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ORIGINAL ARTICLE

Evaluating the Concentration of MMP-9 and TNF- α in Pulpal Blood at Various Stages of Pulpal Inflammation in Diabetics: A Cross Sectional Study

Sanchi AGRAWAL,[1] Sonali TANEJA,[2] Devicharan SHETTY,[3] Velayutham GOPIKRISHNA,[4] Vidhi Kiran BHALLA[2]

[1]Private Endodontic Consultant, India
[2]Department of Conservative Dentistry and Endodontics, I.T.S Centre for Dental Studies and Research, Ghaziabad, India
[3]Department of Oral Pathology, I.T.S Centre for Dental Studies and Research, Ghaziabad, India
[4]Department of Conservative Dentistry and Endodontics, Sri Ramachandra Institute of Higher Education & Research, Chennai, India

ABSTRACT

Objective: To investigate the concentration of Matrix metalloproteinases-9 (MMP-9) and Tumor necroses factor-alpha (TNF- α) in pulpal blood at various stages of pulpal inflammation in diabetics and to establish the relationship between these two biomarkers.

Methods: 77 patients, each having a tooth with pulpal exposure due to caries presenting with distinct stages of pulpitis were grouped into 2 main study groups as based on the HbA1c Levels-Group 1: Non-Diabetics (Control Group) (HbA1c <5.6%) and Group 2: Type 2 Diabetics (Experimental Group) (HbA1c >6.5%; Random Plasma Glucose >200) and diabetes mellitus with less than 10-year history. Depending on the radiological and clinical diagnosis, these two groups were again sub-divided into 2 subgroups: Sub-group A: Tooth with Symptomatic Irreversible Pulpitis. Sub Group B: Tooth with Reversible Pulpitis. Thus, for comparison purposes, a total of 4 sub-divisions were formed: Sub-group 1A- Non-Diabetic, Symtomatic Irreversible Pulpitis, Sub-group 1B: Non-Diabetic, Reversible Pulpitis, Sub-group 2A: Diabetic, Symptomatic Irreversible Pulpitis, Sub-group 2B: Diabetic, Reversible Pulpitis. Blood sample was collected from pulp chamber after partial pulpotomy was done. The total levels of MMP-9 and TNF-α were assessed by enzyme linked immunosorbent assays (ELISA). Inter-group comparison in levels of MMP-9 and TNF- α were conducted using the Kruskal Wallis test and pairwise comparison was done Mann-Whitney U test.

Results: The inter-group comparison in levels of MMP-9 and TNF- α were conducted using the Kruskal Wallis test and pairwise comparison was done using Mann-Whitney U test. Pearson correlations were conducted in order to investigate correlations between the paired TNF-α and MMP-9 values and also their correlation with the blood sugar levels within the pulp diagnosis groups. MMP-9 and TNF-α levels were significantly higher ($p<0.005$) in irreversible pulpits than reversible pulpits and also in Type-2 diabetics than non-diabetics. Highest level of MMP-9 and TNF-α was found in Group 2A (Diabetic, symptomaticirreversible pulpitis) and lowest in Group 1B (Non-Diabetic, reversible pulpitis). There exists a very high significant positive correlation between MMP-9 & TNF-α ($p<0.005$).

Conclusion: These findings show that the inflammatory mediators MMP-9 and TNF-α are significantly increased in pulpal blood samples of diabetic patients. Also, in diabetic patients diagnosed with reversible pulpitis, higher levels of inflammatory pulpal biomarkers were reported that could compromise the success of Vital Pulp Therapy (VPT) and may necessitate endodontic intervention. MMP-9 and TNF- α were reported to have a positive correlation.

Keywords: Diabetes, inflammatory biomarkers, matrix metalloproteinase-9, pulpal inflammation, tumor necrosis factor-α

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Address for correspondence:
Sonali Taneja
Department of Conservative Dentistry and Endodontics, I.T.S Centre for Dental Studies and Research, Ghaziabad, India
E-mail: drsonali_taneja@yahoo.com

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HIGHLIGHTS

- Inflammatory biomarkers especially MMP-9 and TNF-α are significantly elevated in pulpal blood in teeth with symptomatic irreversible pulpitis especially in diabetic patients.
- Significantly higher levels of inflammatory biomarkers were reported for teeth diagnosed with reversible pulpitis having diabetes that could compromise the success of VPT.

INTRODUCTION

The dental pulp is a connective tissue contained within rigid dentinal walls which may be exposed to a variety of insults (1). Inflammation of pulp is a succession of cellular, vascular and immunological processes (2). First, there is acute inflammatory response followed by phase of advanced inflammation which releases a multitude of pro-inflammatory mediators like Tumor necroses factor - α (TNF-α) which in turn stimulates expression of Matrix metalloproteinases (MMPs) (3).

An accuratebiological pulpal diagnosis is a critical issue that needs to be addressed so as to accurately categorize a particular pulpal disease as reversible or irreversible that may further assist in selecting the course of therapy such as Vital Pulp Therapy (VPT) as opposed to non-surgical root canal treatment (NSRCT) with a high degree of precision (4). The lack of a conclusive indicator for assessment of severityof pulpal inflammation provides hope for exploring the option of newer diagnostic tools (5). Endodontic molecular studies provide tremendous scope especially in identification of biological markers that could help to establish the pulpal inflammation level in teeth diagnosed with symptomatic irreversible pulpitis or reversible pulpitis which may serve as a sound diagnostic tool and further assist in deciding the treatment protocol (Pulpotomy/ NSRCT) (6). Considering the unreliability in diagnosing irreversible pulpitis, planning a NSRCT for every case of pulpal exposure does not seem to be justifiable. In such cases, assessment of inflammatory mediators from pulp could provide a supplementary approach in guiding the treatment plan (7).

Existing data fail to address the issue of association of cytokine expression and the extremity of the clinical symptoms of pulpitis (8). Systemic conditions may influence the coronal pulp response to several irritants and influence the pulp immunity (9). Since diabetic patients tend to have a lower responsiveness and a more complicated interaction of inflammatory as well as immune markers within the dental pulp than non-diabetic patients, the quantitative evaluation of the biological markers may provide an additional diagnostic tool (10). Therefore, this study was done to investigate the concentration of MMP-9 and TNF- α in pulpal blood in various stages of pulpal inflammation in diabetics. The first null hypothesis was that there was no difference between level of biomarkers MMP-9 and TNF-α in different stage of pulpal inflammation. The second null hypothesis was that there was no difference between level of biomarkers MMP-9 and TNF-α in non-diabetics and Type 2 diabetics.

MATERIALS AND METHODS

The study followed the design of "Strengthening the reporting of observational studies in epidemiology" (STROBE Statement). The project protocol was independently reviewed and approved by the institutional ethical committee of ITS Centre for Dental Studies and Research under protocol number ITSCDSR/L/2019/093 on May 23, 2019. All the treatment procedures were performed according to evidence based guidelines and declaration of Helsinki.

Sample Size Estimation

Sample size was approximated based on the results of pilot study (16 samples) which were not included in the main study. The effect size was calculated as 0.38. With 80% power of the study, 5% α error, the final sample size was estimated to be 75 using G*power software, version 3.1.9.7 (Franz Fauluniversitat, Kiel, Germany).

Selection of Sample and Randomisation

Patients belonging to either gender, between the age groups 15-35 years referred to the Department of Conservative & Endodontics between February 2019 to April 2019 were screened for enrolment in the study. Patients with clinical and radiologic evidence of a deep carious lesion approaching close to the pulp space were considered potential for recruitment. Two hundred and fifty eight patients were assessed. Patients were excluded if they suffered from a systemic disease comprising other types of diabetes, pre diabetes or other diseases that might disturb the results (e.g. hyperparathyroidism, hypertension, hyperthyroidism, hepatic disease or chronic renal disease) or if they had conditions that could affect the outcome such as pregnancy, cancer or taking medications such as Non steroidal anti-inflammatory drugs, corticosteroids, antibiotics and statins. Further reasons for exclusion were those teeth that gave a negative response to cold test, internal/external root resorption, condensing apical periodontitis, evidence of an endodontic –periodontal communication, longitudinal root fracture, extraoral swelling, teeth with pulpal blood volume of less than 2.5 µL, patients with compromised immunity. After exclusion, 77 patients were enrolled for analysis (Fig. 1). All the patients enrolled for the study gave a written informed consent.

The study population consisted of 77 patients who were grouped into 2 main study groups based on the Random Plasma Glucose [RPG] and Hemoglobin A1c [HbA1c] levels. For determination of type 2 Diabetes, the random blood samples for serum RPG, and HbA1c levels were taken. Laboratory measurements were RPG and HbA1c. Based on this, participants were divided into two main groups–

Group 1: Non-Diabetics (Control Group) (HbA1C< 5.6%)

For the Non-diabetic group, patients were healthy with absence of history of having diabetes and were 15–75 years of age. A total of 39 patients were selected.

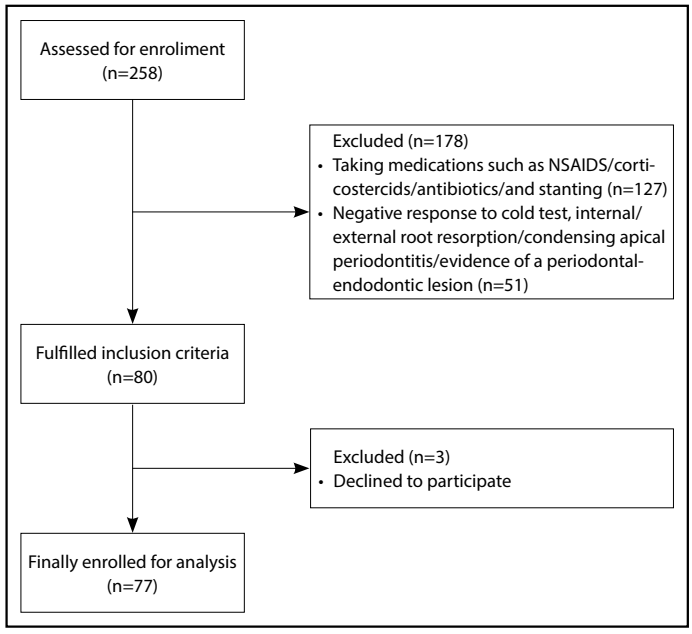

Figure 1. Strobe flowchart

Group 2: Type 2 diabetics (Experimental group) (FPG >200, HbA1C > 6.5%)

For the diabetic group, patients needed to have Type 2 diabetes, Random Blood Glucose level of >200, haemoglobin A1C [HbA1c] >6.5 %, be 15 to 75 years of age and should not have any other systemic disease. A total of 38 patients were selected.

Following this, diagnosis for all the cases was formulated using a systematic approach of history taking, clinical (History and nature of pain, reaction to cold/ hot stimuli, presence or absence of pain on percussion and chewing) and radiographic examination along with pulp sensibility test (Cold test and Electric Pulp Tester). Pulpal and Periapical Diagnosis was defined using American Association of Endodontists (AAE) classification as follows:

Subgroup A (Teeth with symptomatic irreversible pulpitis):

History of continuous moderate to severe pain spontaneous or provoked; pro-longed pain induced by stimulation with carbon dioxide snow; tenderness to percussion or chewing and periodontal ligament (PDL) space widening but no radiolucency periapically.

Subgroup B (Teeth with reversible pulpitis):

Mild clinical symptoms, no history of pain, slightly exaggerated response to sweet or cold stimuli, normal response to cold test, sensitivity to percussion or chewing and no PDL space widening.

This way, the following 4 sub-divisions were formed:

Sub-group 1A- Non-Diabetic with symptomatic irreversible pulpitis

Sub-group 1B: Non-Diabetic with reversible pulpitis

Sub-group 2A: Diabetic with symptomatic irreversible pulpitis

Sub-group 2B: Diabetic with reversible pulpitis.

Clinical Treatment Intervention and Sample Collection

Under rubber dam isolation, a slow-speed round bur was used to remove caries. Peripheral caries wereremoved prior to excavating caries on the pulpal floor. On exposure of pulp, blood samples were obtained from the dental pulp utilizing 10-µL micro capillary tubes which was heparinised (11, 12).

The treatment was then done by the same operator according to the case:

a. For Subgroup A: RCT was performed followed by post-endodontic restoration with composite.

b. For Subgroup B: Partial pulpotomy was performed-The exposed pulp was dressed using ProRoot MTA white (DENTSPLY) over which resin modified GIC liner was given followed by direct composite restoration.

Transport and Storage of Blood Samples

Micro capillary tubes were used to transport pulpal bloodinto labelled reaction tubes (e-cups) consisting saline (50µL). The depth of penetration of the pulpal blood sample in the micro capillary tube was measured and calculated with respect to the micro capillarytubes' maximal volume capacity. Thus, blood sample volume was obtained.

Cool Polar packs were used to transport the e-cups to the laboratory at a temperature of –20°C. Here they were put into centrifugation for 1 minute at1000 rpm and then stored at –20°C in the refrigerator.

MMP-9 and TNF-α Assays

Prior to measuring the quantities of MMP-9 and TNF-α, serum and blood clots were separated by room temperature centrifugation (15 minutes at 1000 rpm). A minimal sample volume of 2.5µL was determined experimentally to permit replicable measurements in duplicates for both MMP-9 and TNF- α. The levels of both these biomarkers were evaluated using *in vitro* enzyme- linked immunosorbent assay (ELISA) kits [TNF- α (Diaclone) & MMP-9 (Qayee-Bio)] by independent observer totally blinded to pulpal inflammatory status of tooth from which sample was taken.

Statistical Analysis

The inter-group comparison in levels of MMP-9 and TNF- α were conducted using the Kruskal Wallis test and pairwise comparison was done using Mann-Whitney U test to check the level of significance, in the differences between these groups. Pearson correlations were conducted in order to investigate correlations between the paired TNF-α and MMP-9 values and also their correlation with the blood sugar levels within the pulp diagnosis groups. $p<0.05$ was considered statistically significant. The data was statistically analyzed using SPSS 21.0. (Statistical package for social sciences; Chicago).

RESULTS

There was absence of markeddifference ($p>0.05$) between the sex and age of the participants in the non diabetic and diabetic groups.

TABLE 1. Mean and standard deviations of TNF- α and MMP-9 value

Sub group no.	Group name	TNF-α (ng/ml)	MMP-9 (ng/ml)
		Mean±SD	Mean±SD
1A (20 patients)	Non-diabetic (Irreversible pulpitis)	11.16±6.54[a]	4.15±0.46[b]
1B (19 patients)	Non-diabetic (Reversible pulpitis)	2.57±1.64	3.23±0.69
2A (19 patients)	Diabetic (Irreversible pulpitis)	22.2±16.62	6.49±2.58
2B (19 patients)	Diabetic (Reversible pulpitis)	9.83±5.21[a]	4.27±0.6[b]

NF- α: Tumor necroses factor-alpha, MMP: Matrix metalloproteinases-9, SD: Standard deviation

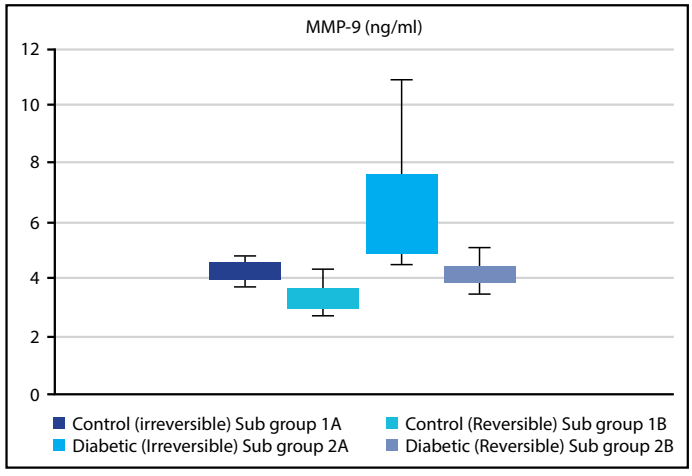

Figure 2. Box plot depicting MMP-9-values (ng/mL) for all 4 diagnosis groups

MMP-9: Matrix metalloproteinases-9

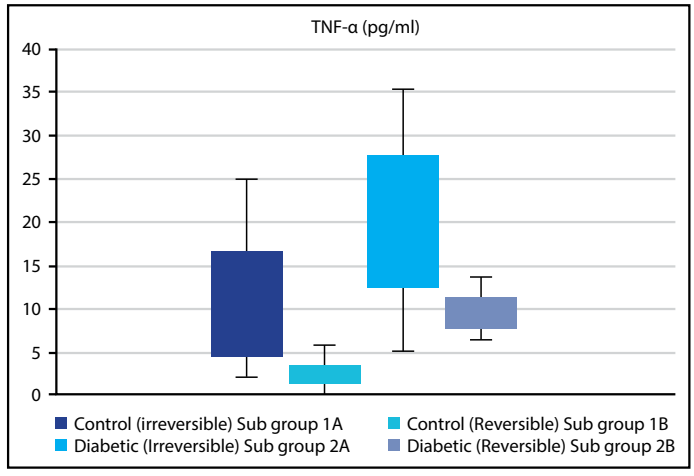

Figure 3. Box plot depicting TNF-α values (ng/mL) for all 4 diagnosis group

TNF-α: Tumor necroses factor-alpha

MMP-9 and TNF-Levels

The mean and Standard Deviation in ng/ml of MMP-9 and TNF-α for all the subgroups has been tabulated in Table 1 and is given as box plot in Figure 2 and Figure 3 respectively. The results for both Kruskal Wallis and Mann Whitney tests show that the Diabeticirreversible pulpitis group (2A) has the highest mean, followed by Non-Diabetic irreversible pulpitis (2B) and then by Diabetic Reversible pulpitis (2B) while the Non-Diabetic reversible pulpitis group (1B) had the lowest mean (Table 2). Pairwise comparison showed a statistically significant difference was present in TNF-α and MMP-9 levels between all the groups ($p<0.05$) except the difference between the values of the Group 1A [Control (irreversible pulpitis)] and Group 2B [Diabetic (reversible pulpitis)]which was non-significant (Table 3).

Interrelation of MMP-9 and TNF-α and their relation with sugar level using Pearson correlation

The blood Sugar Levels (Hb1Ac) showed a positive correlation irrespective of the group assignment ($p<0.001$) with TNF-α levels (positive correlation coefficient of 0.478) and MMP-9 levels (positive correlation coefficient of 0.489).

The MMP-9 and TNF-α levels revealed asignificant correlation irrespective of the groups ($p<0.001$) with a positive correlation coefficient of 0.889 (Table 4).

DISCUSSION

The cascade of tissue inflammation expressed molecules may serve as diagnostic markers for the presence of inflammation (13). The protein markers namely MMPs and TNF-α have been implicated in pulpal inflammation and may have a correlation to the patients systemic health, especially diabetes mellitus (14, 15). The study investigated the correlation of tissue inflammatory markers in patients with pulpal disease.

Results of this study revealed that the mean of TNF- α in irreversible pulpitis group (Subgroup 1) was found to be significantly greater than reversible pulpitis group (Subgroup 2) in all the patients with the Diabetic group showing higher mean values than non-diabetic patients. An inflammatory response by the dental pulp is induced by the bacterial constituents and TNF- α is synthesised as well as released by a broad range of cells involved in the immune-inflammatory process (14, 15). Macrophages/monocytes could be triggered by the localised collection of bacteria and their products in the pulp to produce cytokines (15, 16). The increased number of macrophages than dendritic cells which are present in the advanced phases of caries invasion might be the reason for markedly higher mean of TNF-α in irreversible pulpitis group as compared to its mean in reversible pulpitis group.

Our present finding is in accordance with the study of Pezelj-Ribaric et al. (2002) (17) who stated that the degree of pulpal inflammation may be determined in the laboratory with the help of TNF-αas an objective marker Elsalhy et al. (2013) (18) and Abd-Elmeguid et al. (2013) (19) reported that levels of TNF-α were remarkably higher in samples classified with symptomatic ir-

TABLE 2. Intra-group comparison of TNF-α and MMP-9 values using Kruskal-Wallis test

Sub group no.	Group name	TNF-α		MMP-9	
		Mean rank	p	Mean rank	p
Subgroup 1A (20 patients)	Non Diabetic (Irreversible pulpitis)	42.71	<0.001*	38.29	<0.001*
Subgroup 1B (19 patients)	Non Diabetic (Reversible pulpitis)	13.45		13.05	
Subgroup 2A (19 patients)	Diabetic (Irreversible pulpitis)	60.11		65.84	
2B (19 patients)	Diabetic (Reversible pulpitis)	39.7		38.82	

*: p<0.05 was considered as statistically significant. TNF- α: Tumor necroses factor-alpha, MMP: Matrix metalloproteinases-9

TABLE 3. Pairwise comparison of TNF- α and MMP-9 values using Mann-Whitney test

Sub group no.	Group name	TNF-α	MMP-9
1A vs 1B	Non Diabetic (Irreversible pulpitis) vs Non Diabetic (Reversible pulpitis)	<0.001*	<0.001*
1A vs 2A	Non Diabetic (Irreversible pulpitis) vs Diabetic (Irreversible pulpitis)	0.003*	<0.001*
1A vs 2B	Non Diabetic (Irreversible pulpitis) vs Diabetic (Reversible pulpitis)	0.607	0.857
1B vs 2B	Non Diabetic (Reversible pulpitis) vs Diabetic (Irreversible pulpitis)	<0.001*	<0.001*
1B vs 2B	Non Diabetic (Reversible pulpitis) vs Diabetic (Reversible pulpitis)	<0.001*	<0.001*
2A vs 2B	Diabetic (Irreversible pulpitis) vs Diabetic (Reversible pulpitis)	<0.001*	<0.001*

*: p<0.05 was considered as statistically significant. TNF- α: Tumor necroses factor-alpha, MMP: Matrix metalloproteinases-9

reversible pulpitis as compared to the normal pulp samples thereby partially rejecting the first null hypothesis. Altered immunoinflammatory responses like chemotaxis, phagocytosis and neutrophils lead to an unregulated elevated production of proinflammatory mediators like TNF-α by macrophages and monocytes in diabetics which may increase host tissue destruction (20). Additionally , the initial increase of kallikrein enzymes may occur in the dental pulp as an outcome of the diabetic hyperglycaemic inflammatory process causing an increased level of TNF- α in comparison to Healthy Individuals (21).

Hyperglycaemia also induces oxidative stress, together with soluble advanced glycation end products (AGEs) along with lipid peroxidation products, probably serve as a prime activator of upstream kinases, resulting in induction of inflammatory gene expression (22). Furthermore, since the dental pulp reacts to bacterial infection in a similar manner as any different connective tissue in the organism, it may be extrapolated that greater TNF-α levels in plasma of diabetics as compared to healthy individuals might have led to increased TNF-α levels in pulpitis in diabeticcompared to non-diabetic patients.

The mean oflevels of MMP-9 in pulpal blood in irreversible pulpitis group (SubgroupA) was found to be significantly higher than the mean of reversible pulpitis (Subgroup B) in both diabetic and non-diabetic groups thereby completely rejecting the first null hypothesis. Pulpitis is clearly a PMN-driven inflammation which are primarily released in the advanced phases of inflammation and are the predominant source of MMP-9 (23). Whole-blood fractionation experiments suggest that Neutrophils (PMN) are the predominant cellular source of MMP 9 which are capable of digesting the extracellular matrix (24). Amongst the advanced phases of pulpal inflammation such asirreversible pulpitis, the pulp complex has the potential to increase the release of neutrophils along-with matrix-degrading MMPs, which consecutively can damage the extracellular

TABLE 4. Interrelation of MMP-9 and TNF-α and their relation with sugar level

		HbA1C	TNF-α	MMP-9
HbA1C	Correlation coefficient	1.000	**0.478**	**0.489**
	p	–	<0.001*	<0.001*
TNF-α	Correlation Coefficient		1.000	0.889
	p		–	<0.001*
MMP-9	Correlation Coefficient			1.000
	p			–

*: The mean the result is significant at the 0.05 level. MMP: Matrix metalloproteinases-9, TNF- α: Tumor necroses factor-alpha, HbA1C: Hemoglobin A1c

matrix present in dental pulp (24). As stated earlier, increased levels of cytokines such as TNF-α in advanced stages of pulpal inflammation might also lead to increased levels of MMP-9 (25). Also, as MMP-9 degrades ECM and can be considered as marker of tissue breakdown, its levels are increased in irreversible pulpits as tissue breakdown is more in Irreversible pulpits as compared to reversible pulpits. The study by Mente et al. (2016) (26) has reported similarly.

Another finding in our study was that a statistically significant higher mean of MMP-9 was observed in diabetic group in both irreversible pulpits and reversible pulpits (B1 & B2) as compared to the mean of non-diabetic group (Group 1A&1B). Hence the second null hypothesis was rejected. Thickened basement membrane and angiopathy has been demonstrated in dental pulp vessels as a result of long standing diabetes which impairs leukotactic response, decreasing PMN's killing ability. Hyperglycaemia also reduces G6PDH-mediated entry of glucose into pentose phosphate pathway which in turn has reduced the production of the chief intracellular reductant NADPH, thereby increasing thelevels of oxidative stress (27, 28). Additionally, the elevated NADPH oxidases activated via NA-

DPH oxidases elevates the production of reactive oxygen species (ROS) which further stimulates production of MMPs (29).

MMP-9 and TNF-α levels exhibitedremarkably significant correlation irrespective of the group assignment ($p<0.001$) with a positive correlation coefficient of 0.889. TNF-α is one of many cytokines released at the inflammatory site when the teeth encounters trauma orbacterial infiltration through the dentinal tubules occurs. The elevation of TNF-α in the dental pulp can triggerthe cells in the pulp to produce matrix metalloproteinases (MMPs) (30). TNF-α has also been demonstrated to upregulate MMP-9 production in human monocytes. TNF-α also stimulates MMP-9 production via protein kinase C signal-transduction pathway or increased plasminogen activator which in turn accelerates MMP-9 production (31).

Insignificant differences were found between the mean levels of MMP-9 and TNF-α in diabetic reversible pulpits (2B) and non-diabetic symptomatic irreversible pulpitis (1A) subgroups in our study due to the fact that diabetes may result in additional modifications in the pulp structure like increased basement membrane thickness of blood vessels, reduction in collagen concentration, obliterative endarteritis and angiopathy (32).

Though the present study did not aimto judge the conducted treatment prognosis but it was observed that some of the patients treated with VPT in diabetic reversible pulpits group, who came back for follow-up, had treatment failures which necessitated subsequent RCT. This finding raisesa question on the prognosis of VPT even in reversible pulpitis in diabeticpatients and necessitates clinical studies to assess the treatment outcome on evaluation of biological markers. This may be considered as one limitation of thisstudy.

Further clinical trials should be conducted assessing the long-term outcome of the procedures (VPT/RCT) and correlating it with the inflammatory biomarker levels which may assist in modifying the treatment protocol for the particular clinical scenario. The findings from the study provides a scope to develop a highly specific diagnostic chairside kit that can assist in choosing the precise and accurate therapeutic modality by gauging the severity of pulpal inflammation that may differ in individuals with varying medical history. This would enable the dental practitioner in distinguishing teeth with mild clinical symptoms having reversible inflammation confined to a portion of the pulp from those with severe inflammation and widespread pulp destruction after carious exposure. This valuable additional information can assist the clinician to review the optimal and precise therapeutic regimen for a tooth that has a cariously exposed pulp (e.g. vital pulp therapy [partial pulpotomy] vs RCT). The study highlights the prospective of using the innovation of molecular biology to establish the association of specific biomarkers to the pulpal inflammatory status.

CONCLUSION

The present study indicated that MMP-9 and TNF-α in pulpal blood for teeth diagnosed with symptomatic irreversible pulpitis was significantly higher in diabetic patients when compared to non-diabetic patients. Additionally, teeth diagnosed with reversible pulpitis in a diabetic patient reported with higher levels of inflammatory pulpal biomarkers that could compromise the success of VPT and may necessitate endodontic intervention. A significant positive correlation was observed between MMP-9 and TNF-α.

Disclosures

Conflict of interest: The authors deny any conflict of interest.

Ethics Committee Approval: This study was approved by The ITS Centre for Dental Studies and Research Ethics Committee (Date: 23/05/2019, Number: ITSCDSR/L/2019/093).

Peer-review: Externally peer-reviewed.

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