A comparative analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls

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ABSTRACT

Background and Objectives: Superoxide dismutase (SOD), a metalloenzyme is a powerful antioxidant in the body that acts against superoxide, released in inflammatory pathways and causes extracellular structure degradation. In this study, SOD activities in gingiva and gingival crevicular fluid (GCF) from patients with chronic periodontitis (CP) and periodontally healthy controls were compared. **Materials and Methods:** Thirty patients, involving teeth with moderate-to-severe periodontal breakdown and ≥5 mm pockets that required full-thickness flap surgery in the maxillary quadrants, and in controls, thirty patients with teeth scheduled for extraction for reasons other than periodontal destruction were studied. After clinical measurements, GCF samples were collected. Tissue samples were harvested from the same teeth, during flap operation in CP patients and immediately after tooth extraction in controls. SOD activities were spectrophotometrically assayed. The results were statistically analyzed. **Results:** Gingival SOD activity was significantly higher in the CP group than in the controls ($p<0.001$). Significant difference was found in GCF SOD activity between the groups ($p = 0.012$). Correlations between gingival and GCF SOD were statistically highly significant in CP and control groups ($p = 0.00$). **Conclusion:** In chronic periodontitis, superoxide dismutase activity seems to increase in gingiva, probably as a result of a higher need for superoxide dismutase activity and protection in gingiva in CP, while a lower activity seen in GCF which could be due to the low amounts of superoxide dismutase in the extracellular fluid. The strong correlation between the gingival and GCF SOD activities suggests distinct actions of these superoxide dismutases.

Keywords: Antioxidant mechanism; chronic periodontitis; gingiva; gingival crevicular fluid; superoxide dismutase; superoxide.

INTRODUCTION

Oxygen is an element and a vitally important substance for life on the earth, especially for human life. Living organisms need oxygen for most, if not all, of their cellular functions. On the other hand, oxygen can produce metabolites that are toxic and potentially lethal to the same cells.[1]

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In recent years, the term "**reactive oxygen species**" or "**reactive oxygen intermediates**" is a collective term which has been adopted to include molecules such as hydroxyl radical (·OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) which whilst not radicals in nature are capable of radical formation in the extra- and intracellular environments.[2] They interconvert to multiple species, are cytotoxic, and are able to participate in chain reactions leading ultimately to tissue necrosis unless quenched by specific scavengers.[2]

The human periodontal diseases are inflammatory disorders that give rise to tissue damage and loss, as a result of the complex interactions between pathogenic bacteria

and the host's immune response. It is likely that the role of reactive oxygen species is common to both bacteria and host-mediated pathways of tissue damage. Several studies have shown increased generation rate of reactive oxygen species from peripheral blood polymorphonuclear leucocytes in rapidly progressive periodontitis, juvenile and chronic adult periodontitis.[3]

It is estimated that 1–3 billion reactive species are generated/cell/day, and given this, the importance of the body's antioxidant defense systems to the maintenance of health becomes clear.[4]

The body contains a range of enzymatic and non-enzymatic antioxidant systems to protect them against harmful oxidative reactions. Their specific role is to remove harmful oxidants (or reactive oxygen species) as soon as they form or to repair the damage caused by these reactive oxygen species.[2]

Antioxidants may be regarded as "***those substances which when present at low concentrations, compared to those of an oxidisable substrate, will significantly delay or inhibit oxidation of that substrate.***"[2]

Superoxide dismutase or superoxireductase is a primary oxygen radical scavenging enzyme for eukaryotic, aerobic cells and tissues. Because of this, superoxide dismutase is an important antioxidant enzyme that protects tissues against oxidative injury from free oxygen radicals generated by various metabolic processes. Antioxidant molecules are present in all body fluids and tissues. The function of superoxide dismutase is to remove damaging reactive oxygen species from the cellular environment by catalyzing the dismutation of two superoxide anions to hydrogen peroxide.[5]

In literature, there are only very few studies concerning the relationship between superoxide dismutase and inflammation in the oral cavity or periodontium, and the results are conflicting. The studies concerning the reactive oxygen species and/or antioxidant mechanisms are also limited in number.[3]

The aim of this study was to estimate the superoxide dismutase activity levels in patients with chronic periodontitis (CP) and periodontally healthy controls and with the objectives of to determine the protective anti-oxidant property of superoxide dismutase activity levels in the gingiva in units (U) and in the gingival crevicular fluid (GCF) in units/ml (U/ml) as well as to analyze the correlation between gingival and GCF superoxide dismutase activities levels.

MATERIALS AND METHODS

Source of data

This study was conducted in the Department of Periodontics, KLE University, Belgaum, Karnataka. An ethical clearance was obtained before the study by the Ethical Committee, KLE University, Belgaum.

Sample size

A total of 60 individuals (30 chronic periodontitis and 30 periodontally healthy), both males and females were enrolled for the study. The procedure was explained and a written consent was obtained from the patients prior the study.

Inclusion criteria

Group I (Periodontally healthy controls): comprised of GCF and gingival tissues taken from 30 healthy adult patients (31–52 years) whose teeth were indicated for extraction for reasons other than periodontal destruction.

Group II (Chronic periodontitis): comprised of GCF and gingival tissues taken from 30 adult patients (31–52 years) diagnosed clinically and radiographically as chronic periodontitis according to the criteria currently accepted with

- Gingival inflammation
- Pocket depths ranging from 5–8 mm
- 50% periodontal bone loss
- Patients scheduled for full-thickness periodontal flap surgery in the right or left maxillary quadrant.[6]

Exclusion criteria

1. Patients with a history of any systemic diseases/conditions
2. Patients with history of a periodontal treatment (including scaling) 6 months prior to the study.
3. Patients on any medication (antibiotics/anti-inflammatory drugs) 6 months prior to the study.
4. Patients with a habit of smoking.

Clinical measurements

The patients were seated comfortably on a dental chair with good illumination.

The periodontal status of the subjects were determined by measuring the

- Probing depth (PD)
- Clinical Attachment Level (CAL)

And recording the

- Plaque index (PI) (Sillness & Loe 1964)[7]
- Gingival index (GI) (Loe & Sillness 1963)[8]
- Gingival bleeding index (GBI) (Ainamo 1975)[9]

Full-mouth periapical radiographs were taken from the patients in order to determine the periodontal bone loss.

CLINICAL PROCEDURE

Sampling of gingival crevicular fluid (GCF)

To avoid irritation, sampling was performed 1 week after the clinical measurements were performed. The patients were asked to rinse their mouth with a glass of water so as to cleanse the mouth. The areas were isolated with cotton rolls to eliminate salivary contamination and gently air-dried.

In the control group, GCF was collected from sites showing no signs of gingival inflammation and alveolar bone loss in the right or left maxillary quadrants. In the test group, GCF was collected from teeth that presented with 5–8 mm probing depth and 50% alveolar bone loss in the right and left maxillary quadrants requiring surgery for pocket elimination.

The calibrated micro-capillary tubes were placed extracrevicularly in the mesio-buccal, disto-buccal or mid-buccal surface of the tooth for a standardized time of 1 minute in each site. If plaque or debris clogged the micro-capillary tubes, the GCF collection was rejected and repeated. The collected GCF was then immediately transferred to small sterilized microcentrifuge (eppendorff) tubes and kept at –20ºC until analysis.

Sampling of gingiva

In the test group, the gingival tissue samples were harvested during full-thickness flap operations from the same teeth from which GCF samples were collected. After the initial treatment (scaling and polishing), modified Widman flap procedure was performed for pocket elimination and debridement. The tissues which were excised by internal bevel, sulcular and interproximal incisions were collected as samples. Routine periodontal treatment and oral hygiene procedures were continued after surgery.

In the control group, the same teeth from which GCF samples were obtained were selected for tissue sampling. The gingival surrounding the socket of those teeth was excised into small pieces immediately after extraction.

All the gingival tissue samples were washed in saline solution immediately after excision and dried with filter papers. Tissue samples were stored in small sterile plastic vials and kept at –20ºC until analysis.

LABORATORY PROCEDURE

All the gingival crevicular fluid and gingival tissue samples were sent to the Department of Microbiology and Immunology, Maratha Mandal's Dental College and Hospital, Belgaum for the biochemical laboratory procedures.

Laboratory enzymatic assay of superoxide dismutase activity[10]

Principle

The ability of Superoxide dismutase (SOD) to inhibit superoxide ion catalyzed reactions is used to measure its activity in unknown samples. Specifically inhibition of superoxide ion catalyzed reduction of ferricytochrome c by a cell lysate (or extracellular fluid) is compared to known standards. Superoxide ion is generated by the oxidation of xanthine by xanthine oxidase (XO) to form superoxide ion and urate as products.

Sodium azide is added to inhibit endogeneous catalase and peroxidase activity, and (Ethylene diamino tetra-acetic acid) EDTA is added in order to chelate free copper, which has dismutase activity.

Superoxide dismutase (SOD) assay

The spectrophotometric analysis of SOD activity was determined as described by Spychalla and Desborough. The enzyme reaction mixture containing 20 mM Na_2CO_3 buffer (pH 10), 0.1 mM EDTA, 5 μM ferricytochrome *c* and 50 μM xanthine was prepared. This mixture was used as a blank reading at 550 nm. The assay was initiated with the addition of sufficient xanthine oxidase to produce a basal rate of ferricytochrome *c* reduction. (V1). After verification of VI, the GCF and the gingival tissue samples were added and the resulting reaction velocity (V2) calculated. One unit of SOD was defined as the amount of enzyme that inhibited the rate of ferricytochrome *c* reduction by 50%. Units of activity were calculated as a function of the reaction velocities (units = VI/V2 X concentration of standard).

STATISTICAL ANALYSIS

The homogeneity of variances was tested by Levene test. To compare the variances which were not homogeneous were analyzed by Mann-Whitney U-test. The variances that were homogeneous were tested by t-test for independent samples. The correlations between gingival and GCF SOD activities and between clinical parameters and SOD activities were analyzed by simple correlation analysis (Pearson's correlation coefficient).

RESULTS

Clinical findings

Mean ±standard error values of clinical parameters are given in Table 1. To avoid presenting too much data, only the results of the sampling areas are given in chronic periodontitis and control groups. All of the clinical parameters were significantly higher in the chronic periodontitis group than in the control group ($p < 0.001$) (Table 1). Since the results belong to the same sampling areas only, in the control group, the values of clinical parameters were 0 ± 0 except PI (Plaque index) and PD (Probing depth).

LABORATORY FINDINGS

SOD activity in gingiva

Mean gingival SOD activity levels were 9.50 ± 2.11 U/ml homogenate in the CP group and 5.89 ± 0.74 U/ml homogenate in the control group. The CP group showed significantly higher gingival SOD activity than the control group ($p < 0.001$). The results revealed that SOD activity in inflamed gingival from periodontal disease sites of CP patients was significantly higher than that of healthy gingival in controls. (Table 2, Graph 1).

SOD activity in GCF

Mean total SOD activities were 6.90 ± 1.75 U in CP group and 8.04 ± 1.61 U in the control group. The control group showed higher GCF SOD activity level than CP group which was statistically significant ($p = 0.012$). The results indicated that GCF SOD activity in healthy GCF was significantly higher than that from inflamed GCF from patients with chronic periodontitis. (Table 3, Graph 2).

Table 1 Comparison of clinical parameters between Group I (control) and Group II (Chronic periodontitis)

Clinical Parameter	Groups	Mean of Clinical Parameters	'U' Value	'p' Value	Significance
G.I	I	2 ± 0	0		Highly Significant
(Gingival Index)	II	0 ± 0		<0.001	
P.I	I	2.40 ± 0.50	0		Highly Significant
(Plaque Index)	II	0.43 ± 0.50		<0.001	
PD	I	6.07 ± 1.44	0		Highly Significant
(Probing Depth)	II	1.33 ± 0.48		<0.001	
CAL	I	3.77 ± 1.17	0		Highly Significant
(Clinical Attachment Level)	II	0.0 ± 0.0		<0.001	

Observation:
The Mann-Whitney - 'U' Test showed that the mean clinical parameters between Group I and Group II were highly significant. ($p < 0.001$).

Table 2 Comparison of Gingival Superoxide dismutase activities between Group I (Control) and Group II (Chronic Periodontitis)

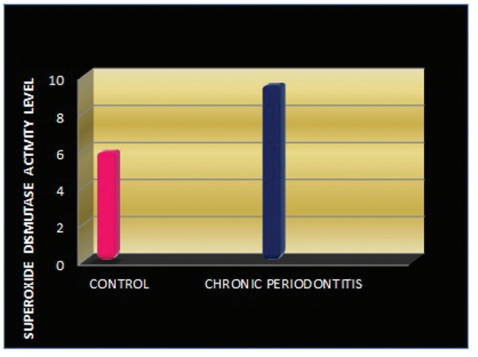
Group	n	U Homogenate	'U' Value	'p' Value	Significance
CP	30	9.50 ± 2.11	50	<0.001	Highly Significant
Control	30	5.89 ± 0.74			

Observation:
The Mann Whitney 'U' test showed that the superoxide dismutase activity levels in the gingival tissues between chronic periodontitis and clinically healthy controls to be highly significant. ($p<0.001$)

Table 3 Comparison of Gingival crevicular fluid superoxide dismutase activities between Group I (Controls) and Group II (Chronic Periodontitis)

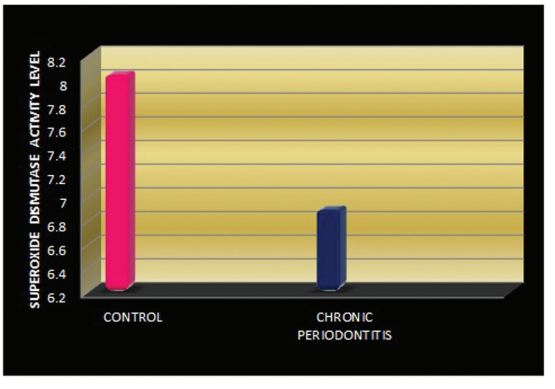
Group	n	U/ml Homogenate	't' Value	'p' Value	Signifance
CP	30	6.90 ± 1.75	2.608	0.012	Signifacant
Control	30	8.04 ± 1.61			

Observation:
The student paired 't' test showed that the superoxide dismutase activity levels in the gingival crevicular fluid between chronic periodontitis and clinically healthy controls to be significant. ($p = 0.012$)



THE DIFFERENCE IS STATISTICALLY HIGHLY SIGNIFICANT BETWEEN GROUP I (CONTROL) AND GROUP II (CHRONIC PERIODONTITIS) (p<0.001).
CHRONIC PERIODONTITIS GROUP, n=30; CONTROL GROUP, n=30.

Graph 1. Comparison of superoxide dismutase activities in the gingival tissues between group I (controls) and group II (chronic periodontitis).



THE DIFFERENCE IS STATISTICALLY SIGNIFICANT BETWEEN GROUP I (CONTROL) AND GROUP II (CHRONIC PERIODONTITIS) (p=0.012).
CHRONIC PERIODONTITIS GROUP, n=30; CONTROL GROUP, n=30.

Graph 2. Comparison of superoxide dismutase activities in the gingival crevicular fluid between group I (controls) and group II (chronic periodontitis).

Correlations

The correlations between gingival and GCF SOD activities and between clinical parameters and SOD activities were analyzed by simple correlation analysis. Negative, weak and statistically non-significant correlations were found between these parameters in both groups by Pearson's correlation coefficients. Also, gingival and GCF SOD activities did not show any significant correlations with the clinical parameters in either group ($p > 0.05$) suggesting a weak correlation among these parameters.

It was also seen that the relationship between gingival and GCF activities were strong with respect to SOD activity levels.

DISCUSSION

Free radicals seem to influence many oral diseases, most commonly periodontitis. Though oxidative stress is implicated in the pathogenesis of periodontitis, it is seen that the total antioxidant capacity of the gingival crevicular fluid (GCF) volume and plasma appears compromised. The periodontal tissues also provide an ideal medium to study the mechanisms of reactive oxygen species mediated tissue damage and of antioxidant defense in response to bacterial colonization.

Superoxide dismutase or SOD is a metalloenzyme whose active center is occupied by copper and zinc, sometimes manganese or iron; is a powerful antioxidant. SOD plays an extremely important role in the protection of all aerobic life-systems, including man, against oxygen toxicity (and the free radicals derived from oxygen). The superoxide dismutase enzyme catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. It is endogenously produced intracellular enzyme present in essentially every cell in the body.

In this study, the total activity of SOD in GCF instead of concentration has been presented in this study. In recent years, concentration is not likely to be the best expression, since GCF volume and composition do not have an absolute correlation.[11] It has been mentioned that, if there is local production, increases in GCF flow will dilute the concentration in GCF, and therefore, greater emphasis should be given to the total amount,[12] and that total activity in the sample is a more appropriate means of reporting GCF constituent data than is concentration. Standardization in sampling is required to allow comparison between samples. This was achieved by sampling all sites for the same length of time (5 minutes).[13,14]

In this study, the results revealed that the activity level of superoxide dismutase was significantly higher in the gingival tissues in the chronic periodontitis group (9.50 ± 2.11 U/ml) as compared to the control group (5.89 ± 0.74 U/ml). This finding seems to confirm several findings in the medical literature about oxidant-antioxidant balance. It has been shown that SOD activity increases directly after occurrence of oxidative stress.[15]

Studies on SOD in relation to periodontal tissues or the oral cavity are limited and the results are conflicting.[4] In contrast to our finding, reactive oxygen species (ROS) have been shown to depolymerise gingival hyaluronic acid and proteoglycans and it has been presupposed that the balance between ROS and the presence of SOD is disturbed.[13] Another study showed a significant and

progressive reduction in SOD activity within gingiva adjacent to deeper pockets.[14] However, consistent with the findings in this study, another study has shown that SOD activity increases with progression of inflammation in the pulp.[15]

The human periodontal ligament has been shown to possess the enzyme SOD, which might afford biological protection against ROS, particularly superoxide ion during the inflammatory response.[16] Bacterial lipopolysaccharide was shown to stimulate superoxide ion release from gingival fibroblast, suggesting that the induction of SOD may represent an important defense mechanism of the fibroblast during inflammation.[17] Mn-SOD in the axon terminals of mechanoreceptors in the periodontal ligament of rats has been shown to exert protective action against nerve injury and neuronal death under severe conditions.[18] In the present study, increased gingival SOD activity level in chronic periodontitis seems to support these findings.[19]

The availability of SOD has provided a tool that allows testing physiological processes for the involvement of superoxide ion.[19] In the present study, increased SOD activity level in inflamed gingival from chronic periodontitis patients may indicate the increased superoxide ion generation by polymorphonuclear neutrophils invaded at the disease site. This increase in superoxide ion may have led to the occurrence of oxidative stress, which in turn caused an increased need for SOD production to establish the ROS-AO (Reactive Oxygen Species-Antioxidant) balance to protect the tissue.

The present findings indicated that the GCF SOD activity was higher in clinically healthy controls (8.04 ± 1.61 U) as compared to patients with chronic periodontitis (6.90 ± 1.75 U) and the difference was statistically significant.

The possible reasons for this low GCF SOD activity in chronic periodontitis in the present study could be:

1. It is known that SODs are mainly found in cells and tissues and there is only a minor activity in extracellular fluids.[2]
2. Another possible reason may be that suppression on SOD production may have occurred in GCF/PMNLs because of the oxidative damage caused by the increased ROS/superoxide ion generation.[20]

Several studies have analyzed SOD in synovial fluid from patients with rheumatoid arthritis whose pathogenesis resembles that of periodontitis where ROS-AO interactions that take part in the pathogenesis of these diseases. These studies have provided contrary results. One study could find no SOD activity in the synovial fluid of rheumatoid arthritis patients,[21] whereas, another study found a fourfold higher SOD concentration in synovial fluid from rheumatoid arthritis patients.[22]

Our finding is consistent with a study that found that the synovial fluid SOD activity in rheumatoid arthritis patients was significantly lower than in healthy controls[14] and another study which showed that the AO activity (SOD activity) able to scavenge superoxide ion in GCF did not differ between controls and chronic periodontitis patients.[20] Also, studies have indicated that total salivary AO activity remains at the same level in periodontal disease[13] or is reduced (similar to our finding).[2]

We also analyzed the relationship between gingival and GCF SOD activities which was statistically significant. Based on this finding, it may be speculated that:

1. The activities of SODs in gingival and GCF might be dependent on each other, or
2. The SOD enzyme from gingival tissues might flow to the GCF.

Longitudinal studies and investigations involving sampling of healthy and diseased sites within periodontitis patients are in progress to more clearly define the role of endogenous antioxidant such as superoxide dismutase in the pathobiology of periodontitis and which may provide a more useful physiological tool than therapeutic options currently available.

CONCLUSION

In conclusion, superoxide dismutase activity seems to significantly increase in gingiva in chronic periodontitis. This increase may be because of the occurrence of increased oxidative stress and the increased need for oxidant/anti-oxidant balance and protection. The superoxide dismutase defense in the gingival crevicular fluid is significantly lower in the chronic periodontitis as compared to that in healthy controls and it is doubtful whether this low SOD activity in GCF can protect against reactive oxygen species.

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