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Original Article

Dentistry Section

Salivary Metallothionein Level in Type 2 Diabetes Mellitus Patients with and without Chronic Periodontitis: A Cross-sectional Study

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ABSTRACT

Introduction: Metallothionein (MT) is a cysteine-rich protein involved in cellular defence mechanisms, including the regulation of oxidative stress and immune responses. Oxidative stress is a common link between Diabetes Mellitus (DM) and periodontitis, two chronic inflammatory conditions with a bidirectional relationship. Although MT has been widely studied in diabetes, its role in periodontal disease and the combined impact of both conditions on MT expression remain unclear.

Aim: To evaluate the levels of MT in the saliva of diabetic patients with and without periodontitis.

Materials and Methods: The present cross-sectional study was conducted at Sri Ramachandra Dental College and Hospital, Chennai, Tamil Nadu India, over two months (February to March 2021), involving 76 participants divided equally into four groups (n=19): healthy controls, Chronic Periodontitis (CP), Type 2 Diabetes Mellitus (T2DM), and T2DM with periodontitis (T2DM+CP). Clinical periodontal parameters-gingival bleeding index, Plaque Index (PI), Probing Pocket Depth (PPD), Clinical Attachment Level (CAL), and Periodontal Inflamed Surface Area (PISA)-were recorded. Salivary metallothionein levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA). Data were analysed using Statistical Package for Social Sciences (SPSS) software with the Shapiro-Wilk test, one-way Analysis of Variance, Dunn's post-hoc test, and Spearman's correlation ($p<0.05$).

Results: The T2DM+CP group showed significantly higher periodontal parameters and glycaemic levels compared to the other groups ($p<0.05$). Salivary MT levels were also highest in this group and were statistically significant, with a p-value of <0.05 when compared to the other groups. A positive correlation was observed between MT and glycaemic levels, and a moderately positive correlation (r-value of 0.507) was noted between MT levels in patients with T2DM and periodontitis.

Conclusion: Elevated salivary MT levels in diabetic patients with periodontitis suggest a potential role as a biomarker of oxidative stress and disease activity. Further longitudinal and interventional studies are needed to validate MT as a diagnostic and prognostic marker.

Keywords: Antioxidants, Biomarkers, Oxidative stress, Periodontal disease, Saliva

INTRODUCTION

Diabetes Mellitus (DM) and periodontitis are among the two most commonly observed chronic conditions in developing countries like India [1]. Both conditions are known to influence each other bidirectionally, with diabetes affecting periodontal health and periodontitis worsening glycaemic control [2]. Longitudinal studies have demonstrated a two-way relationship between diabetes and periodontitis [3-5]. Various mechanistic links have been proposed to explain this association [6]. One such link is oxidative stress, which plays a significant role in how periodontitis impacts DM [7]. Various molecules release antioxidants to prevent tissue damage in an environment characterised by the overproduction of free radicals [8]. Nevertheless, no single antioxidant can effectively counteract these potent hydroxyl radicals. Metallothionein is gaining attention as an effective radical scavenger in several diseases, including DM, periodontitis, cardiovascular disease, and among smokers [9-13].

Metallothionein is a protein that binds to metals and has a low molecular weight along with a high concentration of cysteine. It plays several roles in maintaining cellular balance, supporting the immune system, and protecting against oxidative stress [14]. It can be induced by various situations, including Zinc (Zn) supplementation. Disulfide bond formation and the concomitant release of Zn under oxidative stress contribute to the functions of MT as a promising antioxidant in diseases, including diabetes and periodontitis [15]. In an experimental study, MT induced by Zn supplementation significantly prevented Streptozotocin (STZ) induced diabetes in a rat model [10]. Furthermore, it has been found that patients who smoke and have periodontitis show a greater concentration of MT compared to non smokers [13].

Metallothionein is upregulated in diabetic individuals to counteract the oxidative burst, thus serving as a predictor of Reactive Oxygen Species (ROS) induced inflammatory phenomena [16]. Conversely, periodontitis is also a chronic inflammatory disease associated with oxidative bursts [17]. A biologically plausible mechanism linking diabetes and periodontitis has been established; however, further investigation is needed to understand the extent of this relationship and its clinical implications. Based on the current literature, estimating the level of MT in diabetic patients with and without periodontitis would provide a definitive indication of the role of diabetes in initiating and exacerbating periodontitis.

Therefore, this study is the first to investigate the role of MT in periodontal disease and its association with DM by evaluating the levels of MT in the saliva of diabetic patients with and without periodontitis. Understanding the mechanisms that link diabetes and periodontitis could lead to the development of more targeted and effective treatments for diabetic patients suffering from periodontitis. Additionally, the evaluation of biomarkers aids in the early detection and monitoring of periodontal disease progression in diabetic patients, ultimately improving patient outcomes and quality of life.

MATERIALS AND METHODS

The present cross-sectional study was conducted at the Department of Periodontology and General Medicine, Sri Ramachandra Dental College and Hospital, Chennai, Tamil Nadu, India, India, to evaluate differences in periodontal clinical parameters and salivary metallothionein (MT) levels, involving a total of 76 participants screened between February and March 2021. The institute's project review committee reviewed the study protocol and granted ethical clearance from the Institutional Ethics Committee (IEC) (CSP/22/MAR/106/67). Before participating in the trial, each subject provided a signed informed consent agreement. The present study adhered to the principles outlined in the 2013 revision of the Helsinki Declaration.

Sample size calculation: Power calculations were conducted using statistical tools to determine the sample size. With a power of 0.95 and Cohen's fixed effect size of 0.5, along with an alpha error of α (0.05), the sample size for each group was determined to be 19.

Inclusion and Exclusion criteria: Participants were screened and selected based on specific inclusion and exclusion criteria. They were divided into four groups: Healthy group (PH), Periodontitis patients without T2DM (CP), Periodontitis patients with DM (T2DM+CP), and T2DM. For inclusion in the healthy group, participants must have no attachment loss or history of periodontal disease, full mouth bleeding scores below 10%, and a probing depth of less than 3 mm in all teeth. For the periodontitis group, patients were diagnosed based on the Page and Eke criteria for periodontitis [18]. For inclusion in the diabetes group, patients were diagnosed according to Indian Council of Medical Research (ICMR) criteria [19]. Exclusion criteria for all participants included individuals under 18 years of age, current smokers, patients with systemic diseases other than DM, chronic diabetic patients for more than five years, those with a habit of tobacco chewing or cigarette smoking, patients on any medication in the preceding three months other than oral hyperglycaemic drugs, and immunocompromised patients.

Study Procedure

Periodontal clinical examination: The periodontal clinical parameters assessed in this study included the percentage of Bleeding On Probing (BOP), Periodontal Index [20], Probing Pocket Depth (PPD) [21], Clinical Attachment Loss (CAL) [21], and Periodontal Inflamed Surface Area (PISA) [22] for all teeth except the third molars, using a sterilised William's probe and mouth mirror.

Salivary sample collection: An unstimulated whole saliva sample was obtained from the volunteers. The subjects were instructed to maintain a calm posture with their heads lowered and mouths open, allowing saliva to drip passively from the lower lip into graduated sterile tubes. Approximately 0.5-1.5 mL of pooled saliva was collected by the passive drooling method. The collected saliva samples were stored at -20°C until further analysis.

MT Assay [23]: The collected and stored samples were used to evaluate MT levels using a human ELISA kit. This kit employs a two-sided sandwich ELISA to quantify MT in samples. A microplate was coated with a preselected antibody that specifically targets MT. The standards and samples were transferred into the wells using a pipette, where any MT present would be captured by the immobilised antibody. After removing any unattached compounds, the wells were treated with Horseradish Peroxidase Conjugate (HRP)-conjugated human MT detection antibodies. Following washing to eliminate any unbound HRP reagent, a chromogen solution was added to the wells, resulting in colour development that was directly proportional to the quantity of MT bound during the initial stage. The colour development was stopped, and the intensity of the colour was quantified.

STATISTICAL ANALYSIS

The Shapiro-Wilk test was used to determine whether the data distribution was normal. Non-parametric tests were employed since the data was not normally distributed. To evaluate the differences in parameters across the various groups, a one-way ANOVA test was conducted. This was followed by multiple pair comparisons using Dunn's post-hoc test. The relationship between glycaemic status, MT levels, and clinical periodontal markers was assessed using Spearman's correlation coefficient. A p-value of less than 0.05 was considered the threshold for statistical significance.

RESULTS

A total of 76 individuals (35 females and 41 males) were subjected to the estimation of MT levels in saliva, with an age range of 35-60 years (mean age: 40.67±6.98 years).

Participant Characteristics and Clinical Parameters

Demographic data, including the age and gender of the participants, were collected. There was a statistically significant difference in the mean age among the four groups (p<0.001), whereas the gender distribution did not differ significantly between the groups (p=0.997) [Table/Fig-1].

Characteristics	PH	CP	T2DM	T2DM+CP	p-value
Age (years)	43.13±6.45	44.17±5.24	38.09±5.03	35.56±6.45	0.001
Gender (Male/ Female)	10/8	9/8	11/9	11/10	0.997

[Table/Fig-1]: Demographic characteristics for all groups.

The periodontal parameters (PPD, CAL, PISA, BOP%, PI) are summarised in [Table/Fig-2]. There were significant differences in all the periodontal parameters between the groups (PH, CP, T2DM+CP, and T2DM). Both the CP and T2DM+CP groups had significantly higher BOP%, PI, PPD, CAL, and PISA compared to the PH group. The periodontal clinical parameters were found to be highest in the T2DM+CP group compared to the other three groups, and the difference was statistically significant (p<0.001).

Clinical parameter	Group 1- Healthy	Group 2- Chronic Periodontitis (CP)	Group 3-T2DM	Group 4- T2DM+CP	p-value
BOP%	6.4821± 1.46206	25.2632± 1.18144	9.0053± 4.40839	40.8263± 3.04427	<0.001*
PI	0.437± 0.1674	1.468± 0.2868	0.737± 0.4058	2.095± 0.3391	<0.001*
PPD (mm)	1.8947± 0.65784	5.0053± 0.42094	1.6158± 0.61306	6.2842± 0.47289	<0.001*
CAL (mm)	-	6.0105± 0.41217	-	7.5632± 0.93821	<0.001*
PISA (mm^2)	51.8053± 5.77047	175.9684± 6.16802	61.9842± 29.71594	251.9105± 15.97811	<0.001*

[Table/Fig-2]: Comparison of clinical periodontal parameters among four groups (Mean±SD).
*statistically significant

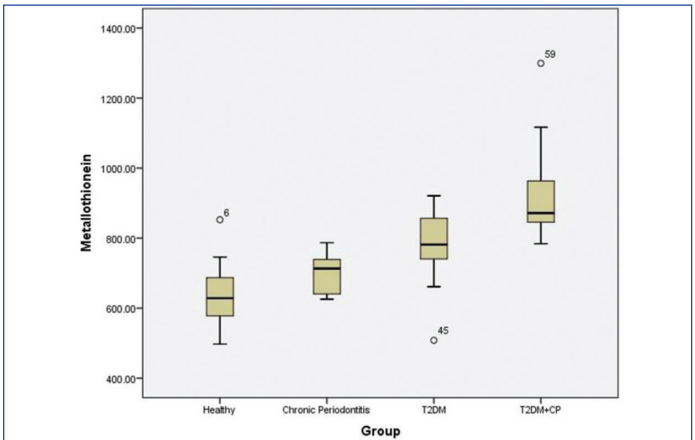
Laboratory findings: The mean FBS and Postprandial Blood Sugar (PPBS) levels were statistically significant between the T2DM+CP group and the T2DM group [Table/Fig-3]. Salivary MT levels were found to be highest in the T2DM+CP group compared with the other three groups (p<0.001) [Table/Fig-4,5]. In pairwise comparisons, significant differences were found between all the groups (p<0.05) [Table/Fig-6].

Parameters	Group	N	Mean	SD	p-value
FBS (mg/dL)	T2DM	19	123.95	6.133	0.005
	T2DM with CP	19	131.16	7.143	
PPBS (mg/dL)	T2DM	19	216.63	14.005	0.015
	T2DM with Chronic Periodontitis (CP)	19	231.47	16.604	

[Table/Fig-3]: Comparison of laboratory parameters between the groups.

Parameter	Group	N	Mean	SD	Mean Rank	p-value
Metallothionein Level (pg/mL)	Healthy	19	639.42	86.375	17.24	<0.001
	Chronic Periodontitis (CP)	19	700.37	51.515	27.79	
	T2DM	19	783.74	99.352	45.74	
	T2DM with Chronic Periodontitis (CP)	19	918.79	128.476	63.24	

[Table/Fig-4]: Comparison of metallothionein levels between the groups.



[Table/Fig-5]: Comparison of MT levels between the groups.

Group I	Group J	Mean difference (I J)	p-value
PH	CP	2.34	0.045
	T2DM	4.67	0.001
	T2DM+CP	6.89	<0.001
CP	T2DM	2.33	0.032
	T2DM+CP	4.55	<0.001
T2DM	T2DM+CP	2.22	0.027

[Table/Fig-6]: Pairwise comparison of MT levels between each groups.

The correlation between clinical parameters and salivary MT levels was also analysed. A statistically significant positive correlation was observed between the levels of MT in saliva and the clinical periodontal parameters in all groups [Table/Fig-7]. A moderately strong positive correlation was found between MT levels and PISA in the T2DM+CP group (r=0.681, p=0.0001) [Table/Fig-8]. MT levels significantly correlated with PPD levels in all four groups (p<0.01) [Table/Fig-9]. A very strong positive correlation was found between MT levels and CAL in the CP group (r=0.942, p=0.0001) [Table/Fig-10]. No significant correlation was found between MT levels and BOP in any of the groups [Table/Fig-11].

Group	Correlation coefficient statistics	PISA
Healthy	Pearson correlation	0.076
	p-value	0.756
	Strength of correlation	*
Chronic Periodontitis (CP)	Pearson correlation	0.388
	p-value	0.101
	Strength of correlation	*
T2DM	Pearson correlation	0.060
	p-value	0.807
	Strength of correlation	*
T2DM+CP	Pearson correlation	0.681**
	p-value	0.0001
	Strength of correlation	Moderately strong positive

[Table/Fig-8]: Correlation between Metallothionien and PISA.
*-Statistically significant; **- highly significant

Group	Correlation coefficient statistics	PPD
Healthy	Pearson Correlation	0.796**
	p-value	0.0001
	Strength of correlation	Strong positive
Chronic Periodontitis (CP)	Pearson Correlation	0.938**
	p-value	0.0001
	Strength of correlation	Very Strong positive
T2DM	Pearson Correlation	0.771**
	p-value	0.0001
	Strength of correlation	Strong positive
T2DM+CP	Pearson Correlation	0.594**
	p-value	0.0001
	Strength of correlation	Moderately strong positive

[Table/Fig-9]: Correlation between Metallothionien and PPD.

The correlation between FBS, PPBS, and MT levels was also examined. A positive correlation was observed between glycaemic status and MT levels (as FBS and PPBS increased, metallothionein levels also increased) [Table/Fig-12].

DISCUSSION

The present study was conducted to evaluate the impact of DM on periodontitis. While numerous other markers have

Spearman's rho		MT level	PISA in sq.mm	PPD in mm	CAL in mm	BOP %	PI
Metallothionein level	Correlation Coefficient	1.000	0.544**	0.583**	0.528**	0.592**	0.578**
	Sig. (2-tailed)		<0.001	<0.001	<0.001	<0.001	<0.001
	N	76	76	76	76	76	76
PISA in sq.mm	Correlation Coefficient	0.544**	1.000	0.808**	0.895**	0.889**	0.834**
	Sig. (2-tailed)	<0.001		<0.001	<0.001	<0.001	<0.001
	N	76	76	76	76	76	76
PPD in mm	Correlation Coefficient	0.583**	0.808**	1.000	0.932**	0.828**	0.803**
	Sig. (2-tailed)	<0.001	<0.001		<0.001	<0.001	<0.001
	N	76	76	76	76	76	76
CAL in mm	Correlation Coefficient	0.528**	0.895**	0.932**	1.000	0.899**	0.877**
	Sig. (2-tailed)	<0.001	<0.001	<0.001		<0.001	<0.001
	N	76	76	76	76	76	76
BOP %	Correlation Coefficient	0.592**	0.889**	0.828**	0.899**	1.000	0.916**
	Sig. (2-tailed)	<0.001	<0.001	<0.001	<0.001		<0.001
	N	76	76	76	76	76	76

[Table/Fig-7]: Correlation between periodontal parameters and MT levels.

Group	Correlation coefficient statistics	CAL
Chronic Periodontitis (CP)	Pearson correlation	0.942**
	p-value	0.0001
	Strength of correlation	Very Strong positive
T2DM+CP	Pearson correlation	0.507*
	p-value	0.0001
	Strength of correlation	Moderately strong positive

[Table/Fig-10]: Correlation between Metallothionien and CAL.

Group	Correlation coefficient statistics	BOP
Healthy	Pearson Correlation	-0.071
	p-value	0.772
	Strength of correlation	*
Chronic Periodontitis (CP)	Pearson Correlation	0.240
	p-value	0.322
	Strength of correlation	*
T2DM	Pearson Correlation	0.221
	p-value	0.362
	Strength of correlation	*
T2DM+CP	Pearson Correlation	0.234
	p-value	0.336
	Strength of correlation	19

[Table/Fig-11]: Correlation between Metallothionien and BOP.

Metallothionein level		FBS	PPBS
Group 3: Spearman's rho	rho	0.805**	0.837**
	p-value	0.0001	0.0001
	N	19	19
Group 4: Spearman's rho	Correlation Coefficient	0.665**	0.726**
	Sig. (2-tailed)	0.002	0.0001
	N	19	19

[Table/Fig-12]: Correlation between FBS, PPBS, and MT levels among four groups (Mean±SD).

been previously studied to identify the link between these two conditions, metallothionein has emerged as an optimal indicator, as it contributes to the severity of periodontitis by influencing the oxidative stress pathway, a common factor in both conditions [24]. Therefore, it was evaluated in this study to determine the impact of DM on periodontitis.

Four groups were included in this study, Periodontally Healthy (PH), CP, T2DM, and T2DM with CP (T2DM+CP). Comparing these groups will provide a definitive indication of the contribution of periodontal disease to oxidative stress and overall health outcomes. The PH and CP groups were included separately to establish baseline differences in oxidative stress markers between healthy individuals and those with CP, independent of diabetes. This allows for a clearer understanding of how periodontitis alone contributes to oxidative stress. Diabetic individuals included in the study had a confirmed diagnosis of T2DM with a relatively recent onset. This criterion was established to eliminate chronic diabetic cases, as periodontitis plays a significant role in those patients. Both diabetes and periodontitis are age-related diseases, and including only recent diabetes cases helps to better isolate the effects of diabetes on periodontitis in the early stages of the disease.

Metallothionein, a protein known for its role in metal ion homeostasis and protection against oxidative stress, serves as a valuable marker in this context. MT's ability to bind to heavy metals and scavenge free radicals makes it crucial in mitigating oxidative damage [24]. By measuring MT levels, this study aimed to elucidate the role of oxidative stress in patients with DM and periodontitis, thereby clarifying the interplay between these conditions.

There are several methods to assess metallothionein levels. In the present study, the ELISA method was employed due to its high sensitivity, specificity, and quantitative capabilities [25]. ELISA allows for precise measurement of MT levels in biological samples, making it an ideal choice for this investigation. Salivary samples were collected for the estimation of metallothionein because saliva is an easily accessible and non-invasive biofluid that reflects local oxidative stress and inflammatory status [26,27].

The salivary levels of MT were significantly higher in patients with CP and T2DM (918.79±128.476 pg/mL) compared with the CP group (700.37±51.515 pg/mL). This finding is particularly notable and unparalleled in any other study, as this is the first study to evaluate salivary MT levels specifically in diabetic patients. However, this result aligns with the study by Yadav VS et al., who reported that when periodontitis and smoking coexist, salivary MT levels are higher in smokers with periodontitis [13]. This is attributed to the role of oxidative stress in both conditions. Similarly, the elevated levels of MT in the T2DM+CP group compared with the CP group suggest that diabetes-induced hyperglycaemia causes the accumulation of oxidative stress products in periodontal tissue. Hyperglycaemia-induced formation of Advanced Glycation End-products (AGEs) can contribute to oxidative stress through various linked pathways, causing cellular malfunction and contributing to the development and progression of a variety of chronic diseases such as periodontitis [28].

In the present study, several clinical periodontal parameters were assessed, including PPD, PI, CAL, BOP, and PISA. CAL is measured as it is considered the gold standard for evaluating the extent of periodontal support loss, providing a reliable indicator of the severity of periodontitis. The PISA Index is intended to quantify the amount of bleeding pocket epithelium and is anticipated to reflect the inflammatory burden associated with periodontitis [29]. According to the present study, clinical parameters such as PPD, CAL, PI, and BOP% were statistically higher in the T2DM+CP group compared to the CP group, reiterating that diabetes worsens periodontal health. Diabetes can modify inflammation-related cytokines such as IL-6, TNF-α, and IL-1β, elevate Reaction Oxygen Species and their association with the oxidative stress index, disrupt the RANKL/OPG axis, stimulate osteoclasts that cause bone resorption, and reduce the activity of polymorphonuclear leukocytes. All of these consequences may expedite the progression of periodontitis [6].

A positive correlation was found between salivary MT levels and clinical periodontal parameters. These relationships imply that both systemic and local levels of MT may serve as potential biomarkers of oxidative stress for periodontitis, as they suggest the inflammatory burden in periodontal tissues. A positive correlation was also observed between MT levels and glycaemic status. As FBS and PPBS levels increase, metallothionein levels rise. This correlation suggests that higher blood glucose levels may stimulate the synthesis of MT, potentially as a protective response against the increased oxidative stress associated with hyperglycaemia, thereby maintaining cellular integrity and function in the face of metabolic disturbances caused by diabetes.

The results of the present study demonstrated that periodontitis and diabetes are associated with an increase in salivary metallothionein levels. The elevated levels observed in diabetic patients with periodontitis suggest a significant contribution of DM to the initiation and progression of periodontal disease. This finding underscores the importance of early detection and management of diabetes to prevent the onset and progression of periodontal disease. Moreover, this study highlights the potential benefits of regular periodontal screening for patients with diabetes. Early identification and treatment of periodontal disease in diabetic patients could reduce

the overall inflammatory burden, which in turn may help in better glycaemic control and management of diabetes. This bidirectional relationship between diabetes and periodontal disease suggests that integrated care approaches targeting both conditions could be beneficial in improving overall health outcomes for patients.

Limitation(s)

Firstly, the study lacked an intervention arm with periodontal therapy, which would have been useful in elucidating the potential changes in MT levels following therapeutic measures, thereby strengthening the evidence for MT as a biomarker. Secondly, the study did not evaluate MT levels after achieving control of diabetes mellitus (DM), which could have helped to determine whether elevated levels of MT are primarily the result of poor glycaemic control or if they persist independently of diabetes management. Lastly, the study was conducted with a relatively small sample size, which may limit the generalisability of the findings.

CONCLUSION(S)

Periodontitis and diabetes were associated with an increase in salivary metallothionein levels. Nevertheless, further research using a larger sample size and an additional intervention group is necessary to validate the significance of metallothionein in periodontal disease. Indeed, a longitudinal study is essential to thoroughly understand the role of metallothionein in the relationship between diabetes and periodontitis.

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