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METABOLIC CHANGES OF SERUM CATHEPSIN D IN PERIODONTITIS WITH AND WITHOUT TYPE 2 DIABETES MELLITUS

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ABSTRACT

Diabetes is shown to have an increased risk for periodontal disease compared to non-diabetes. The present study aimed to evaluate the concentration of serum cathepsin D level in periodontitis subjects with and without type 2 diabetes mellitus. We studied a total of 600 subjects categorized into 4 groups as Group I (150 healthy subjects as control), Group II (150 type 2 diabetes mellitus with periodontitis), Group III (150 type 2 diabetes mellitus without periodontitis) and Group IV (150 non-diabetic patients with periodontitis). Serum concentration of cathepsin D was measured by means of UV absorption spectrophotometer at 280 nm. The activity of cathepsin D was found to be elevated 8 times in non-diabetic periodontitis subjects (group IV) when compared to control and it was 3 times higher when compared to type 2 diabetes mellitus with periodontitis (group III). It gives an evidence for the release of lysosomal enzymes during periodontal tissue damage.

KEYWORDS: Cathepsin D, Lysosomal enzymes, Oxidative stress, Periodontitis, Type 2 diabetes mellitus.

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INTRODUCTION

Type 2 diabetes mellitus is a metabolic syndrome associated with hyperinsulinemia, insulin resistance, hyperglycemia, dyslipidemia and obesity. Uncontrolled or poorly controlled diabetes is associated with an increased susceptibility and severity of infections, including periodontitis. As with other systemic conditions associated with periodontitis, diabetes mellitus does not cause gingivitis or periodontitis, but evidence indicates that it alters the response of the periodontal tissues to local factors, hastening bone loss and delaying postsurgical healing of the periodontal tissues[1]. Polymorphonuclear leukocyte (PMNL), altered collagen metabolism, advanced glycation end products (AGE's) [2] and bacterial pathogens in diabetes mellitus are the factors contributing to the development of periodontal disease. The presence of acute infection can predispose to insulin resistance. The periodontal disease is the major cause of adult tooth loss and it involves irreversible destruction of the tooth-supporting tissues[3].The primary etiologic factor of periodontal diseases are bacteria and their by products, including lipopolysaccharides (LPS). Bacterial endotoxins, toxins and LPS can directly damage connective tissue, which causes certain cells of the periodontium to secrete enzymes. The bacteria in dental plaque when allowed to accumulate produce inflammatory mediators such as cytokines, prostanoids, and lysosomal enzymes[4] including matrix metalloproteinases, collagenase, and cathepsin D. In 1993, Loe[5] described that periodontitis as the sixth complication of diabetes mellitus. Cathepsin D (EC 3.4.23.5) is a lysosomal aspartic proteinase enzyme that belonging to the pepsin family and is widely distributed in almost all mammalian cells. It is the major lysosomal endopeptidase which plays an important role in physiological and pathological breakdown of intracellular and extracellular proteins[6]. Lysosomal enzymes are produced in the reticular network, enclosed in tiny vesicles that fuse with late endosomes or autophagosomes. Cathepsins are lysosomal hydrolases that degrade proteins in lysosomes at an acidic pH[7]. Cathepsin D has been observed in various biological events such as cellular protein turnover, regulation of programmed cell death and degradation of several brain antigen processing[8]. In addition to its role as a protease, it may also have non-enzymatic functions as a mitogen in some cancer cell lines, inflammation and tumour progression and formation of metastasis,[9,10] the majority of cathepsin D is found in soluble parts of most of human cells, about 20% are appears to be membrane bounded[11,12]. Tissue remodeling represents another involvement of cathepsin D in mammalian physiology[13]. Cathepsin D inactivates many inhibitors of proteolytic enzymes. The proteolytic activity of the enzyme is regulated by various intra lysosomal factors such as pH, products of metabolism, hormones, growth factors and specific inhibitors. These enzymes have been found to be up regulated in many acute and chronic pathological conditions such as trauma, sepsis and diabetes mellitus[14].Hence the evaluation of lysosomal enzyme, cathepsin D may provide information on the pathogenesis of enzymatic tissue damage in type 2 diabetes mellitus with periodontitis.

MATERIALS AND METHODS

Study subjects and Ethical Approval

The study group consisted of a total of 600 subjects between the age group 25 to 55 years and were categorized into four groups as control (Group I), type 2 diabetes mellitus without periodontitis (Group II), type 2 diabetes mellitus with periodontitis (group III), and non-diabetes mellitus with periodontitis (group IV). Group II patients are enrolled from SRM Speciality Hospital, India and group III and group IV were selected from the outpatients attending Department of Periodontology & Oral Implantology, SRM Dental College, India. A total of 150 age- and sex-matched non-diabetic healthy individuals were selected as the control group from the general population. The study protocol was approved by the Institutional Ethical Committee of Medical and Health Sciences, SRM University, India and an informed consent was obtained from all the subjects.

Clinical Assessment of study subjects

Information about the age, gender, blood pressure, body mass index (BMI), duration of diabetes mellitus, current medications (insulin supplementation, oral hypoglycemic agents), diet and diabetes mellitus complications were obtained by a standardized questionnaire. For all subjects, the basic clinical history and demographic data were recorded. The clinical assessment for periodontitis subjects included examination of gingiva, intra oral examination-number of teeth present and missing, pathological migration, and probing depth. Mean pocket probing depth, and clinical attachment loss (CAL) were measured using mouth mirror and William's periodontal probe to assess the periodontal status. Pocket probing depth was measured as the distance from the gingival margin to the bottom of the probed pocket. Probing depths were recorded at six sites per tooth, rounded up to the nearest millimetre. Periodontitis was confirmed by bone loss evident on radiographic examination. The periodontal status was examined by a trained Periodontist of SRM Dental College, Department of Periodontology, Chennai -600 089.

Inclusion and Exclusion Criteria

Inclusion criteria include known diabetic type 2 patients of both sexes, for at least the past 5 years diagnosed by a physician by means of the oral glucose tolerance test. All periodontitis individuals included under the category of periodontitis should have more than 30% of the sites with Clinical attachment level (CAL) ≥ 3mm, pocket depth (PD) ≥ 4 mm, and at least 2 teeth in each quadrant with the condition of 20 teeth in all the subjects. The healthy controls were not on any kind of prescribed medication or dietary restrictions. Smokers, alcoholics, drug abused, patients who had periodontal therapy six months prior to the study, patients under antibiotics and having systemic disease other than diabetics, taking hormone drugs, lipid lowering drugs, oral contraceptives, and pregnant women were excluded from the study.

Measurement of clinical parameters and cathepsin D enzyme activity

Blood samples were collected after an overnight fast for each subject. The fasting blood glucose (FBG), measured using the glucose oxidase - peroxidase method, was expressed in milligrams per deciliter (mg/dl) and blood glycated hemoglobin (HbA1c), analyzed by high-performance liquid chromatography method (Biosystems S.A, Costa Brava, Spain) was expressed in percentage. Serum was obtained by centrifuging the blood at 1500 r.p.m for 10 minutes. Cathepsin D was determined by the method of Anson, [15] as modified by Barrett,[16] the assay for cathepsin D involved measurement of TCA-soluble, Folin-reactive products of hemoglobin digestion at pH 3.5 and 37°C. 1 ml of 1% hemoglobin dissolved in 0.2 M acetate buffer (pH 3.8), 0.8 ml of 0.05 M acetate buffer (pH 3.8) and 0.2 ml of the enzyme sample were mixed. After incubation at 37°C for 60 min, the reaction was stopped by addition of 2 ml of 10% trichloroacetic acid solution and the mixture was centrifuged at 1000 g for 15 minutes. The liberated peptides soluble in the presence of trichloroacetic acid were measured in UV absorption spectrophotometer at 280 nm and the cathepsin D activity was calculated and the enzyme unit was expressed by micromoles of tyrosine liberated per minute / 1000 ml serum.

Statistical Analysis

The data are presented as mean ± SD (standard deviation). An unpaired Student's t test was used to evaluate the significance of differences, accepting $P < 0.05$ as the level of significance. Comparisons between control group, type 2 diabetes with periodontitis, type 2 diabetes without periodontitis and non-diabetic with periodontitis group were made using ANOVA. Statistical analysis included Pearson's correlations between cathepsin D with HbA1c and fasting blood glucose in all the 4 groups; $P < 0.05$ were considered significant. Data were analyzed using the Graphpad Prism 6 for windows statistical software package (San Diego, California).

RESULTS

The demographic data, periodontal parameters, and clinical parameters of the study population are summarized in Table 1. As expected the mean levels of periodontal probing depth (PPD) and clinical attachment

level (CAL), were significantly greater than 4mm in type II diabetes mellitus with periodontitis (group III) and in non-diabetic with periodontitis (group IV), compared to healthy subjects. Comparing to control, type 2 diabetic subjects without periodontitis (group II) and type 2 diabetic subjects with periodontitis (group III) presented mean percentage of HbA1c levels 7.74 ± 1.31 and 8.38 ± 1.17 respectively. However, there were no differences in the HbA1c levels between the control (group I) and non-diabetic periodontitis (group IV) subjects.

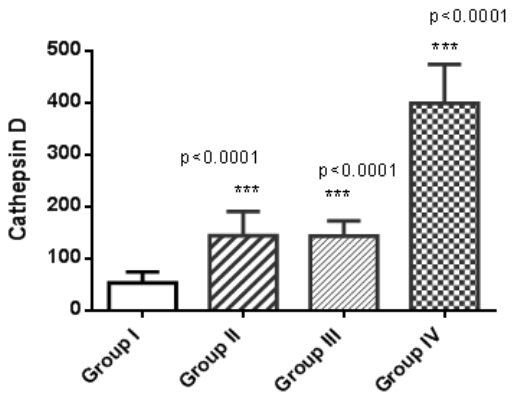
Table 1
Demographic and clinical characteristics of study population

Parameters	Control Group I	Type 2 diabetes without periodontitis Group II	Type 2 diabetes with periodontitis Group III	Non-diabetes with periodontitis Group IV
No of samples	150	150	150	150
Gender (M/F)	80/70	78/72	77/73	75/75
Age, years	35.46 ± 10.74	46.26 ± 10.02***	44.42 ± 10.37***	41.66 ± 10.45***
Duration of diabetes, years	-	8.39 ± 5.35	8.70 ± 4.82	-
HbA1c %	5.20 ± 0.51	7.74 ± 1.31***	8.38 ± 1.17***	5.14 ± 0.56 NS
BMI, kg/m^2	22.72 ± 1.5	23.32 ± 1.49 NS	24.07 ± 1.51 NS	23.93 ± 1.12**
Systolic blood pressure(mm Hg)	119.5 ± 4.65	126.4 ± 5.70	128.8 ± 5.09	126.7 ± 8.39
Diastolic blood pressure(mm Hg)	72.93 ± 2.10	75.14 ± 1.78	79.05 ± 3.03	76.47 ± 4.52
FBG, mg/dl	95.28 ± 12.51	183.7 ± 57.16***	176.7 ± 59.12***	96.88 ± 12.67 NS
PPD (mm)	1.45 ± 0.13	1.42 ± 0.17 NS	4.61 ± 0.51***	4.67 ± 0.46***
CAL (mm)	0.70 ± 0.27	0.64 ± 0.15 NS	4.91 ± 0.37***	4.62 ± 0.58***

Values are expressed as Mean ± SD; except for gender (Male, M / Female, F). Glycosylated hemoglobin, HbA1c; Body mass index, BMI; Fasting blood glucose, FBG; Periodontal probing depth, PPD; Clinical attachment level, CAL. Differences were considered significant level at *** p <0.0001; ** p <0.001;
****p<0.05 of parameters of group II, III, IV vs group I and NS, non-significant***

The mean serum cathepsin D levels in control (group I), type 2 diabetes mellitus without periodontitis (group II), type 2 diabetes mellitus with periodontitis (group III) and non-diabetes with periodontitis subjects (group IV) was shown in Fig. 1. The serum cathepsin D enzyme activity was found to be significantly increased in group II, group III and group IV when compared to control. Very interestingly, the activity of cathepsin D was found to be 8 fold times elevated in non-diabetes with periodontitis (group IV) and about 3 times in type 2 diabetes mellitus without periodontitis (group II) and type 2 diabetes mellitus with periodontitis (group III) when compared to control (group I).

Figure 1



Mean± SD of serum cathepsin D (µmoles/min/L) levels in healthy controls (group I), type 2 diabetes mellitus without periodontitis (group II), type 2 diabetes mellitus with periodontitis (group III) and non-diabetes with periodontitis (group IV) subjects

Pearson correlation data of cathepsin D with HbA1c, FBG, PD, and CAL for the 4 groups were shown in Fig. 2. A significant inverse correlation was obtained between cathepsin D versus HbA1c (r = -0.458, p =0.044) in type 2 diabetes mellitus with periodontitis (group III) and with group II and group IV, it showed positive correlation. Cathepsin D correlates negatively with FBG and periodontal probing depth whereas it correlated positively with clinical attachment level in group III subjects. No significant correlations were observed between serum cathepsin D with other parameters in all the 4 groups.

Figure 2

Correlation between Cathepsin D (µ moles/min/L) in group III subjects with a) Fasting blood sugar (FBG) b) HbA1C c) Periodontal probing depth d) Clinical attachment level

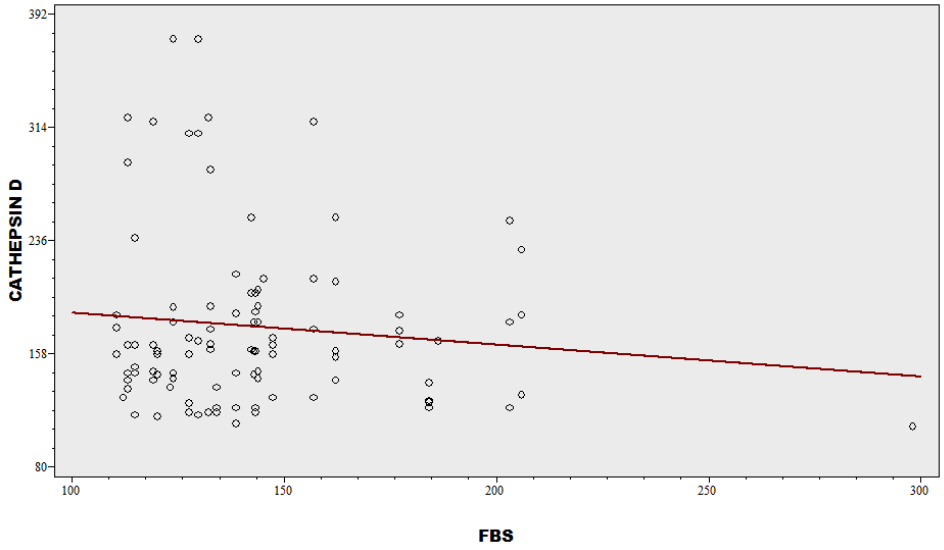


Figure 2 a

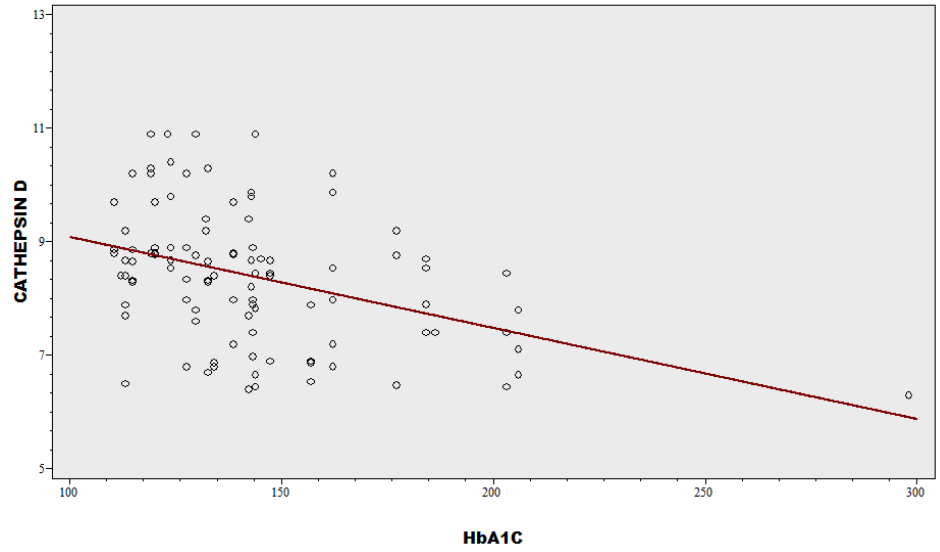


Figure 2 b

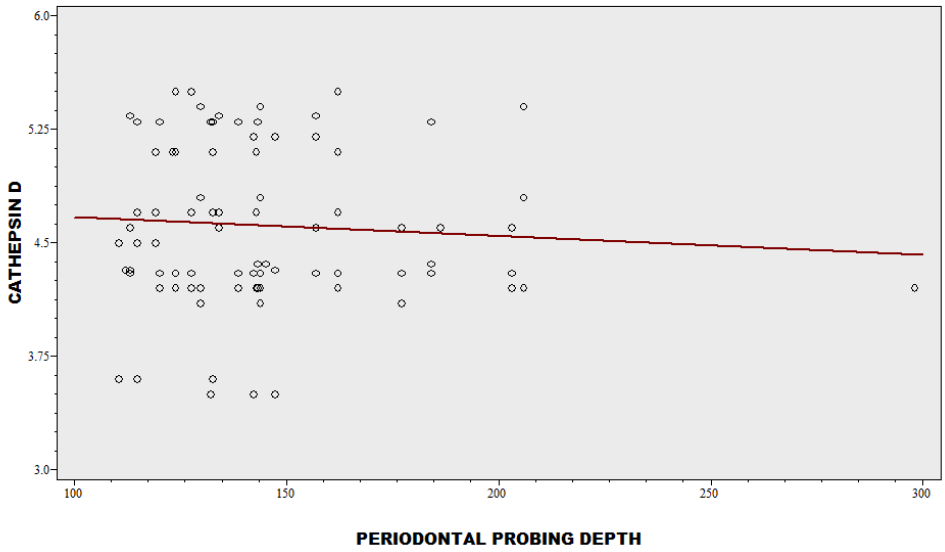


Figure 2 c

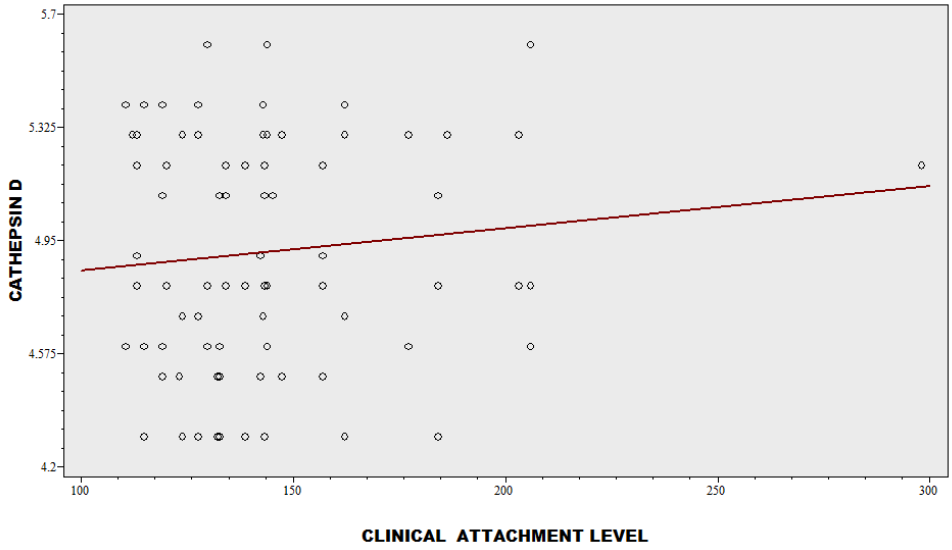


Figure 2 d

DISCUSSION

Lysosomes and endosomes are outfitted with a perplexing variety of endopeptidases. Evidence from a variety of sources demonstrates that, increased production of free oxygen radicals and oxidative stress were seen in non-diabetic with periodontitis subjects. Oxidative stress and free radical generation, both play an important role in the development of systemic diseases such as type 2 diabetes, atherosclerosis, and cardiovascular diseases[17]. The Cathepsin D activity was found to be significantly higher in the serum of non-diabetic subjects with periodontitis when compared to control gives an evidence for the release of lysosomal enzymes during periodontal tissue damage. Human cathepsin D consists of 412 amino acids, with two active site aspartic acid residues essential for its catalytic activity. Important feature of cathepsin D is its selective localization inside acidic compartments of cell. At least two mechanisms of localization of cathepsin D to lysosomes are known. In first

mechanism the terminal end of oligosachcaride is marked with mannose 6-phosphate and along with appropriate concentration of mannose 6-phosphate receptor, mature enzyme is transported from Golgi apparatus into endosomes. In endosomes the acidic medium causes dissociation of this complex and the released receptor comes back to Golgi apparatus, [18, 19] the second mechanism is independent of mannose 6 phosphate receptor where the C-terminus of activating peptide is transported to primary lysosomes[20]. The role of saccharidic moieties, particularly procathepsin D with mannose-6-phosphate receptors interaction was done by Rochefort et al [21]. They found that this interaction is most likely facilitated by the activation peptide that can be a part of normal physiological function of procathepsin D. Glycosylation occurs only in cathepsin D since other members of human aspartic peptidases have not been reported to contain sugar moiety. The highest cathepsin D enzyme activity was observed in subjects with non-diabetic periodontitis (Group IV) and the activity was found to be lowered in subjects with type 2 diabetes mellitus without periodontitis (Group II). Such a decrease in lysosomal cathepsin D activity may be explained by the slower anabolic pathway, including proenzyme of lysosomal enzymes synthesis and/or exhaustion of the energy level in the body supplying adenosine triphosphate (ATP) to the proton pump in the lysosomes. In a cell with high energy charge, acidification of lysosomes would be maximized due to maximal activity of the proton pump, [22] and lysosomal pH would be lowest. The pH optima for cathepsin D suggests that ATP activation of cathepsin D may be sturdily favored under certain conditions and the optimum for cathepsin D is about pH 4 and this is not altered by ATP[23]. Thus, when lysosomal pH drops below 5 the catalytic activity of cathepsin D would increase. Under these conditions increased activation of cathepsin D by ATP would be enhanced. Our data extend and support previous studies indicating the role of oxidative stress in the development of diabetes[24]. Relationship between pH and the availability of lysosomal enzymes suggests that lysosomal membrane can be characterized with the properties of a charged membrane. Alteration in the physicochemical properties of the structural part of the lysosome appears to be the dominant factor in determining the availability of lysosomal enzymes. However, a change in body pH presumably could increase or decrease the availability and activity of lysosomal enzymes leading to further catabolic effects. These enzymes play a role in the catabolism of glycoproteins and aminoacids. The mitochondrion is the organelle that is most influenced by oxidative damage, and lysosomal enzymes have been found to act on mitochondria to promote reactive oxygen species (ROS) generation, thereby creating a feedback loop that leads to additional lysosomal damage[25]. In type 2 diabetes mellitus with periodontitis, increase cathepsin D level and hyperglycemia involves glycosylation of structural proteins and matrix molecules, resulting in the formation advanced glycation end products (AGEs). Recently, it has been shown that the accumulation of AGEs is a major pathogenic process in diabetes in which blood sugar is increased and therefore the glycation reaction is accelerated[26, 27]. In diabetes patients, hyperglycaemia leads to excess ROS production within the mitochondrial electron transport chain, during AGE formation [28]. AGEs are among the factors which influence the periodontal disease development and increases oxidative stress in gingiva in diabetes patients [29, 30]. AGE formation on collagen proteins would lead to the thickening of basement membrane in gingival tissues, impairing the delivery of leukocytes and nutrients into the gingival and periodontal tissues. It results in an irreversible and increased cross-linking of collagen molecules, contributes to other complications of diabetes as a result of decreased elasticity of blood vessels. A number of observations have shown that cathepsins can translocate from the lysosome lumen to the cytosol in response to a variety of death stimuli such as TNF-α and in many cases of apoptosis induction, partial rupture of the lysosomal membrane appears to be an early event, occurring either before mitochondrial transmembrane potential loss or caspase activation[31,32]. The proteases that are released from the lysosomal compartment to the cytosol seem to be able to activate some steps of the death cascade leading to apoptosis. Furthermore, impaired chemotaxis, adherence

neutrophil function and delayed apoptosis have been reported in patients with diabetes[33,34]. If apoptosis is delayed, however, this will lead to increased retention of neutrophils in the periodontal tissues. This in turn could lead to increased tissue damage and formation of more reactive oxygen species with the release of destructive enzymes, cathepsin D by the neutrophils. Increased cathepsin D level among group IV subjects indicates the end point of periodontal tissue destruction[35.] Accumulation of glucose-mediated AGEs in diabetic patients impairs chemotactic and phagocytic function of polymorphonuclear leukocytes[36]. It also impairs the movement of metabolic waste of periodontal pathogens out of the tissue; causing decreased wound healing capacity and increased disease severity. This process occurs in people without diabetes too, but the rate of formation is greatly increased in people with diabetes. ROS are presumably part of the complicated mechanism for the development of insulin resistance and beta-cell destruction in the pancreas.

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CONCLUSION

From our study, it is clear that elevated levels of cathepsin D influence the diabetic condition with periodontitis by impairing insulin signaling and insulin resistance. Periodontitis if untreated leads to tooth loss and hence a proper intervention is required from stage to stage in order to retain the teeth in the oral cavity. The suggestion of a link between cathepsin D in type 2 diabetes mellitus and periodontitis provides a testable hypothesis that may produce a novel close by into the diabetic complications. Increased level of Cathepsin D contributes to the pathogenesis of type 2 diabetes mellitus with and without periodontitis, and non-diabetes with periodontitis. Taking into account that early recognition and treatment of periodontitis are vitally essential for type 2 diabetes patients to have a control over glycaemic effects

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