Original Article

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Dentistry Section

Estimation of Calcium and Iron Levels in Gingival Crevicular Fluid and Serum in Periodontal Health and Disease

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ABSTRACT

Introduction: Gingival Crevicular Fluid (GCF) has been referred to as a promising medium for detection of markers for periodontal disease activity. Analysis of GCF shows minute changes in biomarker levels well before the onset of clinical signs and symptoms; which helps to even predict a person's predisposition towards periodontal disease occurrence. The elemental analysis of human blood serum is noteworthy in routine clinical practice as well as in medical research.

Aim: This study was done to determine the changes in calcium and iron levels in GCF and serum in human subjects with normal periodontal health and those with disease.

Materials and Methods: This was a cross-sectional study conducted from March 2019 to December 2019. Eight study subjects (four healthy subjects and four periodontitis cases) were selected from the patients reporting to the Department of Periodontics at Tagore Dental College and Hospital, Chennai. The subjects were chosen based on inclusion and exclusion criteria and all patients were subjected to a clinical examination wherein the Probing Depth (PD) and Clinical Attachment Level (CAL) were recorded by a single examiner using William's Periodontal probe. The GCF samples were collected by Capillary Tubing method. Blood was collected by venipuncture and centrifuged to provide serum samples. Dual viewing Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was used to estimate Calcium and Iron in GCF and serum. SPSS version 21.0 was used for statistical analysis. Mann Whitney U test was used for comparing the groups. A p-value less than 0.05 was considered statistically significant.

Results: Serum iron levels were significantly less in periodontitis patients than healthy subjects (p-value 0.043). GCF iron level (p-value 0.386), GCF calcium level (p-value 0.149) and serum calcium level (p-value 0.564) did not show any major variation among subjects with normal periodontal health and those with disease.

Conclusion: The findings of this study showed that iron and calcium are present in GCF and serum samples of healthy persons and patients with chronic periodontitis which can be detected using ICP-OES. A significant difference in serum iron levels between health and disease could indicate a patient's predisposition towards developing periodontitis. Calcium levels in GCF and serum do not point towards periodontal disease activity.

Keywords: Blood, Biomarkers, Elements analysis, Inductively coupled plasma optical emission spectrometry, Periodontitis

INTRODUCTION

Specific microorganisms in dental plaque can lead to an inflammatory disease Chronic periodontitis, resulting in loss of periodontium which can be seen clinically as periodontal pocket formation and gingival recession [1]. The combined activity of the pathogenic bacteria and host's immune and inflammatory responses result in tissue destruction in periodontal disease [2]. The host's immune system acts against the local microbial attack and prevents the spread of their damaging products. However, this defense mechanism may destroy the surrounding cells and connective tissue structures of the host [3].

Presently, periodontitis is diagnosed based on clinical measurements like Probing Depth (PD), Clinical Attachment Level (CAL), Bleeding On Probing (BOP), assessing Plaque Index (PI) and radiographic findings [4]. These clinical measurements provide information only on past periodontal tissue destruction, not on the present phase of the disease activity or predict the future course of disease and extent of destruction [4]. Thus, one of the key challenges in periodontology is to discover a method to figure out the present phase of disease activity and to predict the future of periodontal disease [4].

The constituents of the GCF can be used to assess the active phase of periodontal disease. GCF will show elevated levels of bacterial and host-mediated enzymes, connective tissue breakdown products, inflammatory mediators and extracellular matrix proteins during the active phase of periodontitis [5]. The qualitative and quantitative measurement of GCF components can act as a reference for interpreting the extent of gingival and periodontal examination [6]. Hence, GCF analysis can indicate the periodontal disease status of specific sites and potential biomarkers of periodontitis can be accurately identified [7].

Periodontitis can also be linked with various systemic conditions [8-11]. The local and systemic effects of periodontal disease can be seen for many years among older individuals [11]. Chronic periodontitis can be associated with various systemic conditions such as diabetes mellitus, cardiovascular disease, osteoporosis, respiratory diseases, rheumatoid arthritis, malignancies, erectile dysfunction, kidney disease and dementia. Surrogate indicators for chronic periodontitis, like tooth loss, manifest steady but weak connection with various systemic conditions [8,9,11]. The efficient treatment of periodontal infection is essential to attain good oral health and also to decrease the adverse effects of chronic local inflammation and bacteremia [10,11]. The blood drawn for routine diagnostic check-ups can also be assessed for systemic biomarkers which can not only aid in the early diagnosis and monitoring of periodontitis but can also give information at the patient level [12].

Calcium and Iron are essential for the functioning of many physiologic and biochemical reactions. Calcium is a major element of teeth and bone, offering a structural function. Calcium is needed for nerve transmission, maintenance of cell metabolic rate and muscle activity [13]. It also partakes in many indispensable tasks such as synthesis,

release and receptor responsiveness to neurotransmitters [14]. Serum iron is the protein-bound iron in circulating blood. The majority of total-body iron; 60% to 70%, is present in haemoglobin in circulating erythrocytes. Another 10% of essential body iron is present in the forms of myoglobins, cytochromes, and iron-containing enzymes. In healthy individuals, the remaining iron is stored as ferritin and haemosiderin [15].

Iron is an essential element for promoting microbial growth and development because of its requirement at various stages of cell metabolism such as DNA synthesis and aerobic and anaerobic glycolysis [16]. Many in vitro and in vivo studies showed an increased growth of several strains of microorganisms in host cells, tissues, and fluids with the addition of iron [17,18]. Moreover, multitude of studies on experimental infection have shown that iron strengthens bacterial virulence of strains such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Bacteroides melaninogenicus* and *Staphylococcus aureus* [19-22]. Based on the previous literature, this short term clinical study was aimed primarily to find out whether calcium and iron can be detected in healthy and chronic periodontitis patients and whether these elements could be taken as diagnostic markers for periodontitis, which would indicate disease activity in chronic periodontitis and aid in detection, prevention and treatment of periodontal disease.

MATERIALS AND METHODS

This cross-sectional study conducted from March 2019 to December 2019 was performed in accordance to the Declaration of Helsinki, 2008 and was approved by the Institutional Ethical Committee (Ref No: IEC/TDCH/022/2019). This study being a preliminary study, power of the study was kept at 70% and a small sample size was calculated using OpenEpi (The OpenEpi Collection of Epidemiologic Calculators, Version 3.01).

Sample size: The minimum sample size per group (n) was calculated as four and hence the total sample size was eight.

The formula used was- $n=2[(\alpha+\beta)^2\ \alpha^2]/(\mu1-\mu2)^2$ where,

where, n=the sample size in each of the groups

$\mu1$=population mean in treatment group 1=0.0594 (Mean of serum iron of healthy patients)

$\mu2$=population mean in treatment group 2=0.009065 (Mean of serum iron of periodontitis patients)

$\mu1-\mu2=0.05$

$\mu2$=population variance (SD)=0.000668

α=Alpha is 95%-Critical value is 1.96

β=Beta is 20%-Critical value is 0.84

$n=2\{(\alpha+\beta)^2\ \alpha^2\}/(\mu1-\mu2)^2$

$=2\times(1.96+0.84)^2\times0.000668/(0.0594-0.009065)^2$

$=2\times7.84\times0.000668/(0.05)^2$

$=0.0104/0.0025$

$n=4.16$

Subjects reporting to the Outpatient Department of Periodontics at Tagore Dental College and Hospital, Chennai underwent a full-mouth periodontal examination and a total of eight study subjects were randomly recruited for this study based on the inclusion and exclusion criteria mentioned below.

Inclusion criteria: Subjects above the age of 30 years and with a minimum of 20 teeth (excluding the third molars) were selected.

Exclusion criteria: Patients who were under medication for systemic diseases like diabetes mellitus, hypertension and cardiovascular disorders (myocardial infarction, atherosclerosis, stroke) were not included. Subjects with a history of intake of any anti-inflammatory drugs, anti-coagulants, antibiotics, antioxidants or multi-vitamin supplements in the previous six months were excluded. Tobacco users, alcoholics, pregnant and lactating females were also excluded.

The patients were divided into two groups of four patients each as:

Group 1- Healthy- Subjects with no clinical signs of gingival and periodontal inflammation

Group 2- Chronic Periodontitis- Subjects who had clinical signs of gingival inflammation, presence of PD and clinical attachment loss ≥5 mm.

All selected subjects were explained about the study in their regional language and written informed consent was obtained from the participants.

Clinical Examination

The PD and clinical attachment loss was measured at all sites by using a William's Periodontal Probe. The gingival index was assessed for all patients according to the criteria given by Loe H and Silness J (1963) [23]. The gingival units of each tooth (buccal, lingual, mesial and distal) was given a score from 0-3 and the average GI for each tooth was calculated [23]. The GI for the patient was determined by adding the GI for each tooth and dividing by the total number of teeth present in the oral cavity [23].

Sample Collection

Collection of GCF

The GCF samples were collected by the capillary tubing method using 1-5 µL calibrated volumetric micro capillary pipettes (Sigma-Aldrich, St.Louis, MO, USA). This technique gives an undiluted sample of native GCF [24]. In periodontitis patients, the sites showing the highest clinical signs of inflammation and highest CAL were selected for GCF collection. In healthy subjects, GCF was collected from the labial aspect of mandibular anterior teeth. GCF was pooled from multiple sites in healthy participants to get a sufficient quantity.

Cotton rolls were used to keep the site isolated and prevent saliva contamination. A standardised 5 µL volume of GCF was collected from each patient and the collected samples were then stored in Eppendorf Plastic Vials at -20°C till further analysis.

Collection of Serum

Two mL venous blood samples were drawn from patients of both groups. Samples were collected from the antecubital fossa by venipuncture and allowed to clot at room temperature. The collected samples were centrifuged after an hour to separate the serum from the blood component and the separated serum was preserved in plastic vials at -20°C until further analysis.

Biochemical Analysis

Prior analyses, the samples collected were pretreated with double distilled deionised water. The estimation of Calcium and Iron in GCF and serum was done by using Dual-Viewing (DV) ICP-OES (Perkin Elmer Optima 5300).

Qualitative information of element present in the sample, was involved in identifying the presence of emission at wavelength, characteristic of the selected element: calcium-wavelength 317.933 nm, iron-wavelength 238.204 nm. Quantitative measurement of the element in the sample can be obtained using calibration curves [25].

ICP-OES is used for multielement determination over wide range of concentrations. The precision, accuracy of ICP-OES are considered sufficient for most trace elemental analyses. The ICP-OES technique experiences the fewest interferences of the commonly used analytical atomic spectrometry techniques [25]. In ICP-OES, liquid samples were introduced into a radiofrequency (RF)-induced argon plasma and instantly dried, vaporised, and energised through collisional excitation at higher temperature. The resulting atomic emission was

observed in either a radial or axial configuration and imaged onto the entrance slit of a wavelength selection device [26].

STATISTICAL ANALYSIS

Data was presented as mean±standard deviation (SD). Statistical Package for the Social Sciences (SPSS) version 21.0 was used for statistical analysis and data was not normally distributed. The Mann Whitney U test was used for comparing the groups. A probability value (p-value) less than 0.05 was considered statistically significant.

RESULTS

The study subjects were categorised into two groups of four patients each: those with healthy periodontium and those with chronic periodontitis.

PD and CAL were significantly increased in periodontitis patients compared to healthy subjects indicating the severity of the disease, p=0.001, p<0.001, respectively [Table/Fig-1].

Parameter	Healthy (n=4) Mean (±SD)	Periodontitis (n=4) Mean (±SD)	p-value
PD	1.710 (±0.3741)	3.702 (±0.5894)	0.001*
CAL	1.710 (±0.3741)	4.227 (±0.2815)	<0.001**
GI	0.015 (±0.0300)	1.802 (±0.8216)	0.018*

[Table/Fig-1]: Distribution of baseline characteristics between healthy and periodontitis.
Mann-Whitney U test; *Statistically significant (p-value <0.05); **Highly significant; PD: Probing depth; CAL: Clinical attachment loss; GI: Gingival index; SD: Standard deviation

The gingival index was also significantly increased in periodontitis patients compared to the control groups indicating the presence of gingival inflammation, p=0.018 [Table/Fig-1].

Serum iron levels showed a significant reduction in periodontitis subjects compared to healthy patients, p-value=0.043. However, the difference in serum calcium levels between healthy and periodontitis groups were not found to be significant p>0.05 [Table/Fig-2].

There was no significant difference between GCF calcium and iron levels in healthy and periodontitis groups [Table/Fig-3].

Elements (µg/mL)	Healthy Mean (±SD)	Periodontitis Mean (±SD)	p-value
Calcium	2.636 (±2.0444)	1.978 (±1.3161)	0.564
Iron	0.059 (±0.0592)	0.009 (±0.0090)	0.043*

[Table/Fig-2]: Mean distribution of serum calcium and iron in healthy and periodontitis cases.
Mann-Whitney U test. *Statistically significant (p-value <0.05). µg/mL: Microgram per millilitre; SD: Standard deviation

Elements (µg/mL)	Healthy Mean (±SD)	Periodontitis Mean (±SD)	p-value
Calcium	0.0027 (±0.00374)	0.00080 (±0.00091)	0.149
Iron	0.0000 (±0.00008)	0.0000 (±0.00002)	0.386

[Table/Fig-3]: Mean distribution of calcium and iron in GCF of healthy and periodontitis cases.
Mann-Whitney U test; GCF: Gingival crevicular fluid; µg/mL: Microgram per millilitre; SD: Standard deviation

DISCUSSION

The onset and rate of progression of periodontitis is unique and is determined by various factors like microbiological, environmental, immune, and inflammatory [27]. Therefore, it is necessary that the periodontal evaluation of a patient should focus on a comprehensive valuation of the clinical condition and estimation of risk for disease [28]. The complexity of the periodontal conditions demands a high degree of skill by the clinician to understand and detect the disease [28].

The prompt diagnosis of the periodontal disease is extremely challenging because the bone loss, soft tissue loss is progressive and it is also difficult as the initial phase of the disease is painless and patients seldom seek prophylactic care [29].

Useful diagnostic indicators should indicate the presence or absence of periodontal disease, the response to treatment, and the need for supplementary treatment [30]. The discovery of predictive biomarkers is considerably difficult due to the episodic nature of disease [12]. Thus, an idealistic and objective diagnostic method is still being sought to assess the active disease status of periodontitis.

Though GCF provides site specific information, its complexity in collecting a sample without contamination and time collection hinders its use during routine chair-side examination [12]. Likewise, blood cannot provide site-specific information; however, it is simple, rapid, and can be carried out as part of a routine general diagnostic check-up [12].

The GCF is constituted by various indicators and markers of connective tissue and bone destruction, providing a window for non-invasive analysis of periodontitis and ascertaining the severity of gum disease [31]. The biochemical analysis of blood serum also provides a non-invasive means of estimating the host's systemic response in periodontal disease. This is the first study to simultaneously assess the levels of calcium and iron in GCF and serum in patients with and without chronic periodontitis.

The present study did not show any significant difference between calcium levels in both GCF and serum with periodontal health and disease. The present findings are supported by Koregol AC et al., whose study stated that calcium concentration in GCF did not show any significant variations (p-value >0.05) with the various parameters like gingival index scores and pocket depth [32]. Researchers have been examining the role of serum calcium in the etiology and progression of periodontitis for more than four decades [33]. Studies show a relationship between calcium deficient diet and progression of periodontal disease [34,35]. A study by Amarasena N et al., (2008) showed that the serum calcium levels were significantly associated (p-value=0.04) with periodontal disease progression [33]. This is even more plausible considering that Vitamin D and calcium supplements have a positive effect in periodontal treatment [36-38].

The present study showed that serum Iron was significantly decreased (p-value=0.043) in patients with chronic periodontitis in comparison to the healthy controls. The results are very similar to another study by Kalburgi NB et al., where the serum iron levels in periodontitis patients were significantly reduced (p-value 0.013*) as compared to the healthy patients [39]. Thus, the estimation of serum iron may indicate a person's predisposition to chronic periodontitis. However, there are studies with conflicting results, Prakash S et al. found no significant relationship (p-value 0.09) between serum iron and periodontitis [40]. Rao PK et al., has also concluded that periodontitis does not induce anaemia like state [41]. In this study, the mean GCF iron concentration in both healthy and periodontitis patients were found to be very low.

However, a study by Mukherjee S had shown the relationship of iron with periodontal disease progression and revealed that iron concentration in GCF was increased during periodontitis when compared to GCF iron levels during gingivitis [16]. The release of heme compounds also appears to strengthen periodontal infection than the increase in serum iron concentration [16]. All these studies, even though with conflicting results have clearly emphasised the importance of assessing Calcium and Iron levels in GCF and serum in periodontitis. However, the association between these elements and the pathogenesis of periodontitis remains unclear.

Limitation(s)

The main limitation of this study was the small sample size. This was a preliminary study and more research is therefore necessary to monitor the relationship between calcium, iron and their role in periodontal disease activity.

CONCLUSION(S)

Within the limitations of the study, it can be said that the serum iron levels may aid in predicting periodontitis and the calcium levels in GCF and serum do not affect the periodontal disease activity. Further studies with larger sample sizes are required to confirm the findings of this preliminary study. Biochemical analysis of GCF and serum is essential for the diagnosis and assessment of periodontal diseases on a more rational and less empirical basis.

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