Comparative Evaluation of Cathepsin K Levels in Gingival Crevicular Fluid among Smoking and Nonsmoking Patients with Chronic Periodontitis

Abstract

**Background:** The aim of the study is to comparatively evaluate the levels of cathepsin K (CSTK) in gingival crevicular fluid (GCF) among smoking and nonsmoking patients with chronic periodontitis (CP). **Materials and Methods:** A total of 160 systemically healthy male patients were included in the study. Based on probing pocket depth, clinical attachment level, plaque index, and modified sulcular bleeding index, the patients were allotted into four groups: Group I - with forty subjects who were smokers with healthy periodontium, Group II - with forty nonsmoking subjects with healthy periodontium, Group III - forty patients who were smokers with CP, and Group IV - with forty nonsmoking CP patients. Those who claimed to have never smoked were recruited into the nonsmoker group, whereas subjects who reported smoking ≥10 cigarettes per day for more than 5 years were recruited into the smoker group. The GCF samples were collected using microcapillary pipettes and analyzed for levels of CSTK using enzyme-linked immunosorbent assay. **Results:** The GCF concentration of CSTK was expressed in pg/µl. The mean CSTK levels in the groups were Group I - 0.158 ± 0.043 pg/µl, Group II - 0.145 ± 0.026 pg/µl, Group III - 15.768 ± 12.40 pg/µl, and for Group IV - 11.59 ± 12.15 pg/µl, respectively. The levels of CSTK were statistically higher in Group III when compared with Group IV (P = 0.037) (P < 0.05). **Conclusion:** CSTK levels were significantly increased in smokers with CP than nonsmokers, suggesting a positive influence of smoking on CSTK which could possibly play a role in the increased susceptibility for osteoclastic bone destruction in smoker subjects.

**Keywords:** Cathepsin K, gingival crevicular fluid, periodontitis, smoking

Introduction

Our understanding of periodontal diseases has evolved over the years and has transformed from periodontitis being considered an almost ubiquitous condition, in which the role of plaque as the sole etiological factor was unquestioned to the current understanding of the pivotal role of host immune inflammatory response together with considerable knowledge on the influence of various individual risk factors suggesting that periodontitis is a multifactorial polymicrobial disease.

Cigarette smoking is the strongest of the modifiable risk factors for periodontitis secondary to bacterial plaque. Evidence from various studies has shown that adult smokers are about two to four times more likely to have periodontitis than nonsmokers. So far, the effects of smoking on subgingival microflora, gingival vasculature, neutrophils, serum IgG, and circulating levels of cytokines have been reported. Although various studies have attempted to explain the mechanism of action of smoking in the pathogenesis of periodontitis, the molecular basis of how smoking contributes to alveolar bone loss is still poorly understood.

Substances produced by the subgingival bacterial flora and the tissue during inflammation and immune reactions may affect bone turnover by causing the differentiation and stimulation of osteoclasts or by inhibiting bone formation by osteoblasts. Osteoclasts are cells that play pivotal roles in bone morphogenesis, remodeling, and resorption, differentiate from the hematopoietic myeloid precursors of macrophage/monocyte lineage. Proteolytic processes are known to be critical in osteoclastic bone resorption.

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Degradation of bone matrix proteins is initiated by acidic lysosomal proteinases secreted from osteoclasts into the bone resorption lacunae and then is continued and completed in the intracellular endosomal/lysosomal system. So far, a variety of lysosomal proteinases have been identified in both the lacuna and the lysosome-like organelles in actively resorbing osteoclasts.[21‑24] Among the various lysosomal proteinases, recent data, however, strongly implicate cathepsin K (CSTK) as the predominant effector in osteoclastic bone resorption.[25]

CSTK expression is required for normal skeletal development.[26,27] Nonsense, missense, and stop codon mutations in the CSTK gene have been identified in humans with pycnodysostosis, which is an autosomal recessive osteochondrodysplasia characterized by osteosclerosis and short stature.[28‑30]

Literature reports the involvement of CSTK in various disorders associated with bone resorption such as osteoporosis, Paget’s disease,[31] diffuse sclerosing osteomyelitis of mandible,[32] and the critical role of CSTK in the pathogenesis of rheumatoid arthritis.[33,34] Abnormally high CSTK production is reported in ankylosing spondylitis[35] and atherosclerosis.[30] Recent studies have reported elevated levels of CSTK in gingival crevicular fluid (GCF) of patients with periodontitis and peri-implantitis.[36]

Apart from an in vitro study by Tanaka et al., 2013,[37] till date to the best of our knowledge, no human studies have documented the influence of smoking as an environmental factor to regulate the levels of CSTK. Hence, the present study was designed to comparatively evaluate the influence of an environmental factor - smoking on the levels of CSTK in GCF among chronic periodontitis (CP) patients.

Materials and Methods

Patients seeking dental treatment in the department of periodontics were recruited in this study. This study was approved by the Institutional Review Board. All the patients who participated were informed of the nature of the study and those willing to participate, duly signed the consent form.

Eighty systemically healthy male subjects with CP (forty smokers and forty nonsmokers) and eighty male systemically, periodontally healthy subjects (forty smokers and forty nonsmokers) were recruited in the study. The healthy group consisted of patients with ≥90% of the measured sites exhibiting probing pocket depth (PPD) <3 mm and clinical attachment level (CAL) = 0 mm, no bleeding on probing, and no radiographic sign of alveolar bone loss (i.e. a distance of <3 mm between the cementoenamel junction and bone crest at >95% of the proximal tooth sites).[38] Group I had forty smokers with healthy periodontium and Group II had 40 nonsmokers with healthy periodontium. CP patients were diagnosed in accordance with the clinical criteria stated in the consensus report[39] of the World Workshop in Periodontitis. Those who claimed to have never smoked were recruited into the nonsmoker group, whereas subjects who reported smoking ≥10 cigarettes per day for more than 5 years were recruited into the smoker group. Patients who smoked ≥10 cigarettes per day for <5 years and those who smoked <10 cigarettes per day for more than 5 years were excluded to better differentiate smokers from nonsmokers.[40] Group III had forty smokers with CP and Group IV had forty nonsmokers with CP.

Patients with aggressive periodontitis, diabetes, hypertension, gross oral pathology, heart disease, rheumatoid or osteoarthritis, tumors, or any other systemic disease which can alter the course of periodontal disease, on any medication such as phenytoin, cyclosporine, calcium channel blockers, bisphosphonates, vitamin D, or calcium supplements and who had taken antibiotics, anti-inflammatory drugs or received periodontal therapy in the preceding 6 months, and females were excluded from the study.

All patients had a clinical periodontal examination which included the measurement of PPD and CAL at six sites around each tooth with a manual probe University of North Carolina-15. Plaque index (PI) (Silness and Loe, 1964) and modified sulcular bleeding index (mSBI) (A Mombelli, 1987) were also recorded. All measurements were performed by a single calibrated examiner. Only one site with deepest PD per patient was selected as the sampling site in all the groups.

After isolating the tooth with a cotton roll, supragingival plaque was removed with a curette without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected by placing a microcapillary pipette at the entrance of the gingival sulcus, gently touching the gingival margin. From each site in all the groups.

The enzyme-linked immunosorbent assay kit for quantification of human CSTK levels in GCF was purchased from USCN life sciences, Inc., USA, and the assay was performed according to the manufacturer’s recommendations. The detection limit of the assay as
reported by the manufacturer is from 0.156 ng/mL to 
10 ng/mL. Data were then calculated and obtained by 
methods of interpolation of a predetermined standard 
curve. As the samples have been diluted, the concentration 
read from the standard curve has been multiplied by the 
dilution factor. Values of total amounts are expressed as 
pg/µL. The concentration of CSTK in the samples is then 
determined by comparing the optical density of the samples 
to the standard curve.

Statistical analysis

Clinical parameters as well as the total amount and 
concentration of CSTK in healthy and CP groups were 
expressed as mean ± standard deviation. A Levene test 
for equality of variance showed that the CSTK values in 
Group III and IV were not normally distributed. Hence, 
nonparametric statistical analysis was performed when 
necessary.

Student t-test (two-tailed, independent) has been used to 
compare the clinical parameters between Group I/II and 
Group III/IV. Student’s t-test was used to compare GCF 
concentrations of RANKL and CSTK in Group I and II. 
Mann–Whitney U-test has been used to compare the values 
of cytokine CSTK between smoking and nonsmoking 
healthy and CP patients.

The correlation between CSTK with the clinical parameters 
in Group I and II was done using Pearson correlation 
analysis. The correlation between CSTK with clinical 
parameters in Group III and IV was done using Kendall 
Tau-b correlation analysis. P < 0.05 is considered 
statistically significant for all the statistical analyses. 
All data were analyzed using a software program (SPSS 
Version 15, SPSS Inc., Chicago, IL, USA).

Results

Clinical findings of study groups

The descriptive statistics of the study population showing 
mean and standard deviation for age, PPD, CAL, PLI, and 
mSBI and pack years are represented in Tables 1 and 2.

Comparison of clinical parameters among the study 
groups

When clinical parameters were compared between 
Group III and IV, PPD and CAL were significantly higher 
in Group III than Group IV with P = 0.003 and 0.001, 
respectively. The gingival index was statistically higher in 
nonsmokers than smokers with CP (P = 0.014). There was 
statistically no significant difference between Group III 
and IV in plaque index [Table 3].

Analysis of cathepsin concentration in gingival 
crevicular fluid

The mean concentration of CSTK of all the groups is 
shown in Table 4.

### Table 1: Descriptive statistics of Groups I and II

| Clinical parameters | Group I (smokers healthy) | Group II (nonsmokers healthy) |
|---------------------|---------------------------|-------------------------------|
| Age (years)         | 40.9±4.58                 | 40.6±5.23                    |
| PPD (mm)            | 1.58±0.309                | 2.15±0.39                    |
| CAL (mm)            | 0.00                      | 0.00                         |
| PLI                 | 0.83±0.34                 | 0.92±0.21                    |
| mSBI                | 0.00                      | 0.00                         |
| Pack-years          | 3.77±0.73                 | NA                           |

SD=Standard deviation

### Table 2: Descriptive statistics of Groups III and IV

| Clinical parameters | Group III (smokers CP) | Group IV (nonsmokers CP) |
|---------------------|------------------------|-------------------------|
| Age (years)         | 41.42±7.37             | 41.33±9.71              |
| PPD (mm)            | 5.55±0.33              | 5.11±0.84               |
| CAL (mm)            | 5.87±0.42              | 5.41±0.77               |
| PLI                 | 1.67±0.46              | 1.77±0.37               |
| mSBI                | 1.50±0.37              | 1.74±0.47               |
| Pack-years          | 12.39±3.42             | NA                      |

SD=Standard deviation

### Table 3: Comparison of clinical parameters between 
Groups III and IV

| Comparison of clinical parameters | Significant (two-tailed) |
|-----------------------------------|-------------------------|
| PPD                               | 0.003**                 |
| CAL                               | 0.001**                 |
| PLI                               | 0.323                   |
| mSBI index                        | 0.014*                  |

Student’s t-test was used to compare the clinical parameters between 
Groups III and IV. *Significance at the level of 0.05, **Significance 
at the level of 0.01. PLI=Plaque index, PPD=Probing pocket depth, 
CAL=Clinical attachment level

### Table 4: Mean concentration cathepsin K in gingival 
crevicular fluid of all the study groups

| Groups   | Sample size (n) | Cathepsin K (pg/µL) | Mean±SD |
|----------|-----------------|---------------------|---------|
| Group I  | 40              | 0.158±0.043         |         |
| Group II | 40              | 0.145±0.026         |         |
| Group III| 40              | 15.768±12.40        |         |
| Group IV | 40              | 11.59±12.15         |         |

SD=Standard deviation

CSTK levels were detected only in eight patients in each of 
Group I and II. CSTK levels were detected in all patients 
in Group III and IV.

When comparing the CSTK concentration among Group I 
and II patients, there was no significant difference between the
groups \( (P = 0.496) \) \( (P < 0.05) \). There was a significantly higher concentration of CSTK levels in smokers when compared with nonsmoking CP patients \( (\text{Group III and IV}) \) \( (P = 0.037) \) \( (P < 0.05) \) [Table 5]. The CSTK levels were significantly higher in Group III \( (\text{smokers CP}) \) when compared with Group I \( (\text{healthy CP}) \) \( (P = 0.000) \) \( (P < 0.01) \), and similarly, the CSTK levels were significantly higher in Group IV \( (\text{nonsmokers CP}) \) when compared with Group II \( (\text{nonsmoking healthy}) \) \( (P = 0.000) \) \( (P < 0.01) \) [Table 5].

**Discussion**

Smoking influences angiogenesis,[9,41] adhesion molecule profiles and leukocyte recruitment,[9,42] multiple aspects of inflammatory response,[41‑43] and homeostasis and healing potential of the periodontal connective tissue.[16,46] Although smokers have been reported to be more susceptible to advanced and aggressive forms of periodontal disease than nonsmokers,[47‑50] the exact molecular mechanisms by which smoking exerts detrimental effects on the periodontal tissue remains conflicting.

In the present study, females were excluded intentionally as it would be difficult to recruit females who admit that they smoke. The other reasons for excluding them were to avoid potential hormonal influences on the periodontium.[51,52]

In the present study, CSTK levels in the GCF showed a significant increase \( (P = 0.000) \) \( (P < 0.01) \) in the nonsmokers CP \( (\text{Group IV}) \) group when compared with the nonsmoker healthy subjects \( (\text{Group II}) \). This present finding is in accordance with the results of Mogi and Otogoto et al.[53] and Garg et al.[54] reporting elevated concentration of CSTK in the GCF of CP patients when compared with healthy individuals. A decrease in GCF levels of CSTK following nonsurgical periodontal therapy was shown by Garg et al.[55] in patients with periodontitis which further supports the role of CSTK in osteoclastic bone resorption in periodontal disease.

To the best of our knowledge, till date, this is the first study to evaluate the influence of smoking on CSTK levels in the GCF of CP patients. There was a significantly higher concentration of CSTK levels in smokers when compared with nonsmoking CP patients \( (\text{Group III and IV}) \) \( (P = 0.037) \). This finding suggests that smoking has a positive influence on CSTK levels in CP patients; however, the mechanism remains unclear.

Studies done by Cesar Neto et al.[56] and Boström et al.[57] have suggested that smoking favors osteoclastogenesis by an increased production of pro‑inflammatory cytokines such as IL‑6 and tumor necrosis factor‑alpha \( (\text{TNF‑α}) \). An in vitro study by Kudo et al.[58] demonstrated that cytokines, TNF‑α, and IL‑1α directly induced osteoclastogenesis in peripheral blood mononuclear cell which were positive for tartrate‑resistant acid phosphatase and CSTK by a process which is distinct from the RANK/RANKL signaling pathway. In addition, Teng et al.[59] showed that the inflammatory mediators such as IL‑1 β, TNF‑α, IL‑6, IL‑11, IL‑17, and PGE2 can regulate osteoclastic activity through RANK‑independent pathways also. Although the RANKL‑dependent pathway is crucial for osteoclastogenesis, the presence of increased levels of potent osteogenic inflammatory mediators may drive RANKL independent pathway in various chronic pathological bone resorptive conditions. Such RANKL independent‑cytokine dependent pathway could have influenced the CSTK levels in the present study. However, this hypothesis needs to be carefully elucidated by further studies in the future.

In contrary to the present study finding, an in vitro study by Tanaka et al.[57] on the direct effects of nicotine on RAW264.7 cells suggested that nicotine had a suppressive effect on the expression of CSTK in activated osteoclasts. However, this in vitro model which has shown the effects of nicotine on bone marrow osteoclastic cell lines may not reflect or recreate the complex micro/macromolecular nature of the human periodontium and the multifactorial polymicrobial nature of periodontal pathogenesis.

No significant correlations between CSTK levels and any of the clinical parameters were obtained in the nonsmokers CP \( (\text{Group IV}) \) whereas the study by Garg et al.[53] showed a negative correlation between the clinical parameters such as PD and CAL with CSTK levels and the negative correlation was attributed to the consumption of CSTK in degradation of Type I collagen at its noncollagenous termini and release of cross‑linked N and C telopeptides.

In the present study, the oral hygiene status as depicted by plaque scores was almost similar between the smoking and nonsmoking CP group and this finding is in agreement with the other previous studies.[14,46,49,60,61] Contradicting the present study finding, other studies have shown higher plaque levels in smokers.[56,62‑66] The gingival index was statistically higher in nonsmokers than smokers with CP \( (P = 0.014) \) \( (P < 0.05) \) which is in agreement with the earlier studies.[60,64,65]

**Conclusion**

Although smoking has shown a positive influence on CSTK levels, further studies are needed to investigate peptide and

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**Table 5: Comparison cathepsin K levels in gingival crevicular fluid between Groups**

| Group                                   | Mann-Whitney U value | \( P \) |
|-----------------------------------------|----------------------|--------|
| CSTK levels between Groups III and IV   | 583.000              | 0.037* |
| CSTK levels between Groups I and III    | 0.000                | 0.000**|
| CSTK levels between Groups II and IV    | 0.000                | 0.000**|

Mann-Whitney U-test was used for this statistical analysis.

*Significance at the level of \(< 0.05\). **Significance at the level of \(< 0.01\). CSTK = Cathepsin K
mRNA expression levels of CSTK in gingival tissues at various stages of periodontitis, also following conventional and regenerative periodontal therapy in smokers with CP to provide deepest understanding on the role of CSTK in periodontal pathogenesis and if CSTK inhibitors could be used as potential host modulating agents.

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Conflicts of interest
There are no conflicts of interest.

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