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Original Article

Markers of Oxidative Stress in the Saliva of Type 2 Diabetic Patients

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Abstract: *Objective*: Diabetes mellitus is associated with increased prevalence of oral diseases for which reactive oxygen species have been implicated. The saliva contains protective antioxidants which statutorily curtail these destructive oxygen molecules. A functional compromise of the antioxidants may precipitate oxidative stress leading to the increased oral disease susceptibility. However, salivary markers of oxidative stress have not been sufficiently studied in the diabetics. *Methods:* A total of 166 adults were recruited for this study. They comprised of 95 Type 2 diabetic patients and 71 healthy non-diabetic controls. About 3 ml of unstimulated saliva samples were collected from participants and processed, levels of salivary H_2O_2, NO and MDA were measured using spectrophotometry method and compared between the two groups. Data was analysed using t-test, logistic regression and receiver operating characteristics (ROC) with statistical significance set at $p<0.05$. *Results:* Salivary H_2O_2 ($p=0.024$) and NO ($p=0.002$) were significantly higher in the diabetic patients when compared to the healthy non-diabetic control group. Binary logistic regression showed that patients with Type 2 diabetic mellitus are more likely to have elevated salivary H_2O_2 (OR= 1.013; $p=0.025$) and NO (OR=1.016; $p=0.003$) levels. ROC analysis showed statistically significant performance of salivary NO levels in distinguishing between T2DM patients and healthy controls. *Conclusions:* Higher levels of oxidative stress markers including salivary H_2O_2 and NO in the diabetic groups could be a pointer to the characteristic high prevalence of oral diseases in diabetes mellitus, given that oxidative stress predisposes to disease vulnerability. This calls for increased attention to oral health in diabetes management to minimise co-morbidity.

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INTRODUCTION

The oral mucosa is one of the key body sites with major pathologic sequalae of diabetes mellitus (DM). Clinical conditions including halitosis, lichen planus, gingivitis, periodontitis, angular cheilitis etc. occur more frequently in diabetic patients compared to the general population (Al-Maskari, Al-Maskari, & Al-Sudairy, 2011). Although DM which is a chronic disorder of intermediary metabolism, characterised by chronic hyperglycaemia, is associated with generalised immune dysfunction (Berbudi, Rahmadika, Cahyadi, & Ruslami, 2019), several studies have equally implicated reactive oxygen species in both the pathogenesis of the disease and the development of complications including oral diseases (Maritim, Sanders, & Watkins, 2003). These free radicals which result from glucose oxidation, non-enzymatic glycation of plasma proteins and subsequent degradation of such proteins, are injurious to cells, organelles and lipid-rich cell membranes thereby predisposing to the increased inflammatory and infective diseases in the oral cavity (Maritim et al., 2003).

Furthermore, DM is associated with grossly depleted levels of antioxidant enzymes including catalase, glutathione peroxidase, superoxide dismutase and water-soluble vitamins (Trivedi et al., 2014). These agents which primarily function as free radical scavengers are consumed at a higher rate than they are produced in affected patients (Bajaj & Khan, 2012). Thus, low levels encourage the proliferation of reactive oxygen species which are very toxic to cells either directly or through their metabolites (Demircan et al., 2008; Rehman & Akash, 2017). Hydrogen peroxide (H_2O_2), for example, causes irreparable damages to DNA, RNA and lipids (Asmat, Abad, & Ismail, 2016). Also, nitric oxide (NO) which plays a vital role in the relaxation of blood vessels causes vascular injuries in DM because of excessive production (Desmond Jay, Hirofumi Hitomi, & Griendling, 2006; Hoang, Padgham, & Meininger, 2013; Pitocco, Tesauro, Alessandro, Ghirlanda, & Cardillo, 2013; Pitocco et al., 2010). Consequently, there is a significant elevation of malondialdehyde (MDA), a prominent product of lipid peroxidation, and this is a common finding in diabetes mellitus (Nakhjavani et al., 2010; Slatter, C.H. Bolton, & Bailey, 2000; Zygula et al., 2019).

Most of the available diabetes studies interrogating the role of oxidative stress in systemic infections were done in the serum (Bhutia, Ghosh, Sherpa, Pal, & Mohanta, 2011; Blasiak et al., 2004; Gallou et al., 1993). The saliva, which is the main fluid of the oral cavity and has a rich antioxidant capacity, has hardly been sufficiently studied in the diabetics. The saliva has however proven to be resourceful in providing useful information regarding systemic disease. Salivary antioxidant status and lipid peroxidation have been studied in cigarette smokers to determine their contribution to the development of periodontitis (Kosoko, Olayanju, Rahamon, & Arinola, 2017); salivary cortisol have been used to screen, diagnose and monitor patients with Cushing' s disease (Bozovic, Racic, & Ivkovic, 2013; Papanicolaou, Mullen, Kyrou, & Nieman, 2002); and salivary markers of oxidative stress have been studied in several other conditions like ageing, dementia, and acute asthma (Bentur, Mansour, Brik, Eizenberg, & Nagler, 2006; Choromanska et al., 2017; Maciejczyk, Zalewska, & Ladny, 2019). In this study, we aimed to measure salivary H_2O_2, NO and MDA in diabetic patients in comparison to healthy non-diabetic controls.

MATERIALS AND METHODS

Participants

This was a cross-sectional study involving adult Type 2 diabetic patients and healthy non-diabetic control group. The diabetic patients were recruited from the medical outpatient clinic of the University College Hospital, Ibadan, while healthy non-diabetic members of staff served as the control group. A questionnaire was used to obtain demographic and clinical information from the patients and anthropometric measurements including weight and height were taken of all participants. Figure 1 shows the

flow diagram for diabetic patients' recruitment. Ethical approval was obtained from the University of Ibadan/ University College Hospital Research Ethics Committee.

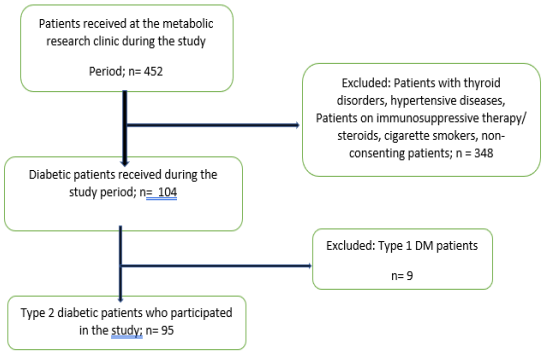

Figure 1: Flow diagram for patients in the study

Sample Collection

About 3 ml of unstimulated saliva samples were collected from all participants after rinsing their mouth with clean water, samples were collected passively in a clean universal bottle by making the participants drool. The samples were centrifuged at a speed of 3000 radian per minute for five minutes, the supernatant transferred to clean Eppendorf bottles and stored at -20 ^{0}C until the time of analysis. Levels of H_2O_2, NO and MDA were measured in all saliva samples. About 3 ml of blood samples were also drawn from all participants into a fluoride oxalate bottle for measuring fasting plasma glucose in the diabetic patients and random plasma glucose in the healthy non-diabetic participants. Samples were centrifuged at a speed of 4000 radian per minute for five minutes, the plasma transferred into a clean plain bottle and stored at – 20 ^{0}C until the time of analysis. The study lasted for three months.

Laboratory Analysis

Malondialdehyde was estimated using the thiobarbituric acid reactive substance (TBARS) test (Bernheim et al., 1947). In this assay, free MDA present in the sample reacts with Thiobarbituric Acid (TBA) and generates MDA-TBA adduct with absorbance maximum at 532nm wavelength. Salivary MDA was determined by comparing absorbance with MDA standard.

Hydrogen peroxide concentration was determined as described by Wolff (1994). The assay is based on peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange to form Fe^{3+}-xylenol orange complex with absorbance maximum at 560nm wavelength. Salivary H_2O_2 was determined by comparing absorbance with standard solutions of H_2O_2.

Nitric oxide concentration in saliva was determined using Griess reagent (Sulpanilamide and N-1-napthyethylenediamine dihydrochloride). The assay is based on a reaction that utilizes sulpanilamide and N-1-napthyethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitrite forms coloured chromophore with reagent, with absorbance maximum at 540nm wavelength. The production of nitrite was quantified by comparing the result with absorbances of standard solutions of sodium nitrite.

Plasma glucose levels in both the patients and the control group were determined using the glucose oxidase method. The assay is based on enzymatic oxidation of glucose into gluconic acid and hydrogen peroxide, which is by a chromogenic oxygen acceptor in the presence of a peroxidase. Glucose concentration is determined by relating the optical density of sample at 500nm to that of a glucose standard.

Statistical Analysis

The effect of the reactive oxygen species was determined by comparative analysis of data between the diabetic patients and healthy non-diabetic adults. Data was summarized as Mean±SEM. Mean comparisons between cases and controls were carried out using Student t-test and test of associations were carried out using binary logistic regression. Area under curve, and best estimates of specificity and sensitivity were evaluated with the Receiver Operator Characteristics (ROC) curve. Statistical significance was set at p≤0.05. Data obtained were analysed using statistical package for social sciences (SPSS) version 20.0 (IBM Inc., USA).

RESULTS

A total of one hundred and sixty-six participants were recruited for this study. While 95 (57.2%) of them were known diabetic patients on treatment, the remaining 71 (42.8%) participant were healthy non-diabetic adults. There were no significant differences in the age and weight between the diabetic and the healthy controls, though the BMI was significantly higher in the diabetic patients.

There was no significant different in the levels of salivary MDA between the two groups (p=0.845), however salivary H_2O_2 (p=0.024) and NO (p=0.002) were significantly higher in the diabetic patients compared to the healthy non-diabetic control group (Table 1).

Table 1: Comparison of mean age, anthropometric parameters, salivary oxidants and MDA levels in Type 2 diabetes patients and non-diabetic controls

Variable	Cases (n=95)	Control (n=71)	t	P
Age	58.89±1.41	55.48±1.10	1.793	0.075
Gender (Male)	31 (32.6)[a]	49 (71.0)[a]	[b]23.568	0.000*
Body weight	73.99±1.76	71.84±1.58	0.895	0.372
Height	1.59±0.01	1.67±0.01	4.926	0.000*
BMI	29.20±0.67	26.03±0.74	3.092	0.002*
Salivary MDA	1.14±0.05	1.13±0.05	0.164	0.845
Salivary H_2O_2	62.90±3.54	52.03±2.81	2.282	0.024*
Salivary NO	42.13±4.43	24.18±2.73	3.181	0.002*

*Significant at p<0.05, BMI= body mass index , [a]frequency (percentage), [b]Chi-Square

Binary logistic regression showed that patients with Type 2 diabetic mellitus are more likely to have elevated salivary H_2O_2 (OR= 1.013; p=0.025) and NO (OR=1.016; p=0.003) levels (Table 2). ROC analysis showed statistically significant performance of salivary NO levels in distinguishing between T2DM patients and controls with AUC of 0.633, sensitivity and specificity of 63.2% and 50.7% respectively (Table 3; Figure 2). There were no significant associations between salivary MDA, H_2O_2 and NO levels with fasting plasma glucose level, duration of T2DM as well as comorbidity in T2DM patients (Tables 4).

Table 2: Binary logistic regression analysis of association between Type 2 Diabetes Mellitus (T2DM) and salivary oxidants and MDA levels

Variable	B	P	Exp β	95% CI
Salivary MDA	-0.140	0.720	0.869	0.404-1.870
Salivary H_2O_2	0.013	0.025*	1.013	1.002-1.025
Salivary NO	0.016	0.003*	1.016	1.006-1.027

*Significant at p<0.05

Table 3: ROC Analysis of salivary MDA, H_2O_2 and NO levels

Variable	AUC	95% CI	p	Sensitivity	Specificity
Salivary MDA	0.507	0.418-0.596	0.879	50.5	54.9
Salivary H_2O_2	0.588	0.500-0.676	0.052	51.6	49.3
Salivary NO	0.633	0.548-0.719	0.003*	63.2	50.7

*Significant at p<0.05

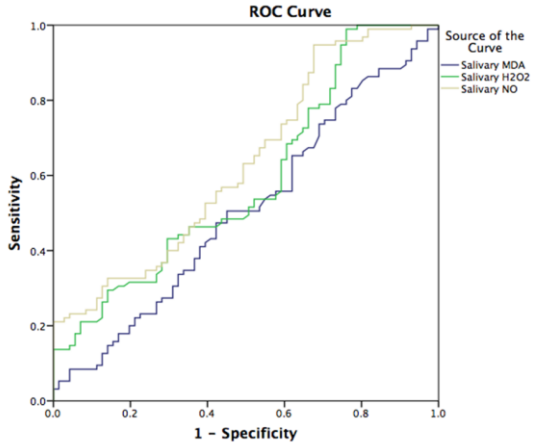

Figure 2: Receiver operating characteristics (ROC) curve of salivary MDA, H_2O_2 and NO levels

Table 4: Binary logistic regression analysis of association between fasting plasma glucose level, DM duration and Co-morbid condition, and salivary oxidants and MDA levels among Type 2 Diabetes Mellitus (T2DM) patients

Variable	B	p	Exp β	95% CI
Fasting plasma glucose				
Salivary MDA	-1.058	0.139	0.347	0.085-1.411
Salivary H_2O_2	-0.002	0.805	0.998	0.982-1.014
Salivary NO	0.010	0.110	1.010	0.998-1.022
DM duration				
Salivary MDA	-0.122	0.826	0.885	0.297-2.634
Salivary H_2O_2	-0.005	0.515	0.995	0.981-1.009
Salivary NO	0.007	0.161	1.007	0.997-1.018
Co-morbid condition				
Salivary MDA	-0.013	0.980	0.987	0.370-2.634
Salivary H_2O_2	0.003	0.643	1.003	0.991-1.015
Salivary NO	-0.005	0.350	0.995	0.985-1.005

DISCUSSION

Diabetes mellitus is associated with increased prevalence of oral disease, and reactive oxygen species have been implicated in their pathogenesis (Indurkar, Maurya, & Indurkar, 2016; Kadir et al., 2002). In this study, we found significantly higher salivary levels of H_2O_2 in diabetic patients compared to the controls. This agrees with the reports of Leoncini et al (Leoncini, Signorello, Piana, Carrubba, & U, 1997) and Awatef et al (Awatef, Abdelh, & Asma, 2013), who found higher levels of H_2O_2 using blood samples from diabetic patients compared to control groups. The impaired secretion of antioxidants in the diabetics have been implicated in the uncontrolled production of H_2O_2 and its deleterious effects (Thomas, Ramesh, Suresh, & Prasad, 2013; Trivedi et al., 2014). This could explain increased prevalence of oral disorders in patients with diabetes mellitus and supports the need for increased awareness of the associations between diabetes and oral health.

Elevated salivary NO levels was implicated in the development of mucosa erosion and ulceration (Sunitha & Shanmugam, 2006). In this study, we found significantly higher levels of salivary NO in the diabetics compared to the healthy non-diabetic controls. This is similar to findings in a previous study where elevated NO was found in plasma and saliva of diabetic patients (Astaneie et al., 2005). The increased secretion was attributed to a reactive response to hyperglycaemia, a cardinal fulcrum in diabetes mellitus (Ceriello et al., 2002; Cosentino, Hishikawa, Katusic, & Lüscher, 1997). Furthermore, due to the inflammatory sequela associated with the etiology of diabetes mellitus, increased salivary NO levels observed in diabetes patients could be indicative of ongoing inflammation in the oral cavity. Although excessive salivary NO has been previously implicated as a physio-pathological regulator in modifying oral mucosal disease, we posit that inflammation within the oral cavity could be the underlying mechanism that links salivary NO with oral mucosal disease.

This study demonstrated a significant association between diabetes mellitus and salivary H_2O_2 level (OR=0.013; p=0.025) and salivary NO level (OR=0.016; p=0.003), but not salivary MDA (OR= -0.140; p= 0.720), although ROC analysis indicated a statistically significant area under curve (AUC) for salivary NO with sensitivity and specificity of 63.2% and 50.7% respectively. This indicates that salivary NO would be a better marker than salivary H_2O_2 for distinguishing between diabetes patients and non-diabetic controls. It has also been suggested to be a good predictor of xerostomia in diabetic patients (Abadi, Koopaie, & Montazeri, 2020). The low specificity of salivary NO observed in this study may be due to varying degrees of oral health in the participants which may have confounded the result. Increased NO synthesis is a frequent finding in periodontal diseases, radicular cysts and apical infections (Paquette & Williams, 2000; Takeichi et al., 1999). Elevated levels have also been reported in periodontitis, benign diseases and malignant tumours of the salivary glands, including squamous cell carcinoma(Bentz, Haines, Hanson, & Radosevich, 1998; Daghigh, Borghaei, Thornton, & Bee, 2002). Further studies of salivary NO levels in diabetic patients with predetermined oral health status would make for better understanding of the relationship between diabetes mellitus and salivary NO.

There were no significant associations between salivary H_2O_2, NO and MDA levels with fasting plasma glucose level, duration of T2DM and comorbidity in T2DM patients in this study. This agrees with the study of Abadi *et al*. who reported no significant association between salivary NO level and blood glucose and HbA1c (Abadi et al., 2020). Although this indicates that salivary levels of H_2O_2, NO and MDA may not be directly influenced by degree of hyperglycaemia, duration of illness or presence of comorbidities. It could also be because of anti-diabetic therapy or lifestyle modifications adopted by diabetes patients.

There are some limitations to this study. Salivary levels of antioxidant markers should have given more credence to this study; however, several studies have shown that serum levels are reduced in diabetes (Trivedi et al., 2014). Serum HbA1c which is a marker of long-term glycaemic control would have also been evaluated rather than fasting plasma glucose to determine how the salivary markers changed with glycaemic control.

CONCLUSION

This study showed that the levels of salivary H_2O_2 and NO were significantly higher in diabetes patients than non-diabetic controls and could be a pointer to the high prevalence of oral diseases in diabetes. There is a need for increased awareness of the associations between diabetes and oral health with integration of oral health management into the management of diabetes mellitus given the associations between salivary levels of H_2O_2 and NO with oral mucosa diseases.

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Conflict of interest

Authors have no conflict of interest to declare

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