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OXIDATIVE STRESS SALIVARY MARKERS IN TYPE-2 DIABETIC MELLITUS

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ABSTRACT:
Background: Type 2 diabetes mellitus (T2DM) is a global health concern associated with systemic as well as oral complications. The preventive antioxidants found in saliva naturally reduce the damaging effects of reactive oxygen molecules. Any disruption to the regular functioning of these antioxidants may lead to oxidative stress, which could boost an individual's vulnerability to oral diseases. Diabetes patients are vulnerable to various dental complications, such as oral mucosal disorders, dental caries, dry mouth, and periodontal disease.
Objectives: To assess the salivary Hydrogen peroxide (H2O2) and Nitric oxide (NO) levels in patients with controlled and uncontrolled type 2 diabetes mellitus.
Materials and methods: The present study included 40 samples aged 45-65 years. Group I consisted of 20 controlled diabetic patients, and Group II consisted of 20 uncontrolled diabetic patients. Unstimulated whole saliva samples were collected from both groups, and laboratory analysis was done. Salivary Hydrogen peroxide (H2O2) and Nitric oxide (NO) levels were quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) and Spectrophotometric assay.
Results: Salivary Hydrogen peroxide levels were found to be higher in the uncontrolled diabetic group than in the controlled diabetic group, and salivary NO levels were found to be higher in the controlled diabetic group than in the uncontrolled diabetic group. The Mean±SD values of Hydrogen peroxide among controlled and uncontrolled diabetic patients are 2.98±0.12 and 2.99±0.11, respectively. The mean±SD value of NO in the controlled group was found to be 2.99±0.15, and in the unchecked group, it had a mean±SD value of NO as 2.64±0.96. However, it has been found that there is no statistically significant difference between Hydrogen peroxide and NO levels among controlled and uncontrolled diabetics, with p-values of 0.867 and 0.419, respectively.
Conclusion: Values of salivary biochemical markers were distinctly different between controlled and uncontrolled diabetic groups, suggesting the usefulness of salivary Hydrogen peroxide (H2O2) and Nitric oxide (NO) levels in Type 2 diabetics.
Categories: Dentistry, Oral Medicine, Diabetes mellitus
Keywords: Antioxidants, Diabetes mellitus; Oxidative stress; Biomarkers

INTRODUCTION:

Diabetes mellitus (DM) is a condition that affects your body's metabolism and causes high blood sugar levels due to a lack of insulin production or resistance to insulin. This condition has significant implications for oral health, giving rise to various oral manifestations and complications. These issues can adversely impact a patient's quality of life and there is a noteworthy connection between chronic oral complications and blood glucose control [1].

Type 2 diabetes has a high global and national prevalence. Globally, 463 million cases of diabetes were projected in 2019, [2] with research predicting that the incidence of type 2 diabetes cases will rise by an additional 130-200 million cases by 2025-2030 [3]. (Figure 1)

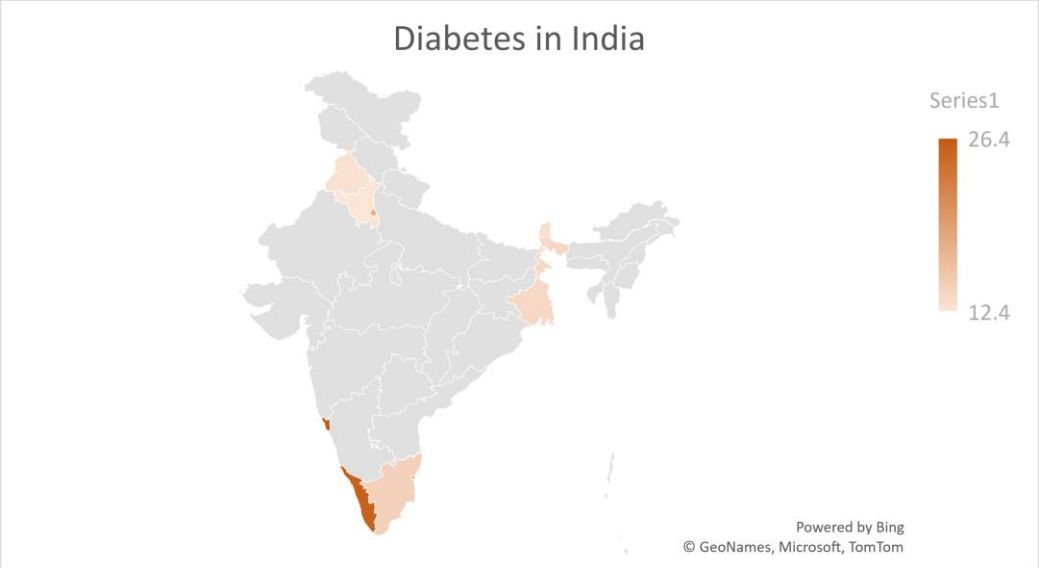


Figure 1 shows the Top 10 cities with high prevalence of diabetes in India [4]

Oxidative stress occurs when there is an unequal number of free radicals being produced compared to the body's ability to neutralize them through antioxidants. This can lead to harmful effects in the body and it has been implicated in the aetiopathogenesis of various oral pathologies. Based on existing literature, it is suggested that oxidative stress plays a significant role in developing both types of diabetes mellitus. Additionally, the oxidative degradation of glycated proteins also contributes to this process [5]. Complications of diabetes mellitus can be promoted by the consequences of oxidative stress. Antioxidants are important in managing diabetes because they can counteract oxidative stress, when the body produces more reactive oxygen species (ROS) than it can neutralize [6].

According to the National Institutes of Health, a biomarker is a measurable characteristic that serves as an indicator of normal biological processes, or a response to a therapeutic intervention, whether pathogenic or pharmacologic. It is important to differentiate between screening biomarkers used to detect individuals who are at high risk for a disease, diagnostic biomarkers that confirm the presence of a disease, and prognostic biomarkers that monitor the effectiveness of therapy [7].

Collecting saliva for medical research is simple and requires minimal training. Saliva contains numerous biological compounds, making it an effective biospecimen for assessing one's health status. Saliva as a diagnostic tool to assess oxidative stress is widely accepted. However,

routine invasive procedures can cause mental trauma, discomfort, and anxiety, particularly for pediatric and elderly patients. As Type 2 diabetes mellitus (T2DM) becomes more common, it's important to have a reliable and non-invasive way to screen for it [8].

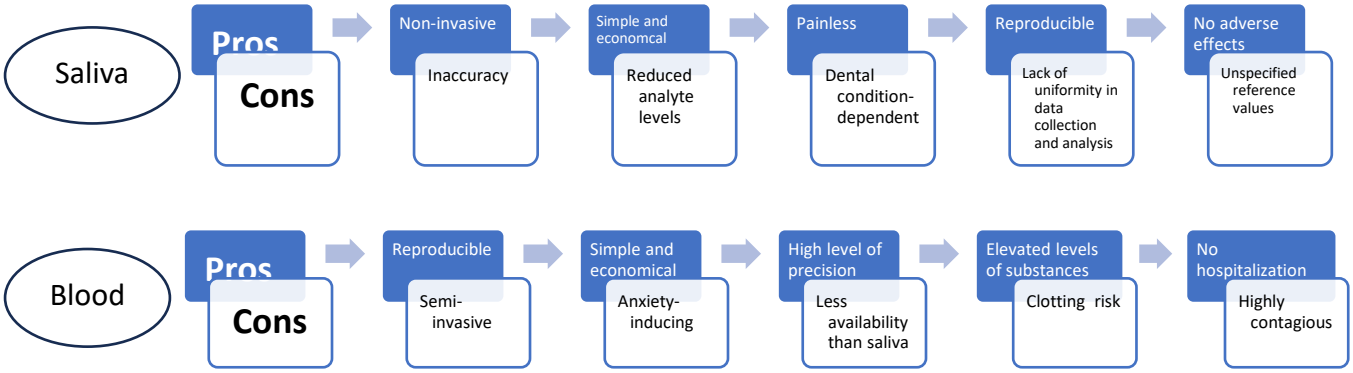


Figure 2 Pros and cons of the use of saliva or blood for the detection of clinically relevant analytes [9].

Nitric oxide (NO) is indeed recognized as an important biomarker in diabetes mellitus. NO is a signaling molecule involved in various physiological processes, including vascular tone regulation, neurotransmission, and immune response. In diabetes mellitus, alterations in NO production and activity are commonly observed and contribute to the development of complications associated with the disease. Researchers use various methods to assess NO levels and activity in diabetes mellitus patients, including measuring NO metabolites (such as nitrite and nitrate) in blood or urine, assessing endothelial function using techniques like flow-mediated dilation, and analyzing NO synthase expression and activity. (Figure 2)

Hydrogen peroxide (H2O2) itself is not typically used as a biomarker for diabetes mellitus. However, oxidative stress, which involves the generation of reactive oxygen species (ROS) including hydrogen peroxide, is implicated in the pathogenesis of diabetes mellitus and its complications. In diabetes mellitus, there is often an imbalance between the production of ROS and the body's antioxidant defenses, leading to oxidative stress. This oxidative stress can contribute to various complications of diabetes, such as cardiovascular disease, nephropathy, retinopathy, and neuropathy. Researchers often measure markers of oxidative stress, including hydrogen peroxide, as well as other ROS and antioxidant enzymes, to assess the level of oxidative stress in diabetes mellitus patients. However, hydrogen peroxide alone is not typically used as a specific biomarker for diabetes mellitus diagnosis or monitoring. Instead, biomarkers commonly used in the diagnosis and management of diabetes mellitus include blood glucose levels, glycated hemoglobin (HbA1c), insulin levels, and markers of insulin resistance such as C-peptide. These biomarkers provide valuable information about glucose metabolism and insulin action in diabetes mellitus patients.

Evaluating salivary antioxidant markers Hydrogen peroxide (H2O2) and Nitric acid (NO) in diabetic patients is important in understanding the antioxidants' role in diabetes. Research has revealed that it can effectively decrease inflammation and oxidative stress in individuals with diabetes. Assessing salivary levels of hydroxytoluene and Vitamin C in diabetic patients can be an aid in gauging the success of antioxidant therapy for managing diabetes. It can also be a useful tool for tracking the effectiveness of dietary changes or antioxidant supplements in reducing oxidative stress in diabetic patients [11,12].

MATERIALS AND METHODS:

Sample size calculation was done by keeping the power of the study at 90% and the significance level at 5% with an effect size of 0.5, the total sample size obtained was 40. Ethical clearance was obtained from IHEC/SDC/OMED-2105/23/216—a Cross-sectional single-centered study with adult type 2 diabetic patients. Seventy-five participants who were known diabetic patients on treatment were recruited and screened based on the inclusion and exclusion criteria. The 40 participants were age and gender-matched and divided into two groups based on metabolic control as evidenced by plasma random blood sugar levels evaluated before the salivary sample collection. Patients with random blood sugar levels 7.8 to 11.0mmol/l or ≥140-199 mg/dL were categorized in group 1(controlled type 2 diabetes group 20), and with values ≥11.1 mmol/l or 200 mg/dl were classified in group 2(uncontrolled type 2 diabetes group 20) [11]. The inclusion and exclusion criteria are mentioned in Table 1.

Table 1: The inclusion and exclusion criteria

INCLUSION CRITERIA	EXCLUSION CRITERIA
Patients diagnosed with Type 2 Diabetes mellitus and under medication.	Patients with other systemic diseases coexisting with diabetes mellitus, a history of radiotherapy for head and neck cancer, and a history of salivary gland disorders.
Patients falling under the age group 45 to 65 years.	Patients diagnosed with type 1 diabetes mellitus and gestational diabetes.

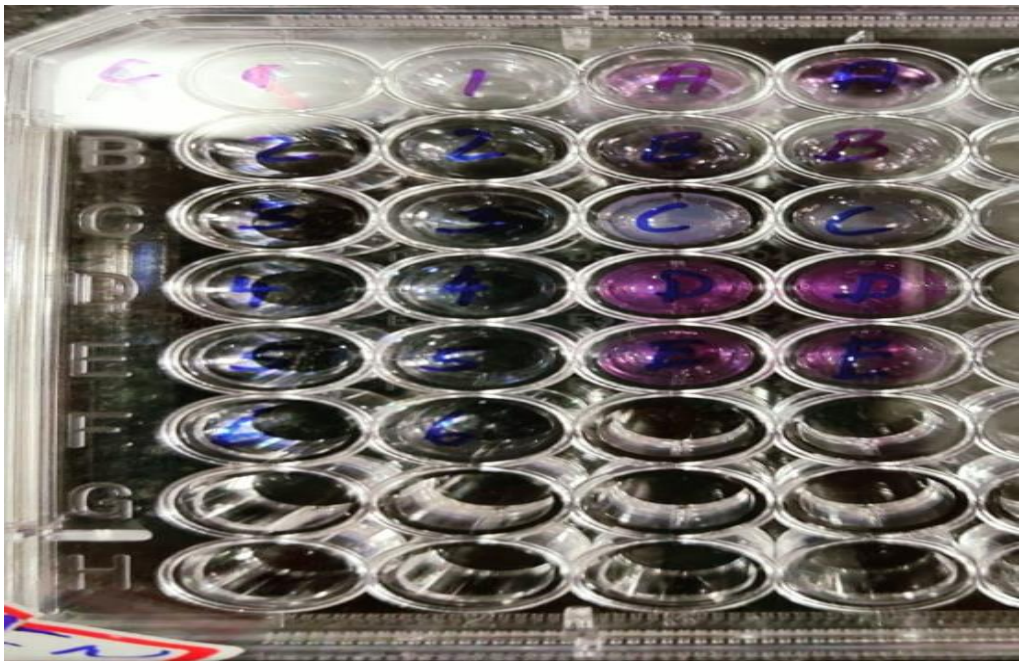
Laboratory assessment:

Sample collection:

Approximately 3 ml of unstimulated whole saliva was collected by allowing participants to drool naturally into a clean, sterile, calibrated universal bottle. The collected sample was centrifuged at the rate of 5000 rpm for five minutes. Then, the separated fluid part was transferred to fresh Eppendorf bottles and stored at a temperature of 20^{o}c until analyzed.

Hydrogen peroxide analysis:

In the collected salivary samples, butylated hydroxytoluene levels were assessed by the indirect method of using a hydrogen peroxide reagent. The concentration of hydrogen peroxide (H_2O_2) was quantified using the method described by Wolff in 1994 [12], which is centered around the peroxide-mediated oxidation of ferrous ions (Fe^{3+}). This oxidation is followed by the reaction of Fe^{2+} with xylenol orange, forming a complex known as Fe^{3+} - xylenol orange.



Experiments were performed at room temperature (98.0°F). Typically, in a 96-well, flat-bottomed polystyrene microtiter plate, a 100µl hydrogen peroxide standard solution was serially diluted. As the standard blank, the diluting medium hydrogen peroxide was utilized. After loading the plate with salivary samples (50µl), hydrogen peroxide (50µl) was added to each well, followed by the Wolff technique (1994) [13]. The absorption intensity was measured at wavelengths of 492 nm and 630 nm. The salivary levels of hydrogen peroxide were determined by comparing the absorbance in the samples to that of standard solutions of H_2O_2.

Nitric oxide analysis:

Nitric oxide concentration in saliva was determined using Griess reagent (sulphanilamide and N-1-napthyethylenediamine dihydrochloride). The assay is based on a reaction that utilizes sulphanilamide and N-1-napthyethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitrite forms colored chromophores with reagent, with absorbance at 490nm and 630nm wavelength. The production of nitrite was quantified by comparing the result with absorbance with standard solutions of sodium nitrite.

The absorption intensity was measured using a Microplate ELISA Plate Analyser, which is automatic, with a linear measurement range from 0.000 to 3.500 absorbance units(A) having four optional wavelengths of 405,450,492,630nm.

Interpretation:

The colorimetric assay is based on the fusion of the reagent with the compounds assessed in the salivary sample. The darker the color of the sample, the more Hydrogen peroxide and nitric acid are left in the evaluated samples, and the lighter the color, the lesser the amount of estimated salivary marker. Then, the ELISA plate was fed into the spectrophotometer (Robonik, Elisa plate analyzer) and assessed at wavelengths of 492 nm and 630 nm, and then the absorbance values were evaluated. The decrease in absorbance rate is denoted by lower values or vice versa. (Figure 3)

Figure 3: Laboratory analysis of saliva samples' hydrogen peroxide and nitric acid levels. White denotes salivary hydrogen peroxide samples, whereas pink denotes nitric acid samples

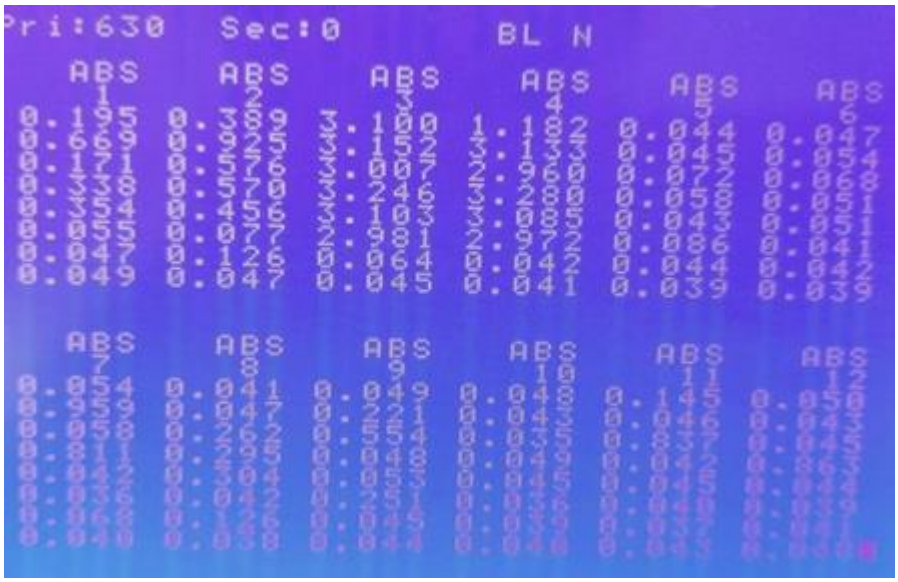


Figure 4: Absorbance values in the salivary samples displayed in the monitor of the ELISA plate analyser

The absorption intensity was measured at 492 nm and 630 nm wavelengths for hydrogen peroxide and nitric oxide. Absorbance values range from 0.000 to 3.500 absorbance units(A). (Figure 4)

STATISTICAL ANALYSIS:

Non-parametric statistical tests were utilized to assess the results because the data evaluated in this study did not adhere to a normal distribution. From the acquired data, an unpaired *t*-test was performed using SPSS 23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp), a statistical software for social sciences. The obtained data was presented in a summarized format as Mean ± standard deviation (SD) of the mean. To compare the means between group 1 and group 2, an independent t-test was conducted (as outlined in Table 1). Statistical significance was determined by setting the threshold at $p \leq 0.05$, indicating that results with significance were attributed to *p*-values that were equal to or less than 0.05. Pearson's correlation test was performed for correlation between hydrogen peroxide, nitric acid, and RBS values.

RESULTS:

The study participants (n=40) were gender-matched and divided into controlled and uncontrolled diabetic groups (Table 2). The mean age of controlled and uncontrolled diabetic patients in the current study are 55.5±1.20 years and 57.5±1.35 years respectively (Table 3). The Mean±SD values of hydrogen peroxide among controlled and uncontrolled diabetic patients are 2.98±0.12 and 2.99±0.11, respectively. No statistically significant difference (p=0.867) was found in the hydrogen peroxide values between these two groups. The mean±SD values of Nitric Oxide (NO) in the controlled group were found to be 2.99±0.15. The uncontrolled group had a mean±SD value of NO as 2.64±0.96. When comparing the Mean±SD values of NO among controlled and uncontrolled diabetic patients, no statistically significant difference (p-0.419) was found. (Table 4, Graph 1)

Table 2: Gender distribution of patients

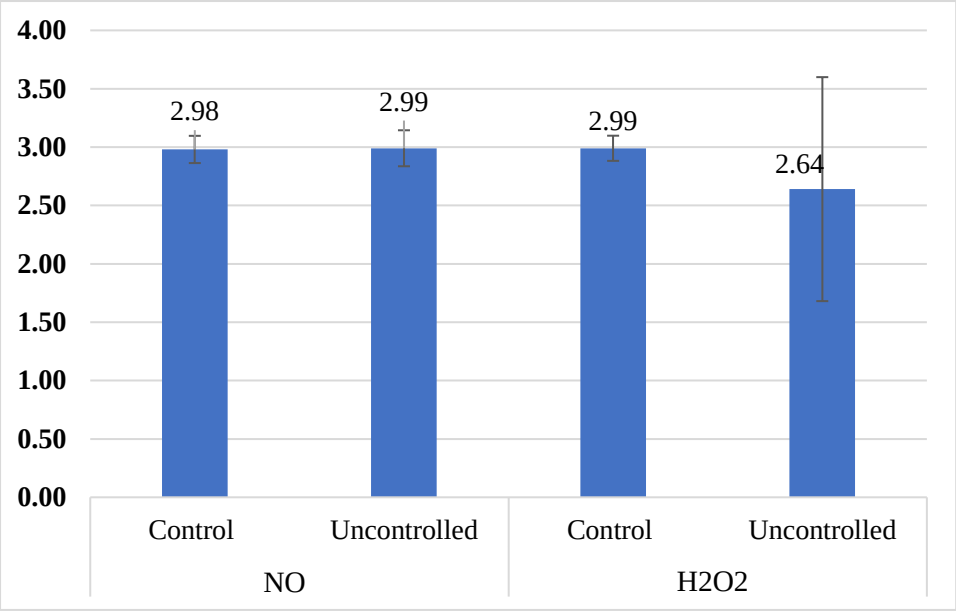
Study population	Total of patients (n=40)	Male	Female
Controlled diabetic group	20	10	10
Uncontrolled diabetic group	20	10	10

Table 3: Mean and standard deviation of age of the participants

Study population (n=40)	Age
Controlled group (n=20)	55.5±1.20 years
Uncontrolled group (n=20)	57.5±1.35 years

Table 4: Distribution of Hydrogen Peroxide (H2O2), Nitric Oxide (NO)

		Mean±SD	t value	p-value
Hydrogen Peroxide	Controlled	2.98±0.12	0.172	0.867
	Uncontrolled	2.99±0.11		
Nitric Oxide	Controlled	2.99±0.15	0.877	0.419
	Uncontrolled	2.64±0.96		



Graph 1: Distribution of Hydrogen Peroxide (H2O2), Nitric Oxide (NO) Mean±SD values among the study population

Graph 1 depicts the mean±SD values of Hydrogen Peroxide and Nitric Oxide among the controlled and uncontrolled diabetic patients. The X-axis indicates the study groups, and the Y-axis indicates the mean absorbance values of Nitric Oxide and Hydrogen Peroxide. The Hydrogen Peroxide in uncontrolled diabetic groups is found to have a more comprehensive standard deviation when compared to other groups.

Tables 5 show the correlation between Hydrogen Peroxide (H2O2), Nitric Oxide (NO), and random blood sugar values in the controlled and uncontrolled diabetic group. When Pearson's correlation test was performed, a positive correlation was found between H2O2 and RBS values in the controlled diabetic group, and this relationship was statistically significant (p-0.01). No statistically significant correlation was found between NO and RBS values in controlled and uncontrolled groups.

Table 4: Correlation between Hydrogen Peroxide and glycaemic values among controlled and uncontrolled diabetic patients

	Random Blood Sugar in Controlled	Hydrogen Peroxide in Controlled	Random Blood Sugar in Uncontrolled	Hydrogen Peroxide in Uncontrolled
Random Blood Sugar in Controlled	1	0.910*	0.894*	-0.333
Hydrogen Peroxide in Control	0.910*	1	0.950**	-0.153
Random Blood Sugar in Uncontrolled	0.894*	0.950**	1	0.048
Hydrogen Peroxide in Uncontrolled	-0.333	-0.153	0.048	1

**Correlation is significant at p<0.01

*Correlation is significant at p<0.05

Table 5: Correlation between Nitric Oxide levels and glycaemic values among controlled and uncontrolled diabetic patients.

	Random Blood Sugar in Controlled	Nitric Oxide in Controlled	Random Blood Sugar in Uncontrolled	Nitric Oxide in Uncontrolled
Random Blood Sugar in Controlled	1	0.692	-0.030	0.596
Nitric Oxide in Controlled	0.692	1	-0.345	0.215
Random Blood Sugar in Uncontrolled	-0.030	-0.345	1	0.675
Nitric Oxide in Uncontrolled	0.596	0.215	0.675	1

DISCUSSION:

Oxidative stress is a mediator of insulin resistance, the development of glucose intolerance, and the establishment of diabetes mellitus, subsequently favoring cell injury leading to diabetic complications. Beta cells are more vulnerable to reactive oxygen species (ROS) because they have lower concentrations of antioxidant enzymes. Thus, oxidative stress can disrupt mitochondrial function and drastically lower insulin production [15]. All substances that prevent or delay substrate oxidation, even at deficient concentrations, can be considered antioxidants. The body uses various mechanisms to produce antioxidants, either endogenous or exogenous. It helps to prevent diseases by shielding cells from their toxic effects and neutralizing the elevated levels of free radicals [16].

Diabetes mellitus is associated with increased prevalence of oral disease and reactive oxygen species have been implicated in their pathogenesis (Indurkar, Maurya, 2016; Kadir et al 2002). In this study, found that significant higher salivary levels of H_2O_2 and NO in diabetic patients. Leoncini et al and Awatef et al found that higher levels of H_2O_2 using blood samples from diabetic patients compared to controlled group. Astaneie et al, found that elevated NO was found in plasma and saliva of diabetic patients. Furthermore, due to the inflammatory sequela associated with the etiology of diabetes mellitus, salivary levels observed in diabetes patients

could be indicative of ongoing inflammation in the oral cavity. The increased prevalence of oral disorders in patients with diabetes mellitus supports the need for increased awareness of the association between diabetes and oral health.

The study of Abadi et al. reported no significant association between NO levels and blood glucose and HbA1c. This study demonstrated a significant association between diabetes mellitus and salivary H_2O_2 and NO levels, although ROC analysis indicated a statistically significant area under the curve (AUC) for salivary with sensitivity and specificity of 30.5% and 9.7% respectively. This indicates that salivary level in diabetic patients would be a better marker than salivary H_2O_2 for distinguishing between diabetes patients. Increased NO synthesis is a frequent finding in periodontal diseases, radicular cysts, and apical infections (Paquette, 2000).

LIMITATION AND RECOMMENDATION:

The study had limitations, including limited sample size, single-centered, and the use of random blood sugar levels instead of the Oral Glucose Tolerance test or HbA1C, which are more precise markers for assessing diabetes control. More extensive research with a multicentre analysis considering a larger sample size, using HbA1c/OGTT as a preferable choice of screening, is recommended. Future studies should look at the relationship between butylated hydroxytoluene and ascorbic acid levels in saliva and serum in people with diabetes mellitus to establish saliva as an alternative helpful marker to serum for tracking the disease

CONCLUSION:

This study demonstrated significantly higher levels of salivary H_2O_2 and NO in diabetes patients, potentially indicating a higher prevalence of oral diseases in this group.. This is a need for increased awareness of the association between diabetes and oral health with the integration of oral health management into the management of diabetes mellitus given the association between salivary levels of H_2O_2 and NO with oral mucosa diseases.

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