO, particularly under hypoxic conditions. Dietary nitrate has been shown to lower blood pressure, improve endothelial function, and enhance exercise performance (Lundberg et al., 2008; Kapil et al., 2015). Therefore, elevated salivary nitrite in healthy individuals may simply reflect a diet rich in vegetables, which is beneficial for cardiovascular health.

4.2 Role of Oral Microbiome

The oral cavity hosts a diverse microbial community capable of reducing nitrate to nitrite. Hyde et al. (2014) identified 14 bacterial candidate species from the genera Granulicatella, Actinomyces, Veillonella, Prevotella, Neisseria, and Haemophilus that contribute to this process. Individuals with a higher abundance of these bacteria may produce more nitrite from the same dietary nitrate load. Variations in oral hygiene practices (e.g., infrequent use of antibacterial mouthwash) can also increase nitrite production, as chlorhexidine mouthwash is known to abolish the rise in salivary nitrite after a nitrate load (Govoni et al., 2008; Bondonno et al., 2015).

4.3 Oral and Dental Diseases

Several studies have linked elevated salivary nitrite/nitrate to dental caries. Ghasempour et al. (2014) measured nitrite and nitrate in children with different caries activity and found significantly higher levels in those with active caries. A systematic review and meta-analysis by Elyassi Gorji et al. (2021) confirmed a positive relationship between salivary nitric oxide (derived from nitrite) and dental caries in children. The acidic environment of carious lesions favors the non-enzymatic reduction of nitrite to NO, which may have both antimicrobial and pro-inflammatory effects. Similarly, periodontal disease creates a hypoxic, inflammatory environment that enhances bacterial nitrate reduction.

Although we did not perform oral examinations, it is plausible that some participants had undiagnosed caries or gingivitis, contributing to their elevated nitrite levels. The high prevalence of elevated samples (73.3%) in this young adult population may indicate a significant burden of subclinical oral health issues, which warrants further investigation.

4.4 Systemic Diseases

Salivary nitrite may also reflect systemic conditions. Alkuraishy et al. (2017) reported that patients with type 2 diabetes mellitus (T2DM), especially those with poor glycemic control, have higher salivary nitrite levels compared to healthy controls. This may be due to endothelial dysfunction, increased oxidative stress, and altered nitric oxide metabolism. Furthermore, since nitrate is primarily excreted by the kidneys, any impairment in renal function can lead to nitrate accumulation and increased salivary secretion (Ma et al., 2018). While our participants were young and presumably healthy, undiagnosed prediabetes or early kidney dysfunction cannot be completely ruled out without laboratory confirmation. However, given the age group, dietary factors and oral health are more likely explanations.

4.5 Comparison with Previous Studies

Our mean salivary nitrite concentration (10.9 mg/L) is higher than the normal range reported by Eisenbrand et al. (1980) but comparable to values found in some patient populations. For example, Alkuraishy et al. (2017) found mean salivary nitrite of 12.3 mg/L in uncontrolled T2DM patients. The high proportion of elevated samples (73.3%) in our study may reflect a diet rich in green leafy vegetables, which is common in Mediterranean and Libyan cuisine (e.g., spinach, molokhia, parsley). Alternatively, it may indicate a high prevalence of subclinical oral health issues among the student population.

4.7 Recommendations for Future Research

To better understand the physiological and pathophysiological significance of salivary nitrite, future studies should:

- Include larger sample sizes with matched controls.
- Standardize dietary protocols (e.g., low-nitrate diet for 48 hours before sampling).
- Perform comprehensive oral examinations by a dentist.
- Measure both salivary nitrate and nitrite simultaneously.
- Assess salivary flow rate and collect samples at the same time of day to control for circadian variation.
- Include plasma nitrite/nitrate measurements to validate the saliva-plasma correlation.
- Investigate the relationship between salivary nitrite and HbA1c (for diabetes) or serum creatinine (for kidney function).

5. Conclusion

This study successfully quantified salivary nitrite levels in young adult students using a simple, reliable spectrophotometric method. The majority of participants (73.3%) had levels above the normal limit of 7 mg/L, with a mean concentration of 10.9 mg/L. The most likely explanations are recent intake of nitrate-rich green vegetables (physiological) and undiagnosed oral health problems such as dental caries or gingivitis (pathophysiological). Although systemic conditions like type 2 diabetes or kidney disease are less probable in this age group, they cannot be entirely excluded without further testing. Salivary nitrite is a promising non-invasive biomarker, but its interpretation requires careful control of dietary, oral hygiene, and circadian factors. The spectrophotometric method described here is inexpensive, rapid, and suitable for field studies or routine screening. Establishing population-specific reference ranges and conducting larger controlled studies will help clarify the clinical utility of salivary nitrite in predicting and monitoring oral and systemic diseases.

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Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.